A STUDY OF BACTERIAL ADHESION ON A SINGLE–CELL LEVEL BY MEANS OF FORCE MEASURING OPTICAL TWEEZERS AND SIMULATIONS

by

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THE PETITE PARADOX

behold a single brush stroke to sincerely appreciate a painting

perceive the motif to truly acknowledge the brush stroke
The intriguing world of microbiology is nowadays accessible for detailed exploration at a single–molecular level. Optical tweezers are a novel instrument that allows for non–invasive manipulation of single cells by the sole use of laser light and operates on the nano– and micrometer scale which corresponds to the same length scale as living cells. Moreover, forces within the field of microbiology are typically in the picoNewton range which is in accordance with the capability of force measuring optical tweezers systems. Both these conformabilities imply that force measuring optical tweezers is suitable for studies of single living cells.

This thesis focuses on the mechanisms of bacterial attachments to host cells which constitute the first step in bacterial infection processes. Bacteria bind specifically to host receptors by means of adhesins that are expressed either directly on the bacterial membrane or on micrometer–long adhesion organelles that are called pili. The properties of single adhesin–receptor bonds that mediate adherence of the bacterium Helicobacter pylori are first examined at various acidities. Further on, biomechanical properties of P pili expressed by Escherichia coli are presented to which computer simulations, that capture the complex kinetics of the pili structure and precisely replicate measured data, are applied. Simulations are found to be a powerful tool for investigations of adhesive attributes of binding systems and are utilized in the analyses of the specific binding properties of P pili on a single–pilus level. However, bacterial binding systems generally involve a multitude of adhesin–receptor bonds. To explore bacterial attachments, the knowledge from single–pilus studies is brought into a full multipili attachment scenario which is analyzed by means of theoretical treatments and simulations. The results are remarkable in several aspects. Not only is it found that the intrinsic
properties of P pili are composed in an optimal combination to promote strong multipili bindings. The properties of the pili structure itself are also found to be optimized with respect to its in vivo environment. Indeed, the true meaning of the attributes derived at a single–pilus level cannot be unraveled until a multipili–binding system is considered. Whereas detailed studies are presented for the helix–like P pili expressed by Gram–negative Escherichia coli, conceptual studies are presented for the open coil–like T4 pili expressed by Gram–positive Streptococcus pneumoniae. The structural and adhesive properties of these two types of pili differ considerably. These dissimilarities have far–reaching consequences on the adhesion possibilities at both single–pilus and multipili levels which are discussed qualitatively.

Moreover, error analyses of conventional data processing methods in dynamic force spectroscopy as well as development of novel analysis methods are presented. These findings provide better understanding of how to perform refined force measurements on single adhesion organelles as well as how to improve the analyses of measurement data to obtain accurate parameter values of biomechanical entities.

In conclusion, this thesis comprises a study of bacterial adhesion organelles and the way they cooperate to establish efficient attachment systems that can successfully withstand strong external forces that acts upon bacteria. Such systems can resist, for instance, rinsing effects and thereby allow bacteria to colonize their host. By understanding the complexity, and thereby possible weaknesses, of bacterial attachments, new targets for combating bacterial infections can be identified.
SAMMANFATTNING

Den fascinerande mikrobiologiska världen är numera tillgänglig för detaljerade undersökningar på en molekylär nivå. Optisk pincett är ett modernt instrument som möjliggör beröringsfri manipulation av enskilda celler genom att enbart använda laserljus och har ett användningsområde på nano- och mikrometerskalan vilket motsvarar samma längdskala som för levande celler. Typiska krafter inom mikrobiologin ligger inom picoNewton-området vilket också lämpar sig för kraftmätande optiska pincetter. Dessa båda överensstämmanden medför att kraftmätande optiska pincetter är användbara för studier på levande celler.

genom teoretiska analyser samt med hjälp av simuleringsar vilket leder till

Utöver analyserna av specifika adhesionsorganeller presenteras felanalyser av konventionella databehandlingsmetoder inom dynamisk kraftspektroskopi samt utveckling av nya metoder. Resultaten ger bättre förståelse för hur kraftmätningar bör utföras på enskilda adhesionsorganeller samt för hur mätdata bör analyseras för att erhålla precisa parametervärden för biomekaniska störheter.

Sammanfattningsvis understryks att denna avhandling innefattar i första hand en studie av adhesionsorganeller och hur dessa samverkar för att etablera bindningssystem som effektivt motstår starka externa krafter. Sådana system kan stå emot kraftiga flöden och därmed utgöra en grund för bakteriell kolonisering. Genom att förstå de komplexa bakteriella bindningarna och därigenom deras eventuella svagheter kan nya angreppssätt identifieras i kampen mot bakteriella infektioner.
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*Journal of Biomedical Optics.* 10(4):044024, 2004
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1. **INTRODUCTION TO THE FIELD OF BIOPHYSICS**

Biophysics is a broad scientific area where the viewpoints and methods from physics are applied on biological fields and biological systems. The interdisciplinary aspect generates opportunities to expand the knowledge of already existing research fields and to approach new challenges.

1.1. **LIFE SCIENCE**

Life science is the study of living organisms and their interactions which on the micrometer scale transits to the field of cell biology. Many interesting queries that involve single molecular interactions are present on a cellular level. This includes the structure, function and properties of, for instance, DNA, microtubules, flagella and adhesion organelles of various types of bacteria. As such, theories from the field of physics are useful to describe observations and predict the outcome of similar processes found in biological systems.

Pathogenic bacteria have been confronted successfully by heavy use of antibiotics during the last century. However, rapid mutations of bacteria have been observed lately which constitutes a serious problem since it leads to bacterial resistance against traditional antibiotics, and therefore necessitates the development of new treatment methods. Understanding of the fundamental mechanisms of bacterial adhesion and colonization is essential to realize new methods of fighting pathogenic bacteria and bacterial infections. One possible target is the adhesive process, which, if prohibited, could result in reduced or annihilated bacterial colonization and invasion. Adhesion takes place on a single–molecule level which has been an
inaccessible research area until recently due to lack of appropriate measurement techniques.

1.2. PROBING METHODS

Instrumentations that allow the experimentalist to not only observe, but also manipulate single molecules with high precision have been developed during the last decades. *Atomic force microscopy* (AFM) is a common tool for scanning surface structures with resolution on the nanometre scale (1) which allows for identification of single atoms. In addition, AFM can be applied for other purposes, for instance force measurements at a single–molecule level. However, the AFM–technique is limited to measurements at relatively high forces as the maximum resolution normally is in the order of ~10 pN. The innovative method of *biomembrane force probe* (BFP) is applicable for force measurements over a wide range of forces, from sub-picoNewton to ~10^3 pN (2, 3). In contrast to AFM and BFP, a *photonic force microscope* (PFM) is capable of scanning in three dimensions, with a resolution of ~1 nm (4). The basic concept of the latter is closely related to that of *optical tweezers* (OT) which is the instrument used in this thesis. Detailed description thereof is given in chapter 2.

1.3. THE SCOPE, TARGET AND CONTRIBUTIONS OF THIS THESIS

The fundamental methodology for force measurements and properties of bacterial adhesion organelles are investigated in detail in this thesis. First, an analysis of isolated adhesin–receptor interactions is laid out. The specific bond of interest is the *Helicobacter pylori* adhesive bond BabA–Lewis b. Second, the theory and biomechanical behaviour of adhesion organelles, with focus on helix–like pili, is presented together with a quantitative investigation of the *Escherichia coli* P pili. Third, the properties of the specific PapG–galabiose binding, associated with specific adhesion of the P pili, are examined using novel analyze methods in combination with detailed knowledge of the P pili structure. Finally, the knowledge of the properties and behaviour of single adhesion organelles is applied in a multipili binding scenario. The *in vivo* situation with multipili attachments exposed to external forces is modeled and the amazing effects of cooperativity in a multipili–binding system are scrutinized.

My main contributions to the field of biophysics in this thesis are; characterization and quantitative data of the specific BabA–Lewis b and the
PapG–galabiose bindings, a new method of performing dynamic force spectroscopy under non–linear loading conditions as well as detailed analysis of how properties of single adhesion organelles strongly affects the binding lifetime of a multipili system.
2. **OPTICAL TWEEZERS**

An optical tweezers system is a tool for measurements and manipulation of objects on a microscopic level. This chapter is dedicated to the basic theory of optical trapping, description of the instrumentation required to realize an optical trap and the force measurement procedure that has been implemented.

### 2.1. Optical Trapping

The pioneering fundamental work of optical tweezers was presented by Ashkin et al. in the seventies and early eighties (5-7). Initially, the radiation pressure of a laser beam was used to balance gravity and thereby to levitate particles. The first optical trap was compounded by two laser beams propagating in opposite directions. A single beam trap was first realized in the mid eighties (8). Practical implementations of the technique by optical manipulations of living objects, such as bacterial cells, were soon presented (9).
Figure 2.1. Dielectric micrometer sized objects can be trapped by strongly focused light when their refractive index is higher than that of the surrounding medium.

The basic concept of an optical tweezers system is relatively uncomplicated. In its most elementary form it can be compounded of a microscope objective with a high numerical aperture by which small dielectric objects can be trapped by a strongly focused laser beam. The theory that describes optical trapping can be divided into two categories— the theory for the Rayleigh regime and the ray optics regime, respectively. When the wavelength of the light is significantly longer than the dimension of the trapped object, the theory of Rayleigh regime can be applied. In contrast, the theory of the ray optics regime is valid when the wavelength of the light is distinctly shorter than the width of the trapped object. The object to be trapped is assumed to be, and is consistently so in this work, a spherical dielectric body. The beads that have been used here have a diameter of ~3 µm which is in the same order of magnitude as the wavelength of the trap laser beam which is ~1 µm and thereby in the low infrared region. This implies that the present case does not fall into any of the two extreme regimes, and the beads are therefore called medium–sized particles in this context. The theory for medium–sized particles is very complicated and even though there has been progress in the field (10-12), it is still not completely developed. However, the phenomenological aspects are well described by both the Rayleigh and the ray optics theory. The latter is the most intuitive and is therefore used in discussions and illustrations in this work.
2.1.1. Rayleigh Theory

The Rayleigh theory is suitable when the size of the object is significantly smaller than the wavelength of the trapping beam. The dielectric object is subjected to an electromagnetic field which induces a dipole property for the object. A gradient in the intensity of the electromagnetic field, as in the case with a laser beam of Gaussian intensity distribution, gives rise to a force acting on the dipole, i.e., the dielectric object. The direction of the force is towards the higher intensity when the refractive index of the dipole is higher than that of the surrounding medium. This implies that the trapping beam induces a three-dimensional trap in the focus of the beam. The magnitude of the trap force is proportional to the dipole moment, which depends on the relative refractive indexes, and the gradient of the electromagnetic intensity. This implies that a highly focused beam induces a strong trap on a dielectric object with a refractive index that differs considerably from that of the surrounding medium (13).

2.1.2. Ray Optics Theory

Light can be described as straight rays that are compounded of small energy quanta. Each quantum, or photon, carries a momentum, $p$, which needs to be considered when the ray is redirected. The momentum can be described by the de Broglie’s relation

$$p = \frac{h}{\lambda},$$  \hspace{1cm} (2.1)

where $h$ is Planck’s constant and $\lambda$ is the wavelength. A dielectric object with a refractive index, $n_{\text{bead}}$, that differs from that of the surrounding medium, $n_m$, refracts the ray of light according to Snell’s law,

$$\frac{\sin \theta_m}{\sin \theta_{\text{bead}}} = \frac{n_{\text{bead}}}{n_m},$$  \hspace{1cm} (2.2)

where $\theta_m$ and $\theta_{\text{bead}}$ refer to the angles of incidence and refraction, respectively. The change in direction of the ray implies a proportional change in momentum. As a consequence of the law of preservation of momentum, this means that the object experiences a change in momentum equal to the change in momentum of the ray but in the opposite direction. This transfer of momentum is illustrated on the left hand side in Figure 2.2 for a spherical bead displaced from the optical axis in the lateral plane.
Figure 2.2. The ray description is schematically illustrated for a spherical dielectric bead with a refractive index higher than that of the surrounding medium. The incoming beam has a Gaussian intensity distribution that is illustrated with yellow and red colors corresponding to low and high intensities, respectively. The green arrow depicts the change in momentum the bead will experience as a result of the refraction of the ray $R_2$. On the left hand side, the rays are collimated which results in a lateral restoring force on the slightly displaced bead. The impact of ray $R_1$ is significantly weaker than that of ray $R_2$ as a consequence of the lower intensity further away from the centre. However, this setup does not constitute a stable trap since the bead is constantly pushed in the propagation direction due to the radiation pressure. On the right hand side, a lens is introduced which leads to a strongly focused beam. The bead is here displaced in the axial direction which leads to a restoring force. The change in momentum from the second ray, $R_2$, cancels the lateral contribution from $R_1$ and doubles the restoring effect in the axial direction. This setup results in a three–dimensional single beam optical trap.
A Gaussian beam intensity profile is normally used in optical tweezers which is illustrated by the dissimilar thickness of the rays in the figure. The resulting momentum transferred from the rays to the bead stabilizes the position of the bead if the refractive index of the bead is higher than that of the surrounding medium. The green arrow in the left hand side of Figure 2.2 show the contribution from ray \( R_2 \) which dominates over the contribution from ray \( R_1 \) since the intensity is higher in the centre of the beam. This means that a gradient beam imposes a trapping effect on the bead in the lateral plane. However, the bead is pushed forward in the propagation direction of the beam as a result of two factors. First, the change in momentum discussed above contributes to a pushing force on the bead. Second, not all rays pass through the bead. A fraction of them scatter off the surface of the bead which adds another pushing effect. This pushing force is referred to as the scattering force and must be balanced to create a stable three dimensional trap.

In order to achieve stable trapping, this problem was first solved by applying two laser beams in opposing directions (5). The total restoring force becomes doubled and the pushing effects cancel out. A more convenient and commonly used method for stable trapping is to use a single beam that is strongly focused. The case where the bead is displaced in the axial direction is illustrated on the right hand side of Figure 2.2. Again, the transferred momentum from ray \( R_2 \) is shown as a green arrow in the figure. The corresponding arrow from ray \( R_1 \) cancels the lateral contribution of the momentum and doubles the restoring effect in the axial direction on the bead. The total transferred momentum induces a conservative force in all three spatial directions on the bead.

The position of the bead is directly correlated to the restoring force from the trapping beam. Moreover, this force is linear, for a limited interval of displacements from the centre of the trap, which is a convenient condition for force measurements. The minute force acting on the bead can be monitored by probing the position of the trapped bead. This setup is called a force measuring optical tweezers (FMOT) and is used throughout this thesis. The forces that are measured are typically in the lower picoNewton range which is suitable for investigating many processes on the molecular level in life sciences.
2.2. Instrumentation

The optical tweezers setup used in this work is installed on an air floating optical table that reduces the impact of external noise sources, e.g., vibrations. The instrumentation is kept to a minimum in the lab by placing as much of the equipment as possible outside. In addition, an air filter and an air conditioning system are installed to ensure clean and stable experimental conditions. A picture of the optical tweezers laboratory is provided in Figure 2.3.

![Figure 2.3. The optical tweezers laboratory. The tweezers system is positioned on an air floating optical table inside a noise secured room for optimal experimental conditions.](image)

The experimental setup for an optical tweezers system is not extensive and can be constructed relatively straightforward in its most rudimentary form (14). However, a certain set of optical components are required to allow for accurate and practical control of the trap position and its quality. Figure 2.4 shows a schematic illustration of the experimental setup. The width of the trap laser beam, which is in the millimetre scale in reality, has been strongly exaggerated to visualize the optical refractions as the light passes through the lenses. The set of mirrors and lenses compounds a system that allows for control of the spatial position of the trap in the focus of the microscope objective. The vertical position of the trap in the sample is set by the axial position of the first lens ($L_z$) in the path while the lateral position of the trap
Optical Tweezers

is controlled by the tilting angle of the Gimble Mounted Mirror (GMM). This mirror allows for an angular alteration of the trapping beam without changing the degree of overfilling of the objective (15), i.e., the amount of light passing through the objective is constant. A fixed degree of overfilling is a prerequisite to keep the trap strength constant.

![Image of optical tweezers system setup](image)

Figure 2.4. A schematic illustration of the optical tweezers system setup. The trap laser beam passes through a set of mirrors and lenses before it enters the microscope objective. The components in the optical path allows for control of the three dimensional position of the trap. In particular, the mirror $L_z$ and the Gimble Mounted Mirror (GMM) allow for axial and lateral translation of the trap, respectively. The probe laser beam passes initially through an optical fibre and is directed into the same path as the trap laser beam by passing the Dichroic Mirror (DM) that is coated to reflect a narrow bandwidth of wavelengths around 1064 nm. The probe laser beam is detected by the Position Sensitive Detector (PSD) which is coupled to a computer via a preamplifier and an A/D–converter.

The position of a trapped bead which serves as a force transducer must be determined to allow for a force measuring optical tweezers system. This can be realized by tracing the scattered light from the trapping beam (4, 16,
17) or introducing a probe laser. The latter alternative is implemented in this setup. The probe laser beam is directed into the same optical path as the trapping beam by means of a dichroic mirror (DM) inside the microscope. This mirror reflects only a narrow bandwidth around the wavelength of the trapping beam (1064 nm). Both beams are conveyed through the microscope but the trap laser beam is filtered out of the optical path after the condenser lens by means of a line laser filter. The probe laser beam is focused to a small spot of which the lateral position is detected by the Position Sensitive Detector (PSD) that generates an analogous voltage signal. A computer receives the signal and calculates the corresponding force acting on the trapped bead using the parameters from the calibration procedure, see section 2.3. The software used was a custom made LabVIEW program that allowed for a various number of measurements types. The experimental data are stored in an extensive database that is accessed by a custom made Matlab program, which allows for selective cross–searches and immediate statistical analysis. This entire software system was adjusted to the prerequisites of the actual measurements and allows for easy manipulations and expansions when needed.

In addition, the visual image of the sample is detected by a charge-coupled device (CCD) camera and displayed on a monitor. This setup provides increased visual resolution and it also allows for capturing of image frames as well as recording of movies.

### 2.2.1. Microscope

An inverted microscope (IX–71, Olympus) with a high numerical aperture oil–immersion objective acted as the centre for the optical tweezers. The sample was inserted into a custom made sample holder that was controlled by an x–y–piezo stage with sub–nanometre resolution. The PSD was mounted in the far field of the microscope objective together with the line laser filter. Further modifications have been exerted to allow lasers and illumination for fluorescence to be implemented.

### 2.2.2. Trap Laser

The trap laser beam is engendered by a continuous wave Nd:YVO₄ laser which supply a Gaussian TEM₀₀ mode beam of high qualitative profile and stable intensity (Millenia IR, Spectra Physics). Heating of the sample by absorption of laser light may constitute a problem (18). This issue has been considered in the setup of the instrumentation and the wavelength of the trap laser was chosen to be 1064 nm which ensures low heat absorption of the
biological sample. With the typically power used in experiments (~100 mW in the sample), the heating effect is negligible. To further prevent damage of cells, e.g., a bacterium, the power was reduced by a factor of ~10 whenever a cell was trapped. The trap laser beam is directed through a system of mirrors and lenses into the microscope where its path is merged with the probe laser beam path via a dichroic mirror, see Figure 2.4. The strength of the trap, i.e., the stiffness, is controlled by tuning the power of the trap laser and is derived by a two-step calibration procedure.

2.2.3. **Probe Laser**

In addition to the trap laser beam, a probe laser beam is directed into the sample with the sole purpose of detecting the position of the trapped bead in the sample. The probe laser operates in the visual regime with a wavelength of 633 nm (1137, Uniphase, Manteca) with a power of only a few µW in the sample. It enters the objective as a collimated beam and becomes focused slightly below the centre of the trapped bead.

![Figure 2.5. A schematic drawing that illustrates how the probe laser beam is redirected by the position of the trapped bead.](image)

The position of the trapped bead determines how the probe laser beam is refracted and, in the end, its position on the PSD, see Figure 2.5. This system allows for minute detection of the lateral position of the trapped bead.
2.2.4. **COMPONENT DETAILS**

A detailed description of the main components that have been presented above is given in Table 1.

**Table 1. The model and manufacturers for the main components in the optical tweezers system.**

<table>
<thead>
<tr>
<th>Components</th>
<th>Manufacturer</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air floating optical table</td>
<td>TMC</td>
<td>Custom made</td>
</tr>
<tr>
<td>Gimble Mounted Mirror</td>
<td>Melles Griot</td>
<td>Model no. 07 MCD</td>
</tr>
<tr>
<td>Trap laser Nd:YVO4</td>
<td>Spectra Physics</td>
<td>Millennia IR</td>
</tr>
<tr>
<td>Probe laser HeNe</td>
<td>Manteca</td>
<td>1137, Uniphase</td>
</tr>
<tr>
<td>Microscope</td>
<td>Olympus</td>
<td>IX–71</td>
</tr>
<tr>
<td>Objective</td>
<td>Olympus</td>
<td>UPlanFl 100X/IR, NA 1.30</td>
</tr>
<tr>
<td>Line laser filter</td>
<td>Omega Optical</td>
<td>Hot mirror, 840</td>
</tr>
<tr>
<td>Piezo stage</td>
<td>Physik Instrumente</td>
<td>P517.2CL</td>
</tr>
<tr>
<td>PSD</td>
<td>Sitek</td>
<td>Model no. L20 SU9</td>
</tr>
<tr>
<td>CCD</td>
<td>Kappa</td>
<td>DX–20h / DX–2h</td>
</tr>
<tr>
<td>Preamplifier</td>
<td>Stanford Research</td>
<td>SR640</td>
</tr>
<tr>
<td>A/D–card</td>
<td>National Instrument</td>
<td>PCI 6259M</td>
</tr>
</tbody>
</table>
2.3. Calibration

The instantaneous position of the trapped bead is acquired by means of the probe laser beam and the PSD. In force measurements, the position is converted to the corresponding force. These two entities are coupled by two calibration constants that must be determined prior to each set of force measurements. In the first step, a relation between the position of the probe laser beam on the PSD and the actual bead position is acquired. This is accomplished by moving the trap in the lateral direction a known distance by means of tilting the GMM. The relation between the tilting angle of the mirror and the lateral translation of the trap could also be considered as a calibration constant. This relation is however relatively stable over time and only has to be measured occasionally. The displacement of the trapped bead results in a corresponding alteration of the detection point on the PSD as is depicted in Figure 2.5. This relation is linear for a limited interval of bead displacements, see Figure 2.6, and can therefore be described by a single parameter.

![Figure 2.6. The voltage signal as a function of the displacement of a 3 µm bead in the calibration procedure. The dashed line represents the slope of the linear region which is used in measurements.](image)
In the second step, the position of the bead is coupled to the external force, i.e., the trap strength is derived. This can be performed in a variety of ways; for instance by use of Boltzmann distribution, Stokes drag force or a power spectrum (19, 20).

The sample is in thermal equilibrium which means that an object, i.e., the bead, will have a distribution of its energy states that follows the Boltzmann distribution. Since the energy potential of the trap is known to be harmonic, a histogram of measured positions will follow a Gaussian distribution which width describes the trap strength (16, 21, 22).

The viscous friction force that the surrounding medium exerts on an object that moves relative the medium is called Stokes force. This is a well known force for spherical objects and can be used to calibrate the trap strength. A bead is dragged by the trap, normally by a controlled sinus wave movement, and the Stokes drag force will displace the bead from the centre of the trap. Stokes drag force can be readily calculated for a spherical object when the viscosity and velocity is known. The strength of the trap can be calculated by relating the displacement in the trap to the friction force (23-25).

Finally, we have the power spectrum method which is also the method used in this work. The botanist Robert Brown discovered in the year 1827, when studying pollen particles that floated in water, that the movements of tiny objects appeared to be of a random nature. Although Brown failed to give a correct explanation for the phenomena, it is still named after him. Any small object, e.g., a bead, in a viscous medium is subject to Brownian motion. The temperature is a measure of the unorganized motion of the molecules in the solvents. The mechanical interaction between the bead and the solvent molecules imposes a geometrically random path for the bead. This can be thought of as small balls, of random velocities which mean velocity is given by the temperature, hitting the bead from all directions. This results in continuous changes of the momentum of the bead which will cause diffusion. The Brownian motion of a free–floating bead is suppressed by the optical trap. The strength of the trap determines to which degree and for which frequencies this suppression effectuates. The Langevin equation describes the equation of motion for the bead under the influence of Brownian motion in a harmonic potential,
where $\gamma$ is the viscous drag coefficient, $x$ is the position of the bead, $t$ is the time, $k$ is the trap stiffness and $F_B(t)$ is the random force due to the Brownian motion. By means of a Fourier transform, a Lorentzian relation is obtained as

$$S_x(f) = \frac{k_B T}{\gamma \pi^2 \left( f^2 + f_c^2 \right)} , \quad (2.4)$$

where $k_B$ is Boltzmann’s constant, $T$ is the absolute temperature, $f$ is the frequency and $f_c$ is the characteristic frequency defined as

$$f_c = \frac{k}{2\pi \gamma} . \quad (2.5)$$

A fitting procedure of calibration data to Eq. (2.4) was automatically executed before every measurement series. The trap stiffness is thereby derived from the parameter value for $f_c$ by means of Eq. (2.5). Typical power spectra from a calibration are shown in Figure 2.7.
Figure 2.7. Power spectra from a calibration procedure featuring four different trapping beam output powers. The characteristic frequency, the “corner” of the power spectrum curves, is clearly shifted towards higher values when the power increases. This frequency is used to determine the stiffness of the trap. The power of the trap laser and the resulting trap stiffness are presented for all four curves.

All three methods mentioned above have been implemented to verify the calibration procedure with a successful outcome. Even though the beam profile is Gaussian and therefore symmetric with respect to the propagation axis, the trap strength will in general deviate between the two lateral directions (26) as a result of the polarization of the light. The details for this phenomenon are not presented here, but it can be concluded that it is important to calibrate along the same direction as the measurements are performed.
2.4. Measurement Procedure

The basic idea of force measurements by means of the optical tweezers system described above is to let an adhesion molecule, expressed by bacterium for instance, attach to a trapped small bead with a diameter of ~3 \( \mu \)m and then pull them apart and probe the resulting force response. This is achieved by immobilizing large beads on the coverslip surface to which single bacteria cells are mounted. The diameter of the large beads (here ~9 \( \mu \)m) determines the vertical distance from the coverslip in the measurements since the bacteria are mounted on the waist of beads. This is an important entity since the strength of the trap depends on the vertical distance from the coverslip due to spherical aberrations (27-29), which implies that the experimentalist must be careful to keep the distance to the coverslip invariant to ensure that the calibration constants are valid. An outline of an experimental setup is shown in Figure 2.8 where a pilus on a bacterium surface is attached to the trapped small bead.

![Figure 2.8](image)

Figure 2.8. An illustration of a typical measurement model system. The large bead is immobilized on the coverslip and a bacterium (green) is mounted on the waist of the large bead. A small bead is trapped by the trapping laser beam (transparent red) and, in this case, bound to a pilus (yellow). The coverslip is translated in the left direction in the figure and thereby displacing the trapped bead from the focus. The probe laser beam (solid red) is redirected by this displacement which is illustrated. The current force and position of the coverslip are measured.
2.4.1. PREPARATIONS

There are a few preparations to perform before measurements can be performed. A convenient way to immobilize the large beads on the coverslip surface is by means of a drying procedure. First, the beads are suspended in a liquid solution from which a small drop is placed on single coverslips. Second, the solutions are evaporated in a laboratory oven at 60°C for 60 minutes. The resulting coverslips feature landscapes of immobilized large beads that congregates in the drying procedure, see Figure 2.9A. The final preparations, depending on the type of measurement, are carried out (including the insertion of bacteria and small beads) and the sample is inserted into the custom made sample holder for the optical tweezers.

![Figure 2.9. Panel A, the large beads form a bead landscape during the drying procedure that is useful in the measurements. Panel B, a snap shot from an experimental situation. A part of the bead landscape is shown on the left hand side. A small bead that may be used as a force transducer is positioned on the right hand side. Finally, a bacterium is moving freely between the two beads.](image)

2.4.2. FORCE MEASUREMENTS

The first step in a typical force measurement is to locate and trap a bacterium, with low trap laser power, and mount it on the waist of a large bead. Second, a small bead is trapped and placed at the same distance to the coverslip and brought into the proximity of the bacterium. The calibration procedure is carried out to determine the two calibration constants as described in section 2.3. The next step is to establish a binding between the small bead and an adhesion organelle. The small bead is brought into close proximity of the bacterium and its adhesion molecules, whereafter the coverslip is translated so that the lateral distance between the trapped bead and the bacterium increases. At this point, the force response reveals if any bonds have been formed. If no bond is established, the bead is carefully moved...
slightly closer to the bacterium than it was the previous time and a new attempt is performed. This procedure is repeated until binding occur. The coverslip is translated in a controlled manner, by means of the piezo–stage, together with the immobilized large bead and the mounted bacterium. This gives rise to a force between the bacterium and the small bead (mediated by the attached adhesion organelle). The force on the small bead displaces it from the focus of the trap which gives rise to a refraction of the probe laser beam that is detected by the PSD. Finally, the force measurement data are acquired and recorded.

2.5. MODEL SYSTEMS

All measurements included in this thesis have been performed with similar, but not identical, models. Three different model systems have been implemented in this work for various studies; receptor–ligand adhesion, biophysical properties of adhesion organelles, and receptor–ligand adhesion including organelles. Illustrations of the different measurement models are shown in Figure 2.10.

The results of primary interest from receptor–ligand adhesion measurements, i.e., bacteria that adhesion organelles, panel A, are a set of rupture forces. Intrinsic properties of the bond can be derived from the distribution of these forces. In the adhesion organelle measurements, panel B, the force–elongation response is of main interest since it reveals information regarding the biomechanical properties of the pili structure. Finally, panel C illustrates a measurement model that is applied when the properties of the bond between an adhesin, that is expressed at the tip of the pilus, and its receptor is to be investigated. Even though all three models are to some extent unique, they all share the same measurement procedure as described above.
Figure 2.10. Schematic illustrations of the three main measurement types performed in this thesis. A force measurement on an adhesion–receptor bond without any pili is shown in panel A. In panel B there is no specific adhesion process involved. Instead the force–elongation response of a pilus is probed. Finally, in panel C, it is a combination of the first two cases with an adhesin–receptor bond on the tip of a pilus.
3. **Bond Kinetics**

Molecules are formed when atoms interact by means of covalent bonds. These bonds are very strong with energies in the range of 100–300 $k_B T$ and distinct bond lengths of 0.1–0.2 nm (30), where $k_B$ is Boltzmann’s constant and $T$ is the absolute temperature. The high binding energy often results in very stable molecules that can be virtually irreversible. In biological systems, many complicated and sensitive interactions take place at molecular level. Molecules or biological surfaces constantly interact with each other, or even with themselves, to create temporary bindings, exchange information or to perform other functions. These tasks require bonds that are reversible and have the ability to break within a relevant time span, i.e., weaker bonds. There are many types of weaker bonds and interactions, for instance ionic bonds, hydrogen bonds, polarity effects, and hydrophobic interactions. In the general case, these interactions are weaker and have longer bond length than a covalent bond. The extended bond length, which can be 10 nm or even more (30), may serve as a guide for the surfaces and molecules to locate each other.

Nature has customized these weaker interactions to amazing compounds where certain structures induce unique energy patterns that require a specific construction to fit into this pattern and thereby create bonds. These bonds are called *specific bonds* and serve as lock–and–key arrangements. With specific bonds, biological structures are able to recognize and bind to the corresponding molecules while other, in many aspects, similar molecules do not bind. This is effectuated in the human body immune system where immunoglobulin binds specifically to extraneous bacteria and viruses, or when bacteria adhere to receptors on host cells in the first step of colonization.
In the general *in vivo* situation, bacterial attachments to host cells are mediated by means of multiple specific bonds. This multivalence prolongs the expected attachment time extensively and the entire binding systems are therefore of great interest to study. However, to be able to understand the complex properties of a multiple binding systems, detailed knowledge of the properties of single bonds must first be acquired.

### 3.1. **Energy Landscape**

A common way to describe the environment of a molecule in a liquid solution that is in thermodynamic equilibrium is to set up an energy landscape for its interaction with the vicinity. This landscape can be used to deduce the probability for the molecule to occupy any given position at an arbitrary time. The energy landscape, which is three–dimensional, arises from the intermolecular potentials of the neighbouring molecules. A bounded molecule has generally, in the force–free case, several different pathways to unbind.

Using this picture, an external force that interacts with the molecule will linearly alter the entire energy landscape. It is reasonable to assume that this force, if it is strong enough, will define the reaction coordinate for any molecular transition. The three–dimensional geometry of the problem can therefore effectively be reduced to only one dimension in this case.

Barriers in the energy landscape define the bond by restricting the molecular transitions. The number of local minima is referred to as the *mode* of the system. The energy landscape that is depicted in Figure 3.1 has a single mode profile.
Figure 3.1. A schematic drawing of a one-dimensional energy landscape for a molecule. Since it has one energy minimum it is a single mode energy profile. The green line represents the energy landscape when no external force is present. In the presence of an external force, $F$, the energy landscape is tilted linearly which alters the transition probability. If the force is high enough, the barrier will diminish and the bond has vanished.

Let us consider the force-free transition for a bond with a single-mode energy landscape as is depicted in Figure 3.1. Kramers (31) deduced that the probability for a transition over an energy barrier, of height $E_T$, can be described by an Arrhenius factor as

$$k_{\text{off}}^{\text{th}} = \nu e^{-\frac{E_T}{k_B T}},$$

(3.1)

where $k_{\text{off}}^{\text{th}}$ is the intrinsic thermal off-rate of the bond. The constant, $\nu$, is the attempt rate which originates from molecular vibrations in an overdamped condensed system and is assumed to be constant in a limited temperature interval (31, 32). This constant is not well defined but is in the interval of $10^9$–$10^{10}$ Hz (3, 33). The thermal off-rate, and thereby the expected lifetime, depends only on the static energy landscape when the temperature is constant. Note that no matter how large energy barrier a bond has to overcome, it will dissociate over a long enough period of time.
Let $P$ be the probability that two single molecules, such as a receptor–ligand pair, are bound with a single–mode energy landscape. The change of $P$ as a function of time, $t$, depends on the off–rate, $k_{\text{off}}$, as,

$$\frac{dP}{dt} = -k_{\text{off}}P.$$  \hfill (3.2)

The solution to this first–order differential equation with a constant $k_{\text{off}}$ is an exponential decreasing function with time. This is the case when no external force is applied.

Information concerning the thermal off–rate, and thereby the energy barrier, would be obtained by probing the average dissociation time. However, many specific bonds have very high binding affinities and the time required for spontaneous breaking the bond would be extensively long and therefore impractical for experimental analyses. A superior approach is to apply an increasing external force to the bond that reduces the lifetime to convenient extents. Moreover, this method allows for determination of the location of the energy barrier, $x_b$, which is difficult or impossible to detect in equilibrium conditions.

In a famous paper about bond dynamics, Bell (34) postulated that the off–rate depends exponentially on an applied force, $F$, as

$$k_{\text{off}} = k_{\text{off}}^{\text{th}} e^{\frac{F x_b}{k_B T}},$$  \hfill (3.3)

where $x_b$ is referred to as the bond length. This equation is a consequence of the fact that the force tilts the energy landscape linearly, $E(F,x) = E(F=0,x) - Fx$, see Figure 3.1. Later, Evans et al. (35) thoroughly examined the bases of this theory. It was shown that a prefactor, $g(F)$, is needed to account for the change of the shape of the barrier in the energy landscape when a force is applied. In addition, the bond length is also dependent on the force in the general case. The equation can then be written as

$$k_{\text{off}} = g(F) k_{\text{off}}^{\text{th}} e^{\frac{F x_b(F)}{k_B T}}.$$  \hfill (3.4)
Equation (3.4) is complicated to analyze since the actual shape of the energy landscape can not be derived. However, the prefactor \( g(F) \) reduces to a weak function of the force under the assumptions of a deep potential well with a sharp energy barrier (36). The contribution from \( g(F) \) can then be merged with the attempt rate, \( \nu \), and thereby removed from the equation. In addition, this assumption, which is generally used, also implies that the bond length does not alter noticeable with force and can be regarded as a constant. It has been shown that the assumption of sharp energy barriers is acceptable since the effect of assigning a harmonic potential to the energy landscape close to the barrier, i.e., allowing the bond length to depend on the force, is marginal (37).

In a typical measurement, the force is increased linearly over time, \( t \), as \( F(t) = rt \) with a constant loading rate, \( r \). Assuming sharp barriers we have in conclusion

\[
k_{\text{off}} = k_{\text{off}}^{\text{th}} e^{k_B T r t x_b}.
\]  

(3.5)

Since \( dP/dF \) describes the distribution of rupture probability density with respect to the force, the most probable rupture force, here defined as the bond strength, can be found at the maximum of the rupture probability density function, i.e., where the second derivative, \( d^2 P/dF^2 \), equals zero. It is here assumed that the rupture probability density function has one maximum, i.e., a single-mode distribution which. The general requirement for the bond strength, obtained by substituting \( dt = (1/r)dF \) in Eq. (3.2) and equating the second derivative with respect to \( F \) to zero, leads to the expression

\[
k_{\text{off}}^2 = r \frac{dk_{\text{off}}}{dF}.
\]  

(3.6)

Finally, by means of Eq. (3.5), an expression for the bond strength, \( F^* \), can be derived,

\[
F^* = \frac{k_B T}{x_b} \ln \left( \frac{r x_b}{k_{\text{off}}^{\text{th}} k_B T} \right).
\]  

(3.7)

Equation (3.7) predicts that the bond strength scales logarithmically with the loading rate. This relationship can be used to explore the properties of the
binding. The bond strength at different loading rates reveals both the bond length and the thermal off-rate. This method is called dynamic force spectroscopy (DFS). The case with a linearly increasing force with time, as presented above, is referred to as linear DFS.

Here it is assumed that no rebinding occurs after a rupture event. This is generally true with a soft force probe like the optical trap. For instance, after a rupture at 40 pN the separation of the two binding molecules is typically ~300 nm. At such large distances no rebinding can physically take place.

3.3. Error Analysis in Linear Dynamic Force Spectroscopy – Paper I

Although linear DFS is a well established method to measure and analyze properties of molecular bonds, there are several possibilities, and even pitfalls, when the experiments as well as the data process are performed. To address these issues, Paper I focuses on the error propagation in a linear DFS study by means of numerical analyses.

In a DFS study, a set of rupture forces is collected and gathered into a frequency distribution by means of a histogram or a density estimator. A fitting procedure is thereafter applied to the distribution to find the bond strength. Two different fitting functions are commonly found in literature. The first is the familiar Gaussian function that is a useful fitting function in many disciplines of physics, and also often applied in DFS studies (38-40). The centre point of the distribution is interpreted as the bond strength, \( F^* \), and the parameter \( \sigma \) describes the width of the distribution,

\[
\rho_{\text{Gauss}}(F) = \frac{1}{\sigma \sqrt{2\pi}} e^{-\frac{(F - F^*)^2}{2\sigma^2}}. \tag{3.8}
\]

The second distribution is the exact solution for the rupture probability density distribution (RPDD), \( \rho_{\text{RPDD}}(F) \) which is given by solving Eq. (3.2) with \( k_{\text{off}} \) given by Eq. (3.5), (41-43)

\[
\rho_{\text{RPDD}}(F) = -\frac{dP}{dF} = \frac{k_{\text{th}}}{k_{\text{off}}} e^{\frac{F_{\text{th}}}{k_B T}} e^{-\frac{F_{\text{th}}}{k_B T} - \frac{F}{k_B T}}. \tag{3.9}
\]
It is important to notice that the expected distribution of rupture forces, obtained by DFS, is not symmetric around the bond strength. This implies that using a symmetric fitting function as a Gaussian, or assigning the mean value of the distribution forces as the bond strength, induces an error, $\varepsilon_{\text{Gauss}}$, which is illustrated in Figure 3.2. In the study presented in Paper I, this error was found to be virtually independent of the loading rate, which implies that the use of a Gaussian fitting function is equivalent to a systematic error in a DFS analysis. In contrast, since the RPDD is the exact theoretically fitting function, it is not expected to give rise to any errors.

![Figure 3.2. A comparison between the two commonly used fitting functions, RPDD (red) and a Gaussian (green). The RPDD is an exact description of the expected rupture force density distribution and provides a bond strength that equals the true bond strength, $F^*_{\text{RPDD}}$. In contrast, the Gaussian fit induces an expected error, $\varepsilon_{\text{Gauss}}$, as indicated in the figure.](image)

The magnitude of the expected error and the standard deviation of the bond strength are shown in Figure 3.3A as a function of the number of measurements for the two fitting functions. It is apparent that the choice of fitting function determines the accuracy while the number of measurements, on the other hand, decides the precision.
Figure 3.3. Panel A, the standard deviations and expected errors of the bond strengths as functions of the number of measurements in the data set. Panel B, the influence of uniformly and normally distributed noise depending on the fitting function. The noise levels refer to one standard deviation for the Gaussian distribution and the mean of the absolute noise for the uniform distribution.

In contrast to errors arising from the use of a Gaussian fitting function, which is induced in the analysis procedure, errors also originate from the experimental instrumentation and the biological sample itself. The magnitude of the errors of the bond strength depends on the fitting function in combination with the level and distribution of noise. Two dissimilar noise distributions have been treated; uniformly and normally distributed noise, respectively. The impact of the noise on the expected error of the bond strength is illustrated in Figure 3.3B. It is worth noticing that normalized noise gives rise to virtually no expected error whatsoever when the RPDD fitting function is applied. The probability distributions for the bond strength, depending on fitting functions and noise, are presented in Figure 3.4.
Figure 3.4. The probability distributions for the bond strength for different combinations of noise levels and fitting functions. The noise has a uniform distribution in panel A and a normal distribution in panel B. Curves in orange shade correspond to results where a Gaussian has been applied while green shade indicates curves that originate from fits with a RPDD function. The dashed red lines indicate the true theoretical bond strength, $F_{\text{True}}$.

In a linear DFS study, the bond strengths are plotted versus the logarithm of the loading rates and are thereafter subjected to a linear fit. The analysis of the error propagation through this fit showed that the use of a Gaussian fitting function leads, alone, to an underestimation of the thermal off-rate by 30%. The bond length is less sensitive to the choice of fitting parameter since it is deduced from the slope which is independent of systematic errors. Instead, it is the precision of the bond strengths that determines the error in the bond length.

Further on, the question of how to choose the number of loading rates was addressed. In an experimental situation, the total number of measurements is a limiting factor due to time consumption and/or cost of conducting the experiment. Collecting many data points at few loading rates improves the precision of the bond strength values as is depicted in Figure 3.3A. However, few loading rates imply few data points in the resulting force spectrum to use in the linear fitting procedure which creates a dilemma and makes the choice non-trivial. The conclusions of the analysis in Paper I, briefly laid out here, show that the best results are achieved by focusing on the bond strengths, i.e., to use few loading rates and thereby have access to many measurements in the derivation of each individual bond strength.

The values of the bond length and the thermal off-rate used in the numerical analysis were 0.5 nm and 0.001 s$^{-1}$, respectively, while the loading rates were chosen in the range from 100 to 10,000 pN/s. The results showed that the analysis of the expected errors in the bond strength is insensitive to changes in the parameters of the analyses. This implies that the results found here can be qualitatively applied to all linear DFS analyses.
3.4. Non–Linear Dynamic Force Spectroscopy – Paper II

The functions presented for the bond strength and the rupture probability density distribution, Eqs (3.7) and (3.9), both originate from the restriction of constant loading rate which is the most common and convenient case in experiments. However, there are situations where the loading rate can not be chosen explicitly by the experimentalist. The loading rate is the rate at which the external force on the bond increases. Elasticity within the model system, caused by the sample, measurement equipment or measurement procedure, may alter the effective loading rate on the bond during the measurement process. In this case, an applied constant loading rate may be decreased or even transformed into a time dependent form. It is possible to undertake a DFS study even in the case with a varying loading rate under the condition that it is continuous with a low second derivative with respect to time. This method was first developed in Paper II.

The off–rate function can be expressed as

\[ k_{\text{off}} = k_{\text{off}}^{\text{th}} e^{B_x b}, \tag{3.10} \]

where \( B \) is a function that describes the time evolution of the force and is defined as

\[ B(t) = \frac{F(t)}{k_B T}. \tag{3.11} \]

At the peaks of the rupture force density distribution histogram, the requirement

\[ k_{\text{off}}^2 = \frac{dk_{\text{off}}}{dt} \tag{3.12} \]

applies. Inserting the expression for the off–rate, Eq. (3.10), into Eq. (3.12) yields

\[ k_{\text{off}} = x_b \frac{dB}{dt} = x_b A, \tag{3.13} \]

where we introduce \( A = dB/dt \) for simplicity reason. Equations (3.10) and (3.13) result in an expression for the thermal off–rate,
This relation only applies at the time of the most probable rupture force, i.e., the most probable rupture time. To conclude this derivation we take the logarithm of both sides of Eq. (3.14),

$$\ln\left(\frac{k_{\text{off}}^{\text{th}}}{x_b}\right) - \ln(\lambda) = -Bx_b.$$  \hspace{1cm} (3.15)

Note that the first term on the left hand side is a constant and the slope of $\ln(\lambda)$ versus $B$ equals $x_b$. Further on, the thermal off–rate $k_{\text{off}}^{\text{th}}$ is given by $k_{\text{off}}^{\text{th}} = A x_b$ where $B = 0$ on the extrapolated linear fit.

The theory for non–linear dynamic force spectroscopy is a generalization of the corresponding linear theory and it can be applied in a similar manner. Nevertheless, attention must be paid to the level of non–linearity in the system. A highly non–linear system may give rise to a multitude of peaks, referred to as peak modes, which need to be coupled correctly. The bond length and thermal off–rate can be derived from each individual peak mode by plotting $\ln(\lambda)$ versus $B$ for that particular mode. However, peak modes that are weak, i.e., vague theoretical histogram maxima, will be hard to identify and peak modes that are positioned close to each other will easily be blurred and impossible to identify in practical measurements.

### 3.5. SLIP–BONDS AND CATCH–BONDS

The theory presented above describing single bond kinetics is valid for so called slip–bonds, i.e., bonds that have an off–rate that increases with an applied force. However, it has been suggested that some bindings prolong their lifetime when they are exposed to an external force in a limited interval. These bonds are referred to as catch–bonds (44-46). This is, of course, a very interesting property that has far–reaching effects in a biological system.
3.6. DYNAMIC FORCE SPECTROSCOPY WITH MULTIPLE BONDS

Studies to characterize specific receptor–ligand interactions are often intended to target single bonds. However, the ubiquity of multiple bond attachments in experimental situations must be considered in both the experimental execution and the subsequent analyses.

Figure 3.5. A schematic illustration of a bacterium with surface attached adhesins that binds by multiple bonds to receptors on host cells.

Consider a multiple binding complex that attaches with a parallel configuration, see Figure 3.5, where the applied force is shared equally between all individual bonds. We assume that the bonds can fail independently, i.e., they are coupled only through the sharing of the force. A first assumption, previously used in several force measurement analyses (47-50), is simply to assume that the bond strength of the complex, $F^*_N$, is related to the bond strength of an individual bond, $F^*$, by the integer, $N$, representing the number of bonds in parallel, i.e., $F^*_N = NF^*$. However, this straightforward assumption leads to a significant overestimation of the bond strength of the complex.

For a system with no applied force, the bonds in a multiple binding complex are completely uncorrelated. In this case, the lifetime of the complex equals the lifetime of the last bond that fails. Since the probability function for each bond is exponentially decreasing with time,

$$P = e^{-k_{off}t},$$  \hspace{1cm} (3.16)

the lifetime of the complex will not increase linearly with the number of bonds. This system is analogous to any mechanical or electrical network with
parallel redundancy. Tees et al. (51) showed that the expected lifetime, $\langle t \rangle_N$, for a complex, under these circumstances, can be written as the expected lifetime for one binding, $\langle t \rangle_1$, multiplied with the $N$th harmonic number, $H_N$,

$$
\langle t \rangle_N = \langle t \rangle_1 H_N = \frac{1}{k_{\text{off}}^{\text{th}}} \sum_{i=1}^{N} i^{-1}.
$$

(3.17)

For instance, a system with two bonds ($N = 2$) is therefore expected to stay attached 50% longer time than a single bond.

When multiple bonds are exposed to a linearly increasing external force, for instance in a DFS study, they establish a cooperative coupled system. All attached bonds, $N_{\text{Bound}}$, will divide the force, $F$, in such a way that each bond is exposed to an individual force equal to $F/N_{\text{Bound}}$. The probability distribution for rupture forces is widely spread (35), which means that it is probable that, in a multiple binding complex, at least one of many individual bonds will rupture at a low force. As a result, the force acting on each of the remaining attached bonds increases. This enhanced force amplifies the off-rate for the remaining bonds and the process of unbinding thereby accelerates, which reduces the lifetime of each individual bond. Williams et al. (52) presented an implicit relationship between the bond strength and the number of bonds for a given loading rate, $r$,

$$
\frac{1}{r} = \frac{x_h}{k_{\text{off}}^{\text{th}} k_B T} \sum_{n=1}^{N} \frac{1}{n^2} e^{-F_n x_h / n k_B T}.
$$

(3.18)

Equation (3.18) provides the bond strengths for parallel systems but does not describe other aspects of the rupture probability distributions. Numerical methods by means of Monte Carlo simulations were conducted to illuminate this issue.

Consider a binding system with six possible binding states: a single bond state and five states with two, three, four, five and six parallel bonds, respectively. The simulated distributions are shown in Figure 3.6 and the bond strengths of the simulated distributions agree well with the prediction of Eq. (3.18). The superimposed probability distribution, i.e., the sum of all six individual distributions, is the distribution the experimentalist would obtain if all six states were equally likely to occur.
Figure 3.6. The rupture probability distributions for 1–6 parallel bonds as well as the (unnormalized) superimposed distribution that is shown in light grey. Maxima originating from multiple bonds are difficult to distinguish for \( N > 3 \). It is also apparent that the positions of the peaks are shifted toward higher forces due to the presence of binding system states of high order. The distributions were compiled from one million simulated samples for each state by means of density estimators. The bond length, thermal off-rate and the loading rate were 0.5 nm, 0.001 s\(^{-1}\) and 1000 pN/s, respectively.

Figure 3.6 shows that consecutive individual distributions overlap each other which causes severe blurring of higher order maxima in the superimposed distribution. The blurring effect originates from the decreasing distance between successive peak forces and from the broadening of the distributions for larger number of bonds in parallel. Another problematic effect with multiple bonds is that high order maxima are shifted toward higher forces in the superimposed distribution. This effect is clearly visualized from the simulations presented in Figure 3.6, by comparison of the location of the peaks for \( N \geq 3 \) with the corresponding peaks in the superimposed distribution. In this example, the distributions were compiled by one million simulated samples where the bond length, thermal off-rate and the loading rate were set to 0.5 nm, 0.001 s\(^{-1}\) and 1000 pN/s, respectively. Alteration of these parameter values will affect the results in Figure 3.6 quantitatively but not qualitatively.

The difficulties originating from the presence of the higher order maxima arise even though we are here considering the ideal situation with no non–specific forces, no signal noise and effectively an infinite statistical background. Every aspect of the problem is further intensified in the case of a practical situation, i.e., non–ideal situation. Under experimental conditions, it is unlikely that maxima of third or higher order could be distinguished in a force distribution based on a set of measurements that contains rupture forces.
from different parallel binding states. Even though the single–bond distribution appears to be relatively stable in Figure 3.6, the contribution from frequent occasions of attachments with two bonds can easily shift the bond strength towards higher forces.

The present number of bonds in a measurement can actually be determined for certain systems where every bond is associated with a soft polymer with known stiffness. A multitude of such bonds in a parallel configuration exhibit a higher stiffness than a single bond. This increased stiffness can be quantified in the analysis and the number of attached bonds appearing in different measurements can thereby be determined (53). However, this method requires that the stiffness of the bonds (with their polymers) is the dominating factor of the stiffness of the entire binding system and, further on, that all bonds exhibit equal stiffness values. For instance, if measurements are performed in situ, the varying stiffness between the bodies of different individual bacterium cells is expected to cause the resulting stiffness in the measurements to vary substantially and thereby obstruct this kind of analysis method. It is therefore often difficult, or impossible, to determine the number of bonds in a particular measurement which implies that individual rupture forces can not, in general, be contributed to a certain number of bonds.

It is concluded that high order states should be avoided in analyses and that it is important to minimize the occurrence of multiple bonds in the measurement system to be able to evaluate the bond strength of the single binding state correctly. In an experimental situation, this can be achieved by means of short contact times and by careful control of the contact force. For a suitable measurement setup, the majority of the measurements should result in no binding at all to ensure a negligible occurrence of multiple bindings.

The contact time in force measurements, conducted on surface attached adhesins, that are presented in this thesis was in general less than 0.1 second to minimize the risk of multiple bindings. The fraction of measurements that resulted in any binding at all was ~20%. In contrast to the case with surface attached adhesins, multiple bindings is directly identified for helix–like pili with force plateau responses since the unfolding force level of $N$ pili equals $N$ times the unfolding force level for a single pilus. Therefore, occasional multipili attachments constitute no difficulties in analyses of single pilus properties.
3.7. HELICOBACTER PYLORI BINDING BABA–LEWIS B – PAPERS III–V

In the mid eighties, Robin Warren and Barry Marshall (54) suggested that several gastric diseases, including gastritis and peptic ulcers could be attributed to the bacterium *Helicobacter pylori*. At first, this proposal was met with scepticism since there was little evidence that the bacteria actually caused the diseases. However, further research supported their theory and in the year 2005 they were awarded the Nobel Prize in Physiology or Medicine for this discovery.

![Image of H. pylori bacterium](image)

**Figure 3.7.** The *H. pylori* is a spiral shaped Gram–negative bacterium with 5–7 flagella than enables rapid movement. Image courtesy Luke Marshall, Perth Australia.

In general, it is impossible for bacteria to survive in the natural inhospitable environment in the stomach. Nevertheless, *H. pylori* have adapted to the challenging prerequisites and approximately 50% of the world population hosts the bacterium today. The first step of an infection is the adherence of bacteria to host cells. *H. pylori* express outer membrane proteins of which two, BabA and SabA, have been identified as adhesins (55-57). The specific binding of the former to its receptor, the fucosylated ABO blood group antigen Lewis b, has been investigated here and are presented in detail in *Papers III–V*. 


The bond length and thermal off–rate of the BabA–Lewis b bond was derived by applying a linear DFS as described in section 3.2. The elasticity of the bacterium body reduced the effective loading rate but did not induce any non–linear effects. It is of interest to study how the bond depends on the acidity since the pH level varies considerable, ranging from pH 2 to 7, in the stomach. DFS was conducted at four different acidity levels; pH 3.6, 4.0, 5.5 and 7.4 and at four different loading rates; 6.8, 68, 480, and 2700 pN/s. More than 2700 single force measurements were performed. In addition, 1300 control measurements were conducted on a mutant, devoid of the BabA adhesin with little or no binding which validated the specificity. Figure 3.9A shows the resulting kernel density estimate (KDE) for the case of pH 7.4 and a loading rate of 2700 pN/s. The dashed line indicates the best fit using the RPDD function, Eq. (3.9), by which the bond strength for this setup was found. The tail of high rupture forces is attributed to multiple bonds which are discussed in section 3.6.

Moreover, it can be readily concluded that the negation of the right hand side of Eq. (3.2), which corresponds to the vertical axis in Figure 3.9A, would never increase if \( k_{\text{off}} \) was to decrease with time. This would be the case for a catch bond when the force is a monotonically increasing function with respect to time. Therefore, the existence of distinct peaks in the rupture probability density functions implies that the BabA–Lewis b bond can be classified as a slip–bond within the force interval applied here.
Figure 3.9. Panel A, the measured rupture probability is represented by the solid area while the best fit, using a RPDD function, is plotted with a dashed line. Panel B, the force spectra for the different acidity levels are illustrated by squares (pH 7.4), diamonds (pH 5.5), circles (pH 4.0) and triangles (pH 3.6). The lines show the best linear fits for the four different cases.

The same procedure was conducted for the other combinations of pH and loading rates which resulted in four different force spectra, see Figure 3.9B. Note that the two spectra for pH 5.5 and 7.4 almost overlap and their linear fits are difficult to distinguish from each other in the figure. The linearity is interpreted as a single mode energy landscape for which the thermal off–rate and bond length were obtained for each spectrum using Eq. (3.7) and are displayed in Figure 3.10. Further on, the linearity suggests that the bond is well described by Bell’s kinetic theory, i.e., the off–rate follows the relationship of Eq. (3.3).
Figure 3.10. Panel A, the bond length (squares) and thermal off-rate (circles) change drastically when the acidity drops below pH 5.5. The error bars mark the 95% confidence intervals. Panel B, the basic outline of the energy landscape can be estimated from the values in panel A.

*H. pylori* bind to the epithelium cell wall and the gastric mucosa where the acidity is nearly neutral. The epithelium cells are continuously shedded which would act as an effective rinsing mechanism if the bacteria would continue to bind to shedded host cell. This event is depicted as step 1 in Figure 3.11 that also shows the following steps. The acidity rises when a bacterium, together with its shedded host cell, is transported from the epithelium towards the corpus, step 2. As Figure 3.10A shows, the thermal off-rate increases extensively as the acidity approaches ~pH 3. The bacterium will then quickly detach, step 3, and return, step 4, using the acidity gradient as guide and its flagella as propeller, to the epithelium cell wall and find a new host cell to bind to. This elegant survival strategy, based on a high selective off-rate, is a prerequisite for the *H. pylori* habitation of the stomach.
Figure 3.11. Schematic illustration of the regulation mechanism that allows for permanent attachment of *H. pylori* to the epithelium. The host cell is shedded in step 1. The shedded cell is, in step 2, transported towards the corpus and the acidity level drops drastically. In step 3, the off-rate is monumentally increased and the bacterium quickly detaches. In step 4, the bacterium is free to swim back and attach to a new host cell.

### 3.8. **Concluding Remarks**

The theory of bond kinetics can successfully be implemented in data analyses of force measurement data where dynamic force spectroscopy is an efficient method to extract the intrinsic properties of molecular bonds. However, to further increase the understanding of bacterial attachments, the complex biomechanical properties of the adhesion organelles must be scrutinized.
4. SINGLE BACTERIAL ADHESION ORGANELLES

Many types of bacteria express cell surface organelles that are called pili (or fimbriae). The pili may possess many different functions where adhesion to specific receptors on host cells is one of the most important. Pili are protein filaments constituted of repeated subunits and are often referred to as macromolecules even when their subunits are not linked by covalent bonds. The internal structure and properties varies substantially between different types of pili whereof two are addressed in this thesis; helix–like and open coil–like pili. By the use of FMOT, detailed information about their biomechanical properties is revealed.

Measurements were first customized to focus on the internal properties of the pilus, i.e., the biomechanical properties of the pili structure. The specific adhesin–receptor bonds were therefore not addressed in this analysis. The pili were attached to the small bead by means of non–specific interactions that were sufficiently strong to withstand the relevant forces in this study, see Figure 4.1.
Figure 4.1. The pilus is attached by non–specific interactions to the trapped small bead. This type of measurements is suitable for investigations of the internal properties of the pilus rod.

The force and elongation are two convenient parameters to measure in physical experiments and they also reveal rich information of the biomechanical properties of the pili. Their relation depends strongly on bond kinetics but also on entropic resistance. Any thermodynamical system always strives to maximize its entropy, e.g., the number of geometric configurations for semi–flexible macromolecules. This results in an effective restoring force when the molecule is stretched. The relation between the restoring force and the elongation can be described by the wormlike chain (WLC) model.

4.1. THE WORMLIKE CHAIN MODEL

The wormlike chain model was first presented by Bustamante et al. (58) and later derived in detail by Marko et al. (59). The WLC describes the force needed to stretch a polymer in liquid solution where the force equals the resistance of entropy that constantly seeks to minimize the end–to–end distance of the polymer to maximize the entropy. The WLC uses a continuous description of a flexible polymer with low curvatures and a bending stiffness defined by the persistence length, $p$. Another model, with a different approach, is the freely jointed chain (FJC) model where the polymer is described by a series of rigid subunits that can rotate freely around the connecting joints (60). It was found that the theoretic expression of the WLC diverged from the prediction given by the FJC model for low forces. To solve this problem, the WLC were interpolated with the FJC in this force regime by means of an Ad hoc contribution which resulted in a modified and very useful WLC function to describe the force–elongation of semi–flexible polymers in liquid solutions,
\[ F = \frac{k_B T}{p} \left[ \frac{1}{4} \left( \frac{1 - L}{L_c} \right)^2 - \frac{1}{4} \frac{L}{L_c} \right], \]  

(4.1)

where \( L \) is the projected length of the polymer in the direction of the force and \( L_c \) is the contour length. The latter is the true length of the curved polymer which sets the upper limit for \( L \). The WLC model has proven to be very successful and is frequently used for various types of macromolecules (61-64).

**4.2. BIOMECHANICAL PROPERTIES OF HELIX–LIKE PILI – PAPER VI**

*Escherichia coli* is a Gram–negative ubiquitous pathogen. The uropathogenic *E. coli* (UPEC) expresses the helix–like P pilus that are compounded by several different types of subunits. The two types of subunits of interest in this thesis are PapG and PapA. The former subunit functions as an adhesin at the distal end of the pili while the latter dominates the structure of the pili. Every P pilus has one PapG adhesin and \(~1000\) PapA subunits. The PapA subunits have lengths of \( >4.1\text{ nm} \) and diameters of \( <2.7\text{ nm} \) and are linked into a pilus by means of *head–to–tail* (HT) bonds between consecutive subunits. The HT–bonds can be either opened or closed which alters the effective length of the unfolded subunits, e.g., the contour length of the pilus.

Further on, in its relaxed state, the PapA subunits are folded into a right–handed helical rod, with 3.28 subunits per turn, that has a diameter of \( \sim 6.8\text{ nm} \) (65). The rod is held together by *layer–to–layer* (LL) bonds that act between PapA subunits three steps away, i.e., unit \( n \) binds to unit \( n+3 \). This means that each layer is stabilized by 3.28 bindings which provide a high bending stiffness to the rod. In contrast, subunits that are unfolded constitute a linearized soft polymer that is well described by the WLC model. Figure 4.2 illustrates the two types of internal bonds in a helix–like pilus, such as the P pilus, that is partly folded and partly unfolded.
Figure 4.2. The model of a helix–like pilus involves a rod with a high bending stiffness and a flexible chain held together by layer–to–layer bonds and head–to–tail bonds, respectively. This figure is a modified reprint from reference (66).

Both these bonds can be described by one single energy landscape with three local minima, one for each state, see Figure 4.3A. A closed LL–bond is in state A, and transits to state B when it opens. In state B, the LL bond is open while the HT bond is closed. The unit transits into state C when the HT bond opens. Since the model allows each PapA unit to occupy one of three possible binding states (relative to its left hand side neighbour), it constitutes a three–state model. This triple state energy landscape describes the transition probabilities between the three states as well as the change in contour length when bonds opens or closes.
Figure 4.3. Panel A, a schematic drawing of the energy landscape for a three-state model. The transition from state $i$ to state $j$ is denoted by $k_{ij}$. The steady-state unfolding takes place when the applied force tilts the landscape in such a way that the energy of state A and state B is equal which is the case for the curve labelled With force $F$. Panel B, a conceptual illustration of the force–elongation response of a helix–like pilus with its three regions.

In its relaxed state, a P pilus is folded into a ~1 µm long rod. When a relaxed pilus is exposed to a forced elongation it will first show a linear elastic response. This is referred to as region I in Figure 4.3B and is relatively steep, ~500pN/µm (67), which indicates that both the rod and the bacterium body are relatively resistant to deformation. Moreover, measurements on purified pili have shown a similar slope of region I which implies that the elasticity of the bacterium body has a negligible influence on the force–elongation response (68).

When the applied force, $F$, reaches a certain level, the pilus enters region II. Except for the outermost LL–bond, an external force is divided equally between the 3.28 LL–bonds for each layer. In addition, an internal LL–bond that opens will quickly close again if its neighbouring bonds are still closed. This implies that an external force will first open the outermost bond, followed by the neighbouring bond since it, at this point, has become the outermost bond. The bonds will open in a sequential manner and the pilus will thereby undergo a zipper–like unfolding, which results in a constant force plateau (67, 68). The constant unfolding force depends on the velocity of the forced elongation but for P pili it always equals or exceeds the steady state unfolding force of ~28 pN.

Further elongation will force the pilus into region III which is governed by the transitions of the HT bonds. These bonds are stronger than the LL
bonds and therefore require a higher force to open. In opposite to the LL bonds, the HT bonds are independent of each other which implies that all bonds in state B have an equal probability to open or close at all time. This property induces an entropic effect that causes the soft shape of region III in Figure 4.3B (67, 68). The biomechanical properties of helix–like pili are analyzed further in Paper VI.

Finally, it is observed that the pili can be elongated a large distance while taking a significant force. This distance is referred to as the force exposure length which is addressed in forthcoming discussions. To set the nomenclature in this thesis, the phrase helix–like refers ultimately to the structure and binding characteristics represented by the P pili. The qualitative results and conclusions are though expected to apply to other helix–like pili that have been shown to possess similar architecture and biomechanical properties, such as type 1 and S pili (69, 70).

4.3. MONTE CARLO SIMULATIONS – PAPER VII

The influence of an external force on a helix–like pilus depends to a high degree on the probabilistic nature of state transitions described by bond kinetics. This suggests that the system can with advantage be modeled with a Monte Carlo approach.

Paper VII focuses on the construction of a Monte Carlo simulation method that describes and replicates measured force–elongation data for P pili. The simulations are based on Bell’s bond kinetics in a first order Markov Chain Monte Carlo Metropolis algorithm. With the exception of internal bonds in the folded rod, all bonds have possibilities to undergo transitions during each timestep. The current state of all bonds determines the contour length, $L_c$, of the pilus. The forced elongation length is always known which allows for determination of the force response of the pilus according to the WLC relation, Eq. (4.1).

The Monte Carlo simulations provide excellent fits to measured data as is illustrated by the black curves in Figure 4.4 for both elongation and retraction. The force response for unfolded subunits is described by the WLC relation. In the figure, this is mainly observable when the entire pilus is unfolded and no transition takes place, which is the case in the limiting sections of region III, at elongations of ~9 and ~12 µm in panel A and at ~7.5 and 10 µm in panel B, respectively.
Figure 4.4. The measured force–elongation responses are plotted in grey and data from Monte Carlo simulations are plotted in black. The two panels show measurement data for two different P pili. The pili are subjected to elongation and retraction in panel A and panel B, respectively. This figure is a modified reprint of figures from reference (71).

The primary reason to consider a Monte Carlo model is the new possibilities it presents. It was shown that the model replicates measured data with high precision which suggests that the model is accurate and could be used to target problems that are impossible to address analytically or experimentally, e.g., the complex situation with multiple pili binding systems. This simulation method was also implemented in Paper II, Paper IX and Paper X that are described in detailed in section 4.4 and in chapters 5 and 6, respectively.
4.4. P PILI SPECIFIC BINDING PapG–galabiose – PAPER II

P pili bind specifically to the glycolipid galabiose receptor (72-75) with their adhesins PapG that are located at the distal end of the pili. Although the internal structures of the pili are not directly correlated to the adhesins, knowledge thereof is required to be able to analyze the rupture force data from experiments including PapG–galabiose bonds performed \textit{in situ}.

Properties of specific adhesin–receptor bonds are normally investigated by means of linear DFS, which requires a linear or negligible elastic damping from the additional components in the measurement system. This is however not the case for the specific PapG–galabiose binding measurements performed \textit{in situ} which are analysed in \textbf{Paper II}. The biomechanical properties of the pili give rise to non–linear loading conditions, as Figure 4.4 indicates, which calls for alternative methods of analyzing the measured data.

![Diagram](image)

\textbf{Figure 4.5.} The PapG adhesin is located at the tip of the pilus. The small bead is coated with galabiose which is the specific receptor for PapG.

A total of 720 measurements were performed, according to the model that is depicted in Figure 4.5, at three different velocities, \(v = 0.5\), \(v = 5.0\), and \(v = 50\ \mu\text{m/s}\). The measured data, in form of histograms of rupture forces, are presented in Figure 4.6 for all three measurement velocities.

Previous work by Andersson et al. show that the unfolding force in region II for the P pilus scales with the logarithm of the unfolding velocity for velocities above \(\sim 0.4\ \mu\text{m/s}\) (76). Since the force is approximately constant during the relatively long time the pilus is in region II, the probability for rupture at these forces is high which induces peaks in the rupture probability histograms. The constant force plateaus were found to be 32, 45, and 63 pN and can be identified as they correspond to the low–force peaks in Figure 4.6. The high–force peaks, on the other hand, originate from the strongly increased off–rate induced by the quickly progressing force in region III.
Figure 4.6. Histograms of the rupture probability distributions for the three different velocities as well as for the negative control measurements. This figure is a modified reprint from reference (77).

The specificity of the binding data was tested by 120 negative control measurements where solvent galabiose was added to block the PapG adhesins. The presence of the blocking agent resulted in virtually no binding events which validates the specificity of the binding system.

Effects of region I were neglected in the analyses since the rupture probability is small during the short period of time the pili spend in this region. Moreover, all bonds are assumed to be history independent which implies that the obtained relative rupture probability of the bond was unaffected by the neglect of possible rupture events during region I. The prerequisites for rupture events alters when the pili transits from region II into region III which implies that the rupture data have to be divided into two separate categories; data for rupture in region II and region III, respectively. The data are analyzed by three different methods that are described separately below whereafter the results from all methods are summarized.
4.4.1. RUPTURE IN REGION II

The experimental data showed that a significant fraction of the measurements resulted in rupture in region II as well as in region III for all three velocities. Nevertheless, the thermal off–rate can be deduced from the rupture data in region II solely as long as the pili that enters region III are accounted for to obtain an unbiased statistical data set. The binding probability is derived from the complete set of measured data of individual binding lifetimes, and is tracked as it diminishes with time due to pili detachments. Since the adhesin–receptor bonds are assumed to be history independent and the force and thereby the off-rate is constant, the probability, \( P \), for a pilus to be attached at the time \( t + t_0 \) is

\[
P(t + t_0) = P(t_0) e^{-k_{\text{off}}(t-t_0)}, \quad (4.2)
\]

where \( t_0 \) is an arbitrary time and \( P(t_0) \) is the probability that the pilus is attached at time \( t_0 \). This implies that the contribution from a specific pilus, when it has entered region III, at time \( t_{III} \), to the total binding probability can be readily replaced with an analytic function according to Eq. (4.2) with \( t_0 = t_{III} \) and \( P(t_{III}) = 1 \). A description of the contribution to the binding probability from two pili is illustrated in Figure 4.7A. Pilus #1 detaches in region II while pilus #2 enters region III, at time \( t_{III} \), and is subjected to the replacement procedure.
Figure 4.7. Panel A, a conceptual drawing of the contributions to the total binding probability of two different pili. The first pilus detaches during region II while the second pilus enters region III and is from this point replaced by an analytic function that described the theoretical binding probability of that pilus. Panel B, the obtained binding probabilities for the three unfolding velocities (grey) and the analytic solution with the best fit in dashed (black). Panel C, the linear fit of the three off–rates as function of the corresponding force level for the velocities. This figure is a modified reprint from reference (77).

The total binding probabilities decrease exponentially with time as is shown in Figure 4.7B. The best fits, obtained by the implicit fitting procedure, are drawn with black dashed lines and show good agreement with measured data. The starting offset, indicated in the figure, originates from the fact that rupture events that take place before a distinct force plateau can be identified are neglected since they can not be distinguished from possible multiple pili attachments. However, the starting offset does not influence the results since the data sets have been normalized.

The measurement data and the corresponding fit for each velocity give rise to an off–rate that is associated with the force level for that particular velocity. The off–rates are plotted against the force in Figure 4.7C which allows for the determination of the bond length and thermal off–rate according to Eq. (3.3).
4.4.2. RUPTURE IN REGION III

The loading force on the adhesin–receptor bond changes from constant to non–linear when the pilus enters region III which complicates the analyses of the rupture events in this region. However, since the force has a smooth variation with time, the theory of non–linear DFS, see section 3.4, can be applied. The high force peaks, presented in Figure 4.6, originate from rupture events in region III and give rise to three independent sets of values for the parameters $A$ and $B$ in the non–linear DFS theory. However, since the rupture forces for the highest velocity, $50 \mu m/s$, occasionally emanate from the non–linear region of the measurement system (forces above $\sim 100$ pN), they are not reliable and therefore excluded from the analysis. Instead, a linear fit of the parameters from the two lower velocities is used to supply values of the bond characteristic parameters.

![Graph showing the rupture data for region III.](image)

**Figure 4.8.** The rupture data for region III provide values for the parameters $A$ and $B$ in the non–linear DFS theory. The data for the highest velocity are excluded from this analyze since it is perturbed by the non–linearity of the measurement system at high forces. The best fitting straight line, dashed, provides the bond length and thermal off–rate for the bond. This figure is a modified reprint from reference (77).
4.4.3. RUPTURE IN BOTH REGION II AND REGION III – MONTE CARLO SIMULATIONS

The rupture force data collected when the pili resided in region II and III, respectively, has been scrutinized separately above. However, a third approach is also feasible by means of Monte Carlo simulations. The entire set of rupture data for each velocity was processed simultaneously to obtain a binding probability as a function of the applied force, see Figure 4.9A. Monte Carlo simulations were applied to find the best fit to these functions, by varying the bond length and thermal off–rate. The mean square error of the fit, Figure 4.9B, was used as an estimate of the accuracy of the parameters. The binding probability with the parameters that provides the best fit is plotted as black dashed lines in Figure 4.9A.

---

**Figure 4.9.** Panel A, the binding probability based on the entire set of measurement data for the three velocities. The black dashed curves represent the best fitting binding probabilities found by Monte Carlo simulations. Panel B, the mean square error curves for different bond lengths as functions of the thermal off–rate. The lowest value of the mean square error corresponds to the best set of parameter values. This figure is a modified reprint from reference (77).

4.4.4. CHARACTERISATIONS OF THE SPECIFIC PapG–GALABIOSE BOND

Three different analysis methods were applied, where each of them provides parameter values for the PapG–galabiose bond which show good agreement with each other. The resulting final parameter values were taken as the mean and is $x_b = 0.50 \pm 0.02$ nm and $k_{\text{off}}^{\text{th}} = 0.0026 \pm 0.0005$ s$^{-1}$. This implies that the specific bond has an off–rate that is approximately 2000 times lower than the opening rate for the LL bonds at the lowest unfolding force (~28 pN). The specific PapG–galabiose bond is therefore strong enough to allow the P pili
to utilize their unfolding property and thereby share an external force with other pili to prolong the attachment time of the bacterium.

Moreover, the monotonically increase in off-rate as the applied force increases, see Figure 4.7C, suggests that the PapG–galabiose bond can be characterised as a slip–bond in this force regime.

The structural architecture varies considerably between different types of pili. The pneumococcal pili, T4, expressed by the Gram–positive *Streptococcus pneumoniae* possess so called open coil–like superstructure (78). It is assembled by at least two protofilaments that are coiled around each other and thereby form a micrometer long stable but flexible structure. Each protofilament has a backbone of covalently bounded RrgB subunits. Small clusters of RrgA are distributed along the pili and have been identified to mediate adhesion to extracellular matrix proteins (79). In addition, there are also scattered RrgC subunits which role is still unknown. The structure of the T4 pili is schematically illustrated in Figure 4.10.

![Figure 4.10](image.png)

**Figure 4.10.** The T4 pili consist of at least two protofilaments constructed by repeated RrgB subunits that are covalently linked. The subunit RrgA has adhesive properties while the role of RrgC is unknown.

To further investigate the T4 pili structure and its biomechanical properties, force elongation measurements were performed on a number of pili that were non–specifically linked to the trapped small bead, see Figure 4.11. This attachment is assumed to predominately be constituted by a multitude of anchoring points, i.e., attachment points.
Figure 4.11. The T4 pili were non–specifically attached to the trapped small bead whereafter force measurements were conducted.

In contrast to the helix–like pili, the open coil–like pili showed no indication of state transitions. Instead their force responses are well described by the WLC model which confirms the linear confirmation of the internal structure of the macromolecule suggested by Hilleringmann et al. (78) and illustrated in Figure 4.10. Elongation curves and the following retraction curves regularly overlapped each other which suggest that no dynamic effects were present. A typical force measurement curve is shown in Figure 4.12A together with the best fitting WLC curve (black dashed line).

Figure 4.12. A typical force measurement is presented in panel A where the elongation (red) and retraction (green) curves follows each other and show no hysteresis. In some measurements there were discontinuities as panel B shows. More discontinuities took place for high forces which are not observable in the figure. These features resulted in alterations of the contour lengths which cause the hysteresis seen in panel B.

Occasionally, discontinuities in the force–elongation curves were observed, as is shown in Figure 4.12B. These discontinuities were attributed
to partial detachments of the pili from the small bead, i.e., one or more of the anchoring points suddenly detached when exposed to force. A partial detachment results in an increased contour length of the part of the pilus that is stretched. This change in contour length was confirmed by the three WLC–fits drawn in Figure 4.12B. At forces above those presented in the figure, more discontinuities were present that further increased the contour length which is the reason that the retraction curve deviates significantly from the elongation curve. It was postulated that these partial detachments also occur in vivo which allows a single pilus to prolong its attachment time with a factor equal to the number of anchoring points (adhesin–receptor bonds), i.e., a pilus with \( N \) anchoring points has a lifetime that equals \( N \) multiplied by the lifetime of a single adhesin–receptor bond since the adhesins can be expected to detach in a zipper–like manner. The details regarding this mechanism will be addressed in future studies.

Figure 4.13. A conceptual image of how the zipper–like detachment of an open coil–like T4 pilus might take place. The high flexibility of the pili improves the ability to attach with a multitude of anchoring points, i.e., adhesins in vivo.

From repeated measurements, the persistence length of the T4 pili was found to be \( 2.1\pm1.7 \) nm suggesting that the pili have a flexible structure which is advantageous when attaching to host surfaces with rough topography. Figure 4.13 shows a conceptual image that illustrates both the zipper–like detachment and that the high flexibility is advantageous to acquire a multitude of adhesin–receptor bonds.

The usage of the expression open coil–like in this thesis specifically addresses the pneumococcal T4 pili although the analyses and conclusions are expected to apply to open coil–like pili in general.
4.6. COMPARISON BETWEEN HELIX–LIKE AND OPEN COIL–LIKE PILI

While the helix–like pili can be elongated up to ten micrometers, the open coil–like pili are more limited with an elongation ability of approximately one micrometer. Further on, the main part of the elongation for open coil–like is characterized by very low force exposure. The force exposure length of open coil–like pili is actually only ~100 nm as can be seen from Figure 4.12A. This is to be compared to the force exposure length of P pilus that is ~10 µm, see Figure 4.4, i.e., 100 times longer. These differences have little importance on a single–pilus level, but they are highly significant in a scenario with a multitude of binding pili as is discussed in chapter 5. In addition, the results suggest that the two types of pili have completely different adhesion strategies. The helix–like pili only have a single adhesin, positioned at the distal tip, and therefore detach with the adhesin. In contrast, the open coil–like pili have a number of adhesins distributed along the protofilaments which means that many adhesin–receptor bonds are required to rupture before the pilus detaches entirely.

4.7. CONCLUDING REMARKS

The structure of pili possesses various architectures that lead to completely different properties of the pili. These biomechanical properties as well as the adhesin–receptor bonds associated with the pili can be investigated by means of force measuring optical tweezers and dynamic force spectroscopy. The information provided by such studies presents deep understanding of the fundamental biomechanical properties on a single–pilus level. The next step in the analysis of adhesion organelles is to investigate how they cooperate in a binding system constituted by multiple adhesion organelles.
5. MULTIPLE BACTERIAL ADHESION ORGANELLES

In the general in vivo situation, bacteria bind to host cells with a multitude of specific bonds. The significance of the multiplicity is highly dependent on whether the bacteria express their adhesins directly on the bacterial surface or by means of pili. In the former case, which is discussed to some extent in section 3.6, the bacterial binding lifetime is exclusively a function of the direct binding properties, i.e., the adhesin–receptor bonds, and the applied force. In contrast, when bacteria bind by means of multiple pili, referred to as multipili attachments, derivation of bacterial lifetimes becomes more challenging. Detailed knowledge of the biomechanical properties of single pili and their adhesin–receptor bonds is a prerequisite to understand complicated multipili attachments. While the lifetime dependence on multiplicity for bacteria expressing surface attached adhesins is well described by analyses by Tees et al. (51) and Williams (52), multipili attachments of helix–like pili are for the first time scrutinized in Paper IX.

The lifetime of a multipili binding system depends on many factors, such as the properties of the adhesin–receptor bond, the number of pili, the external force and the geometry of the binding system, but also on the internal properties of the pili structure. These issues are here discussed in detail for helix–like pili and conceptually, and somewhat hypothetically, for open coil–like pili. This study deals exclusively with slip–bonds and excludes effects of possible rebinding.
5.1. HELIX–LIKE PILI – PAPER IX

The extensive theory of multipili attachment of helix–like pili in general and P pilus in particular is laid out in Paper IX while the main features are encapsulated here.

Consider a bacterium that expresses helix–like pili with single adhesins on their distal ends that are attached to receptors on host cells. Further on, the bacterium is exposed to a constant external force distributed among its pili. The relaxed lengths of pili vary considerable and they can also bind to the host cells at arbitrary angles. These two geometrical properties of a multipili system imply that the pili possess highly diverse force onset positions, i.e., the positions of the bacterium where the individual pili become exposed to force. The concept of force onset positions is presented in Figure 5.1 where panels A and B illustrate that pili can be exposed to force in different ways and panel C visualizes the definition of the force onset positions. For instance, pilus 2 has a larger force onset position than pilus 1 and therefore becomes exposed to force later, i.e., pilus 1 has to elongate a distance that match this difference before pilus 2 experiences any force. The geometry of the system is in this model reduced to one spatial dimension defined by the direction of the external force as is depicted in panel C.

Figure 5.1. A conceptual illustration of the high variety of the force onset positions of different pili. Panel A, a bacterium is attached by three pili to the host tissue. Panel B, an external force is introduced that completely unfolds pilus 1 and partly unfold pilus 2 while pilus 3 remains folded. Panel C, the system is reduced to one spatial dimension in the same direction as the external force. The force onset positions are defined as the different positions of the bacterium where the pili first become exposed to force. These positions depend on the attachment positions of the pili as well as their relaxed lengths.
There are two extreme cases of force distribution; *sequential force exposure* and *simultaneous force exposure*. With a sequential force exposure, one pilus is exposed to the entire force until it detaches whereby another pilus becomes exposed to the entire force, i.e., the pili are exposed to force in a sequential manner. On the other hand, if several pili share the force equally they have simultaneous force exposure. Binding systems will in general vary over time between these two scenarios. For instance, the system depicted in Figure 5.1C can first be considered as a sequential system since only pilus 1 is exposed to force. Pilus 2 and pilus 3 will though after a while, assuming that no adhesin–receptor bond has ruptured, share the force and the binding system experiences a transition to simultaneous force exposure.

### 5.1.1. Dual–Pili Binding System

A bacterium that binds to host cells by means of two pili is referred to as a *dual–pili attaching system*. The two pili can be of different relaxed lengths and have different force onset positions. The theory of bond kinetics elucidates that the lifetime of adhesin–receptor bonds are strongly dependent on external forces wherefore it is expected that the two limiting types of force exposure (sequential and simultaneous) give rise to diverse lifetimes. Indeed, the expected bacterial adhesion lifetimes for the sequential, $\langle t(F) \rangle_{\text{Bac}}^{\text{Seq}}$, and simultaneous, $\langle t(F) \rangle_{\text{Bac}}^{\text{Sim}}$, force exposures were derived in *Paper IX*,

\[
\langle t(F) \rangle_{\text{Bac}}^{\text{Seq}} = \frac{2}{k_{\text{off}}^{\text{th,AR}}} e^{\frac{F_{\text{x}}^{\text{AR}}}{k_{\text{B}} T}} \quad (5.1)
\]

and

\[
\langle t(F) \rangle_{\text{Bac}}^{\text{Sim}} = \frac{1}{2k_{\text{off}}^{\text{th,AR}}} e^{\frac{F_{\text{x}}^{\text{AR}}}{2k_{\text{B}} T}} + \frac{1}{k_{\text{off}}^{\text{th,AR}}} e^{\frac{F_{\text{x}}^{\text{AR}}}{k_{\text{B}} T}} , \quad (5.2)
\]

respectively, and have dissimilar force dependencies. Note that the nomenclature in this chapter has been somewhat altered since there are now two different types of bonds involved. The superscript $\text{AR}$ and $\text{LL}$ refer to Adhesin–Receptor and Layer–to–Layer bonds, respectively. In general, the lifetime of a system with simultaneous force exposure widely exceeds the one with sequential force exposure, i.e., $\langle t(F) \rangle_{\text{Bac}}^{\text{Sim}} \gg \langle t(F) \rangle_{\text{Bac}}^{\text{Seq}}$. This implies that
a binding system benefits from a high degree of cooperativity, i.e., many pili that easily share an external force. The unfolding velocity of a pilus depends on the bond length, the thermal off–rate and on the bond opening length of the internal LL–bonds, where the latter equals the distance between state A and state B in Figure 4.3. A high force will increase the unfolding velocity exponentially, weighted by the bond length of the LL–bonds, which means that a system with sequential force exposure will transit to a simultaneous force exposure in a short period of time. However, a high force also increases the off–rate of the adhesin–receptor bond exponentially, weighted by the bond length of the adhesin–receptor bonds, which decreases the probability that pilus 1 remains attached during the entire unfolding time. To comprehend the opposite effects of these two factors, it is helpful to derive the expected unfolding length, $<L(F)>_II$, which is

$$<L(F)>_II = \frac{x_{AB}^{LL} (1-\eta_x) F_{\theta x}^{LL}}{\eta_k k_n T} ,$$

where $x_{AB}^{LL}$ is the bond opening length for a LL–bond. The parameters $\eta_k$ and $\eta_x$ are defined as

$$\eta_k \equiv \frac{k_{th,AR}^{th,LL}}{\eta_k ^{th,AR}}$$

and

$$\eta_x \equiv \frac{x_b^{AR}}{x_b^{LL}} ,$$

respectively. This expression describes how far the pilus is expected to unfold under a constant external force before it detaches. No restriction of the length of the pilus is included in the derivation. The parameter $\eta_x$ is of special importance since it determines whether the expected unfolding length increases ($\eta_x < 1$) or decreases ($\eta_x > 1$) with an external force.

A comparison of the lifetimes for a dual–pili system for the different force exposure situations are presented in Figure 5.2 for four combinations of $\eta_x$ and $\eta_k$. The limiting cases of sequential, Eq. (5.1), and simultaneous, Eq. (5.2), force exposures are plotted with thick blue and red lines, respectively.
In addition, a set of situations with dissimilar force onset positions of the pili are presented, illustrated by thin black lines that are defined in panel D.

**Figure 5.2.** The lifetime of a dual–pili system is highly reliant on the parameters $\eta_x$ and $\eta_k$. These two parameters are varied in the four panels where the blue and red lines indicate the limiting cases with sequential and simultaneous systems, respectively. The thin black lines show the lifetime of the system for a set of differences in the force onset positions, $x_2^0$, between the two pili, defined in panel D. In panel A, all parameter values are set to those of P pili. The bond length and thermal off–rate for the LL-bonds are then varied in the other panels.

Panel A shows the lifetimes for dual–pili systems based on the P pili properties which are obtained at a single–pilus level (69, 77), while panels B–D show lifetimes when $\eta_x$ and $\eta_k$ have been varied. Effects of region III have not been included in these calculations wherefore the results are to be considered as lifetime estimations. The parameters that define the adhesin–receptor bond were kept constant in all situations. Instead, the parameters $\eta_x$ and $\eta_k$ were varied by tuning the bond length and thermal off–rate of the LL–bonds. In other words, only the internal parameters of the pili structure have been modified in the calculations presented in the four different panels. Since the limiting cases with sequential and simultaneous force exposures are independent of the internal parameters they give rise to the same curves in all panels. In contrast, systems with dissimilar force onset positions depend to a
high degree on the internal properties. In all panels, the expected unfolding length increases with the force since $\eta_\nu$ consequently falls short of the limiting value 1. This implies that simultaneous force exposure occurs more frequently for higher forces which also can be observed in Figure 5.2. A dual–pili system constituted of P pili exhibits high cooperativity which means that the pili share an external force in an efficient way. In contrast, the cooperativity has been decreased in panel B–D, by means of increased $\eta_\nu$ and $\eta_k$, which results in strongly reduced lifetimes of the systems.

These results show that it is essential to consider the cooperativity of the binding system, defined to a high degree by the pili internal properties, when evaluating the binding capability of a system that includes more than a single pilus.

5.1.2. MULTIPILI BINDING SYSTEM

It is evident that the lifetime for a dual–pili system depends not only on the adhesin–receptor bonds but also on the cooperativity set by the internal properties of the pili. When the system is expanded into a full multipili scenario there is no reason to expect that this phenomenon will abate or desist. A detailed theoretical treatment quickly becomes overwhelming and even though rough estimations of the lifetime can be derived (and are presented in Paper IX), the complexity of the system suggests that a numerical approach is to be considered. Therefore, a Monte Carlo method, similar to the one described in section 4.3, is introduced. The simulations here are not used to replicate a particular experimental result from a FMOT measurement where the lengths of the pili were set by the experimental procedure. Instead, these simulations use similar constraints as are present in the in vivo situation, which means that the system is subjected to an external force rather than to a forced elongation. The system will therefore occupy the configuration of states in each individual pilus needed to acquire steady–state, i.e., the total force taken by all pili will sum up to the external force. The external force is constant throughout every simulation which corresponds to the case with a bacterium attachment to a cell wall during constant flow of the surrounding medium. Further on, the simulated multipili systems are chosen to represent P pili. The binding systems are defined by a number of pili, $N$, of equal length that have evenly distributed force onset positions. The difference in force onset positions are set to 500 nm for $N<12$ and to $5000/(N−1)$ nm for $N>=12$ which implies that the maximum difference is 5 $\mu$m. In general, a set of thousand simulations were conducted for each data point to obtain an average lifetime with high accuracy.
Figure 5.3. The simulated lifetime for different external forces as functions of the number of pili in the system. The open red circles indicate the forces that equal the product of the number of pili and the steady–state unfolding force level, $F_{\text{SS}}^{\text{UF}}$ which equals ~28 pN. The lifetime corresponding to these circles are approximately 3 seconds for all number of pili when $N>2$.

Figure 5.3 shows the lifetime of the binding system for various numbers of pili as functions of the external force. For each curve, the force that equals the product of the number of pili and the steady–state unfolding force are marked with an open red circle. This corresponds to the case where all pili can share the force equally over time, i.e., the cooperativity is maximized. These forces are high enough for all pili to reach the steady–state unfolding force, region II, but low enough so that no pilus becomes exposed to a higher force when it enters region III. It is observed that, for $N>2$, the lifetimes corresponding to these markers are consistently ~3 seconds. Interestingly, this can be coupled to the in vivo environment of UPEC. P pili are mainly expressed in the ureter where the urine flow is constituted by repeated boluses that are pushed towards the bladder by means of peristaltic motion (80, 81). Moreover, the boluses have an average length and velocity of 7 cm and 3 cm/s, respectively, which implies that the boluses are applying a rinsing force for ~2 seconds on bacteria that are attached to the cell wall (82). This correlation in time scales suggests that the steady–state unfolding force (~28 pN) of P pili has been optimized to withstand the rinsing effect of boluses with as few pili as possible. A higher steady–state unfolding force level would result in fewer pili that share the external force, whereby the force on each individual pilus increase which implies that the off–rate of each
individual adhesin–receptor bond would be too high for the system to withstand the stress for 2 seconds. On the other hand, a lower steady–state unfolding force would entail that more pili are needed to sum up to the external force. If this larger number of pili would be inaccessible, one or more pili would enter region III and quickly detach followed by the remaining pili. The steady–state force of the P pili seems have adapted to the requirements set by the in vivo conditions.

Figure 5.4. The simulated (open circles) lifetimes and estimated lifetimes (dashed lines) of multipili systems. The number of pili for every system is depicted next to the lines.

Simulated lifetimes (circles) and theoretical estimations thereof (dashed lines) are presented in Figure 5.4 for systems with various numbers of pili (indicated in the figure). The theoretical estimations are capable of describing the general decay patterns of the lifetimes even though some discrepancies are present. Note that the lifetime–axis spans over not less than 10 orders of magnitude. From these results it is evident that the number of attached pili has a decisive role in the bacterium attachment time.
5.1.3. **INTERNAL PROPERTIES**

As have been discussed above, the internal properties of the pili structure are decisive for the cooperativity of a binding system and it is required that $\eta_x < 1$ to obtain a system that handles high forces efficiently. To investigate further on this matter, simulations were performed with a multipili system constituted by 10 pili that had evenly distributed force onset positions with a difference of 500 nm. The values of the two parameters $\eta_x$ and $\eta_k$ were varied by tuning the properties of the LL–bonds while the properties of the AR–bonds were at all instances kept constant, i.e., in the same manner as for the dual–pili system in section 5.1.1. The intriguing results are presented in Figure 5.5.

![Figure 5.5](image_url)

**Figure 5.5.** The lifetime of a multipili binding system with 10 pili depends strongly on the internal properties of the pili. Panel A, the bond length of the LL–bonds were varied. Panel B, the thermal off–rate of the LL–bonds were varied. The results in both panels show that P pili have optimal internal properties for strong multipili attachment in this extensive force interval.

The bond length of the LL–bonds is varied in the simulations presented in panel A. The system possesses low cooperativity for $\eta_x > 1$ which results in short lifetimes close to that of a system with sequential force exposure. The curves for $\eta_x < 0.9$ are similar for low forces but start to diverge when the force increases. Further on, the thermal off–rate of the LL–bonds is varied in the simulations presented in panel B. High thermal off–rate of the LL–bonds gives rise to long lifetimes for low forces. However, for high forces it can actually be disadvantageous with a high thermal off–rate.
Some general conclusions can be drawn from the results in both panels. For low forces, the lifetime profits from high unfolding velocity since that will increase the cooperativity of the system. However, a high unfolding velocity also implies that one or more pili quickly unfold and enter region III where the force on those particular pili increases rapidly and the cooperativity, and thereby the lifetime, actually decreases. The unfolding velocity increases strongly with the force when $\eta_x < 1$ which implies that a low $\eta_x$ will induce a lifetime that decrease quickly when the force increases. In addition, a low $\eta_k$ gives rise to a high unfolding velocity for all forces, and will therefore more rapidly lead to a decreased lifetime when the force increases (assuming that the system also has an expected unfolding length that increases with force, i.e., $\eta_x < 1$). This phenomenon is visible in both panels for the curves with the lowest $\eta_x$ or lowest $\eta_k$ where the lifetimes quickly decrease with the force. The optimal parameter values depend on which force interval the binding system is to endure in. As is indicated in the Figure 5.5, it was found that P pili actually possess such beneficial internal properties that successful formation of a strong multipili binding system is possible throughout this extensive force interval.

The internal properties of the pili, that are completely independent of the adhesin–receptor bond, are actually crucial to the fate of the multipili binding system. These properties can prolong or reduce the lifetime of a system with several orders of magnitude. Another fascinating finding is that the internal properties of P pilus are in fact optimized, with respect to the binding strength of the specific bonds, to ensure strong multipili attachment of the bacteria.

It has been demonstrated in this study that the internal properties of the pili have a decisive role in multipili attachments by helix–like pili. This implies that the LL–bonds could constitute a possible target for new medications to prevent or neutralize colonization of bacteria which is the first step in an infection process. Moreover, it was elucidated that P pili possess internal biomechanical properties that optimize its multipili attachment over a wide range of forces and that it has an unfolding force level that is adjusted to its in vivo environment.
5.2. OPEN COIL–LIKE PILI

The open coil–like T4 pili expressed by *Streptococcus pneumoniae* lack the ability to elongate by means of unfolding. This can lead to the presumption that open coil–like pili constitute weak multipili attachments since it has been shown that large elongation ability promotes high cooperativity. However, the absence of an efficient elongation property of the T4 pili structures has, to some extent, been compensated for by other functionalities. The open coil–like pili express adhesins that are distributed along the entire protofilaments that build the pili, see Figure 4.10. It is hypothesized that the individual pili *in vivo* establish multiple adhesin–receptor bonds in a manner that is similar to the description of anchoring points in section 4.5. This means that the adhesin–receptor bonds that mediate attachment of a pilus can detach in a zipper–like manner when the pilus is exposed to force. The unbounded part of the pilus is well described by the WLC model and has a contour length that increases in discrete steps every time one or more specific bond detaches. These stepwise increases in contour length may actually be equivalent to a certain degree of elongation in the system.

Consider a dual–pili system where the second pilus has a force onset position that is 500 nm larger than that of the first pilus. The contour lengths of the unbounded parts of the pili are 200 and 500 nm for the first and second pili, respectively. The binding system is exposed to a constant external force of 100 pN which, as a consequence of the difference in force onset position, is initially completely taken by the first pilus. The unbounded part of the pilus will instantly be stretched according to the WLC description until the force response equals the external force whereby the elongation halts. The external force will cause the outermost adhesin–receptor bond on this pilus to rupture, i.e., the pilus undergoes a partial detachment, which implies that the contour length increases and the pilus is elongated furthermore. This procedure continues until the first pilus has elongated long enough for the second pilus to share the force. Figure 5.6A illustrates the force–response curve as the contour length increases by discrete steps as a consequence of partial detachments. The black and blue curves show the force responses from the first and second pili, respectively. The first pilus is in this conceptual study set to increase its contour length by 200 nm for each partly detachment. The sum of the force responses of the two pili corresponds to the total force acting on the bacterium when the pili are stretched and is plotted as a dashed green line which for small elongations follows the black curve closely since the second pilus takes a negligible force for these elongations.
The red curve shows the force that the first pilus must withstand to not undergo any detachments, which equals the difference between the external force and the force taken by the second pilus. This force is nearly equal to the entire external force until the first pilus has elongated a distance in close proximity to 1000 nm which is the sum of the difference in force onset positions and the contour length of the second pilus. In other words, the first pilus has at this point increased its contour length with ~800 nm which corresponds to the sum of the original differences in force onset positions and contour lengths of the two pili. The structural composition with distributed adhesins allows the open coil–like pili to extend their force exposure length from ~100 nm to ~1 µm which strongly enhance the cooperativity of a multipili system.

Figure 5.6. Panel A, the force response of a single T4 pilus when its contour length increases by discrete steps of 200 nm. The pilus can adjust its force exposure region by partly detachments and thereby effectively inhibit a force exposure length of approximately one micrometer. The black and blue lines correspond to the force responses of the first and second pilus, respectively. The green dashed line is the sum of these two responses which always sum up to the constant external force of 100 pN when the pili are stretched. The red curve shows the force acting on the first pilus for different elongations thereof. The force is efficiently shared between the two pili only when the first pilus has increased its contour length by ~800 nm. Panel B, the off–rates for any detachments in the systems as functions of the elongation of the first pilus. The off–rates have been normalized by the off–rate for a sequential system to eliminate the unknown thermal off–rate. Initially, the system acts as a sequential system while it transits to a simultaneous system when the force is shared equally. This transition is distinct and results in a decrease of the off–rate in the system by several orders of magnitudes depending on the bond length of the specific bond.
A significant external force acting on a bacterium is transmitted to the attached pili and ultimately to the outermost specific bonds. The sum of the off–rates for these bonds can be considered as the total off–rate in the system. The effect of the elongation of the first pilus by means of partly detachments can be analyzed by normalizing this total off–rate by the total off–rate of an equivalent system under sequential force exposure. This ratio is independent of the thermal off–rate but depends on the bond length that has been varied which resulted in a set of different ratio–elongation responses that are depicted in Figure 5.6B. It is evident that the first pilus will initially undergo partial detachments in a relatively rapid pace, corresponding to a sequential system, until the second pilus starts to share the force. At this point the total off–rate of the system decreases significantly since the system approaches the simultaneous force exposure mode, and the dual–pili system becomes more stable. The total off–rate for the system differs with several orders of magnitude between the sequential and simultaneous force exposures.

This conceptual study suggests that a multipili binding system of open coil–like T4 pili, that are unable to unfold, still possesses a cooperative property by partly detaching its adhesin–receptor bonds.
5.3. COMPARISON BETWEEN HELIX–LIKE AND OPEN COIL–LIKE PILI

At a single–pilus level, the structural compositions as well as the biomechanical properties of helix–like and open coil–like pili are completely different. These differences are reflected also in a multipili binding scenario where systems of these two types of pili have dissimilar mechanisms of handling large external forces. There are two key differences between the two systems. First, whereas helix–like pili express one adhesin at the tip of the pili, open coil–like pili express a multitude of adhesins distributed along their protofilaments. Second, helix–like pili can elongate by unfolding which open coil–like pili cannot.

The multitude of adhesins on single open coil–like pili increases the binding lifetime linearly with the number of adhesins since they can be expected to detach sequentially and have negligible rebinding probability under significant force exposure. In a comparison between the two cases, this property favours the open coil–like adhesion mechanism.

Single open coil–like pili can elongate ~1 µm but have a force exposure length of only ~100 nm. However, the multitude of adhesins can be assumed to effectively prolong the force exposure length by a factor of 10 as has been described in section 5.2. Even so, the force exposure length of the open coil–like pili clearly falls short of that of helix–like pili that is ~10 µm. This implies that both systems possess cooperativity to some extent even though the cooperativity of helix–like pili is evidently more prominent. Since the cooperativity is the key factor for a multipili binding system to withstand high forces, a system of helix–like pili is more likely to withstand high external forces.

There are other aspects that may be important in an in vivo situation. For instance, the difference in adhesin distribution implies that the Streptococcus pneumoniae is likely to be located close to the host cells while E. coli could be found several micrometers from the host cells while staying attached. The closeness to the surface may be of various significances depending on the divergence of the external force in the surrounding medium. The many factors involved in bacteria attachments give rise to complex analyses which is an intriguing challenge.
5.4. CONCLUDING REMARKS

Indeed, it is intriguing to observe how similar problems under different circumstances can be solved by completely different mechanisms. To understand multipili attachment, detailed knowledge of the biomechanical properties of single pili is required. Curiously, to understand the consequences of single pili properties, multipili attachment must be investigated.
Enterotoxigenic *Escherichia coli* (ETEC) are found in the intestinal tract where it causes diarrhea. It binds to the host epithelium by means of CFA/I pili that have an architecture that reminds of that of P pilus (83). The pili are mainly constructed by the composition of the subunit CfaB in a helix–like manner. Further on, the pili express, at the distal end, the subunit CfaE which has been found to mediate adhesion to host cells (84, 85).

Force spectroscopy was applied to single CFA/I pili to investigate their biomechanical properties. As in the case with other helix–like pili, the force response of the CFA/I pili displays a constant force plateau which corresponds to sequential unfolding of the helix–like structure. Despite this resemblance, significant dissimilarity of the constant force level is observed. The CFA/I pili have a steady–state unfolding force of only $7.5 \pm 1.5 \text{ pN}$ which implies that they start unfolding already at weak external forces. A typical force–elongation curve is displayed in Figure 6.1A. Moreover, WLC fits were performed for the part of the force–elongation response that corresponds to linearized pili, i.e., in the region where the constant force plateau has ended. The persistence length was found to be $4.5 \pm 1.4 \text{ nm}$ which is somewhat higher than the persistence length for P pilus that is $3.3 \pm 0.6 \text{ nm}$ for instance (71). However, this difference is within the margin of error.

Monte Carlo simulations were able to replicate measured data with high accuracy. The simulated data are depicted in panel A by a black dashed line that follows the grey curve perfectly.
Figure 6.1. Panel A, a typical force–elongation response of a CFA/I pilus is plotted in grey. A fit from a computer simulation is displayed in black which shows good agreement with the measured data. Panel B, a histogram of the distribution of steady–state unfolding forces, of which the mean value amounts to 7.5 ± 1.5 pN.

There are many resemblances between the force–elongation curved of the different helix–like pili presented in this thesis. The region I and region II are qualitatively equal to those of P pili even though the slope of region I and the force level of region II are different. However, there are distinguished dissimilarities in the responses in region III. Whereas P pili have a soft shape that corresponds to a conformational change of the HT–bonds, CFA/I pili show no such effect. The full understanding of the \textit{in vivo} consequences of the similarities and, perhaps even more interesting, the dissimilarities between these two types of pili remains a target for future work.
7. SUMMARY OF THE PAPERS AND MY CONTRIBUTIONS

This thesis is based on ten papers that are briefly described below together with a statement of my main contributions to each of them. In addition to the contribution explicitly stated for each paper, I have been responsible for extensive development of software for measurement procedures, data collection, and for the subsequent data analyses. Moreover, I contributed to a substantial part of the writing of the papers.

Paper I

Methods and Error Estimations in Single–molecule Dynamic Force Spectroscopy

The resulting errors in the acquired parameters in dynamic force spectroscopy were analyzed with respect to both measurement procedures and analyze methods. This includes the effect of the number of measurements, number of loading rates, choice of frequency function, choice of fitting function, as well as different types of noise.

My contributions to this paper involve all parts in the work; development of the theory, Monte Carlo simulations, and data analyses.
Paper II

Physical properties of the specific PapG–galabiose binding in *E. coli* P pili–mediated adhesion

This paper investigates the specific PapG–galabiose binding associated with the P pili expressed by *Escherichia coli*. The analysis is conducted by three separate methods to manage the non–linearity of the loading force that arises from the dynamic force–elongation response of the pili structure.

My contributions to this paper are development of novel data analysis methods, the related Monte Carlo simulations, and data processing.

Paper III

Measurements of the binding force between the *Helicobacter pylori* adhesin BabA and the Lewis b blood group antigen using optical tweezers

A model system for force measurements of the specific BabA–Lewis b binding was developed. This includes measurement procedures, custom made control programs for the hardware and data acquisition as well as software for the data analyses. When a model system were established that allowed for accurate binding measurements in an efficient manner, a large set of measurements were conducted.

My contributions to this paper include all parts of the project with emphases on software development, conducting measurements and analyzing the data.
Summary of the Papers and My Contributions

Paper IV

Dynamic Force Spectroscopy of the Helicobacter pylori BabA–Lewis b Binding

Dynamic Force Spectroscopy was applied by performing rupture force measurements on the specific BabA–Lewis b binding at different loading rates. The specific bond was characterized and intrinsic properties were obtained with this technique. This includes derivation of the bond length, the thermal off–rate, and classification of the bond as a slip–bond.

My contributions to this paper involved all parts.

Paper V

pH Regulated H. pylori Adherence: Implications for Persistent Infection and Disease

Extensive investigations of the acidity sensitivity of the Helicobacter pylori attachment is presented in this paper. Strains extracted from patients from different parts of the world were analyzed. The influence of acidity on the attachment and the way the bacterium has adopted to different environments were discussed. Measurements were performed in vitro, in situ, and in vivo. In particular, the specific BabA–Lewis b binding for one strain was characterized by dynamic force spectroscopy performed at four different pH–levels. This provides insight the way the specific bond is affected by the surrounding acidity.

My contributions reside to the dynamic force spectroscopy which includes measurement execution and data analyses. Moreover, I was involved in the quantification of fluorescence data by image analysis procedures.
Paper VI

Unraveling the Secrets of Bacterial Adhesion Organelles using Single Molecule Force Spectroscopy

The bond kinetics of helix–like pili was reviewed in detail including the theoretical models of the different force response regions and dynamic effect of the constant force plateau. Different types of pili were compared with respect to their biomechanical properties. A brief introduction to the multipili scenario was finally given.

My contributions to this paper include processing data and collecting information as well as performing analyses with emphasis on the multipili scenario.

Paper VII

Modeling of the elongation and retraction of *Escherichia coli* P pili under strain by Monte Carlo simulations

Previously obtained force–elongation response data of P pili was subjected to fitting procedures by a novel Monte Carlo simulation program. This program operates on a single bond level and includes all layer–to–layer bonds as well as all head–to–tail bonds of the pili structure. The method was shown to be able to reproduce the measured data with high accuracy.

My contributions to this paper were the construction of the Monte Carlo program as well as most of the data analyses.
Paper VIII

Characterization of the Biomechanical Properties of T4 Pili Expressed by *Streptococcus pneumoniae*

The open coil–like T4 pili expressed by *Streptococcus pneumoniae* were analyzed by means of force–elongation measurements. The resulting biomechanical properties were compared in detail to those of helix–like pili in general and P pili in particular.

My contributions to this paper involve conceptual data analyses of the force responses as well as determination of the persistence lengths by wormlike chain fits.

Paper IX

Multipili Attachment of Bacteria Exposed to Stress

The knowledge of the biomechanical properties of single pili and their adhesin–receptor bonds were introduced into a multipili scenario. The properties of the pili structures, defined by its architecture and internal bonds, were found to have a monumental influence on the binding capabilities of a multipili system exposed to an external force. The analyses were performed by analytic derivations complemented by Monte Carlo simulations.

My contribution includes development of the theory and the Monte Carlo program. The analyses were performed in cooperation with O. Axner.
**Paper X**

**Differentiating Pili Expressed by Enterotoxigenic and Uropathogenic *Escherichia coli* with Optical Tweezers**

The CFA/I pili are expressed by *Escherichia coli* in the intestinal tract where they mediate adherence to the epithelium. The biomechanical properties of the pili structure were scrutinized by means of force–elongation measurements.

My contributions to this paper include data analyzes and in particular determination of the steady–state unfolding level as well as investigations of the slope and derivation of the persistence length of the fully linearized pili. Moreover, my contribution includes the Monte Carlo simulations and analyses thereof.
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9. REFERENCES


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