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This thesis was imbedded into the SHENC research project, which focusses on examining vWF using different techniques (more information can be obtained at <http://www.shenc.de/>).

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1. INTRODUCTION

1.1. Aim of the study

This thesis was imbedded into the SHENC project (**S**hear flow regulation of **H**emostasis - bridging the gap between **N**anomechanics and **C**linical presentation), which is composed of twelve research groups from Austria and Germany. The aim of this project is to unravel the role of von Willebrand factor (vWF) in the human body, including pathological conditions. Due to the broad expertise of the various groups, a wide spectrum of methods and technologies is used to investigate vWF and its impact on human diseases. In this way, novel and improved treatment options should be found to help patients affected by pathological conditions concerning vWF. More information can be obtained at <http://www.shenc.de/>.

My part of this project was to study the interactions between DNA and vWF on a single molecular level and to find appropriate imaging conditions for the characterization of λ -DNA strands. Those tasks were carried out using AFM techniques at the Institute of Biophysics (JKU Linz), which is specialized in these techniques.

This thesis continues the work done by Grässle et al who showed that vWF is able to interact with protein-free DNA via vWF domain A1.¹ Nevertheless, no data exists about the acting forces of this interaction. Therefore single molecule force spectroscopy was the method of choice to identify the behavior of this biological interaction on a single molecule level. Different wild type domains and two different gain of function-mutations (GOF-mutations) in the vWF domain A1 of A1A2A3 construct were used to examine and compare their binding behaviors. In total, six vWF constructs were used. Ristocetin, an antibiotic, was used to expose the binding site in vWF domain A1 in measurements without shear flow. The effect of ristocetin on the constructs and the ristocetin dependent recovery was tested. Heparin was chosen to block specific interactions.

Besides force spectroscopy methods, AFM magnetic alternating current (MAC) mode imaging was used to characterize the λ -DNA used in this thesis. The goal was to find an appropriate protocol which allows high resolution imaging of DNA strands.

1.2. Atomic force microscopy

Atomic Force Microscopy (AFM) is a powerful technique to study different materials, for example biological samples, at a molecular basis; either via interaction studies or using high resolution imaging. In this thesis, both methods were used. The most important components of an atomic force microscope are: a scanner with piezoelectric properties, a laser, a photodiode to detect the laser signal, feedback electronics and a chip exhibiting a cantilever with a sharp tip. ²

Cantilevers are one of the most important parts of AFM measurements. Usually they are made of silicon, silicon oxide or silicon nitride, with a highly reflective backside to allow laser light reflection to the photodiode. A cantilever's spring constant and resonance frequency define its performance in measurements. ^{3,4} There are many chip types for different measuring modes which carry differently shaped cantilevers (rectangular or triangular - see figure 1). In force spectroscopy experiments, molecules can be chemically attached to the tips of the cantilevers, allowing interaction studies of specific molecules. Here, so called MSCT tips were used. Feedback electronics are necessary to detect and adjust the force which the cantilever is applying to the sample, to avoid destruction.

AFM scanners work on the principle of inverse piezoelectric effect. This means, that if electricity is applied, the crystal inside the scanner shows mechanical stress: it is either expanding or contracting, depending on the voltage. In this way it is responsible for tip positioning.

According to common literature, there are three main operating modes for AFM: the contact, the non-contact and the tapping mode. Each mode has its own advantages, which qualifies it for special experimental setups and sample properties.

During contact mode imaging, the tip remains in contact with the sample. Here, the tip literally scans the sample like a needle scans the disc in a record player. Different forces, like van der Waals interactions, may repel or attract the tip. This mode can be sub classified in the constant height mode, where the distance from tip to sample stays the same; or constant force mode, where the force between sample and tip is kept constant. The constant force mode is mostly used; as constant height may destroy

sensitive biological samples.⁵ Force spectroscopy is also operated in contact mode. When performing force spectroscopy, the tip of the cantilever is moved in the Z-direction, which means it is approached to or retracted from the sample surface. In contrast, during imaging with the AFM, the tip is moved in the XY-direction to scan the sample.⁴

In the non-contact mode, the cantilever is oscillated near its natural resonance frequency which is determined before. If forces from the sample act on the cantilever, this will result in a change of the cantilevers frequency, which will be converted into topographic sample information.

The tapping mode is more or less a combination of contact and non-contact mode. The tip is oscillating and constantly tapping onto the surface, but does not have immediate contact with it. Most often this technique is used to image very soft, biological samples.⁵

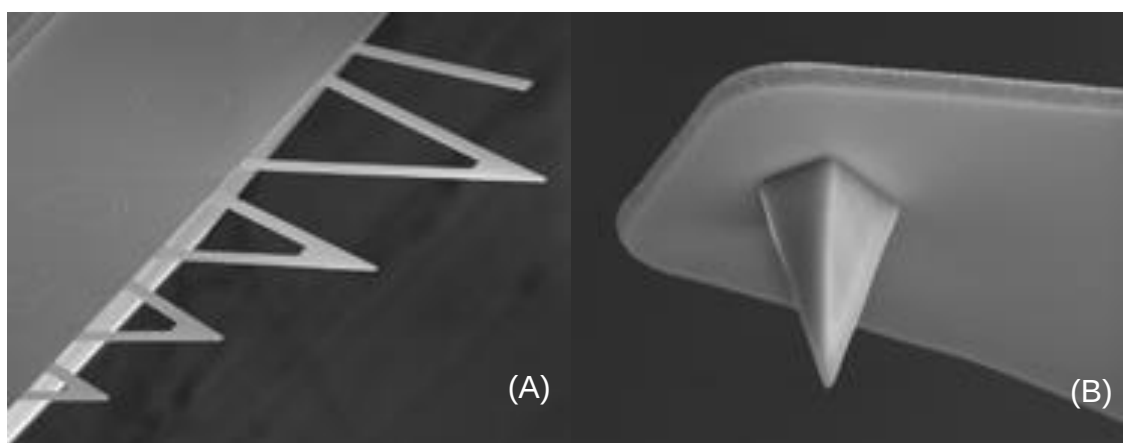


Figure 1: MSCT chip schematics. (A) Schematics of the cantilevers present on an MSCT chip used for force spectroscopy. The rectangular cantilever is called “B”, the following triangular ones from the biggest to the smallest “C”, “D”, “E” and “F”. Length of the B cantilever is approximately 210 μm . Cantilever “A” is located on the opposite side of the chip and not shown on the image. **(B)** Each cantilever has a tip. This is where molecules are bound during tip functionalization.

(Images obtained from: <http://www.brukerafmprobes.com/Product.aspx?ProductID=3419>⁵⁰)

At the institute of Biophysics, Department of Applied Experimental Biophysics of the Johannes Kepler University (JKU) Linz, only contact and tapping mode are used. Besides that, also magnetic alternating current – mode (MAC mode) is of importance. It belongs to the category of tapping mode. During MAC mode imaging, a magnetic field is applied. This field accelerates a magnetically coated cantilever to oscillate. The advantage is a better signal-to-noise ratio, therefore lower forces and less vibration amplitudes are applied to the samples. This makes it a perfect technique for sensitive biological samples.⁶

AFM has many benefits compared to for example surface plasmon resonance methods (Biacore), as it enables measurement of single molecules. Further, it allows to measure at high resolution but sample preparation remains uncomplicated. Measuring is non-destructive, which makes it a perfect tool for biological samples. Measurements can be performed in air, in liquid, in vacuum or in gas, which allows the investigation under different physiological conditions and in real time.^{3,7}

1.2.1. Single molecule force spectroscopy

In this thesis, one special operation mode of AFM is most abundantly used: the single molecule force spectroscopy (SMFS), often abbreviated as force spectroscopy. Force spectroscopy makes it possible to explore the energetic properties between two binding partners at a single molecular level. The binding between such molecules underlies distinct energy surfaces. The properties to describe such an energy landscape were determined here.

The setup is quite easy to understand: one binding partner is immobilized on a sample surface, while the other one is bound to the tip of a cantilever. By bringing those two in contact, a bond might be formed. If there is bond formation, the linker will be stretched and the bond will cause the cantilever to bend. The bond will rupture when the applied force in the other direction becomes too high. The bending of the cantilever changes the deflection of the laser beam which is focused onto the back of the cantilever and reflected to a photodiode (see figure 2). The photodiode converts

this signal and the unbinding event can be observed in a so called force distance cycle.

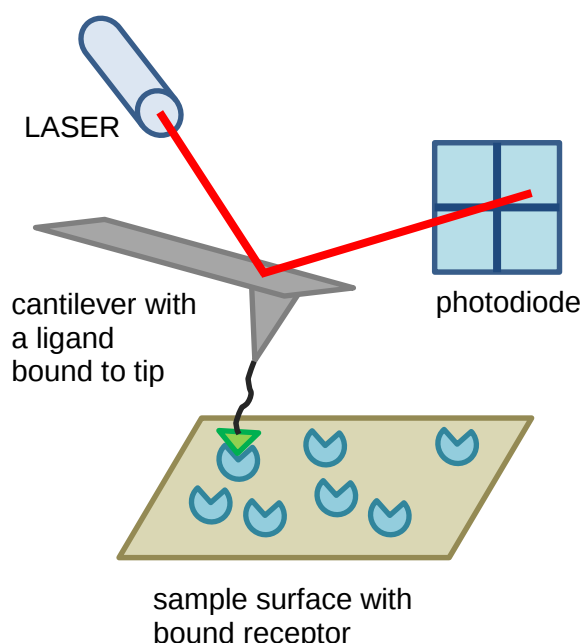


Figure 2: Schematics of a force spectroscopy measurement. A laser is focused onto the reflective backside of the cantilever. For simplicity, the cantilever is pictured as rectangular, although a triangular one was used for force measurements in this thesis. An interaction between receptor and ligand leads to a distinct bending of the cantilever and hence to laser beam deflection. A photodiode records the laser deflection and converts the signal to a force value.

A force distance cycle displays the different forces acting onto the cantilever in relation to the distance travelled. A schematic description is shown in figure 3. As the tip approaches the surface, either forces attracting or repelling the tip will start to act on it. The cantilever will bend according to the force acting between the sample surface and the tip. While the tip is in contact with the surface, chemical interactions of molecules could arise and bonds could be formed. During the following retraction, bending of the cantilever gets less and less again; but if a bond has formed before, the cantilever will bend again until the applied force is high enough to rupture the bond. The deflection of the cantilever can be converted into forces by knowing its spring constant, which can be experimentally determined. The recorded force distance cycles display unbinding or unspecific adhesion, which can be distinguished by the shape of the unbinding event. Specific interactions show a parabolic shape, while unspecific adhesions have linear appearance.⁸ The velocity used for approaching and retracting the tip also has an impact on the chemical bonds. This is called the loading rate dependence (LRD) (see chapter 2.2.4.2. – Loading Rate Dependences).^{9,10}

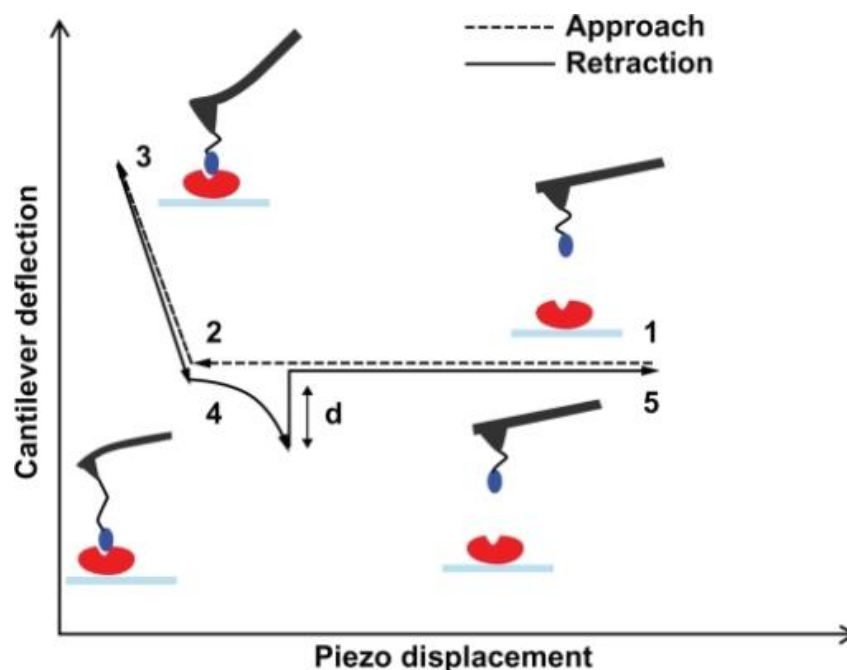


Figure 3: Schematic description of a force distance cycle. (1)

The dotted line shows the approach of the tip (where one binding partner is bound), to the sample surface, where the other one is bound. **(2)** Repulsive forces between the molecules cause the cantilever to deflect. When the maximum force is reached **(3)**, the cantilever is retracted again. The molecules are able to interact. **(4)** When the retraction forces are too big, the binding is dissolved again and the cantilever reaches its original position again **(5)**. The unbinding force is indicated by “d”. Image obtained from: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3230568/> ⁵¹

1.2.2. Tip and sample functionalization

In order to make interaction studies possible, molecules have to be properly attached to the tip and the sample surface. For force spectroscopy, MSCT chips were used.

For the tip functionalization, various protocols for different ligand attachments exist (all protocols are available online at <http://www.jku.at/biophysics/content/e257042>). Polyethyleneglycol (PEG) chains, for example, are flexible polymer chains which are

often attached to the tip before adding the specific ligand. PEG chains are, aside from the two end groups (which are *N*-Hydroxysuccinimid (NHS) on one end and one variable group at the other end), chemically inert. They allow the ligand to orient freely which supports it to find its binding partner on the sample surface. A further advantage is that the PEG chain is highly elastic, which makes it easy to distinguish adhesions from specific bindings in force distance cycles.⁸ In order to attach a PEG chain to a cantilever tip, aminofunctionalization of the tip is necessary beforehand. Therefore, (3-Aminopropyl)triethoxysilane (APTES) is used to introduce amino groups (NH₂) onto the silicon nitride tip. The APTES procedure is carried out in the gas phase. Instead of APTES, also ethanolamine could be used to attach amino groups to the surface. The NHS-ester end of the PEG chain will bind to the NH₂ groups of the MSCT tip via an amide bond.¹¹

In this thesis, maleimide-PEG-NHS linker molecules were used. After binding to the tip, a maleimide group is free for further reactions (also see 2.2.2. Tip Functionalization). Next, disulfide-Tris(hydroxymethyl)aminomethane-nitrilotriacetic acid (disulfide-TRIS-NTA) is attached to the linker molecule. [Intellectual Property Rights: Working with TRIS-NTA is covered by patents (for example US 2008/0038750 A1), the inventor is Prof. Dr. Robert Tampé, Biocenter, Frankfurt am Main, Germany.] As a last step, proteins with a His₆-tag are bound. Thus, Ni is added together with the protein (see figure 4). This procedure results in stable linker-bound molecules. Such tips can be stored in buffer at 4 °C until further use.¹²

As a support for functionalized surfaces, mica is commonly used. Mica is a silicate mineral with a negatively charged, hydrophilic and atomically flat surface. It can be cut with normal scissors to have it in a proper size for the AFM sample stage. By use of normal adhesive tape, the top layer of a mica support can be cleaved off, resulting in a clean and atomically flat surface. This can be done many times – until there are no layers left to cleave. Other materials suitable as sample support are highly oriented pyrolytic graphite (HOPG), glass, silicon and silicon nitride. However, in this thesis, mica was used. Again, different protocols of surface chemistry exist to basically bind every protein of interest.⁸

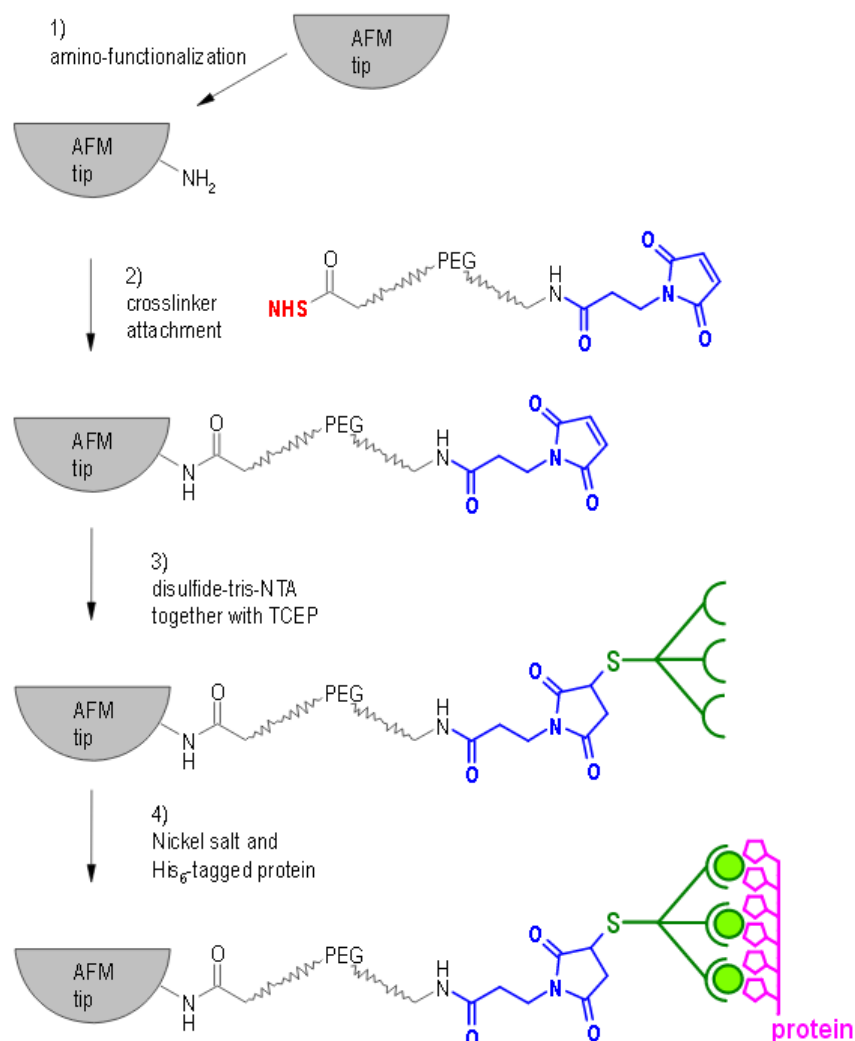


Figure 4: Tip functionalization with His₆-tagged proteins. First, aminofunctionalization of the tip was done, followed by attachment of the PEG-linker. A maleimide group with disulfide-TRIS-NTA allowed the attachment of proteins with a histidine tag. (Image available at <http://www.jku.at/biophysics/content/e257042> ¹²⁾)

1.3. Deoxyribonucleic acid and proteins

1.3.1. Structure of deoxyribonucleic acid

In 1953 a fundamental breakthrough in science was achieved – John Watson and Francis Crick discovered the double helical structure of the carrier of our hereditary information, the deoxyribonucleic acid (DNA).¹³

Thinking of the extensive amount of information stored in our DNA, it seems amazing that only four different nucleotide subunits make up this molecule. Each nucleotide is composed of a deoxyribose (sugar), a phosphate group and one of the four bases: adenine (A), thymine (T), guanine (G) or cytosine (C). Attachment between a base and a deoxyribose is formed via a glycosidic linkage. Altogether, the two polynucleotide chains create the three dimensional double helical structure. The deoxyribose units with the phosphates form the backbone, and the bases pointing inside from each chain form hydrogen bonds between each other, therefore holding the two strands together. Base pairing always follows one rule: only A and T can bind with each other, whereas C only binds with G. C and T are so-called pyrimidines (one ring bases), whereas A and G are purines (two ring bases), showing that always a purine and a pyrimidine form hydrogen bonds. Between A and T two bonds are formed, while C and G form three, hence representing the stronger bond (see figure 5). All base pairs have identical geometry, so that DNA structure remains unaffected by the nucleotide sequence.

The two strands are antiparallel to each other because of their different chemical polarities. Each strand's backbone has two different ends: one with the 5' phosphate and one with the 3' hydroxyl-group (OH-group). This means, the base at the very end of the 5' end pairs with the base at the end of the 3' end and vice versa. Inside the backbone, each 3' OH-group of the ribose forms a covalent phosphodiester bond with the 5' OH-group of the following ribose, and so on. Every ten nucleotides the helix makes one complete turn, resulting in two differently sized grooves: the major and the minor groove.

The important thing about DNA is the sequence of the bases. Parts of this sequence (specific genes) are transcribed and translated into an amino acid sequence, leading

to certain three-dimensional shaped proteins. The entity of those proteins defines literally everything in an organism, hence making us what we are.

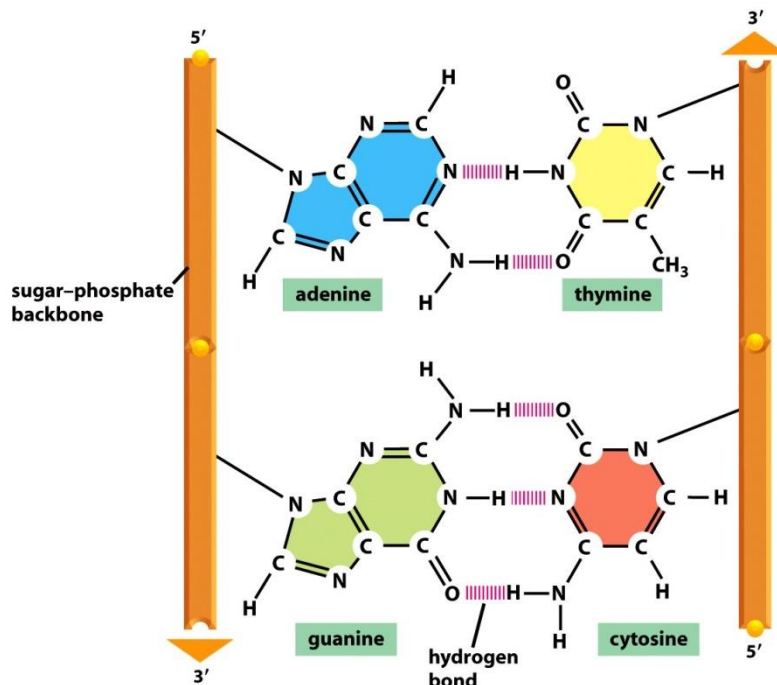


Figure 4-4 Molecular Biology of the Cell 5/e (© Garland Science 2008)

Figure 5: The structure of DNA.

The backbone of the double helical structure is formed by deoxyribose and phosphate. Base pairing is achieved via hydrogen bonds and is only possible between adenine / thymine and between guanine / cytosine. Guanine and cytosine are bound more stable as three hydrogen bonds hold them together. Image obtained from Molecular Biology of the Cell, Alberts et al, Figure 4-4 ¹⁴⁾

The production of a protein out of a DNA sequence happens in two steps: transcription and translation. During transcription, the relevant DNA sequence is transcribed into a single strand of ribonucleic acid (RNA). In contrast to DNA, RNA contains ribose as sugar component, and uracil (U) instead of T. The reaction is catalyzed by an enzyme called RNA polymerase and the produced molecule is called pre-messenger RNA or heterogeneous nuclear RNA (pre mRNA or hnRNA). The pre mRNA contains the same information as the DNA sequence itself, just written in a slightly different form. As a next step, RNA processing steps make a final mRNA out of the primary transcript. In fact, a cap containing a modified G is added at the RNA's 5' end, and at the 3' end, a poly A tail is formed by the poly A polymerase. Those modifications make sure that both ends are intact. Further, during RNA splicing, non – coding sequences (the introns) are cut out by the so called spliceosome and the coding ones (exons) are ligated, leading to the final mRNA.

The mRNA is transported out of the nucleus to the ribosomes for translation. To translate the nucleotide sequence into an amino acid sequence, another form of RNA

is needed: the transfer RNA (tRNA). The tRNAs have a clover leaf like structure and can bind an amino acid corresponding to a nucleotide triplet on their anticodon loop. This nucleotide triplet, the anticodon, is complementary to the codon triplet on the mRNA. Therefore, as codon and anticodon match, the correct amino acid is brought for the corresponding triplet. This process is carried out in ribosomes; they catalyze the peptide bond formations between the amino acids, leading to a fully assembled protein after folding. ^{14–16}

1.3.2. Bacteriophage lambda DNA

For this thesis, DNA of the bacteriophage lambda (λ) was used for interaction studies with von Willebrand factor and for imaging. A bacteriophage is a virus using bacterial hosts; bacteriophage λ infects *E.coli*. ¹⁷

In 1951, Ester Lederberg was the first to discover λ phages from her *E.coli* culture. Since then, its genome was used to bring insight into the physical properties of DNA.¹⁸ A special thing about the bacteriophage is its two forms of development: the virus can either exist in a lytic or lysogenic state. In lysogenic growth, the virus genome is integrated into the host genome. This leads to a quiescent status: no gene expression of the virus is detectable – the virus simply divides with the bacteria (to be accurate, there are two genes expressed: one codes for a repressor, which is assuring the lysogenic state, and one allows integration into the host genome). This leads to amplification of bacteria with integrated virus DNA. ¹⁹

During lytic growth, which is controlled in a timely manner, genes are expressed. Some genes are transcribed immediately, (“immediate early transcription”); the following ones are produced in the “delayed early transcription” and the last ones get transcribed in the “late transcription”. This ends in formation of new virus particles inside the host cell, which finally leads to lysis of the bacterium and sets the new viruses free. ¹⁸

1.3.3. DNA in the blood stream - neutrophil extracellular traps

Necrosis and apoptosis are the two commonly known processes leading to cell death. But also a third process is known – NETosis, referring to neutrophil extracellular traps (NETs). As the name already indicates, NETs are produced by neutrophils, which are important cells in our innate immune system.

If pathogens enter the body, neutrophils are able to release such NETs, which are chromatin fibers loaded with antimicrobial proteins. In this way, viruses, bacteria and fungi can be trapped and killed. Although neutrophils get denucleated, they are still able to phagocytose trapped microorganisms. This practice is called “vital NETosis”. The major factor initiating the formation of NETs is the enzyme peptidyl arginine deiminase 4 (PAD 4), which leads to decondensation of the chromatin via histone modifications.²⁰ In a mouse model it was shown, that a knockout of PAD 4 results in a disability of performing NETosis.²¹ Apparently, before PAD 4 comes into account, the first stimuli for NETosis are reactive oxygen species (ROS). Those are produced because of several triggers like tumor necrosis factor (TNF), the outer membrane component of gram negative bacteria lipopolysaccharide (LPS), and others.²²

Besides the protecting nature of NET formation, there is also a backside: even without presence of pathogens, NETosis was observed in several diseases like preeclampsia, systemic lupus erythematosus or small vessel vasculitis.²⁰ As a high amount of extracellular DNA is a symptom of cystic fibrosis, there is also the question if this DNA comes from NETs or belongs to cellular debris. Cystic fibrosis patients often deal with colonization of *P.aeruginosa*, and it was proposed that NETs promote this colonization. Apparently the bacteria are too dominant as that the antimicrobial proteins of NETs could have an effect on them.²²

Red blood cells and platelets can also be bound by the released DNA fibers; this shows the link to blood coagulation. Activated platelets are even thought to increase the amount of NET production. Moreover, NETs seem to bind to von Willebrand factor (vWF) and there is also evidence that vWF somehow links the NETs to blood vessel walls.²⁰

1.3.4. Proteins

The amino acid sequence of a protein contains all the information for correct folding and posttranslational modifications. The distinct three dimensional shape of a protein is a prerequisite to carry out its task properly. Misfolded ones could also be a danger to an organism.

After translation, polypeptides are described as their primary sequence, referring to the amino acid linked together in a linear form. As each amino acid can form two peptide bonds, linear chains are produced without branching regions. In the genetic code, there are 20 amino acids which are used as building blocks for proteins, each of them exposing a different side chain. According to the properties of the side chains, amino acids are classified into four groups: uncharged polar, nonpolar, acidic and basic. The backbones of the amino acids are linked via peptide bonds, the side chains facing outwards. The varying properties of the side chains, van der Waals forces, hydrogen bonds and electrostatic attractions help to fold the protein into its predetermined shape.

Secondary structures of polypeptides are mainly formed by two patterns: α helices and β sheets. These patterns occur locally in some parts of the peptide. In combination with the forces mentioned above, they are responsible for the unique 3D structures of proteins, indicated as tertiary structure. So called chaperone proteins help proteins to fold properly and also assist in refolding if something went wrong. As two or more polypeptide chains often work together to form a proper protein, there is also a fourth level of organization: the quaternary structure.

Besides the 3D structure of a protein, also posttranslational modifications contribute to its unique properties, which result in one of their various functions in the cell.

Proteins usually bind other proteins in a very specific way. This specificity is important to ensure stable bonds, as the two interacting partners need to fit together to be close enough to form bonds. Directly at the binding site, there are distinct amino acids necessary to form a specific interaction. The bonds are usually of quite weak, noncovalent nature; but as many of them form at once, stable bonds are achieved. Noncovalent bonds are for example electrostatic attractions, van der Waals interactions and hydrogen bonds. ^{14,16}

1.4. Von Willebrand factor

1.4.1. Structure and maturation of von Willebrand factor

Von Willebrand factor (vWF) is an adhesive, multimeric plasma glycoprotein which is circulating in our blood and is playing a major role in hemostasis.^{1,23,24} In non-pathological conditions, plasma concentration of vWF is approximately 10 µg/mL.²⁵ In 1926, von Willebrand factor was discovered by the Finnish physician Dr. Erik von Willebrand. He first described a bleeding disorder, which is caused by lack of vWF, which he found in families of the Åland islands.²⁶

Encoded by chromosome 22, the synthesized pre-pro-vWF has a length of 2813 amino acids with a 22 amino acid signal peptide at its N terminus. The vWF gene is quite complex with 180 kb and 52 exons.^{24,27} The signal peptide leads the protein's way to the endoplasmatic reticulum for processing. At the endoplasmatic reticulum, two vWFs dimerize via disulfide bonds at their C terminal ends, leading to further transport to the Golgi apparatus. At the Golgi, multimerization happens via further disulfide bonds and the propeptide, which has a length of 741 amino acids, is cleaved off. However, it seems that the propeptide remains non-covalently bound to vWF and leads its way to the Weibel Palade bodies, where mature protein is stored. Multimers of vWF can grow up to 500 – 20000 kDa, consisting of many subunits with 250 kDa each.²⁷

Generally, vWF is stored in two different places: in α -granules if it was produced by megakaryocytes; and in Weibel-Palade bodies if it was produced by endothelial cells. As already mentioned, the propeptide is necessary for storage in Weibel-Palade bodies, which only form in the presence of vWF (in contrast to α -granules).²⁸

Besides storage of vWF for regulated release, some molecules are released via the constitutive secretion pathway, which is only done by endothelial cells and not by platelets.^{28–30}

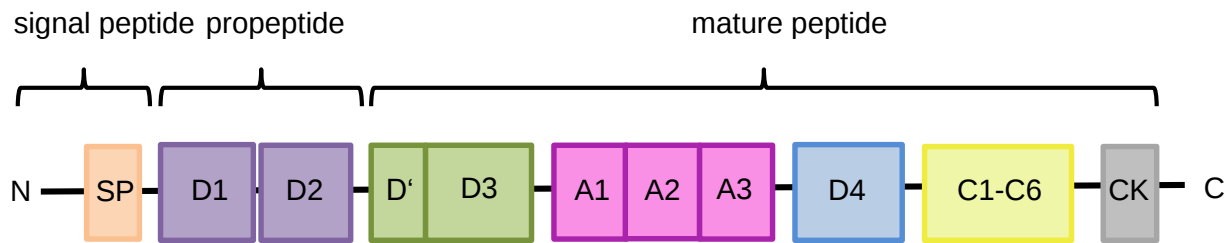


Figure 6: Structure of von Willebrand factor. The signal peptide at the N terminus has a length of 22 amino acids and guides the transport to the endoplasmatic reticulum. Multimerization at the Golgi apparatus is accomplished via vWF's D3 domains. The propeptide, with its length of 741 amino acids, is cleaved at its furin cleavage site.²⁹

In addition to multimerization, another essential event in vWF maturation is O- and N-linked glycosylation. Altogether, vWF gets 17 N-linked glycans (which is the addition of a sugar molecule to a nitrogen atom) and 10 O-linked glycans (where the sugar molecule is attached to an oxygen molecule). The glycans which are present on the mature protein apparently also carry carbohydrate residues which determine blood groups.²⁸

The various domains, which evolved by exon shuffling during evolution, have different functions and different binding partners. As already mentioned, the CK domain at the C terminal end is important for vWF dimerization, and the D3 domain for multimerization of the dimers. Multimerization also seems to be dependent on D1 and D2, which are the two domains in the propeptide. The D' domain is known to interact with factor VIII, A1 with GPIIb α and collagen, and A3 also with collagen. C1 and C2 show evidence to be important for vWF binding to fibrin, but this remains quite unclear to date. C1 is also thought to stabilize platelet adhesion to A1.³¹ Generally, there seems to be a lot to still be discovered concerning the functions of the domains and their interactions. However, what is for sure is that mutations in any of those can have severe effects on the structural and functional properties of the protein.²⁹ The structure of vWF is displayed in figure 6.

1.4.2. Hemostasis and von Willebrand factor

Blood vessels make sure that our blood is transported everywhere in the body where it is of need, without leaving its defined compartment. However, vessels may rupture, leading to leaking out of this most important fluid of the body. In such a case, it is necessary to quickly stop this bleeding – here hemostasis comes into account. The process of hemostasis is essential for survival; any disorders – no matter if enhancing or lowering the ability of clotting - will lead to problems.

Hemostasis, which is the stopping of bleeding, is divided into three steps: vascular spasm, platelet plug formation and coagulation. All of these steps need to happen in a tightly controlled but fast manner to ensure proper action at the correct time and in the correct place.

Vascular spasm is the first mechanism against extensive blood loss. During vasoconstriction, blood loss is lowered and platelet plug formation and coagulation can start. Blood vessels will promptly constrict in response to any injury – the higher the extent of injury the more constricted the vessels will be, as more blood needs to be prevented from flowing out. ¹⁵

As a next step, a plug of platelets will be formed; this is where vWF comes into account. Megakaryocytes are the production units for platelets, which are made by fragmentation of their cytoplasm. ²⁸ Under physiological conditions and with intact blood vessels, platelets travel within the blood stream and do not stick to the inner linings. This is accomplished by endothelial cells releasing nitric oxide (NO) and prostacyclin (PGI₂). ¹⁵ However, if the vessel is injured and its inner linings are exposed (like collagen and laminin), platelets get slowed down to allow adherence. Platelets cannot adhere on their own: vWF binds to collagen at the vessel wall and to activated platelets, acting as a kind of bridge between them. ³² Apparently, vWF binds with its A3 and A1 domains to the exposed vessel subendothelium. ³³ Activated platelets themselves play an important role in activating the complement system by activating coagulation and starting degranulation to release factors for early wound healing (like for example growth factors, cytokines, prostaglandins, histamine and many more). ³⁴ VWF adheres to the vessel walls and gets stretched by the blood

passing by. This allows exposing its A1 domain, which is able to bind with the GPIIb/IIIa receptor of platelets, leading to their activation. Another binding site in vWF can interact with platelet integrin $\alpha IIb\beta 3$, which binds to the RGD motif in vWFs C terminus (RGD stands for arginine, glycine, and aspartic acid, the three amino acids in this motif).²⁸ There are not only vWF monomers. VWF also dimerizes and later forms multimers. The binding of vWF and platelets is required for further steps of platelet plug formation. As already mentioned before, high shear flow of the blood stream also assists in exposing binding sites of vWF to activated platelets, thereby activating it.^{32,35} Platelets bind with their receptor and a tether is formed at site of binding. Tethers are membrane extensions, and as the platelets are moving quite fast in the blood stream, the tethers are elongated parallel to the direction of blood flow. Only one such extension is formed at a time, but the tether can form multiple contacts with vWF molecules. The tether is either strong enough to keep the platelet in place or the contact to vWF is released. In this case, a new tether may be formed, allowing interaction with another site of vWF.³⁶ Besides the interaction of vWF with platelets, platelets also interact with each other. Fibrinogen is able to bind to integrin $\alpha IIb\beta 3$, which has various ligands when activated. As fibrinogen has two binding sites for this integrin, it is able to crosslink two platelets and therefore help in formation of aggregates.^{28,37} These processes all belong to the primary hemostasis.

Coagulation is the third step in hemostasis, and referred to as secondary hemostasis. During this phase, blood consistency is altered: from a really liquid state it is converted into a quite gel-like form. This is achieved by different clotting factors, also called coagulants.¹⁵ The key molecule in this step is thrombin, which is activated either by the intrinsic or extrinsic pathway.³⁸ Besides recruitment of blood platelets, vWF has another role concerning hemostasis, namely to carry the blood coagulation factor VIII.³⁹

1.4.3. Clearance of von Willebrand factor

Macrophages in the spleen and liver are the major factor when it comes to clearance of freely flowing vWF, experiencing only a low amount of shear stress.²⁸

Interestingly, it seems that the blood group affects the half-life of vWF in plasma. As mentioned before, blood group determining residues are present on the glycans, and apparently they have an effect. Blood group O seems to be linked to lower vWF plasma levels; individuals with non-O have higher concentrations. Also in von Willebrand disease type 1 patients (a disease affecting the vWF gene which will be explained more detailed later in this thesis), a high number of patients had blood group O.⁴⁰

For vWF which is not freely flowing in the bloodstream, another mechanism is necessary. The metalloprotease ADAMTS13 (**A** Disintegrin **A**nd **M**etalloproteinase with **T**hrombo**S**pondin type 1 motif, member **13**) is able to cleave vWF under shear flow and during thrombus formation (therefore it is also called von Willebrand factor cleaving protease, vWFCP).⁴¹ ADAMTS13, which is present in the blood stream with a concentration of approximately 1 µg/mL, only targets vWF.⁴² Hepatic stellate cells and endothelial cells are the production sites of ADAMTS13.²⁸

During growth of a thrombus with vWF and activated platelets, vWF is stretched by blood flow. This exposes its A2 domain hence uncovering the cleavage site for ADAMTS13. Without shear stress, for example because of no blood flow, vWF seems to be resistant against cleavage. The feedback mechanism of this cleaving protease is of crucial importance, as deficiency allows the formation of dangerous thrombi. This may lead to death when untreated, as in patients of thrombotic thrombocytopenic purpura (TTP), which is the deficiency of ADAMTS13.^{33,42} Symptoms of TTP include neurological and renal dysfunctions, fever, thrombocytopenia (which is a low platelet count) and others.⁴³ Apparently, reduced levels of ADAMTS13 contribute to occurrences of stroke and myocardial infarction.²⁸ All these facts demonstrate that a balance of vWF and ADAMTS13 is of huge importance. Besides the fact that a deficiency of vWF cleaving protease results in too big and too much vWF – which leads to life-threatening symptoms – also a deficiency in vWF itself results in problems. This bleeding disorder is called von Willebrand disease.⁴³

1.4.4. Von Willebrand factor mutations and von Willebrand disease

Von Willebrand disease (vWD) is an inherited disorder and shows different symptoms, like heavy menstrual bleeding in women, extensive bleeding after surgeries, epistaxis, easy bleeding in the oral cavity and others. Symptoms may become more severe with increasing age of patients, thus the disease may not be discovered in an early stage. ⁴⁴

There are three major types of vWD which need to be distinguished: vWD type 1, type 2 and type 3.

The most frequent form is vWD type 1 (with 70 % of all vWD cases ⁴⁴), whose patients have reduced levels of vWF. In contrast to that, the deficiency in type 2 vWD is not of quantitative but of qualitative nature. This type is further divided into four subtypes, which are called 2A, 2B, 2M and 2N. Type 3 patients show a severe reduction of vWF levels, but only 1 % of all vWD cases are a type 3 vWD. ²⁸

The major type of vWD, type 1, shows the mildest symptoms. Characteristics of this form are decreased synthesis and secretion, and also an increased clearance. ²⁹

As already mentioned, type 2 vWD is distinguished in four subtypes. Subtype 2A appears most often in all type 2 patients. Qualitative deficiencies are due to increased cleavage by ADAMTS13, reduced assembly to dimers and multimers, and impaired secretion of vWF to the extracellular space. These deficiencies happen because of missense mutations, which altogether lead to ineffective clump formation of platelets and vWF. In 2B, vWF binding to the platelet receptor GP1b α is enhanced, and the complexes are removed, which may lead to thrombocytopenia. In contrast to that, a characteristic of 2M is that binding to GPIb α is reduced. In subtype 2N, binding to factor VIII is reduced. ⁴⁴

The rare type 3 form of vWD is characterized by missense mutations, which may also lead to premature stop codons. This may have several consequences; however, the most common mutation is linked to an accelerated clearance from the blood stream. ²⁹

Besides those classical forms of vWD, there is also another one: the acquired von Willebrand syndrome (AvWS). It is not caused by mutations of the vWF gene itself and it is mostly found in older people without former bleeding problems. This syndrome is often linked to other diseases, like for example lymphoproliferative, cardiovascular and immunological disorders. Symptoms occur most often because vWF is removed from blood too fast. But also decreased synthesis, conformational changes, and others seem to contribute. ^{44,45}

Generally, vWD is an inherited disorder. Both autosomal recessive and autosomal dominant inheritance are existent. VWD type 1 and most types of type 2 (namely 2A, 2B and 2M) are inherited in a dominant manner, whereas 2N and type 3 are inherited in a recessive way. Some forms of type 1 and a subtype of 2A were also seen to be autosomal recessive, but generally most cases show dominant inheritance. ^{29,44}

The good thing is that there is a treatment available for affected people. Besides ongoing research seeking therapeutics, there are already two strategies being used. One is the administration of desmopressin, which is a synthetic analog to the human vasopressin. Desmopressin increases vWF and factor VIII levels for up to eight hours. Direct infusion of vWF and factor VIII is another approach being used. Compared to synthetic desmopressin, this strategy is dependent on human plasma; which bears the (small but existing) risk of transmissible diseases. However, research is ongoing to find other suitable and more efficient treatment options. ⁴⁶

1.5. Interaction studies of vWF and DNA

In order to reveal the mechanisms of interactions that happen between DNA and vWF, molecular force recognition studies were the main topic of this thesis. Characteristic forces and dynamics were analyzed on a molecular level.

Interactions of vWF and DNA are not well characterized. As mentioned, extracellular traps of DNA (NETs) are present in our blood stream. It is hypothesized that vWF binds this DNA via its A1 domain. Apparently, GPIIb α , DNA and heparin (which is an anticoagulant) compete for the same binding site; located in vWF's A1 domain.

As a part of the A1 domain is positively charged, and DNA of course is negatively charged, there is already an explanation of how they interact – namely in a charge based manner. However, as the DNA released by neutrophils also consists of proteins like for example histones, one could not clearly state if vWF binds to the DNA itself, or to attached proteins. Besides DNA, also GPIIb α and heparin are negatively charged, further confirming the hypothesis of the interaction being of electrostatic nature. This was also shown by Grässle et al by blocking with polycationic chitosan. The polycation was interacting with the DNA, thereby blocking its negative charges and the outcome was inhibition of binding to vWF. Grässle et al describe the interactions between vWF and DNA as being nonspecific but of high affinity.¹

Physiologically, shear stress is needed to activate vWF for GPIIb α or DNA binding. In the body this is achieved by the blood flow, which stretches the vWF molecule. However *in vitro*, with no shear available, chemical compounds can be used to enable those interactions. Such compounds are for example ristocetin and botrocetin.⁴⁷ Botrocetin is a snake venom and ristocetin a glycopeptide produced from bacteria. Ristocetin, which was also used in this thesis, seems to bind to sequences in the A1 domain rich in prolines.²⁴ In this way the A1 domain is exposed and activated vWF is available for binding DNA, completely without shear stress acting on the protein. To block this interaction, heparin seems to be a good choice as it was shown to compete with DNA for the A1 binding site. Therefore, if vWF is pre-incubated with heparin, DNA binding should disappear.¹

To sum up, according to recent research, A1 domain of vWF is thought to be the main interacting partner of DNA. It seems that ristocetin could be used to mimic physiological conditions *in vitro* to enable binding without shear flow. Heparin seems to be useful to block this interaction. Interaction of vWF with NETs shows that vWF also plays a role in inflammatory processes. Apparently, this happens via acting as a linker for leukocytes to endothelial cells, which leads to the leukocyte's extravasation to surrounding tissues and inflammatory conditions.

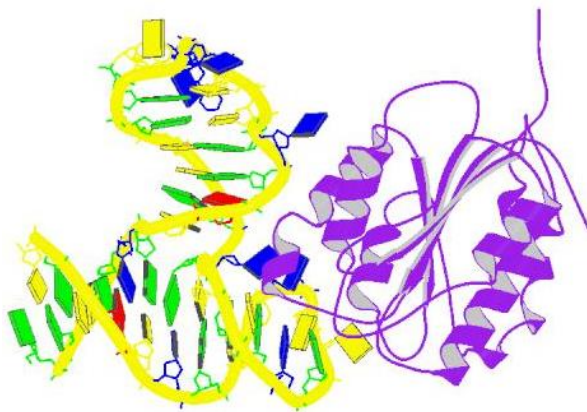


Figure 7: Crystal structure of vWF A1 domain in complex with a DNA aptamer. The A1 domain is thought to be the main interacting partner with for example GPIb α , heparin and also DNA. Electrostatic interactions seem to be relevant for this binding, as DNA is negatively charged and vWF A1 domain mainly positive.

Obtained from:

<http://www.rcsb.org/pdb/explore.do?structureId=3HXO> ⁵²

2. MATERIAL AND METHODS

2.1. Imaging of λ -DNA

2.1.1. Sample preparation

To get an idea of the distribution and structure of λ -DNA (New England Biolabs Inc., Ipswich, USA) on mica, AFM imaging was done before using it for force spectroscopy measurements.

In order to get a good image of DNA, several protocols were tested. Finding appropriate imaging conditions turned out to be quite a challenge. The first protocols are based on the imaging experience of the institute; protocols 5 – 8 were kindly provided by my colleague Anny Fis and her former laboratory (Laboratoire de Physique, ENS Lyon, France).

For the first three imaging approaches, DNA was **purified** with two different methods:

- Imaging protocol 1

DNA was purified using a DNA purification quick kit (Hi Yield® Gel/PCR DNA Fragment Extraction Kit, Süd-Laborbedarf GmbH, Gauting, Germany), to get rid of ethylenediaminetetraacetic acid (EDTA).

Then the DNA was incubated for 15 minutes at a concentration of 50 $\mu\text{g/mL}$ on freshly cleaved mica in the presence of 100 mM TRIS and 100 mM Ni. After that, the sample was washed using 5 mM TRIS and 1 mM Ni in MQ- H_2O . This buffer was also used for imaging.

The same protocol was also tried using 3 $\mu\text{g/mL}$ DNA.

- Imaging protocol 2

As a second approach, DNA was purified with ethanol precipitation.

Therefore, 100 μL λ -DNA was mixed with 250 μL absolute ethanol and 38 μL sodium acetate, and incubated for 2 hours at - 20 °C. To obtain a pellet, the solution was centrifuged at 13000 rounds per minute (rpm) for 15 minutes at

- 4 °C, and the supernatant was decanted away. As a washing step, 1 mL 70 % ethanol was added, spun down briefly and the supernatant was decanted again. After air drying the pellet was re-suspended in 100 µL MQ-H₂O and the concentration was measured using the NanoDrop device. Then, 10 µg/mL of the purified DNA was incubated on mica under the same buffer conditions as in imaging protocol 1.

- Imaging protocol 3

As a third approach, 3 µg/mL of the ethanol precipitated DNA were used. The buffer for washing and measuring was changed to 15 mM TRIS and 0.5 mM Ni, all other parameters remained unchanged.

The following protocols used **unpurified** DNA in different concentrations and with different buffers and incubation parameters:

- Imaging protocol 4

Unpurified DNA (3 µg/mL) was incubated on freshly cleaved mica for 15 minutes in the presence of 100 mM TRIS and 100 mM Ni. To wash the sample and carry out measurements, 15 mM TRIS and 0.5 mM Ni were used.

- Imaging protocol 5

DNA was diluted to a concentration of 0.2 µg/mL, using a buffer solution of 10 mM TRIS pH 7.5 and 10 mM MgCl₂. Then 5 µL of the DNA solution was deposited on freshly cleaved mica and incubated for 1 minute. For imaging, TRIS buffered saline (TBS) pH 7.4 buffer was used.

TBS buffer:

50 mM TRIS

150 mM NaCl

Prepared in MQ-H₂O

- Imaging protocol 6

The cleaved mica was pre-incubated with 10 mM NiCl₂ for 1 minute. Then the mica was washed with MQ-H₂O and dried with nitrogen gas.

DNA was diluted to 0.2 µg/mL, using a buffer solution containing 10 mM TRIS, 2 mM MgCl₂ and 5 mM NaCl. The solution was incubated for 10 minutes on the mica. Measurements were performed in TBS.

- Imaging protocol 7

0.2 µg/mL DNA in 10 mM TRIS-HCl pH 7.4 and 5 mM MgCl₂ were incubated on freshly cleaved mica for 10 minutes. Afterwards, the mica was rinsed with MQ-H₂O and dried with nitrogen gas carefully. Imaging was done in TBS.

- Imaging protocol 8

20 µL TE buffer containing 1 nM unpurified DNA were incubated for 5 minutes on cleaved mica and then the mica was washed with MQ-H₂O. Before mounting in the fluid cell, the sample was dried with nitrogen gas. Imaging was also done in TE buffer.

TE buffer:

10 mM	TRIS
2 mM	MgCl ₂
1 mM	EDTA pH 8.0
Prepared in MQ-H ₂ O	

2.1.2. Imaging procedure

For all imaging experiments, the Pico SPM Plus AFM setup (Agilent technologies, Chandler, USA) was used. To image λ-DNA, MAC-mode imaging was conducted. For MAC-mode imaging, magnetically coated MACLevers were used, which carry one rectangular cantilever.

For MAC-mode imaging, besides a magnetically coated cantilever also a MAC nose for the scanner was necessary to enable magnetic excitation. In the software (PicoView 1.12, Agilent Technologies) the mode “ACAFM” was chosen. Six different

images were shown by the software: topography (trace and retrace), amplitude (trace and retrace) and phase (trace and retrace).

After measurements, the program Gwyddion 2.39 was used for visualization and evaluation of the obtained data.

2.2. Force spectroscopy measurements of vWF and DNA

2.2.1. Photo-biotinylation of λ -DNA

5 mg of Psoralen-PEO4-Biotin (Santa Cruz Biotechnology Inc., Dallas, USA) were suspended in 2.5 mL isopropanol. Ten aliquots, containing each 250 μ L (= 0.5 mg Biotin) were filled into Crimp-vials and were evaporated using the rotary evaporator. To completely dry them, they were put onto the vacuum pump overnight. Afterwards the vials were flooded with argon, closed tightly and stored at -20 °C for further use.

λ -DNA was frozen at -20 °C in 50 μ L aliquots and slowly thawed on ice. To prevent oxidation, Psoralen-PEO4-Biotin was re-suspended in 500 μ L 1 mM EDTA pH 7.4 to reach a final concentration of 1 mg/mL. Each 50 μ L aliquot of DNA was mixed with 50 μ L Psoralen-PEO4-Biotin.

The solution was irradiated at 360 nm for 20 minutes (lids of the Eppendorf tubes were open), the distance from the UV light source (UV Lamp dual wavelength, serial number 022.9120, CAMAG®, Berlin, Germany) to the fluid not exceeding 2 cm.

After irradiation, dialysis at 4 °C against PBS pH 7.3 was carried out for approximately 20 hours. Dialysis tubes with a molecular weight cut-off of 10 kDa were used and buffer was exchanged four times.

Buffer for dialysis: PBS pH 7.3

16.36 g	NaCl
0.403 g	KCl
3.560 g	Na ₂ HPO ₄ · 2H ₂ O
0.490 g	KH ₂ PO ₄

Filled up to 2 L with MQ-H₂O

After dialysis, DNA was distributed into aliquots and stored at -20 °C until further use.

In order to check if this method of photobiotinylation worked, an electrophoretic mobility shift assay (EMSA) was carried out with the help of colleagues from the single molecule genetics group of the institute. Therefore, the DNA was once digested with DpnI and once sonicated, to get smaller parts.

Pre-electrophoresis in a 5 % polyacrylamide gel was done for 30 minutes at 100 V in 0.5x TBE buffer. DNA plus 10x EMSA-buffer was filled to a final volume of 20 µL using H₂O and 4 µL of 6x EMSA loading dye was added to each reaction.

The samples were loaded onto the 5 % polyacrylamide gel and electrophoresis was run for 50 minutes at 100 V in 0.5x TBE buffer. After that, the gel was blotted on a nylon membrane for 80 minutes at 100 V, again in 0.5x TBE buffer. To covalently bind the DNA fragments to the membrane, crosslinking using UV-light was carried out for 12 minutes at 254 nm. DNA-free sites were blocked with 1 % casein in 1x TBS for 15 minutes. Afterwards, the reaction was incubated for 15 minutes with blocking solution and horseradish peroxidase-labelled streptavidin. Unbound streptavidin was washed away with washing solution (TRIS-HCl + NaCl + SDS at pH 8.0). Then the membrane was incubated in equilibration solution (TRIS-HCl pH 8.0) before treatment with 3 mL WestFemto Luminol – enhancer solution for 5 minutes. The chemiluminescent signal could then be detected.

2.2.2. Tip functionalization

All protocols for the different tip functionalizations can be obtained online at the webpage of the biophysics institute from the Johannes Kepler University Linz (<http://www.jku.at/biophysics/content/e257042>).

As all vWF constructs which were used in this study featured a His₆-tag, only the protocol for maleimide tips was used, which is the following.

As a first step, amino-functionalization of the MSCT chips (Bruker AFM Probes, Camarillo, USA) using APTES in the gas phase was done. Therefore, the cantilevers were placed in a desiccator filled with argon after a washing step in chloroform. Two

cut lids of Eppendorf tubes, one filled with 30 μ L APTES and the other with 10 μ L triethylamine (TEA), were also put into the desiccator and the lid was closed. After two hours, the reactants were removed again, the desiccator was flooded with argon and the chips stayed in there for three days to cure before use. This procedure was carried out once a week by one person of the research group. After amino-functionalization, the tips are stable for several weeks if stored under argon.

Next, 1 mg Maleimide-PEG-NHS linker was dissolved in 0.5 mL chloroform and transferred into a Teflon reaction chamber. 30 μ L triethylamine was added, carefully mixed, and the chips were put into the solution immediately as TEA directly starts the chemical reaction. A lid was put onto the Teflon chamber to prevent evaporation of the chloroform. After an incubation time of two hours the chips were washed three times for 5 minutes in pure chloroform.

During the washing procedure, the following components were pre-mixed:

- 50 μ L disulfide TRIS NTA (1 mM)
- 2 μ L EDTA pH 7.5 (100 mM)
- 5 μ L Hepes pH 7.5 (1 M)
- 3 μ L TCEP hydrochloride (100 mM) and
- 3 μ L Hepes pH 9.6 (1 M)

The chips were dried using nitrogen gas and put into a polystyrene Petri dish which was covered with a piece of Parafilm inside. All chips were arranged in a way on the Parafilm that the drop of pre-mixed liquid would incubate on all cantilevers at once.

The Petri dish was covered with the lid and the cantilevers were incubated for two hours.

After incubation, washing was done again in TBS pH 7.4, three times for 5 minutes. Chips were placed on a new Petri dish without drying, and pre-incubated with 50 μ L TBS and 2 μ L NiCl_2 (5 mM). After 5 minutes, the droplet was removed again and 50 μ L His₆-tagged protein (0.5 μ M) with 2 μ L NiCl_2 (5 mM) were put onto the cantilevers and incubated for two hours.

As a last step, the chips were washed again three times for 5 minutes in TBS, and then also stored one by one in multi-well-plates in TBS at 4 °C until use. This way, functionalized tips are stable for several weeks.

2.2.3. λ -DNA sample preparation for force measurements

The components of the sample plate were washed using SDS, isopropanol and MQ-H₂O. Freshly cleaved mica was assembled into the sample plate. Lysozyme-biotin, streptavidin and λ -DNA were all stored at $-20\text{ }^{\circ}\text{C}$ before use and thawed at room temperature.

Binding of the biotinylated λ -DNA to the supporting sample surface was carried out in 3 steps:

1. Lysozyme-biotin ($1\text{ }\mu\text{M}$) was incubated for 15 minutes on the mica. Due to its positive charge lysozyme-biotin adhered to the negatively charged mica. A washing step using PBS made sure that unbound lysozyme-biotin was washed away before continuing with the next step.
2. Streptavidin with a concentration of 0.5 mg/mL was also incubated for 15 minutes and the mica was washed again with PBS. Streptavidin then was assumed to be tightly bound to the lysozyme-biotin surface.
3. λ -DNA in PBS ($25\text{ }\mu\text{g/mL}$), which was beforehand biotinylated with Psoralen-PEO4-Biotin, was incubated for 30 minutes onto the streptavidin layer. Streptavidin has four binding sites for biotin, therefore binding to the first layer (lysozyme-biotin) and to the biotinylated DNA was possible. After the last step, washing was carried out with TBS.

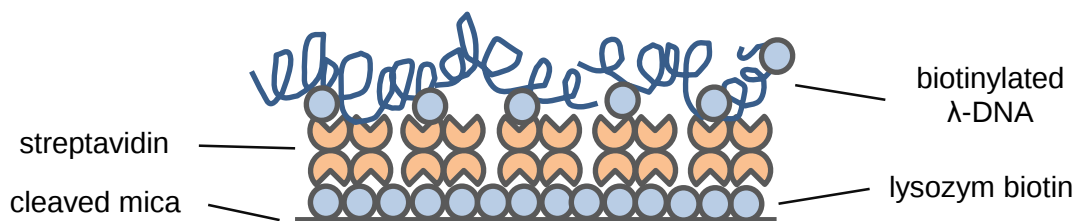


Figure 8: Scheme of a DNA sample consisting of four different layers. Cleaved mica was used as a support. Lysozyme biotin was incubated on the mica, binding due to its positive charge. Streptavidin, the next layer, binds both lysozyme biotin and the biotinylated DNA.

2.2.4. Obtaining force distance cycles

All force measurements were performed on a Pico SPM Plus atomic force microscopy setup. Measurements were carried out in TBS pH 7.4 unless indicated differently. The software PicoView 1.12 from Agilent Technologies was used, and operated in contact mode.

Functionalized chips were stored at $-4\text{ }^{\circ}\text{C}$ in TBS and were taken out of the fridge some minutes prior to measurements to reach room temperature. To avoid contamination, the sample plate was cleaned with SDS, isopropanol and MQ- H_2O , dried with a tissue and assembled with the appropriate sample. The chip was inserted onto the scanner in the correct position, and then the scanner was mounted into the AFM setup. The sample plate holding the sample was put onto the appropriate place beneath the scanner. As a next step, the laser was correctly adjusted to focus on the chosen cantilever for the measurement. For all force measurements in this thesis, MSCT cantilever D was used. After focusing the laser (reflected from the cantilever tip) into the center of the photodiode, approaching could be started by pressing the “approach” button in the software. Approaching was done with 5 nm/s and stopped automatically when the sample surface was reached. Stopping was enabled by setting a threshold of 0.8 V for the feedback electronics. The scanning range of the piezo was opened to the maximum value to find the force-distance-curve on the screen. Then the desired parameters (like number of force distance cycles, speed and range) could be set.

In order to avoid position dependent artefacts, the position of measurement was changed roughly every 200 force distance cycles.

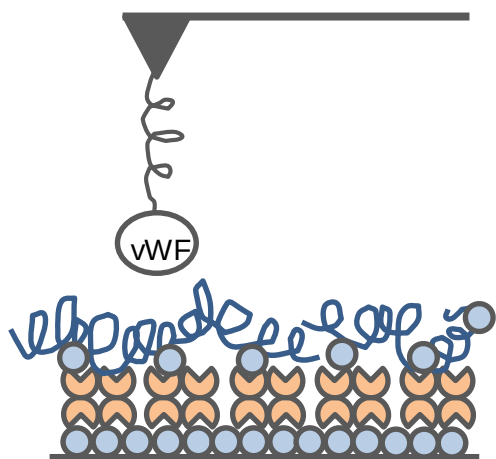


Figure 9: Schematics of force spectroscopic measurements. VWF was attached to the MSCT cantilever tip and DNA was immobilized on the sample surface. By approaching the cantilever tip to the surface, binding was made possible and rupture forces could be recorded.

2.2.4.1. Pre-testing with and without ristocetin

To study binding probabilities of different vWF molecules to DNA, three to four chips were used for each system. With each chip, one measurement in normal TBS, one with 1.5 mg/mL ristocetin (möLab GmbH, Langenfeld, Germany) in TBS and one again with normal TBS (no ristocetin) was carried out. To get valuable results, approximately 1000 curves were done per measurement. This means that per chip 3000 curves were recorded, leading to 9000 - 12000 force-distance-cycles per system. Using ristocetin, shear stress could be mimicked; this pre-testing should reveal the dependence of ristocetin for binding events vWF and DNA. Figure 10 shows the experimental setup.

In total, six vWF-systems were investigated:

- Monomer
- Dimer
- A1 domain
- A1A2A3 wild type (wt)
- A1A2A3 A1461D GOF-mutant
- A1A2A3 R1308C GOF-mutant

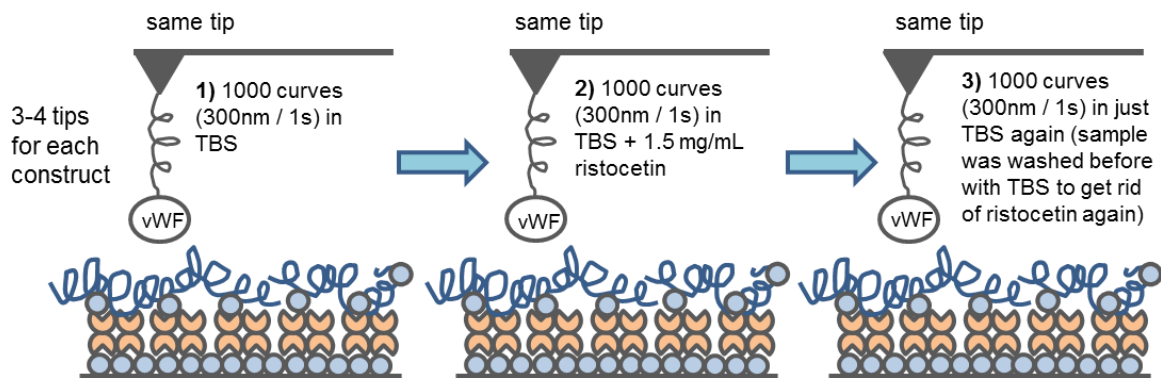


Figure 10: Experimental setup of pre-testing experiments. For each of the six vWF systems (monomer, dimer, A1, A1A2A3 wt, A1A2A3 A1461D and A1A2A3 R1308C) 3-4 tips were used. For each tip, the experiment was carried out as shown in the figure. First, 1000 curves in normal TBS were measured, then in TBS + ristocetin. As a third step, the ristocetin buffer was washed away and measurement was again performed in TBS only.

During some measurements, the force distance cycles looked as if something was sticking to the tip. After literature search it was concluded that this was due to ristocetin, as it is able to form dimers when used in a concentration higher than 0.4 mg/mL. The problem could be solved by retracting the tip and approaching again. ⁴⁷

2.2.4.2. Loading rate dependences

To investigate the dynamics of the vWF / DNA interaction, loading rate dependence (LRD) measurements were performed for each of the six systems. To do that, nine different speeds were measured: 300 nm / 1 s; 300 nm / 0.5 s; 300 nm / 0.2 s; 200 nm / 1 s; 200 nm / 0.5 s; 200 nm / 0.2 s; 100 nm / 1 s; 100 nm / 4 s and 75 nm / 1.5 s. From each of the six systems, a well working cantilever was chosen to measure the LRD.

To block specific interactions of vWF with DNA, heparin sodium salt (Sigma-Aldrich, St. Louis, USA) was used in varying concentrations (50 U/mL were recommended by Grässle et al ¹). Therefore the vWF-tip was pre-incubated in a heparin solution and also the AFM measurement was done in a TBS + heparin buffer. Blocking was checked at a velocity of 300 nm / 1 s, and the binding probability value was compared to the value obtained with the same parameters without blocking agents. A significant reduction in binding probability ensured specific interactions between vWF and DNA.

2.2.4.3. Analysis of force distance cycles

Analysis of the measured force curves was done using an in-house written routine for the program MATLAB R2013a (8.1.0.604). With the command “kspec19p” each single curve was analyzed and unbinding events were marked using first or second order polynomial fit. After marking all approximately 1000 curves from one measurement, the binding probability, which is an important parameter, was automatically calculated and MATLAB displayed a file showing 6 different graphs (see figure 11):

- A) a probability density function of rupture forces
- B) a probability density function of length
- C) unbinding force versus the unbinding length
- D) a probability density function of k_{eff}
- E) the probability of unbinding events per curve
- F) the force over Piezo movement (overlay of all curves)

This information was saved in result files, which were necessary for additional evaluation of the data.

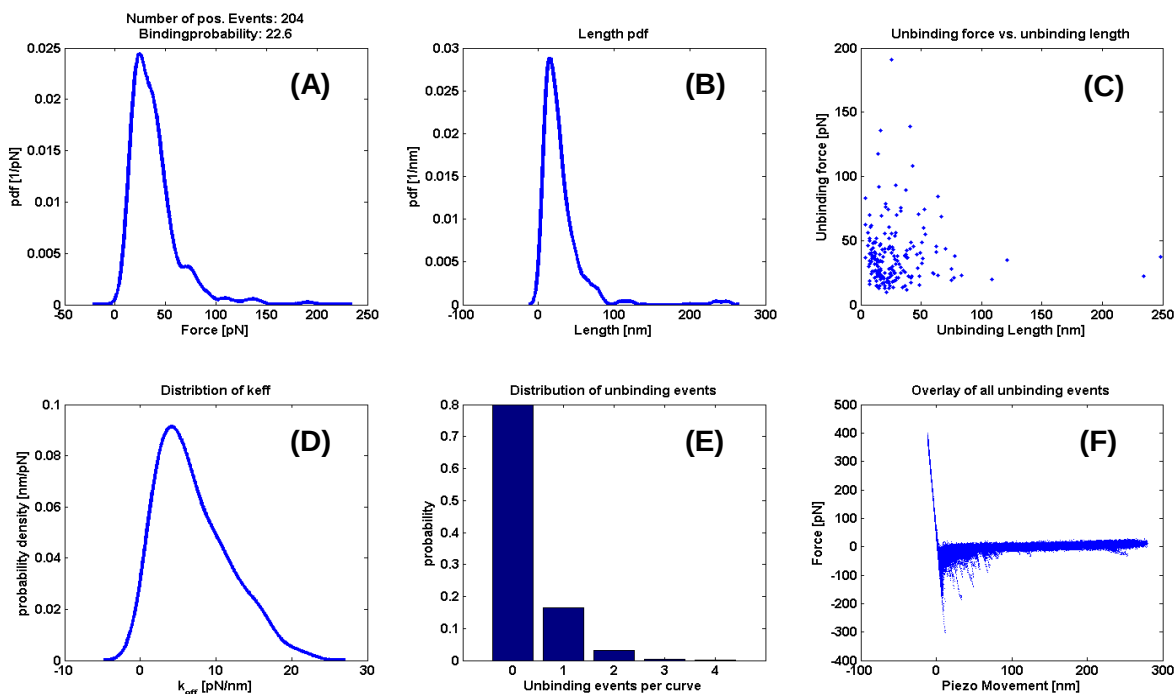


Figure 11: Example of a MATLAB result file after analysis of force distance cycles. In this case vWF A1 measurement on λ -DNA with ristocetin is shown. **(A)** The peak shows the distribution of rupture forces. On the upper left corner in can be read that of the approximately 1000 curves 204 showed unbinding events, which resulted in 22.6 % binding probability. **(B)** The distribution of bond length is shown. **(C)** This scatter blot shows the unbinding force in relation to the unbinding length. **(D)** The distribution of k_{eff} , which is the combined spring constant (cantilever and linker) at rupture ⁸, is shown. **(E)** This histogram shows the probability of unbindings per curve. In this case, most curves showed no unbindings. **(F)** The movement of the Piezo in relation to the force is illustrated as an overlay of all curves.

For LRD blots, these result files were used again for different MATLAB commands. Actual spring constants were experimentally determined as the actual values usually differ a bit from the values indicated on the chip box by the manufacturer. This is also due to the molecules attached to the tips. For this procedure, the tips were washed in MQ-H₂O to avoid crystallization of buffer salts during spring constant determination in air. The thermal noise method was used, which uses a thermal noise to allow fluctuation of the cantilever. A power spectral density plot was used to find the actual spring constant. ⁴⁸ Spring constant measurements were the last thing done, as the tip is measured on a glass surface and may break during this procedure. The theoretical

values given by the manufacturer were adjusted in MATLAB using the command “spcadjust”.

Next, the command “spdst” enabled to mark maxima on the force pdf function (valid for the Bell-Evans-model), itself producing new result files. Those new result files were used for the command “dfs_ml”, which finally exported the LRD plot (see figure 12) and thus determined the energy barrier (X_β) and the dissociation constant (k_{off}), which are the most meaningful parameters to describe biological interactions. X_β basically describes the distance between the energy minima to the maxima and can be seen as an estimate of the bond length. Therefore, higher values of X_β can suggest a more stable the bond compared to lower values. In contrast, the lower k_{off} , the more stable the bond.

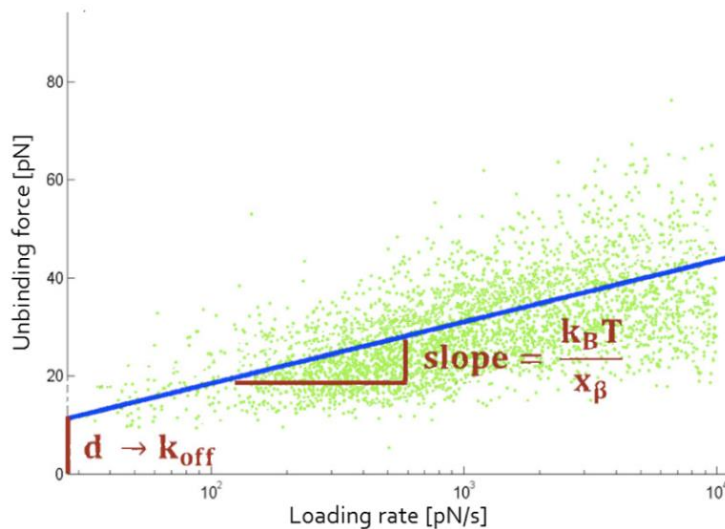


Figure 12: Schematics of a loading rate dependence plot. The unbinding force (pN) over the loading rate (pN/s) is shown. The figure indicates that X_β can be calculated from the slope of the plot and k_{off} from the interception with the y-axis.

The image was kindly provided by Sandra Posch.

3. RESULTS

3.1. Imaging of λ -DNA

In order to characterize the structure of λ -DNA prior to using it for force measurements, AFM MAC mode imaging was performed. Moreover, it was tried to find an appropriate protocol for imaging DNA.

It turned out that obtaining an image of λ -DNA strands on mica is not as easy as thought in the beginning. Therefore, seven protocols were tried without leading to an image of single λ -DNA strands. Finally, with the eighth protocol, a successful outcome was achieved – strands of DNA could be observed.

The protocols already differ in the purification method of the λ -DNA. In the beginning, two different purification methods were used; later, unpurified DNA was used. Buffer conditions, incubation times, washing steps and other factors were constantly changed to find optimal conditions.

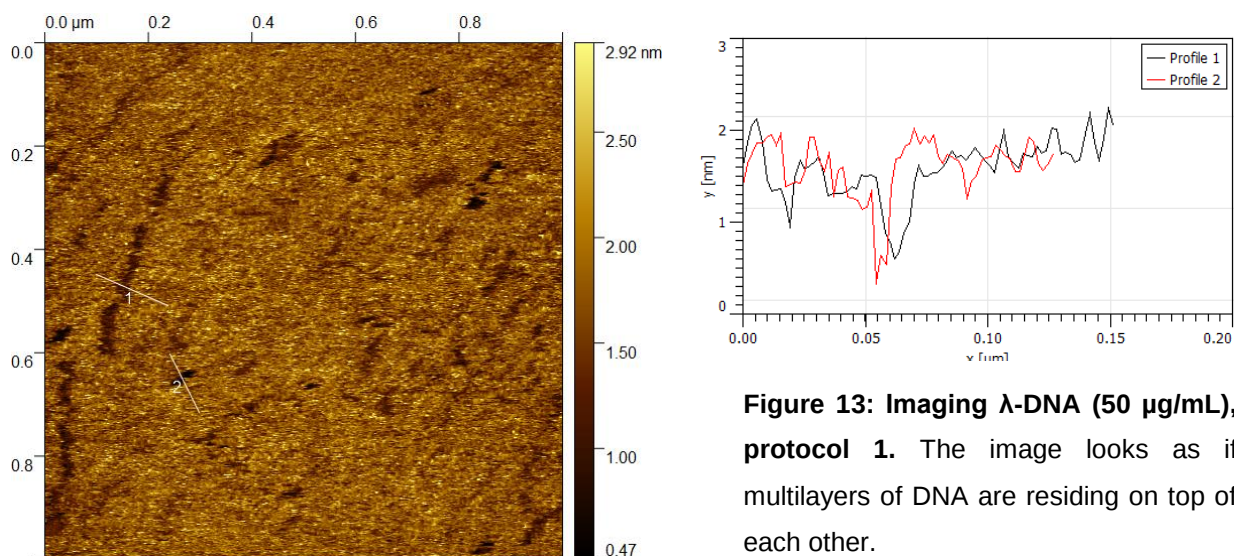
For each protocol, several images were taken on different positions of the sample to avoid position dependent artefacts. In some cases, several samples were prepared and imaged per protocol. Representative images are shown here.

- Imaging protocol 1

For protocol 1, the quick purification kit was used to purify the DNA, to eliminate the EDTA contained, which might have interfered with imaging experiments. Either 50 $\mu\text{g/mL}$ or 3 $\mu\text{g/mL}$ DNA were used for the sample preparation on mica. Incubation was done for 15 minutes and with 100 mM TRIS and 100 mM Ni. After incubation, the sample was washed with 5 mM TRIS and 1 mM Ni, which was also used for imaging.

Looking at the images obtained using 50 $\mu\text{g/mL}$ DNA, one can probably see the multilayers on the sample surface (figure 13). The height profile shows that the

scratches have a depth of approximately 1 nm. DNA was reported to be about 2 nm in height, so these images suggest a dense and tightly bound DNA layer.



50 $\mu\text{g/mL}$ were concluded to be a too high concentration of DNA, thus a lower concentration of 3 $\mu\text{g/mL}$ was used as a next step; all other conditions remained unchanged.

Figures 14 and 15 also do not show distinct strands of DNA. Either bare mica (figure 14) or somehow strange knobs (figure 15) were observed. Figure 14 shows a surface roughness of about 0.2-0.3 nm, which is comparable to literature values of mica roughness (which is 0.3-0.4 nm⁴⁹).

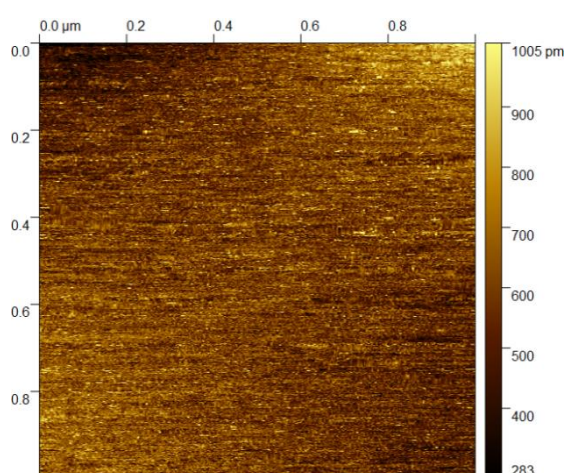


Figure 14: Imaging λ -DNA (3 $\mu\text{g/mL}$), protocol 1. Apparently only bare mica is observable when using the lower concentration of DNA.

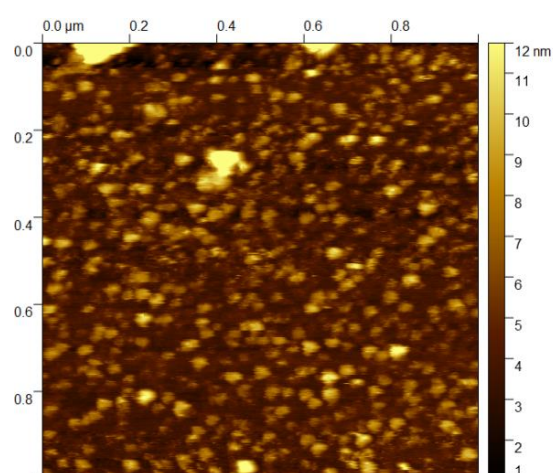


Figure 15: Imaging λ -DNA (3 $\mu\text{g/mL}$), protocol 1. In this case, knobs are visible. This may be aggregates of λ -DNA, or maybe some other contamination.

The fact that at one time many aggregates and another time nothing was observed, using the same sample of DNA, suggests an uneven distribution of DNA molecules on the surface.

- Imaging protocol 2

In protocol 2, DNA was not purified using the quick kit, but with the EtOH precipitation method.

This method was chosen because obtained DNA is supposed to be very clean and in a high yield. So it was thought that maybe this way knobs, which were observed when using protocol 1, could be avoided. As 3 $\mu\text{g/mL}$ DNA were seemingly too little and 50 $\mu\text{g/mL}$ too much, 10 $\mu\text{g/mL}$ were chosen for this protocol. To know the concentration of the DNA after purification, measurement with the NanoDrop was necessary. The concentration of the DNA was 400.5 $\text{ng}/\mu\text{L}$. To assess purity, the 260/280 absorbance ratio was measured. Purity of DNA was 1.84, which is considered as “pure” for DNA. (The 260/280 ratio is commonly used to determine purity of nucleic acids – absorbance is measured at 260 nm and 280 nm.)

All other parameters remained equal to protocol 1.

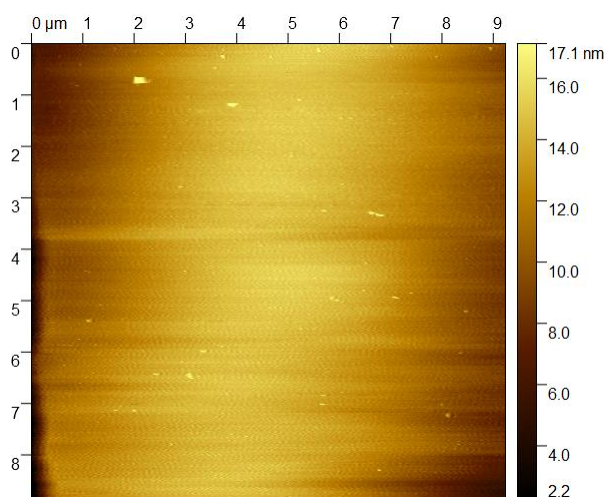


Figure 16: Imaging λ -DNA (10 $\mu\text{g/mL}$), protocol 2. Using this method, where DNA is purified using EtOH purification, nothing was observed during imaging.

Looking at figure 16, the mica surface seems empty. Some structures were observed, however this might probably be dust or other contaminations. As the purity

measurement gave a really good value, the EtOH precipitation method was not thought to destroy the DNA. Therefore it was surprising that the sample seemed to be empty.

- Imaging protocol 3

Also for protocol 3, DNA purified with EtOH-precipitation was used – this time in a concentration of 3 $\mu\text{g/mL}$ and with a different measurement buffer: 15 mM TRIS and 0.5 mM Ni.

Although lowering the concentration of DNA compared to protocol 2, the mica was not looking empty this time – it was full of small high structures (figure 17).

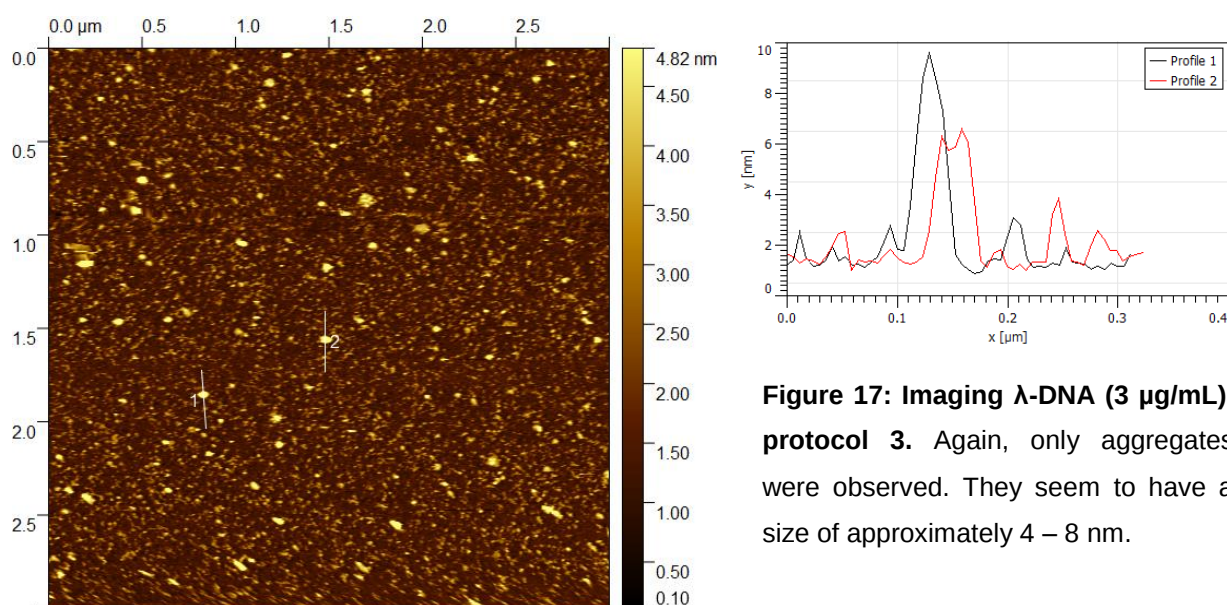


Figure 17: Imaging λ -DNA (3 $\mu\text{g/mL}$), protocol 3. Again, only aggregates were observed. They seem to have a size of approximately 4 – 8 nm.

It was unsure if the aggregates were accumulated DNA, however this seemed to be a plausible explanation.

- Imaging protocol 4

From imaging protocol 4 on, unpurified λ -DNA (3 $\mu\text{g/mL}$) was incubated for 15 minutes on mica. This was done in 100 mM TRIS and 100 mM Ni, however to wash

and image the sample 15 mM TRIS and 0.5 mM Ni were used instead. Looking at figure 18, again few high structures can be seen.

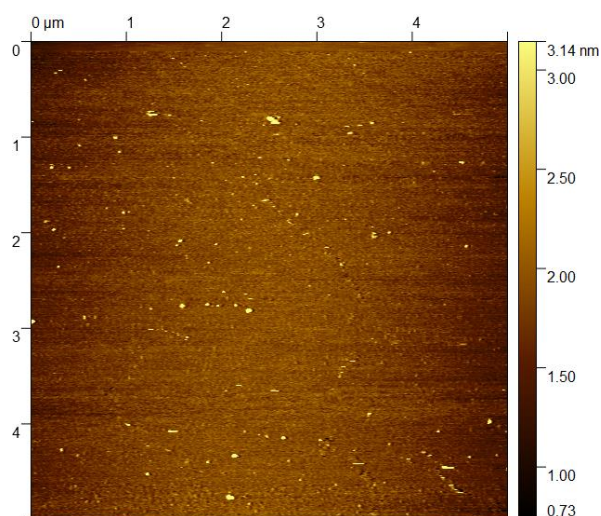


Figure 18: Imaging λ -DNA (3 $\mu\text{g/mL}$), protocol 4. Like in the previous images, only aggregates are seen, however quite sporadically distributed.

- Imaging protocol 5

For protocol 5, the DNA concentration was even lowered to 0.2 $\mu\text{g/mL}$; in a buffer with 10 mM TRIS and 10 mM MgCl_2 . Incubation time was decreased to 1 minute and imaging was done in TBS pH 7.4 (containing 50 mM TRIS and 150 mM NaCl in MQ- H_2O). A representative image is shown in figure 19.

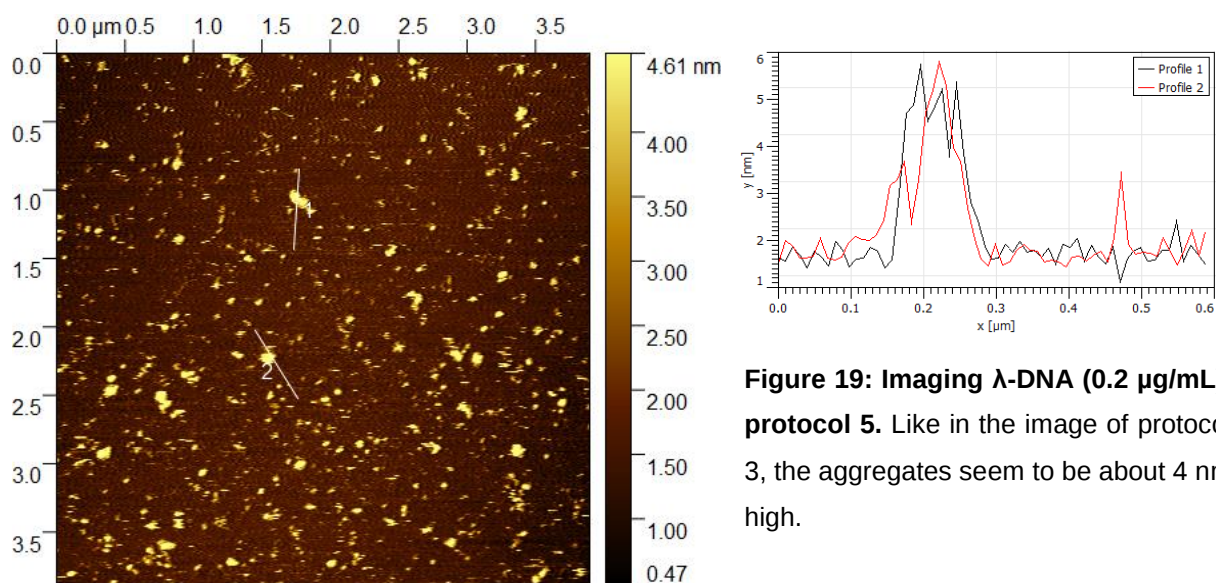


Figure 19: Imaging λ -DNA (0.2 $\mu\text{g/mL}$), protocol 5. Like in the image of protocol 3, the aggregates seem to be about 4 nm high.

Here again, only knobs were observed. The bigger ones had a size of about 4 nm but there were also many smaller ones.

- Imaging protocol 6

For this approach, cleaved mica was pre-incubated for 1 minute using 10 mM NiCl_2 and afterwards washed (with MQ- H_2O) and dried with nitrogen gas.

DNA, again in a concentration of 0.2 $\mu\text{g/mL}$ was diluted in a buffer with 10 mM TRIS, 2 mM MgCl_2 and 5 mM NaCl and incubated for 10 minutes. The measurements were performed in TBS. Figure 20 again shows no DNA strands.

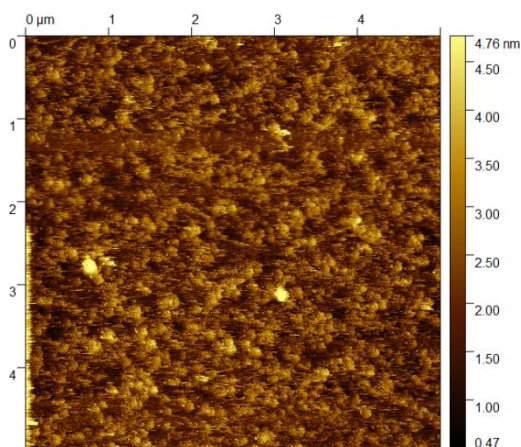


Figure 20: Imaging λ -DNA (0.2 $\mu\text{g/mL}$), protocol 6. The regular structures seen are probably due to a tip artefact.

- Imaging protocol 7

This time DNA was diluted to a concentration of 0.2 $\mu\text{g/mL}$ using 10 mM TRIS-HCl and 5 mM MgCl_2 . The solution was incubated on freshly cleaved mica for 10 minutes. After incubation, the mica was rinsed with MQ- H_2O and dried with nitrogen gas. The outcome can be seen in figure 21.

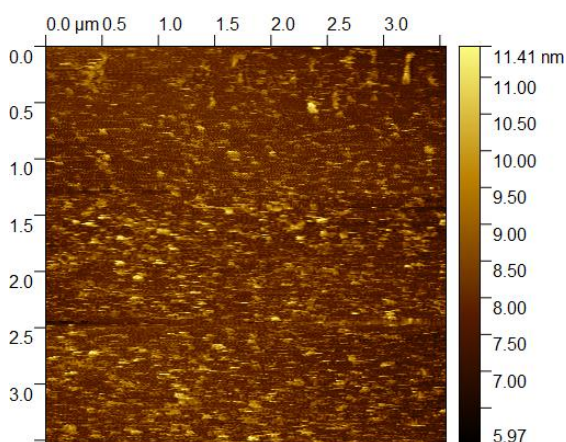


Figure 21: Imaging λ -DNA (0.2 $\mu\text{g/mL}$), protocol 7. Besides the aggregates, it also looked as if some strands were present, however, no distinct strands could be visualized.

- Imaging protocol 8

For protocol 8, the DNA concentration was lowered even more: 20 μL TE buffer (consisting of 10 mM TRIS, 2 mM MgCl_2 and 1 mM EDTA) containing 1 nM DNA was used. Incubation on mica was done for 5 minutes; afterwards the mica was washed with MQ- H_2O and dried with nitrogen gas. For imaging, again TE buffer was used. Finally, protocol 8 seemed to have all the appropriate conditions to obtain proper images of λ -DNA strands. Figures 22 and 23 still show only slightly visible strands and differently sized aggregates. In contrast, in figure 24, DNA strands can be clearly observed. However, besides the strands, huge aggregates, which could be any dirt or dust, are visible.

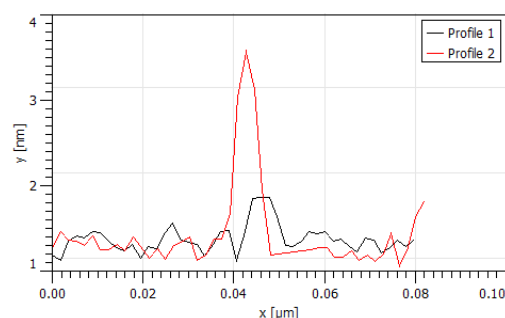
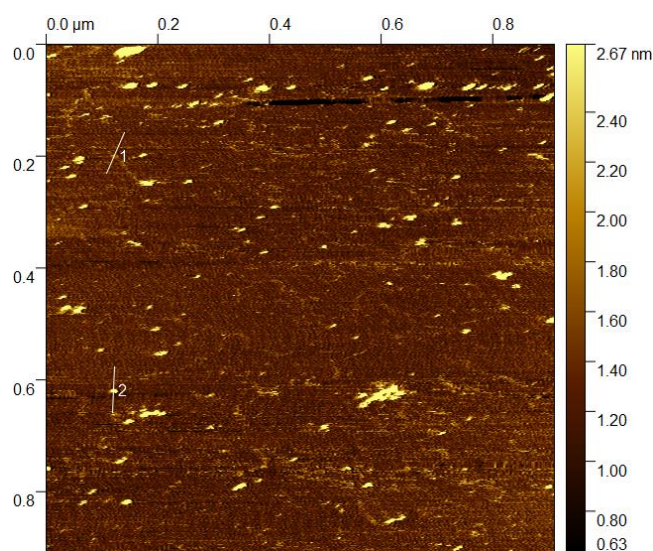


Figure 22: Imaging λ -DNA (1 nM), protocol 8. Looking at the height profile shows that there are also some lines observed, with a diameter of less than 1 nm. Besides that, aggregates can also be seen.

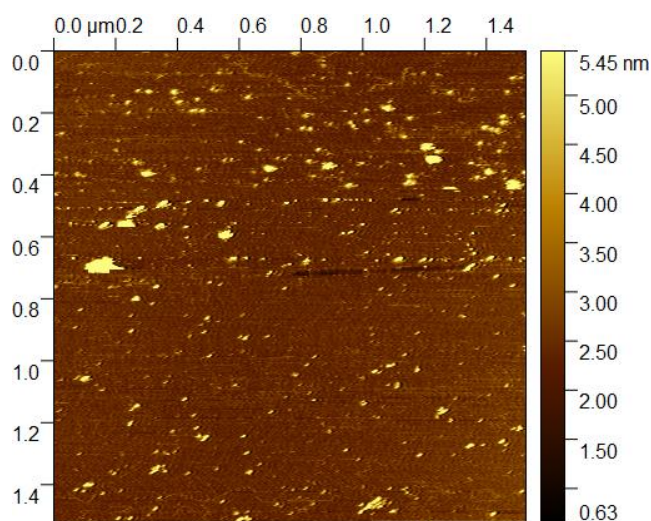


Figure 23: Imaging λ -DNA (1 nM), protocol 8. Another position on the sample surface also shows differently sized aggregates. In some places they seem to be located in line, therefore having the appearance of DNA strands with knobs.

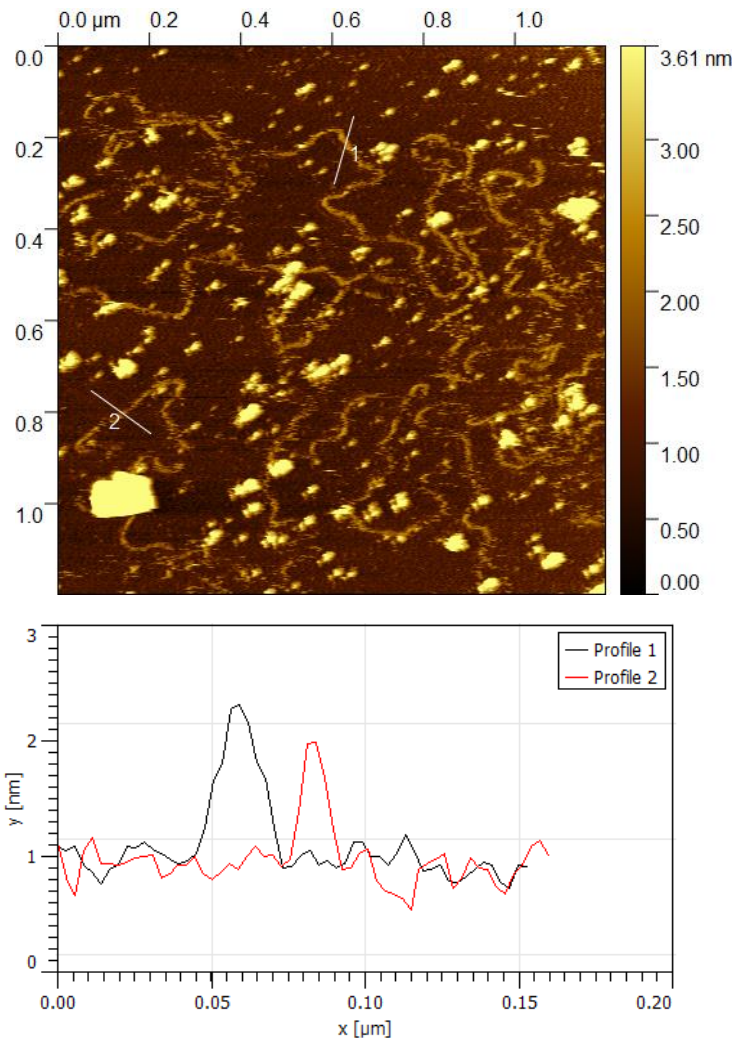


Figure 24: Imaging λ -DNA (1 nM), protocol 8. In the eighth protocol, DNA strands could finally be observed. However, besides the strands, again aggregates show up. The height profile shows that the strands are roughly 1 nm high. DNA is thought to have a diameter of about 2 nm, however as it adheres tightly to the mica it may appear smaller. The aggregates could be accumulated DNA.

To sum up, getting a good image of DNA is harder than it may seem. DNA seems to distribute very unevenly on the mica, therefore the position on the sample might affect what can be observed on the image. Aggregation of DNA seems to also be a major problem, although it is not known for sure if the aggregates really are DNA.

3.2. Force spectroscopy measurements of vWF and DNA

3.2.1. Verifying photo-biotinylation of λ -DNA

For force spectroscopy measurements, DNA was biotinylated to enable binding to mica via a streptavidin linker and lysozyme biotin. This method was thought to be the most appropriate one to bind DNA in a stable manner but to still allow interaction in force experiments. To verify that the λ -DNA was biotinylated an EMSA was carried out.

The relatively huge λ -DNA (about 48.500 base pairs) was once digested with the enzyme DpnI and once sonicated, to get smaller parts. As only biotinylated DNA was able to bind the horseradish peroxidase-labelled streptavidin, a chemiluminescent signal is a clear indicator that there is biotin bound to the DNA (see figure 25).

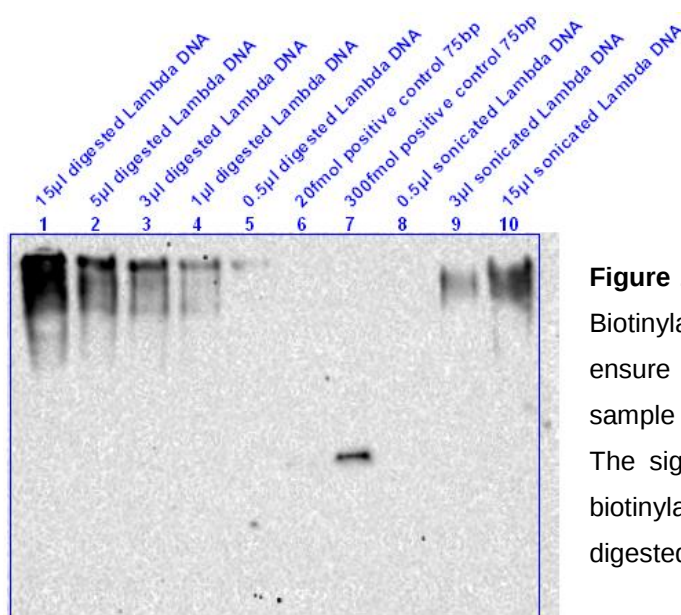


Figure 25: EMSA verifying DNA biotinylation.

Biotinylation of the λ -DNA was checked to ensure its binding to the streptavidin on the sample for force spectroscopy measurements. The signal clearly indicates that the DNA was biotinylated. Intensity of the signal is higher for digested DNA compared to sonicated DNA.

It seems that the DNA fragments were still quite big, as they did not migrate into the gel very well. Nevertheless, this does not influence the positive result. Figure 25 unmistakably shows that the DNA was biotinylated as a signal was detectable; the signal is also increasing with the concentration. In lane 6, the positive control did not work, however the positive control in lane 7 shows a nice band.

Lanes 1-5 show a stronger signal compared to lanes 9 and 10. Apparently, sonication destroyed more DNA than the enzymatic digest did.

All in all, the fact that a chemiluminescent signal was detectable is proof that DNA biotinylation was successful. Hence it could be concluded that DNA bound to streptavidin and is present on the sample surface.

3.2.2. Pre-testing with and without ristocetin

As the antibiotic ristocetin is thought to be of necessity for the vWF – DNA interaction in the absence of shear flow, its impact on different vWF constructs was examined. The six different vWF constructs (monomer, dimer, domain A1, domains A1A2A3 wt, A1A2A3 + A1461D mutation and A1A2A3 + R1308C mutation) were all tested for their binding probabilities with λ -DNA. Both mutations are found within the A1 domain of vWF and are GOF-mutations, hence enhancing vWFs interactions with domain A1 and its binding partners.

To do so, force spectroscopy measurements were first carried out in normal TBS to look at the binding behavior without ristocetin being present. Ristocetin is thought to enable interaction of the molecules without shear flow by exposing vWFs binding domain in the A1 domain. Therefore, as a next step, ristocetin was added to the buffer; to see if it has a beneficial effect on the binding probabilities of the constructs. To see whether this effect is reversible or not, the third measurement was again carried out in normal TBS lacking ristocetin.

Figure 26 shows the binding probabilities of the different vWF constructs with λ -DNA.

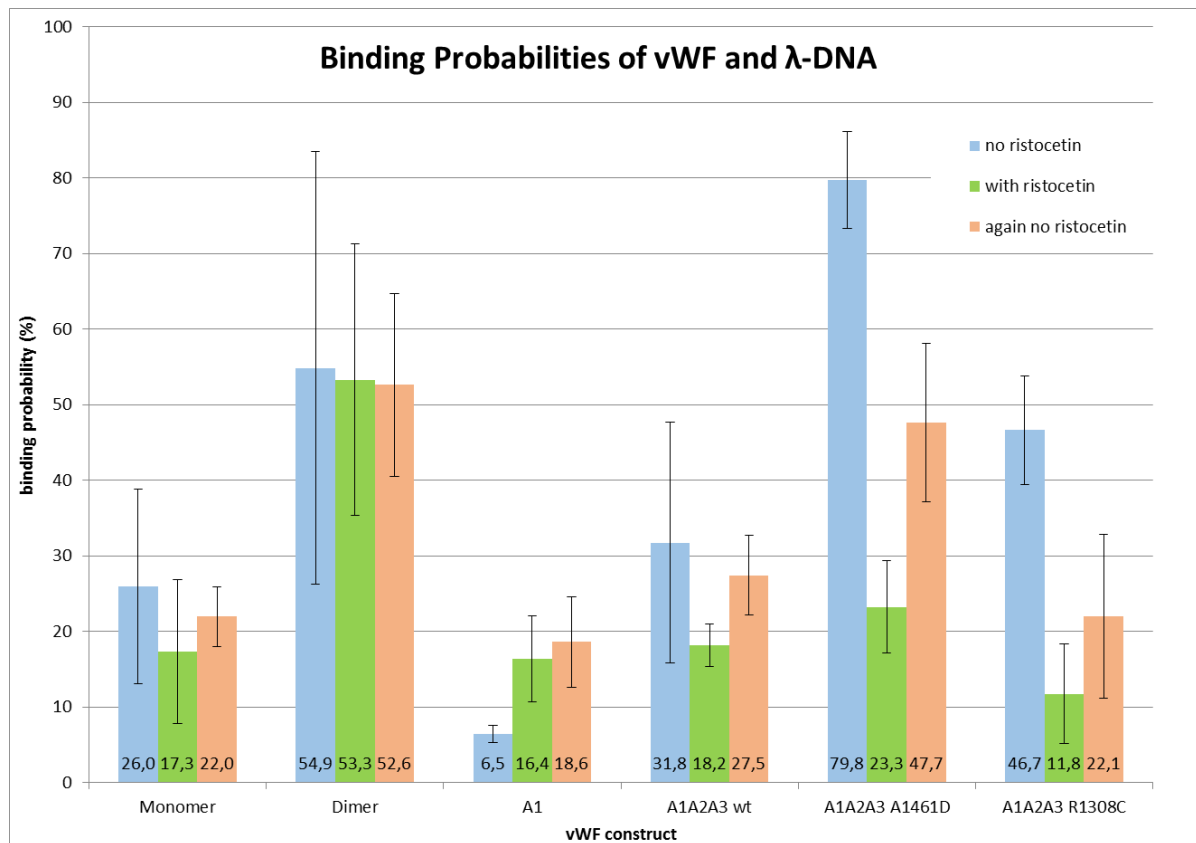


Figure 26: Binding probabilities of vWF constructs with DNA. Blue bars indicate measurements without ristocetin; the green ones represent measurements with ristocetin and the orange ones again without ristocetin. Mean values of at least three tips per construct are shown. Obvious outliers were excluded from the evaluation. With each cantilever, about 1000 curves were measured in each buffer condition. So per construct, between 9000 and 12000 curves were used for analysis.

A1A2A3 + A1461D showed the highest value in binding probability (79.8 %) without ristocetin added. This was expected, as it is a strong GOF-mutation which should bind to DNA really well. Interestingly, the addition of ristocetin did not further increase the value – the amount of binding events dropped from about 80 % to 23.3 % (which was a drop of about three fourths). However, after removing ristocetin again, the binding probability value rose again to 47.7 %. It seems as if ristocetin somehow had an interfering effect for the interaction between this construct and λ -DNA.

For **A1A2A3 + R1308C**, which is the second and weaker GOF-mutation, the numbers looked similar. Although the starting value at a binding probability of 46.7 % was lower compared to the A1461D mutation, the drop to 11.8 % also accounts for

approximately one fourth of the starting value. Removing ristocetin doubled the value again to 22.1 %, which was again similar compared to the other GOF-mutation.

Compared to the vWF **A1A2A3 wt** system, both GOF-mutations showed a higher binding probability. The A1A2A3 wt started (as expected) with a lower value than the mutations. Also the drop in binding probability was not that big, it dropped from 31.8 % to 18.2 %, which was still more than half. Exchanging ristocetin buffer into TBS resulted in 27.5 %, so the value of the beginning was nearly reached again. However, taking also the error bars into account, no significant differences were observed with and without ristocetin.

Interestingly, the vWF **dimer** seemed to not be influenced by ristocetin. No significant difference could be observed.

For the **monomer**, addition of ristocetin resulted in a drop of binding probability from 26.0 % to 17.3 %. Afterwards, the value rose again to 22.0 %. So also for the monomer, ristocetin seemed to slightly inhibit vWF binding to DNA. Removing ristocetin could restore this a bit. But also in this case the error bars were quite big, so that the differences were not significant.

Comparing the binding probabilities of monomer and dimer shows that the value is approximately twice as high for the dimer. A possible explanation is because the monomer has one A1 domain, and the dimer two.

The **A1 domain** seemed to be the only vWF construct which is dependent on, or at least benefits of, ristocetin in the measuring buffer. Binding probability rose from 6.5 % to 16.4 %, which is more than twice as high. After removing ristocetin again from the measuring buffer the binding probability did not decrease. Apparently in this case, the positive effect of ristocetin (which is exposing the A1 domain in the absence of shear flow to enable binding to DNA) was stable and not reversible. It seemed once the DNA binding site was made accessible by ristocetin, it stayed in a DNA binding conformation.

As A1 is thought to be the part responsible for vWF binding to DNA, it may seem strange that it showed the lowest binding probability compared to other constructs.

However, it was concluded that the neighboring domains which are present in the other construct may stabilize the structure of A1, hence contributing to stable interactions with DNA.

The results make clear that only the binding probability of vWF domain A1 is enhanced in the presence of ristocetin. All other constructs seemed not to be dependent on this compound. Moreover, the two mutations were confirmed to be GOF-mutations, as they both showed higher binding probabilities compared to the A1A2A3 wt.

To prove that DNA solely interacts with domain A1, test measurements with a vWF construct lacking domain A1 (A2A3) were performed. As the A1 domain is thought to be the main interacting partner with DNA, low binding probability was expected. Indeed, the values were really low for all tips (two tips were tested). It did not matter if ristocetin was present or not; none of the values exceeded 2.5 % at binding probability (see figure 27). That ristocetin had no effect was also expected, as it is thought to work only on the vWF A1 domain.

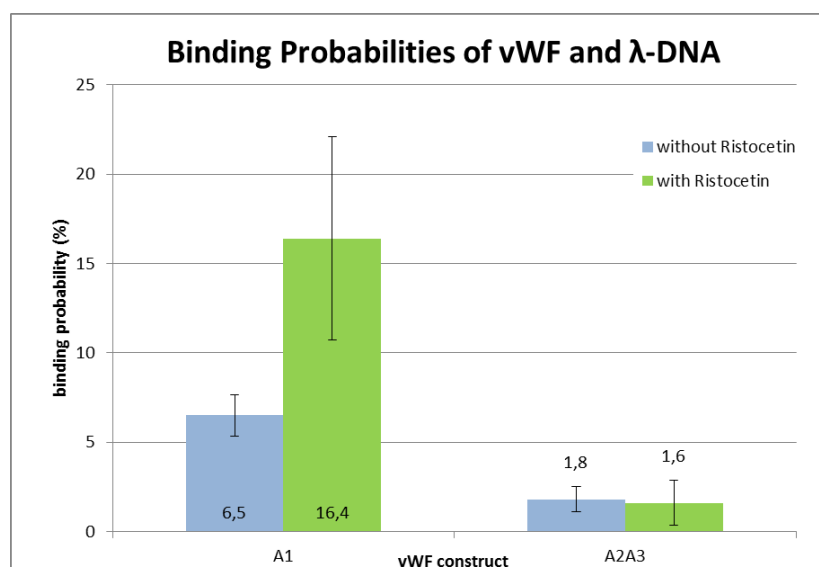


Figure 27: Binding probabilities of vWF A1 and A2A3 to λ -DNA. It is observable that force spectroscopy experiments using construct A2A3 only showed negligible binding probability compared to the A1 construct.

As the pre-tests lead to the conclusion that only the A1 domain is dependent on ristocetin, only the LRD measurement of A1 was carried out in the presence of ristocetin. All other constructs were measured in normal TBS.

3.2.3. Loading rate dependences

For LRDs, a well working cantilever for each construct was chosen. With this one tip, nine different velocities were measured, again 1000 curves for each velocity. Blocking experiments with heparin were performed to block specific interactions between DNA and vWF.

Figure 28 shows an example of a result file MATLAB establishes after the entire curve analysis. X_β and k_{off} were also calculated automatically and give an estimate of the length of the binding pocket and the bond lifetime, hence the stability of the biological interaction, respectively.

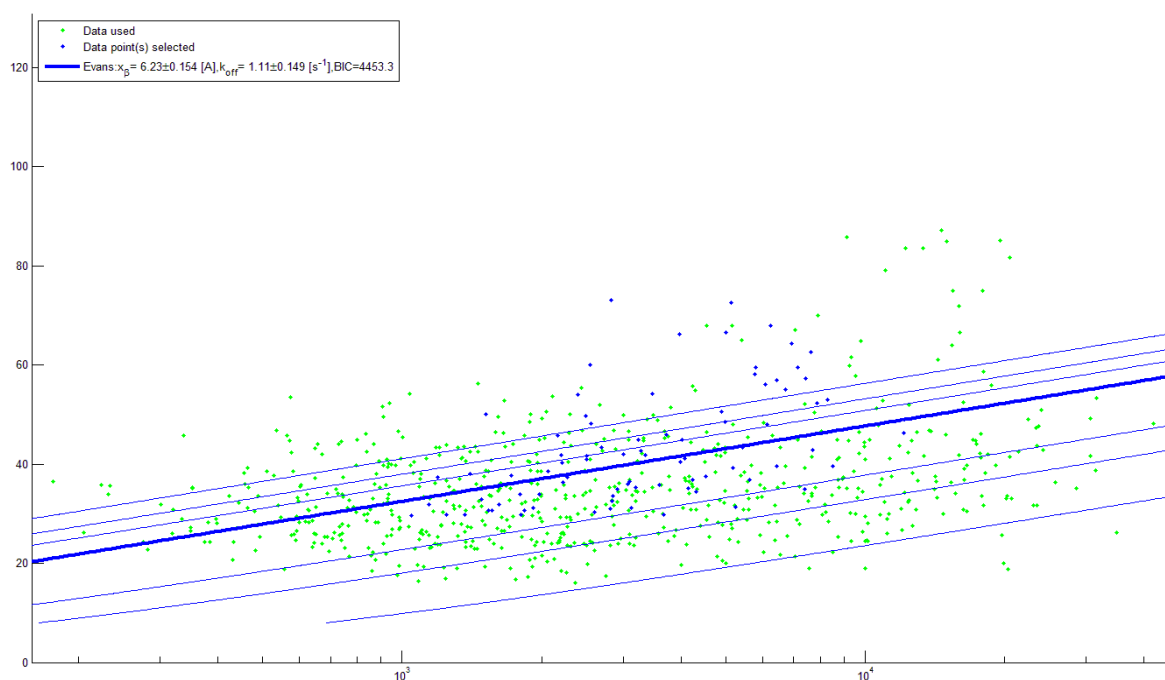


Figure 28: Example of a MATLAB LRD result file using the Evans model. MATLAB fits the values obtained from the LRDs into a blot and uses it to calculate X_β and k_{off} . The thick blue line indicates the most probable fit. At the upper left corner, X_β (in this case 6.23) and k_{off} (1.11) can be seen. Those two values are the most important ones when describing biological interactions. The example shows the fit of vWF A1 domain.

In total, the results of six LRDs as shown in figure 28 are presented (one for each vWF construct), so also six different X_β and k_{off} values. Those values allow interpreting the different constructs according to their binding behavior and bond formation properties with DNA. All values can be read at table 1.

X_β defines the energy barrier of the bond, it can also be seen as an estimate of the bond length in ångström (Å). The bond lifetime was calculated from k_{off} (in s^{-1}). The lower k_{off} , the more stable is the bond. Therefore, k_{off} was reverted into τ , for better graphical illustration of the bond stability. The higher τ , the higher is the lifetime of the bond (see figures 29, 30 and 31).

The following simple formula was used to calculate τ :

$$\tau = \frac{1}{k_{off}}$$

Table 1: X_β , k_{off} and τ values for all six vWF systems, including error values. For graphic illustration of the values see figures 29, 30 and 31. All values are spring constant adjusted.

vWF system	X_β (Å)	error X_β	k_{off} (1/s)	error k_{off}	τ (s)	error τ
monomer	4.10	0.06	1.27	0.11	0.79	0.07
dimer	3.09	0.04	0.99	0.07	1.01	0.07
A1	6.23	0.15	1.11	0.15	0.90	0.12
A1A2A3 wt	1.85	0.03	0.25	0.03	4.03	0.47
A1A2A3 A1461D	2.52	0.03	0.39	0.03	2.55	0.21
A1A2A3 R1308C	3.96	0.06	0.58	0.05	1.73	0.15

Figure 29 shows the dissociation constant values. As already stated, k_{off} is normally converted to τ , for better graphical illustration of the bond lifetime (figure 30).

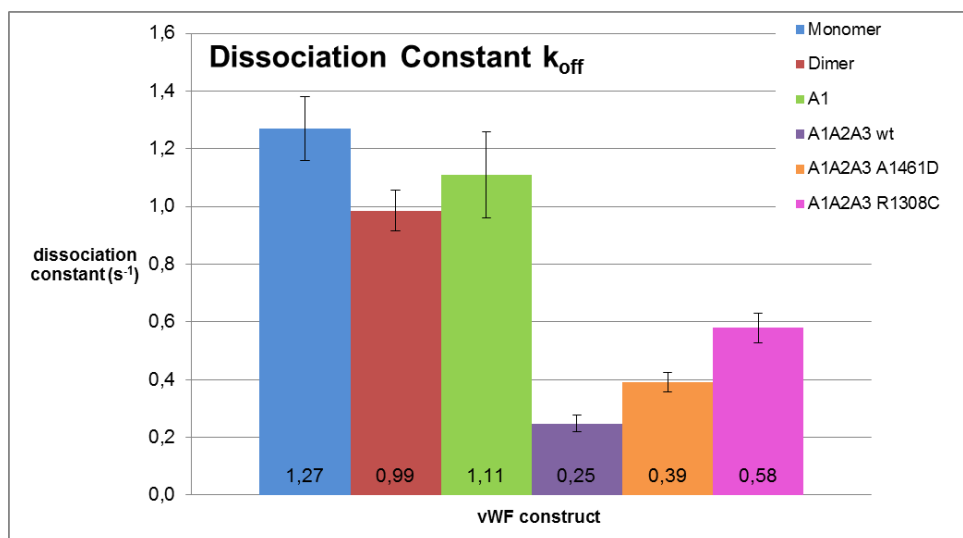


Figure 29: Graphical illustration of the dissociation constant values. In this case, a lower value means that the bond has a higher lifetime, as a high value means fast dissociation. To make the results more clear, figure 30 shows the inverse (τ), so that a higher value really indicates a longer lifetime.

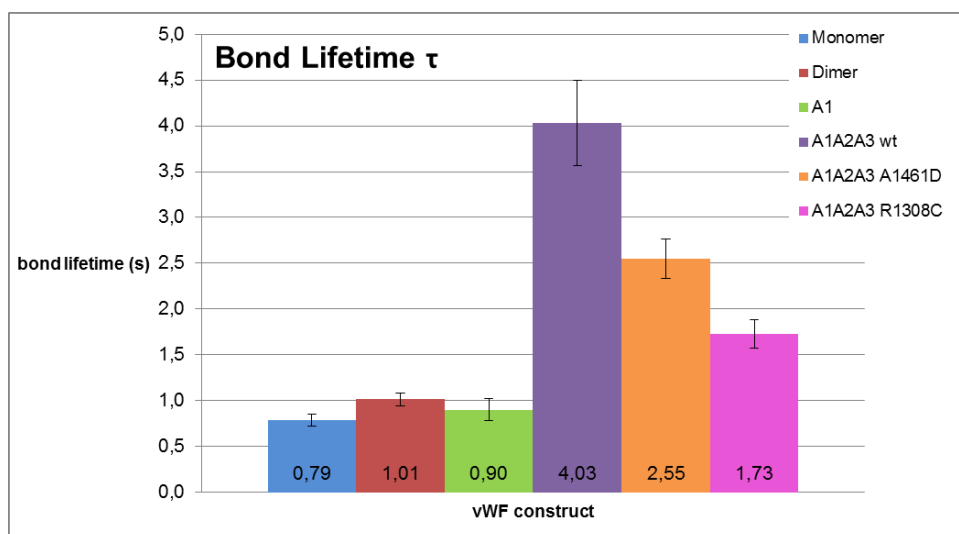


Figure 30: Graph illustrating bond lifetimes (τ) of the vWF constructs. A higher value means that the bond has a higher lifetime. A1A2A3 wt seems to be the most stable bond.

Bond lifetimes are a very interesting factor, as they directly correlate to the stability of the bond. Figure 29 shows the kinetic off values of the interactions while in figure 30 the corresponding lifetimes are shown as τ . Figure 30 illustrates that A1A2A3 wt had the highest lifetime, with a value of approximately 4 seconds. The next highest value is already more than one second less, which was the A1461D GOF-mutation of A1A2A3. The next one in this sequence is the other GOF mutation R1308C with 1.73 seconds. The three smallest values were obtained by measuring the monomer, the dimer and the A1 domain on DNA. This does not really correlate with the binding probability values. The GOF-mutations bound to DNA more often, but the interactions were less stable than the interactions between A1A2A3 wt and DNA. Further, the monomer and the dimer showed quite high binding probabilities but the interactions were not really stable, as indicated in figure 30.

Besides the bond lifetimes, also the energy barrier values (X_β) were determined. The values can be seen in figure 31. The vWF domain A1 reached the highest value with 6.23 Å, followed by the monomer and A1A2A3 GOF-mutation R1308C. Next lowest values were reached by the monomer and the other A1A2A3 GOF-mutation A1461D. With 1.85 Å the A1A2A3 wt construct showed the lowest energy barrier value.

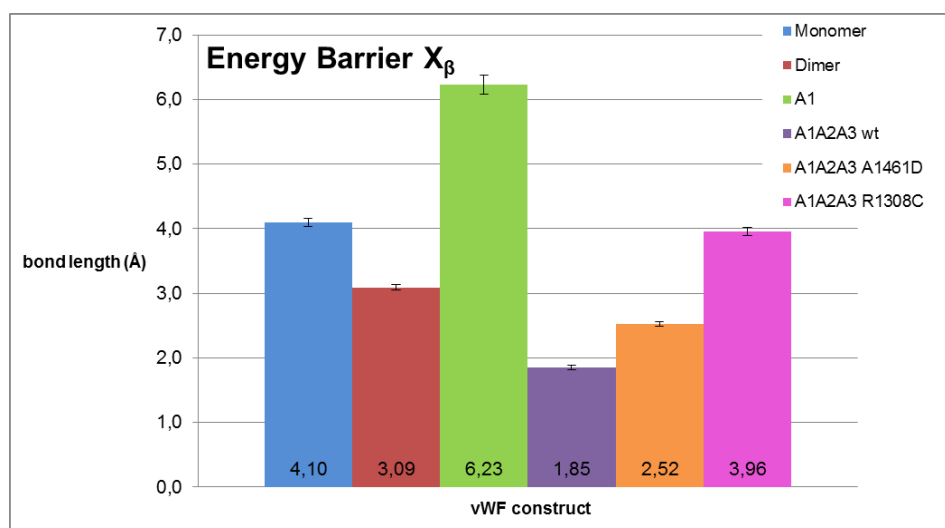


Figure 31: Graph showing the approximated bond lengths / energy barriers (X_β). A higher energy barrier value means that the bond is more stable. However, those values are quite hard to interpret.

To combine all the six LRD fits (as in figure 28) in one graph, the software OriginPro 9 was used. A plot showing the loading rate in relation to the force was generated and is shown in figure 32.

To calculate the most probable force (F^*) for each loading rate, the following formula was used ⁸:

$$F^* = \frac{k_B T}{x_\beta} \ln \left(\frac{x_\beta r}{k_B T * k_{off}} \right)$$

x_β (in nanometers) and k_{off} (in s^{-1}) were used for each LRD. $k_B T$ is the Boltzmann constant in dependency of the temperature (which is 4.11 J/K).

All forces were directly calculated using the formula in OriginPro and are illustrated in the graph below (figure 32). The loading rate ranges (x-axis) are all shown in the range of 100 – 100000 pN/s.

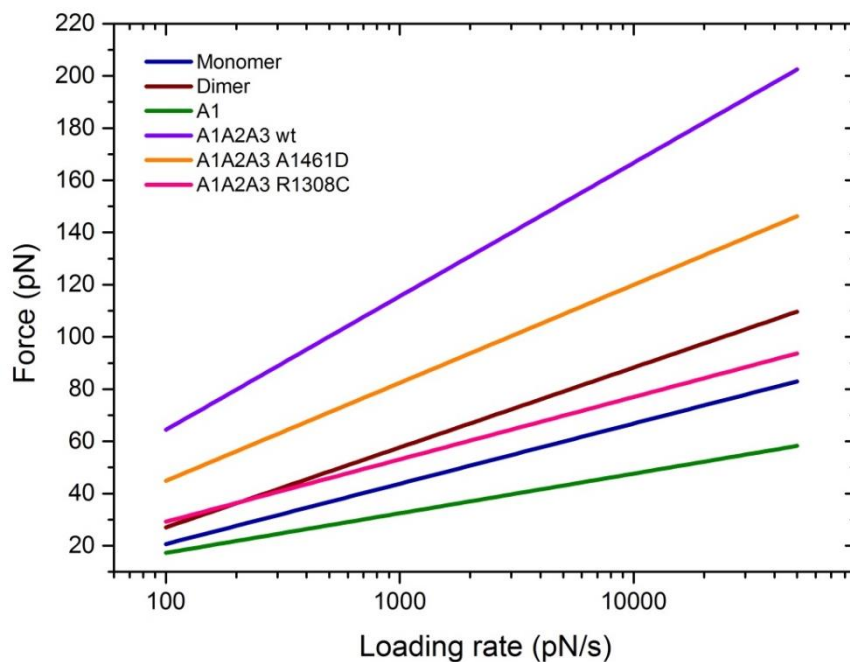


Figure 32: LRD blot showing all six vWF systems. The forces which were necessary to dissociate the receptor from its ligand in pN in relation to the loading rate can be read out of the graph. A1 shows the lowest and A1A2A3 wt the highest values in terms of acting forces.

The y-axis depicts the distinct forces of each vWF construct. It is illustrated that the DNA interaction with the vWF A1 domain showed the smallest forces, followed by the monomer. A1A2A3 wt clearly showed the strongest acting forces, followed by its

GOF-mutation A1461D. The dimer and A1A2A3 + R1308C lie in between. The values obtained directly correlate with the τ values, as is shown in figure 33.

To block all specific interactions between vWF and λ -DNA, heparin was used. As Grässle et al. state, heparin is highly negatively charged, and therefore competes with DNA for the same binding site in the positively charged A1 binding domain.¹

In this thesis, blocking experiments for all constructs showed a reduction in binding probabilities, except for the monomer. All the bindings of the other constructs were at least reduced by 50 %. However, the dimer was the one which showed the least efficient blocking: only 50.6 % blocking was achieved. As monomer and dimer showed such a weak blocking in terms of tip block experiments with heparin, it was concluded that they have too many binding sites. Compared to A1A2A3 constructs and only A1, they consist of many more domains, thus having more sites which may contribute to DNA interactions, which may not be able to be blocked, due to electrostatic interactions. Blocking was carried out in varying concentrations of heparin for varying amounts of time, starting from 50 U/mL for 2 hours.

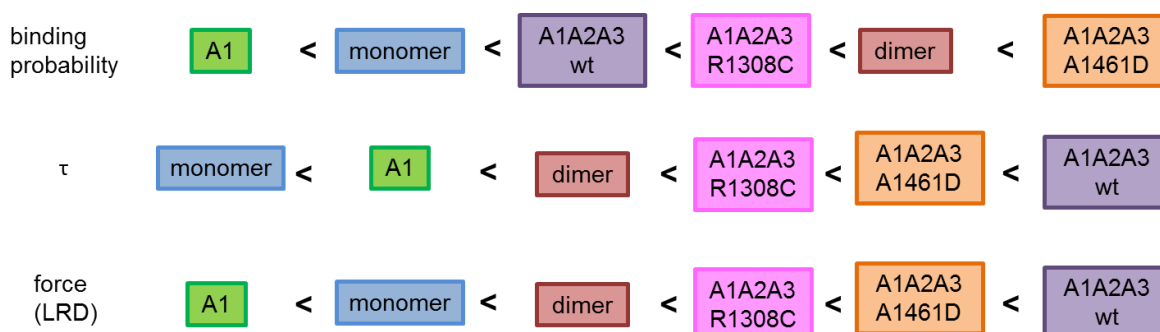


Figure 33: Comparison of all values. It can be seen that A1 and monomer have the lowest values in all cases. The graph makes clear that A1A2A3 wt for example has a quite low binding probability, but once it is bound, it is very stable – shown by the high lifetime.

Figure 33 shows an overall graphical view of the values obtained in force spectroscopic measurements of vWF and DNA.

Binding probabilities, bond lifetimes and acting forces are shown, starting from the lowest value on the left.

The graph points out again that the domain A1 and the monomer showed the lowest binding probabilities and also reached the lowest values in term of bond lifetime (τ). Also the forces necessary to dissociate the complexes (see figure 32) were the lowest for these constructs.

A1A2A3 wt has the next lowest binding probability with 31.8 % using no ristocetin. However, the lifetime and the force were the highest for this construct. This means that the complex between A1A2A3 wt was really stable and high forces were necessary to dissociate it again.

4. DISCUSSION

This thesis was grouped into two main parts: finding appropriate imaging conditions for λ -DNA strands and identifying interactions between wt and mutant VWF constructs and λ -DNA.

Imaging strands of DNA was quite a challenge. Eight different protocols were tested to finally achieve a good image of λ -DNA strands bound to mica.

In the first approach a DNA purification quick kit was used, to get pure DNA without EDTA. Different concentrations were tried; however it was impossible to find DNA strands on the obtained images. The mica looked either as if it was fully packed with DNA or aggregates showed up. For protocol 2, DNA was EtOH precipitated for purification. Although the obtained DNA seemed to be really pure and the concentration was also fine, only bare mica was observed during AFM imaging. So the conclusion was that either the DNA strands were destroyed during precipitation and dissolving or DNA was so unevenly distributed on the surface that it was just not found with AFM imaging. Lowering the concentration of the EtOH purified DNA and taking a different buffer (protocol 3) had the effect that the mica was not looking empty anymore. However, also no strands were observed but many small aggregates. The aggregates also could have been TRIS / Ni complexes.

As the purification methods brought no satisfying results, it was decided to use the unpurified λ -DNA for all following experiments. For protocols 4 and 5, DNA concentration and buffer conditions were different, but again both just showed the same result: aggregates. The aggregates seemed bigger in protocol 5, although the concentration was lower. For protocols 6 and 7 both 0.2 $\mu\text{g/mL}$ DNA was used. The difference to the previous protocols was that the sample was dried with nitrogen. It was thought that the DNA would maybe adhere better to the sample surface if it was dried before and buffer is added for imaging later on. However, in both cases there was no DNA observable.

In the end, when using protocol 8, it was possible to observe DNA strands. Here also the sample was dried with nitrogen. Next to the strands, again aggregates were

observable. This led to the conclusion that some part of the DNA maybe formed aggregates while some percentage stayed in a linear conformation. Maybe the DNA distributed really unevenly on the mica, so that the correct position for imaging had to be found in order to get a satisfying image. However, with protocol 8 it was finally possible to get an image of the strands. The strands were determined to have a diameter of about 1 nm, which is in good correlation with literature.

It was not quite clear if sample preparation had the biggest influence or if it was due to the imaging procedure that imaging proper DNA strands was that hard to achieve. It was probably a combination of both. As DNA is sensitive and observing of intact strands was the focus, it was important to be very cautious. Moreover, the purity of all components was important to get a good image. The aggregates which were always seen may be due to some impurity or complexes formed by some chemicals. But as aggregates were also seen next to intact strands, it seemed that they were no big problem. Moreover, during imaging there were many parameters which had to be adjusted in a correct way to get a good picture. Also the magnetically coated tip had to be of good quality, which was not always the case.

It is known that AFM imaging is never an easy task. Moreover, DNA seems to be a quite difficult sample, so that in the end everything has to be perfectly adjusted and good positions on the sample have to be chosen to get a nice image. So in the end it can be concluded that some luck is also necessary to get an image of DNA strands.

The other and most important section of this thesis were the force spectroscopy measurements of vWF and DNA interactions.

As a first part, a suitable surface chemistry had to be found to bind DNA to the mica, but in a way that it is still able to interact with a binding partner. DNA as prepared for imaging was not dense enough for single molecule force spectroscopy, thus another DNA sample chemistry for force measurements had to be developed. Therefore it was chosen to photo-biotinylate it, so that it can be bound via streptavidin and lysozyme-biotin to cleaved mica. To verify that DNA was biotinylated, an EMSA approach was being performed. A chemiluminescent signal indicated successful

biotinylation of the DNA. The signal of digested DNA was better than that of the sonicated DNA, apparently sonication destroyed too much. Nevertheless, there was an intense signal, which is definitely verifying biotinylation of the λ -DNA. Since a strong interaction between avidin and biotin is known, it was sure that the DNA would bind to the sample surface.

Concerning binding vWF constructs to cantilever tips a suitable chemistry for His₆-tagged proteins was already well established at the institute of Biophysics in Linz. The functionalization also involved attachment of a linker molecule, which enables ligands to orient freely. This should facilitate bond formation. Any badly functionalized tips were recognized during measurements because of a different behavior compared to other tips (for example completely different binding probability values) and were excluded.

Pre-testing with and without ristocetin was conducted to elucidate the effect of ristocetin on the different constructs (monomer, dimer, A1, A1A2A3 and two A1A2A3 GOF-mutations). Ristocetin is thought to enable binding of vWF to DNA without the normally necessary shear stress by exposing its A1 domain to allow interaction.¹ The binding probabilities of all constructs were determined first without ristocetin, then with buffer + 1.5 mg/mL ristocetin, and finally again without ristocetin. The final step was necessary to clarify if the effect of ristocetin is reversible. The experiments showed that the A1 domain is the only domain dependent on ristocetin (see figure 26). The binding probability rose from 6.5 to 16.4 %; in contrast to all other constructs where ristocetin seemed to disturb interactions. It was concluded that A1 alone is dependent on the positive effect of ristocetin. The rather big monomers and dimers were able to interact solely in buffer to a high extent. For the GOF-mutations it was already expected beforehand that the interaction would be good.

Quite often when doing measurements with ristocetin, problems occurred. Force distance cycles sometimes looked as if something was sticking to the tip. The only way to get rid of this was to stop the cycles, retract the tip and approach again at a different position. However this was a recurrent phenomenon. After literature search it was concluded that this may be due to the ristocetin forming dimers. According to Hoylaerts et al, a concentration exceeding 0.4 mg/mL ristocetin led to high amounts of unspecific bindings between vWF and GPIIb, which might have also been the problem here.⁴⁷

A LRD was done with each of the six constructs. However ristocetin was only used for A1, as the others seemed not to be dependent on it. Nine different velocities were measured with each tip (which means approximately 9000 force distance cycles per tip) and analyzed in MATLAB. The two most important parameters X_β and k_{off} were calculated by the software and were later used for graphical illustration of the acting forces (see figure 32). X_β and k_{off} (which was converted into τ) did not really correlate with each other, however as they are two independent parameters there is no need for them to correlate. Also comparing the binding probabilities to the lifetimes showed a high amount of bindings does not necessarily mean that the lifetimes are high. However, in case of domain A1 and monomer there is a good correlation, as the binding probability of those two constructs was the lowest, and also the lifetimes were the lowest. Moreover, they also showed the lowest force values.

Also blocking with heparin was not that easy to achieve, this might also be due to unspecific interactions and several binding sites between DNA and vWF, based on electrostatic phenomena. Maybe finding another blocking agent would be a benefit here.

Altogether, one can state that single molecule force spectroscopy is a really sophisticated method. Forces in the pico Newton range can be measured, which shows how sensitive the whole system is. As it is that sensitive, there are of course many parameters that can influence the experiments. This is the same for AFM imaging methods. Therefore sample preparation needs to ensure proper quality to obtain good images.

5. OUTLOOK

There are many things which still could be done to completely reveal the vWF – DNA interaction.

Single molecule force spectroscopy is a very sophisticated and sensitive method. So of course more statistics of the measurements could refine the outcome of this thesis and would be a good thing to do.

Also varying concentrations of ristocetin would be another factor which could influence the outcome of binding probability measurements. Maybe measuring the same vWF construct on DNA with varying concentrations of ristocetin would be a nice experiment.

Moreover, as blocking was not as successful as hoped in some cases, other blocking methods, like antibodies targeting vWF domain A1, could be found and tested for their potency. As there are also many vWF mutations known (besides those two which were used in this thesis) it could also be interesting to test their reaction to ristocetin and their force profile when interacting with DNA.

All in all there are many possibilities to continue and thoroughly examine the results achieved in this Master's thesis.

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7.2. List of Tables

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7.3. List of Abbreviations

A	adenine
ADAMTS13	a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13
AFM	atomic force microscopy
APTES	(3-aminopropyl)triethoxysilane
AvWS	acquired von Willebrand syndrome
C	cytosine
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Factor VIII	factor eight
G	guanine
GOF	gain of function
His6	hexahistidine
hnRNA	heterogenous nuclear RNA
HOPG	highly oriented pyrolytic graphite
kDa	kilo Dalton
k_{off}	kinetic off rate
LPS	lipopolysaccharide
LRD	loading rate dependence

MAC	magnetic alternating current
MQ	MilliQ®
mRNA	messenger RNA
NET	neutrophil extracellular trap
NHS	N-Hydroxysuccinimid
NO	nitric oxide
NTA	nitrilotriacetic acid
OH	hydroxyl group
PAD4	peptidyl arginine deaminase 4
PBS	phosphate buffered saline
PEG	polyethyleneglycol
PGI ₂	prostacyclin
RNA	ribonucleic acid
ROS	reactive oxygen species
SMFS	single molecular force spectroscopy
T	thymine
TBS	TRIS buffered saline
TE	TRIS EDTA
TEA	triethylamine
TNF	tumor necrosis factor
TRIS	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
TTP	thrombocytopenic purpura
U	uracil
vWD	von Willebrand disease
vWF	von Willebrand factor
vWFCP	von Willebrand factor cleaving protease
wt	wild type
X _β	energy barrier

7.4. Abstract

Von Willebrand factor (vWF) is a protein in blood that plays an essential role in our primary hemostasis. By forming a mesh and anchoring activated blood platelets to injured vessel walls, continuous bleeding can be prevented in a fast way, as vWF contains binding sites for e.g. platelets and collagen.

Recently, also binding of vWF to DNA was revealed. DNA is the central molecule in biology, as it is the carrier of the genetic information. In the bloodstream, DNA may be present as neutrophil extracellular traps. Those traps are produced by immune cells to trap and kill invading microorganisms, like viruses and bacteria. The interaction between vWF and DNA suggests that vWF, next to its huge importance in hemostasis, may also play a role in inflammatory responses.

vWF domain A1 was reported to be the binding domain for DNA within vWF. Further, vWF binds to DNA in a ristocetin dependent manner.

Ristocetin is an antibiotic which is thought to mimic shear flow conditions normally present in the blood stream, and therefore vWF-DNA interaction is possible without the presence of shear flow. Besides the vWF wildtype constructs, also two mutations (R1308C and A1461D) in the vWF A1 domain were investigated.

To investigate the interactions between vWF and DNA, force spectroscopy experiments were carried out with six different vWF constructs. Ristocetin was used to test the binding behaviors of the different vWF constructs to DNA. Here it was shown that vWF A1 domain is the only construct which is dependent on ristocetin for DNA binding. All other constructs were able to interact without its presence. The presence of ristocetin might even disturb the interactions of the other domains and DNA. Loading rate dependence measurements were performed for each of the six constructs to reveal the dynamics behind the interactions. In this way, the energy barrier X_β and the dissociation constant k_{off} (and subsequently the bond life time τ), which are very important parameters for describing biological interactions, could be determined.

Bond lifetimes were for example compared to binding probabilities and it turned out that a high binding probability does not necessarily correlate with a long lifetime.

To characterize the λ -DNA used in this study, AFM imaging was another important part of this thesis. As DNA imaging turned out to be quite a challenge, different incubation conditions were tested to find an appropriate protocol for DNA imaging. In the end, we succeeded in imaging λ -DNA strands with a high resolution.

7.5. Zusammenfassung

Der von Willebrand Faktor (vWF) ist ein Protein im Blut welches eine lebenswichtige Rolle in unserem primären Blutgerinnungssystem spielt. Um ungewollten Blutfluss stoppen zu können, bildet der vWF ein dichtes Netzwerk mit seinen Bindungspartnern (zB. Kollagen und Blutplättchen) und verankert dieses im verletzten Blutgefäß.

Vor kurzem wurde herausgefunden, dass der vWF auch mit DNA interagieren kann. DNA ist bekannt als wichtigstes Molekül in der Biologie, da sie die Trägerin der Erbinformation ist. Im Blutstrom kann DNA als sogenannte „neutrophil extracellular traps“, also von Immunzellen fabrizierte Fallen aus DNA, vorliegen. Diese „traps“ haben die Aufgabe eindringende Pathogene wie Viren oder Bakterien zu erfassen. Die Interaktion von vWF und DNA verdeutlicht dass der vWF anscheinend auch eine Rolle in Entzündungsprozessen spielt.

Die Bindungsstelle für DNA liegt anscheinend in der A1 Domäne des vWF. Außerdem bindet vWF DNA abhängig von Ristocetin. Ristocetin ist ein Antibiotikum welches die Interaktion der beiden Bindungspartner ohne Scherstress ermöglichen soll. Neben vWF Wildtyp-Konstrukten wurden auch zwei Mutationen (R1308C und A1461D) der A1 Domäne untersucht.

Um den Bindungseigenschaften zwischen DNA und vWF auf den Grund zu gehen, wurden Kraftspektroskopie-Experimente mit sechs verschiedenen vWF Konstrukten durchgeführt. Die Evaluierung der Bindungsanzahlen der verschiedenen Konstrukte wurde unter anderem im Beisein von Ristocetin durchgeführt. Es stellte sich heraus, dass A1 die einzige Domäne war welche von Ristocetin profitierte, bei allen anderen schien es eher einen negativen Effekt zu haben. Auch „loading rate dependences“ wurden für alle sechs Konstrukte gemessen, um die Bindungsdynamik genauer untersuchen zu können. So wurden die Energiebarrieren X_β und die Dissoziationskonstanten k_{off} (und somit in weiterer Folge auch die Lebenszeiten τ) berechnet. Dies sind die wichtigsten Parameter um biologische Interaktionen beschreiben zu können. Bindungsanzahlen und Lebenszeiten konnten zum Beispiel verglichen werden, was zeigte, dass diese Werte nicht unbedingt zusammenhängen müssen.

Zusätzlich zu den Kraftspektroskopie-Experimenten war auch Imaging der verwendeten λ -DNA ein Teil dieser Masterarbeit. Dies stellte sich leider als nicht ganz so einfach dar, sodass verschiedene Inkubationskonditionen getestet werden mussten um ein passendes Protokoll zu finden.

Letztendlich wurden aber passende Konditionen gefunden, welche es ermöglichten, DNA Stränge mit hoher Auflösung zu imagen.

CURRICULUM VITAE

Verena Pfarrhofer



Personal Information

Date of Birth	01. April 1991
Place of Birth	Linz
Nationality	Austrian

Education

2013 – 2015	University of Vienna Master's degree program: Molecular Biology Chosen focus on Molecular Medicine
2010 – 2013	University of Applied Sciences IMC Krems Bachelor's degree program: Medical and Pharmaceutical Biotechnology (in English) Passed with distinction
2009 – 2010	University of Applied Sciences in Wels Bio- and Environmental Technology Cancelled before completion
2001 – 2009	Secondary school BRG Hamerling, Linz Focus on sciences (biology) Passed with distinction

● ● Working Experience

- | | |
|-----------------------|---|
| Nov. 2014 – Oct. 2015 | Master's internship at Johannes Kepler University in Linz
Institute of Applied Experimental Biophysics

Master's thesis: Investigating interactions between von Willebrand factor and DNA using single molecule force spectroscopy |
| March & April 2014 | Internship at Max F. Perutz Laboratories in Vienna, Group of Alexander Dammermann

Gene Editing in <i>C. elegans</i> using CRISPR/Cas9 Technique |
| July 2012 – Feb. 2013 | Bachelor's internship at Upper Austrian Red Cross in Linz
Department of Tissue Engineering

Bachelor's thesis: Isolation and Characterization of Mesenchymal Stem Cells from Human Umbilical Cord and Human Umbilical Cord Blood |

● ● Scholarships

- Merit Scholarship University of Vienna
- Merit Scholarship University of Applied Sciences IMC Krems

● ● Conferences

- | | |
|----------------|---|
| September 2015 | 1 st international SHENC Symposium: Function of von Willebrand factor in primary and secondary hemostasis, Hamburg |
| January 2015 | Winter School and Winter Workshop: Advances in Single Molecule Research in Biology and Nanoscience, Linz |