Novel single-molecule force spectroscopy approaches to characterize structure and stability of transmembrane proteins

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NOVEL SINGLE-MOLECULE FORCE SPECTROSCOPY
APPROACHES TO CHARACTERIZE STRUCTURE AND
STABILITY OF TRANSMEMBRANE PROTEINS

A thesis submitted to attain the degree of
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presented by
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Summary

Proteins are one of the four important groups of biological macromolecules that enter in the composition of living matter. Transmembrane (TM) proteins are an important class of proteins that are spanning cell membranes completely. Since TM proteins are involved in many vital functions, about half of all drug targets are TM proteins. Although 20 to 30% of all proteins are TM proteins, only about 1% of all solved protein structures comprise TM protein structures. This implies that protein structure determination with current methods is extremely challenging and some other techniques for studying TM proteins are desperately needed.

The atomic force microscope was invented about 30 years ago, initially, as a tool for obtaining high-resolution topographs of samples surfaces. However, it has evolved since then and found new applications in various fields. One of those is single molecule force spectroscopy (SMFS). It gives opportunity to apply force in a well-controlled fashion on single biopolymers such as proteins, ribonucleic acids and polysaccharides and to study their behavior under mechanical stress. From these experiments one can extract, for instance, important structural information on the stability of protein segments, as well as on the thermodynamic and kinetic parameters of the free energy profile of the proteins.

In order to perform SMFS on TM proteins, membranes in which TM proteins are being embedded are adsorbed on solid supports. This brought up an argument that the results of SMFS on TM proteins could be affected by the interactions or interference with the support. To eliminate this issue, we performed SMFS on TM proteins that are embedded in membranes freely spanning across nanoscopic pores. The analysis of SMFS data from freely spanning membrane required taking into account the deflection of the freely spanning membrane when force is applied on TM proteins. For the TM protein we studied, there were no significant differences between the unfolding distances and forces of structural segments of TM proteins from the freely spanning and directly supported membranes. This setup demonstrated for the first time, that SMFS can be used to study other TM proteins in a similar fashion and it opens new possibilities for experiments that were previously impossible.
Zusammenfassung


verwendet werden kann, um andere TM-Proteine auf gleiche Art und Weise zu untersuchen und eröffnet neue Möglichkeiten für Experimente, die bisher unmöglich waren.
"As a graduate student your most important responsibility to yourself is not just to learn some technical things ether experimental techniques or calculational techniques it is to get yourself into a field that is developing that is going to prosper in the next 5 to 10 years. If you get into such a field you are likely to grow with that field and that is the best situation. The most important contribution a professor can make to his graduate student is to launch him into the right area."

Interview to Simons Foundation Science Lives
18 “Commonality between Gauge theory and statistical mechanics”
https://www.simonsfoundation.org/science_lives_video/chen-ning-yang-2/

Chen-Ning Yang (1 October 1922 – )
1. **Cells, membranes and transmembrane proteins**

1.1 The cell as the fundamental unit of living systems

With the invention of the optical microscope around 1595 by the Jansens (father and son) [1] and its further development by Antonie van Leeuwenhoek and others, it became possible to look for “the secret of life” at a smaller scale. One of the first users of the optical microscope was Robert Hooke who observed piece of cork under the microscope and noticed compartments of similar shape and size. He called them cells. Hooke published his observations in 1665 in his book called “Micrographia”.

Today we know that the cell is a common characteristic of all living systems. Our body has about $10^{13}$ cells of more than 200 different types [2]. Moreover we carry about 10 times more bacterial cells than our own cells. Different types of cells have different functions and different shapes. For example *Escherichia coli* (*E. coli*), a bacterium in our gut has a cylindrical shape and is few microns in length [3]. At the same time, we have neuron in our body stretching from our feet to the spinal cord that is more than a meter in length. Despite these overwhelming differences, cells have similarities in their organization and building blocks. All of them have cell membranes, which separates the inner part of the cell (cytoplasm) from the environment. Eukaryotic cells contain organelles, which are also separated from the cytoplasm by membranes. Most of the cellular structures (membranes, cytoskeleton, cell organelles etc.) are composed of 4 types of molecules: proteins, lipids, polysaccharides and nucleic acids [3].

There is no fundamental difference between the atoms composing living matter and non-living matter. For instance, cells can be viewed as machines, which are “informed” what to do, how to respond to the external influences. According to our current understanding, the entire complexity of our body, our thoughts and that of rest of known life on Earth is an emergent phenomenon of a large number of interacting cells.
1.2 Membranes

We generally refer to a membrane as to an object in which two spatial dimensions are much larger than the third one. Simply it can be understood as something, which is long, wide and thin.

1.2.1 Cell membranes

With the invention of the transmission electron microscope (TEM) in 1932 by Max Knoll and Ernst Ruska [4] and its further development in 1958 it became possible to see the cell membrane [5]. Cell membrane structure was correctly determined by David Robertson [6] who did TEM studies of Schwann cells and proposed that the cell membrane is composed of a lipid bilayer and proteins.

\[ \text{Figure 1. Fluid mosaic model (A) and artistic view of the cell membrane (B).} \]

\[ ^* \text{Here and after the invention years are taken the years of the publications where the invention became publicly announced.} \]
One of the models which had success in describing the cell membrane was the fluid mosaic model [7, 8] (Figure 1A). This model describes the cell membrane as a fluid lipid bilayer with proteins embedded in it and assumes that the whole system is in a thermodynamic equilibrium. The next event, which changed our view of the cell membrane, was the discovery of lipid rafts [9-11], although there are still some disputes regarding existence [12]. Lipid rafts are relatively ordered domains mostly composed of sphingolipids, cholesterol and specific proteins. They are not in a thermodynamic equilibrium [13].

The cell membrane is composed of 3 types of molecules: lipids, proteins and polysaccharides. About half of the cell membrane mass is represented by membrane proteins. In most eukaryotic cells cholesterol constitutes about half of the number of lipid molecules of the cell membrane. The main functions of the cell membrane are selective permeability and direct involvement in cell–cell interaction. These functions are mostly maintained by membrane proteins, which will be discussed in 1.3.

1.2.2 Synthetic lipid membranes

To mimic the cell membrane, synthetic lipid membranes have been widely used [14, 15]. There are more than 500 types of lipids and all of them are amphiphilic molecules. They have hydrophobic tails composed of hydrocarbon chains and hydrophilic heads (Figure 2A). Due to their amphiphilic nature lipid molecules can self-assemble into a variety of structures depending on different factors (lipid concentration and type, temperature, solvent pH etc.) [16].

\[ \text{Figure 2. Schematic representations of a lipid molecule (A) and of a vertical cross section of a lipid bilayer (B).} \]
Irving Langmuir in 1917 suggested that lipids could assemble into a bilayer (Figure 2B). A lipid bilayer has a hydrophobic core and a hydrophilic surface. Again, depending on the lipid composition of the membrane and the external parameters, lipid bilayers can be in a liquid or in gel phase. In the liquid phase lipids are diffusing with translational diffusion coefficients of $10^{-9}$–$10^{-7}$ cm$^2$/s [17, 18]. Furthermore, lipids can also “jump” from one monolayer to another although this is a relatively rare event [19, 20]. Based on the abovementioned, a lipid bilayer should be understood as a two-dimensional liquid where lipids or objects embedded in the lipid bilayer are free to “swim” along the bilayer.

1.3 Transmembrane proteins

Proteins are polymers composed of amino acids and folded into specific three-dimensional structures. Proteins can be associated with membranes in various ways (Figure 3) [23]. An important class of membrane proteins is represented by transmembrane(TM) proteins. These are proteins that span the membrane completely (Figure 3).

TM proteins are involved in many vital functions such as transport of ions, water, urea and proteins in and out of the cells, electron transfer and light harvesting. More than half of known drug targets are TM proteins [24, 25].

Since the core of a lipid bilayer is hydrophobic it is energetically unfavorable to have polar groups exposed to the hydrophobic tails of the lipids. Hence these polar groups of the peptides should face each other and form hydrogen bonds. This is the reason why in the membrane core proteins are in α-helical or β-barrel conformation. Furthermore this hydrophobic effect can cause also a tilt of the proteins in the bilayer or a change in the bilayer thickness around the protein (Figure 4) in case of a mismatch between the thickness of the hydrophobic TM domain of the protein and the lipid bilayer core [26].

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*There are 20 common proteinogenic amino acids encoded in the genomes of organisms however due to post-translational modification more of them may occur [21, 22]. We still do not know whether this choice of amino acids as well as their left-handed chirality is a historical accident.*
Figure 3. Proteins can associate with lipid membranes in various ways. Proteins, which span the bilayer completely, are called transmembrane proteins.

Figure 4. Schematic examples of hydrophobic mismatch between TM proteins and lipid bilayer. The hydrophobic TM domain is in-between the dashed gray lines.
With respect to their structural units TM proteins can be classified into two groups: one composed of α-helices and one composed of β-sheets. α-helices and β-sheets are stabilized by hydrogen bonds between amino acids. These secondary structure units of proteins were first rigorously described in 1951 mostly by the efforts of Linus Pauling [27]. Interestingly β-barrel TM proteins have been found only in the outer membranes of some prokaryotes.

An example of an α-helical TM protein is bacteriorhodopsin (BR) (Figure 5A), a 27 kDa protein found in *Halobacterium salinarum*. An example of a β-barrel TM protein is outer membrane phospholipase A (OmpLA) (Figure 5B) a 31 kDa protein found in the outer membrane of gram-negative bacteria. Both proteins were visualized using data from the protein data bank (PDB)*.

![Figure 5](image_url)

**Figure 5.** Representatives of two groups of TM proteins in side view: (A) BR (PDB code 1FBB) [30] composed of 7 TM α-helices and (B) OmpLA (PDB code 1QDS) [31] composed of 12 antiparallel β-sheets. α-helices, β-sheets, single helical turns and loops are colored purple, yellow, blue and gray respectively. Loops are unstructured parts of polypeptide chain. Both proteins are facing extracellular side with their top.

Many TM proteins are functional only as oligomers. For example OmpLA can hydrolyze lipid molecules only as a dimer [32, 33]. In contrast, BR that is a light driven proton pump, can function as a monomer [34]. The seven helices of this protein enclose a retinal molecule, which induces a conformational change in the protein once it absorbs a photon. Due to this conformational change, the proton is being pumped after which BR

* Protein data bank (PDB) is an archive for structural data of proteins [28, 29].
accepts its initial conformation. This cycle repeats when new photon is being absorbed [35-38]. The study of the membrane of *Halobacterium salinarum* showed that almost half of it is covered with BR. BR molecules assemble together into 100-1000 nm patches [39, 40]. These patches have a distinctive purple color and are called purple membrane (PM). The purple color is originating from the retinal molecule in each BR. The studies of the PM structure showed that BRs are organized in a hierarchical order in PM. Three BRs are assembled in a trimer (Figure 6) [40-43]. There are 6 lipid molecules in the center and 24 surrounding each trimer [43-46]. However, the 75% of the PM mass is due to BRs and only 25% is due to lipids. The stability of a BR in trimer is higher compared to the stability of monomeric BR [47]. The next hierarchical step in the architecture of the PM is the arrangement of trimers into two-dimensional hexagonal lattices.

![Figure 6. Top view (from the cytoplasmic side) of BR trimer (PDB code 1BRR) [42] (lipids are not shown). Trimers are arranged into the hexagonal lattice in the native membrane of *Halobacterium salinarum*. α-helices, β-sheets and loops are colored purple, yellow and gray respectively.](image)

The arrangement of trimers, the association of three BRs into a trimer and the arrangement of the helices within the BR are all governed primarily by hydrophobic interactions. Mutations studies helped determine that the aromatic interaction between Tryptophan 12 and Phenylalanine 135 located at the outer rim of the BR trimer are responsible for intertrimer association into the hexagonal lattice. The association energy of trimer-trimer single interaction is around 0.9 kcal/mol in its absolute value [48-50].
1.3.1 Insertion and folding of TM proteins and hydrophobicity scales

Knowing the structure of the proteins is useful for understanding their function, the mechanisms of achieving this function and for structure based drug design. TEM, nuclear magnetic resonance spectroscopy [51] and X-ray crystallography are used to achieve this goal. The overwhelming majority (about 89 %) of the protein structures’ known today were resolved by X-ray crystallography. The X-ray diffraction method was invented in 1912-1913 by Max von Laue and Braggs (father and son) [52-54]. Later, researchers applied this method for revealing the structures of biological molecules such as nucleic acids [55-57] and proteins [58-65]. The structures are desired to be determined with less than 5 Å resolution i.e. the positions of atoms in the protein are desired to be determined with that resolution. The achievement of this result is a laborious task and can take decades for single protein structure determination even today with all our technological advancements [66, 67].

Protein structure determination is especially challenging for TM proteins above all due to the difficulty of obtaining large enough diffraction quality crystals from these proteins for late diffraction analysis [68-71]. Enough to mention that, today out of solved protein structures (104417) less than 0.97 % are TM protein structures (1008)”. This is a miserable number if one takes into account that 20 to 30 % of all proteins encoded in the genomes of both prokaryotes and eukaryotes are predicted to be TM proteins [72]. If the TM protein structures will be determined with the same methods, as it has been done so far, then by extrapolation it was guessed that by 2025 we will have around 2200 TM protein structures determined [73].

In contrast, the determination of the protein amino acid sequence is much easier [74, 75]. Moreover, protein sequences can be obtained from the nucleic acid sequence, which encodes it. Additionally there are no extra difficulties for TM protein sequencing. Due

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* By protein structure we mean its native i.e. functional three-dimensional structure (tertiary structure), unless otherwise mentioned. Clearly, atoms in protein are not static and hence the structure is not static, nevertheless in native state fluctuations of protein structure are small.

** Statistics on protein solved structures have been taken from PDB on 28.09.2015.
to this, we know about fifty million protein sequences'. There is a two orders of magnitude difference between the number of known protein structures and the number of known protein sequences. Furthermore, for TM proteins this gap is of 4 orders of magnitude. Since the determination of protein structures is such a challenging problem compared to protein sequencing and it stays so as far as there are no technological breakthroughs, the protein structure prediction from sequence became a useful alternative.

One of the most successful methods for protein structure prediction is the comparative structure prediction method [77-83]. The basic idea is to use the known protein structures in order to predict the unknown structures. This is a pattern recognition based technique and it was successful since there are families of structurally related proteins [84]. For example similarly to BR, guanine-nucleotide-binding protein coupled receptors (GPCRs)" have 7 TM α-helices. Nevertheless, they have different functions and, furthermore, they don’t have sequence similarities. In such cases, homology modeling (a type of comparative structure prediction) can be a useful strategy. It models the new structure of a protein based on its homologues with known structures [83, 86-88]. These methods are getting more powerful with the increasing number of protein structures in PDB. Nonetheless the number of TM protein structures is relatively small hence these methods are limited to give reliable results for TM proteins.

TM proteins unlike water-soluble proteins are constrained by the lipid bilayer, hence they have large parts that are embedded within the hydrophobic environment and most of them are α-helical. These facts motivated researchers to perform the so-called hydropathy analysis for protein sequences in order to obtain some structural information. The approach is first to quantify the partitioning of each amino acid side chain from aqueous phase into the hydrophobic core of the lipid bilayer.

* Similar to PDB there is an archive for protein sequence data [76]
http://www.ebi.ac.uk/uniprot/TrEMBLstats. The statistical data on protein sequences was taken from the release of 16.09.2015 according to which there are 50825784 sequence entries.

** GPCRs are highly desired drug targets [24, 25, 85].
The free energy difference between having amino acid in the aqueous phase and in the lipid bilayer core ideally is used to quantify this partitioning. However, due to its practical difficulty, most of the hydrophobicity scales instead of lipid bilayer hydrophobic core used various solvents such as ethanol, dioxane [91], methanol [92], carbon tetrachloride [93], alkane [94], cyclohexane [95] and octanol [96]. Once such a hydrophobicity scale is available, hydropathy index plots can be generated (Figure 7).

*In thermodynamics the free energy is the amount of the internal energy of the system that can be converted into work. If the system has a constant temperature and volume, Helmholtz free energy is meant, if the system has a constant temperature and pressure Gibbs free energy is meant [89, 90]. Systems, which can exchange energy (but not matter) with the surrounding environment are being characterized with the free energy minimum principle that is: a system self organizes in a way, which has the minimum free energy. The hydrophobic effect is one manifestation of this principle. For systems, which do not exchange neither energy nor particles (isolated systems) with the surrounding environment this principle reduces to the second law of thermodynamics that is: the entropy of isolated systems never decreases.
The hydropathy index plot is generated using the amino acid sequence of a protein and corresponding hydropathy index values for amino acids from particular hydrophobicity scale [97, 98]. A certain window size is used to plot the hydrophatic index. The window moves from the N-terminal end to the C-terminal end along the sequence. Each time by leaving one amino acid out and adding another into the window a new average hydrophatic index is being determined and marked on the graph.

If the TM α-helix spans the lipid membrane perpendicular to the membrane surface it will be composed of around 20 to 30 amino acids. This knowledge combined with the hydropathy index plot can give some clues on the TM protein structure. Different hydrophobicity scales have been developed through experiments [91, 93-97, 99-103] and molecular dynamic simulations [104, 105]. Next we will discuss two hydrophobicity scales, which had a large impact in this research area [106-109].

1.3.1.1 The biophysical hydrophobicity scale and self-inserting peptides

Wimley, Creamer and White studied the partitioning of the pentapeptides AcWL-X-LL where X is one of 20 naturally occurring amino acids, between water and octanol [96]. This particular design of peptides was chosen to have no aggregation of peptides in any of the two phases: water and octanol up to 1 mM of peptide concentration for all 20 naturally occurring amino acids in the central position. Octanol is a water immiscible fatty alcohol and it was chosen to mimic the hydrophobic core of the lipid bilayer. Peptides were dissolved in HEPES buffer (hydrophilic phase) after which partitioning of peptides between hydrophilic and hydrophobic phases occurred. The peptide concentration in both phases was measured by reverse-phase HPLC [110]. The difference between the free energies of peptide positioning in the octanol phase and in the aqueous phase has been calculated using the ratio of peptide concentrations in aqueous and octanol phases and the expression for calculating the free energy difference $\Delta G$ in equilibrated systems is as follows:

$$\Delta G = -RT \ln \left( \frac{c_{\text{wat}} v_{\text{wat}}}{c_{\text{oct}} v_{\text{oct}}} \right)$$  \hspace{1cm} \text{Eq. 1}

where $R$ is the universal gas constant, $T$ is absolute temperature, $c_{\text{wat}}$ and $c_{\text{oct}}$ are peptide concentrations in aqueous and octanol phases and $v_{\text{wat}}$ and $v_{\text{oct}}$ are the molar volumes of
water and octanol. This hydrophobicity scale became known as the “biophysical hydrophobicity scale”.

The biophysical hydrophobicity scale gives a quantitative measure of peptide insertion into the lipid bilayer however it says nothing about the insertion mechanism. A two-stage [111-113] and a three [114] and a four-step [115] thermodynamic models have been developed to describe the partitioning, folding, insertion and association of TM α-helices (Figure 8). The four-step model contains both previously proposed two-stage and three-step models and, hence, it is more comprehensive. These models describe the process only from the thermodynamic point of view and do not necessarily reflect the real membrane integration mechanism.

![Diagram of partitioning, folding, insertion, and association processes](image)

**Figure 8.** Four-step model of peptide partitioning, folding, insertion and association [115]. The two-stage model contains only the last two steps of the four-step model [111-113]. The three-step model contains only the first, second and the last steps of the four-step model [114]. The indices of free energy differences are abbreviations from the terms shown in the left part of the figure. These models describe the process only from the thermodynamic point of view and they do not necessarily describe the actual insertion and/or folding mechanisms. Furthermore different peptides can have different membrane integration mechanisms.

The importance of understanding of the peptide and lipid bilayer interactions can be reflected in the fact that some diseases such as Alzheimer and Parkinson involve amyloid peptides, which may aggregate and disrupt the neuronal cell membranes. Moreover, there is also a large group of antimicrobial peptides, which disrupt bacterial cell membranes. These peptides can have different mechanisms of cell membrane disruption [116]. For example melittin is a 26 amino acid long peptide found in bee venom [117]. This peptide partitions
and folds on the lipid bilayer surface [118, 119]. When peptide to lipid ratio is increased and several melattins come together at the lipid bilayer interface they form a pore in the lipid bilayer of 4.2 nm in diameter by bending lipid monolayers [120]. Another example is gramicidin a 15 amino acid long peptide synthesized by *Bacillus brevis* [121, 122]. Two gramicidin peptides together form a TM channel. Each one spans half of the lipid bilayer as a helix and meet the other one with its N-terminal end [123, 124]. Monovalent cations can freely pass through these channels. As a result, the ion gradient between the cytoplasm and the extracellular environment destabilizes and the cell dies. These peptides are being actively used in drug and vaccine development. Furthermore, researchers design peptides that insert into the lipid membranes for drug, gene or imaging agent delivery into cells [125-127]. For instance family of pH low inserting peptides (pHLIPs) was designed based on the C helix of the BR [128]. These peptides are water-soluble at neutral pH. They as well can bind to lipid bilayer surface at neutral pH. At lower pH this peptides insert into lipid bilayer as TM α-helices and when the pH is increased the peptides unfold and exit the lipid bilayer [127, 129]. At low pH the protonation of aspartic acid residues in the pHLIP sequence makes possible the folding and insertion of pHLIP [127]. The insertion thermodynamics [130] as well as kinetics [131, 132] for this peptides have been studied in detail. Based on this data a pHLIP insertion model has been proposed as follows: first, the peptide is binds to the lipid bilayer surface, when pH is lowered the helix on the surface forms which then gradually inserts into the lipid bilayer [131]. This peptides have been used for targeting acidic tissues for imaging agent or drug delivery [133-135].

As it was discussed different peptides can have different membrane integration mechanisms. Moreover, these also depend on the lipid composition, temperature and pH value of the medium. Hence, the four-step model does not describe the actual pathways for these peptides. However it is still proves useful for understanding the integration process from the thermodynamic point of view.
1.3.1.2 *In vivo* insertion and the biological hydrophobicity scale

In the previous subsection we discussed several examples of self-inserting peptides however most TM proteins are being inserted cotranslationally*. This happens with the participation of a group of enzymes and other proteins. A simplified version of this process is the following: when the protein is being synthesized by the ribosome, first, the signal sequence is being synthesized and then it is recognized by the signal sequence recognition protein, which binds to it and to the ribosome. Next, this complex formed by the ribosome, the signal sequence recognition protein and the nascent polypeptide binds to the signal sequence recognition protein receptor, which is localized in the endoplasmic reticulum membrane in eukaryotes or in the cytoplasmic membrane in prokaryotes. Afterwards this membrane bound complex connects with the translocon complex, which resides in the same membrane. Then signal sequence recognition protein and signal sequence recognition protein receptor detach (Figure 9A). The function of the signal sequence recognition protein and signal sequence recognition protein receptor is to bring together the ribosome, which started translating the new protein and the translocon complex. The translocon complex is a key player since it “decides” whether a particular newly synthesized peptide should go through it into the endoplasmic reticulum lumen (or the prokaryote periplasm) or whether it should became a TM protein. If a particular sequence should be part of the TM protein then the translocon opens laterally and releases the helix into the lipid membrane. Some small α-helical TM proteins are inserted via the YidC pathway [137, 138] however most of α-helical TM proteins are being inserted via the heterotrimeric Sec61αβγ in eucaryotes or its homologues SecYEG in bacteria and SecYEβ in archaean (Figure 9B,C). The Sec61α, Sec61β and Sec61γ subunits in eukaryotes correspond to the SecY, SecE and SecG subunits in the

*β-barrel TM proteins which are in the outer membranes of some prokaryotes are an exception [136].
The structure of the SecYEβ translocon complex of *Methanococcus jannaschii* has been solved in 2004 with a resolution of 3.2 Å [139]. It has been shown that SecY is composed of 10 TM α-helices, SecE is a single TM α-helix and another α-helix lying on the cytoplasmic surface of the membrane, Secβ is a single TM α-helix. In addition, Secβ and SecE subunits are forming β-sheets with the SecY subunit (Figure 9B,C) [139, 141]. The structure of the Sec complex can be slightly different for different organisms [142, 143].

Since the Sec translocon complexes are themselves α-helical TM proteins they are also inserted into the lipid bilayer by means of other Sec translocon complexes. This raises a “chicken and egg” type of dilemma. If lipid bilayers and proteins evolved together, then self-inserting peptides could have evolved into the first translocon complexes or they could have
assisted insertion and stabilization of the first multi-span TM α-helical proteins, which then evolved into the first translocon complexes [144].

The insertion and folding mechanism of the nascent polypeptide chain through Sec complex is still a mystery. This mechanism has been actively investigated recently by means of X-ray crystallography [139, 141], cryo-electron microscopy [140, 145-148] and molecular dynamics (MD) simulations [149-153]. There are reviews in which researchers proposed different models for Sec translocon assisted insertion and folding of the nascent polypeptide chain based on previous studies [154, 155]. The main challenges in determining the exact mechanism of this process are involvement of large protein complexes such as ribosome and the timescale of translation. The translation is relatively slow process compared to the other enzymatic processes at same length scale, it takes about 50 ms to add a single amino acid to the newly synthesizing polypeptide chain.

Since the in vivo insertion of TM α-helices is mediated by a sophisticated biological machinery, Tara Hessa and her colleagues in Gunnar von Heijne group addressed the question of constructing a biological hydrophobicity scale based on the insertion of peptides co-translationally via the Sec translocon complex. Particularly they studied the insertion of designed peptides with the formula GGPG-(LₐA₁₉₋ₙ)·GGPG where n = 0,…,7, and then, of the same peptides by replacing the central amino acid by each of the 20 naturally occurring amino acids into the membrane of dog pancreas rough microsomes. These peptides were designed to be part of the luminal P2 domain of the integral membrane protein leader peptidase. In order to find out whether the designed peptide was inserted glycosylation sites were arranged before and after the peptide sequences. Since glycosylation can only happen in the lumen of microsomes, peptide insertion likelihood was measured by determining the fractions of single (1g) and double (2g) glycosylation through a gel assay [102, 103]. Then the free energy difference between the peptide being inserted or secreted was calculated using the ratio of fractions of single and double glycosylation:

$$\Delta G = -RT \ln \left( \frac{f_{1g}}{f_{2g}} \right)$$

Eq. 2

where R is the universal gas constant, T is the absolute temperature, f₁g and f₂g are fractions of singly and doubly glycosylated molecules respectively. Further, they investigated the role of the amino acid position in peptide insertion. In the first set of experiments, the amino acid of interest was placed at different positions along the sequence of the designed
peptides. In the second set of experiment a pair of amino acids were moved symmetrically from the center and insertion likelihood was determined for all cases. Conclusion of these studies was that there was a strong dependence of the insertion likelihood on the position of particular amino acids. As a result the TM helix prediction method was developed based on the biological hydrophobicity scale and amino acid position dependent insertion [103].

**Figure 10.** (A) Hydropathy plot for BR based on the biophysical hydrophobicity scale [96] (green) and translocon TM analysis for BR (blue) based on the biological hydrophobicity scale and position dependent membrane insertion studies [102, 103]. A window size of 19 amino acids was used in both cases, calculations were done with the Membrane Protein Explorer [156]. The TM helixes can be guessed by local maxima and length of TM segments. (B) There is surprisingly good correlation between biophysical and biological hydrophobicity scales. The slope of the linear fit is 1.07 and the residual sum of squares is 0.7.
Figure 10 shows how well the biological and biophysical hydrophobicity scales are correlated. This remarkable correlation is evidence that the complexity of the sophisticated biological machinery has not been entirely encoded into the biological hydrophobicity scale. Furthermore, the hydropathy plots presented so far are for BR which is composed of just 7 TM α-helices and this α-helices are tilted from the bilayer normal by a maximum of 20°. Though from the determined structures of the TM proteins we can conclude that they contain less divers structural motives compared to water-soluble proteins they can still

\[ \text{Figure 11. Examples of TM proteins with nontrivial structural arrangements. LeuT (A) (PDB code 3F3A) [157] and ApcT (B) (PDB code 3GIA) [158] both have tilted and disrupted TM α-helices. Photosystem II (C) (PDB code 3A0B) [159] is a large heterodimer and it has both TM as well as water soluble regions. α-helices, β-sheets, single helical turns and loops are colored purple, yellow, blue and gray respectively. All proteins are oriented to the extracellular side with the top.} \]
exhibit complex arrangements of α-helices (Figure 11). For example leucine transporter LeuT [157] (Figure 11A) and amino acid transporter ApcT [158] (Figure 11B) both have tilted as well as disrupted TM α-helices. Another example is the photosystem II complex [159] (Figure 11C) that has a molecular mass of 350 kDa which is more than ten times that of BR. These examples are from TM proteins whose structures are determined, but, as it was mentioned, this is a tiny fraction of all TM proteins. There are TM proteins which have an even higher molecular mass for example mouse Piezo1 has molecular mass of about 1200 kDa and thought to span the lipid bilayer over 30 times [160]. These examples completely demolish the somewhat naive expectation of predicting protein structures from their sequence with such a simplistic means as hydropathy plots.

1.3.2 Protein-folding problem

Comparative structure prediction, hydrophobicity scales as well as the study of the mechanism of co-translational protein insertion and folding are attempts towards solving the so-called “protein-folding problem” [161, 162]. The problem is frequently stated in the following way: find out the structure of the protein from its amino acid sequence. Once one states this question one assumes that the whole information required to determine the protein structure is in its amino acid sequence. Confidence in the later statement arose from the experiments by Anfinsen’s group in 1950s and 1960s [163-165]. They studied the unfolding and refolding of ribonuclease A, a 14 kDa enzyme with 124 amino acids that cleaves single stranded ribonucleic acid (RNA). In their famous experiment they reduced the disulfide bonds with 2-mercaptoethanol and denatured ribonuclease A with 8 M urea. Afterwards by removing the denaturants they observed that the protein can perform its enzymatic function, hence they concluded that it refolded into its native structure. This experiment supported the thermodynamic hypothesis, which states that in the native environment (solvent, temperature, pH etc.) proteins fold spontaneously into their native structure. Spontaneous folding means that folding is governed purely by physicochemical interactions between amino acids and between amino acids and the solvent.

De novo protein structure prediction is another attempt of solving “protein folding problem”. In this case, researchers try to simulate the actual folding process in silico – using computers. To achieve this goal first they define the energy of interaction between atoms –
the so called “forcefield” [166-168]. Next, they computationally solve Newton’s law of motion or Schroedinger’s equation, for polypeptide chains in solvent, until they fold into a stable structure. However, structure prediction with this method requires vast computational power. Even with the massively parallel supercomputer Anton specifically created for simulating biopolymers [169-174] and distributed computing projects such as Folding@home [175, 176] which uses volunteers’ computers all over the world, it was possible to obtain structures in silico only for small proteins. In another attempt for protein structure prediction researchers created a videogame where people who play – “citizen scientists” should look for a right way to fold a given polypeptide chain [177]. Humans have intuition and in certain cases they are much better in recognizing patterns than today’s computers – this property is made use of in such a “citizen science” projects.

The thermodynamic hypothesis is not universal. As it was already mentioned in the last section, α-helical TM proteins are being inserted and folded co-translationally via the Sec translocon complex. It is still debated whether translocon assisted TM protein insertion is an equilibrium process [102, 106, 149, 178, 179]. Furthermore, it has been shown that by changing the Sec translocon sequence the structure of α-helical TM proteins inserted through this translocon could be changed. Hence the same sequence of TM protein can lead to different structures depending on the translocon amino acid sequence [180]. Other diverging examples are dual-topology TM proteins [181, 182]. An example from this group is the EmrE dimer. Each subunit of EmrE is about 12 kDa and has 4 TM α-helices. Subunits have identical sequences but inverted topologies [183].

Similarly, many water-soluble proteins are folded with the help of other proteins called chaperons [184, 185]. In fact, Anfinsen-style experiments with relatively larger water soluble proteins showed that they failed to refold back into their native structure [186, 187]. Proteins, which failed to fold into their native structure, are called misfolded. Such misfolded proteins can become a cause of diseases [188, 189]. It is clear that, in such cases, the protein sequence does not contain the entire information to determine the native structure – part of the information is contained in other proteins and molecules, which assist its folding. On the other hand, it is also clear that the protein sequence does not contain the information on the physical laws of interactions between amino acids and solvent molecules. Then how does the sequence fold into a specific structure without containing information on its native environment? This puzzle is resolved in the next section.
1.3.3 Levinthal’s paradox

In 1969, at a conference in Monticello, Illinois Cyrus Levinthal stated the following paradox [190]: He estimated that a 300 amino acid long polypeptide chain can arrange into about $10^{300}$ possible configurations. Time spent for changing a configuration is on the order of picosecond. If we consider that the polypeptide chain screens randomly through configurations to reach its native state then it will take much longer than the age of universe for protein to fold. Yet proteins fold even faster than they are being synthesized, the typical folding time is ranging from microseconds to seconds. This indicates that natural polypeptide chain does not screen through all possible configurations randomly. Several suggestions have been made to resolve this paradox having the basic idea of hierarchical folding. Polypeptide chains folds step by step by not reconsidering the already folded parts [194-202]. In this scenario the folding time drastically reduces since, with each step, the number of next possible configurations reduces. This hypothesis stimulated the experimental search for structures of polypeptide chains in intermediate states of folding [203-208]. An examples of such a folding intermediate in water soluble proteins is the so called “molten globule” which is the unspecific arrangement of the secondary structure elements of that protein [204, 209-211].

However, it can be asked next why should a polypeptide chain follow this hierarchical folding mechanism? Maybe before this, it makes sense to ask why at all such a native state should exist? The vast majority of natural polypeptide chains of the same sequence but different initial configurations fold into the same structure after some time under the native conditions. This is a disorder to order transition. Isn’t this surprising? Well, the above-mentioned statements would be surprising if natural polypeptide chains were just random sequences of naturally occurring amino acids. However protein sequences are not

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Levinthal assumed that we can determine dihedral angles of a polypeptide chain within less than 10th of a radian, however there are about 3 stable states per dihedral angle in a polypeptide chain [191, 192] and in the case of a 300 amino acid long chain there will be 299 dihedral angles hence one will have in total $3^{299}$ that is on the order of $10^{142}$ possible configurations if we assume that dihedral angles are independent from one another, which is not entirely true [193]. If the protein randomly changes through configurations till it gets to the native state it will spend on the order of $10^{142}10^{-12} = 10^{130}$ seconds or on the order of $10^{122}$ years when the age of the universe is on the order of $10^{10}$ years.
random, just as random sequences of letters and spaces rarely form words or a sentences. Rather, protein sequences are “designed by evolution” i.e. the polypeptide chains, which did not fold in a biologically relevant time into a functional state and made the host organism less competitive, disappeared due to natural selection. Natural protein sequences are the result of many trials and errors. From this perspective the puzzle stated at the end of section 1.3.2 (if protein sequences do not contain the full information regarding other proteins and molecules present in the native environment and influencing its folding then how do natural polypeptide chains fold into specific structures?) vanishes. Important to notice that folding into specific structures in a biologically relevant time is a necessary but not a sufficient condition for an amino acid sequence to be a protein sequence [212, 213]. There is more information encrypted in protein sequences that needs to be understood.

1.3.4 De novo protein design

In the previous section we argued that proteins found in vivo are evolutionarily designed. Researchers, inspired by nature, decided to design proteins, which would fold into specific structures and perform the desired functions in the desired environment. This is an inverse problem to the “protein folding problem” since here we have a structure model and we would like to find an amino acid sequence which will fold into this particular structure [214, 215]. De novo protein design is not a trivial problem since there is an astronomical number of ways one can make a chain of amino acids of particular length. If we assume dealing with 20 amino acids, then, for a protein with N residues, there are 20^N possibilities, a very large number even for a 100 amino acid long small proteins. To overcome this, researchers use novel algorithms and computational methods [216-220]. Once one obtains the sequence of a “virtual protein”, it can be encoded into the bacterial genome and the protein can be produced in vitro. Next, the structures can be studied experimentally to see how well they are matching with the designed structures and protein functionality can be tested as well. Using this method, proteins have been designed for novel therapeutic applications [221-226]. Particular examples are HB36 and HB80 that target evolutionally conserved parts of the influenza virus and block their function [222]. Repeat proteins and synthetic protein cage-like assemblies have been synthesized that have potential for material science applications [227, 228] and for targeted drug delivery [229, 230]. The next step...
could be the de novo creation of a synthetic virus by having a nucleic acid inside the cage-like protein assembly, which can have a wide range of applications, such as gene therapy.

1.3.5 The concept of free energy landscape

The free energy landscape of a system is a surface in N+1 dimensions where N is the number of degrees of freedom of the system and the additional dimension is the free energy axis i.e. this is a mapping where, for each configuration of the system, the free energy is represented. This concept has been adopted for proteins to offer a visual demonstration of protein (un)folding and for a better understanding of the related problems [231-233]. Each configuration of a polypeptide chain will have a corresponding free energy, which will be represented as a point on the free energy landscape.

The sequential change of the polypeptide chain configurations with their corresponding free energy values will form a line on the free energy landscape, which is called a free energy profile (Figure 12). The projection of the free energy profile on the plane of degrees of freedom is the reaction coordinate. The reaction coordinate in other words is the line in the space of degrees of freedom constructed by sequential changes of the polypeptide chain configurations.

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* The number of degrees of freedom is the number of independent parameters required to fully describe the configuration of the system. For example a point in three dimensions has three degrees of freedom, since, to describe its position, three numbers are required such as Cartesian or spherical or any other coordinates. A cylinder in three dimensions has five degrees of freedom since except three numbers which are required to describe the position of its center, two numbers are required to describe its orientation. Due to the axial symmetry of the cylinder there is no need of a third number to describe its orientation.
As it was mentioned previously, systems which can exchange energy with the surrounding environment, tend to obtain a configuration, which has a minimal free energy. The proteins evolved in a way that their energy landscapes have a funnel shape (Figure 13C,D) [231, 233-238] with the global minimum corresponding to the native state of the protein [239]. This ensures the stability of the native state and folding into this state in a biologically relevant time. The question “how is it possible to start with different configurations and to fold into the same native state” is equivalent to asking “how is it possible to put the ball in the different points on the rim of the funnel and to end up with it in the center (if the system is under the influence of gravitational field)”.

Free energy landscapes of natural proteins are not ideally smooth. They have local minima, maxima, saddle points – in other words, they are rugged. This roughness of the landscape resists the process of folding into the native state. In analogy to a ball rolling into the funnel, sometimes, this phenomenon is called “friction”. The misfolding of a protein can occur if it is “stuck” on the way to the native state due to ruggedness. Chaperons could be

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**Figure 12.** Qualitative free energy landscape for a system with two degrees of freedom (A). The black line is the free energy profile of the system between the initial and the final configurations that represents the sequential changes of the polypeptide chain configurations with their corresponding free energy values. The projection of the free energy profile on the plane of degrees of freedom (gray line) is the reaction coordinate. Equivalently, the reaction coordinate is the line on the plane of degrees of freedom formed by the sequential configuration changes of the system. From another perspective, the relation between the free energy profile and the reaction coordinate can be described in the following way: the free energy of the system as a function of the reaction coordinate is the free energy profile (B).
involved in assisting the folding of proteins, which are stuck due to the ruggedness of the landscape. It is also evident why in the native state proteins are structurally stable. In this energy minimum thermal fluctuations cannot induce large structural changes due to the depth and narrowness of the “funnel” around the minimum. The unfolded polypeptide chain is in the wider region of the free energy landscape and this is the reason why it can change configurations relatively faster and with a low energy cost.

One should keep in mind that the figures of free energy landscapes presented here are for systems with 2 degrees of freedom. Even for small proteins and even if we do not take into account the degrees of freedom of the solvent molecules, the energy landscape will be a surface in a space of several hundred dimensions. We cannot imagine which kind of complex topological arrangement is possible in such a high dimensional spaces.
Figure 13. Qualitative examples of free energy landscapes in three dimensions (for a system with two degrees of freedom). (A) Thought free energy landscape for a polypeptide chain which does not have a distinguished global minimum. A polypeptide chain with such a free energy landscape will have several structures of similar stability. Most of the energy landscapes of random amino acid sequences belong to this category. (B) A free energy landscape with a flat surface and one distinct minimum. A polypeptide chain with this free energy landscape might take very long to fold into its stable structure since in the flat region of the free energy landscape it will change configurations “randomly”. (C) A funnel shaped, smooth free energy landscape. With such a free energy landscape proteins can fold into the specific stable structure in a biologically relevant time. (D) A more realistic funnel shaped, rugged free energy landscape that has a general funnel shape but also local maxima, minima and saddle points. To overcome these obstacles during folding chaperones might be required.
1.3.6 Unfolding of proteins

Protein controlled unfolding is a powerful method for studying protein stability and exploring the properties of their energy landscape. Proteins can be unfolded or denatured by adding denaturant to the medium, by changing the external parameters (temperature, pressure etc.) or by applying mechanical force on single proteins.

As it was mentioned, Anfinsen and his colleagues have used 2-mercaptoethanol and urea to denature ribonuclease A [163-165]. 2-mercaptoethanol is used for reducing disulfide bonds between cysteine residues. Dithiothreitol and tris(2-carboxyethyl)phosphine can be used for the same purpose. Urea has been used for disrupting hydrogen bonds between amino acids [240]. Another widely used denaturant for hydrogen bond disruption is guanidine hydrochloride [241]. Methods such as size exclusion chromatography, circular dichroism, fluorescence resonance energy transfer and nuclear magnetic resonance spectroscopy can give an insight into the (un)folding intermediates [242-244]. TM proteins are not exception and they can also be chemically denatured [245-247]. Proteins can be protected from chemical denaturation by stabilizing osmolytes [248]. From the free energy landscape perspective denaturants raise the free energy of the unfolded state relative to the free energy of the folded state[249].

Thermal denaturation of proteins is frequently used along with chemical denaturation. A common domestic example of thermally denatured proteins is boiled egg. An increase in the temperature breaks the hydrogen bonds in proteins and can cause aggregation of polypeptide chains. Interestingly, cooling can as well denature proteins [250]. Cooling denaturation is caused by temperature dependent interactions of protein nonpolar groups with water. High pressure denaturation of proteins is primarily caused by hydrogen bond disruption [251, 252]. The pressure-temperature denaturation diagram for proteins has an elliptic shape (Figure 14) [253-255].
With the development of single molecule force spectroscopic techniques at the end of the last century, it became possible to unfold single proteins applying force to the protein terminal ends [256-258]. With this method unfolding steps could be observed and some information on protein structure and energetics has been obtained. This will be discussed in more detail in chapter 3.
“Rules of discovery. The first rule of discovery is to have brains and good luck. The second rule of discovery is to sit tight and wait till you get a bright idea.”

“How to Solve It” Princeton University Press, 1945, pg. 172

George Pólya (13 December 1887 – 7 September 1985)
2. The atomic force microscope

In contrast to previous versions of microscopes, which used light or a beam of subatomic particles to study the structure of matter, scanning tunneling microscope, invented in 1982 by Binning, Rohrer, Gerber and Weibel, had a conceptually different working principle [259]. It was using tunneling current between a conductive surface and an atomically sharp conductive tip to measure the distance between these two objects in vacuum and then to construct the atomic resolution topography of the surface by moving the tip over the surface while keeping current constant. The atomic resolution is due to the fact that the tunneling current decades exponentially with the increase of distance between the tip and the surface [260]. Four years later in 1986 two co-inventors of the scanning tunneling microscope Gerd Binning and Christoph Gerber along with Calvin Quate came up with a more widely applicable microscopy technique which they called atomic force microscope (AFM) [261]. Instead of using tunneling current, AFM uses the interaction force between tip and the surface to obtain surface topography while keeping the force constant by controlling the distance between the tip and the sample during scanning. Again, the high resolution is due to the sensitive dependence of the interaction forces on the distance between the tip and the surface [16]. AFM’s further fantastic applications were and are yet to come...

2.1 Setup and working principle

In fact, AFM invention was inspired by the stylus profilometer. The AFM has four main components: a pezoelectric scanner, a cantilever, a laser and a photodetector (Figure 15). The cantilever and the sample are manipulated relative to each other by means of a piezoelectric scanner. As a result of the interactions with the sample the cantilever can be deflected. This deflection changes the path of the laser beam reflected from the top of the cantilever, a change which is recorded by a photodetector. As the spring constant of the cantilever is known along with the extent of deflection, the force acting on the cantilever can
be determined using Hooke’s law, i.e. assuming that the absolute value of the force is proportional to the displacement of the cantilever:\[
F = \kappa \cdot \delta z
\]  
Eq. 3
where \( F \) is the absolute value of the force, \( \kappa \) is the spring constant of the cantilever and \( \delta z \) is the extent of deflection. In order to be able to scan the surfaces at constant force, a feedback controller is used.

\* This assumption is valid for relatively small deflections. Deflection is defined as \( \delta z = |z - z_0| \) where \( z_0 \) is the equilibrium position of the cantilever end and \( z \) is the final position. We can write the Taylor series for the potential energy of the cantilever or spring around it’s equilibrium position
\[
U(z) = U(z_0) + \left. \frac{1}{1!} \right|_{z_z}^{z_0} dU(z) + \left. \frac{1}{2!} \right|_{z_z}^{z_0} \frac{d^2 U(z)}{dz^2} + \ldots
\]
and assuming small deflections we can take only the first three terms. The first term is constant and it can be eliminated by taking the appropriate zero level for the potential energy. The second term is equal to zero since \( z_0 \) is an equilibrium position and hence corresponds to the minimum of the potential energy, which means that the first derivative of the potential energy at that point is equal to zero. Next, the relation between force and potential energy
\[
F(z) = -\nabla U(U(z))
\]
is used, which can be written for absolute values
\[
F(\delta z) = \frac{dU(U(z))}{d\delta z} = \left. \frac{d^2 U(z)}{dz^2} \right|_{z_z}^{z_0} \cdot \delta z
\]
where one can make a notation \( \left. \frac{d^2 U(z)}{dz^2} \right|_{z_z}^{z_0} = \kappa \) and obtain Eq. 3.

In the future, when force is being mentioned, its absolute value will be meant.
A Piezoelectric scanner, sometimes shortly called a piezo, is made of a piezoelectric material. Piezoelectric materials have the property to generate voltage under mechanical stress. This effect has been discovered in 1880 by brothers Curie [262]. An inverse piezoelectric effect gives the opportunity to finely control the change in size of the piezoelectric material by applying voltage on it. This means it is possible to move the cantilever relative to the sample in three spatial dimensions with subnanometer precision. In some setups the cantilever base is controlled by piezo while the sample position is fixed, while in others the reverse arrangement is possible.

Cantilevers are produced with various microfabrication techniques and are part of an AFM cantilever chip (Figure 16). To have a proper reflection of the laser beam from the cantilever, the top of the cantilever can be covered with a thin layer of material with a high reflection coefficient such as gold. On the end of the bottom side, depending on its applications, cantilevers can have a sharp tip. A rectangular cantilever is a long stripe typically made of silicon or silicon nitride. Besides rectangular cantilevers there are also V-shaped cantilevers that thought to be more resistant to torsional load, however the detailed calculations showed that this is not the case [263, 264]. Cantilevers, depending on their application, can be different in size, shape, tip type etc. One of the widely used rectangular cantilevers is the 200 micron long, 20 micron wide and 800 nm thick silicon nitride cantilever with a pyramidal tip of 2.9 micron in height and with a tip radius of less than 30 nm (OMCL-RC800PSA). Depending on the applications the AFM cantilever tip can be modified or substituted [265-268]. In AFM cantilevers are mounted under certain angle with respect to sample plane, this is done to avoid possible contact of the AFM cantilever chip with the sample.

Use of a laser in AFM, for determining the deflection and hence the force acting on
the cantilever, was first proposed in 1988 [269]. Mostly infrared lasers are used with a wavelength of about 850 nm. The optical path varies for different versions of AFM. There can be additional prisms and mirrors included for directing the laser beam to the photodetector. These parts are important since, for example, the refractive index can be different for different environments, which can affect the path of the laser beam, and, by rotating the mirror, the laser spot can be brought back to the photodetector. Most of the commercial AFMs do not have control of the laser spot size. Since cantilevers can have different width, the laser spot can sometimes be larger than the cantilever width and that can cause unwanted heating of the sample.

A quadrant photodiode serves as a photodetector in most AFMs. A photodiode is converting light into the electrical current. The amount of laser light that hits each of the quadrants of the quadrant photodiode is being converted into currents with corresponding voltages $A, B, C, D$. The signal used to measure the deflection is $((A + B) - (C + D)) / ((A + B) + (C + D))$. Initially, the laser spot is aligned to be in the center of the quadrant photodiode. Once the cantilever deflects, the laser spot moves from the center of the quadrant photodiode, which leads to voltage changes obtained from different quadrants. For large deflections of the cantilever, the quadrant photodiode signal changes nonlinearly with deflection, for such cases array detectors are advantageous [270].

2.1.1 Photodetector calibration

Photodetector or photodiode calibration means determination of the dependence of the output current on the input amount of light, in other words one needs to have a wavelength dependent responsivity of the photodiode, which is represented as the ratio of generated current and incident light power. The straightforward way of calibration is to use a light source of a known wavelength and power and to measure the output current generated by the photodiode when light is shining on it.
2.1.2 Piezoelectric scanner calibration

Piezoelectric scanner calibration can be done using a grid of known dimensions. The grid needs to be imaged and the dimensions of the grid from the AFM image should be compared with manufacturer’s reported dimensions of the grid. If there is a difference between these two values, then a correction factor needs to be introduced. By these means, the measurements of all three spatial dimensions of the piezoelectric scanner can be calibrated.

2.1.3 Sensitivity and spring constant calibration

The calibration of the cantilever spring constant\(^1\) is being done more frequently than piezoelectric scanner or photodetector calibration, since cantilevers are being changed much more frequently. In most of the cases the cantilever spring constant needs to be calibrated before each experiment. Even though cantilever manufacturers provide values for spring constants, these values can differ with more than 50\% due to production imperfections and laser spot positioning on the cantilever back. The laser spot should be positioned at the end of the cantilever on the backside of the tip. If the laser spot is positioned closer to the cantilever base, then the cantilever will appear stiffer and will have a higher apparent spring constant.

Before calibrating the cantilever spring constant, the sensitivity of the cantilever and photodetector system should be determined. This is done by pressing the cantilever on a hard surface for example mica. It is required to convert the voltage signal generated from the movement of the laser spot on the photodetector into the deflection of the cantilever measured in length units. The conversion factor is simply the slope of the force vs. the pizo movement curve (Figure 17) where the cantilever is in contact with the hard surface and is being deflected. It is clear that sensitivity will depend on the position of the laser spot on the cantilever from where it is being reflected onto the photodetector. It is desired to have the laser spot as close to the end of the cantilever as possible.

\(^1\) Here we mean the normal spring constant of the cantilever. Cantilevers also have a torsional spring constant which is the proportionality coefficient between the torsional force and the rotation angle for small rotations.
Once the sensitivity has been determined, the next step is to measure the cantilever spring constant. The most frequently used method for cantilever spring constant calibration is the so-called thermal noise calibration method. In this method, one assumes that the cantilever is a harmonic oscillator with one degree of freedom – first elastic mode \[271\]. Next, from the equipartition theorem for harmonic oscillator Eq. 4 cantilever spring constant can be obtained Eq. 5:

\[
\frac{\kappa \langle \delta z^2 \rangle}{2} = \frac{k_B T}{2} \quad \text{Eq. 4}
\]

\[
\kappa = \frac{k_B T}{\langle \delta z^2 \rangle} \quad \text{Eq. 5}
\]

where \(\kappa\) is the cantilever spring constant and \(\langle \delta z^2 \rangle\) is the mean square displacement of the cantilever end for the first elastic mode of the cantilever. The mean square displacement of the cantilever end is being calculated from the area under the power spectral density of the cantilever deflection and piezo movement.

\[\text{Figure 17. Qualitative curves of single approach and retract cycle of the AFM cantilever on hard surface. First piezo expands and cantilever moves towards the sample surface (1). Then, at a certain distance from the surface, intermolecular forces between the cantilever tip and the sample surface are strong enough to attract the cantilever tip and slightly bend the cantilever (2). Subsequent expansion of the piezo increases the bending of the cantilever until predefined cantilever deflection is reached (3). Then the retraction starts, the piezo contracts and the reverse process happens (4-5-6). If there is no drift and/or dissipation, the approach and retraction curves coincide, here we draw them shifted from one another to make them better visible.}\]
thermodynamic fluctuations of the cantilever. The power spectral density of the thermal fluctuations of the cantilever should be obtained for the cantilever when it is at least 100 microns away from the sample surface, this distance depends also on cantilever type. However, the shape of the cantilever during dynamic fluctuations is different from its deflected shape on the hard surface when sensitivity is being determined, this requires a correction factor to be introduced [272]. With this method cantilevers can be calibrated with around 10% of error.

AFM cantilevers can be modeled as harmonic oscillators for small deflections. In this case the angular resonance frequency of the cantilever $\omega_0$ will be given by the well-known equation for the harmonic oscillator Eq. 6:

$$\omega_0 = \sqrt{\frac{\kappa}{m_{\text{eff}}}}$$  
\text{Eq. 6}  

$$\omega_0' = \sqrt{\frac{\kappa}{m_{\text{eff}} + M}}$$  
\text{Eq. 7}  

where $\omega_0$ and $\omega_0'$ are angular resonance frequencies of the cantilever before and after adding the particle of mass $M$ to the cantilever end, $m_{\text{eff}}$ is the effective mass and for a homogenous rectangular cantilever it is approximately equal to 0.24 times the cantilever mass [273]. By adding a small object of known mass $M$ to the end of the cantilever the angular resonance frequency will decrease Eq. 7. The spring constant can be calculated if one knows the angular resonance frequencies before and after adding the object of known mass. By solving the system of Eq. 6 and Eq. 7 the spring constant $\kappa$ can be easily calculated Eq. 8:

$$\kappa = \frac{(\omega_0 \omega_0')^2 M}{\omega_0^2 - \omega_0'^2} = \frac{M}{\omega_0'^2 - \omega_0^2}$$  
\text{Eq. 8}  

This method can be used for cantilevers of any shape if they can be assumed to be harmonic oscillators. The complications here are related to the act of attaching particles of known mass to the end of the cantilever, which can be time consuming and can damage the cantilever. Also, one should aim to have the particle as close to the end of the cantilever as possible. The error of this method will come from the error of the mass determination of the reference particle and the errors of angular resonance frequencies.

The spring constant of the rectangular cantilever can be theoretically calculated if one knows the shape, dimensions and material of the cantilever. For the rectangular cantilever the spring constant can be calculated with the Eq. 9:
\[ \kappa = \frac{Ewt^3}{4l^3} \]  

Eq. 9

where \( E \) is the Young’s modulus of the material from which the cantilever is made, \( w, t \)
and \( l \) are, respectively, the width, thickness and length of the rectangular cantilever. The
Young’s modulus is a measure of the material resistance to elastic deformation, it can be
calculated from the slope of the stress-strain curve of particular material. Eq. 9 is derived
from the Euler-Bernoulli beam theory [274-276]. An analogous formula can be obtained for
V-shaped cantilevers, first, by assuming that the spring constant of a V-shaped cantilever is
equivalent to the spring constant of a rectangular cantilever with the width equal to the
double of the width of each of the beams of a V-shaped cantilever [277]. A more accurate
result for a V-shaped cantilever spring constant based on its dimensions and Young’s
modulus was obtained later by Sader [278]. Due to errors in determining cantilever
dimensions (1 to 3 %) and due to error in Young’s modulus determination (around 3 %)
this method can give spring constants with around 10 % error.

A straightforward method for finding a cantilever spring constant is to use a
reference cantilever with a predefined spring constant [279]. One cantilever base is
immobilized while the other cantilever is approached (Figure 17). The cantilever spring
constant can be determined using the slope of the force distance curve on the reference
cantilever \( \frac{\delta F}{\delta z} \) and the spring constant of reference cantilever \( \kappa_{\text{ref}} \) the by plugging it into
Eq. 10.

\[ \kappa = \kappa_{\text{ref}} \cdot \frac{1 - \frac{\delta F}{\delta z}}{\kappa_{\text{ref}}} \]  

Eq. 10

If cantilevers initially are not parallel to each other then the tilt angle should be taken into
account and Eq. 10 should be modified [280]. The drawbacks of this method are the need
for precise determination of the spring constant of the reference cantilever as well as the

\[ \text{Figure 18. The cantilever with an unknown spring constant is pressed against the cantilever with a predefined spring constant.} \]
need for precisely matching the point of contact of the two cantilevers. A small difference in contact point of the two cantilevers can lead to large errors in spring constant determination due to the change of the effective cantilever length [281].

Another method to determine the spring constant of the rectangular cantilever was proposed by Sader. Here, one needs to know the resonance frequency and the quality factor of the rectangular cantilever, its length and width as well as the properties of the surrounding fluid. The spring constant can be obtained by the Eq. 11 [282]:

$$\kappa = 0.1906 \rho w^2 l Q \left( \text{Re}(\omega_0) \right) \omega_0^2$$

Eq. 11

Where $\rho$ and Re are the density and Reynolds number of the surrounding fluid respectively, $w$, $l$, $Q$ and $\omega_0$ are the width, the length, the quality factor and the angular resonance frequency of the rectangular cantilever, respectively and $\Gamma_i$ is the imaginary part of the hydrodynamic function [283]. The Reynolds number is representing the ratio of inertial forces to viscous forces, in our case it depends on the angular resonance frequency. The quality factor is representing the ratio of stored energy to that of dissipated energy during oscillation. The quality factor as well as the resonance frequency depends on the medium in which the cantilever is embedded. This method gives around 5 to 10 % error due to cantilever dimension determination and Reynolds number determination.

2.2 Applications

After its invention about 30 years ago AFM underwent drastic improvements. Nowadays, it has applications that were not even dreamed of when it was invented.

2.2.1 Imaging

The first straightforward application and the reason why AFM has been developed was to obtain high-resolution topography images of various, not necessarily conductive surfaces. AFM topographs can be obtained with subnanometer resolution, that is two orders of magnitude better than the resolution of super-resolution microscopy techniques [284-286].
A common mode of imaging is to scan the sample surface while keeping the cantilever at constant deflection with the feedback loop. The feedback system adjusts the height of the piezo to keep cantilever deflection and hence the force acting on it constant during the scanning process. This is the so-called contact mode imaging. With this method first high-resolution topography images of TM proteins were obtained in the groups of Mueller and Engel [287-290]. An example of contact mode images of the two-dimensional crystals of the outer membrane protein FuhA [291] from gram-negative bacteria is shown in Figure 19. Furthermore, it was possible to image in contact mode single cells and to visualize the movement of their lamellipodia and filopodia with time-lapse contact mode imaging [292].

Another imaging mode is the so-called intermittent contact mode where the AFM cantilever oscillates and the tip makes contact with the sample surface at the lowest point of the oscillation. This mode is particularly useful when one works with relatively fragile samples, since, in this case, generally, smaller forces are applied for shorter time than in the case of the contact mode.

A high-speed AFM imaging method was pioneered by Ando and colleagues [293, 294]. This required the development of a special high-speed scanner and small cantilevers. Here, one gains speed at the cost of resolution, particularly, it was reported that 100 by 100 pixel images were recorded within 80 ms [293]. This made it possible to study the dynamics of macromolecules by visualizing their motion with AFM [48, 295-298]. For instance, images of myosin V walking along actin filaments were recorded where one can observe how myosin V heads interchangeably bind to the actin filament and how the molecular motor moves along the filament [299, 300].
Imaging artifacts in AFM topographs are frequent and due to this they are well characterized [301-304]. One group of artifacts is related to the finite size of the AFM tip (Figure 20A). If the shape of the tip is known, then the possible artifacts can be determined. Generally, for high resolution imaging, one should use cantilevers with sharp tips and small tip radius to minimize this effects. AFM cantilevers that do not have single distinguished endpoint on the tip can result in image multiplying. Images, which were obtained with dirty
tips, can have repetitive features of same orientation. Another type of artifact can occur if the sample surface has peaks that are exceeding the height of the AFM tip (Figure 20B). In this case, the tip will not touch the sample surface but the cantilever can still be deflected.

![Figure 20](image)

**Figure 20.** Possible artifacts in contact mode AFM imaging. (A) The red curve is the actual profile of the sample surface and the gray curve is the profile obtained by AFM imaging. The differences are caused by the tip size and shape. (B) An example of possible artifact when scanning rough surfaces, if the sample surface has peaks that are exceeding the height of the tip, then an artifact can occur. Such artifacts are possible, for example, during cell imaging.

### 2.2.2 Sensing

In the beginning of 1990s researchers have realized that AFM cantilevers can be used as microsensors. Notably, it was demonstrated that added mass, a change of temperature, a change of electromagnetic field and some other external factors influence the resonance frequency and/or bending of the cantilever [305]. For such “sensing” proposes there is no need for the cantilevers to have a tip. Mass measurements with these sensors is based on Eq. 7 [306]. With microcantilever array sensors, measurements of the concentration of molecules of interest in the medium, of the viscosity of the medium as well as quantification of protein ligand interactions have been achieved [307-312].

### 2.2.3 Force spectroscopy

Another important application of AFM is its ability to measure the mechanical properties of objects and materials. By pushing or pulling on samples, until a predefined
force or pizo height displacement is achieved, the AFM can record how the sample responded to the force applied by the cantilever tip. The force distance (FD) curves, generated as a result, contain information on the mechanical properties of the sample and hence, the factors that influence these properties can be indirectly measured. For instance, the Young's modulus for carbon nanotubes [313-315], graphene [316] and dentinoenamel junction [317] have been determined with this method. Furthermore, mechanical properties of the living cells [318, 319] and the interaction between cells and between cell and substrates have been measured [320]. AFM was as well applied for measuring the interactions of single molecules, which will be discussed in greater detail in the next chapter.
“Deducing models of function from the behavior of a complex system is an invers problem that is impossible to solve.”

“Sequence and consequence” Phil. Trans. R. Soc. B (2010) vol. 365, pg. 207-212

Sidney Brenner (13 January 1927 –)
3. Single-molecule force spectroscopy

Average values of properties may be useless in some cases (Figure 21). Similarly, average values, of certain properties obtained from ensemble measurements of large number of molecules may not contain the necessary information. For instance, these averages may result from wide and/or multimodal distributions. A particular example in which ensemble measurement techniques fail are low affinity interactions. Furthermore, many cellular processes are often carried out by one or few biomolecules and these processes can be directly studied with single molecule techniques. For these reasons single molecule measurements are indispensable.

![Average depth of the lake](image)

*Figure 21. The knowledge of the average depth of the lake may not be enough for a brave walk across the lake.*

Single-molecule force spectroscopy (SMFS) is a powerful tool for the direct measurement of interaction forces between single molecules and of their motion. Furthermore, with rigorous theoretical treatment, the parameters describing the free energy profile of the interacting molecular system can obtained from these forces.

3.1 SMFS techniques

At the end of the 20th century, several SMFS techniques have been invented. First, it became possible to measure directly the tiny interaction forces exerted by single molecules. Namely, it was possible to either apply a constant force on the studied molecules or to pull them with constant speed. Later, with an appropriate theoretical treatment, some structural
information on the stability segments of biopolymers, as well as on the thermodynamic and kinetic parameters of the corresponding free energy profile could be obtained. There are three most frequently used SMFS techniques: optical tweezers, magnetic tweezers and AFM that will be described in the next sections.

3.1.1 Optical tweezers

First demonstration of optical trapping of micron sized particles with a laser beam was in 1970 by Arthur Ashkin [325, 326]. Later, Ashkin and colleagues demonstrated optical trapping of bacterium, virus and dense array of viruses [327, 328].

The principle of trapping colloidal particles in the laser beam can be explained with the means of classical physics if the size of the particle is large relative to the wavelength of the incident light. The light beam that passes through the particle is being refracted since the particle and the medium have different refractive indices (Figure 22A). Light has the momentum associated with it and a change in the direction of light propagation means also a change in that momentum. A change of the momentum represents force according to the second law of Newton. According to the third law of Newton, there should be an opposite force of equal value acting on the particle where light refracts.

Optical tweezers typically use micron sized glass or polystyrene beads that are trapped in a laser beam with a Gaussian intensity profile. Biopolymers can be covalently attached to these beads and their properties can be studied by applying a well-controlled force by moving the laser beams. The forces that can be applied in conventional optical tweezers range from 0.1 to 100 pN. The spatial and temporal resolutions of the optical tweezers are 0.1 nm and $10^{-4}$ s respectively [329].

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* Later, mostly by the efforts of Chu, Phillips and Cohen-Tannoudji a similar principle has been used for trapping single atoms and cooling gases down to a few microkelvins [321-324]. After all temperature is an average measure of the kinetic energies of the atoms.

** For single atoms, this phenomenon can be explained by the means of quantum mechanics. The adsorption and re-emission of photons by atoms changes the momentum of atoms. The laser light wavelength as well as the arrangement of the laser light beams can be tuned to reduce the speed of atoms.
A wide variety of optical tweezers assays are known that allowed testing forces and lengths of individual steps of single molecular motor proteins [330-332], creating and running synthetic rotary molecular motors [333], measuring the force required to pack nucleic acids inside a bacteriophage capsid [334, 335] etc. Optical tweezers have been widely used for (un)folding nucleic acids since the force required for this is on the order of 10 pN and is in the operation range of this technique [336-339]. Furthermore, probe manipulation in three dimensions allowed researchers to tie knots of biopolymers of the desired topology and test the mechanical stability of knotted biopolymers [340].

Optical tweezers have some limitations, which should be taken into account before designing the experiments. The stiffness range of optical tweezers is from 0.005 to 1 pN/nm and depends on the intensity and gradient of the beam. If the intensity of the laser beam is being affected by particles in the solution, this will also affect the stiffness of the optical tweezers.

**Figure 22.** Schematic representation of the basic working principle of optical trapping. (A) beam of light passing through the colloidal particle is being refracted. The initial and the final directions of the beam are different, which means that the momentum associated with the incident light has changed. The change of the momentum represents force according to the second law of Newton. According to the third law of Newton there should be an equal force acting on the particle from the light beam that is opposite in direction to the momentum change of the light beam. (B) The colloidal particle can be trapped in the laser beam that has a Gaussian intensity profile based on the aforementioned principle. Lasers are arranged such that the beam has the highest intensity in the central part and the particle tends to localize in that specific part, any deviation from the center will cause imbalance in the refracted laser light which will generate force tending to bring the particle back to the center of the beam.
tweezers and hence, the experimental measurement. As it was mentioned above, optical tweezers can test usually forces up to 100 pN, the forces required for unfolding most of the proteins are higher than 100 pN, so that optical tweezers are rarely used for this purpose. Since there are laser beams involved in this technique there will be some heating of the sample and there is a risk of radiation damaging it.

3.1.2 Magnetic tweezers

Magnetic tweezers technique is based on the ancient knowledge of interaction of magnetic particles though it took several hundred years until this knowledge was implemented at the micro and nano scales for stretching and twisting polymers in a well-controlled manner [341]. The basic setup of magnetic tweezers is composed of two permanent magnets\(^*\) that can be rotated, of a magnetic particle that is covalently attached to one end of the polymer of interest, the other end of that polymer is attached to the coverslip, under which the objective of the inverted microscope is localized (used for visualizing the magnetic bead). The forces that can be applied by conventional magnetic tweezers range from 0.001 to 100 pN. The spatial and temporal resolutions of the magnetic tweezers are 5 nm and 10\(^2\) s respectively [329].

The magnetic tweezers have been extensively used for studying nucleic acid associated molecular motors [343-345]. Also, they have been used to produce adenosine triphosphate (ATP) by attaching a magnetic bead to the γ subunit of F\(_1\)-ATPase and by its mechanical rotation [346, 347]. Besides this, magnetic tweezers are probably the best candidates for manipulating objects inside the cell, since they cause least damage and are the easiest to control in dense media compared to the other two techniques discussed here [348, 349].

\(^*\) In order to generate rotation of the magnetic bead at least two permanent magnets are required, for pulling magnetic bead obviously one magnet is enough. There are also setups with electromagnet instead of permanent magnet that can generate higher force on the magnetic bead [342]. Electromagnetic setup is not widely used and its characteristics will not be discussed here.
Despite these advantages, magnetic tweezers are limited with regard to the video detection of the magnetic bead position, namely, detection of very fast and very small displacements of the bead are not possible. Also the permanent magnets involved in this technique offer limited possibilities of manipulation compared to the lasers in the optical tweezers or the piezo in AFM.

### 3.1.3 AFM based SMFS

In less than 10 years after its invention, AFM was already being used for SMFS. First, AFM based SMFS experiments focused on measuring the interaction force between strands of deoxyribonucleic acid (DNA) [350] and between biotin-avidin pairs [351]. The forces that can be applied in AFM range from 10 pN to 10 nN. The spatial and temporal resolutions of AFM are 0.5 nm and $10^{-3}$ s respectively. Thanks to these characteristics, AFM made it possible for the first time to test the strength of the covalent bond directly [352]. Soon, it was realized that an external pulling force applied through AFM can be used to unfold proteins [256, 258]. For water-soluble proteins, the support is functionalized with the protein of interest. Then, the AFM tip is approached towards the support until the setpoint force is reached, is kept in this position for a predefined time, after which the

**Figure 23. Schematic representation of the basic setup of the magnetic tweezers composed of permanent magnets that can rotate causing in turn the rotation of the magnetic bead, which is covalently attached to the polymer of interest. By manipulating the permanent magnets, the magnetic particle can be manipulated and the polymer under investigation can be twisted or stretched.**
retraction of the tip starts. There is a certain chance that the non-functionalized terminal end of the protein will bind nonspecifically to the AFM tip during this process. If this is the case, the protein can unfold during retraction. For TM proteins, membranes with the embedded TM proteins, are adsorbed to the support [290] and the same approach-retraction cycle is performed. In the case of ligand-receptor interactions, one assay is to functionalize the support with ligands and the AFM tip with receptors. Then, by bringing the tip and the sample in close vicinity and keeping them at this distance for a predefined time there is a certain chance that the ligand-receptor bonding will occur. Afterwards, by retracting the cantilever, the force necessary to break the bond can be measured. Another assay is to directly test ligand-receptor interaction with a ligand-functionalized tip directly on the cells [353]. Furthermore, in recent years, it became possible to simultaneously image and perform SMFS with AFM [354, 355].

The molecular unbinding or unfolding processes are stochastic, due to this around 100 such events have to be recorded to draw statistically sound conclusions. Frequently, interactions between the tip and the terminal end of the protein or between the ligand and the receptor are rare. Hence, many thousands of approach and retract cycles are required to obtain the desired number of FD curves with unfolding or unbinding events. Another challenge of AFM based SMFS is the isolation of the relevant interaction. Since there are many molecules in the fluid cell, the AFM tip can interact with some of them and we need to distinguish the interactions that we are looking for. In case of multidomain protein unfolding, this problem is solved by the specific pattern of the FD curves with the unfolding events. It is highly unlikely that unspecific interactions will produce a similar pattern, namely FD curve with multiple peaks at specific positions. However, in case of ligand-receptor interactions, where FD curves will typically have one peak, we need different criteria for isolating specific interactions. One, commonly used, method to do so is to use polymeric handles tethering the ligand to the tip and the receptor to the support. Once the specific shape of the force vs. extension dependency of the polymeric tether is known, the FD curves having that shape are considered to be resulting from specific interactions [356]. Nevertheless, in the case of multidomain protein unfolding, soft cantilevers or introduction of other elements, such as long polymeric handles, that soften the system will also decrease the likelihood of minor intermediates detection.
3.2 Polymer extension models

As it was discussed previously, during SMFS, force is applied on polymeric handles. In techniques such as optical tweezers and AFM the SMFS, data is obtained in the form of FD curves. From this data, some characteristics of the polymeric handle can be obtained by using an appropriate model. Next, we will discuss three polymer extension models and their relations to each other.

3.2.1 The freely jointed chain model

A freely jointed chain (FJC) is a number of successively connected segments of the same length that can freely rotate relative to one another (Figure 24). Segments do not have thickness, mass and charge and sometimes are referred to as repeating units. Also in this model segments are free to pass through each other without any resistance\(^\ddagger\). This means that two neighboring segments can make any angle and there is no force required to change this angle. The contour length of a chain is the sum of the lengths of all the segments or repeating units, i.e. if segment length is \(b\) and there are \(N\) such segments the contour length will be \(L = bN\).

\[\text{Figure 24. A freely jointed chain is a succession of solid segments with free joints. Each segment is having length } b. \text{ The end-to-end distance of the chain is denoted as } x. \text{ In most configurations the end to end distance of the FJC is much smaller than its contour length.}\]

For a large number of repeating units the FJC is making a random coil. This is happening because a random coil can be achieved with a much larger number of configurations than for example a straight chain when \(x = L\). Since the coil conformation can be obtained with more configurations than a straight one, it is highly likely that in the

\(^1\) In case of the magnetic tweezers one typically analyzes extension vs. magnet rotations curves.

\(^\ddagger\) This statement is true for all models discussed here.
absence of an external force, for a large enough number of segments, the FJC end to end distance $x$ is much smaller than its contour length. Although there is no force required for changing the angle between two neighboring segments in the FJC, there is a force required for stretching the FJC since by fixing its ends and stretching them we reduce the number of configurations that the chain can take. If one assumes small extensions and small forces acting on the FJC – so small that the FJC can still be assumed to be a random coil, then the force dependency on extension is linear Eq. 13 [357].

$$F(x) = \frac{3k_B T}{b} \frac{x}{L} \quad \text{Eq. 13}$$

This shows that for small forces, the FJC obeys the Hook’s law with the spring constant proportional to the temperature, i.e. for higher temperatures the resistance of the FJC to stretching is higher. This is a consequence of the purely entropic nature of the resistance of the FJC to external stretching force and commonly referred to as entropic elasticity. The factor 3 in Eq. 13 is a consequence of space dimensionality.

There is an exact analytical solution for this model for arbitrary forces acting on the FJC Eq. 14:

$$\frac{x(F)}{L} = \coth \left( \frac{Fb}{k_B T} \right) - \frac{k_B T}{Fb} \quad \text{Eq. 14}$$

It can be obtained from the partition function of the FJC, from which the free energy of the FJC can be calculated, then the extension as a function of force can be obtained by taking the derivative of that free energy with respect to force with an opposite sign [357, 358]. Figure 25A shows the Eq. 13 and Eq. 14 plotted in one graph and Figure 25B shows the relative error of Eq. 13 as a function of force for $b = 0.8\ nm$.

The FJC model has been successfully applied for describing FD curves of polyinosine [350] and polymethacrylic acid [359] in an aqueous medium and polydimethylsiloxane in heptane [360]. Generally, the FJC model is used with one fitting parameter which is the contour length of the polymer. The length of the repeating unit of the FJC should not be confused with the length of the repeating unit of a polymer under study. After fitting the FJC to the FD curve obtained from stretching the real polymer chain, the segment length of FJC should be substituted by the Kuhn length* of the polymer. The Kuhn length is the minimal length of the polymer segment for which that segments can be

*Henceforth we will denote Kuhn length with $b$. 
considered to be freely jointed. Another measure of the stiffness of the polymer chain is the persistence length denoted with $p$. It can be understood as the minimal length starting from where polymer can be bent at 90° without breaking. The persistence length is directly proportional to the Young’s modulus and inversly proportional to the temperature. Persistence length can also be considered to be equal to half of the Kuhn length.

For large stretching forces acting for example on polyethylene glycol in an aqueous medium, the FJC model fails to describe experimental FD curves. For this case the FJC model has been extended by taking into account the supermolecular reorganization and bond angle deformations [361, 362].

![Figure 25](image.png)

**Figure 25.** (A) linear approximate solution (blue) and exact result (red) of the FJC relative extension vs. force dependency for $b = 0.8 \text{nm}$. (B) the relative error of the linear approximation – for a 50 pN force applied at the FJC ends, the relative error of linear approximation is more than 200 %. Graphs are for FJC in three-dimensional space.

3.2.2 The freely rotating chain model

The freely rotating chain (FRC) model is a same as FJC model but with one additional constraint: each segment can rotate freely while keeping the angle with its neighboring segment constant (Figure 26). In contrast to the FJC, there is cooperativity between repeating units of the FRC and hence, the response to the external stretching force is not purely entropic.
The closed form analytical expression for force extension dependency for this model is not known. Certainly, this is one of the reasons why it was not used as widely as the other two models. Though, with the transfer-matrix calculation method, it was possible to obtain the force response of the freely rotating model for three distinct force ranges Eq. 15 [363].

\[
\frac{x}{L} = \begin{cases} 
\frac{Fk}{3k_BT} & \frac{Fb}{k_BT} < \frac{b}{p} \\
1 - \left(\frac{Fp}{4k_BT}\right)^{1/2} & \frac{b}{p} < \frac{Fb}{k_BT} < \frac{p}{b} \\
1 - \left(\frac{Fb}{2k_BT}\right)^{-1} & \frac{p}{b} < \frac{Fb}{k_BT} 
\end{cases}
\tag{Eq. 15}
\]

Clearly, the persistence length \( p \) and the Kuhn length \( b \) both depend on the angle between the two neighboring repeating units of the FRC.

3.2.3 The worm-like chain model

The worm-like chain (WLC) model, also referred to as the Kratky-Porod model or the elastic rod model, was proposed by Kratky and Porod in 1949 and treats the polymer as a long elastic rod without thickness [364, 365]. The deformation of a rod can be described with three parameters: bend, stretch and twist density [365]. In this model only bending is allowed and it is assumed that there is no twisting or stretching of the chain (no stretching means that the chain cannot be stretched beyond the value of its contour length).

Interestingly, the WLC model can be obtained from a completely different perspective, namely, by taking an FRC with an infinitely small repeating unit length and with
an infinitely many repeating units and assuming that the angles between the repeating units tend to be straight (Figure 27) [357].

This is a phenomenological model and similarly to previously discussed models it is generally used to fit experimental FD curves with one fitting parameter – the contour length.¹

\[ F(x) = \frac{k_BT}{p} \left( \frac{1}{4} \left(1 - \frac{x}{L}\right)^2 + \frac{x}{L} - \frac{1}{4} \right) \]

Eq. 16

The WLC model has been successfully applied for describing the extension of double stranded DNA [371] and polypeptide chains [256, 258]. However, at higher forces, due to changes in bond angles, polymers extend more than their initial contour length. As it was discussed previously, for the FJC model, as well as for the WLC model corrections have been introduced for such cases [367, 372].

¹ In some cases, the persistence length has also been used as a fitting parameter, in fact, it has been argued that persistence length itself is a function of force [366, 367].
But then, a natural question arises: how can the WLC model, which does not take into account any information regarding the sequence of the biopolymer or the solvent can successfully describe FD curves of different biopolymers such as double stranded DNA or polypeptides? First, there is a misleading statement in this question, namely, there is some information about the biopolymer type, its sequence, as well as about the solvent in the value of the persistence length used in the WLC model. The persistence length depends on the sequence of the biopolymer [373] as well as on the solvent [369] in which the biopolymer finds itself. Persistence length is used as a second fitting parameter in the WLC model in some cases, nevertheless there are a variety of methods for determining biopolymer persistence lengths [373-377]. Secondly, models do not need to take into account the whole complexity of the reality. Polymer extension models should be understood as tools for extracting some useful information from experimental data. Validation of polymer extension models today is straightforward by means of SMFS techniques: to test whether specific polymer extension model is usable for a particular biopolymer in certain external conditions, first, the biopolymer of a known contour length needs to be stretched to a certain degree. Then, the resulting FD curve can be fitted with a specific model. Consequently the contour length resulting from the fit can be compared to the known contour length and, depending on the difference between these two numbers, a decision can be made whether the model is usable for this specific case.

Figure 28. The FJC Eq. 14 that has a purely entropic response and the WLC Eq. 16 that has entropic and elastic bending responses to external forces are compared. For small extensions, the response is dominated by the entropic factor and both models show similar behavior. However, for larger extensions the WLC shows a higher force then the FJC. A persistence length of 0.4 nm for the WLC and Kuhn length of 0.8 nm for the FJC was used.
3.3 Dynamic force spectroscopy

Part of the free energy profile of the proteins or of the ligand-receptor bond can be characterized with several parameters. Those are: the distance between the native (bound) and the transition states $x^\ddagger$, the free energy difference between the native (bound) and the transition states $\Delta G^\ddagger$ and the free energy difference between the native (bound) and the unfolded (unbound) states $\Delta G$ of protein (ligand-receptor pair) (Figure 29A). Another characteristics of these systems is the transition rate $k_0$, analogous, to the reaction rate in chemical reactions (it is measured in inverse time units).

In case of externally applied force that tends to unfold the protein or to dissociate the ligand-receptor bond, the free energy landscape changes and hence, the free energy profile changes. The free energy difference between the native (bound) and the transition states decreases (Figure 29B). As a result, the probability of protein unfolding or ligand-receptor bond dissociation increases. In case of constant speed pulling, this process is continuous and eventually, leads to the transition from one state to another.

Systems that are at the free energy minima are not static at that position in time, rather, they fluctuate around that position. As a consequence, these fluctuations can move the system to a different minimum on the free energy landscape. By considering this, it is easy to understand, why in case of pulling with high loading rates, the rupture forces are generally higher. Assume the same system is being pulled with two different loading rates, one being 10 times smaller than the other (Figure 29C). After 10 s, the force on the system with the low loading rate equals to the force on the system with the higher loading rate after 1 s. This is represented by the identical free energy profiles of these systems after different time segments. In case of a slow loading rate, the transition already has occurred, since there was enough time for random fluctuations to move the system to the different minimum over the free energy barrier. By contrast, in the case of the fast loading rate, the transition has not occurred yet, since there was not enough time for random fluctuations to move the system to the different minimum over the free energy barrier. Hence, the process of pulling continues in the second case and the unfolding (unbinding) will occur at higher force values.

---

1 The loading rate is the rate of change of the force, in other words it is the first derivative of force with respect to time.
The unfolding (rupture) forces of the same system under the same conditions are generally different, since they are conditioned by the random fluctuations. By testing the same system under loading rates varying by several orders of magnitude it is possible to “sense” the free energy profile and extract some of the parameters characterizing it. This approach of testing the same system under different loading rates is known as dynamic force spectroscopy (DFS). Several models have been proposed that are used for extracting some of the parameters characterizing the free energy profile of the system – these models will be discussed next. The approach is to find the dependency of the unfolding (rupture) force probability density function on the parameters of the free energy profile and the loading rate, then fit that function to the experimental data using the parameters of the free energy profile as fitting parameters.
Figure 29. The simple free energy profile can be characterized with several parameters; these are the distance between the native (bound) and the transition states $x^\pm$, the free energy difference between the native (bound) and the transition states $\Delta G^\pm$ and the free energy difference between the native (bound) and the unfolded (unbound) states $\Delta G$ of the protein (ligand-receptor pair). The transition rate constant $k_0$ is another kinetic characteristic of the system (A). Under the external force that tends to unfold (dissociate) the protein (ligand–receptor pair), the free energy profile changes in a way that the free energy difference between the native (bound) and the transition states decreases (B). (C) A simple argument that explains why, in case of a slow loading rate, rupture forces are generally lower. The same free energy profile is being tested under a low loading rate (slow pulling) on the left and a high loading rate (fast pulling) on the right. The numbers near the dashed lines show the first passage time i.e. the average amount of time that is required for the system to reach to certain free energy level first time due to random thermal fluctuations. The same force is applied to the system on the left after 10 s and to the system on the right after 1 s due to the 10-fold difference in loading rates. As it can be noticed, at that force, system on the left is likely to have passed over the transition state. By contrast, the system on the right is still in the same free energy well, the constant loading rate pulling continues the force increases and the transition will occur at higher force values.
3.3.1 Bell-Evans model

The first DFS model was proposed by Evans and Ritchie in 1997 [378]. The probability density function of unfolding (rupture) forces $p(F)$ is related to the survival probability $S(t)$ as in Eq. 17.

$$p(F)dF = -\frac{dS(t)}{dt}dt$$  \hspace{1cm} \text{Eq. 17}

This is the mathematical formulation of the simple argument in Figure 29C. Next, it is assumed that the unfolding (unbinding) is irreversible – this means that we deal with processes far from equilibrium. Unfolding (unbinding) events are irreversible particularly in the case of a high pulling speed and/or in case of using long polymeric linkers that tether the system to the pulling device [356]. In case of irreversible unfolding (unbinding), the survival probability satisfies to the first order rate equation Eq. 18:

$$\frac{dS(t)}{dt} = -k \cdot S(t)$$  \hspace{1cm} \text{Eq. 18}

where $k$ is the transition rate constant". In case of an externally applied force, that tend to unfold (disrupt) protein (ligand-receptor bond), transition rate is a function of force. In order to determine the unfolding (rupture) force probability density function, the transition rate constant dependency on force needs to be known. For this purpose, Bell’s expression has been used, Eq. 19 [379]:

$$k(F) = k_0 e^{F_x^d/k_B T}$$  \hspace{1cm} \text{Eq. 19}

where $k_0$ is the transition rate constant in the absence of an external force. This expression is valid when the free energy difference between the transition and the native (bound) states is large. It also assumes that the distance between the transition and native (bound) states is independent of force.

By plugging in Eq. 19 into the Eq. 18 and then using the result for solving the Eq. 17 for the unfolding (rupture) forces, the probability density function can be achieved, Eq. 20:

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*The probability that the protein is still folded or that the ligand–receptor bond is still intact.

**The word constant here is misleading since its value depends on force and hence, on time.
\[ p(F, r; k_0, x^\ddagger) = \frac{k_0}{r} \exp \left( \frac{Fx^\ddagger}{k_B T} - k_0 x^\ddagger \left( e^{\frac{Fx^\ddagger}{k_B T}} - 1 \right) \right) \]  

Eq. 20

where \( r = \frac{dF}{dt} \) is the loading rate. Eq. 20 is representing the unfolding (rupture) force probability density function depending on two variables, namely, force and loading rate and on two parameters, namely, transition rate constant in the absence of an external force and the distance between the transition state and native (bound) state. Ideally, after a large number of unfolding (rupture) events has been observed, Eq. 20 should be fitted to the unfolding (rupture) force probability mass function using \( k_0 \) and \( x^\ddagger \) as fitting parameters [380]. However, often, other more easily implementable approaches have been taken. Namely, from Eq. 20 it is straightforward to calculate the most probable unfolding (rupture) force \( F^\ast \) Eq. 21.

\[ F^\ast(r; k_0, x^\ddagger) = \frac{k_B T}{x^\ddagger} \ln \left( \frac{x^\ddagger r}{k_0 k_B T} \right) \]  

Eq. 21

On a linear-lg scale the most probable rupture force vs. loading rate is a straight line. Then, the most probable forces for particular loading rates can be determined from the data and that can be fitted by Eq. 21 using \( k_0 \) and \( x^\ddagger \) as fitting parameters. The unfolding (rupture) force probability density function is negatively skewed, hence, its mean and mode are different. In this case the mode, i.e. the most probable unfolding (rupture) force is used, since there is a simple exact analytical expression for it.

This phenomenological model assumes that the reaction coordinate is the same as in the case of absence of the external force, i.e. it is assumed that in the absence of the force, the spontaneous unfolding (unbinding) of the protein (ligand-receptor pair) is happening along the same reaction coordinate on the free energy landscape. Further, it assumes that unfolding (unbinding) is irreversible and the distance between the native (bound) state and the transition state is independent on the external force – this sets the lower bound for pulling speed. Bell’s expression for the force-dependent reaction rate is valid for small forces, so that the free energy difference between the transition and the native (bound) states is large – this sets an upper bound for the loading rate. The biggest disadvantage of the model, however, is that in some cases, the experimentally obtained most probable forces, as well as
the average forces on the linear-lg scale don’t lie on a straight line. It has been speculated, that this could be a consequence of multiple transition barriers in the free energy profile [380].

This model assumes that the force is directly being applied on the system without any intermediate elements such as springs of polymeric handles. However in real experiments these elements are present. Corrections to the Bell-Evans model have been introduced by taking into account springs and polymeric handles [381, 382].

3.3.2 Dudko-Hummer-Szabo model

One of the drawbacks of the Bell-Evans model, namely, the use of Bell’s expression that assumes that the distance between the transition and the native (bound) states does not change in case of an external force, was attempted to be avoided by Dudko, Hummer and Szabo [383-385]. To achieve this, they assumed that the shape of the free energy profile is known and used Kramers’ theory to find the force-dependent transition rate constant [386-388], Eq. 22:

\[
k(F) = k_0 \left(1 - \frac{\nu F x^+}{\Delta G^\ddagger} \right)^{\frac{1}{\nu}} e^{\frac{\Delta G^\ddagger}{k_B T} \left(1 - \frac{\nu F x^+}{\Delta G^\ddagger} \right)^\nu}
\]

Eq. 22

where \( \nu \) is a factor that defines the shape of the free energy profile, for example if \( \nu = 1/2 \), the free energy profile is a parabola with a cusp-shaped transition barrier, if \( \nu = 2/3 \), the free energy profile is given by the sum of the linear and cubic functions of the reaction coordinate. The phenomenological Bell’s expression is a special case of Eq. 22 and it corresponds to the case when \( \nu = 1 \) [384].

In the same manner as before, by plugging in Eq. 22 in Eq. 18 and then using the result for solving Eq. 17 for the unfolding (rupture) forces probability density function, Eq. 23 can be obtained:

\[
p(F,r;k_0,x^+,\Delta G^\ddagger) = \frac{k(F)}{r} e^{\frac{k(F)}{2} \left(1 - \frac{\nu F x^+}{\Delta G^\ddagger} \right)^\frac{1}{\nu}}
\]

Eq. 23

where \( k(F) \) is given by Eq. 22. Unlike the Bell-Evans model, here we have three fitting parameters \( k_0, x^+ \) and \( \Delta G^\ddagger \).
The Dudko-Hummer-Szabo model, similarly to the Bell-Evans model, assumes that the reaction coordinate is the same as in the case of absence of the external force. Further, it assumes that unfolding (unbinding) is irreversible. This sets the lower bound for pulling speed. Kramers’ theory is valid when the free energy difference between the transition and native (bound) states is large, i.e. there is an upper bound for the externally applied force and hence, for the loading rate.

This model was developed without taking into account any intermediate elements such as springs of polymeric handles. The corrections to the Dudko-Hummer-Szabo model have been introduced by taking into account springs and polymeric handles [389, 390].

The Dudko-Hummer-Szabo model has been extended also for free energy profiles with multiple transitions [391]. During mechanical unfolding of TM proteins, the unfolding is generally happening stepwise, i.e. when the force gradually increases, first, some part of the TM protein unfolds, then, other parts [258]. This can be seen in the FD curves as multiple force peaks. Previously, these events were considered independent from each other and each unfolding event was treated separately as a separate free energy profile with a single transition state.

3.3.3 Friddle-Noy-De Yoreo model

The irreversibility assumption of the previous two models, which is mathematically formulated in Eq. 18 was challenged by Friddle, Noy and De Yoreo. They accounted for the reversible unfolding (unbinding) which is mathematically formulated in eq () [392]

\[
\frac{dS_b(t)}{dt} = -k_u \cdot S_b(t) + k_u \cdot S_u(t)
\]

Eq. 24

where \( S_b(t) \) is the survival probability (same as \( S(t) \) in the Eq. 18), \( S_u(t) \) is the unfolding (unbinding) probability and \( k_u \) and \( k_b \) are the unfolding (unbinding) and refolding (rebinding) rate constants that can depend on the force if there is an external force applied to the system.

As an unfolding (unbinding) rate constant in this model, the same Bell’s expression [379] has been adopted, however it was assumed that the pulling is done through a spring Eq. 25:
\[ k_u(F) = k_{0u} \exp \left( \frac{1}{k_b T} \left( Fx^\ddagger - \frac{1}{2} \kappa x^\ddagger^2 \right) \right) \]  

Eq. 25

where \( k_{0u} \) is the unfolding (unbinding) rate constant in the absence of an external force, same as \( k_0 \) in Eq. 19 and Eq. 22.

The unfolding (rupture) force probability density function has not been determined for this model. However, by considering that the bond loading starts at a threshold force called equilibrium force \( F_{eq} \), it was possible to truncate Eq. 24 and by plugging in Eq. 25, the average force expression for this model was obtained Eq. 26 [392]:

\[ \langle F(r; k_0, x^\ddagger, \Delta G) \rangle = F_{eq} + \frac{k_b T}{x^\ddagger} \ln \left( 1 + \frac{r x^\ddagger}{k_u(F_{eq}) k_b T} e^{-\gamma} \right) \]  

Eq. 26

where \( F_{eq} = \sqrt{2k \Delta G} \) [393] and \( k_u(F_{eq}) \) can be obtained from Eq. 25 by substituting \( F \) with \( F_{eq} \). Here the fitting parameters are \( k_0, x^\ddagger \) and \( \Delta G \).

The Friddle-Noy-De Yoreo model has the same limitations as the Bell-Evans model except for the assumption of irreversibility. It assumes that the reaction coordinate is the same as in the case of an absence of external force. Bell’s expression for force dependent reaction rate is only valid for small forces, so that the free energy difference between the transition and the native (bound) states is large – this sets an upper bound for the loading rate. Another consequence of the use of Bell’s expression is the assumption of independence of the distance between the transition and the native (bound) states on external force. Finally, the unfolding (rupture) force probability density function is not determined and there are no corrections introduced for polymeric tethers connecting the system to the pulling device.
3.4 Fluctuation theorems

Thermodynamics has been developed in 19th century to describe systems with a large number of identical components. However, for small systems, its limits have been pushed down only relatively recently [394, 395]. As a result, a new equality and a theorem have been formulated, for systems driven arbitrarily far from equilibrium, such that the laws of “macroscopic” thermodynamics (e.g. the second law) follow from these more generally formulated statements. These great achievements have been largely applied for analyzing SMFS data and for gathering information on the free energy profiles in a model free approach. But first, these theoretical achievements had to be confirmed by experiments.

3.4.1 Jarzynski equality

A significant breakthrough in nonequilibrium thermodynamics has been achieved by Jarzynski in 1997 for describing a system that is initially in a thermal equilibrium with the environment and then driven out of it due to, for example, an externally applied force, afterwards it brought to another state and equilibrated there [396, 397]. Jarzynski obtained an equality which made it possible to determine the free energy difference between the initial and the final equilibrium states from the works of repeated nonequilibrium processes that have led system from one state to another – Eq. 27

\[
\frac{\Delta G}{k_B T} = \left\langle e^{\frac{-W}{k_B T}} \right\rangle
\]

where \( W \) is the work done during a single nonequilibrium process and averaging is done over all such processes’. The equality holds in the case of averaging over infinitely many repeated nonequilibrium processes, nonetheless, the systematic error arising from a finite number of such processes is easy to estimate [398]. This simple and elegant equality demonstrates that the information on equilibrium properties of the system can be obtained from the ensemble of nonequilibrium measurements.

\[
\left\langle W \right\rangle \geq \Delta G
\]

The second law of thermodynamics can be stated as \( \left\langle W \right\rangle \geq \Delta G \). It can result from Eq. 27 and Jensen’s inequality.
More straightforward derivation of Eq. 27 has been demonstrated by Hummer and Szabo in 2001 by using the Feynman-Kac theorem for path integrals. In the same work, they also provided a method for reconstructing the free energy profile of the system based on the Jarzynski equality [399-401]. This approach has been applied for reconstructing the free energy profiles of proteins [402, 403] and nucleic acids [404, 405].

The Jarzynski equality found its experimental confirmation in 2002 by Liphardt and his colleagues in Bustamante’s group [398] and the Hummer-Szabo approach for free energy profile reconstruction found its experimental confirmation in 2011 by Gupta and colleagues in Woodside’s group [406]. In both cases optical tweezers-based assays were used.

3.4.2 Crooks fluctuation theorem

Two years later, Crooks came up with another fundamental result, for a system that is initially in a thermal equilibrium with the environment and then driven out of it due to, for example, an externally applied force, transitioning to another state where equilibrium is reached and then the reverse transition is carried out, again, arbitrarily far from equilibrium [407]

\[
\frac{p_F(W)}{p_R(-W)} = \exp\left(\frac{W - \Delta G}{k_B T}\right)
\]

Eq. 28

where \(p_F(W)\) and \(p_R(W)\) are the work probability density functions of the forward and reverse processes respectively.

The Jarzynski equality is a special case of the Crooks fluctuation theorem. It can be obtained by rearranging Eq. 28 into \(p_R(-W) = p_F(W)\exp\left(-\frac{W - \Delta G}{k_B T}\right)\) and then, by integrating both sides with respect to \(W\) with integration limits from \(-\infty\) to \(\infty\).

The Crooks fluctuation theorem found its experimental confirmation in 2005 by Collin and colleagues in Bustamante’s group [408]. Similar to the previous experiment probing the Jarzynski equality, an optical tweezers-based assay was used.
“In fact the hardest part of research is always to find a question that’s big enough that is worth answering but little enough that you actually can answer it.”

https://www.youtube.com/watch?v=06yXsnTFF-U

Edward Witten (26 August 1951 –)
4. Single-molecule force spectroscopy from freely spanning purple membrane


SMFS provides detailed insight into the mechanical (un)folding pathways and structural stability of membrane proteins. So far, SMFS could only be applied to membrane proteins embedded in native or synthetic membranes adsorbed to solid supports. This adsorption causes experimental limitations and raises the question to what extent the support influences the results obtained by SMFS. Therefore, we introduce here SMFS from native purple membrane freely spanning across nanopores. We show that correct analysis of the SMFS data requires extending the WLC model, which describes the mechanical stretching of a polypeptide, by the cubic extension model, which describes the bending of a purple membrane exposed to mechanical stress. This new experimental and theoretical approach allows to characterize the stepwise (un)folding of the membrane protein bacteriorhodopsin and to assign the stability of single and grouped secondary structures. The (un)folding and stability of BR shows no significant difference between freely spanning and directly supported purple membranes. Importantly, the novel experimental SMFS setup opens an avenue to characterize any protein from freely spanning cellular or synthetic membranes.
4.1 Introduction

Membrane proteins play crucial roles in the living cell and are involved in various cellular processes including signal transduction, energy conversion, molecular transport, osmoregulation, cell–cell communication, cell shape regulation, cell adhesion, cell migration, and cell sorting. To fulfill their functional tasks, newly synthesized polypeptides of membrane proteins must insert and fold into the cellular membranes, where they adopt three-dimensionally precisely defined structures [8, 115, 409, 410]. Destabilized membrane proteins can lose their structure–function relationship, which can cause cellular dysfunction and diseases. Factors that can lead to such destabilization and malfunction include the lipid composition of the cellular membrane, mutations, misfolded proteins, chemicals, electrolyte, pH, or mechanical forces [411-413]. Hence, the understanding of how intra- and intermolecular interactions contribute to the dynamic (de)stabilization and (mal)function of membrane proteins is of considerable interest [411, 414, 415]. Many biophysical and biochemical tools are available to characterize the folding and stability of membrane proteins, which have been isolated from the cellular context [410, 416, 417]. However, to describe folding and stability accurately, membrane proteins should preferably reside in their native cellular membrane or at least in lipid membranes that mimic the amphiphilic nature of cellular membranes [8].

In the past 15 years, AFM-based SMFS has been particularly useful to characterize the (un)folding pathways of membrane proteins and to quantify the strength of the interactions stabilizing their individual secondary structural elements [416-418]. In contrast to ensemble-measurement techniques, which suffer from problems associated with population averaging, SMFS quantifies the folding and stability of single membrane proteins. To conduct AFM-based SMFS, protein membranes are preferably adsorbed on a chemically inert and hydrophilic support (most frequently muscovite mica) and imaged by scanning the AFM tip over the sample [419]. After this, the AFM tip is pushed into contact with the membrane protein by applying a well-controlled force (in the range of 100–1000 pN) for a defined dwell time. This forced contact facilitates the nonspecific attachment of the membrane protein polypeptide to the AFM tip. Then the AFM tip is retracted and the cantilever deflection recorded to measure the forces required to mechanically unfold the single membrane protein. The force sensitivity of this approach lies in the piconewton (pN)
range, which is sufficient to quantify the contribution of single polypeptide loops or secondary structure elements to the overall stability of the membrane protein. Because of the high spatial precision of the AFM, these forces can be localized onto the primary, secondary, or tertiary structure of the membrane protein [416, 418, 420]. With this approach, the stability and unfolding pathway of various transmembrane proteins including human aquaporin [421], BR [422, 423], sodium proton antiporter (NhaA) [424], proteorhodopsin (PR) [425], outer membrane proteins A (OmpA) and G (OmpG) [426, 427], bovine and mouse rhodopsin [428], opsins [429], sensory rhodopsin [430], human β2 adrenergic receptor (β2AR) [431], FhuA [432], amino acid transporter (SteT) [433], betaine symporter (BetP) [434], dipeptide and tripeptide permease A (DtpA) [435], and lactose permease (LacY) [436] have been characterized.

Frequently neglected, it was for a long time questioned whether the support carrying the protein membrane for AFM and SMFS measurements influences the stability and unfolding pathways of membrane proteins [422]. Indeed, the distance between the adsorbed protein membrane and the support is sufficiently close (≈0.5–2 nm) to allow unspecific interactions [437, 438]. For example, these unspecific interactions, although in most cases being rather small, have been shown to reduce the diffusion coefficient (e.g., mobility) of proteins in membranes adsorbed to mica [439]. Hitherto, in several attempts, SMFS has been applied to characterize the influence of the support on the unfolding and stability of supported membrane proteins [422, 440, 441]. These controls unfolded the same kind of membrane protein directly supported by different supports. As the controls revealed no differences on the membrane protein stability, it was concluded that the hydrophilic mica support, which is most frequently used for biological AFM and SMFS [442], has negligible influences on the membrane protein folding and stability. Nevertheless, the final control would be to characterize the stability and (un)folding of membrane proteins directly supported by mica with those not supported at all by a solid surface. This latter possibility is given by membranes supported by polymer cushions or freely spanning across nanoscopic holes [438, 442]. Moreover, if it would be possible to characterize the stability and (un)folding of proteins embedded in a freely spanning membrane, this could open an exciting avenue to characterize how membrane proteins interact with soluble proteins or molecules from both membrane surfaces. Conducting SMFS from freely spanning membranes would also allow exposing both membrane surfaces to different environmental
conditions (e.g., buffer, proteins, molecular compounds) or establishing an electrochemical gradient across the membrane. For these reasons, we use SMFS here to investigate whether the support influences the intra- and intermolecular interactions and, hence, the (un)folding of membrane proteins. As a reference membrane protein, we have chosen the structurally and functionally exceptionally well-characterized light-driven proton pump BR from the PM of *Halobacterium salinarum* [443-445]. Using SMFS, we characterize native PM adsorbed either to mica (directly supported PM) or to porous poly(methyl methacrylate) (PMMA), being perforated by nanoscopic holes (freely spanning PM).

4.2 Experimental procedure

4.2.1 Preparation of porous substrates

Porous PMMA substrates were prepared as described [446]. Briefly, PMMA ($M_w = 9.59$ kg/mol, PDI = 1.05) and polystyrene (PS, $M_w = 96$ kg/mol, PDI = 1.04) were purchased from Polymer Standards Service GmbH and dissolved in methyl ethyl ketone (MEK, Sigma-Aldrich). The polymers had a total concentration of 15 mg/mL with the mass ratio between PS and PMMA being 3:7. The polymer blend films were spin-cast at a speed of 1500 rotations per minute (rpm) at room temperature and at a relative humidity of 45%–50% onto silicon substrates cleaned by the snow-jet method [447]. In order to create nanoscopic holes on the PMMA support, PS was selectively dissolved by dipping the support into a cyclohexane bath three times for 30 s and drying it in a stream of nitrogen in between and at the end.

4.2.2 Sample preparation

PMs from *H. salinarum* were basically prepared as described [39, 448]. Briefly, the BR overexpressing *H. salinarum* strain S9 was grown in L37 medium (4.3 M NaCl, 80 mM MgSO$_4$, 10.2 mM Na$_3$ citrate, 27 mM KCl, 1% Pepton L37 (Oxoid)) at 40 °C and 120 rpm to an $OD_{600}$ of about 1 (3–4 days). To induce high BR expression, rpm were reduced to 80 and extra light was turned on. After another 3–4 days, cells were pelleted (10000g; 20 min;
room temperature) and washed once with basal salt (4.3 M NaCl, 80 mM MgSO₄, 27 mM KCl) and then cell pellet weight was determined. Cells were lysed by resuspending the pellet in 25 mL of H₂O per g cells and stirring overnight at 4 °C with 0.02 % NaN₃ and DNase I (1 mg/mL cells). Cell debris were removed by centrifugation: once at 4300 g (10 min; 4 °C) and twice at 7600 g (10 min; 4 °C). PM was pelleted (55 000 g; 1 h; 4 °C), resuspended in H₂O, and centrifuged again as before. Two more washing steps with H₂O followed (60 000 g; 1 h; 4 °C) with subsequent resuspending of the PM at 1 mL/g cells with 0.02 % NaN₃. The BR concentration was determined by measuring the absorption at 568 nm and using the absorption coefficient 2.35 mL mg⁻¹ cm⁻¹. Purified PM in distilled water and 0.02 % NaN₃ was frozen and stored at −80 °C. After thawing, the PM stock solution (4–8 mg/mL) was kept at 4 °C.

Porous PMMA supports were treated using a plasma cleaner (PDC-32 G, Harrick Plasma, U.S.A.) for 10 s (18 W). Then, 20 μL of PM diluted to 5 μg/mL in buffer solution (300 mM KCl, 20 mM Tris–HCl, pH 7.8) was adsorbed on the support (either porous PMMA or freshly cleaved muscovite mica) at room temperature (≈25 °C). After an adsorption time of 20 min, the samples were gently rinsed with the imaging buffer (150 mM KCl, 20 mM Tris–HCl, pH 7.8) to remove nonadsorbed and weakly attached membranes.

### 4.2.3 AFM imaging and SMFS description

AFM imaging and SMFS was carried out using a JPK NanoWizard II Ultra (JPK Instruments, Germany). The 200 μm long AFM cantilevers having nominal spring constants of 60 pN/nm (OMCL-RC800PSA, Olympus, Japan) were calibrated in buffer solution using the thermal noise calibration method [272, 449]. Samples were first imaged using contact mode AFM while keeping the applied force < 200 pN. After having localized a PM completely spanning a nanopore, the AFM tip was gently pushed onto PM applying a maximal force of 300 pN for 0.5 s. This mechanical contact of AFM tip and PM facilitated the nonspecific adsorption of the terminal end of BR [419, 422]. The probability of nonspecifically attaching the AFM tip to the C-terminal end of BR is much higher than to the N-terminal end [450]. FD curves recorded upon unfolding BR from the C-terminal end show a different pattern compared to FD curves recorded upon unfolding BR from the N-terminal end. Thus, the pattern of the FD curves can be used to identify from which PM
surface BR has been unfolded. Alternatively, one could use high-resolution AFM imaging to identify the cytoplasmic and the extracellular PM surfaces [419]. In this work, we used SMFS to record FD curves from the cytoplasmic surface of PM at which BR exposes the C-terminal end. Approach and retraction velocities while recording FD curves were 500 nm/s. All experiments were conducted in imaging buffer at room temperature.

4.2.4 FD curve selection and analysis

Mechanical unfolding of BR was recorded by FD curves. Each force peak of a FD curve denoted the unfolding of a structural segment of BR. The pulling distance at which a force peak was detected assigned the contour length of the unfolded and stretched polypeptide that tethered the AFM stylus and the anchoring structural segment. The very last force peak of a FD curve represented the unfolding of the last structural segment remaining anchored to the membrane. Overcoming the stability of this last segment led to complete unfolding of BR, followed by extraction of it from the membrane. A full mechanical unfolding of BR shows four major peaks with the last peak located at a distance between 60 to 70 nm [422, 440], in the case of freely spanning membrane this peak is observed between 80 to 90 nm due to the bending of the PM.

All unfolding FD curves recorded from PM were manually aligned at the characteristic second major force peak as described [422, 440]. The aligned FD curves recorded from PM adsorbed directly onto mica were analyzed using the WLC model (eq 1) and those recorded from freely spanning PM were analyzed using the WLC model combined by the cubic response model (eq 3).
4.3 Results and discussion

4.3.1 Mechanical unfolding of single BR from freely spanning PM

To be able to characterize PM freely spanning across nanoscopic holes, we first produced porous PMMA supports using polymer blend lithography as described [446] and characterized them by AFM (Figure 30). Polymer blend lithography is based on the structure formation via polymer demixing in spin-cast films [451]. PMMA is hydrophilic and exposes a negatively charged surface, both of which properties can be adjusted by oxygen plasma treatment [452]. Comparably, muscovite mica, which is frequently used as support for the adsorption of PM for AFM studies, is also hydrophilic and negatively charged [290]. Thus, we thought that PMMA could be a good candidate to absorb PM under similar conditions [290, 419]. The AFM topographs show porous PMMA supports, which were peppered by nanoscopic holes that had mean diameters of 381 ± 103 nm (mean ± SD, n = 83). The depth of the holes ranged between 40 and 60 nm and the mean surface roughness of the porous PMMA was ≈1.2 nm. PM patches extracted from *H. salinarum* exhibit diameters ranging from 100 to 1000 nm [287] and, thus, could nicely cover some of these nanoscopic holes.

For SMFS, native PM was adsorbed onto porous PMMA supports and imaged by AFM in buffer solution (Figure 31). The PM concentration was chosen so that individual PMs sparsely and heterogeneously distributed over the PMMA support. After having localized a PM covering an entire pore of the support (Figure 31A), the AFM tip was gently pushed onto the PM to force the nonspecific attachment of the BR polypeptide to the tip [422]. During the approach of the AFM tip to the freely spanning PM, we recorded a FD curve (Figure 31B). As soon as the tip came into contact with the PM the force increased nonlinearly. The slight slope of the FD curve indicated the bending of the freely spanning PM, which is quite soft and elastic [453]. To avoid damage to the freely spanning PM, the applied force and the contact time were limited to 300 pN and 0.5 s, respectively. After this forced contact, the AFM tip was retracted from the membrane and a retraction FD curve recorded. Rarely, in ≈0.54 % of all cases (n = 54439), the retraction FD
Figure 30. Characterization of porous polymethyl methacrylate (PMMA) supports imaged by AFM. (A) Topograph and (B) deflection error image of a porous PMMA support. (C) Histogram of topography height of the red rectangle (358 nm × 438 nm, 900 pixels) indicated in (A). (D) Mean diameter of holes of the PMMA support (n=83). Bin size, 20 nm. (E) Topograph of a single hole of the PMMA support. (F) Cross section height analysis along the white line in (E). Contact mode AFM was conducted in buffer solution (300 mM KCl, 20 mM Tris-HCl, pH 7.8) applying forces of ≈200 pN.
curve showed a sawtooth-like pattern of four major force peaks (Figure 31B). FD curves showing similar patterns were previously assigned to the mechanical unfolding of a single BR from PM adsorbed onto mica [422]. For comparison, we recorded a FD curve on the PMMA support next to the freely spanning PM (Figure 31B). The approach FD curve showed a sharp transition as soon as the AFM tip came in contact with the PMMA. The steep slope of the tip-sample contact region indicates that the PMMA support is much stiffer compared to the freely spanning PM. The retraction FD curve recorded no adhesive force peak pattern such as observed on PM. After recording the FD curves, we reimaged the PMMA support (Figure 31C). The PM from which BR has been unfolded remained intact, indicating that the membrane did not rupture upon pushing and stressing with the AFM tip.

Figure 31. SMFS of BR from freely spanning PM. (A) AFM topograph (left) and deflection error image (right) of a PM (see the arrow) spanning over a nanoscopic hole in the PMMA support. After localizing a freely spanning purple membrane (arrow) the AFM is used for SMFS. Therefore, the AFM tip is approached and retracted to the center of the purple membrane (label 1 in (A)) while recording an approach (blue) and retraction (red) FD curve (labeled 1 in (B)). The retraction FD curve shows the typical sawtooth-like unfolding pattern of a single BR [422, 440]. For comparison, the FD curve below has been recorded on the PMMA surface labeled 2 in (A). (C) Topograph (left) and deflection error image (right) of the same area after force spectroscopy showing that the structural integrity of the freely spanning purple membrane is preserved. Experiments were conducted in buffer solution (150 mM KCl, 20 mM Tris–HCl, pH 7.8) at room temperature (≈25 °C).
After having recorded one FD curve showing the characteristic sawtooth-like pattern of BR [422], we decided to significantly increase the number of FD curves (Figure 32). Therefore, we repeated the SMFS experiment several thousand times on freely spanning PMs (Figure 32A,C and Figure 33A). In our experiments, we only recorded FD curves from PM exposing the cytoplasmic surface to the AFM tip. This criterion ensured that we unfolded only BR by mechanically pulling the C-terminal end. The sawtooth-like pattern of the individual FD curves recorded was highly reproducible (Figure 32A), which is also highlighted by the superimposition of 163 FD curves recorded from at least 20 different sample preparations (Figure 32C). It was described previously that the single force peaks of this sawtooth-like pattern record the individual unfolding steps of BR [416, 420, 440]. In each of these steps, BR unfolds a structural segment until the entire membrane protein has been unfolded and extracted from the PM. It has been shown that by using the WLC model each of these major force peaks can be fitted to estimate the contour lengths of the polypeptide stretch unfolded in each of these unfolding steps. The contour length of every unfolded polypeptide stretch assigns a structural segment of the membrane protein [440]. Thus, to estimate the structural segments of BR unfolded from freely spanning PM each of the unfolding force peaks was fitted by the WLC model (Figure 32C). For these fits, we used the same WLC parameters (e.g., persistence length of 0.4 nm) as previously established for BR unfolded from directly supported PM [422, 440]. Upon fitting, the four major unfolding force peaks the WLC model obtained contour lengths of 90, 165, 225, and 296 amino acids (aa). However, because the overall contour length of BR is 248 aa, these fits indicate that the WLC model does not describe the experiment correctly.

4.3.2 Deflection of freely spanning PM moves force peaks to larger distances

Fitting the force peaks recorded upon unfolding BR from freely spanning PM by the WLC model revealed contour lengths much longer than the overall polypeptide length of BR. On the search of the origin of this discrepancy, we decided to unfold BR from the same PM preparation but adsorbed onto freshly cleaved mica (Figure 32B,D). All other experimental conditions including the AFM, contact force, contact time, pulling velocity, cantilever type, buffer solution, and temperature were kept exactly as used to characterize
Figure 32. BR unfolded from freely spanning and mica-supported purple membrane show the same major unfolding intermediates. Single FD curves (A, B) and superimposition of FD curves (C, D) recorded upon unfolding of BR from freely spanning purple membrane (A, C) or from directly supported purple membrane (B, D). n gives the number of FD curves superimposed. Green curves in (C, D) are calculated using the WLC model (Eq. 16) to fit the major force peaks. Red curves in (C) are calculated combining the WLC model and the cubic response of purple membrane (Eq. 30) to fit the force peaks. The numbers at the top of each fitted curve give the contour lengths in amino acids (aa) unfolded at each force peak. These unfolding peaks assign the unfolding steps of the BR molecule [422, 440]. (E) These contour lengths obtained by fitting the force peaks, thus, are used to assign the structural segments unfolded in each unfolding step of BR. Such structural segments are either formed by paired or single transmembrane α-helices and their connecting polypeptide loops. (F) Secondary structure model of BR [454] with the structural segments unfolding in each unfolding step being colored differently.
freely spanning PM. FD curves recorded on mica-supported PM showed the specific sawtooth-like pattern of four major force peaks. Upon fitting this pattern using the WLC model we revealed the contour length of the major unfolding force peaks of 17, 88, 148, and 219 aa. These contour lengths matched those published before for BR [422, 440] but were much shorter compared with those determined upon unfolding of BR from freely spanning PM.

We wanted to understand why the unfolding force peaks recorded on BR from freely spanning PM occurred at larger distances and, thus, can be fitted by the WLC model only when using much larger contour lengths. Cellular membranes are soft and flexible [455, 456]. When pushing the AFM tip onto the freely spanning PM, we recorded a soft response indicating the bending of the membrane under the external load (Figure 31B and Figure 33). Thus, we hypothesized that upon applying a mechanical pulling force, the freely spanning PM bends as well (Figure 34). Consequently, every unfolding force peak of BR would measure the stretching of the already unfolded BR polypeptide and of the PM. The stretching of a freely spanning PM, thus, alters the shape of the unfolding force peaks and must be considered when analyzing the individual force peaks. To take into account the elastic response of PM under an applied force, we applied a thin plate theory describing the deflection of a circular membrane (or plate) [457]. According to this theory, if a thin membrane is deflected more than the thickness of the membrane, the deflection of the membrane is proportional to the cube root of the force. Because PM shows a thickness of ≈5.6–5.8 nm [458] and because the force peaks are shifted by more than 20 nm (Figure 32C,D), the approximation can be applied to our experiment. Thus, pulling BR from a freely spanning PM can be described by two springs in series (Figure 3B). One spring is given by the stretched BR polypeptide and can be described by the WLC model Eq. 16. The other spring in series can be described by the cubic response of the membrane Eq. 29

$$F(x_{membrane}) = m \cdot x^{3}_{membrane}$$  \hspace{1cm} Eq. 29

where $x_{membrane}$ is the bending of the membrane and the coefficient $m$ describes the membrane properties and pore diameter [457, 459, 460]. Both, the WLC model and the cubic response model of the membrane, thus, can be combined to describe the extension of the unfolded BR polypeptide and of the freely spanning membrane.
Next, we applied the combination of the WLC model with the membrane cubic response Eq. 30 to fit every major force peak of the FD curves recorded upon unfolding BR from freely spanning PM (Figure 32C). Best fits were obtained for \( m \approx 0.0075 \) pN/nm\(^3\). These fits nicely follow the slope of the force peaks and reveal for each peak the contour length of the unfolded polypeptide stretch and the bending of the PM. Within the accuracy of the fits, which ranged from \( \approx 2 \) to 8 aa, the contour lengths obtained for BR unfolded from freely spanning PM (Figure 32C) were the same as those observed for BR unfolded from PM directly supported by mica (Figure 32D). The similar contour lengths observed for both PM preparations demonstrate that the major unfolding steps, and thus, the major unfolding intermediates of BR do not differ. The structural segments unfolded in each of the major unfolding steps were mapped to the secondary structure of BR (Figure 32E,F). Finally, the agreement between both experimental setups suggests that the stable structural segments shaping the major unfolding steps of BR in native PM are influenced neither by interactions with the directly supporting mica nor by the mechanical bending of the freely spanning PM.

\[
F(F) = x_{WLC}(F) + x_{membrane}(F)
\]

Eq. 30

\( x_{WLC}(F) \) and \( x_{membrane}(F) \) are the WLC and membrane cubic response functions, respectively.

**Figure 33.** FD curves recording the unfolding of single BR from freely spanning PM (A) and FD curves recording no specific interactions with the freely spanning purple membrane (B). Shown are approach (blue) and retraction (red) FD curves recorded on purple membrane freely spanning across nanoscopic PMMA hole. (A) Approach FD curves show no specific interaction of AFM tip and purple membrane. Retraction FD curves show a sawtooth-like force peak pattern characteristic for the complete unfolding single BR. (B) Approach and retraction FD curves recorded on purple membrane showing no specific interactions or unfolding events. The data was recorded in buffer solution (150 mM KCl, 20 mM Tris-HCl, pH 7.8) at room temperature as described in the main text.
4.3.3 BRs from mica-supported and freely spanning PM show the same major and minor unfolding intermediates

In the previous sections, we have addressed the apparent problem that force peaks of BR unfolded from freely spanning PM shift to longer distances. These major force peaks of the FD curves described the major unfolding steps of BR. Combining the WLC model with a model describing the cubic response of the freely spanning PM showed that the major unfolding steps of BR were the same as for PM adsorbed directly to mica. Next, we wondered whether the minor unfolding steps between both samples were the same too. The minor unfolding steps are detected by the side force peaks following the major ones [423, 440]. To answer this question, we first displayed the superimposed FD curves in different color codes that allowed some of these side peaks to be observed (Figure 35A,B).
superimposed FD curves recorded from directly supported PM revealed one or two side peaks per major force peak. However, these side force peaks were less evident from freely spanning PM. To understand the origin of this difference we went back and analyzed single FD curves (Figure 35C,D). It appeared that each of the major force peaks detected could show two side peaks independent of which PM preparation BR was unfolded. This analysis highlights that unfolding BR from freely spanning PM reveals the same major and side force peaks as observed upon unfolding of BR from directly supported PM. Because these unfolding force peaks detect the major and minor unfolding steps of BR (Figure 35E,F), the results show that the mechanical unfolding intermediates of BR do not depend on whether PM has been directly supported by mica or freely spanning a nanopore. We can thus conclude that the mechanical unfolding intermediates are intrinsic to BR embedded in native PM.
Figure 35. BR unfolded from freely spanning and mica-supported PM shows the same minor unfolding intermediates. Superimposition of FD curves recorded upon unfolding of BR from freely spanning PM (A) and directly supported PM (B). The density plots colored in the frequency color spectrum allow estimating major and minor (or side) unfolding force peaks. n gives the number of FD curves superimposed. Red curves in (A) are calculated by combining the WLC model with the model describing the elastic response of the freely spanning PM (eq 3). Green curves in (B) represent WLC curves calculated to fit the force peaks. Curves fitting the side force peaks are represented as dotted lines. Numbers at the top of each fitted curve give the contour lengths in amino acids (aa) unfolded in each unfolding step detected by a force peak. Zoom on individual side peaks of single FD curves recorded on freely spanning PM (C) and directly supported PM (D). As described, the red and green curves were calculated to describe the unfolding intermediates of the side force peaks. (E) Reconstructing the stepwise unfolding pathway of BR. The contour lengths determined are used to assign the structural segments unfolded in each unfolding step of BR. Such structural segments are either formed by parts of single, single, or paired TM α-helices and their connecting polypeptide loops. (F) Secondary structure model of BR with the structural segments unfolding in each unfolding step being colored differently.
4.3.4 BR in mica-supported and freely spanning PM shows similar mechanical stability

We wondered whether the stability of the structural segments detected upon mechanically unfolding BR from freely spanning and mica-supported PM differs. Therefore, we analyzed the force required to unfold every structural segment of BR (Figure 36). The analysis showed that unfolding the structural segments of BR from freely spanning PM (Figure 36A) required less force compared to those unfolded from mica-supported PM (Figure 36B). Superficially, this result leaves the impression that BR in freely spanning PM is less stable compared to mica-supported PM. However, unfolding BR from freely spanning PM involves stretching of the BR polypeptide and of the PM (Figure 34). In contrast, unfolding BR from mica-supported PM involves the stretching of the BR polypeptide and at a negligible level the bending of the supported PM. More importantly, the force required to unfold a structural segment of a membrane protein depends on the loading rate, which describes the time scale (e.g., velocity) at which a force is applied to the structural segment [461, 462]. Applied to membrane proteins, this loading rate dependency of the unfolding force has been shown for BR [463], bovine and mouse rhodopsin [428], the sodium-coupled betaine symporter BetP [434], the peptide transporter DtpA [435], the lactose permease LacY [436], and the human β2-adrenergic receptor [431]. Thus, if the freely spanning PM bends in response to the pulling force applied, it lowers the loading rate applied to the unfolding BR. Therefore, to compare the forces at which a structural segment unfolds the loading rate applied must be specified [378, 385, 392, 464]. Next, we determined the loading rate and the unfolding force for each major structural segment unfolded from freely spanning PM and mica-supported PM (Figure 36C). As expected, the loading rates detected upon unfolding BR from freely spanning PM were significantly lower (range ≈ 5×10^2 – 8×10^3 pN/s) compared to the loading rates detected for directly supported PM (range ≈ 1×10^3 – 2×10^4 pN/s). This comparison shows that bending of the freely spanning PM lowers the loading rate applied to BR, which as a consequence unfolds structural segments at lower forces. To compare the unfolding forces of the structural segments detected from freely spanning PM, we went back to the literature and looked for forces required to unfold the structural segments of BR at similar loading rates (Figure 36C). This comparison showed that at similar loading rates the structural segments of BR from directly
supported PM and from freely spanning PM unfold at the same forces. Thus, we can conclude that structural segments of BR show similar stabilities independent of whether PM is directly supported by mica or freely spanning across a nanoscopic pore.

**Figure 36.** Forces required to unfold structural segments of BR from freely spanning PM (A) and directly supported PM (B) depend on loading rate (C). (A, B) Unfolding forces detected for the first force peak were binned by 10 pN, whereas unfolding forces of all other peaks were binned 5 pN. Average values given for each graph present mean and SD. \( n \) gives the number of force peaks analyzed. Every unfolding force peak describes the unfolding of a structural segment of BR (see Figure 32F). The first force peak characterizes the unfolding of TM \( \alpha \)-helices (F) and (G), the second force peak characterizes the unfolding of TM \( \alpha \)-helices (D) and (E), the third force peak characterizes the unfolding of TM \( \alpha \)-helices (B) and (C), and the second force peak characterizes the unfolding of TM \( \alpha \)-helix (A). ***, **, and * indicate \( P \) values being highly significant (<0.001), very significant (<0.01), and significant (<0.05), respectively. \( P \) values were determined between unfolding force distributions of force peaks detected from freely spanning (A) and directly supported (B) PM using the Student’s \( t \) test. (C) loading rate dependent unfolding forces of the second, third, and fourth unfolding force peak. Every data point represents the measurement of a single force peak. The loading rate for every single unfolding force peak was determined as described [463] by multiplying the slope of the force peak by the pulling velocity. The black line serves as a reference and gives the loading rate dependency of the unfolding force of the peak as measured previously by dynamic SMFS [463].
4.4 Conclusion

Here, we introduce SMFS of single membrane proteins from native cellular membranes freely spanning across nanoscopic pores. The SMFS experiments show that BR molecules embedded in freely spanning PM and that are mechanically pulled from the C-terminal end unfold in sequential steps. These mechanically induced unfolding steps describe the unfolding of secondary structure elements of BR. It has been debated for a long time whether the unfolding steps and, thus, the unfolding intermediates of BR as detected by SMFS are influenced by the support to which the PM has been adsorbed [422]. Our experiments reveal no differences of the unfolding pathways of BR unfolded from freely spanning PM and from PM directly adsorbed to mica. Furthermore, we could not detect any differences in the strength of the interactions stabilizing individual structural segments of BR. However, mica is a frequently used support for the preparation of biological systems because it interacts weakly (electrostatic and van der Waals interactions) with the biological sample [290, 442]. Thus, it may not be excluded that supports more strongly interacting with membrane proteins can influence their unfolding pathways and stability [438]. Importantly, our novel experimental SMFS setup opens an avenue to characterize any membrane protein from freely spanning cellular or synthetic membranes. Currently, SMFS is applied to characterize the unfolding and folding pathways of membrane proteins or to quantify and structurally localize the inter- and intramolecular interactions that stabilize their structural segments [416, 418, 420]. Using freely spanning membranes, it may in the future become possible to characterize the chaperones preventing membrane proteins from misfolding or how membrane proteins are inserted and folded by translocases. Because we used an AFM-based SMFS setup here, it also may be beneficial to employ the imaging capabilities of AFM to directly observe the proteins of freely spanning membranes [465]. Thus, AFM imaging combined with AFM-based SMFS may enable us in the future to observe single native proteins of freely spanning membranes and to quantify their inter- and intramolecular forces while interacting with other proteins (e.g., G-proteins, actomyosin cortex, etc.).
“Long ago it was said: if Tycho had instruments ten times as precise, we would never have had a Kepler, or a Newton, or Astronomy. It is a misfortune for a science to be born too late, when the means of observations have become too perfect. That is what is happening at this moment with respect to physical chemistry; the founders are hampered in their general grasp by third and forth decimal places; happily they are men of robust faith.”

“Science and Hypothesis” The Walter Scott Publishing Co. LTD. 1905, pg. 181 (translated by W. J. G.)

Jules Henri Poincaré (29 April 1854 – 17 July 1912)
5. General discussion and outlook

In this thesis, the importance of TM proteins and some open questions related to TM protein research have been discussed. Next, AFM was introduced as a technique that can assist in finding the answer to some of these questions. Particularly, the AFM-based SMFS method and the theoretical framework for analyzing the data generated by this method, have been reviewed. Finally, a novel SMFS assay has been introduced with the corresponding model for data analysis for TM protein unfolding from freely spanning membranes [466].

Plenty of things can be improved in the AFM-based SMFS method both with regard to data analysis (the theoretical aspects) as well as to the technological aspects of the method - some of these I would like to discuss below.

In AFM-based SMFS data analysis, first, the curves showing the characteristic pattern of protein unfolding are selected and corrected for the cantilever deflection, after which the alignment of the obtained FD curves is done. The necessity of alignment is primarily due to the fact that during some approach-retract cycles the AFM tip can nonspecifically attach to different regions of the TM protein’s unstructured peptide chain. As a consequence, during retraction, each time, we will stretch a polypeptide chain of a different contour length, which means that the shapes of the FD curves will differ. However, it is desired to show the superimposition of all FD curves as a density plot with an average fit to force peaks. To overcome the mismatch between FD curves they are being moved along the distance axes in order to be aligned [47, 440, 461]. In most of the cases this is done manually, however several studies have been carried out leading to the introduction of automatic, reference free alignment methods of FD curves [467-470] some of which are unpublished. Unfortunately, there is still no general agreement on the universal alignment methodology. Once such a methodology is found, the raw data of previously published mechanical unfolding studies of TM proteins can be reanalyzed and the results can be compared. Another method to overcome this problem, from the point of view of experimental design, is to introduce a binding site at one of the terminal ends of the TM protein that would drastically increase the probability of its binding to the AFM tip. Hence,
in the vast majority of cases, during retraction, a polypeptide of the same contour length will be stretched and there will be no requirement for FD curve alignment.

Another issue in data analysis is the fitting of an appropriate polymer extension model to the FD curves. As it was mentioned in section 3.2.3, the Marko-Siggia approximation (Eq. 16) for the WLC model, which is widely used for fitting polypeptide force versus extension curves has around 10% relative error for an extension that equals to half of the contour length [369, 370]. This can be critical since FD curves obtained from TM protein unfolding are representing the finite region of force versus extension dependency, typically around extensions of about half of the contour length. This issue can be solved by obtaining a more accurate analytical expression for force versus extension dependency for the WLC model.

In DFS studies, as it was discussed in subchapter 3.3, DFS models are used in the final step. It is still argued which model should be used and in which cases. To answer this question, one needs a model independent measurement of free energy profile parameters ($\chi^\beta$, $\Delta G$ and $\Delta G^\beta$) and unfolding (unbinding) rate ($k_0$) and a comparison between them and the results obtained by applying these models to DFS experiment data. Moreover, it is likely that for different systems, different models are applicable. Furthermore, the fitting method that is commonly used is fitting average (or in some cases, most probable) unfolding (rupture) force dependency on the loading rate. However, it has been suggested that fitting of the unfolding (rupture) force probability density function (as a function of unfolding (rupture) force and loading rate) to the data as a surface to the 2D histogram [380, 383, 384] is more informative. The key result of a DFS model is providing the dependency of unfolding (rupture) force probability density function or/and the average (or most probable) unfolding (rupture) force on the loading rate and on the parameters of the free energy profile. Attempts should be made for deducing the unfolding (rupture) force probability density function from fluctuation theorems [395, 471, 472]. This result will be model-free and current DFS models could be viewed as special cases of this more general result.

From the technological perspective, probably, the weakest point of AFM based SMFS is cantilever calibration that is introducing an error of 10 to 20%. More accurate and easier to carry calibration methods are desired. One idea could be calibration by using a reference force. For instance if the AFM cantilever tip has well characterized magnetic
properties, then the reference magnet can be placed at a fixed distance from the tip so that the value of the force exerted on the tip can be calculated by knowing the strength of the magnetic field and the distance between the tip and reference magnet. Then, the spring constant can be calculated by dividing this force by the cantilever deflection. A similar procedure can be done with an external electric field, if the electric properties of the tip are well characterized.

An interesting experiment can be conducted for protein (un)folding studies. Using cantilevers that are functionalized with the sequence of amino acids of TM proteins and study their folding and unfolding. This might sound straightforward however reliable functionalization protocols are required since TM proteins have large hydrophobic regions, hence, probably, non-aqueous solvents should be used during functionalization. Furthermore, membranes containing Sec61αβγ or its homologues can be spanned freely over nanoscopic pores and the folding-unfolding of polypeptide with particular amino acid sequence can be studied by approaching functionalized cantilevers and waiting above the membrane for a predefined time and then retracting. Surely, there would be also an option to add chaperons or other molecules on both sides of the freely spanning membrane.
6. Appendix

6.1 Abbreviations

ms \hspace{1em} \text{Millisecond}

s \hspace{1em} \text{Second}

min \hspace{1em} \text{Minute}

h \hspace{1em} \text{Hour}

Å \hspace{1em} \text{Angstroem}

nm \hspace{1em} \text{Nanometer}

μm \hspace{1em} \text{Micrometre or micron}

cm \hspace{1em} \text{Centimetre}

kDa \hspace{1em} \text{Kilodalton}

μg \hspace{1em} \text{Microgram}

mg \hspace{1em} \text{Milligram}

g \hspace{1em} \text{Gram}

kg \hspace{1em} \text{Kilogram}

pN \hspace{1em} \text{Piconewton}

μL \hspace{1em} \text{Microliter}

mL \hspace{1em} \text{Milliliter}

L \hspace{1em} \text{Liter}

mM \hspace{1em} \text{Millimolar (} \times 10^{-3} \text{ mole/liter)}

M \hspace{1em} \text{Molar (mole/liter)}

kcal \hspace{1em} \text{Kilocalorie}

W \hspace{1em} \text{Watt}

° \hspace{1em} \text{Degree (angle)}

°C \hspace{1em} \text{Degree Celsius}

\textit{E. coli} \hspace{1em} \textit{Escherichia coli}

TEM \hspace{1em} \text{Transmission electron microscope}

TM \hspace{1em} \text{Transmembrane}

BR \hspace{1em} \text{Bacteriorhodopsin}
PDB  Protein data bank
PM   Purple membrane
GPCR G protein-coupled receptor
RNA  Ribonucleic acid
AFM  Atomic force microscope
SMFS Single-molecule force spectroscopy
DFS  Dynamic force spectroscopy
ATP  Adenosine triphosphate
DNA  Deoxyribonucleic acid
FJC  Freely jointed chain
FRC  Freely rotating chain
WLC  Worm-like chain
MEK  methyl ethyl ketone
PMMA poly(methyl methacylate)
PS   polystyrene
Tris tris(hydroxymethyl)aminomethane

6.2 Symbols

$\Delta G$  Free energy difference between two equilibrium states
$R$    Universal gas constant
$T$    Absolute temperature
$c_{\text{wat}}$ Peptide concentration in aqueous phase
$c_{\text{oct}}$ Peptide concentration in octanol phase
$v_{\text{wat}}$ Molar volume of water
$v_{\text{oct}}$ Molar volume of octanol
$F$    Force
$\kappa$ Spring constant
$l$    Length of the cantilever
$w$    Width of the cantilever
$t$    Thickness of the cantilever in chapter 2 otherwise time
\( E \)  
Young’s modulus

\( Q \)  
Quality factor

\( \rho \)  
Density

\( \Gamma_i \)  
Imaginary part of the hydrodynamic function

\( \delta z \)  
Deflection

\( z \)  
Final position of the cantilever

\( z_0 \)  
Initial position of the cantilever

\( U \)  
Potential energy

\( \text{grad} \)  
Gradient operation

\( \langle \rangle \)  
Average

\( k_B \)  
Boltzmann constant

\( \omega_0 \)  
Angular resonance frequency

\( \omega_0' \)  
Angular resonance frequency after adding mass

\( m_{\text{eff}} \)  
Effective mass

\( M \)  
Particle mass

\( L \)  
Contour length of the polymer

\( N \)  
Number of repeating units of the polymer

\( b \)  
Kuhn length

\( p \)  
Persistence length

\( x \)  
Extension

\( \Delta G^{\ddagger} \)  
Free energy difference between transition and native (bound) states

\( x^{\ddagger} \)  
Distance between transition and native (bound) states

\( k \)  
Transition rate constant

\( p(F) \)  
Unfolding (rupture) force probability density function

\( S(t) \)  
Survival probability

\( k_0 \)  
Transition rate constant in the absence of external force

\( F^* \)  
Most probable unfolding (rupture) force

\( v \)  
Free energy profile shape factor

\( r \)  
Loading rate

\( S_b(t) \)  
Folding (binding) probability
$S_u(t)$  Unfolding (unbinding) probability

$k_u$  Unfolding (unbinding) rate constant

$k_b$  Folding (binding) rate constant

$k_{bu}$  Unfolding (unbinding) rate constant in the absence of external force

$F_{eq}$  Equilibrium force

$\gamma$  Euler’s constant

$W$  Work

$p_F(W)$  Work probability density function during forward process

$p_R(-W)$  Work probability density function during revers process

$x_{membrane}$  Membrane extension

$m$  Proportionality coefficient for membrane cubic response

$\equiv$  Identical

$\approx$  Approximately equal

$\infty$  Infinity

6.3 Acknowledgements

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7 Bibliography


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Summary

A physicist by training I have been doing research for 6 years in the fields of experimental and theoretical molecular and membrane biophysics. Currently, I am interested in collective behavior / swarming, non-equilibrium statistical mechanics and fluid dynamics.

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Russian full professional proficiency
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Excellence scholarship in Yerevan State University / 15.01.2008 - 15.05.2009

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Organizer of seminar talks of renowned researchers for students of Systems Biology PhD Program / Since June 2014

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Outside interests

I love classical music and frequently attend classical music concerts. I am also a fun of art exhibitions. These help me to think more broadly. Hiking and traveling are other amusements of mine. I feel and learn nature, take photographs and enjoy my free time with friends. I also like doing sports. Since the age of five I have participated in gymnastics, volleyball, basketball, track and field, swimming, diving and freestyle wrestling classes. During my university studies, I have become weightlifting champion of YSU twice in the lightest weight. While I was in Dresden I attended chess training course, and here in Basel I was attending rowing training course in Basel rowing club.