



APPLICATION NOTE 1

Interactions of biomolecules in cell membrane models

This application note illustrates how the analytical instruments from KSV NIMA can be used to create cell membrane models and study biomolecular interactions in these models.

Introduction

Most biochemical reactions in nature take place at membranes composed of phospholipid bilayers on or inside cells. The membrane affects protein folding and creates specific microenvironments where the reactions take place. In order to understand and mimic real biological systems, it is essential that interactions are studied in an environment as close as possible to nature.

Langmuir monolayers of membrane phospholipids have been verified as excellent model systems for biological membranes [1]. The main alternative method is the supported lipid bilayer (SLB), where a bilayer is created on a solid support by the Langmuir-Blodgett, Langmuir-Schaefer or self assembly technique. Disadvantages with these techniques include unwanted interactions of lipids and biomolecules with the support, where the diffusion of molecules is hindered. In the worst case, this can lead to denaturation of the biomolecules in question [2]. By contrast, no such interactions occur for floating monolayers.

These model systems are employed in several application areas, such as drug development, food technology, and biological and biochemical research. We present two case studies to show how model membranes are utilized. The first case is about the development of antiparasitic drug and the second is about research into methods for removal of lipophilic allergenic compounds from food. Other application areas that could be studied with model membranes include interactions of drugs in cell membranes with proteins, nucleic acids, polysaccharides, and the membrane itself. This method can also be used to study migration of drugs or targeted drug carriers into membranes, native synthesis of biomolecules and drugs in membranes, and biosensor applications with naturally occurring proteins.

Case study I: The interaction of an antiparasitic peptide with cell membrane models

The full study was published in Colloids and Surfaces B: Biointerfaces 74 (2009) 504–510 by Pascholati et al.

The interactions of molecules in membranes are important for many applications outside basic research in biochemistry. In drug discovery the permeation of the drug into cells through cell walls and the reaction of the drug within the cell membrane are important factors for drug efficiency. The KSV NIMA PM-IRRAS can be used for screening drug candidates *in vitro* before embarking on expensive *in vivo* tests. The following example is a drug candidate for the Human African Sleeping Sickness (HAT - *African Trypanosomiasis*) caused by protozoan parasite and studied in a model cell membrane.

Methods

The interactions of an oligopeptide-based drug that has shown activity in the treatment of HAT, S-(2,4-dinitrophenyl)glutathione di-2-propyl ester, was studied in Langmuir monolayer model membrane of DPPC (dipalmitoyl phosphorylcholine), an abundant lipid in the protozoan membrane. The monolayer properties of the drug itself and its interactions with DPPC monolayers were studied with Langmuir equipment (KSV NIMA Langmuir Trough) with a surface potential meter (KSV NIMA SPOT) and polarization modulation surface infrared reflection absorption spectrometer (KSV NIMA PM-IRRAS). Additionally Brewster angle microscopy (KSV NIMA BAM) and dynamic surface elasticity experiments were performed on the system.

Results and discussion

Langmuir isotherm experiments of the pure drug showed an unusual decrease in the surface pressure at molecular area of approximately 145 \AA^2 (Figure1). By contrast, the surface potential did not undergo a decrease at the same molecular area (Figure1). The behavior of the pressure and potential isotherms suggest that the drug could undergo aggregation, dimerization or conformational change at this surface pressure.

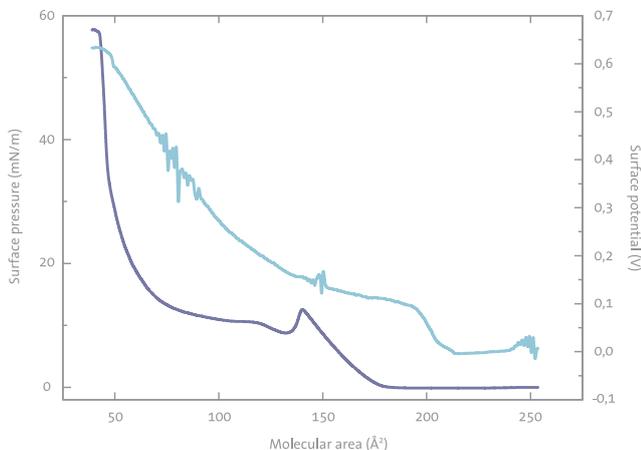


Figure1. Surface pressure-area and surface potential-area isotherms of a HAT-drug monolayer at air-buffer interface.

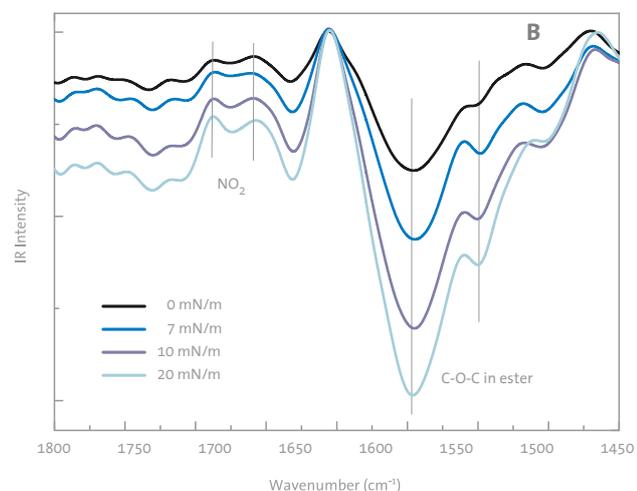
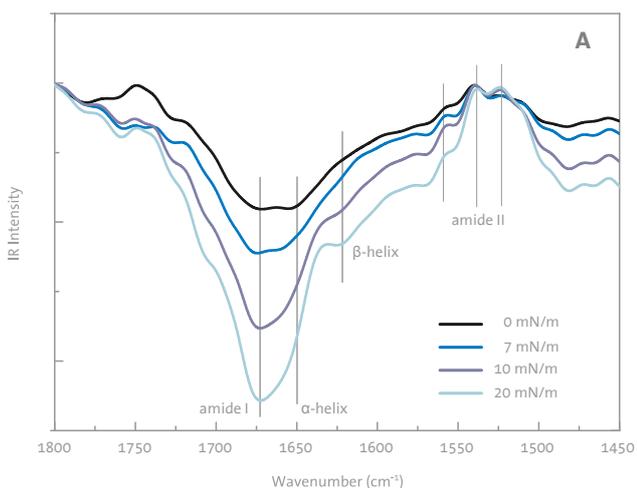


Figure2. PM-IRRAS spectrums of drug monolayer at air buffer interface with different surface pressures. A) From 1450 to 1050 cm^{-1} B) From 1800 to 1450 cm^{-1}

The PM-IRRAS measurements of this monolayer show negative peaks for amides indicating mainly disordered amide groups when the surface pressure is 7 mN/m or less (Figure2.A). The negative intensity for the amide I (C=O stretch) indicates that the C=O groups lie parallel to the surface in the monolayer. When the surface is compressed further, the amide I band for beta-sheet grows more pronounced, indicating intermolecular association between the drug molecules. Because the bands of nitro- and ester groups (Figure2.B) do not show change in their position or sign, the orientational changes of the drug can be ruled out as a reason for the decrease in the surface pressure isotherm. The BAM and dynamic surface elasticity measurements also support the formation of ordered domains in the monolayer at the peak in the isotherms.

The drug was found to interact with DPPC monolayers, even in minute quantities. The isotherm data (Figure3) indicates a repulsive behavior between DPPC and the drug for all concentrations, as the relative excess area is always positive for the drug. Such small concentrations of the drug as of 0.1 mol\% caused changes in the isotherm, meaning that even DPPC that was not in the direct vicinity of the drug was also affected, a phenomena referred to as repulsive cooperative interactions. The drug was not excluded from the monolayer even at large surface pressures corresponding to cell membranes (30 mN/m). The surface potential isotherms followed the behavior of the pressure isotherms. The PM-IRRAS spectra (not shown here) indicates that there is no orientational change upon compression of the monolayer. The amide I for beta-sheet is distinguishably promoted in the same way as in the pure drug monolayer.

Conclusions

These experiments of HAT drug interacting with protozoan cell model membrane have shed light on the mechanism of action of the drug. The packing of the DPPC monolayer is affected even in minute quantities of the drug. In addition, the drug was shown to associate upon increasing surface pressure. Biologically this implies that the drug incorporated into membranes is not expelled at surface pressures found in biological systems, and the interactions are repulsive which points to a possible disruptive action of the drug towards the membrane. Formation of clusters of drug and possibly lipids in the membrane could decrease the compression-decompression response needed for many biological reactions the cell undergoes. The method can be applied for future studies of drug candidates interacting with cell membrane as an additional screening before expensive and time consuming in vivo testing of the drugs.

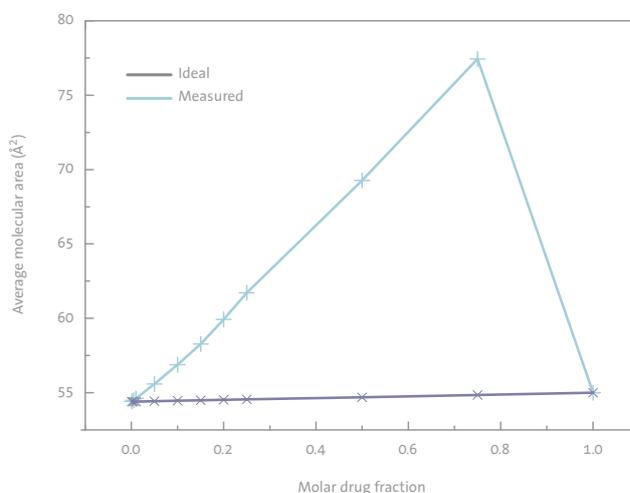


Figure3. A plot of average molecular area of DPPC-drug mixed monolayers at a 30 mN/m surface pressure. The ideal mixtures area is the arithmetic sum of the individual components at the consecutive mixing ratio.

Case study II: Chitosan as a removing agent of β -Lactoglobulin from membrane models

The full study was published in *Langmuir* 24, (2008) 4150–4156 by Caseli et al.

Lipophilic proteins often reside in cell membranes, and the floating monolayer models can be used for studying their interactions in a close to native environment. The removal of allergenic proteins from food is a highly beneficial technology for food processing in order to produce healthy foods like milk products for people who are allergic some components in them. Aside from lactose intolerance, allergy to β -Lactoglobulin found in milk can prevent consumption of milk products. In the following example chitosan was studied as a removal agent for β -Lactoglobulin using the KSV NIMA PM-IRRAS and a KSV NIMA Langmuir Trough.

Methods, results and discussion

The adsorption behavior of β -Lactoglobulin (BLG) to the liquid-water interface was studied with the KSV Minitrough with and without lipid monolayers present. The adsorption was monitored by following the evolution of surface pressure at constant barrier position and injecting the BLG sample to the subphase buffer. When dimyristoyl phosphatidic acid (DMPA) monolayer was present on the surface the adsorption to the monolayer was much more rapid than without the monolayer, which indicates affinity of BLG to monolayers.

Chitosan in the subphase did not cause an appreciable change in the surface pressure of pure buffer, but when a DMPA monolayer was present injection of chitosan to the subphase led to an increase in the surface pressure.

When BLG was injected into a subphase containing chitosan but without a monolayer, a rapid increase in the surface pressure was initially observed, followed by a slow increase before reaching equilibrium. The initial rapid rise may be due to chitosan complexing with BLG and migrating to the surface, and the following slow increase may be attributed to adsorption of uncomplexed BLG. The formation of chitosan-BLG complexes was confirmed also by dynamic light scattering measurements in bulk solution.

When the DMPA monolayer was present, the same experimental setup led also to a rapid increase in the surface pressure at first, which changed to a decreasing trend after the initial period. The total change in the surface pressure was negative (Figure4). This would suggest that soluble chitosan-BLG complexes appear at the interface. The magnitude of the change suggests that some DMPA was also

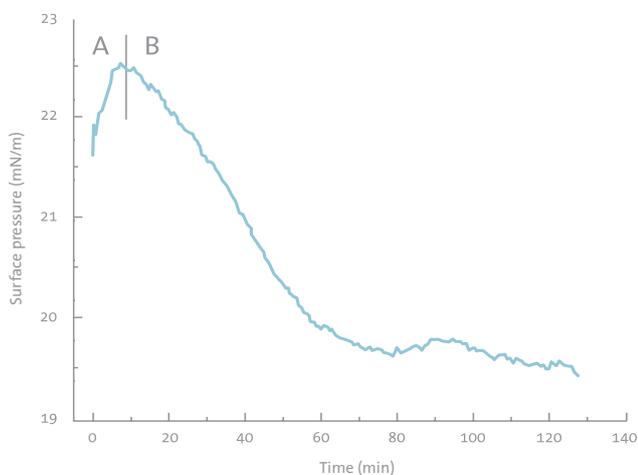


Figure4. Adsorption kinetics of BLG to the DMPA monolayer when chitosan is present in the surface. A) BLG absorption B) BLG-chitosan complex desorption

removed from the monolayer in this interaction.

The same system was tested with different lipids in the monolayer and protein in the subphase. Chitosan could not remove BLG from a neutral phospholipid monolayer (dipalmitoyl phosphorylcholine, DPPC), but similar behavior to DMPA monolayer was found for another negatively charged lipid, dipalmitoyl phosphatidyl glycerol (DPPG). This indicates that the negative charge of these phospholipids is important in the removal of the BLG-chitosan complexes from the layer. Chitosan could not remove other proteins from monolayers (horseradish peroxidase, urease), meaning that the removal is somewhat selective.

The absorption of BLG to a DMPA monolayer was studied with PM-IRRAS as a function of time (Figure5.A). Typical bands for polypeptides, the amide I (C=O stretch) and amide II (NH bending) were negative while the typical DMPA (CH₂ scissor) peaks in 3000 cm⁻¹ region were positive.

In PM-IRRAS measurements on monolayers, resonance parallel to the surface are seen as positive peaks, and perpendicular to surface negative. Figure5.A therefore indicates that the C=O and NH of amide groups preferentially orient perpendicular to the membrane surface, parallel to the acyl chain. This indicates that the polypeptide α -helix axis is inserted to the membrane, a conclusion that is supported by earlier literature. The evolution of the peak intensity over time supports the conclusion made from the Langmuir isotherms that the BLG adsorbs more and more to the surface over time, or undergoes reorientation.

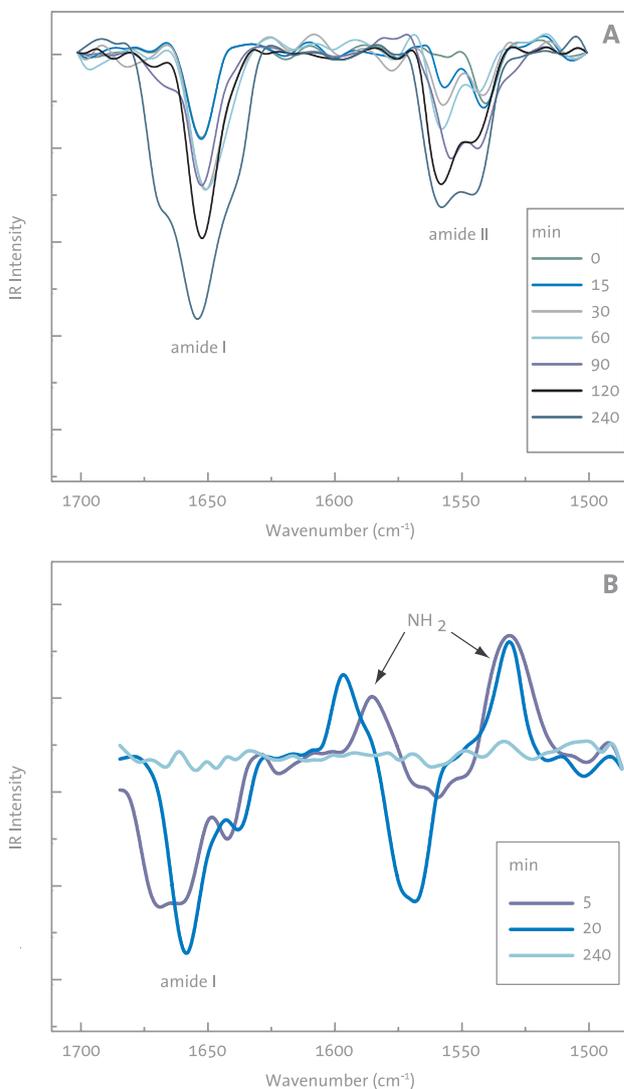


Figure5. A) Change in the amide-region of PM-IRRAS spectrum of the BLG absorbing into DMPA monolayer with time. The initial surface pressure was 20 mN/m before BLG injection. B) Removal of BLG from DMPA monolayer when chitosan is injected to the surface.

Figure 5.B shows the evolution of the PM-IRRAS signal in the amide region after chitosan was introduced to the subphase of DMPA-BLG monolayer. After 20 minutes both amide I and II adsorptions had shifted, indicating a change in the BLG conformation. After 240 minutes from the chitosan addition the amide adsorptions had completely disappeared, meaning that the chitosan had removed the BLG from the membrane completely.

Other experiments on the system such as rheological measurements and fluorescence from deposited LB films support the findings above.

Conclusions

The Langmuir isotherm studies indicate that both chitosan and BLG adsorb to the DMPA monolayer. Chitosan is able to remove BLG from the monolayer, and even some of the DMPA is removed at the same time. From the tested phospholipid monolayers, chitosan was able to remove BLG only from those made with anionic charge. The PM-IRRAS measurements made it possible to see the complexing behavior between BLG and chitosan in the monolayer prior to the removal of the complex, and is, to our knowledge, the first molecular level evidence for the protein-removal mechanism of chitosan.

Summary

Model membranes provide means to study molecular interactions in an environment closely resembling natural cell membranes. Floating monolayers are thought to be the best model for real cell membranes in terms of molecular packing, mobility and dynamics. The two case studies highlight well the key research areas where floating model membranes can be used. Applications of particular interest include design and development of new peptide drugs and drug carriers that interact with cell membranes where the Langmuir film method can be used as a screen to find effective derivatives of molecules before going to in vivo tests.

[1] Brockman, H., *Curr. Opin. Biol.*, 1999, 9, 438-443

[2] Castellana, E.T., and Cremer, P. S., *Solid supported lipid bilayers: From biophysical studies to sensor design*, *Surface Science Reports*, 2006, 61, 429-444



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