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Interactions of an Antimicrobial Peptaibol with Amphiphilic Block Copolymers

Das Leben ist wert, gelebt zu werden,
sagt die Kunst, die schönste Verführerin;
das Leben ist wert, erkannt zu werden,
sagt die Wissenschaft.

Friedrich Nietzsche

CHAPTER 1

INTRODUCTION AND OBJECTIVE

1. Introduction and objective

In living systems, communication and interaction between cells and their environment is provided through membrane proteins. Transmembrane proteins transport various species across the lipid bilayers, either ions and larger substances *[Nikaido 1992, Eisenberg 1998, Sansom 1999, Nardin 2001b, Braun 2002, Klebba 2002]* or genetic material *[Graff 2002, Duckely 2003, Abu-Arish 2004]*. Also, proton gradient can be controlled to regulate the cells' energetic machinery, ATP synthesis etc. *[Friedrich 1998]*.

Motivated by Nature, material science has recently taken advantage of certain solutions to implement membrane proteins in creating new materials with novel functions. The key issue is to create submicron-scale devices to serve as an innovative interface for controlling processes either in artificial systems, or complicated environments like cells. They also offer a new perspective in controlling or introducing new types of interactions at the boundary between natural and synthetic species.

It is well known that proteins' application could not always be straightforward due to their intrinsic properties: poor stability, changes in functionality in certain conditions, folding processes, solubility problems, etc. In cells, membrane proteins are embedded in or attached to lipid membranes, which provide protection and ensure functional conformation of the protein. Therefore, the most straightforward approach to produce membrane protein-based nanostructures is the use of artificial lipid membranes. Indeed, multiple examples of successful protein incorporation in lipid membranes were presented, employing various morphologies of the self assembled lipid superstructures.

Scotto et al. reported that bacteriorhodopsin incorporated spontaneously into both large unilamellar and multilamellar vesicles of various lipid compositions (liposomes) (Fig. 1.1a), including dimyristoyl phosphatidylcholine (DMPC), DMPC and cholesterol, dioleoyl phosphatidylcholine (DOPC), and DOPC and cholesterol. The examinations were made under either fluid-phase or gel conditions. The lipid/protein ratio as well as the vesicle size in function of protein content was investigated *[Scotto 1990]*. The insertion of membrane proteins depending on the lipid bilayer composition was successfully determined employing liposomes pointing that the highest incorporation of multiple proteins was found with dipalmitoylphosphatidylcholine (DPPC) *[Daghastanli 2004]*.

Van Gelder et al. successfully employed free standing lipid membranes (black lipid membranes) (Fig. 1.1b) to detect the single channel activity of OmpF, a bacterial outer membrane porin *[van Gelder 2000]*.

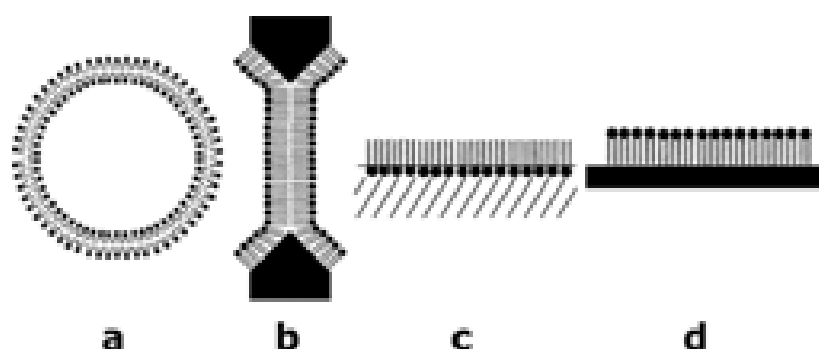The protein structure within a membranelike environment was

Figure 1.1. Artificial membrane systems to produce membrane protein-based nanostructures; a) vesicle, b) free standing membrane, c) Langmuir film, d) solid supported planar film.

investigated by Zheng et al. employing Langmuir films at the air-water interface (Fig. 1.1c). The transmembrane domains of Vpu, a HIV-1 accessory protein, were unidirectionally incorporated in lipid monolayers and probed by x-ray reflectivity and grazing incidence diffraction *[Zheng 2004]* Specific membrane interactions of model cell membranes with blood-clotting proteins in Langmuir films at the air-water interface were reported by Brancato *[Brancato 2001]*.

Rhodopsin has been reconstituted into supported planar lipid membranes (Fig. 1.1d) to measure coupling reactions with transducin to mimic receptor activation and interaction of a membrane receptor with its G protein *[Heyse 1998]*.

In such materials, the membrane proteins were shown to remain functional and that some of them could serve for biomimetic transportation of different species.

Living cells, however, are highly dynamic systems, where every membrane defect will immediately be detected and repaired. In laboratory conditions and applied science any replacement of damaged or oxidized lipids is not achievable. Additionally, lipids themselves lack long-term stability and rigidity, which would render them applicable in biomaterials engineering. The most disadvantageous feature of (fluid) lipid bilayers is their rather high permeability, especially in what may seem the most obvious application of liposomes, i.e. drug delivery. Uncontrolled leakage through liposome membranes additionally poses storage problems.

For these reasons, it has been challenging to find other environments, in which the proteins would remain in their 'native' conformation, thus retaining functionality, and be protected from the hostile surrounding by a compatible matrix. A solution to this problem has been the use of other amphiphilic species, for example amphiphilic block copolymers. Shortly, they are built from at least two chemically incompatible parts (blocks) of different affinity to water. With a plethora of possibilities to create such polymers, in the context of block compositions, block lengths, and polymer architecture, polymer science offers the potential to engineer the most suitable polymers for specific applications. As macromolecules, such polymers may be very well suited to mimic biological amphiphiles and therefore are subject to studies as a complementary component in various bio-composite materials *[Discher 2002]*.

There already exist a few literature reports proving experimentally successful incorporations of proteins into purely polymeric membranes, including evidence of protein functionality in such an artificial environment. Nardin et al. successfully reconstituted OmpF, a channel-forming protein from the outer cell wall of Gram-negative bacteria, into self-assembled membranes from amphiphilic PMOXA-PDMS-PMOXA triblock copolymers. Although two to three times thicker than biological membranes, the polymer membranes serve as a functional matrix for membrane-spanning proteins *[Nardin 2000b, Nardin 2001b]*.

Employing vesicles made from the same PMOXA-PDMS-PMOXA block copolymer, Graff et al. proved that reconstituted LamB λ phage receptors effectively serve as binding site for phage transfection and DNA translocation over the artificial membrane barrier *[Graff 2002]*.

PMOXA-PDMS-PMOXA triblock copolymer membranes were further reported to be successfully functionalized by bacteriorhodopsin and cytochrome c oxidase ion transport proteins *[Ho 2004]*.

Pata and Dan *[Pata 2003]* proved theoretically that protein insertion into polymer membrane at least two-fold thicker than lipid bilayers is possible.

Even though the literature evidence proved successful incorporations of proteins into polymer matrices, nearly nothing is known on the physical chemistry of the insertion process, as well as the material properties of protein-reconstituted polymer membranes. Therefore, the motivation of this thesis is to investigate the behavior of a membrane active peptide, alamethicin, in the membranes from amphiphilic ABA triblock copolymers. As a model system, poly(2-methyloxazoline)-block-poly(dimethylsiloxane)-block-poly(2-methyloxazoline) (PMOXA-PDMS-PMOXA) copolymers were used, well characterized in previous studies concerning protein insertions *[Nardin 2001b, Graff 2002]*.

The choice of alamethicin is justified by the fact that it is a relatively small amphiphilic peptide and could serve as a starting point for further work employing more complex proteins. Alamethicin is a peptide antibiotic naturally produced by trichoderma viride, which contains the non-proteinogenic amino acid, 1-amino isobutyric acid (Aib), inducing α-helical peptide structures. The peptide sequence is: Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-PhI, where PhI is phenylalaninol. As a polyene ionophore, in cell membranes it is reported to form voltage-gated non-specific anion or cation transporting pores by aggregation of four to twelve molecules *[Marsh 1996]*. For further details and specifications on membrane materials and peptaibol employed I refer to chapter two of this thesis.

So far, organization of alamethicin in lipid membranes is still under discussion, even though data from various groups are available *[Aguilella 2001, Taylor 1991]*. The commonly accepted barrel stave model *[Duclohier 2001]* has recently been confronted with a different explanation by Ionov et al. *[Ionov 2000]*, who proposed a lipid-covered ring model. In the latter, alamethicin helices adopt stable planar orientation at the air-water interface, form aggregates with a ring-shaped hole and insert at one side of the lipid membrane. This model has been supported by AFM and X-ray diffraction experiments *[Ionov 2004]*.

A recent report *[Vijayan 2005]* presents the influence of alamethicin on stability of membranes from amphiphilic diblock copolymers. Fluorescence dye leakage and micropipette manipulation studies showed that the membrane permeability strongly depends on its thickness and therefore on the size of the constituting blocks.

The purpose of this work is to characterize peptide-polymer composite materials in terms of miscibility (or phase separation), aggregation behavior and ion permeability, firstly to find the most favorable conditions for the insertion, and secondly, to get more insight into the process itself and the material properties further on. Investigations on planar membranes at the air-water interface were performed employing the Langmuir monolayer technique, supported by Brewster angle microscopy imaging, and further solid supported Langmuir-Blodgett films in combination with topography analysis by atomic force microscopy. The membrane interactions of alamethicin with block copolymer membranes, especially in the context of membrane permeability, were probed in addition employing fully hydrated bilayer systems. Experiments were carried out with giant and small polymersomes characterized by transmission electron microscopy and dynamic light scattering.

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Wer sich Steine zurechtlegen kann,
über die er stolpert, hat Erfolg
in den Naturwissenschaften.

Erwin Chargaff

CHAPTER 2
THEORETICAL BACKGROUND

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2. Theoretical Background

2.1. Molecular interactions at the air-water interface

2.1.1. History of monolayer research

The effects of oil on water surfaces have been known for many centuries *[Gaines 1983, Tredgold 1987]*. Babylonians in the eighteenth century BC already practiced divinity by observing effects of oils spread on water. From the shape and movement of olive oil stains poured on water surfaces, the future of both the country and individual citizens was predicted *[Tabor 1980]*.

The first definite historical reference was made by Aristoteles (BC 384-322). He stated, that 'oil poured on to water makes it more transparent' *[Hett 1937]*. Gaius Plinius Secundus (Fig. 2.1)was rather more explicit when he noted in AD 77 in his famous encyclopedia Naturalis Historia 'again everybody is aware that … all sea water is made smooth by oil, and so divers sprinkle oil from their mouth because it calms the rough element and carries light down with them…' *[Rackham 1964]*. Similar observations were made by Plutarch (AD 45-125) in Greece *[Tabor 1980]*.

Figure 2.1. Gaius Plinius Secundus Maior (23-79) [Rackham 1964].

One of the first technical applications of floating monolayers reported was the Japanese printing art of sumi-nagashi of the 12th century, in which a suspension of colloidal carbon particles and proteins was spread on water, mixed to give diffuse patterns, fixated with gelatine and then transferred onto a sheet of paper *[Albertova 1987]*.

In 1757, aboard one of 96 ships traveling together from America to Europe, Benjamin Franklin (Fig. 2.2) noticed, that ships in the back seemed to have smoother sailing than ships in the front. The captain explained that 'The cooks have been just emptying their greasy water through the scuppers, which has greased the sides of those ships a little.' *[Bigelow 1966]*. In 1762 Franklin was told by an old sailor, that Bermudians put oil on water to smooth the surface when they would strike fish which they could not see when the water was ruffled by the wind. The same gentleman told him that it was a practice with the fishermen in Lisbon when about to return into the river to empty a bottle or two of oil into the sea to suppress the breakers and allow them to pass safely. He further learned from a friend, Sir John Pringle, that those employed in herring fishery in Scotland could at a distance see where the shoals of herring were by the smoothness of the water over them, which might possibly be occasioned, he thought, by some oiliness proceeding from their bodies *[Fulford 1968]*.

All those informations catched Franklin's scientific interest and he wondered to find no mention of them in the books of experimental philosophy of that time. Once in London he spread oil onto a pond and observed a calming influence on the water

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surface. In 1774 he reported to the Royal Society of London that 'At length at Clapham where there is, on the common, a large pond, which I observed to be one day very rough with the wind, I fetched out a cruet of oil, and dropped a little of it on the water. I saw it spread itself with surprising swift-ness upon the surface… the oil, though not more than a teaspoon-

Figure 2.2. Benjamin Franklin (1706-1790) posing on the US one hundred dollar bill.

ful, produced an instant calm over a space several yards square, which spread amazingly, and extended itself gradually, making the pond as smooth as a looking glass.' He suggested that due to a mutual repulsion between the oil particles and no attraction between oil and water, the oil film expands on the surface to an extension until the repulsion between the oil particles is minimized. If wind is blowing over the water, the oil film prevents friction and the wind cannot catch the water to raise wrin-kles. Further he described the 'interesting effect of monolayer films on supposedly dead flies' *[Franklin 1774]*, inventing the first fruit fly trap *[Ringhof 2004]*.

However neither Aristoteles, nor Plinius, nor Franklin recognized the monolayers for what they were, and they could scarcely have had a realistic idea of the true nature of what they were seeing, because they did not have the scientific concept of atoms and molecules.

Figure 2.3. Agnes Pockles 1862-1935 [Beisswanger 1991].

Modern investigations began with Agnes Pockles (Fig. 2.3) experimenting with a very rudimentary setup (an ordinary bak-ing tray, waxed to make it hydrophobic, as trough and a button as duNoüy ring) in her kitchen. She was the first to measure the film behavior depending on the amount of olive oil spread. Her surface pressure versus area per molecule isotherms are in good agreement with those obtained presently using mod-ern experimental techniques. Her findings on technical and practical aspects were published in Nature in 1891 *[Pockles 1891]*.

Scientific activity in thin film studies increased after the reports of Lord Rayleigh (Fig 2.4). One of his most important conclu-sions was that the surface tension of an aqueous solution decreases as the surface is 'contaminated' with olive oil. This effect was noted to be accompanied by a change in molecular area values *[Rayleigh 1890]*. He addi-tionally suspected that the maximum extension of an oil film on water represents a layer one molecule thick *[Rayleigh 1899]*. Further investigations by Devaux *[Devaux 1913]*, Hardy *[Hardy 1911]* and Harkins *[Harkins 1917]* confirmed the mono-molecular nature of such films *[Giles 1971]*.

However it was Irwing Langmuir (Fig. 2.5) who contributed the greatest advances. In his experiments, he used chemi-cally pure substances, both solids and liquids, instead of oil or olive oil used previously. By investigating the pressure-

Figure 2.4. John William Strutt Lord Rayleigh (1842-1919) [AIP].

area relationship of molecules on aqueous surfaces he found, that the areas occupied by molecules such as acids, alcohols and esters are independent of the hydro-carbon chain length, thus showing that only the hydro-philic head groups are immersed in the subphase *[Langmuir 1916, 1917a, 1917b]*. For his discoveries and investigations in surface chemistry he was awarded the Nobel Prize in Chemistry in 1932.

Figure 2.5. Irwing Lang-muir (1881-1957) posing on a Time Magazine cover (August 28, 1950).

Katherine Blodgett (Fig. 2.6), who worked with Langmuir, developed the technique of transferring the films on solid substrates and hence building up multilayer films in 1934 *[Blodgett 1934, 1935]* and published its first application as anti-reflection coatings on glass *[Blodgett 1939]*. Such built-up monolayer assemblies transferred to a solid substrate are therefore referred to as Langmuir-Blodgett (LB) films. The term Langmuir film is normally reserved for a floating monolayer.

Interest in Langmuir-Blodgett films subsided with the outbreak of the Second World War and remained low until the 1960's when Hans Kuhn showed how monolayer films with molecular-scale pattering could be fabricated industrially and further ma-nipulated to build up more complex systems *[Kuhn 1967, 1971, 1972]*. His work and the publications of Gaines *[Gaines 1966]* initiated a revival of interest in the field.

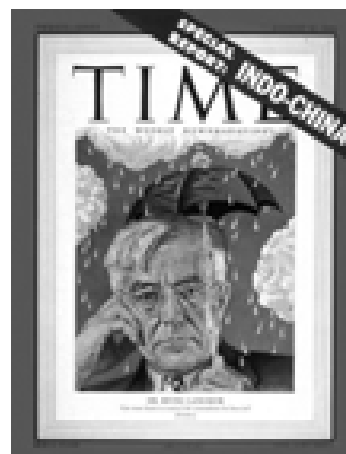*Figure 2.6. Katherine Blodgett (1898-1979) working in her laboratory [GE 1992].*

The first international conference on LB-films was held in 1979 and since then many possible investigations us-ing Langmuir and Langmuir-Blodgett films in the fields of chemistry, physics, biology and engineering for applica-tions such as sensors, detectors, dis-plays and electronic circuits are performed by researchers throughout the world.

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2.1.2. Forces at the air-water interface

Molecules in a solution are subject to attractive forces. In the bulk, these forces are equal. However, at an interface the forces are dissimilar and the net effect is to pull the peripheral molecules into the bulk of the solution. At the interface there are fewer molecules on the vapor side than on the liquid side. This leads to a net attractive force towards the bulk and hence the density increases gradually from the surface to the bulk. Therefore liquids tend to decrease the surface energy by minimizing the surface (Fig. 2.7) *[Adamson 1976, Wu 1982]*.
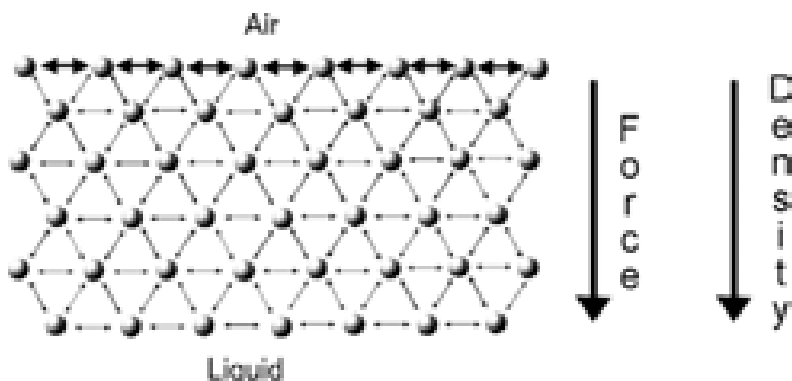

Figure 2.7. Schematic illustration of the interactions of molecules at an interface and in bulk [KSV].

The surface tension γ is a measure of the cohesive energy present at an interface and can be defined as the work dW required to expand the surface isothermally by unit area dA. The tendency of surface active molecules to accumulate at interfaces favors expansion of the interface and hence lowers the surface tension.

dW = γdA   (Eq. 2.1)

Surface tension readings are made by means of Wilhelmy plates *[Wilhelmy, 1863, Dettre 1966, Pallas 1983]* or DuNoüy rings *[Harkins 1930, Huh 1975]* attached to a sensitive electrobalance (Figures 2.8 – 2.10). The force necessary to detach the Wilhelmy plate or the DuNoüy ring from the liquid surface is measured. The Wilhelmy plate is usually a strip of chromatography paper or a platinum plate whereas the DuNoüy ring is made of platinum. When such a probe is suspended at an air-water interface, it is pulled down into the bulk of the subphase by the surface tension of the water. The forces upon the probe are gravity and surface tension acting downwards and buoyancy due to displacement of water acting upwards.

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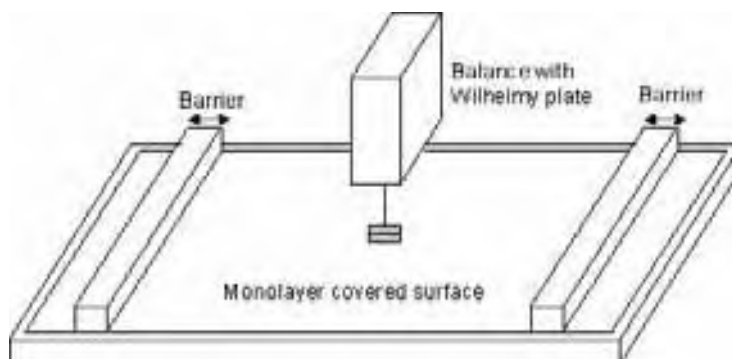

Figure 2.8. Schematic representation of a Langmuir film balance [KSV].

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