Atomic Force Microscopy on biomolecular building blocks: protein channels, peptides and vesicles

by

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Abstract

The Atomic Force Microscope (AFM) is an instrument which offers the opportunity to enter the nanometer-scale world of biophysics and nanotechnology. Within this thesis, the AFM is used to investigate and characterise polymer supported liposomes and engineered channel proteins for their applicability in nanocompartment systems as well as $\beta$-sheet forming polypeptides with respect to their possible application in nanoelectronics.

The molecular systems of interest are polymer supported liposomes, which we characterise, as well as the transmembrane protein FhuA. Therefore, a protein purification process is established and protein crystallisation experiments are performed. Additionally, high resolution measurements on the test system bacteriorhodopsin are carried out in order to optimise imaging conditions. Furthermore, we investigate different types of artificial polypeptides which self-assemble into $\beta$-sheets. The resulting $\beta$-sheets are characterised and tested for their suitability as building blocks for nanoelectronic purposes.

For these different research foci, suitable sample preparation methods are developed and the measurement settings are optimised. The quality of the results is directly influenced by the choice of substrate, the immobilisation method, the measurement conditions (air, solution, buffer composition) and the measurement modus (tapping mode, contact mode) as well as by the nature of the investigated object itself.
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CHAPTER
ONE

Introduction

In December 1959, Richard Feynman gave a talk at the annual meeting of the American Physical Society at the California Institute of Technology with the title "There's plenty of room at the bottom" [46]. Herein, he discussed the possibilities and opportunities which science might offer in the nanometer scale. With his ideas he inspired the scientific community at that time and also nowadays. He initiated the development of tools which allow working in the nanometer scale, asking for novel approaches to build these tools and ideas for their possible use. Especially in the last 15 years, this field of research developed very fast in several directions, for example towards improvement of medical devices, development of future computers as well as increase of knowledge and manipulation of the building blocks of life.

There are two different approaches to achieve the goal of nanometer-sized devices. The so called *top-down approach* shrinks already existing components. This approach is used for miniaturisation and improvement of electronic devices. Up to now, this method reveals the expected results, but, according to Moore's law [76], this development will reach physical limits within the next decade. Therefore, science asks for the development of new designs and materials, which is the starting point for the so called *bottom-up approach*. Here, nature's technology is used to design novel devices and finally modifications are introduced in order to optimise these devices. Examples are molecular transport devices driven by kinesin motor proteins or the use of transmembrane proteins
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as channels in nanocompartment systems for drug delivery. This approach is in general based on molecular self-assembly or molecular recognition of the used building blocks [118, 108].

Within this work we focus on the investigation of nanocontainers which offer a wide range of applications and on self-assembling polypeptides which can be used for nano-electronic purposes.

Nanocontainers are hollow spheres which are build up either from liposomes [50], polymers [88] or single molecules [68]. In these spheres different types of transmembrane proteins are inserted which serve as channels e.g. OmpF [50], FhuA [86]. It is possible to encapsulate proteins, enzymes or drugs within the nanocontainers and thus they can be used for drug delivery [24], medical diagnostics and as intracellular reporters as well as defined reaction space or cargo carrier. Because of their obvious application nanocontainers are promising objects. Their physical properties must be improved in order to fulfil certain requirements such as biocompatibility, addressing, volume, channel size. One crucial aspect of nanocontainers is their stability and especially liposome-based nanocontainers need to be fortified. The optimisation of liposome fortification is one of our research interests. We follow the approach of induced polymerisation within the hydrophobic interior of the lipid vesicles. Here, the polymer acts as a scaffold which stabilises the membrane while the lipid molecules can move freely. The goal of these investigations is to find the optimal experimental settings, e.g. ratio of mono- and bifunctional polymers, ratio of lipid to monomers in order to achieve a homogeneous polymer sphere. The atomic force microscope (AFM) provides us with the three-dimensional structure of the liposomes prior and after polymerisation. For these products, a statistical data analysis can be performed and then these data can be compared with the results of other techniques. Another important aspect of the nanocontainers are the proteins which are used as channels for the passage of specific target molecules. The proteins' stability and transport properties are also crucial for a successful implementation of this universal carrier system. So different types of proteins are engineered and investigated for their functionality. We are focussing on the optimisation of the gating behaviour of the transmembrane protein FhuA. In [86], comparative studies on different protein mutants are carried out. It is our aim to characterise the functional wild type protein.
and the designed mutants with the AFM, since the use of this method allows to directly monitor the influence of changing outer conditions to the protein structure. With respect to the bottom-up approach we are interested in using self-assembling materials for nanoelectronic purposes in order to circumvent Moore's law. We investigate β-sheets which could be used as conducting nanowires. These β-sheets consist of several polypeptide strands which self-assemble into this structure. The polypeptides can be composed of a large variety of amino acids and therefore it is possible to adjust the sequence to the given requirements i.e. charge of substrate and peptide, thiol bond to gold surface. Our aim is to find polypeptide sequences which are suitable for the use as nanowires, and to characterise the resulting β-sheets.

The instrument which allows to investigate such different research objects is the AFM, which was developed by G. Binnig, C.F. Quate and C. Gerber in 1986 [16]. With this instrument it is possible to resolve shape, structural details and interactions of single molecules under natural conditions. Furthermore, material differences can be detected and the direct manipulation of single molecules is possible. The AFM data can be complemented with results of other investigation methods e.g. transmission electron microscopy, dynamic light scattering and thus it is possible to fully characterise the research objects.

These promising research interests can be realised at Jacobs University Bremen as collaboration of three groups combining the expertise of biophysicists and biochemical engineers. The biochemical engineering deals with the protein production and the design of protein mutants, whereas the biophysical aspects are related to the investigation method and to the accessibility of the protein with the chosen method.

Our aim with this work is to contribute to the advance of the bottom-up approach. We are interested in the characterisation of different types of building blocks, the investigation of their assembly and in controlling the intrinsic functionality as well as the directed improvement of this functionality. Furthermore, this also allows to access details about the structure-function relationship of these building blocks which will additionally provide insights into the behaviour and understanding of other building blocks. We are interested in establishing a robust protocol which allows us to prepare transmembrane proteins in such a way that reproducible conditions suitable for AFM investigations are
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achieved. Furthermore, we want to support results of complementary measurements for the polymer stabilised liposomes and it is also our goal to provide sample details which are not accessible with other methods. Finally, we are pursuing the characterisation and the gain of new insights of the artificial $\beta$-sheets for their application within a nanoelectronic system.

In the following chapters a general introduction into the different research objects is given (see chapter 2) as well as more detailed introduction into the Atomic Force Microscope (see chapter 3) and further applied methods and instruments (see chapter 5). Furthermore, a short overview about the possible sample preparation methods which are considered to access the channel protein with the AFM will be given (see chapter 4). In chapter 6 the results are presented and discussed. Additional information on the used materials and protocols will be given in the appendix.
CHAPTER
TWO

Molecular systems of interest

As already mentioned our research interest is focussed on the different components of nanocontainers and the possible building blocks for nanoelectronic purposes.

Here, a short introduction into the polymer supported liposomes is given [35] as well as some basic information about amino acids and peptides. Furthermore, the transmembrane proteins bacteriorhodopsin and FhuA are presented in combination with an introduction into lipid bilayers.

2.1 Nanocontainers from polymer supported liposomes

The so called nanocontainers are objects of increasing interest, because of their possible application in drug delivery, medical diagnostics or their use as intracellular reporters. Nanocontainers are hollow nanometer-sized objects in which proteins, enzymes or drugs can be encapsulated. They offer the possibility to provide an optimised microenvironment for specific objects different from the outer surrounding [53] and thus protect the interior against chemical and biological degradation. It is also possible to address specific cells and then release the content in a controlled manner.

Liposomes, which are made of natural or synthetic lipids, are one example of such
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nanocontainers. They are usually composed of phospholipids or surfactants and form closed vesicle-like aggregates. Due to energetic reasons, the organisation as closed vesicles is favoured compared to any open structure. Advantages of liposome-based nanocontainers are their ability to carry hydrophilic as well as lipophilic particles, the easy adjustment of their size and their biocompatibility. Unfortunately, liposomes are quite sensitive to the outer environment e. g. pH, osmotic stress, lipases and detergents and thus their use as nanocontainers is limited. For this reason, different approaches have already been tested to fortify the liposomes; e. g. the use of surfactants which could be polymerised or the incorporation of polymers during vesicle formation [101, 96, 71, 70, 60, 59, 52, 38, 33, 23].

In polymer chemistry, the polymerisation in surfactant phases using a template is a useful method to achieve ordered nanostructured materials. Using this method it is possible to turn a dynamic self-assembling system into a material which is mechanically and chemically stable. When the so-called direct templating is used, the morphology of the polymerised product will represent the structure of the template [53, 52, 57]. Using these polymerisation reactions is a relatively simple approach to achieve ordered phases; a lyotropic liquid crystalline phase is used as template and loaded with monomer molecules, after initialising the polymerisation reaction a polymer is achieved which ideally preserves and stabilises the structure of the template [53].

In the cases that liposomes are used as polymerisation template, it is possible to dissolve hydrophobic monomers in the hydrophobic part of the lipid bilayer. Then, the formation of the polymer network could be achieved with the help of a radicalic polymerisation process which can be induced by UV light radiation as sketched in fig. 2.1. Using intense UV light offers the advantage of a high polymerisation rate and thus the polymerisation process is finished within a fraction of a second. To achieve a hollow polymer sphere, it is necessary that mono- and bifunctional monomers are distributed homogeneously within the lipid bilayer. If the distribution of the monomers is not homogeneous or an excess of monofunctional monomers is present, a closed polymer sphere cannot be achieved. The polymer network inside the liposome bilayer should allow the lateral movement of the lipids as well as diffusion of low molecular weight substances through the bilayer. Furthermore, the functionality of membrane proteins
should be preserved and also leakage should be reduced.

![Diagram](image)

**Fig. 2.1:** Formation of a nanocontainer by induced polymerisation of monomers within a liposome template [35].

The aim of these investigations is to define the crucial parameters which are necessary to obtain two-dimensional polymerisation of the hydrophobic monofunctional butyl methacrylate or hydroxyethyl methacrylate with the bifunctional ethyleneglycol dimethacrylate within the template liposome bilayer. The factors which are important in order to achieve stable nanometer-sized capsules are: the point of monomer addition, the molar ratio of lipid to monomer, the overall concentrations, and the intensity of UV radiation. Therefore, formation of polymer spheres is controlled at different experimental stages.

The AFM provides the possibility to access the three-dimensional structure of these liposomes before and after polymerisation. These data can be used to perform a statistical data analysis and finally the results can be compared to measurement results of complementary characterisation techniques such as dynamic light scattering and transmission electron microscopy.

### 2.2 Self-assembling polypeptides

For the design of new materials and devices, it seems to be a promising approach to use self-assembling monomeric molecular units such as peptides [85, 94, 22, 40, 63, 112, 118, 119, 43]. Depending on the amino acid sequence, peptides self-assemble into \( \beta \)-sheet structures. Peptides possess a protein-like response and in an artificially designed nanostructure they can provide control over the macroscopic properties and the func-
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It is possible to adjust the peptides to certain requirements by either choosing suitable ones from a large number of natural peptides or designing new artificial peptides. In addition to possible applications in nanotechnology, β-sheet structures also play an important role in amyloid-related diseases and thus a better understanding of the structure-related behaviour might help curing these diseases [44, 102].

Peptides and proteins are mainly build up from 20 different standard amino acids which offers a large number of possible combinations. All amino acids contain an α-amino group (-NH₂) and an α-carboxyl group (-COOH) as well as a side chain R connected to this α carbon. Thus amino acids are chiral molecules whereas the naturally existing ones are all L-enantiomers. According to the composition of the side group (regarding the polarity and charge at pH 7) the amino acids are devided into different groups.

- Non-polar, aliphatic side chains: Glycine (G), alanine (A), proline (P), valine (V), leucine (L), isoleucine (I) and methionine (M). The side chains of alanine, valine, leucine and isoleucine stabilise the protein structure via hydrophobic interaction. Glycine does not really contribute to hydrophobic interactions.

- Aromatic side chains: Phenylalanine (F), tyrosine (Y) and Tryptophan (W). Side groups are quite polar and participate in hydrophobic interactions. These amino acids all absorb UV light (λ ~280 nm) which is used to determine sample concentrations within solutions.

- Polar, uncharged side chains: Serine (S), Threonine (T), Cysteine (C), Asparagine (N) and Glutamine (Q). These amino acids are soluble in water, since they contain functional groups that can form hydrogen bonds. Cysteines form covalent disulfide bonds with each other and thereby stabilise the existing structure.

- Negatively charged side chains: Aspartate (D) and glutamate (E). Soluble in water, possess second carboxyl group which is negatively charged at pH 7. Stabilising, since charge favours hydrogen bonds, additionally coulombic attraction. In the case of coulombic repulsion another stable conformation will be achieved.

- Positively charged side chains: Lysine (K), arginine (R) and histidine (H). Charge favours interaction via hydrogen bonds and coulombic attraction.
In peptides and proteins the single amino acids are connected and form a so called peptide bond. These bonds result from a condensation reaction between the carboxyl group of one amino acid and the amine group of the neighbouring amino acid. Due to the relative distribution of the electron wave function in between the carboxyl oxygen and the amid nitrogen the peptide bond is rigid and planar. This influences directly the secondary structure of peptides.

The sequence of amino acids in such polypeptides is called **primary structure**. The **secondary structure** describes the conformation of certain parts of the polypeptide backbone. The possible structures are limited, due to the properties of the peptide bond. The most stable conformations of the possible secondary structures are the $\alpha$-helix and the $\beta$-sheet and thus occur in many proteins. The **tertiary structure** defines the completely folded polypeptide which is a subunit of the **quaternary structure**.

The structures of an $\alpha$-helix and $\beta$-sheets are shown in fig. 2.2.

![Fig. 2.2](image_url)

As indicated in the figures these secondary structures are stabilised via hydrogen bonds. In an $\alpha$-helix structure this are intramolecular hydrogen bonds which are formed in between the carbonyl oxygen and the amid hydrogen of different amino acids which are neighbouring after a full turn of the backbone. The turning direction of the $\alpha$-helix usually is right-handed – observation direction: from amino terminus towards carboxyl terminus. Additionally the interactions between the amino acid side chains can influence the stability of the helix. Within the $\beta$-sheet structure, the hydrogen bonds may occur
between several parts of one or even between different sequences which need to arrange next to each other. These sequence parts must have the same relative orientation so that bonds can form between the neighbouring carbonyl oxygen and the amid hydrogen. Like this it is possible that sequences which have the same or even the opposite direction of amino terminus towards carboxyl terminus form either parallel or antiparallel sheet structures.

The arrangement of the peptide monomers into tapes, ribbons, fibrils and fibers depends on the peptide concentration [7, 91, 117]. Depending on the concentration of $\beta$-sheets in solution different types of superior structures are formed. These were modeled by [9] and the sketches are shown in fig. 2.3. Here, rod-like monomers (a) self-assemble into long twisted tapes (b). The tape possesses distinct faces when the upper and lower part of the monomer are chemically different. Due to the peptide configuration and the peptide-solvent interactions the resulting tape is curled into a helical configuration. With higher peptide concentration and intertwine attraction ribbons (c) are formed. The stacking of ribbons results in the formation of fibrils (d) and finally fibres (e). The starting point of our investigation is a peptide sequence published by Aggel et al. [9] which self-assembles into antiparallel $\beta$-sheets. The sequence of this 11mer polypeptide is

$$\text{CH}_3\text{CO-QQRQQQQQQQQQ-NH}_2$$
and with the program Rasmol [104] its structure can be modeled. Fig. 2.4(a) shows the structure of a single polypeptide while fig. 2.4(b) shows the arrangement of the resulting $\beta$-sheet.

Fig. 2.4: (a) Space-filling model of the used 11mer polypeptide [104]; (b) Arrangement of 11mer polypeptide into $\beta$-sheet [47].

The polypeptide sequence is set-up from Q for glutamine, R for arginine and E for glutamate as mentioned above.

The $\beta$-sheets formed by this polypeptide possess hydrophilic character on both sides of the tape, because of the polar glutamine side chains [47] which allow to form hydrogen bonds. Since the side chains of arginine and glutamate are oppositely charged, they also increase the hydrophilicity and additionally favour the self-assembly into antiparallel oriented $\beta$-sheets. Due to the chirality of the single polypeptide strands their assembly into a $\beta$-sheet tape shows a left-handed twist as sketched in fig. 2.4(b). The helical pitch of this tape was determined experimentally to $h_{tape} \sim (30 \pm 15)$ nm.

With respect to a possible application of the $\beta$-sheets in the field of nanoelectronics it is of interest to achieve long $\beta$-sheet strands (in the micrometer range) and to control these comparable to the manipulation of DNA, proteins or nanoparticles [120]. The $\beta$-sheets could serve as wires [39] or as templates for metallisation [72]. Then they could be used to connect electrodes on a standard microfabricated system [65, 78, 11].

Therefore, our first modification approach of the basic 11mer polypeptide is to connect two polypeptides using a hairpin sequence (GD) which should additionally support the arrangement as antiparallel $\beta$-sheet. The sequence of the created 24mer polypeptide is:
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\[ CH_3CO-QQRQQQQOEQQ-GD-QQRQQQQEQQQ-NH_2 \]

With G for glycine, D for aspartate and the other amino acids as mentioned above.

In a second modification 11mer polypeptides are connected via two hairpin loops. The sequence of the resulting 37mer polypeptide is:

\[ CH_3CO-QQRQQQQOEQQ-GD-QQRQQQQEQQQ-PG-QQRQQQQEQQQ-NH_2 \]

With all amino acids as given above.

Furthermore, it seemed favourable to modify the basic peptide sequence in such a way that amino acids which possess a higher number of "free" electrons are inserted into the sequence which could facilitate the conductivity through that \( \beta \)-sheet. Therefore, a new 11mer polypeptide and also a 15mer polypeptide were designed both containing the aromatic amino acid phenylalanine (F). The sequence of the 11mer polypeptide is:

\[ CH_3CO-QQRQQQFQQEQQQ-NH_2 \]

Its structure can be modeled using the program Rasmol and is shown in fig. 2.5.

![Space-filling model of the designed 11mer polypeptide.](image)

**Fig. 2.5:** Space-filling model of the designed 11mer polypeptide.

The sequence of the 15mer polypeptide is similar to the sequence of the 11mer but with additional glutamine residues at the ends. This design was chosen in order to find out if the length of the peptide chain influences the length of the resulting \( \beta \)-sheets. The sequence of the 15mer polypeptide is:

\[ CH_3CO-QQQQRQQFQQEQQQQ-NH_2 \]
A space-filling model of this sequence designed with the program Rasmol is shown in fig. 2.6. In the following these polypeptides will be named according to their sequences 11mer, 24mer, 37mer, 11fmer and 15fmer.

The polypeptides are synthesised upon request by the company Biosyant GmbH, Berlin, Germany. The sample purity is controlled by HPLC. The purity of the different polypeptides is always >90% except for the 37mer which is >80%. The peptides are provided as lyophilised powder which is used to prepare a polypeptide stock solution in deionised water.

The use of the AFM allows to monitor the accessible parameters such as helical pitch, width and height of the $\beta$-sheets. Using molecular dynamics simulations (for details see chapter 5.3), it is possible to model the behaviour of short $\beta$-sheets (consisting of for example twelve polypeptide strands) and thus to confirm or to question the results of the AFM measurements.

### 2.3 Transmembrane Proteins

With respect to the application of transmembrane proteins in the nanocontainers, it is of interest to optimise their performance as universal channels. Since the protein FhuA is among others a promising candidate for this application, it is engineered in order to learn about its gating behaviour and to improve its activity as non-selective channel. Additionally, the two-dimensional protein array bacteriorhodopsin is investigated, since this system is well characterised by high-resolution atomic force microscopy and thus
allows to gain experience in handling and adjusting the parameters which influence the measurements.

2.3.1 Basics on membranes

Membranes form the boundary of cells and regulate the interaction with the environment i.e. the traffic of molecules into and out of the cell. The membrane composition differs for different types of organisms and is also depending to the designated function. Some general properties of membranes are e.g. their impermeability to most of polar or charged solutes, their insulating character and their thickness of approximately 5 nm to 8 nm. The structure of the membrane can be described by the fluid mosaic model which is sketched in fig. 2.7. The membrane is build by a lipid bilayer with incorporated

![Diagram of membrane structure](image)

Fig. 2.7: Model of the membrane structure [89].

proteins, sterols and associated lipopolysaccharides. Each lipid consists of a polar head group and a fatty acid chain. While the head groups are hydrophilic, the side chains show hydrophobic behaviour. Due to energetic reasons, lipids form micelles in aqueous solutions, bilayers or liposomes as sketched in fig.2.8. The resulting structure depends on the diameter ratio between the head group and the side chain as well as on the total lipid concentration in the solution.

The lipid bilayer provides an environment which combines a hydrophilic surface with
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![Diagram](image)

Fig. 2.8: (a) Lipid micelle; (b) Lipid bilayer; (c) Liposome [89].

a hydrophobic core. According to the properties of their tertiary structure proteins are either attached (peripheral protein) to the lipid bilayer or inserted (integral protein) into it. Most of the membrane molecules can move freely within the membrane, because the prevailing interactions are of hydrophilic and hydrophobic nature instead of chemical bonds.

The proteins which are present in the membrane can be divided into different groups. There are receptor proteins, recognition proteins and transport proteins, whereas the latter ones distinguish carrier proteins and channel proteins. Molecules can be transported through the membrane either passively or actively. The passive transport moves molecules along a concentration gradient and does not require energy. While the active transport requires energy, since molecules are moved against an outer concentration gradient.

In general, proteins can be extracted from the membrane with the help of detergents whereas it is also possible to solubilise a complete membrane. Detergents are molecules which possess amphiphatic character and thus are able to mediate between hydrophilic and hydrophobic molecules. There is a large variety of detergents which suit different requirements, i.e. anionic, cationic, zwitterionic and non-ionic detergents. The detergent molecules also form micelles in solution and thus offer a similar environment as the lipid bilayer. At detergent concentrations below the critical micellar concentration (cmc) of a specific detergent the detergent molecules interact with the environment and thus insert into the lipid bilayer. With increasing concentrations the bilayer becomes saturated and at concentrations above the cmc detergent micelles are formed in which lipids and
proteins can be integrated. It is possible to separate these different types of micelles using differential centrifugation.

2.3.2 Bacteriorhodopsin

Bacteriorhodopsin (BR) is a transmembrane protein which is a photosynthetic pigment and acts as proton pump. It is the simplest known light-driven proton pump. It transports protons across the membrane out of the cell against an outer gradient. The necessary energy is provided by the light which illuminates the protein. Finally, the achieved proton gradient is transferred into chemical energy.

BR consists of 247 amino acids and its molecular weight is 26 kDa. The polypeptide chain is folded into seven hydrophobic α-helices which are connected via nonhelical loops. To each chain one molecule of retinal is connected. A pathway for the proton movement is formed by the relative orientation of the α-helices to each other and their arrangement within the membrane. Excited by light the retinal molecule changes its conformation and thus induces a conformational change in the surrounding protein. This finally results in the proton pump action. Fig. 2.9 shows a model of the BR structure prepared using the protein data bank-file (pdb code 1JGJ) [14, 6] and the program Rasmol.

![Fig. 2.9: Structural models of bacteriorhodopsin; (a) side view; (b) top view.](image)

BR naturally occurs in large two-dimensional crystalline patches the so called purple membrane. Within this membrane BR is arranged in a hexagonal lattice with a unit
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cell size of 6.2 nm [25, 84]. The lattice is composed of three identical protein chains each
rotated for 120° relative to the others. The BR molecule is purple and most efficient
in the absorption of green light (λ=500 nm - 650 nm) with an maximum at 568 nm. The
wild-type bacteriorhodopsin is usually isolated from *Halobacterium salinarum* strain S9.

Since the purple membrane provides optimal conditions for atomic force microscopy
measurements the protein Bacteriorhodopsin is well characterised by high resolution
measurements with the AFM [84, 83, 82, 48]. It is therefore an ideal test system.

2.3.3 FhuA

The protein FhuA (ferric hydroxamate uptake protein component A) is an active and
selective transporter for the ferrichrome-iron complex across the outer membrane of
gram-negative bacteria. It also transports the structurally related antibiotic albomycin
as well as the unrelated antibiotic rifamycin CGP 4832. Additionally, it acts as a receptor
for bacteriophages (T1, T5, φ80, UC-1), for the bacterial toxin colicin M and the peptide
antibiotic microcin 25 [45].

FhuA belongs to the family of porins which are β-barrel forming proteins such as
OmpF. At the COOH-terminus a barrel domain is formed by 22 antiparallel β-sheet
strands (residues 161-723) while at the NH2-terminus a cork domain (residues 1-160) is
located. The β-sheets of the barrel are connected via short periplasmic turns and longer
surface-located loops. The cork domain consists of a four-stranded β-sheet and four
short helices. They are connected to the barrel and to loops via hydrogen bonds and
are involved in ligand binding. The β-sheet plane is tilted for ~ 45° to the membrane
normal and thus the cork domain nearly blocks the inner cross-section. Fig. 2.10 shows
a structural model of the protein prepared using the protein data bank-file (pdb code
1BY3) and the program Rasmol.

The molar mass of FhuA is 78.9 kDa [34]. It possesses an elliptical cross-section of
3.9 nm × 4.6 nm and a height of 6.9 nm [45]. FhuA is a very large and robust channel. It
is stable at higher temperatures up to 65°C [18]. As other porins, it can resist chaotropic
salts, detergents and proteolysis. Unlike other porins FhuA exists in monomeric form
instead of assembling to trimers.

When FhuA is reconstituted into planar lipid bilayers it does not show channel conduc-
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Fig. 2.10: Structural models of FhuA wild-type; (a) side view; (b) top view.

tance [58, 19]. For the conversion into a passive diffusion channel several protein mutants have been created in which parts of the protein were deleted e.g. loop: FhuA Δ322-355 [58] or cork domain: FhuA Δ5-160, FhuA Δ5-160Δ322-336, Δ5-160Δ335-355 [21, 113], FhuA Δ1-160, FhuA Δ1-129 [86]. In combination with TonB FhuA Δ5-160 seems to be a functional channel with an increased permeability [21]. Whereas the isolated mutant FhuA Δ5-160 does not form stable channels within a planar lipid bilayer. The mutants FhuA Δ5-160Δ322-336 and FhuA Δ5-160Δ335-355 show an increased permeability, but no FhuA specific functions which might be related to an altered conformation [113]. Contrary to these results the mutants FhuA Δ1-160, FhuA Δ1-129 seem to be functional [86] in a polymer membrane [87] although they did not form stable channels within planar lipid bilayers [92].

Up to now the structure of FhuA wild type has been characterised by crystallographic methods [45, 69] while its gating behaviour has been studied by electrophysiological measurements [58, 19] and transmission electron microscope investigations on proteoliposomes [95, 62] and two-dimensional protein crystals [61].

Under optimal imaging conditions the AFM allows to perform high resolution measurements on several transmembrane proteins [84, 83, 74, 15]. Furthermore, the AFM offers the possibility to easily modify the environmental properties of the set-up and thus to investigate the response of the protein channel under changed conditions. Addi-
tionally, the AFM can be used to perform force spectroscopy measurements (details see section 3.1.4). Here the AFM tip is functionalised with specific molecules and so it is possible to investigate the occurring forces between the probe molecule and the protein channel. Offering such unique possibilities we are interested in characterising the protein FhuA using Atomic Force Microscopy.
2 Molecular systems of interest
Atomic Force Microscopy

The atomic force microscope (AFM) is the main instrument used in this work. The focus is set on finding the ideal sample preparation for the different types of objects as well as optimising the AFM measurement settings.

The Atomic Force Microscope is a versatile tool to examine and to manipulate samples in the nanometer scale. Imaging biomolecules with the AFM is one of the most often used applications which allows to resolve the three-dimensional surface of the specimen under natural conditions [42]. Depending on the sample composition and the goal of the investigation, the AFM offers several working modes in order to provide the most suitable measurement conditions for the samples. The mainly used ones are contact mode, tapping mode and force spectroscopy mode. The latter one allows to investigate the interaction forces between single molecules or forces within complex molecules [36, 49]. For the most investigations it is important to use an atomically flat sample substrate and additionally it is helpful to immobilise the biomolecules on the substrate. In this chapter basic information about the AFM set-up, different measurement modes and the used sample preparation will be given.
3 Atomic Force Microscopy

3.1 AFM basics

The atomic force microscope was developed in 1986 by G. Binnig, C.F. Quate and C. Gerber [16] based on their invention of the scanning tunneling microscope [17] in 1983. The functional principle of the AFM is based upon atomic interactions between a probe and a surface. This interaction only takes place in a close proximity, i.e. a few nanometers. The AFM probe is mounted on a small cantilever which is scanned over the specimen surface. The force between tip and sample causes the cantilever to bend. This force can be attractive or repulsive. One method to detect the bending of the cantilever is to observe the deflection of a laser beam focused on the backside of the cantilever. The deflected beam is detected with a position sensitive photo diode. While scanning the sample, the interaction forces change, due to the locally different surface structure and so the cantilever deflection changes. This change is recorded and serves as feedback signal for a piezoelectric actuator which is used to adjust the tip-sample distance in order to maintain the specific measurement conditions. Using a constant interaction force as parameter for the measurement recording allows to obtain a "constant force" map of the specimen surface. In the case a homogeneous sample substrate is used this constant force map corresponds to the real topography of the specimen. A piezoelectric actuator tube is used for the fine adjustment of the relative position between probe and sample, since it allows smallest movement steps in x-, y- and z-direction. The movement of the piezoelectric actuator is controlled by a computer using the feedback signal from the position sensor.

3.1.1 AFM set-up

Fig. 3.1(a) shows the general set-up of an atomic force microscope. The prepared specimen is mounted on the piezoelectric actuator. The cantilever is positioned above the sample and scanned over the surface. Then the laser beam which is focussed on the cantilever backside and reflected onto the position sensitive photodiode. In fig. 3.1(b) the AFM used in this work is shown.

The microscope used is a MultiMode Atomic Force Microscope with a Nanoscope IIIa controller (Veeco Instruments GmbH, Germany) equipped with a J-scanner which
3.1 AFM basics

Fig. 3.1: (a) General set-up of an AFM [90]; (b) Currently used AFM [2].

offers a maximum scan size of 125 μm × 125 μm and 5 μm vertical range. For imaging
the typically used scan sizes range from 50 nm × 50 nm to 50 μm × 50 μm with a x-y-
resolution of 1 nm and a z-resolution of 0.1 nm using 512 pixels per image. The recording
time for one image varies from 5 to 10 minutes. For force spectroscopy measurements a
MultiMode Picoforce System is combined with the standard AFM set-up.

The cantilever probes are kept in measuring position using a cantilever holder in
which they are fixed with a spring. The cantilever holders have different designs for
measurements in air and in solution. For measurements in air, the sample is not covered
and the path for the laser beam is kept clear. For the measurements in solution the 'fluid
cell' made of glass is used as cantilever holder. The sample is covered with solution and
in order to provide a defined path for the laser beam the plain glass surface of the liquid
cell is also in contact to the liquid. Additionally, this design minimises the surface of
the liquid and thus reduces the evaporation of the solution. It is also possible to close
this set-up with an O-ring and so solutions can be exchanged without unmounting the
sample. This allows to investigate the influence of changing conditions on specific areas
of the specimen. For the used type of AFM the typical sample size is limited by the size
of the sample support and should not exceed a diameter of 15 mm and 8 mm height.
3.1.2 AFM probes

The AFM probes are usually manufactured using well established microfabrication techniques from semiconductor industries. Like this, it is easily possible to prepare a large amount of cantilevers even with different designs in a high and reproducible quality. Since the probes are an essential part of the microscope, it is important that probes with different properties are available. Hence, a cantilever can be chosen according to the requirements for the imaging mode which seems to be suitable for the specific sample. The probes usually vary in size, tip shape and the used material. An important detail is the achievable resolution which depends on the shape and the sharpness of the probe tip. Depending on the aspect ratio of the used tip details of investigated objects are visible or not. Fig. 3.2 shows examples of different AFM probes; (a) triangular or V-shaped silicon nitride cantilevers with mounted tip; (b) rectangular or beam cantilever made from silicon with tip.

Fig. 3.2: Different AFM probes; (a) Silicon nitride cantilever; (b) Silicon cantilever (from [1]).

For beam cantilevers the properties such as spring constant $k_{spring}$ and resonance frequency $f_{res}$ can be determined from the cantilever geometry.

$$k_{spring} = \frac{E}{4} \cdot \frac{w l^3}{t^3} ; f_{res} = 0.162 \sqrt{\frac{E}{\rho} \cdot \frac{t}{l^2}}$$

With $E$ and $\rho$ being Young’s modulus and the density of the cantilever material respectively and $w, t, l$ being width, thickness and length of the cantilever. For commercially available cantilevers, these data are given by the manufacturer, but then an error of
\( \approx 10\% \) should be considered. It is also possible to determine the spring constants experimentally as discussed in [32, 109, 29]. This is necessary for example in the case of force spectroscopy measurements when interaction forces are investigated.

**Silicon nitride probes**

For contact mode measurements, triangular cantilevers from silicon nitride (\( \text{Si}_3\text{N}_4 \)) are preferred, because they possess a better mechanical stability with respect to torsional forces which can occur during the scanning process. Additionally, these cantilevers provide a high flexibility which allows scanning with minimal forces exerted by the tip. The cantilever backside is coated with gold to increase the reflectivity for the laser beam. There are also several cantilevers available on one chip (as it can be seen in fig. 3.2 (a)) which allows a simple exchange of the scanning tip if necessary. It is also possible to use these cantilevers for tapping mode measurements, but they are not optimal for this purpose.

The standard cantilevers we use are **MSCT probes** provided by Veeco Probes and the **OMCL-TR400PSA**- from Olympus. The respective cantilever properties of these silicon nitride cantilevers are given in tab.3.1. Wherein \( f_{\text{res}} \) is the resonance frequency, \( k_{\text{spring}} \) the spring constant, \( r_{\text{tip}} \) the nominal tip radius and \( h_{\text{tip}} \) the tip height.

<table>
<thead>
<tr>
<th>type</th>
<th>thickness / ( \mu\text{m} )</th>
<th>width / ( \mu\text{m} )</th>
<th>length / ( \mu\text{m} )</th>
<th>( f_{\text{res}} ) / kHz</th>
<th>( k_{\text{spring}} ) / N/m</th>
<th>( r_{\text{tip}} ) / nm</th>
<th>( h_{\text{tip}} ) / ( \mu\text{m} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSCT</td>
<td>0.6</td>
<td>18/22</td>
<td>85-320</td>
<td>7-120</td>
<td>0.01-0.05</td>
<td>10</td>
<td>2.5-3.5</td>
</tr>
<tr>
<td>OMCL-TR400PSA</td>
<td>0.4</td>
<td>13.4/27.9</td>
<td>100/200</td>
<td>34/11</td>
<td>0.08/0.02</td>
<td>20</td>
<td>2.9</td>
</tr>
</tbody>
</table>

**Silicon probes**

Silicon probes are ideal for the performance of tapping mode measurements in air. These cantilevers are relatively stiff and possess a high resonance frequency. They are usually made from crystal silicon and covered with a layer of silicon oxide.

The RTESP cantilevers, purchased from Veeco probes, are made from phosphorous doped silicon and their specifications are given in tab.3.2.
Tab. 3.2: Physical properties of the used silicon probes.

<table>
<thead>
<tr>
<th>type</th>
<th>thickness / μm</th>
<th>width / μm</th>
<th>length / μm</th>
<th>$f_{res}$ / kHz</th>
<th>$k_{spring}$ / N/m</th>
<th>$r_{tip}$ / nm</th>
<th>$h_{tip}$ / μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTESP</td>
<td>4</td>
<td>35</td>
<td>125</td>
<td>300±100</td>
<td>40 (20-80)</td>
<td>&lt;10</td>
<td>15-20</td>
</tr>
</tbody>
</table>

3.1.3 Interaction forces

Since the AFM cantilever is used to monitor the net interaction force between the specimen and the scanning probe it is important to know which forces contribute to this net force. Therefore, different origins of forces have to be considered [73].

The short-range forces which occur between neutral atoms at distances < 1 nm can be either repulsive or attractive forces. They are due to the overlap of the atoms' electron wave functions and the repulsion of the ion cores. In the case, that the overlap of the electron wave functions minimises the total energy of this system the resulting force is attractive - comparable to the chemical bond. If a strong overlap of the wave functions is present the resulting force is repulsive because of the Pauli exclusion principle. Additionally, at these small distances the positively charged ion cores repel each other, since the shielding by the electrons is missing. For a quantitative description, the Lennard-Jones potential or the Morse potential can be used, but their validity is limited to pairwise interaction. Therefore, the interaction with the neighbouring atoms and their displacement, due to the interaction needs to be considered as well.

The van der Waals force is an attractive force between neutral atoms or molecules which is based on induced dipoles and their interaction. Due to fluctuations of the electron distribution in atoms, it is possible that short time dipoles are formed and polarise the neighbouring atoms. This finally leads to an attraction between the involved atoms or molecules. The range of the van der Waals force is limited to ~5-7 Å. This distance results from the transmission velocity (velocity of light for an electromagnetic wave) and the lifetime of the electric field fluctuations. Hence, at further distances the strength of the van der Waals force is reduced. This force can be calculated using two different approaches. It also has to be considered that the strength of this force is influenced by the surrounding medium which might cover tip and sample. One of the calculation approaches, the Lifshits theory, states that the van der Waals force is
3.1 AFM basics

depending on the dielectric constant $\epsilon$ and the refractive index $n$ of this medium. For measurements in water the $\epsilon$- and $n$-values of the interacting materials are quite similar and thus the van der Waals force is much smaller than in vacuum.

The electrostatic forces act between isolated charges and follow Coulomb's law. Such charges may come up during the sample preparation and can attract the conductive tip. This also applies for neutral, but polar sample surfaces. The force can be calculated using mirror charges in the tip or sample. In the case of interaction between conductive tips and conductive samples with both showing different potentials the force can be calculated as a capacitor showing a distance-dependent capacitance.

When performing measurements in ambient conditions water vapour is always present and may condense on top of the sample surface. Furthermore, AFM tips with a radius below 100 nm can act as condensation nuclei under such conditions. In the case a meniscus is formed, due to surface tension, additional capillary forces are acting on the tip. This force can be much stronger than the van der Waals force and cause a strong adhesion of the tip to the surface. Additionally, the capillary force can directly influence the size of the contact area and thus increase the force exerted during scanning between tip and sample. It is possible to reduce this force by modifying the hydrophilic properties of the surface. To completely overcome them, one has to totally immerse the sample and tip in a solution.

The occurring forces in solutions differ significantly from the forces which are present in the measurements performed in vacuum or air. In liquids the strength of the van der Waals force is reduced and the capillary force does not exist anymore. But then other types of forces such as solvation and additional electrostatic forces become apparent. These forces are due to the interaction between the immersed surface and the surrounding solution. It is possible that single atoms or functional groups are solubilised or dissociated from the surface. Even the adsorption of molecules to the surface is possible. These interactions finally cause the fact that all types of surfaces immersed in solution are charged. This charge is compensated by the arrangement of counter ions next to the surface. The layer of ions next to the surface is relatively stable and is called Helmholtz layer. In the closer surrounding another diffuse layer of counter ions is arranging and thus an electric double layer is formed which is sketched in fig. 3.3(a). The thickness of
this double layer is defined by the *Debye-length* $\lambda_D$. The Debye-length is the distance over which mobile charges can screen out the potential of an electric field. $\lambda_D$ is indirectly proportional to the molar ion concentration and so $\lambda_D$ can be decreased with increasing salt concentration. The DLVO (Derjaguin-Landau-Verwey-Overbeek) theory is used to describe the forces in aqueous solution semi-quantitatively. In this theoretic description only the van der Waals forces and the interactions of electrostatic double layers are considered.

In the case of the AFM probe scanning the surface two of these electric double layers overlap as indicated in fig. 3.3(b) and thus repel each other, due to the surface charges [26, 27, 28]. This double layer interaction limits the achievable resolution, but it can be optimised by adjusting the salt concentration as discussed in [81].

![Diagram](a) ![Diagram](b)

**Fig. 3.3:** Interactions of solids within solution; (a) Formation of an electric double layer; (b) Repulsion of two electric double layers (from [10]).

Additional to the formation of the Helmholtz layer, it has to be considered that thermic interactions between cantilever and surrounding solution occur which may cause a bending of the cantilever i.e. the so called thermal drift. This cantilever drift directly influences the exerted force during scanning [79] and therefore the scanning parameters have to be adjusted continuously.

### 3.1.4 AFM measurement modes

As already mentioned, the atomic force microscope can be operated in different measurement modes. These modes are based on the control of specific settings, i.e. constant
deflection, constant amplitude. In this section the mainly used measurement modes, i.e. contact mode, tapping mode, force spectroscopy mode will shortly be explained.

Contact mode

In this mode the measurements are performed in such a small distance between tip and sample that in principle the AFM tip is in continuous contact with the sample and thus directly following the surface contour. The cantilever is scanned over the sample surface at a constant surface potential. Changes in the surface potential exert a force which causes the cantilever to bend. Using this mode the bending of the cantilever is monitored and ought to be kept constant. In the case the cantilever deflection signal changes this change is monitored and used as feedback signal. This signal is sent to the piezo and the distance between probe and sample is adjusted in order to maintain the initial deflection set-point. The adjustment is recorded and used create the constant force map which is ideally identical with the surface structure of the specimen.

The contact mode is based on the repulsive short-range interaction forces between the probe and specimen atoms. When measurements are carried out in ambient conditions in air the influence of the capillary force has to be considered. In this case it is possible to adjust the set-points in order to minimise the exerted forces. For measurements in solution the interaction between solution, tip and specimen influences the results as stated above. Since here the buffer composition becomes important it is advantageous to immerse the cantilever in the respective measurement buffer and additionally this reduces thermal drift.

Advantages of the contact mode are for example that this measurement mode offers a very high image resolution and that especially hard samples can be easily investigated in air or solution or any other suitable conditions. One disadvantage is that depending on the applied force soft samples like biological samples may be modified or even destroyed during scanning. To prevent sample destruction and also to monitor the influence of the scanning force it is important to control the force which the tip exerts to the sample. With the used Si$_3$N$_4$ cantilevers the forces are ranging from nN to $\mu$N in solution. Details on the force control will be given in the force spectroscopy-section.
3 Atomic Force Microscopy

Tapping mode

When the AFM is operated in tapping mode in air the cantilever is oscillating at a frequency close to its resonance frequency with an amplitude ranging from 20 nm to 100 nm and is scanned over the sample surface. The cantilever oscillation is induced by a separate piezoelectric actuator. The scanning tip is located close to the sample surface and touches the sample frequently at the lower turning point of the oscillation. In this mode the amplitude is monitored and a certain set-point is maintained by fine adjustment with the main piezoelectric actuator. This guarantees a constant tip-sample interaction. The oscillation amplitude is set in such a way that the cantilever tip lightly touches the sample surface. Like this it is possible to reduce significantly the force exerted to the specimen by the scanning tip which finally prevents sample destruction.

In this mode different types of forces act on the probe during the oscillation. When the tip is touching the surface the atomic interaction is the same as in the contact mode and the tip feels a repulsive force. In the 'upper part' of the oscillation the tip is located within the attractive regime of the van der Waals force. Since the used cantilevers are relatively stiff and oscillating the capillary force is not strong enough to drag the tip down. When tapping mode measurements are done in solution the resonance frequency of the cantilever changes significantly, due to viscous damping and the increased effective mass. The choice of the right excitation frequency is tricky, since in solution several harmonics of the basic oscillation frequency [111] are present which all allow relatively stable or instable imaging conditions.

An advantage of the tapping mode is that soft biological samples are nearly not affected by the scanning process. Additionally, it is possible, with the help of the phase contrast image, to distinguish between different materials, due to their different phase response even if there are no topographical changes on the surface. A disadvantage may be the lower resolution compared to the contact mode measurements.

Force spectroscopy mode

Force spectroscopy measurements are usually carried out within solution in order to minimise the occurring forces as well as to provide a uniform environment. For the performance of force spectroscopy measurements the AFM tip and the sample surface
are brought into contact and then are separated again. With consecutive cycles of approaching and retracting the tip and the sample their local interaction forces are recorded with respect to the distance. Fig. 3.4 shows such a force-distance curve and how it is generated.

![Force Calibration Plot](image)

**Fig. 3.4:** Generation of a force-distance curve (from [4]).

When the tip is approaching the sample from a large distance there is no interaction. Thus the cantilever is not deflected and this defines the baseline of the force-distance curve (see "A"). With reducing distance the van der Waals interaction is becoming strong enough to attract and permanently bend the cantilever. At this distinct distance the tip 'jumps' into contact (see "B") and just reaches the sample surface. With further approach the cantilever reaches the repulsive contact regime (see "C") and the monitored cantilever bending is changing in accordance with the ongoing distance reduction. When the tip is retracted from the surface an adhesion force between probe and sample occurs (see "D"). The probe remains in contact with the sample surface, because of this force. Due to the strength of the adhesion force, tip and sample are finally separated at a distance which is larger than the 'jump into contact' distance (see "E").

The composition of sample, probe and the occurring interactions between probe, sample and surrounding solution strongly influences the resulting force distance curve [116]. Fig. 3.5 shows examples of force distance curves with different types of possible interac-
tions such as large and small adhesion, hard and soft samples, long-range repulsion and attraction.

Fig. 3.5: Different types of force-distance curves (from [2]).

Using this measurement mode it is possible to investigate various biological systems and to gain insights into their existing intra- or intermolecular binding forces and/or information about molecular elasticities \([49, 98, 97]\). Additionally, it is possible to investigate the interaction of lipid bilayers on defined substrates and thus to determine the bilayer elasticity and thickness and also determine the influence of inserted proteins on the lipid layer stability \([93]\).

Furthermore, force-distance curves are used to determine the force which a scanning tip in contact mode exerts to the sample. Since the cantilever deflections are small (typically Å-range) Hooke’s law \(F = -k \cdot z\) is valid and can be applied for the force calculation. Here, \(k\) is the spring constant of the used cantilever and \(z\) is the monitored cantilever deflection. This value can be read from the force-distance curve - it is the offset value of the baseline from the setpoint-line.
3.1.5 Image processing

In contrast to the AFM images shown during the scanning process the saved AFM data are raw data which do not include any corrections for an optimal graphical display. Therefore, corrections need to be carried out such as offset and plane tilt or bow which are due to the properties of the piezoelectric actuator and the set-up of the AFM. This image processing was usually carried out using the Nanoscope v5.12r5 programme with which the AFM is run. The image processing included a zeroth, first or second order flattening procedure according to the requirements.

Furthermore, for the detection of a regular structure the "correlation averaging" method was used which is provided by the programme SPIP Image pro. Here, the unit cell of the investigated structure is either determined automatically by the programme or manually set by the user. Then the program searches within the image of interest for the defined unit cell and finally averages over all detected structures. This results in an averaged image of the unit cell, a standard deviation image, a cross correlation image of the complete image, a fourier analysis image and information about the basic constants of the unit cell.

3.2 AFM sample preparation

For significant and reliable AFM measurements it is important that the used experimental conditions can be easily reproduced. Therefore a high quality of the substrate surface as well as an appropriate sample immobilisation are essential. The surface should provide a low roughness so that small object details can be detected and it also should offer the possibility to interact with the sample so that a fixation is achieved. To improve the sample immobilisation it is possible to modify the surfaces chemically. Furthermore, it can also be advantageous to chemically modify the AFM tips.
3.2.1 Standard substrates

Mica

For biological applications of AFM mica is the most commonly used substrate. Mica is usually used as synonym for muscovite mica; Al₂O₃·K₂O·6SiO₂·2H₂O. It offers an atomically flat surface, due to its layered sheet structure which is shown in fig. 3.6 [79]. The octahedral organized Al₂O₃ (some oxygens are replaced by OH-groups) is connected to two layers of SiO₂ tetrahedrons. These sheets are connected via K₂O-groups. The bonds of the potassium atoms are quite weak and therefore can be easily broken.

![Fig. 3.6: (a) General sheet structure of mica; side view. (b) Basal plane and layer of cations; top view. From [79]](image)

In order to produce a fresh and flat surface the uppermost mica sheets are removed with adhesive tape. The new surface is then negatively charged. The attachment of negatively charged molecules like DNA to the surface can be mediated by a buffer solution that contains mono- or divalent cations in millimolar concentration.

Silicon wafer

Silicon wafers are a well known material from semiconductor technology. The wafers have a crystalline structure, the surface is covered with a oxide layer and exhibits a very low roughness. The processing of this material is following established techniques. The silicon dioxide surface is a hydrophilic surface while the pure silicon surface is hydrophobic. When the silicon dioxide surface is treated with a reducing agent then the
pure silicon is exposed, but under ambient conditions it oxidises again. Before the use as sample substrate the wafer was once cleaned with piranha solution (H₂SO₄ and HCl; 4:1) following the given protocol. Then the wafer was cut into pieces (≈ 1cm × 1cm) which had a suitable size for the used AFM. These wafer pieces were stored in a closed box in the cleanroom and taken out when needed. Before usage the silicon wafer pieces were sonicated in ethanol, then rinsed with deionised water and finally dried with nitrogen.

**Template stripped gold**

Gold is also quite a common substrate for biological samples, since it is an ideal binding partner for thiols and therefore can be used for many different applications. Template stripped gold is an ultraflat substrate with the roughness comparable to mica, since this is the template for the gold preparation. The preparation follows the methods as described in [51]. A thin layer of gold is sputter-coated onto a layer of freshly cleaved mica, then annealed at 300°C for 15 min and slowly cooled down. Then the gold is glued with epoxy glue onto a support which is either glass or the standard AFM steel plates. With the mica exhibited to the air the prepared substrates can be easily stored for several months. When a gold substrate is needed the mica can be removed either with adhesive tape or THF solution. To make sure that all mica is removed the conductivity of the gold is measured at several spots.

**Highly ordered pyrolytic graphite**

For certain types of samples the use of "highly ordered pyrolytic graphite" (HOPG) is an option. HOPG is a certain form of high purity graphite. It is a non-polar and chemically inert material. Like mica it also consists of a lamellar structure where the different layers can easily be separated. In order to achieve an atomically flat and clean surface the HOPG can also be cleaved with adhesive tape.

**3.2.2 Selective surface modifications**

In order to perform high resolution AFM measurements and to achieve reproducible results it is important that optimal conditions for the investigations are found. The
sample should be firmly attached to the substrate and this can be reached by surface functionalisation. Furthermore, it is possible to modify the AFM probes or the composition of the environment i.e. the buffer solution to improve the achievable quality and thus to improve the achievable image quality and resolution.

**Functionalisation of substrates**

Surface properties can be changed by self assembling monolayers (SAMs) which arrange themselves in an ordered way on the substrate and provide the possibility to exchange the outstanding chemical endgroup to meet the necessary requirements i.e. a negatively charge endgroup to immobilise a positively charged sample. For the modification of gold surfaces preferably thioles are used since SH-groups form covalent bonds to the gold substrate. The surface charge of mica and silicon dioxide surfaces can be influenced with the help of amino-terminated silanes. In fig. 3.7 the constitution of the substrate surfaces after the functionalisation process is sketched.

![Fig. 3.7: Surface modification with (a) silanes and (b) thioles.](image)

The substrates were freshly prepared directly before the sample was immobilised for the AFM measurements. The template stripped gold surface was passivated using a 10 mM β-mercaptoethanol solution. The surface was covered with 10-20 μl mercaptoethanol solution and incubated in a closed vial for 20 min. Then rinsed with each 3×100 μl ethanol and water. The silanisation process of other substrates is quite similar to this process. The substrate is thoroughly cleaned and then also incubated in a closed vial. The 3-Aminopropyl-methyl-diethoxysilane solution is used as purchased from the
supplier and a droplet of approximately 30 µl is put next to the sample into the vial. Like this a saturated silane atmosphere is created. After 20 min incubation the substrate is taken out and can be used.

Functionalisation of AFM probes

As already mentioned in section 3.1.2 AFM probes are usually made from silicon or silicon nitride. Similar to the functionalisation of substrates, also AFM probes can be functionalised and so the properties of the tips can be adjusted individually. Especially for force spectroscopy measurements specific probe or linker molecules are attached to the tip via strong covalent bonds. Then interaction force between the probe molecule and the sample can be monitored when this force is weaker than the net force between tip and probe molecule [55]. Furthermore, intramolecular forces of complex molecules can be determined by unfolding or folding experiments [97]. Here specific binding sites for probe and substrate are addressed. Additionally, functionalisation can be used to passivate the tip and thus to prevent sticking of the sample to the probe.

The AFM probes were functionalised by immersing them in chosen solution, i.e. 0.1 mM undecanethiole solution or 1 mg/ml BSA. For BSA functionalisation the incubation time was approximately 20 min. For incubation in undecanethiole solution the time varied from 20 min to over night incubation depending on the intended quality of the thiole layer. First the prepared solution was put into a small closeable petri dish and then AFM cantilevers were inserted. After the incubation the AFM probe was taken out and rinsed with ethanol or water. Thiolated AFM tips could be stored in ethanol for a few days while BSA-cantilevers were always freshly prepared.

Buffer composition

When mica is immersed in solution at neutral pH, the surface is negatively charged because potassium ions dissociate [79]. Under the same conditions also biological macromolecules are charged because of the dissociation of outstanding acidic and basic functional groups. For both surfaces in solution Helmholtz layers and electric double layers are formed (see section 3.1.3) which interact with each other when the sample is to be immobilised on the substrate. The addition of ions into the solution reduces the
Debye-lengths of these layers and thus the sample immobilisation is improved. For this purpose divalent cations in millimolar concentration are more effective than monovalent ions [79, 81].

Usually the samples were immobilised using a buffer solution of high salt concentration. For some AFM measurements this immobilisation buffer was also used for imaging while for some experiments lower concentrated imaging buffers were used. The preparation and imaging conditions for each sample were chosen due to the best results. The respective details are given in combination with the achieved results in chapter 6.
Preparation methods for AFM investigations on membrane proteins

For the investigation of functional transmembrane proteins with the AFM, it is important that a preparation method is developed which allows to achieve conditions, suitable for high-resolution imaging. These must prevent the proteins from denaturation and provide an environment so that the natural protein structure is preserved. In recent literature three different preparation methods can be found which seem to provide suitable imaging conditions for the investigation of transmembrane proteins.

- Covalent binding of the protein to a gold surface by thiols
- Insertion of the protein into a preformed lipid bilayer
- Crystallisation of the protein into a two-dimensional array

In this chapter, these possible preparations will shortly be explained and potential advantages and disadvantages for AFM investigations of the protein FhuA will be discussed.
4 Preparation methods for AFM investigations on membrane proteins

4.1 Covalent binding to gold surface

This method is based on the covalent immobilisation of the protein in combination with a support layer to stabilise the protein. Here the proteins are covalently bound to the gold substrate via thiol bonds. Therefore the protein should either possess a prominent thiol-group or a cystein must be introduced into the molecule. The stability of the assembled proteins is then guaranteed by a supporting layer of thiolipids. This method is described by [110, 31, 114, 54] whereas Cisneros et al. [31] are the first to perform AFM measurements on such samples. They prepared a cysteine mutant of the outer membrane protein OmpF and immobilised it on a gold substrate. The sample preparation process consists of three main steps as sketched in fig. 4.1: (A) Passivation of the gold surface, (B) Incubation and immobilisation of the protein OmpF-Cys and (C) Protein stabilisation by thiolipid monolayer.

![Diagram showing steps A, B, and C]

Fig. 4.1: Attachment of OmpF porin trimers to a gold surface [31]; (A) Passivation of the gold surface, (B) Incubation and immobilisation of protein OmpF-Cys, (C) Protein stabilisation by thiolipid monolayer.

The template stripped gold is passivated with a 10 mM β-mercaptoethanol solution. After 20 min incubation and washing, the substrate is prepared for the protein immobilisation. The protein is incubated at room temperature for at least 1 h. It is solubilised in buffer with a concentration of 0.25 mg/ml. Then chemisorption of the thiolipid (1,2-Dipalmitoyl-sn-Glycero-3-Phosphothioethanol (DPPE); 0.5 mg/ml) at 45 °C for minimum 1 h. The detergent and excess lipids are removed by washing with buffer solution. AFM imaging is done in buffer solution using tapping mode.
For this method the critical steps are the surface pretreatment and the thiolipid assembly. After the surface passivation and protein stabilisation steps the scanned surface is no more even and flat. Finally, the OmpF proteins have to be identified by their characteristic arrangement as trimers.

4.2 Protein insertion into lipid bilayer

This sample preparation approach is used for the successful immobilisation of several transmembrane proteins e.g. alkaline phosphatase, LH1-RC and LH2 core complexes as published by Milhiet et al. [74, 75, 15].

Here, a solid supported lipid bilayer is formed on mica by vesicle fusion. In fig. 4.2 this procedure is sketched. Part (a) shows the destabilisation of the bilayer by incubation of a detergent below the cmc, in (b) solubilised membrane proteins are added, while in (c) protein incubation and their insertion into the lipid bilayer are sketched and (d) shows the removal of detergent and excess proteins by washing with buffer.

![Diagram of protein insertion into lipid bilayer](image)

Fig. 4.2: Insertion of proteins into a lipid bilayer [15]; (a) Destabilisation of bilayer by incubation of detergent below the cmc, (b) Addition of solubilised membrane proteins, (c) Incubation and insertion of proteins into lipid bilayer, (d) Removal of detergent and excess proteins by washing.

The lipid bilayer is made from a 1:1 (mol/mol) mixture of dioleoyl-phosphatidylcholine (DOPC) and dipalmitoyl-phosphatidylcholine (DPPC). Then this bilayer is destabilised
to facilitate the insertion of membrane proteins by incubation with detergent. The bilayer is incubated at 4°C for 30 min with detergent containing buffer at a concentration twice below the particular cmc. Then the solubilised protein is incubated for 15 min at the same temperature. The detergent concentration of the solubilisation buffer is set to the cmc. Finally, the sample is rinsed with excess of detergent-free buffer in order to remove all detergent molecules, since their presence disturbs AFM measurements.

For this preparation method the formation of a closed lipid bilayer and the determination of the optimal detergent concentrations for the different steps might be problematic.

4.3 Two-dimensional protein crystallisation

For the amphiphilic transmembrane proteins, 2D crystallisation is known as suitable sample preparation method for structure revealing measurements with the transmission electron microscope [41, 100, 61, 66, 64]. Also atomic force microscopy investigations have been performed on natural protein crystals as Bacteriorhodopsin [84, 83] as well as on reconstituted protein crystals such as OmpF [80] and LH2 [105]. A general introduction into the crystallisation method and an overview about the important experimental details is given in [56, 99, 77, 106]. Whereas especially Rigaud et al. worked on the 2D crystallisation of FhuA [95, 66, 61, 30].

In general, 2D protein crystallisation is based on two main steps. First the mixing and equilibration of solubilised proteins and lipid-detergent micelles. Second the removal of the detergent and the simultaneous formation of the protein crystals. But up to now the crystallisation process is not fully understood. In fig. 4.3(a) three possible crystallisation mechanisms are shortly described. Starting with a mixture of lipid-detergent and lipid-protein-detergent micelles successively the amount of detergent is reduced. Mechanism (I) proposes the formation of a lipid layer and simultaneous protein insertion, since two types of micelles coalesce upon detergent removal followed by crystallisation. Mechanism (II) considers as first step the lipid bilayer formation and upon detergent removal proteins insertion into the bilayer and then crystallisation. (III) Crystal contacts are established when micelles are detergent-depleted. Thus the formation of the bilayer, the protein insertion and the crystallisation happen at once. In fig. 4.3(b) the possible types
Fig. 4.3: (a) Possible mechanisms for 2D crystallisation; (I) Formation of lipid layer, simultaneous protein insertion - two types of micelles coalesce upon detergent removal, crystallisation; (II) Lipid bilayer formation, upon detergent removal insertion of proteins, crystallisation; (III) Establishment of crystal contacts when micelles are detergent-depleted, at once formation of the bilayer, protein insertion and crystallisation; (b) Possible types of 2D crystals (A) planar crystals; (B) stacked planar crystals; (C) vesicular crystals; (D) tubular crystals [99].

of resulting 2D crystals are shown. These could be planar 2D crystals (A), stacking planar 2D crystals (B) which are equivalent to thin 3D crystals or vesicular crystals (C) and tubular crystals (D). For AFM investigations the planar 2D crystals are preferred.

For this preparation method a stock solution containing the purified protein at a concentration of $c = 1 - 2 \text{mg/ml}$ in storage buffer (20 mM Tris–HCl, pH 8, 150 mM NaCl, 0.05% LDAO, and 0.02% NaN₃) is used. For the crystallisation process the protein is diluted in 50-100 µl buffer (20 mM Tris–HCl, pH 8, 100 mM NaCl, 0.1% LDAO, 0.02% NaN₃) to a concentration of $c = 1 \text{mg/ml}$. Then *E. coli* lipids are added at a lipid to protein ratio of 0.5 (w/w). This mixture is equilibrated and gently stirred for 30 min at room temperature. In order to increase the size of the reconstituted protein patches the detergent OTG is added with a final concentration of 20 mM and further incubation
for 30 min. Then the detergent is removed with successive addition of Bio-Beads. For the total removal of the detergent using the Bio-Beads an incubation time of 5-6 h at room temperature is necessary. The reconstituted material is kept at 4°C and investigated with the transmission electron microscope.

Here, the critical point is the purity of the protein solution, since this directly influences the result. Additionally, the lipid to protein-ratio is important, since the formation of a crystalline structure is depending on this otherwise reconstituted proteins in bilayer are achieved.

4.4 Comparison of methods

Covalent binding to gold surface

The advantage of this approach is that the proteins are covalently fixed to the surface and can not be removed during the scanning process. But using this method, the identification of the FhuA proteins might be problematic. In [31] the OmpF protein is clearly identified, because of its trimeric arrangement. The protein FhuA is organised as monomers, the differentiation between protein and artefact is expected to be difficult.

We tried to repeat these experiments, but were not successful. Problems occurred with the preparation of the template-stripped gold substrate, because mica was not completely removed from the gold layer. Furthermore, the surface of the passivated gold substrate was not of comparable quality as in [31]. Finally, the incubation with FhuA-Cys resulted in an undulated and rough surface which could not be clearly resolved. The achieved results were not satisfying and thus other sample preparation methods were tested.

Protein insertion into lipid bilayer

This method provides a flat and defined surface so that inserted proteins could be easily identified if the lipid dimensions fit the protein dimensions. Unfortunately the proteins do not have fix positions in the bilayer and so can easily move. Depending on the protein size and structure it is possible to identify proteins within the lipid bilayer, but
high resolution imaging might be challenging, because of the possible lateral protein motion.

Repeating this recipe, the preparation of a closed lipid bilayer turned out to be problematic and a complete covering of the surface could not be achieved. AFM imaging of the sample after FhuA incubation and detergent removal also revealed an undulated and rough surface, whereas using this method the resulting rough surface structure was more distinct and consisted of spherical aggregates.

Two-dimensional protein crystallisation

In a protein crystal the single proteins are fixed in certain positions and cannot move freely. The close packing of the proteins additionally improves the molecules’ stability. Furthermore, the protein crystal can be easily immobilised on mica or any other suitable substrate since it is possible to individually adjust the immobilisation buffer. Additionally, the crystal structure allows to perform correlation averaging and thus the data evaluation can be improved.

Résumé

When considering all theoretical pro and contra arguments of these methods and additionally taking the achieved practical results into account, we finally decided to focus on the preparation of 2D protein crystals for AFM sample preparation.
4 Preparation methods for AFM investigations on membrane proteins
CHAPTER
FIVE

Additional instruments and methods

Apart from the AFM and the related protein preparation method, several other instruments and techniques are used in this work for which a short introduction will be given in this chapter. In the protein purification process a High Performance Liquid Chromatography is performed which uses an anion exchange and a size exclusion column. Basic biochemical methods such as SDS-PAGE, BCA assay, centrifugation and dialysis are necessary steps within this process. Furthermore, the quality of the protein solution and the protein crystals is controlled with a Transmission Electron Microscope.

5.1 Transmission Electron Microscopy

The Transmission Electron Microscope (TEM) is nowadays a widely used instrument in the field of physics, material science and biology. It offers a high resolution nowadays up to 0.1 nm and allows a fast screening of samples (2-10 min) combined with a relatively simple sample preparation. Thus it is an ideal instrument to perform quick control experiments. Biological specimen normally need to be stained in order to be clearly visible with the TEM.
5 Additional instruments and methods

5.1.1 TEM basics

As indicated by the name the functional principle of the Transmission Electron Microscope (TEM) is based on an electron beam which is transmitted through a thin sample and then monitored onto a fluorescent screen or photographic element. Figure 5.1 shows the basic set-up of a TEM.

![TEM diagram](image)

Fig. 5.1: Basic set-up of an Transmission Electron Microscope (from [90]).

Electrons are generated in the cathode, then accelerated and focussed onto the sample with the help of a magnetic lens system. The electron beam that shines through the specimen is then magnified and projected onto a fluorescent screen which is used to visualise the detected sample structure. The interaction between the sample and the electron beam produces electrons which are scattered either elastically, inelastically, back-scattered or unscattered. The interference of elastically scattered and unscattered electrons produces the phase contrast image which is seen on the screen. The inelastically scattered electrons can create a diffuse background image which reduces the contrast of the achieved image, since these electrons change their velocity and direction. If a sample contains a crystalline structure this can be seen as defined diffraction pattern which is detected in the focal plane.

For TEM measurements the samples have to be thin (<1 μm) and especially biological
samples need to be prepared to withstand the vacuum. To enhance the contrast of biological samples negative staining is used. Negative staining means that atoms with a high atomic number are allowed to adsorb to the biologic material and thus scattering behaviour of the electrons is improved. Furthermore, it has to be paid attention that biological samples can be destroyed if the electron energy is too high. For biological samples the energy of the electron beam is in the range of 80 - 120 keV, whereas for material science samples energies up to 200 keV are used. While the energy of a covalent bond is in the range of $\approx 1.5 - 8.8$ eV and thus can be easily destroyed.

The instrument we use is a *EM 900* (Carl Zeiss AG, Germany) equipped with a differential vacuum pump system (minimal high vacuum $2 \cdot 10^{-6}$ mbar), 80 keV high-voltage and a Wide-angle Dual Speed Slow Scan CCD-camera with a High-Speed Slow Scan Camera controller using the Image SP controller program.

### 5.1.2 TEM sample preparation

For TEM measurements a copper grid which is covered with a carbon film is used as sample substrate. To clean the substrate surface and to improve the adhesion conditions for charged samples the grid is treated 2.5 min with an argon-oxygen plasma (16% O$_2$). Immediately after this the protein solution is deposited on top. After an incubation time of 1-2 min the solution is removed with a tissue. The grid is then washed with water to avoid artefacts such as salt crystals coming from dried buffer solution. For negative staining of the protein sample two droplets of 1% uranyl acetate solution are pipetted onto the grid and directly removed with a paper tissue. After drying the sample is ready to be inserted into the TEM. The more detailed procedure is given in the appendix G.

### 5.2 High Performance Liquid Chromatography

For the investigation of specific proteins a high purity of the protein solution is crucial to avoid artefacts caused by other solubilised molecules. A mixture of solubilised proteins can be separated using chromatographic methods by addressing the different physicochemical properties of the proteins e.g. molecular weight (→ size exclusion chromatography), electric charge (→ ion exchange chromatography), hydrophobicity. To achieve a
5 Additional instruments and methods

higher grade of purity it is advisable to combine several chromatographic methods.

The chromatography is based on the interactions between functionalised polymer beads and specific protein properties. The polymer beads are packed into a column (glass tube; diameter: ≈10 mm; height: 2-30 cm) and the protein solution is washed through this column. Using a closed set-up as in the High Performance Liquid Chromatography (HPLC) system it is possible to achieve a better resolution and a higher material throughput compared to open chromatographic systems. This set-up allows to apply a higher pressure and smaller column materials which consequently causes a different flow behaviour and finally improves the interaction between sample and functionalised beads.

In the following section the basics of HPLC and the used chromatographic methods will be explained. Furthermore, a detailed discussion of example chromatograms is given.

5.2.1 HPLC basics

A standard HPLC system is set-up from a column, containing functionalised polymer beads, which is connected to a reservoir of buffer solution via pumps. The pumps allow to regulate the pressure or the buffer flow in the system and also to generate a buffer gradient by gradually mixing two different buffers. When the sample is eluted from the column the protein concentration is determined by measuring the absorption of UV-light \( \lambda = 280 \text{ nm} \) and then it is fractionated. The basic set-up of a HPLC is sketched in fig.5.2.

![Fig. 5.2: General set-up of a HPLC system (adapted from [90]).](image-url)
5.2.2 Ion exchange chromatography

Ion exchange chromatography (IEC) is one of the most often used techniques to purify proteins, peptides or other biomolecules, since this method offers a high resolution combined with a high sample loading capacity. The IEC is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium as sketched in fig. 5.3(a). For a specific protein the relation between net surface charge and pH of the surrounding medium is unique and thus can be used to separate different proteins solubilised in one solution. When the pH value of the solution is equal to the molecule’s isoelectric point (pI) then the net surface charge is zero and the molecule does not bind to a charged column medium. In the case pH > pI then the molecule is negatively charged and thus binds to a positively charged column medium - an anion exchange medium. If pH < pI the molecule will bind to a cation exchange medium.

In the first step different proteins are loaded into the HPLC and bind to the column material. In the second step the column is flushed with elution buffer whose ionic strength is gradually increased. Thus an increasing number of salt ions competes with the proteins for the anion or cation binding sites. At specific salt concentrations the different proteins are released from the ion exchanger and eluted from the column. Since these exchanging concentrations are dependent on the individual protein properties a mixture of proteins can be separated with such a salt gradient. A typical IEC chromatogram is depicted in fig. 5.3(b).

In this graph the blue line represents the protein concentration determined by the absorption measurement at λ=280 nm. Whereas the red line indicates the ionic strength of buffer composition which is passing the column. It can be seen that in the beginning the sample is injected and immediately some of the proteins are washed out - the so called flow through. Afterwards the salt concentration of the buffer is gradually increased. First proteins with low ionic strength are eluted while molecules with higher ionic strength are washed from the column with higher salt concentrations. Finally, the column is washed using a buffer with high salt concentration (1 M NaCl) and then re-equilibrated again.

The column medium is a matrix consisting of porous spherical particles. These particles possess a large surface area and are functionalised with negatively or positively charged ionic groups, providing a high interaction probability. For the purification of
Fig. 5.3: (a) Sketch of basic interaction of IEC; (b) Typical chromatogram of IEC using a linear gradient elution. (from [5])

PhuA we use a strong anion exchanger, the so called Q-material. This material is functionalised with a quarternary ammonium, -O-CH$_2$N$^+(CH_3)_3$ being the functional group. This anion exchanger is positively charged over a large range of pH. Consequently, it does not act as buffer, but provides stable interaction conditions during the anion exchange chromatography (AEC).

The resolution of a column is depending on the selectivity and efficiency of the used column material as well as on the amount of applied sample. The selectivity is equivalent to the degree of separation between two peaks. It depends on the functional groups with which the matrix is modified as well as on the experimental conditions (pH, ionic strength, elution conditions). The efficiency of a column is depending on the properties of the column material (bead size, pore size, ion exchange between solution and pore) and the packing of the column (even flow of buffer, small diffusion volume). If all settings are optimised it is possible to separate proteins which only differ for the charge of one amino acid. But it is also necessary to be aware of the fact that a single well resolved peak is not necessarily representing a pure substance. Such a peak can also be the result of several components which were not separable under the chosen elution conditions.
5.2.3 Size exclusion chromatography

Size exclusion chromatography (SEC) is the simplest chromatographic method and separates molecules based on their sizes. It is also known as gel filtration or gel permeation chromatography. Compared to other chromatographic techniques its resolution is rather low. It is often used for the final polishing step during a purification because then most of the impurities are already removed. Here, the separation of solubilised molecules is based on size related interaction ability. With smaller size the molecules can enter more easily the porous column material as sketched in fig. 5.4(a). Thus smaller particles remain longer in the column, since larger molecules can not enter these pores.

Since in SEC the buffer composition does not influence the resolution the buffer can be chosen to suit further processing steps. In fig. 5.4(b) a typical chromatogram of a SEC is shown.

![Diagram](image)

**Fig. 5.4:** (a) Sketch of basic interaction of SEC; (b) Typical chromatogram of a SEC elution. (from [3])

Here, the blue line represents the detected protein concentration at $\lambda=280\,\text{nm}$ depending on the volume of the buffer that already flushed the column. For this type of chromatography the order of elution is dependent to the molecular size. The larger molecules (i.e. molecules with high molecular weight) can not enter the pores of the col-

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53
umn material and thus elute first from the column. The smaller molecules have a longer interaction time with the porous material, since they can easily enter the material pores and are eluted at the end.

The column material we used is Superose 12 which is based on highly cross-linked porous agarose particles. This medium possesses a high physical and chemical stability. Usually non-specific interactions between proteins and superose can be neglected when buffers with an ionic strength in the range of 150 mM - 1.5 M are used.

5.3 Molecular dynamics simulations

Some initial molecular dynamics (MD) simulations were carried out by Dr. Danilo Roccatano for the designed 11fmer $\beta$-sheet with a simulation length of 15 ns. The $\beta$-sheet was built up from twelve polypeptide strands which are initially set-up from 2 hexamer polypeptides, whereas this hexamer is built up from 3 polypeptide dimers. The $\beta$-sheet behaviour is modeled in water, treated as simple point charge (SPC) [12], using an octahedral box with periodic boundary conditions. The total number of atoms is 38958. The simulations are carried out with the program Gromacs [13, 67] and the Gromos 43a1 [115, 107] force field, preserving a constant pressure of 1 bar and a constant temperature of 300 K. The electrostatic interactions are calculated using the Particle Mesh Ewald (PME) method [37].

5.4 Basic biochemical methods

In the following the standard biochemical methods which we used during the protein preparation process are shortly explained. SDS-PAGE is the standard control to monitor the purity of the protein solution as well as to control the size of the proteins present in the current solution. For the determination of the protein concentration the BCA Protein Assay Kit (Pierce, Rockford, USA) is used as well as the Nanodrop ND-1000 Spectrophotometer (Fisher Scientific GmbH, Germany). Furthermore, basic information about centrifugation, dialysis and Bio-Beads are given.
5.4.1 SDS-PAGE

SDS-PAGE (Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis) is a method which is used to characterise the protein content of a solution. This method is based on the mobility of polypeptide chains within an electric field through a gel matrix. The natural protein charge is masked by SDS (an anionic detergent) and thus the separation of different sized proteins is based on the mass related mobility within the polyacrylamide gel. The gel is immersed in buffer solution and an electric field is applied. After finishing the protein separation the proteins in the gel are stained and their size can be determined in comparison with known marker proteins which also have to be loaded. Staining is usually done with coomassie brilliant blue or silver stain. Experimental details are given in the appendix H.

5.4.2 BCA assay

The BCA (bicinchoninic acid) assay is used to determine the total amount of protein in solution. The quantification is based on the light absorption of a complex whose concentration is protein-dependent. The used wavelength is \( \lambda = 562 \text{ nm} \) and the absorbance of light is directly proportional to the protein concentration. The protein concentration is determined by comparing the absorbance of the investigated solution with the absorbance of a BSA standard. Therefore, the error of this measurement is influenced by the quality of the BSA standard dilution curve. Furthermore, the formation of the light absorbing complex is time-dependent and so accurate execution of this experiment is important. The sample volume needed is 10 µl.

5.4.3 Nanodrop

With the NanoDrop ND-1000 Spectrophotometer (Fisher Scientific GmbH, Germany) the protein concentration of one sample is quantified using the UV-light absorption of aromatic amino acids at the wavelength \( \lambda = 280 \text{ nm} \). The sample volume needed is \( \sim 1 \mu l \). For this method an error can be determined statistically.
5.4.4 Centrifugation

Centrifugation is a technique to separate the insoluble components of a solution based on size and density. We use it in the protein extraction process for separating solubilised proteins and fragments of the cell membrane as well as for concentrating protein samples and exchanging of buffer solution via filters. The parameters which define a centrifugation are the acceleration, given as multiple of \( g \), the centrifugation time and the temperature.

5.4.5 Dialysis

Dialysis is another method to separate molecules in solution. We apply this technique in order to exchange the buffer conditions of the protein sample, e.g. salt reduction between anion exchange steps, detergent removal for protein crystallisation. The separation is based on different diffusion rates of several molecules through a semipermeable membrane. Critical aspects are the volume of the exchange solution and the interaction time.

5.4.6 Bio-Beads

Bio-Beads are used for the adsorption of nonpolar substances or surface active agents from aqueous solutions. Bio-Beads SM-2 (Bio-Rad, München, Germany) are chemically neutral, macroporous polymeric beads made from polystyrene divinylbenzene. The bead size varies from 300 \( \mu m \) to 1180 \( \mu m \). Single beads are formed from of a large number of highly crosslinked microspheres. This results in a large surface area (300 \( m^2/g \)) and uniform pore sizes (\( 90 \AA \) for dry beads). They have a good physical stability and can withstand temperatures to 250\(^\circ\)C.

We use these beads for the removal of detergent within the protein crystallisation process. Before use Bio-Beads have to be washed thoroughly with methanol and water.
CHAPTER
SIX

Results and Discussion

In this chapter the results from the different nanobiological systems are presented. For the investigation of the polymer supported liposomes and the β-sheet forming polypeptides these results include AFM images and the acquired evaluation data from these images. In the case of the transmembrane protein FhuA the focus is to establish a purification protocol which provides a high sample quality suitable for protein crystallisation. The main goal is the protein crystallisation and then the AFM investigation of the crystals which could have not been reached yet.

6.1 Nanocontainers from polymer supported liposomes

The goal is to determine the parameters which are necessary to achieve two-dimensional polymerisation of the certain polymers within the template liposomes. In order to proof that the chosen settings are suitable for this approach the results of different preparation steps are monitored using several complementary investigation methods.

In this context the AFM is used to determine the shape and the size distribution of the prepared polymer supported liposomes. Stable measurement conditions were achieved with contact mode measurements in air. Contact mode measurements in solution did
6 Results and Discussion

not produce satisfactory results, since these polymer vesicles were easily moved across the mica substrate. Furthermore, tapping mode measurements in liquid were tested, but stable imaging conditions could not be achieved.

With the AFM investigation the three-dimensional spherical shape of the polymer stabilised liposomes was verified. Additionally the vesicle size distribution was determined for a limited amount of vesicles (approximately 50 different vesicles within different measurements) and found to be in agreement with the results achieved by dynamic light scattering and TEM measurements.
Stable Polymethacrylate Nanocapsules from Ultraviolet Light-Induced Template Radical Polymerization of Unilamellar Liposomes

Joana Filipa Pereira da Silva Gomes, Andreas F.-P. Sonnen, Astrid Kronenberger, Jürgen Fritz, Manuel Álvaro Neto Coelho, Didier Fournier, Clara Fournier-Nöel, Monique Mauzac, and Mathias Winterhalter

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6 Results and Discussion

6.2 Self-assembling polypeptides

The aim of these investigations is to explore the possible use of polypeptides as building blocks for nanoelectronic purposes. Therefore, the structure of the self assembled peptides is characterised in order to judge if the peptides are suitable for the use as nanowires.

First, different sample preparation methods and measurement settings are tested for the basic 11mer polypeptide. The accessible characterisation data of the β-sheets (i.e. width, height, length, helical pitch, direction of helicity) are gathered from AFM topography images as indicated in fig. 6.1 and compared for the different conditions. The next step then is the determination of structural details of β-sheets from all polypeptide modifications and the comparison of these results.

Fig. 6.1: Accessible β-sheet characterisation data; scale bar: 10 nm.

6.2.1 Sample preparation and imaging conditions

AFM settings

For the investigation of the β-sheets tapping mode AFM measurements were performed in air at room temperature. The standard scan field size was 1 μm × 1 μm. This resolution was sufficient to detect the pitch of the β-sheet strands. For higher resolution the scan field size was decreased down to 100 nm × 100 nm. The typical scanning tip velocities ranged from 0.3 μm/s to 4 μm/s.
6.2 Self-assembling polypeptides

Substrate

As already mentioned in chapter 3.2.1, several different substrates might be suitable supports for the β-sheets. We tested mica, silicon and HOPG, but only for mica stable AFM imaging conditions were achieved independent from the sample deposition conditions.

Sample deposition

The β-sheets were used as provided from the supplier. A stock solution with a concentration of 1 mg/ml was prepared using deionised water and stored at 4°C. This concentration was equivalent to the following molarities: c₁₁mer = 0.67 mM, c₂₄mer = 0.32 mM, c₃₇mer = 0.21 mM, c₁₁₁mer = 0.66 mM, and c₁₅₁mer = 0.49 mM. For AFM sample preparation, the respective stock solution was vortexed, aliquots were taken and diluted to a final concentration of 10 μg/ml. This dilution was prepared using either deionised water pH 7, PBS buffer, buffer01 (50 mM Tris-HCl, pH 7.8, 100 mM NaCl, 0.1% OPOE), buffer02 (10 mM Tris-HCl, pH 7.6, 150 mM KCl), mix01 (isopropanol-water, 9:1, pH 7), mix02 (isopropanol-water, 9:1, pH 5) or mix03 (isopropanol-water, 9:1, pH 3). Then, 30 μl of this 10 μg/ml β-sheet solution was deposited onto the substrate. After an incubation time of 1-5 min the sample was rinsed with 3 x 100 μl deionised water or isopropanol-water mixture and dried in a flow of nitrogen.

Samples prepared with buffer solutions often contained salt crystals of different sizes. In order to prevent such artefacts, a more intense rinsing with deionised water is necessary or it is even better to avoid the use of such buffers. Furthermore, samples prepared with the isopropanol-water mixture provided quite often unstable measurement conditions, while the structural details of these samples did not differ significantly from samples prepared with deionised water. For reasons of simplicity and also in order to reduce the potential influence of ions to nanoelectronic devices, the sample preparation was finally done with deionised water at pH 7.

6.2.2 AFM results - 11mer, 24mer and 37mer polypeptides

In this section, typical AFM topography images of the 11mer, 24mer and 37mer polypeptides are presented. The gathered evaluation data for these different peptides are pre-
Results and Discussion

Presented and discussed. Here, we first focussed on proof of principle measurements and thus a limited number of experiments and evaluation data is considered. It is clear that the achieved data only represent indicators for the possible behaviour of the final results. The number of evaluated β-sheets can be determined in tab. 6.1 from the column "handed" which describes the relations of the detected helicities.

11mer polypeptides

![Image](a)

![Image](b)

**Fig. 6.2**: 11mer β-sheets on mica; (a) Scale bar: 250 nm, height scale: 8 nm; (b) Scale bar: 25 nm, height scale: 7 nm.

24mer polypeptides

![Image](a)

![Image](b)

**Fig. 6.3**: 24mer β-sheets on mica; (a) Scale bar: 250 nm, height scale: 11 nm; (b) Scale bar: 25 nm, height scale: 11 nm.
6.2 Self-assembling polypeptides

37mer polypeptides

Fig. 6.4: 37mer β-sheets on mica; (a) Scale bar: 250 nm, height scale: 12 nm; (b) Scale bar: 25 nm, height scale: 7 nm.

Tab. 6.1: Comparison of the structural data of the 11mer-, 24mer-, 37mer-β-sheets.

<table>
<thead>
<tr>
<th>sample</th>
<th>length /nm</th>
<th>pitch length /nm</th>
<th>height /nm</th>
<th>width /nm</th>
<th>handed (left:right:none)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11mer</td>
<td>63±6</td>
<td>15.3±0.9</td>
<td>3.3±0.2</td>
<td>15.7±1.1</td>
<td>4:3:2</td>
</tr>
<tr>
<td>24mer</td>
<td>82±14</td>
<td>15.8±2.0</td>
<td>3.3±0.2</td>
<td>13.4±1.1</td>
<td>5:1:4</td>
</tr>
<tr>
<td>37mer</td>
<td>59±6</td>
<td>12.2±0.6</td>
<td>3.1±0.2</td>
<td>12.0±0.6</td>
<td>4:1:4</td>
</tr>
</tbody>
</table>

From these data, it can be seen that the average length of the resulting β-sheets is not influenced by the length of the polypeptide sequence. The average length of all polypeptides varies between 60 nm and 82 nm. Whereas, only some of the 24mer β-sheet strands have a total length above 100 nm. The length distribution of the 11mer β-sheets and the 37mer β-sheets is quite similar. This sequence-independence of the β-sheet length can be explained by the fact that the chosen extension of the initial 11mer polypeptide mainly supports the formation of antiparallel β-sheets. The use of 24mer or 37mer polypeptides does not imply that the resulting β-sheets form longer strands. It could be that the energetic optimal arrangement of the β-sheet forming peptide sequences is hindered, due to the inserted loop sequence. Thus, the formation of longer β-sheets might not be possible, since the peptide possesses an internal tilt as it can
be seen in fig. 2.4(a). The determined heights and widths of the \( \beta \)-sheet strands of the three polypeptides agree with each other within the calculated statistical errors. Only the value of the helical pitch of the 37mer polypeptide deviates from the helical pitch size of the 11mer and the 24mer polypeptides. Also, the mainly detected left-handed helicity of the three different polypeptides is in agreement with the theoretical expectations [47]. In some cases, the helicity can not be indentified unambiguously although a regular pitch structure can clearly be seen. Maybe the immobilised \( \beta \)-sheet is tilted in such a way that the orientation of the groove to the main axis of the \( \beta \)-sheet is changed. It can be said that in general our results are in agreement with the TEM measurement results of Fishwick and co-workers [47].

These results were achieved for \( \beta \)-sheet stock solutions which are older than 14 days. This detail is important since in the next section it is discussed that the length of the formed \( \beta \)-sheets is time-dependent on the scale of a few days.

We conclude that the simple extensions of the peptide sequence do not significantly increase the total length of the resulting \( \beta \)-sheets. Thus these are no critical parameters for the formation of nanowires. Furthermore, it can be said that the \( \beta \)-sheets formed from these polypeptides are not suitable for the use as nanowires since their lengths do not reach the micrometer range. For these and economical reasons, we decided to use the short and simple 11mer peptides for further use and modification.

With respect to the use as nanoelectronic wires, it seems reasonable to support the electron transport through \( \beta \)-sheets via "free" electrons. These could be provided by aromatic amino acids, since they contain delocalised \( \pi \)-electron systems. We chose the most simple aromatic amino acid - phenylalanine. Furthermore, the introduction of an aromatic amino acid might also influence the structure and length of the resulting \( \beta \)-sheets. Additionally, we combine this modification with another extension of the peptide sequence consisting of four glutamine residues in order to find out, if this influences the length of the resulting \( \beta \)-sheets.

6.2.3 AFM results - 11fmer and 15fmer polypeptides

Now, typical AFM topography images of the 11fmer and 15fmer polypeptides are presented. When we found out that the structure of the 11fmer polypeptide changes sig-
nificantly with the age of the stock solution, the investigation of this time dependent behaviour was initialised for both peptides. Therefore, we first investigated the time-induced structural change of the $\beta$-sheets. Then, we focussed on the structural details of these peptides, performed a data evaluation and discussed the gathered data.

In order to investigate the influence of the age of the $\beta$-sheet stock solution on the total length of the $\beta$-sheets, a new stock solution was produced and AFM samples were prepared and investigated after different times. The sample prepared immediately after the production of the stock solution is labelled "day 1". The AFM sample prepared the following day is labelled "day 2" and so on.

Also here, proof of principle measurements were performed and for the most promising objects more detailed investigations need to be carried out. Thus the resulting data only indicate the potential behaviour of the final results. In the following tables the number of evaluated $\beta$-sheet strands is given in column "$\#$".

**11fmer polypeptide**

Fig. 6.5 shows typical AFM images for the samples prepared with different age of the $\beta$-sheet stock solution, whereas in fig. 6.6 a series of images of the sample "24 months" with increasing resolution is presented. In tab. 6.2, the evaluation data of the 11fmer polypeptide $\beta$-sheets are given.

<table>
<thead>
<tr>
<th>time period</th>
<th>length/nm</th>
<th>pitch/nm</th>
<th>height/nm</th>
<th>width/nm</th>
<th>$#$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>30 ± 8</td>
<td>22.8± 1.9</td>
<td>3.5 ±0.8</td>
<td>16.0±2.4</td>
<td>7</td>
</tr>
<tr>
<td>2 days</td>
<td>12 ± 1</td>
<td>-</td>
<td>1.7 ± 0.5</td>
<td>11.1±0.8</td>
<td>7</td>
</tr>
<tr>
<td>5 days</td>
<td>577 ±552</td>
<td>-</td>
<td>3.2 ± 0.4</td>
<td>15.9±1.3</td>
<td>5</td>
</tr>
<tr>
<td>12 days</td>
<td>1036 ±532</td>
<td>-</td>
<td>3.1 ± 0.1</td>
<td>32.4 ±7.9</td>
<td>4</td>
</tr>
<tr>
<td>24 months</td>
<td>&gt;2000</td>
<td>13.4±1.2</td>
<td>4.6 ±0.2</td>
<td>12.4±0.4</td>
<td>6</td>
</tr>
</tbody>
</table>

It is evident that the number and the average length of detected $\beta$-sheet strands generally increases with the age of the polypeptide stock solution. In the first days, nearly no extended $\beta$-sheet strands are formed. Most probably, the many little circular
structures which are clearly visible are short β-sheets which self-assemble into longer β-sheets with increasing time being kept in solution. This seems to be a reasonable explanation, since the amount of these circular structures is reduced with increasing age of the stock solution. Additionally the lengths of the visible β-sheets increase. This can be seen best at fig. 6.6 (a), since this image is of 5 μm × 5 μm scan field size. Furthermore, in fig. 6.6 (c) a regular pitch structure can be seen, especially when the topography along the β-sheet is observed, but the helicity can not be identified. This helical pitch is only observed in two out of five samples and it is not clear why this is the case.
6.2 Self-assembling polypeptides

Fig. 6.6: Typical 11fmer \( \beta \)-sheets (sample "24 months") on mica; (a) Scale bar: 1.25 \( \mu \)m, height scale: 12 nm; (b) Scale bar: 250 nm, height scale: 12 nm; (c) Scale bar: 25 nm, height scale: 10 nm.

It can be said that all data for pitch length, height and width are neither in agreement with each other nor do they show a certain structural development. Thus, we can conclude that at least a time dependent development of \( \beta \)-sheet growth is clearly visible, but more measurements have to be performed to clarify the structural details. Furthermore, it seems possible that this undefined or not detected helical pitch is related to the inserted phenylalanine residue, since its hydrophobic character might influence the \( \beta \)-sheet formation and finally modifies the complete structure.

If parallels are drawn to the behaviour of a similar, but not identical polypeptide (containing three aromatic amino acids) which is discussed in [8], then it seems possible that the investigated sample solution contains \( \beta \)-sheets which possess a different structure. In [8], it is stated, that for their specific polypeptide at pH 2 and concentrations above \( c \approx 0.09 \text{ mM} \) ribbons are formed, and with increasing concentrations \( c \geq 0.6 \text{ mM} \) these ribbons associate into fibrils (see fig.2.3). The concentration of the prepared 11fmer polypeptide stock solution is \( c_{11\text{fmer}} = 0.66 \text{ mM} \) and thus this structural change appears to be possible. But these concentration dependent transitions still need to be verified for the present experimental conditions. Additionally, it might be that, for a higher polypeptide concentration, the relative arrangement of the 11fmer \( \beta \)-sheets to each other differs from the comparable organisation of the initial 11mer polypeptides. Due to hydrophobic interactions of the side chains, a different arrangement of the peptides might be induced.
so that finally the helicity of the resulting $\beta$-sheet is changed or even impossible to detect.

First molecular dynamics (MD) simulations (using the same assumptions as [47]) of this 11mer polypeptide verified, that a $\beta$-sheet which consists of twelve polypeptide strands ($2 \times$ hexamer $\beta$-sheets) is stable in water for at least 15 ns which is a usual time scale for MD simulations. This indicates that the arrangement of these peptides into $\beta$-sheets is dominant and not disturbed by the properties of the phenyl-rings. Furthermore, this configuration seems to be stable while other possible orientations such as a clogging of hydrophobic subunits do not occur within this time-scale. The resulting $\beta$-sheet of the modelled system is shown in cartoon representation with and without highlighted phenyl-rings in fig. 6.7.

Fig. 6.7: (a) Model of 11mer $\beta$-sheet consisting of 12 polypeptide strands; (b) Same model, different viewing angle with highlighted phenyl-rings.

Here, it can be seen that the orientation of the phenyl-rings of each peptide strand is different for neighbouring strands. In order to control the influence of this amino acid to the structure of the complete $\beta$-sheet more simulations are necessary. Therefore, the next step is to model the behaviour of two $\beta$-sheets facing each other with the hydrophobic side chains, which is currently being done. These $\beta$-sheets consist of four polypeptide strands and are modelled in water.
15fmer polypeptide

In the following, the same type of AFM results as for the 11fmer is presented for the investigation of the 15fmer polypeptide. Fig. 6.8 presents the time dependent structure of the 15fmer β-sheets and fig. 6.9 shows topography images with higher resolution. In tab. 6.3, the determined evaluation data for the 15fmer polypeptides are listed.

Fig. 6.8: Time dependent change of 15fmer β-sheet structure on mica; scale bar: 250 nm; (a) sample "day 1", height scale: 3 nm; (b) sample "day 2", height scale: 3 nm; (c) sample "day 5", height scale: 9 nm; (d) sample "day 12", height scale: 12 nm; (e) sample "24 months", height scale: 3 nm.

Also, the AFM images of this 15fmer β-sheet show that amount and average length of β-sheets generally increase with the age of the polypeptide stock solution. In sample "day 1" little circular structures are visible. But for sample "day 2", some longer β-sheets already are present which was not the case for the 11fmer polypeptide. With increasing
Fig. 6.9: Typical 15fmer β-sheets (sample "24 months") on mica; (a) Scale bar: 250 nm, height scale: 3 nm; (b) Scale bar: 25 nm, height scale: 10 nm.

Tab. 6.3: Structural data of the 15fmer β-sheets for changing age of stock solution.

<table>
<thead>
<tr>
<th>time period</th>
<th>length/nm</th>
<th>pitch/nm</th>
<th>height/nm</th>
<th>width/nm</th>
<th>#</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>46 ± 20</td>
<td>21.4±1.5</td>
<td>2.8 ± 0.6</td>
<td>15.6±1.6</td>
<td>6</td>
</tr>
<tr>
<td>2 days</td>
<td>43 ± 6</td>
<td>16.7±0.6</td>
<td>3.3 ± 0.2</td>
<td>12.3±0.6</td>
<td>8</td>
</tr>
<tr>
<td>5 days</td>
<td>116 ± 33</td>
<td>15.3 ± 0.8</td>
<td>3.7 ± 0.1</td>
<td>9.1 ± 0.2</td>
<td>7</td>
</tr>
<tr>
<td>12 days</td>
<td>190±66</td>
<td>15.2 ± 1.3</td>
<td>2.9 ± 0.2</td>
<td>9.3 ± 0.8</td>
<td>4</td>
</tr>
<tr>
<td>24 months</td>
<td>171 ± 27</td>
<td>15.2 ± 1.3</td>
<td>3.5 ± 0.1</td>
<td>12.8±0.8</td>
<td>8</td>
</tr>
</tbody>
</table>

\[\text{age, the average length of the determined β-sheets also increases, whereas for this peptide the average lengths of sample "12 days"- and sample "24 months"- β-sheets do not differ significantly. Whereas this might be due to the fact that for the measurements of the 15fmer sample "24 months" only β-sheet strands were considered which did not exceed the scan field size. Also here, for all 15fmer samples the pitch length could be determined, but the helicity is not clearly defined.}\\
\text{Generally, it can be said that the 11fmer β-sheets grow faster and longer than the 15fmer β-sheets when considering the results of the samples "5 days", "12 days" and "24 months". This difference might be caused by the four more glutamine residues in which these polypeptides differ. When comparing the molecular structure of the 11fmer (see fig. 2.5) and the 15fmer (see fig. 2.6), it can be seen that these additional residues}\]
enlarge the total length of the peptide and also increase the total tilt of the amino acid sequence. This might denote that β-sheets made from this 15fmer peptide finally possess a maximum length, because of the changed interaction forces in between neighbouring peptides and also over the whole β-sheet. This might also cause the bending or curvature of the 11fmer β-sheets on larger scale (see fig. 6.9(a)). Furthermore, the helicity of the investigated β-sheets could not be detected for both polypeptides.

6.2.4 Résumé

We can summarise that five different polypeptides were designed which all form β-sheets. Whereas, the β-sheets formed by the 11mer-, 24mer-, 37mer polypeptides are not suitable for the use as nanowires since their lengths are only in the range of 100 nm and do not reach the micrometer range. Furthermore, the characteristics of these β-sheets do not differ significantly. Therefore and for economical reasons the 11mer polypeptide was used as starting point for further modifications.

In order to provide "free" electrons and thus to possibly improve the conductivity through the β-sheets, two polypeptides were designed which both contain an aromatic amino acid. For the 11fmer and 15fmer polypeptides the obtained β-sheets are both longer than the β-sheets of polypeptides without aromatic amino acid. Whereas for the β-sheets of the 11fmer this difference is significant and the length reaches the micrometer range. Furthermore, we found out that the length of the formed β-sheets is depending on the age of the polypeptide stock solution.

Finally, we can conclude that the 11fmer polypeptide seems to be the ideal candidate for further measurements. The next steps which have to be taken are conductivity measurements and additional characterisation experiments. The conductivity measurements have to prove if the 11fmer β-sheets can be used as nanowires. Furthermore, additional characterisation measurements are important as basis for statistical data evaluation.

6.3 Transmembrane proteins

In order to perform high resolution AFM measurements on the protein FhuA it is important, as it was already pointed out, to fulfil certain conditions.
6 Results and Discussion

- Optimisation of the biochemical sample preparation process
- Protein crystallisation and optimisation
- Optimisation of AFM imaging on a test system: bacteriorhodopsin

6.3.1 Biochemical sample preparation

FhuA cloning and expression were performed in the laboratory of Prof. U. Schwaneberg. Details are published in [86].

The starting point of this work is a *E. coli* cell pellet which contains the over-expressed protein in the outer membrane. In this section, all steps of the processing such as protein extraction, purification and control methods are described. All buffers are prepared with deionised water, filtered and additionally degassed for using the HPLC. The detailed protocols are given in the appendix.

Protein extraction

The cells were resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 2.5 mM MgCl₂, 0.1 mM CaCl₂). RNase and DNase were added to catalyse the degradation of RNA and DNA. To prevent degradation of the proteins the protease inhibitor PMSF (phenylmethanesulfonyl fluoride) was used. The solution had to be cooled on ice. The next step was the disruption of the cells using a high-pressure homogeniser at 1200 bar. This suspension was mixed with extraction buffer (20 mM Tris-HCl, pH 8.0, 2.5 mM MgCl₂, 0.1 mM CaCl₂, 2% Triton X-100) and incubated at room temperature for 30 min. In this extraction step lipids and proteins which are loosely bound to the membrane are removed. The solution was vortexed for 1 min every 5 minutes. Centrifugation for 45 min at 48384 × g, 4°C was used to separate the membrane debris. The pellet was washed with water before it was resuspended in solubilisation buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% OPOE) and incubated for 1 h at 37°C, shaking at 150 rpm. Another centrifugation step (45 min at 48384 × g, 4°C) was used to separate the cell membrane. The pellet was washed with water and stored at -20°C for further protein extraction. The supernatant contained the solubilised protein FhuA. The sample was concentrated by ultrafiltration (*Centricon YM-30*, Millipore) and SDS-PAGE was performed.
In order to directly repeat an already published protocol [20], the protein extraction procedure was also carried out using another detergent and a different buffer composition (buffer composition: 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% OG). This protocol was disregarded because of economical reasons since the achieved results for both extraction buffers were comparable. The detailed protocols are given in the appendix D.

![Fig. 6.10: SDS-PAGE control of protein extraction; (1)=protein ladder, (2)=protein sample.](image)

In fig. 6.10 the control gel of the protein extraction is shown. In lane (1) the protein ladder is loaded and in lane (2) the concentrated protein extraction sample. It can be seen that one of the main components of the solution is a protein with a size of about 80 kDa which is close to FhuA (78.9 kDa). But this solution contains a large variety of other proteins and so protein purification is of high importance.

**Protein purification**

The protein purification procedure was carried out by basically following [20, 19]. Whereas, the chromatofocusing step of this protocol was replaced by a second anion exchange chromatography, because this equipment was not available. As final step, a size exclusion chromatography was performed. The results of each purification step were monitored by SDS-PAGE of the relevant concentrated fractions. In the following section, the results of this protein purification process are presented, whereas the detailed protocols are given in appendix E.
6 Results and Discussion

Anion exchange chromatography

The sample was loaded onto a self-packed anion exchange column (Toyopearl SuperQ 650C, Tosoh Bioscience) which was equilibrated with buffer A (25 mM Tris-HCl, pH 7.8, 20 mM EDTA, 1% OPOE). The proteins were eluted with a linear gradient of buffer B (25 mM Tris-HCl, pH 7.8, 20 mM EDTA, 1 M NaCl, 1% OPOE). The buffer of the protein containing fractions was exchanged with buffer A using ultrafiltration (Microcon YM-10, Millipore). Those fractions with highest protein content and purity were subjected to dialysis and the second purification step repeating AEC with the same conditions as before.

1st purification step

The chromatograms of all samples show similar features, but are never identical, because the composition of each sample differs slightly. Fig. 6.11 shows a typical chromatogram of the first AEC.

![Chromatogram of 1st AEC](image)

**Fig. 6.11:** Chromatogram of 1st AEC; blue line: protein concentration, green line: % B in elution buffer, brown line: conductivity eluted sample, pink marker: sample injection, red markers: fractions of eluted sample.
The chromatogram shows the protein concentration of the eluted sample in arbitrary units (blue line), %B in elution buffer (dotted green line), the conductivity of the eluted sample (dotted brown line), the sample injection (pink marker) and the fractions of the eluted sample (red markers). It can be seen that the amount of protein which does not bind to the column material is higher than the amount of attached protein - the so called flow through. Furthermore, it seems that the flow through is not completely washed out when the salt gradient is started. Increasing the washing volume will prevent this. In the case there is already one protein eluted, this is masked by the flow through and can not be detected. At a conductivity of $\sigma=5.8\, \text{mS/cm}$, protein elution starts and ends at $\sigma=15.7\, \text{mS/cm}$ resulting in a broad peak (peak 1, fractions [7-14]) indicating a high protein concentration of these fractions. With the buffer conductivity ranging from $\sigma=18.9\, \text{mS/cm}$ to $27\, \text{mS/cm}$, a second peak (peak 2, fractions [17+18]) occurs indicating that these fractions possess a significantly lower protein concentration. From this first anion exchange separation, the fractions [7+8], [9-11], [12-14] and [17+18] are selected. Fig. 6.12 shows the resulting SDS-PAGE control. The loading order of the gel is: (1)=protein ladder, (3)=fractions [7+8], (4)=fractions [9-11], (5)=fractions [12-14], (6)=fractions [17+18].

Fig. 6.12: SDS gel result of 1st AEC; (1)=protein ladder, (3)=fractions [7+8], (4)=fractions [9-11], (5)=fractions [12-14], (6)=fractions [17+18].

It can be seen that fractions [17+18] are relatively pure, i.e. a clear band at about 80 kDa (FhuA) and a few bands of other proteins with lower intensity. Whereas, the fractions [9-11] and [12-14] show a broad band of FhuA, but also contain are mixture
6 Results and Discussion

of several proteins in higher concentration. The fractions (7+8) seem to be relatively pure, but are contaminated with proteins of lower weight at approximately the same concentration. Therefore, the purest fractions (17+18) are favoured for the second purification step.

2nd purification step

Fig. 6.13 shows the result of the second run of the AEC for the sample fractions (17+18).

![Chromatogram of 2nd AEC](image)

**Fig. 6.13:** Chromatogram of 2nd AEC; blue line: protein concentration, green line: % B in elution buffer, brown line: conductivity eluted sample, pink marker: sample injection, red markers: fractions of eluted sample.

Here, the protein elution starts at a conductivity of $\sigma=4.6 \text{ mS/cm}$ and this peak ends at $\sigma=15.7 \text{ mS/cm}$. Such a broad peak (peak 1, fractions (7-13)) is usually a successive elution of different proteins. A distinct second peak (peak 2, fraction 18) occurs at a conductivity ranging from $\sigma=20.3 \text{ mS/cm}$ to $29.4 \text{ mS/cm}$. The protein concentration of these fractions is higher than in all other fractions.

For the control the following fractions are loaded on the gel: (1)=fraction 7, (2)=fraction 8, (3)=fraction 9, (4)=protein ladder, (5)=fraction 10, (6)=fraction 11, (7)=fraction
6.3 Transmembrane proteins

12, (8)=fraction 13, (9)=fraction 18. The result is shown in fig. 6.14.

![Image](image.png)

**Fig. 6.14:** SDS gel result of 2nd AEC; (1)=fraction 7, (2)=fraction 8, (3)=fraction 9, (4)=protein ladder, (5)=fraction 10, (6)=fraction 11, (7)=fraction 12, (8)=fraction 13, (9)=fraction 18.

It seems that fractions 7, 8, 9 are relatively pure with a slight double band at about 80 kDa, whereas fraction 8 possesses the highest protein concentration. The protein size and purity of the content of fraction 10 is comparable to the first fractions, but here the protein concentration is quite low. In fraction 11 several proteins are eluted whereas here the FhuA concentration is negligible in comparison to the proteins of lower molecular weight. Surprisingly, the content of fraction 18 is so low concentrated that no protein band can be detected. Finally, the fractions 7, 8, 9 are selected for the SEC.

**Size exclusion chromatography**

For the performance of the final purification step the purest fractions of the second AEC were selected. The sample was loaded onto a self-packed size exclusion column (Superose 12, Amersham Bioscience). For equilibration and sampling buffer C (25 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% OPOE) was used. The most purified fractions were selected and the protein concentration was determined by BCA assay or the Nanodrop spectrophotometer.
3\textsuperscript{rd} purification step

In fig. 6.15, the chromatogram of the SEC for the fractions \((7, 8, 9)\) from the 2\textsuperscript{nd} AEC step is presented.

![Size exclusion chromatogram](image)

**Fig. 6.15**: Size exclusion chromatogram; blue line: protein concentration, brown line: conductivity eluted sample, pink marker: sample injection, red markers: fractions of eluted sample.

The chromatogram shows the protein concentration of the eluted sample in arbitrary units (blue line), the conductivity of the eluted sample (dotted brown line), the sample injection (pink marker) and the fractions of the eluted sample (red markers). With a buffer volume of 16 ml (fraction 32), the particle elution starts. This first peak (peak 1, fractions (32-35)) is small and flat compared to the following very distinct peak (peak 2, fractions (36-40)). In the following, three more peaks are detected (peak 3, fractions (44-47); peak 4, fractions (53-54); peak 5, fractions (56-58)) before with a buffer volume of 31 ml the smallest molecules (EDTA-complexes) are eluted. Its elution is indicated by the drastic change in conductivity (fractions (60-61)).

The control gels of the relevant fractions showed that the protein content of peak 3, peak 4 and peak 5 is below the detection limit. In fig. 6.16, the fractions of peak 1 and peak 2 are loaded. The loading order of the gels is as follows: (1)=fraction
32, (2)=fraction 33, (3)=fraction 34, (4)=fraction 35, (5)=fraction 36, (6)=fraction 37, (7)=protein ladder, (8)=fraction 38, (9)=fraction 39, (10)=fraction 40.

![Fig. 6.16: SDS gel result of SEC; (1)=fraction 32, (2)=fraction 33, (3)=fraction 34, (4)=fraction 35, (5)=fraction 36, (6)=fraction 37, (7)=protein ladder, (8)=fraction 38, (9)=fraction 39, (10)=fraction 40.](image)

The control indicates that SEC fraction 32 contains the purest FhuA sample. Fraction 33 is contaminated with a large amount of proteins of lower molecular weight and also fractions 34-37 contain smaller proteins. All other fractions did not contain proteins in an amount that could be visualised with the silver staining method.

Due to the small volume of the sample (only 80 µl after concentration), the protein concentration of this sample was determined as c=0.6 mg/ml using the Nanodrop machine, whereas the BCA assay usually provides more reliable data.

**Résumé - Biochemical sample preparation**

Within this work, at least seven protein extractions and complete protein purification runs were carried out. Within the first experiments, problems (i.e. buffer composition, change of conditions, fraction size, handling of fractions) of the extraction and purification were detected and successively solved. Finally, it can be stated that the purification process for the protein FhuA is established. A general problem of this process, which
still needs improvement, is the low efficiency. Starting point is a cell culture with a volume of 1.2l and after harvesting this results in a cell pellet with a mass of about 4 g. This cell pellet is used for the protein extraction process and after the purification typically a sample volume of 80 µl - 400 µl with a protein concentration of about 0.3 mg/ml to 0.6 mg/ml is achieved. Here, the most effective improvement could be achieved by an optimisation of the protein extraction procedure. Furthermore, it must be considered that the protein extraction and purification processes are very time consuming procedures and so it takes 1-2 weeks to receive a purified sample.

6.3.2 Protein crystallisation

TEM control of protein solution

As already indicated, in order to achieve good results it is useful to control the quality of the protein solution with the TEM before the protein crystallisation is started. Besides the fact that the protein solution has to be purified, it is also important that the proteins are completely solubilised and that a homogenous protein distribution is present. This control is a quick quality check, but it is not mandatory to perform it before a crystallisation attempt is started.

For this check, the solution is diluted to a protein concentration of about 20 µg/ml and a droplet of 4 µl is deposited onto the TEM grid and protein staining is done. The detailed TEM sample preparation is given in section 5.1.2. Fig. 6.17 shows TEM pictures of (a) a protein solution which possesses suitable quality for a protein crystallisation as well as (b) an example of protein aggregates which are not sufficient for crystallisation experiments.
6.3 Transmembrane proteins

Fig. 6.17: TEM images of solubilised proteins; (a) single proteins in solution – suitable for crystallisation; (b) aggregated proteins in solution – not sufficient for crystallisation.

Due to experimental problems, i.e. grid cleaning, it was not possible to achieve satisfying TEM pictures of the protein solution. Therefore, example images are shown which were provided by Dr. Mohamed Chami, Biozentrum Basel. Fig. 6.17 (a) shows a purified FhuA sample with which a protein crystallisation was successfully performed while (b) shows the quality control of one of our own earlier, not purified FhuA samples which did not crystallise.

Crystallisation procedure

For the execution of the protein crystallisation, we followed the procedure as described by [30, 61] as well as direct advice of Dr. D. Lévy and Dr. M. Chami. Liposomes were prepared from *E. coli* lipid extract in buffer C without detergent (25 mM Tris-HCl, pH 7.2, 150 mM NaCl) using a tip sonicator (*UP 200H, Hielscher Ultrasonics GmbH*). The protein solution and the liposome solution were mixed gently and equilibrated at room temperature for 30 min. The mainly used lipid to protein-ratio was 0.5 (w/w) but also the ratios 0.25, 0.4 and 0.7 were tested. In order to increase the crystal size OTG (final concentration of 20 mM) was added. After addition of OTG, the solution was incubated for 30 min at the same conditions. For detergent removal, dialysis was performed for 72 h at 4°C in 11 detergent free buffer. An alternative method was the treatment with three
successive additions of Bio-Beads SM-2 at room temperature and a total incubation time of 5 h. The detailed protocols are given in appendix F.

**TEM control of protein crystallisation**

TEM is a convenient method to control performance and quality of the protein crystallisation. The TEM samples were prepared as described in chapter 5.1.2, but the measurements did not give the expected results. Also here the problems were most probably related to grid preparation. In order to have an impression how a crystalline sample looks like visualised by TEM, an example is shown in fig. 6.18. This sample was prepared by Dr. Mohamed Chami as proof of principle experiment for control purposes. He also provided this TEM image. This image shows the features which describe a successful crystallisation, i.e. the outer shape and the inner crystalline structures. For this example the crystalline structure can be easily identified, but it is not expanded over the complete patch since the crystallisation procedure was aborted.

![Crystalline structures](image)

*Fig. 6.18:* FhuA reconstituted in *E.coli* lipids, vesicle with highlighted crystalline structures; scale bar: 250 nm.

Since the existence of protein crystals could not be shown by TEM measurements, AFM measurements should at least give an indication if the proteins were reconstituted into lipids and typical lipid patches should be visible. Therefore, AFM measurements were performed and when protein crystals were present, these could then be resolved by high resolution images.
AFM sample preparation and imaging conditions

From the solution which contained the reconstituted FhuA aliquots were taken and diluted in buffer solution 1:50 or 1:30 (25 mM Tris-HCl, pH 7.2, 150 mM NaCl). Mica was used as substrate, freshly cleaved and incubated for 30 min with 50 mM MgCl₂ solution. 25 μl of the prepared FhuA crystal solution sample were incubated for 70 min at room temperature. The sample was rinsed with 3 x 100 μl buffer solution. Contact mode AFM measurements were performed in buffer solution (25 mM Tris-HCl, pH 7.2, 150 mM NaCl; 20 mM Tris-HCl, pH 7.8, 150 mM KCl, 5 mM MgCl₂) using the MSCT tip and the fluid cell. Due to thermal drift, the applied force was constantly adjusted and kept as low as possible for stable imaging.

AFM control of protein crystallisation

Fig. 6.19 shows a representative topography image of the reconstituted FhuA patches. The size of these patches typically range from 1 μm to 4 μm. The average height of different patches recorded under the same imaging conditions is (2.7 ± 1.5) nm which is just in agreement with the typical height of an *E. coli* lipid bilayer (≈4-6 nm, in 20 mM Tris-HCl, pH 7.8, 150 mM KCl, 5 mM MgCl₂). An inner structure is not detected for these first measurements.

Fig. 6.19: Representative AFM image of a reconstituted FhuA patch; scale bar: 2.5 μm.
6 Results and Discussion

Résumé - protein crystallisation

Due to the small volume of the purified FhuA samples, only a few variables of the crystallisation settings were modified up to now. These are for example the lipid to protein ratio, the method of detergent removal and the dialysis conditions. AFM topography measurements confirmed that the protein FhuA was reconstituted in *E. coli* lipids, but crystalline structures were not observed within the first measurements. It is not clear if the correct crystallisation parameters were found. Furthermore, it might be difficult to distinguish between "high resolution cannot be realised" and "no crystalline structure present" by AFM measurements. Therefore, it is necessary to solve the problems with TEM imaging in order to possess a reliable tool with which a quick control of protein crystals is possible.

6.3.3 Test system bacteriorhodopsin

In order to gain experience for the optimisation of the AFM measurement conditions, bacteriorhodopsin is investigated as test system, following the procedure given in [81]. In this publication the rational exchange of single buffer parameters is combined with force spectroscopy and topography measurements. These measurements for different types of settings are the basis for finding the optimal imaging conditions.

Sample preparation and imaging conditions

The BR was used as provided from the supplier. A stock solution with a BR concentration of 1 mg/ml was prepared using deionised water and stored at 4°C. From this solution aliquots were taken and diluted in the chosen buffer to a final concentration of c = 20 μg/ml. For sample preparation 30 μl BR solution were incubated at freshly cleaved mica for 30 min at room temperature and then rinsed with 4 × 100 μl buffer solution. Contact mode measurements were performed in buffer solution using the fluid cell set-up. Due to drift of the cantilever, the applied measurement force was constantly adjusted and kept as low as possible for stable imaging. Also tapping mode measurements were tested, but here stable imaging conditions were possible and thus this mode was not considered any further.
In order to find optimal imaging conditions, a large variety of sample preparation conditions was tested: sample concentrations $c= 5 \mu g/ml$, $20 \mu g/ml$ and $50 \mu g/ml$; incubation times $t=15-75$ min; incubation temperature $T=4^\circ C$ - room temperature. The tested buffer compositions usually contained 10 mM Tris-HCl, pH 7.6 supplemented with a combination of sodium chloride, potassium chloride and magnesium chloride. (+10 mM MgCl$_2$; +50 mM MgCl$_2$; +50 mM KCl; +150 mM KCl; +300 mM KCl; +50 mM NaCl; +150 mM NaCl; +300 mM NaCl; +10 mM MgCl$_2$, 50 mM KCl; +10 mM MgCl$_2$, 150 mM KCl; +10 mM MgCl$_2$, 300 mM KCl). Additionally, different incubation and imaging conditions were tested (incubation: +150 mM KCl, imaging: +50 mM KCl; incubation: +300 mM KCl, imaging: +50 mM KCl). Finally, for measurements on BR the best results were achieved for samples prepared with high salt concentrations i.e. 10 mM Tris-HCl, pH 7.6, 300 mM KCl as well as for incubation with buffer consisting of 20 mM Tris-HCl, pH 7.8, 150 mM KCl, 50 mM MgCl$_2$ and imaging with buffer containing 20 mM Tris-HCl, pH 7.8, 150 mM KCl, 5 mM MgCl$_2$.

**AFM results**

In fig. 6.20(a), a typical bacteriorhodopsin patch is shown. The usual patch size varies between $\approx 0.4 \mu m^2$ and $\approx 2 \mu m^2$ with a height of 5 nm to 7 nm depending on the actual imaging conditions (i.e. scanning force, buffer composition). This height is in agreement with literature giving a height of $(5.6 \pm 0.2)$ nm [79]. These measured patches possess a distinct size and also the internal structure can be seen. When the scan field size is decreased, the inner structure of the BR can be resolved. An exemplary result is shown in fig. 6.20(b). Here, it is important to gradually adjust the applied scanning force, because of the occurring drift.
6 Results and Discussion

Fig. 6.20: Typical BR patch in buffer solution; (a) Scale bar: 500 nm, height scale: 11 nm; (b) Scale bar: 25 nm, height scale: 2 nm.

In fig. 6.21, the correlation averaging process which is carried out for data analysis is demonstrated. Subfigure (a) shows the flattened topography image of the inner structure of a BR patch - initial fig. 6.20(b). In (b) the pink triangle indicates the unit cell which is used as basis for the correlation averaging. This unit cell is either defined by the user or the evaluation programme. The blue triangles are highlighting the substructure of the unit cell. After the processing the average over all unit cells is displayed as shown in subfigure (c). Here, the identical pink and blue triangles highlight the unit cell and its substructure. Subfigure (d) shows the cross correlation image which is achieved by comparing the averaged unit cell (c) to the overall structure shown in (a).
Fig. 6.21: Correlation averaging process; (a) BR patch, scale bar: 25 nm, height scale: 2 nm (b) Chosen unit cell (pink marker) with substructure (blue marker), scale bar: 2.5 nm, height: 0.35 nm; (c) Averaged unit cell (pink marker) showing substructure (blue marker), scale bar: 2.5 nm, height: 0.21 nm; (d) Cross correlation image of initial image (a) using averaged unit cell (c), scale bar: 25 nm, height: 100 nm.

From the very regular and not blurred cross correlation image (fig. 6.21(d)), one can conclude that the lattice constant determined from this topography image is very reliable. In agreement with literature [82, 48], the lattice constants of the trigonal lattice unit cell...
were determined to be $a = b = 6.3 \pm 0.1\text{ nm}$ (lit. $a = b = 6.2 \pm 0.2\text{ nm}$). Additionally, the resolved topographical details of the averaged unit cell (fig. 6.21(c)) are in good agreement with similarly processed measurements of the BR native cytoplasmic surface by Müller et al. [83]. Furthermore, we can also detect that the structure of the BR is changing depending on the strength of the scanning force exerted by the AFM tip. This influence is demonstrated in fig. 6.22.

![AFM images of BR surface](image)

**Fig. 6.22:** Scan of BR performed with varying scanning force; (left) total image, scale bar: 50 nm, height scale: 3 nm; (top) higher force, partial zoom, scale bar: 5 nm, height scale: 0.25 nm; (down) lower force, partial zoom, scale bar: 5 nm, height scale: 0.12 nm.

Here, a force curve was taken directly before the topography scan started and indicated a scan force of $F=110\text{ pN}$ at the begin of the image. Immediately after finishing the topography image, another force curve was taken and a scan force of $F=70\text{ pN}$ was determined. During the topography scan, the set-point is manually adjusted in order to achieve best image quality. Therefore, it is not possible to determine the force values at the specific areas of interest. With an exchange of the scanning modes, the topography images are always started anew at that particular coordinates and former topography
data is lost. Therefore, the forces at the zoom areas are justified approximations, but not absolute values.

Résumé - bacteriorhodopsin

We can summarise that bacteriorhodopsin was successfully used as test system for high resolution AFM measurements. In order to find optimal experimental settings, various sample preparation methods and imaging conditions were tested. The next step was the application of image processing for data analysis, i.e. correlation averaging which finally allowed to reveal structural details of the investigated objects. To conclude we were able to reproduce published results such as the BR lattice constant and the influence of the scanning force to the BR surface structure.

Using this knowledge, we are confident to adapt the sample preparation technique and the data evaluation method to successfully investigate two-dimensional FhuA protein crystals.
CHAPTER
SEVEN

Summary

In this thesis, several preparation methods for AFM investigations have been developed to provide optimised conditions for high resolution imaging of different biomolecular systems. The first investigation subject dealt with imaging of the nanocontainer system. Here, polymer supported liposomes were characterised by AFM measurements. The second subject of interest was also related to the nanocontainer system - the channel protein. In order to find a sample preparation method which allows the characterisation of the transmembrane protein FhuA by AFM, several methods were discussed. Finally, following the aim of 2D protein crystallisation, a robust protocol for the protein purification was developed. First crystallisation experiments were performed, but it is not clear if crystals were formed, since up to date only poor resolution AFM results were achieved. Nevertheless, high resolution measurements were successfully performed on the membrane protein bacteriorhodopsin which was used as a test system. The achieved results are in agreement with already published results, showing the potential to investigate FhuA. Furthermore, with respect to novel approaches in nanoelectronics, new types of β-sheet forming artificial polypeptides were designed and characterised for their suitability towards use as nanoelectronic wires. A promising candidate was identified and further measurements are pending.

The next challenges related to this thesis are the optimisation of the 2D crystallisation of FhuA and then the characterisation of these crystals by high resolution AFM
measurements. Furthermore, for the $\beta$-sheets it is important to perform conductivity measurements for the 11mer polypeptide and then, to do further characterisational AFM measurements for a reliable statistical data analysis.
APPENDIX

ABBREVIATIONS

AFM atomic force microscope
APS ammoniumpersulfate
BCA assay bicinchoninic acid assay
BR bacteriorhodopsin
BSA bovine serum albumine
DOPC dioleoyl-phosphatidylcholine
DPPC dipalmitoyl-phosphatidylcholine
DPPE 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol
DTT 1,4-dithio-DL-threitol
E.coli Escherichia coli
EDTA ethylene diaminetetraacetate
FhuA ferric hydroxamate uptake protein component A
A Abbreviations

HOPG highly ordered pyrolytic graphite
HPLC high performance liquid chromatography
OG octyl-β-D-glucopyranoside
OmpF outer membrane protein F
OPOE octyl-polyoxoethylene
OTG octyl-β-D-1-thioglucoanopyranoside
PBS phosphate buffered saline, pH 7.8
PMSF phenylmethanesulfonyl fluoride
SDS sodium dodecyl sulfate
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEM transmission electron microscope
TEMED tetramethylethylenediamine
THF tetrahydrofuran
Tris tris(hydroxymethyl)aminomethane
APPENDIX

B

Instruments

- AFM: MultiMode AFM with Nanoscope IIIa controller, Veeco Instruments GmbH, Germany; equipped with a Picoforce System and Quadrex Box
- TEM: EM 900, Carl Zeiss AG, Germany; equipped with a CCD-camera
- HPLC: ÄKTA purifier system, Amersham Bioscience - GE Healthcare Europe GmbH, München, Germany
- Nanodrop: NanoDrop ND-1000 Spectrophotometer, Fisher Scientific GmbH, Schwerte, Germany
- centrifuges:
  - High-speed: Avanti J-20XP, Beckman Coulter, Fullerton, USA
  - Use of Centricons: Eppendorf 5810R, Eppendorf AG, Hamburg, Germany
  - Use of Microcons: Eppendorf 5415D / Eppendorf 5415R, Eppendorf AG, Hamburg, Germany
- high-pressure homogenizer: Emulsiflex-C3, Avestin Inc., Ottawa, Canada
- tip sonicator: UP 200H, Hielscher Ultrasonics GmbH, Teltow, Germany
- Plasma cleaner: Model 1020 Plasma Cleaner, E.A. Fischione Instruments, Inc., Export, USA
Materials

- Bacteriorhodopsin: Sigma B0184/Fluka 11708, Sigma-Aldrich Chemie, Taufkirchen, Germany
- β-sheets: synthesized upon request, Biosynthan GmbH, Berlin, Germany
- Chemicals: analytical reagent grade or purer quality;
  Sigma-Aldrich Chemie, Taufkirchen, Germany and Applichem, Darmstadt, Germany
- Lipids: Sigma-Aldrich Chemie, Taufkirchen, Germany and Avanti Lipids, Inc., USA
- BioBeads: Bio beads SM-2, Bio-Rad Laboratories GmbH, München, Germany
- Dialysis tubing: Spectra/Por Membranes, MWCO 12-14 kDa, Spectrum Laboratories, Inc., USA
- β-sheets: synthesised upon request by Biosyntan GmbH, Berlin, Germany
- Toyopearl SuperQ 650C: Tosoh Bioscience GmbH, Stuttgart, Germany
- Superose 12: Amersham Bioscience - GE Healthcare Europe GmbH, München, Germany
- Centricons/Microcons: Millipore Corporation, Billerica, USA
- BCA Protein Assay Kit: Pierce Biotechnology, Rockford, USA
Protein extraction protocol

- Cell suspension
  For the resuspension of a cell pellet from 200 ml culture media:
  - Add 10 ml lysis buffer (20 mM Tris-HCl, pH 8.0, 2.5 mM MgCl₂, 0.1 mM CaCl₂)
  - Add 100 μl RNase (freshly prepared solution; c=10 mg/ml)
  - Add 10 μl DNase (freshly prepared solution; c=10 mg/ml)
  - Add 0.1 μl PMSF (from 0.1 M stock solution in 100% Ethanol); final concentration 1 μM
  - Resuspend pellet using a homogenizer or a pipet until homogeneity is achieved
  - Keep solution on ice

- Disrupt cell walls
  Treat solution:
  - 2 × high-pressure homogenizer at 1200 bar
  or
  - 10 × 15 cycles tip sonicator at amplitude=40%, cycle=0.55; permanently on ice
**Protein extraction protocol**

- Remove lipids and other proteins
  - Add *extraction buffer* (20 mM Tris-HCl, pH 8.0, 2.5 mM MgCl₂, 0.1 mM CaCl₂, 20% Triton X-100) - 1 ml per 10 ml cell suspension solution
  - 30 min incubation at room temperature, 1 min. vortexing all 5 minutes
  - 45 min centrifugation at 45384 x g, 4°C
  - Pellet contains broken cell walls with membrane proteins
  - Supernatant contains proteins and lipids loosely bound to cell membrane
  - Keep 100 μl of supernatant for control

- **FhuA extraction**
  - Wash pellet with water; 3 x 15 ml gentle shaking, not resuspending
  - Add 9 ml *solubilisation buffer* (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% OPOE) to each vial
  - Add 0.09 μl PMSF (from 0.1 M stock solution in 100% ethanol); final concentration 1 μM
  - Resuspend pellet
  - 1 h incubation at 37°C, shaking at 150 rpm
  - 45 min centrifugation at 45384 x g, 4°C
  - Supernatant contains FhuA
  - Wash pellet with water; 3 x 15 ml gentle shaking, not resuspending; freeze pellet

- Sample concentration: 20 min centrifugation at 3220 x g, 4°C *Centricon YM-30,*

Protein purification protocol

The protein purification procedure is adopted from [20, 19], but modified, due to the available equipment. The chromatofocusing step is replaced by a second anion exchange chromatography step. So protein purification is implemented by the use of two runs of anion exchange chromatography and one size exclusion chromatography.

Anion exchange chromatography

Buffer A: 25 mM Tris-HCl, pH 7.8, 20 mM EDTA, 1% OPOE
Buffer B: 25 mM Tris-HCl, pH 7.8, 20 mM EDTA, 1 M NaCl, 1% OPOE
Anion exchange column: self-packed; material: Toyopearl SuperQ 650C, Tosoh Bioscience
HPLC: run program anionexchange within the ÄKTA program Unicorn
HPLC procedure:
- Wash column using water
- Wash column using buffer A
- Prepare glass vials for fraction collection
- Load sample into 'loading loop'
Protein purification protocol

- Run the programme
  - Sample is injected and thus loaded on the column
  - Unbound sample is washed out using buffer A
  - Amount of buffer B is gradually increasing
  - Fraction collection starts; fraction size is 0.5ml
  - Protein concentration and conductivity is measured

- Collect relevant fractions

- Wash column using buffer B

- Wash column using water

- Wash column using ethanol

Washing and concentration of fractions: buffer A, Microcon YM-10, centrifugation filters; 20 min, 4°C, ~9448 × g (13000 rpm)

Perform SDS-PAGE to monitor the protein content of all relevant fractions

This anion exchange chromatography step is repeated with sample fractions which are relatively pure. Before this second run is carried out it is important to exchange the buffer so that the samples are loaded in buffer A otherwise the proteins will not bind to the column due to the high salt concentration.

After the second AEC step the purest fractions are used for the following size exclusion chromatography step. Here, the it is not necessary to exchange the buffer solution but a sample concentration is recommended since the sample volume which can be loaded for the SEC is limited.
Size exclusion chromatography

Buffer C: 25 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% OPOE
Size exclusion column: self-packed; material: Superose12, Amersham Bioscience.
HPLC: run programme sizeexclusion within the ÄKTA program Unicorn.
HPLC procedure:

- Wash column using water
- Wash column using buffer C
- Prepare glass vials for fraction collection
- Load sample into 'loading loop'
- Run the programme
  - Sample is injected and thus loaded on the column
  - Fraction collection starts; fraction size is 0.5 ml
  - Column is flushed with buffer C
  - Proteins pass the column
  - Protein concentration and conductivity is measured
- Collect relevant fractions
- Wash column using water
- Wash column using ethanol

Concentrate fractions: *Microcon YM-10*, centrifugation filters; 20 min, 4°C, ~9448 ×g (13000 rpm)
Perform SDS-PAGE to monitor the protein content of all relevant fractions.
E Protein purification protocol
Protein crystallisation

- Use protein concentration of 1 mg/ml
- Wash Bio-Beads:
  - Bio-Beads + 3 ml methanol; shaking 10-15 min
  - Bio-Beads + 15 ml water; shaking 10-15 min
  - Bio-Beads + 3 ml methanol; shaking 10-15 min
  - Bio-Beads + 15 ml water; shaking 10-15 min
  - Bio-Beads + 15 ml water; shaking 10-15 min
  - keep Bio-Beads in water ~15 ml
- Prepare lipids:
  - *E. coli* lipids in chloroform
  - Evaporate chloroform, keep in exxsicator for 1 h
  - Solubilise lipids in water or buffer using tip sonicator; c=10 mg/ml
- FhuA-liposomes-equilibration
  - Prepare mixture in 2 ml glass vial or eppendorf tube
F Protein crystallisation

- Ideal lipid to protein-ratio: 0.5
- 30 min gentle stirring at room temperature

- Increase crystal size - add OTG to 20 mM final concentration
- Equilibration - 30 min gentle stirring at room temperature

- Detergent removal - version A
  - Add ~8 mg Bio-Beads
  - Gentle stirring at room temperature
  - Add ~8 mg Bio-Beads
  - Gentle stirring at room temperature
  - Add ~8 mg Bio-Beads
  - Gentle stirring at room temperature
  - Total incubation time ~5-6 h

- Detergent removal - version B
  - Prepare dialysis equipment
  - Fill protein solution into dialysis vial
  - Fill lipid solution into dialysis vial
  - Close dialysis vial with dialysis membrane and O-ring
  - Insert dialysis tubing into 1 l detergent-free buffer solution
  - Incubation time ~72 h at 4°C

- Pipet crystal solution off and transfer into a new vial
- Keep crystal solution at 4°C
TEM sample preparation

For TEM measurements a copper grid which is covered with a carbon film is used as sample substrate. The grid is cleaned and made hydrophilic with the help of a *Fischione* plasma cleaner using the following conditions: Ar-O₂ mixture of 16% O₂, cleaning for 150 seconds. The prepared grid is directly used for sample deposition. Sample preparation procedure:

- Prepare several water droplets (ca. 100μl) on a clean surface

- Give 4μl protein solution (c=(10-20)μg/ml) respectively protein crystal solution onto the grid; incubation time 1-2 min

- Remove solution using a tissue.

- Wash sample by dipping grid 3× into a water droplet

- Stain the proteins using 2× 3μl 1% uranyl acetate solution; keep grid vertical, drop staining solution onto grid from the side, directly remove staining solution with a tissue

- Wash tweezer with water
The polyacrylamide gel is usually prepared directly before use. This type of gel can also be stored in humid and cool conditions for approximately one week.

The polyacrylamide gel is composed out of the following components:

- acrylamide:bisacrylamide (0.292 g/ml : 0.008 g/ml) solution
- Tris-HCl (1.5 M, pH 8.8; 0.5 M, pH 6.8)
- H$_2$O
- 10% SDS solution
- 10% APS solution
- TEMED

Exact volumes depend on the number gels that are produced and can be found in [103].

Loading dye composition: 150 mM Tris-HCl, pH 6.8, 100 mM DTT, 6% SDS, 0.3% bromophenol blue, 30% glycerol

SDS-PAGE:

- Mix 10 µl sample + 5 µl loading dye
**H SDS-PAGE**

- Heat mixture 3 min at 95°C
- Prepare gel chamber set-up, fill with Tris-glycine buffer
- Load 10-12 µl of sample-dye solution
- Load 3 µl of prepared protein ladder
- Close chamber, connect to power supply
- Settings: 15 mA per gel, 80 min

**Staining of gel:**

- 10 min used dark destaining solution
- 10 min staining solution
- 10 min used dark destaining solution
- 30 min fresh destaining solution
- Put into storage solution or water


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