Time-dependent tethered particle motion for measuring dissociation kinetics of short complementary DNA oligonucleotides

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Abstract

Tethered particle motion (TPM) is a well-known method for studying mechanical and dynamical properties of biological molecules on a single-molecular level. In TPM experiments, a microparticle is tethered to a surface via the molecule under study. The motion of the microparticle serves a reporter of the properties of the molecule. In this research we explore the possibility to use TPM for studying interactions between complementary short single stranded DNA oligonucleotide. For this purpose the tethered particles and the surface are functionalized with complementary oligonucleotides. The lifetimes of the binding events are analyzed to obtain a dissociation rate constant for the oligonucleotide interaction. This method allows to observe the kinetics of single-molecule binding events, it is possible to measure in very low concentrations and a robust signal is obtained from the microparticles.

The interaction between short complementary single stranded DNA oligonucleotides of 8 and 10 base pairs (bp) is measured and compared with literature values. Oligonucleotides are used because their interaction can be tuned by increasing or decreasing the amount of base pairs. Two systems are explored: The short-tether model system (STMS) consisting of 1000 nm magnetic particles and 40 nm (120 bp) dsDNA tethers and the long-tether model system (LTMS) consisting of 800 nm magnetic particles and 330 nm (1008 bp) dsDNA tethers. Multiple binding events of single molecules are observed for multiple particles. A homemade Matlab algorithm is used to discern binding events in the time-dependent motion of the tethered particles.

We succeeded to find dissociation constants for both oligonucleotide lengths in both model systems: For the 8bp oligonucleotide a $k_{off,8bp,STMS} = 0.10 \ (0.08 - 0.15) \ s^{-1}$ is found and a $k_{off,8bp,LTMS} = 0.065 \ (0.051 - 0.083) \ s^{-1}$ is found relative to a literature value $k_{off,8bp,litt.} = 0.1 \ s^{-1}$. For the 10bp oligonucleotide a $k_{off,10bp,STMS} = 0.0039 \ (0.0031 - 0.0054) \ s^{-1}$ is found and a $k_{off,10bp,LTMS} = 0.0026 \ (0.0019 - 0.0047) \ s^{-1}$ is found relative to $k_{off,10bp,litt.} = 0.01 \ s^{-1}$. In the measurement of the 8bp oligonucleotide in the LTMS two populations of binding events are observed. One population is explained by the oligonucleotide interaction, the origin of the other population with $k_{off} = 0.055 \ (0.0040 - 0.0088) \ s^{-1}$ remains unknown. Direct rebinding of the oligos after dissociation due to the slow diffusion of the particle might be the origin of this double exponential behaviour. A more probable possible explanation is non-specific binding between the particle and the surface. The surface functionalization of the particles in the LTMS is not optimized to suppress non-specific interaction whereas for the particles in the STMS they are. This hypothesis has been checked by repeating the 60 minutes measurement without both the particle-oligos and the surface-oligos. Only a very low amount of non-specific interaction was observed and no dissociation constant could be determined. More experiments would need to be performed to draw definite conclusions from this.
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1. Introduction

Due to the increasing load on the medical health care system, fast and easy-to-use diagnostic tools called point-of-care biosensors provide an effective way for diagnosing patients\(^1\). A point-of-care biosensor is a small, handheld device with which the presence and concentration of a target molecule in body fluid can be detected\(^2\). These target molecules, called analytes, are mostly diagnostic markers for diseases\(^3\). Examples of analytes are antibodies, proteins that are produced by the immune system to identify bacteria and viruses, or troponin, a marker for heart disorders in particular for myocardial infarction\(^4\). Point-of-care biosensors should work fast, that is, within a visit at the general practitioner. They should also be sensitive up to pM to fM concentrations. Also they should be able to measure in small volumes of body fluids, for example in a droplet of blood, urine or saliva. An example of such a point-of-care biosensor is a glucose sensor\(^5\). It measures the concentration of glucose in the blood of a diabetic person. Based on the value measured by the sensor the diabetic person knows if he needs to inject insulin into his body. Other examples of point-of-care biosensors are a pregnancy test and an alcohol test.

An assay is a bioanalytical test on which a biosensor can be based. In a sandwich immunoassay the analyte is sandwiched between a sensor surface and a label by coating the sensor surface and the label with a specific binding agent for the analyte. The label is used for detecting the number of analyte molecules that binds to the sensor surface. This label can for example be a micro- or nanoparticle\(^6\), a fluorescent molecule\(^7\) or an enzyme\(^8\). Figure 1.1 shows a schematic representation of a sandwich immunoassay in which magnetic microparticles are used as the label. The bond between microparticle, analyte and sensor surface needs to be specific for the analyte. For example antibodies are produced by the immune system specifically against an antigen and, therefore, they are often used in immunoassays. The biosensor system in figure 1.1 works as follows: The fluid containing the analyte is introduced in the system and the microparticles bind to the analyte, figure 1.1a. The microparticles are pulled to the sensor surface by a magnet such that the analyte can bind also to the sensor surface, figure 1.1b. The particles that have not bound an analyte are pulled to the upper surface, figure 1.1c. The bound particles stay on the sensor surface. In the end the number of particles bound to the sensor surface is a measure of the analyte concentration.

![Figure 1.1 Schematic representation of a sandwich immunoassay in which analyte molecules are sandwiched between a sensor surface and a label\(^4\). Here, magnetic microparticles are used for labelling. (a) The fluid containing the analyte is introduced in the system and the microparticles bind to the analyte. (b) All microparticles are magnetically pulled towards the sensor surface and subsequently pulled towards the upper surface several times. (c) Particles that have not bound an analyte after several cycles are magnetically pulled towards the upper surface.](image)

However, not all particles that have bound to the sensor surface are bound via an analyte molecule. Some particles bind non-specifically to the sensor surface without an analyte molecule in between. One
would like to distinguish between specifically bound particles and non-specifically bound particles to obtain a better accuracy of the analyte concentration. A closer look is taken at the bound microparticle in figure 1.1c. The microparticle has some freedom of motion. Due to the antibody-analyte-antibody complex the microparticle is not stuck on the surface. This antibody-analyte-antibody complex works effectively as a tether and the microparticle makes tethered particle motion, as can be seen in figure 1.2a. Recent experiments in our laboratory have shown that it is possible to distinguish between specifically bound particles and non-specifically bound particles by looking at the tethered particle motion.

Tethered particle motion (TPM)\textsuperscript{9,10,11} is a well known technique for studying the properties of molecules. In this technique the molecule of interest, often DNA, is sandwiched between a sensor surface and a particle so that the molecule effectively works as a tether. The particle undergoes Brownian motion limited by the length of the tether, as shown in figure 1.2b. In TPM experiments the tether is typically larger than the diameter of the particle. The particle’s motion is influenced by the mechanical properties of the tethering molecule and the environmental conditions. In this way the motion of the particle is a reporter of the conformation and behaviour of the molecule.

Tethered particle motion is used in many different types of research. Examples are quantification of the rate of synthesis of proteins by ribosomes\textsuperscript{12}, measurement of twist and torque of single DNA molecules\textsuperscript{13}, measurement of the kinetics of DNA looping by LAC repressor\textsuperscript{14} and measurements of the persistence length of double stranded DNA molecules\textsuperscript{15}. In this report TPM is used in a different fashion. In contrast to the experiments described above our interest lies not in the properties of the tethering molecule but in the motion of the tethered particle and the interaction between the particle and its local environment on the sensor surface. The tethers used in this study are relatively short compared to common TPM studies.

In tethered particle motion experiments the motion of a particle is observed using standard dark field or bright field microscopy and recorded with a camera. The in-plane position, in terms of x and y coordinate, of the particle is determined from the recordings of the camera. The collection of positions of the particle during the measurement is represented in a motion pattern. Figure 1.2c shows such a motion pattern for one particle. Each dot represents the position of the particle at a certain point in time. The motion pattern has a circular geometry, because the motion of the particle is restricted by the finite tether length.

![Figure 1.2](image_url)

**Figure 1.2**  (a) Schematic representation of a particle that is attached to a surface via an antibody-analyte-antibody complex and therefore has some freedom of motion. (b) A schematic representation of a particle tethered to a sensor surface. (c) A motion pattern of the microparticle. This graph shows the x-and y-coordinate of the positions of the particle over 60 s. Each dot corresponds to the centre of the microparticle being at a certain position at a certain point in time.
Recent experiments have shown that a motion pattern of a particle can change its shape over time. In other words, the motion of the particle is non-uniform in time. The observed time-dependent behaviour suggests the formation and breakdown of bonds between the particle and the surface. Several research questions can be: How can we identify this time-dependent behaviour? What criteria should be used to discern time-dependent behaviour? The first goal of this research is to examine the time-dependent behaviour in a tethered particle motion system by analyzing the motion of the particle in terms of several functions, like mean squared displacement and the step size.

The time-dependent behaviour observed in tethered particle motion experiments provides the opportunity to use the tethered particle as a probe for its environment. Specific binding between the tethered particle and a surface with can be probed. It is interesting to design a model system with which it is possible to observe the particle binding and unbinding with the surface in a controlled way. In this thesis we explore the potential of this system to be used for measuring dissociation rate constants of short single stranded DNA oligonucleotides on a single-molecular level. The advantages of this technique over other techniques that are able to measure dissociation rate constants would be: Firstly that it would be possible to measure in very low concentrations since one target molecule would suffice, secondly that the analyte molecule does not need to be labelled and thirdly a clear and stable signal is obtained using the micrometre sized particles.

The time-dependence in the TPM system is also interesting for application in biosensors. For example, imagine a microparticle that is coated with a binder for a certain analyte tethered to a surface that is also coated with this binder. When the analyte is now present in the sample, the particle could become bound to the surface by catching an analyte molecule from the solution and binding with it to the surface. The motion of the particle in time will then depend on the concentration of analyte in the solution. Low affinity binders can be studied with this biosensor, since bonds can be probed that have a too short lifetime to be probed in existing biosensor technologies.

This report consists of 8 chapters. In chapter 2 the theory of tethered particle motion is described. Also a phenomenological description of binding in the TPM system is given. Finally Monte Carlo simulations and simulations on dissociation, that are compared with the experimental results, are explained. Chapter 3 contains a complete description of the measurement system, the experimental setup and the analysis method of the raw data. Subsequently in chapter 4 the analysis of the time-dependent behaviour in the tethered particle motion system is described. Chapter 5 describes the experimental results on the model systems and chapter 6 sums the conclusions of this report. In chapter 7 and chapter 8 a list of the used literature and the appendix are given, respectively.
2. **Theory**

Tethered particle motion (TPM) is a technique by which usually mechanical properties of tethering molecules are studied as a function of time and on a single-molecular level\(^{16}\). However, the research described in this report focuses on the time-dependent behaviour observed in tethered particle motion. Our aim is to use TPM for studying binding properties on a single-molecular level. This chapter serves to give a more detailed description of how tethered particle motion can be used to obtain quantitative information about binding and unbinding.

### 2.1 Tethered particle motion for studying dissociation kinetics

The model system that is being studied in this research, represented in figure 2.1, consists of a tether and a particle. The particle is bound to a surface by the tether. The properties of the particle, the tether and the combination of tether and particle are discussed in section 2.2. The particle and the surface both contain a binding site with a specific and selective affinity for each other. The number of binding sites on the surface is low, ideally one, and the number of binding sites on the particle is large. When these binding sites are close together a bond can be formed. Due to the tether the particle is kept close to the surface such that the chance of binding is higher and the same binding sites are being probed. In section 2.3 more information about binding will be given and in section 2.4 a description of binding is given for the TPM system. The goal of this system is to observe binding and unbinding between a binding site on the particle and the binding site on the surface to obtain quantitative information on a single-molecular level about the interaction that is being studied. Monte Carlo (MC) simulations and simulations on dissociation, used to support the experimental observations, are explained in section 2.5.

![Figure 2.1](image)

Figure 2.1 Schematic representation of the tethered particle motion system that is being studied in this research consisting of a particle tethered to a surface. Both on the particle and the surface there are binding sites with a specific interaction. The goal of this system is to measured binding and unbinding of the particle with the surface to obtain quantitative information on the interaction between the binding sites.

### 2.2 Properties of particle and tether

The potential to use TPM to study binding and unbinding between particle and surface is exploited in this research. In this section the properties of the particle, the tether and the combination of particle and tether are explained in separate subsections.
2.2.1 Properties of the particle

In conventional tethered particle motion experiments, the diameter of the particle that is used ranges from several nanometres to a few micrometres. These particles are often referred to as beads, however, in this thesis the word particle will be used. Different types of particles are used in TPM experiments, for example polystyrene particles, fluorescent particles and magnetic particles. The size and the type of particles determines the possible ways to actuate and detect the particles in the experiment. These parameters need to be chosen to match with your bioanalytical system. In the system exploited in this research magnetic particles are used that consist of a polymer matrix with many small magnetic iron oxide ($\text{Fe}_2\text{O}_3$) grains. Magnetic particles can be actuated with a magnet and can be detected with standard light microscopy.

A free particle in solution moves due to random thermal fluctuations: Brownian motion. When the particle is tethered it still makes a random thermal motion, however, within a confined volume. This volume is restricted by the length of the tether. When the particle reaches a position at which the tether is fully stretched, the entropic spring effect will make the particle experience the presence of the tether as a pulling force and change its direction. The influence of the tether on the random thermal motion of the particle in non-stressed configurations of the tether may also be significant.

The diffusion coefficient quantifies the diffusive motion of the particle. The diffusion coefficient for spherical particles in a solution with a low Reynolds number is given by the Stokes-Einstein equation shown in equation 2.1. The diffusion coefficient depends on the ratio of the thermal energy $k_B T$ and a drag term. Here $R$ is the particle radius and $\eta$ is the dynamic viscosity of the solution. This equation shows that diffusion is slower for larger particles and more viscous fluids.

$$D_0 = \frac{k_B T}{6\pi \eta R}$$  \hspace{1cm} (2.1)

It is important to note that the Stokes-Einstein equation is only valid when the particle is far from any surface. However, in TPM experiments the particle is close to a surface. The distance between the particle and the surface can become smaller than the diameter of the particle, i.e. the distance at which surface induced drag effects start to play a role. The deviation of the Stokes-Einstein equation close to a surface can be described by an effective diffusion coefficient. Close to a surface the diffusion coefficient diverges into two components, one perpendicular and one parallel to that surface. Equation 2.2 and 2.3 show approximate analytical solutions of the parallel and the perpendicular diffusion coefficient, respectively. This separation between perpendicular and parallel is due to a wall drag effect.

$$D_\parallel = \frac{D_0}{\lambda_\parallel} = \left[1 - \frac{9}{16} \frac{R}{Z} + \frac{1}{8} \left(\frac{R}{Z}\right)^3 - \frac{45}{256} \left(\frac{R}{Z}\right)^4 - \frac{1}{16} \left(\frac{R}{Z}\right)^5\right] \frac{k_B T}{6\pi \eta R}$$  \hspace{1cm} (2.2)

$$D_\perp = \frac{D_0}{\lambda_\perp} = \left(1 + \frac{R}{Z - R}\right)^{-1} \frac{k_B T}{6\pi \eta R}$$  \hspace{1cm} (2.3)

Here $Z$ is the height of the centre of mass of the particle above the surface and $D_0$ is the diffusion coefficient for particles far from the surface, as defined in equation 2.1. For example, a spherical particle with a radius of 500 nm, at a height of 520 nm above the surface, in water, at a temperature of 293 K, has diffusion coefficients equal to $D_0 = 4.29 \cdot 10^{-13} \text{m}^2\text{s}^{-1}$, $D_\perp = 1.65 \cdot 10^{-14} \text{m}^2\text{s}^{-1}$ and $D_\parallel = 1.58 \cdot 10^{-13} \text{m}^2\text{s}^{-1}$. This shows that the particle diffuses faster parallel to the surface than perpendicular to the surface, but move slower in any direction than when the particle is far from the surface. From the
diffusion coefficient the average net displacement $\bar{x}$ that a particle makes, in 2 dimensions, in a time $t$ can be calculated with equation 2.4.

$$\bar{x} = \sqrt{\langle x^2 \rangle} = \sqrt{4D t} \quad (2.4)$$

Particles are in general not perfect spherical objects, they have a certain surface roughness. The surface roughness mainly influences the effective tether length. The effective tether length is defined as the actual tether length plus the deviation of the local radius of the particle from the minimum radius of the particle. The effective tether length will depend on the position of the attachment point of particle and tether. If the tether attaches to a protrusion on the particle, the effective tether length will be larger than the actual tether length. But if the tether attaches to an indentation on the particle, then the effective tether length is about equal to the actual tether length. For rough particles the distribution of effective tether lengths will be broader than for particles with a smooth spherical shape. Figure 2.2a and 2.2b show this effect schematically for the attachment point on an indentation and a protrusion on the particle, respectively. This broadened tether length distribution will lead to a distribution in the size of motion patterns, as will be shown in chapter 4.

![Figure 2.2](image.png)

Figure 2.2 Schematic representation of the variation in effective tether length due to surface roughness of the particle. Two extreme cases are shown here: (a) The effective tether length is equal to the actual tether length for the attachment point of the tether on the particle in an indentation. (b) The effective tether length is larger for the attachment point of the tether on the particle on a protrusion.

The density of the particle determines if gravitational effects will influence the dynamical properties of the particles on the typical time tethered particle motion is studied in an experiment. The sedimentation velocity $v$ of a spherical object can be calculated with Stokes’ law, see equation 2.5. Here $g$ is the gravitational acceleration, $R$ is the radius of the spherical object, $\mu$ is the dynamic viscosity of the fluid and $\rho_p$ and $\rho_f$ are the mass density of the particles and the fluid, respectively. For particles with a higher mass density than the fluid ($\rho_p > \rho_f$) the velocity is negative and thus the particle will sediment.

$$v = -\frac{2}{9} \frac{(\rho_p - \rho_f)}{\mu} g R^2 \quad (2.5)$$

### 2.2.2 Properties of the tether

In conventional TPM experiments the tethering molecule is being studied. The purpose of the tether in our system is just to keep the particle close to the surface, so that interactions between particle and surface are probed very efficiently. In many TPM studies a double stranded DNA chain with a length
between hundreds of nanometres and a few micrometres is used as the tethering molecule. Other molecules like polymers or single stranded DNA or RNA can also be used. A lot is known about the properties of dsDNA as a tether and many protocols for designing dsDNA have been established. For these reasons a dsDNA tether is used in this research.

The persistence length is an important parameter which characterizes the flexibility of a linear macromolecule. On a scale far below the persistence length a polymer acts stiff, whereas on a scale much larger than the persistence length the tether acts as a flexible polymer. In other words, using a dsDNA chain with a length much longer than the persistence length does not influence the motion of the particle drastically. However, a tether with a length much shorter than the persistence length is not flexible anymore, but acts more like a stiff rod. The tether now energetically favours a stretched configuration and thus the particle’s motion also has a preference for being far away from the attachment point of tether on the surface. The persistence length of dsDNA is 50 nm\textsuperscript{18,19}.

Ideally the tether has no influence on the motion of the particle. The tether should only keep the particle in a small hemispherical volume close to the surface. However the tether does influence the motion of the particle. An important effect is tether exclusion from the surface and the particle. When the particle comes close to the attachment point of the tether on the surface, the tether needs to fold up due to the steric hindrance of the particle. Only few configurations of the tether are possible when the tether is folded up. This effect gives rise to an entropic stretching force on the tether. In other words, the particle being close to the attachment point of tether and surface is possible, but it is not very probable. Therefore the particle will not be observed very often at that position. This also applies for stretching the tether. As the particle is very far away from the surface, the tether is fully stretched and only one configuration of the tether is possible. In stretched configurations the tether experiences an effective pulling force. The tether has an entropic preference for intermediate configurations.

2.2.3 Combination of particle and tether
In the last two subsections the properties of the particle and the tether have been discussed separately. However, it is the combination of particle and tether that determines the dynamics of the system. If the size of the particle is very large compared to the length of the tether, then the particle’s motion is very much restricted and in the case of a small particle on a large tether, the particle is relatively free to move. Illustrations of both cases are shown in figure 2.3.

The combination of particle and tether also determines several key parameters for binding in the TPM system. From pure geometrical reasoning it follows that the area on the particle that can bind to the surface is very different for the two extreme cases shown in figure 2.3. For a large particle on a short tether, only a fraction of the area on the particle can interact with the surface. For a small particle on a long tether, the whole particle can interact with the surface since the particle can fully rotate while not interrupting the tether. The rate at which a particle hits the surface decreases for longer tether lengths, because the particle probes a larger volume. But this rate is increased for smaller particles since they diffuse much faster. More details on the binding properties of the TPM system will be given in section 2.3.

Segall et al.\textsuperscript{20} have shown that the presence of the particle exerts an effective force on the tethering molecule. Due to the proximity of the particle to the surface which gives rise to the volume-exclusion effect, the tether experiences an entropic stretching-force. This entropic effect arises from the fact that the closer the end-point of the tether is to the surface the less configurations there are to place the particle on the tether without overlapping the particle with the surface. The presence of the particle
results in a less dense phase-space for the tether at close proximity to the surface. This can be interpreted as an effective entropic stretching force acting on the tether. According to Segall et al., the interplay between the tether motion and the particle motion is described by a dimensionless parameter, the excursion number $N_R$. This excursion number is a measure of the friction coefficient of the particle and the tether. Equation 2.6 gives the definition of the excursion number $N_R$. Here $R_b$ is the radius of the particle, $l_c$ is the contour length of the tether and $l_p$ is the persistence length of the tether. If $N_R > 1$ then the dynamics of the system is dominated by the particle. If $N_R < 1$ then the dynamics is dominated by the motion of the tether.

$$N_R \equiv \frac{R_b}{\sqrt{l_c l_p}}$$

$\{ \begin{array}{ll}
N_R > 1 \rightarrow & \text{dynamics is particle dominated} \\
N_R < 1 \rightarrow & \text{dynamics is DNA dominated}
\end{array}$

(2.6)

Figure 2.3  Schematic representation of two tether-particle systems with different geometries. On the left a large particle on a relatively short tether is shown and on the right a small particle on a relatively large tether. These two extreme cases of TPM systems have different properties in terms of particle-tether interplay.

### 2.3 Binding kinetics

The goal of this research is to study binding within the tethered-particle system at a single-molecule level. In this section a brief theoretical background on binding kinetics is given. The first subsection deals with ensemble reaction kinetics, the second subsection focuses on single-molecule binding and the third subsection is about association and dissociation rate constants.

#### 2.3.1 Reaction kinetics

The classical theory of reaction rate kinetics describes the reaction rates for a large ensemble of molecules. In a system where molecule $A$ can bind with molecule $B$ to form the complex $AB$. The rate at which $A$ and $B$ bind to form $AB$ is called the association rate, $k_{on}$. The rate at which $AB$ complexes breakdown into $A$ and $B$ molecules is called the dissociation rate, $k_{off}$. Equation 2.7 shows the reaction equation of this general binding process.

$$A + B \rightleftharpoons AB$$

$$k_{on} \quad k_{off}$$

(2.7)

The association rate constant, $k_{on}$, is defined as the rate at which $AB$ complexes are formed per unit time, per mole of $A$ and per mole of $B$, as defined in equation 2.8. The unit of the association rate is $M^{-1}s^{-1}$, it depends on the concentration of the reactants. The dissociation rate constant, $k_{off}$, is defined
as the rate at which \( AB \) complexes dissociate into \( A \) and \( B \), as defined in equation 2.9. The unit of the off rate is \( \text{s}^{-1} \). The dissociation rate constant is a measure of the interaction strength of the bond. The overall affinity of the reaction is quantified in the affinity or equilibrium association constant \( K_a \) with unit \( \text{M}^{-1} \), see equation 2.10.

\[
\frac{d[AB]}{dt} = k_{\text{on}} [A] [B] \tag{2.8}
\]

\[
\frac{d[A]}{dt} = \frac{d[B]}{dt} = k_{\text{off}} [AB] \tag{2.9}
\]

\[
K_a = \frac{k_{\text{on}}}{k_{\text{off}}} = \frac{[AB]}{[A][B]} \tag{2.10}
\]

### 2.3.2 Single-molecular bonds

The interest lies in probing single-molecular bonds and not in ensemble averages. The ergodic theorem tells us that the time average of a single bond is equal to the ensemble average. The classical description for the dissociation of single-molecular bonds under an external force is thoroughly described in a paper by Evan Evans\(^{21}\). An external force can be applied to break the bond, but also thermal fluctuations can be strong enough to break the bond. Figure 2.4 shows the potential landscape of a single-molecular bond as a function of the molecular coordinate \( x \). There is an activation energy, \( E_a \), that needs to be overcome in order to form a bond. When the bond is formed, the potential energy will be at its lowest. The deeper this potential well, the stronger the bond is and hence the less probable it is that the bond is broken by thermal fluctuations.

\[
\begin{align*}
\text{Figure 2.4} & \quad \text{Schematic graph of the potential energy landscape of a single-molecular bond as a function of the molecular coordinate } x. \\
& \quad \text{There is an activation energy } E_a \text{ that needs to be overcome to form the bond and an energy barrier } E_b \text{ that needs to be overcome to dissociate the bond.}
\end{align*}
\]

In some molecular bonds the interaction is very weak\(^{21,22}\), that is to say \( E_b \sim k_b T \). Thermal fluctuations can break these molecular bonds on the time scale of seconds. Forces do not need to be applied to break the bond. The dissociation rate \( k_{\text{off}} \) is a measure of the rate at which bonds are broken. It represents the number of bonds broken per second. The parameters that determine the \( k_{\text{off}} \) will thus be the energy barrier of the bond \( E_b \), the thermal energy of the molecules \( k_b T \) and the attempt frequency with which the molecules are trying to pass the energy barrier \( v \). Equation 2.11 shows the
The Arrhenius equation for the dissociation rate. Here, the attempt frequency $\nu$ is set equal to the vibrational frequency of the molecules which is about $10^9$-$10^{10}$ s$^{-1}$. The characteristic binding time $\tau_{off}$ is the reciprocal of the dissociation rate, see equation 2.12.

$$k_{off} = \nu \cdot \exp \left( \frac{-E_b}{k_BT} \right) \quad (2.11)$$

$$\tau_{off} = \frac{1}{k_{off}} \quad (2.12)$$

The association rate $k_{on}$ is a measure of the rate at which a bond is formed per second per molar. Association consists of a diffusive hitting step and an intramolecular chemical reaction. The diffusive rate is determined by the concentration of the reactants, whereas the rate of the chemical reaction is determined by activation energy $E_a$. The diffusive hitting step is different in the TPM system compared to the system of free particles in solution, as will be described in section 2.4.

### 2.3.3 Dissociation rate constants

The system that is being studied consists of a tethered particle that can make an additional bond with the surface with the binding sites on the particle and the surface. Therefore the system can only be in two states: Bound or free. As the system evolves over time, the system will visit each of the two states multiple times. Figure 2.5a shows what the evolution of the system over time could look like. Here the system starts off in the free state, after which binding occurs. After a certain amount of time, called the bound lifetime, the bond dissociates and the system ends up in the free state again. From the evolution of the system over time the bound lifetimes can be overlaid as shown in figure 2.5b. From the bound lifetimes of many of these events the dissociation rate constant is determined.

The process of unbinding is an example of a stochastic process, hence the time it takes for a bond to break is random and can be described by a distribution of times with a well defined characteristic time. For a single bond system dissociation can be described as follows: At time $t = 0$ a bond is formed, the system is in the bound state. At this point the probability that the system is in the bound state is equal to one, see equation 2.13.

$$P_b(t = 0) = 1. \quad (2.13)$$

The probability for the bond to dissociate is in every time interval $\Delta t$ the same. This is where the stochasticity of the dissociation process comes into play. The process does not have a memory. The probability to unbind is equal for $t = 0$ to $t = \Delta t$ and $t = t_1$ to $t = t_1 + \Delta t$ where $t_1 \gg 0$. The time evolution of the probability that the system is still in the bound state at time $t$ decreases proportional to the probability itself, see equation 2.14. Solving this differential equation gives the probability to observe the system in the bound state as a function of time, see equation 2.15. The integration constant $P_b(0) = 1$ was already shown in equation 2.13. The probability that a bond has already dissociated, $P_d(t)$, is shown in equation 2.16.

$$\frac{dP_b(t)}{dt} = -k_{off}P_b(t) \quad (2.14)$$

$$P_b(t) = P_b(0) \cdot \exp(-k_{off} \cdot t) \quad (2.15)$$

$$P_d(t) = 1 - P_b(t) = 1 - \exp(-k_{off} \cdot t) \quad (2.16)$$
When performing measurements a series many binding events of a single tethered-particle will be measured. These binding events can be translated in time to regard them as set of bound particles, \( N_b \). The evolution of the number of bonds that are still bound at time \( t \), \( N_b(t) \), is called the cumulative decay function. Equation 2.17 shows the cumulative decay function. Figure 2.5c shows the graph of the cumulative decay schematically. Fitting the cumulative decay function with equation 2.17 gives the dissociation rate constant. Figure 2.5d shows a semi-logarithmic representation of the cumulative decay. The logarithm of the number of bonds scales linearly with the dissociation constant, see equation 2.18.

\[
N_b(t) = N_b(0) \cdot \exp(-k_{off} \cdot t) \tag{2.17}
\]

\[
\log[N_b(t)] = \log[N_b(0)] - k_{off} \cdot t \tag{2.18}
\]

Figure 2.5 Schematic representation of: (a) The time trace of the state in which the system is, bound or free. (b) Overlaying the bound lifetimes obtained from a single time trace, the data is analyzed as if many binding events are measured at the same time. (c) The cumulative decay of the number of bonds as a function of the bound lifetime, which follows equation 2.17. (d) Logarithm of the number of bonds as a function of the bound lifetime, which follows equation 2.18.

### 2.4 TPM as a system for studying molecular bonds

In this thesis tethered particle motion is exploited as a system for studying molecular bonds by probing specific binding between a binding site on the particle and a binding site on the surface. The process of binding can be separated into two separate processes: Two binding sites need to come close enough to each other, called hitting, and subsequently a bond needs to be formed, called sticking. With both of these processes a different characteristic time scale is associated. The process of hitting is, in the TPM system, limited by the diffusion of the tethered particle. The characteristic time scale on which hitting will occur is therefore equal to the radius of the particle squared divided by the diffusion coefficient of the particle, see equation 2.19.

\[
\tau_{hit} = \frac{R^2}{D_L} \approx 10^{-1} \text{ s} \tag{2.19}
\]
When two binding sites are close enough to each other, there is a possibility for making a bond. This process of attempting to make the bond is governed by the molecular vibrations of the reacting molecules. This frequency is about $10^9 - 10^{10}$ s$^{-1}$ and thus the time scale for attempting to make the bond is about $10^{-10} - 10^{-9}$ s. The processes of hitting and sticking happen on different time scales, they differ about eight orders of magnitude. This means that when the binding sites are at some point close enough to each other to form a bond, they are constantly attempting to actually form the bond while the particle effectively stands still. Since the processes of hitting and sticking are on totally different time scales, they can be separated and treated independently.

The binding of the particle to the surface is modelled by a two step binding process. Figure 2.6 shows a schematic representation of the two step binding process. At first the system is in the free state. The first step in forming a bond is to get the binding sites close together. This diffusional hitting step between the two binding sites, the particle binding site (B) and the surface binding site (S), happens at a rate $k_{hit}$. The system is now in the transient state. Subsequently there are two possibilities: Either a bond is formed or the particle diffuses away such that the binding sites are separated. For each possibility a rate can be defined, respectively, the rate of sticking $k_{stick}$ and the rate of separation $k_{sep}$. If the bond is formed the system enters the bound state and there is a certain rate at which the bond dissociates $k_{off}$. This is the dissociation rate constant that was already defined in equation 2.11.

![Diagram of the two step binding process](image)

Figure 2.6  Schematic representation of the two step binding processes in the TPM system. Firstly there is a diffusional encounter step, called hitting, and secondly there is a intramolecular binding step, called sticking. The rates describing the transitions of the system from the free state to the transient state to the bound state and vice versa are also indicated.

The goal is to obtain the dissociation constant $k_{off}$. However, in the experiment only the free state and the bound state can be separated. Not the dissociation rate but the unbinding rate is measured. Equation 2.20 show the dependency of the unbinding rate on the dissociation rate. Note that the unbinding rate is equal to the dissociation rate times one minus the probability to rebind directly after dissociation, $P_{rebind}$. In the limit of a zero rebinding probability the measured unbinding rate is equal to the dissociation rate.
In the tethered particle motion system the hitting step is different from the case of free particles in solution while the sticking step is equal since the intramolecular chemical reaction is not modified. The hitting rate depends on several parameters: Tether length, particle size, distance between the binding site on the surface and the tether point on the surface and the distance between the binding site on the particle and the tether point on the particle. Simulations on the optimal parameters for obtaining the highest association rate in the TPM system are performed by Koen Merkus, a companion of the theoretical group on polymers and soft matter (TPS)\(^{13}\).

\[
\kappa_{\text{unbind}} = k_{\text{off}} \cdot \frac{k_{\text{sep}}}{k_{\text{stick}}+k_{\text{sep}}} = k_{\text{off}} \cdot \left(1 - \frac{k_{\text{stick}}}{k_{\text{stick}}+k_{\text{sep}}}\right) = k_{\text{off}} \cdot (1 - P_{\text{rebind}}) \quad (2.20)
\]

2.5 Simulations on tethered particle motion

The experimental work performed during this research is accompanied with a theoretical counterpart. Monte Carlo simulations have been performed by Emiel Visser (PhD student, supervisor) on the tethered particle motion system. In the first subsection a basic description will be given of how this simulation method works, how TPM is implemented in the simulations and for what purpose these simulations will be used. Also simulations on the stochastic process of dissociation are performed. In the second subsection a description of these simulations is given.

2.5.1 Monte Carlo simulations of TPM

The Monte Carlo (MC) simulation method is generally used to determine the partition function of a system. In other words, the Monte Carlo method samples the distribution of all possible states that a system can be in with the probability corresponding to that state. The Monte Carlo method used in this research does not give the evolution of the system over time, but only the equilibrium distribution of the system. With the Monte Carlo method one can simulate the tethered particle system in order to obtain the equilibrium distribution of the positions of the particle. The goal of using the Monte Carlo simulations is to check if the experimentally observed motion patterns agree with the theoretically obtained equilibrium distribution simulated with the Monte Carlo method. Also surface roughness effects and multiply tethered particles can be simulated with this MC method.

In the Monte Carlo method used two parameters need to be inserted; first the program needs to be told how the system is built up and secondly a number of boundary conditions should be given. The program now randomly builds up a state of the system and checks if that state satisfies the boundary conditions. If all boundary conditions are met, the state is accepted and the program starts over. But if at least one of the boundary conditions is not met, the state is rejected and the program tries again. After having obtained \(N\) accepted states, the program gives the distribution of these states, which is, for infinitely large \(N\), the equilibrium distribution.

The Monte Carlo simulations used in this research are structured as follows. At \(z < 0\) there is a solid substrate, representing the surface. At the origin of the coordinate system a chain of elements is built up, representing the tether. The tether is simulated as \(N_{WLC}\) worm-like-chain elements with each a length of 1 nm. The first element of 1 nm points exactly perpendicular to the solid substrate. The angle between consecutive chain elements is randomly chosen but is weighted by the ratio of the length of one element and the persistence length. In this way the tether has the amount of stiffness corresponding to the persistence length. The last step is to add a solid sphere that represents the particle. The angle that the particle makes with respect to the last chain element is free, because in the experimental setting the particle is coupled to the tether by a carbon-carbon linker which has full rotational freedom.
The chain that has now been built up is checked on three boundary conditions. Firstly, particle-surface overlapping is checked, secondly particle-tether overlapping is checked and thirdly tether-surface overlapping is checked. If only one of these three boundary conditions is not satisfied, the state will be rejected. If all conditions are met, the state is accepted and the program continues to find another possible state. The program stops as soon as it has found the total number of possible states it has been told.

### 2.5.2 Simulation of dissociation process

A short Matlab script has been written to simulate the process of dissociation of the bond between particle and surface. In subsection 2.2.3 the stochastic dissociation process has already been explained. Due to the random character of dissociation, statistics plays an important role. To examine the statistical variations in dissociation events these simulations are made.

The simulations are build up as follows: At time $t = 0$ the system is in the bound state. Each time step $\Delta t$ the bond has a probability to dissociate equal to the product of the rate of dissociation and the length of the time step: $k_{off} \cdot \Delta t$. These two parameters are input parameters of the program. It is checked if dissociation has occurred with a random number. If the bond has dissociated the binding time is equal to one time step. However, if the bond has not dissociated there is an equal chance to dissociate during the second time step. As soon as a bond has dissociated, the simulation is repeated until a certain amount of dissociation events are simulated. This number of events is also an input parameter of the simulation.

The input parameters in the simulations are chosen to match with your experimental results. The time step is determined by the rate at which dissociation can be observed in the experiment, for example the frame rate of a camera. The number of events can be chosen to be equal to the number of events that are detected in the experiment. In this way the influence of statistical variations of dissociation can be determined. To account for direct rebinding due to the slow diffusion of the particle, a rebinding probability can be inserted which can nullify dissociation. If dissociation is denied, the simulation will continue.
3. Experimental setup

This chapter contains a description of the experimental setup of the measurements performed on two model systems to explore the potential of time-dependent tethered particle motion for measuring dissociation rate constants. The model systems are described in the first section of this chapter. Subsequently the sample preparation protocol is explained in detail and a complete description of the measurement procedure and the raw data analysis is given.

3.1 The measurement systems

Two TPM model systems are exploited in this research. The main difference between the two model systems is the length of the tether. To make a clear distinction between the two model systems throughout the remainder of this report, they will be referred to as the ‘short-tether model system’ (STMS) and the ‘long-tether model system’ (LTMS). The binding between particle and surface is acquired with the model system where the interaction between short single stranded DNA oligonucleotides is probed. On both the particle and the surface-oligonucleotides are attached. A more detailed description of this oligonucleotide interaction will be given in subsection 3.1.3.

3.1.1 Short-tether model system (STMS)

The short-tether model system is shown in more detail in figure 3.1. The STMS roughly consists of a surface, a tether and a particle. For the STMS the Dynabeads® MyOne™ Streptavidin C1 magnetic particles with a diameter of 1 μm are used. These particles consist of many iron oxide (Fe₂O₃) grains in a polymer matrix. The average surface roughness is defined as the average deviation of the radius of the particles from the minimum radius. The average surface roughness of the MyOne™ particles is estimated to be about 150 nm, but this is not a hard number. The value of the average deviation from the particle radius is estimated by eye from the scanning electron microscopy (SEM) image of the MyOne™ particles in figure 3.2a using the scale bar. The particles have a coating of covalently bound streptavidin. Streptavidin is a protein known for its strong binding affinity for biotin. The binding between biotin and streptavidin will be used to attach the tether to the particle as will be discussed below.

As the tethering molecule a double stranded DNA chain of 120 base pairs (bp) is used. This 120 bp has a contour length of \( l_c = 40 \text{ nm} \). The persistence length of dsDNA is 50 nm, so the tether will have a preference to be stretched. On both ends of the tether a functional molecule is covalently bound: On one end it has a biotin molecule and on the other end a Texas Red marker, an organic dye molecule. The tether and the particle are coupled via the strong biotin-streptavidin bond. The tether is bound to a glass surface via an antibody-antigen bond. On the surface antibodies against Texas Red are physisorbed, a binding that is based on a combination of van der Waals forces, electrostatic forces and hydrophobic forces between the surface and a molecule. The DNA tether is bound to the surface via the Texas Red anti-Texas Red bond. The excursion number, defined in subsection 2.1.3, \( N_R \approx 39 \) for the STMS. This means that the dynamics of the tethered particle system is clearly particle-dominated.

Apart from the attachment of the tether, also the molecules for studying association and dissociation kinetics have to be placed on both the particle and the surface. Therefore the surface is also functionalized with antibodies against digoxigenin (anti-DIG), a plant steroid. The area that is not covered with antibodies is blocked by a bovine serum albumin (BSA) protein. This protein is known to physisorb very efficiently and is widely used in biophysical research. As the binding molecules, two
complementary oligonucleotides with a functional group on one end are used. One oligonucleotide has a biotin group on its end and is bound to the particle, the ‘particle-oligo’. The other oligonucleotide has a DIG group on its end and is bound to the surface via the anti-DIG antibody, the ‘surface-oligo’. The oligonucleotides have a length of about 3 nm (discussed in subsection 3.1.3) whereas the tether has a length of 40 nm. When the particle comes close to the surface the particle-oligo and the surface-oligo can bind specifically to each other. When this bond is formed the motion of the particle will become more confined and indicative for the association of two complementary oligos. Subsequently the bond can dissociate and the full tethered particle motion is restored.

Figure 3.1 Schematic representation of the short-tether model system (STMS). The magnetic particles have a significant average surface roughness. The dsDNA tether has a length of 40 nm. The system is in the limit of a large particle on a small tether. Specific binding of the particle with the surface is probed with short complementary ssDNA oligonucleotides.

Figure 3.2 Scanning electron microscopy (SEM) images of two different types of particles. (a) Dynabeads® MyOne™ Streptavidin C1 micro particles with a diameter of 1000 nm and an estimated average surface roughness of about 150 nm. (b) Dynabeads® 800 nm carboxylic particles with an estimated average surface roughness of 30 nm.
3.1.2 Long-tether model system (LTMS)
The long-tether model system resembles the short-tether model system in many ways, as shown in figure 3.3. However, the particles and the tether that are used in the LTMS are different. Again magnetic particles are used: prototype particles functionalized with carboxylic groups and a diameter of 800 nm that are not commercially available but have been provided by Dynal, Thermo Fisher. These particles have an estimated average surface roughness of about 30 nm and are much more smooth than the MyOne™ particles, as can be seen in figure 3.2b. The 800 nm carboxylic particles are functionalized with anti-biotin in order to attach the biotinylated tethers to the particles. This functionalization is performed following an EDC coupling protocol. More details on this protocol is given in subsection 3.2.3.

The tether that is used in the LTMS is dsDNA of 1008 bp corresponding to a contour length of 330 nm, hence the name long-tether model system. The particle has much more translational and rotational freedom due to the smaller ratio of particle size over tether length and the long contour length compared to the persistence length of dsDNA. A much larger part of the particle will therefore be able to probe the surface. The excursion number \( N_R \approx 11 \) for the LTMS. This implies that the dynamics of the tethered particle system is still particle-dominated.

3.1.3 Complementary oligonucleotide interaction
In this research, specific binding of complementary ssDNA oligonucleotides attached to the particle and to the surface is used as a model system for the more general case of two reacting biochemical molecules on the particle and on the surface. Oligonucleotides are used for this purpose because their interaction strength can be tuned by increasing or decreasing the number and types of complementary bases. These oligonucleotides are commercially available with functional group at the end of the chain. Figure 3.4 shows a schematic representation of a dsDNA chain. The dsDNA chain is built up of two ssDNA chains that are complementary to each other. Each ssDNA chain is built up of nucleotides which
consist of a five-carbon sugar, a phosphate group and a nitrogen base. In DNA four different nitrogen bases exist: Adenine (A), thymine (T), cytosine (C) and guanine (G). These nitrogen bases form pairs, adenine with thymine and cytosine with guanine. The interaction between the two ssDNA chains in a dsDNA chain is due to the interaction between the nitrogen bases. The nitrogen base pairs are held together by hydrogen bridges. The adenine-thymine bond forms two hydrogen bridges where the cytosine-guanine bond forms three hydrogen bridges. For this reason the C-G bond is stronger than the A-T bond and therefore the relative G-C to A-T content of a dsDNA chain is an important parameter for characterizing the interaction strength.

![Schematic representation of a double stranded DNA chain with two G-C bonds and one A-T bond. Each nucleotide consists of a sugar, a phosphate group and a nitrogen base. The nitrogen bases form bonds with two (A-T) or three (G-C) hydrogen bridges. A ssDNA chain has a 3’ and a 5’ end which correspond to ending with a sugar group or a phosphate group, respectively.](image)

A single hydrogen bond is not a very stable bond \( (E_b \approx 9 \, k_B T) \), but when a chain of nucleotides bind with their complementary chain, the multitude of hydrogen bridges together forms a stable bond. For the dsDNA chain to fully separate, each single bond between base pairs needs to be broken at the same moment. In the time that it takes for the bonds to break, other bonds can already reform. Because as long as not all the bonds are broken, the nucleotides are close to each other and there is thus a significant chance for rebinding. The bond strength of complementary oligonucleotides increases super linearly as a function of the number of base pairs in the chain. This relation has been quantified by Strunz et al.\textsuperscript{26} They measured the dissociation rate, \( k_{off} \), of complementary oligonucleotides of 10, 20 and 30 nucleotides (nt). They kept the G-C content in the sequences equal to about 60% to only measure the dependence on the amount of nucleotides \( n \). From the measurements they determined a semi-empirical relation between the \( k_{off} \) and \( n \), as shown in equation 3.1. In this equation \( \alpha = 3 \pm 1 \) and \( \beta = 0.5 \pm 0.1 \).

\[
k_{off} = 10^{\alpha n - \beta n} \cdot \text{s}^{-1}
\]

(3.1)

In the research described in this thesis two sequences with different dissociation constants are designed. One combination consists of a particle-oligo and a complementary surface-oligo with 8 nucleotides and the other combination consists of a particle-oligo and a complementary surface-oligo with 10 nucleotides. The surface-oligos have a DIG group on the 3’ end of their chain and the
complementary particle-oligo has a biotin group on the 3' end. Table 3.1 shows the sequences of the particle- and surface-oligos, the G-C content, dissociation constant and the characteristic binding time. The dissociation constant is calculated from the semi-empirical relation obtained by Strunz et al, equation 3.1. The dissociation constants are chosen in this range because binding events of a shorter time are hard to detect (as will be explained in chapter 4) and a longer binding time would lead to very long measurements to observe enough binding events.

Table 3.1  Overview of the main properties of the oligonucleotides used in this research. The G-C content is kept close to the 60% with which equation 3.1 is determined.

<table>
<thead>
<tr>
<th></th>
<th>8bp oligo</th>
<th>10bp oligo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle-oligo sequence</td>
<td>3'-GCAACGCT-5'</td>
<td>3'-TTCGGAGCTA-5'</td>
</tr>
<tr>
<td>Surface-oligo sequence</td>
<td>3'-AGCGTTGC-5'</td>
<td>3'-TAGCTCCGAA-5'</td>
</tr>
<tr>
<td>G-C content (%)</td>
<td>62.5</td>
<td>50</td>
</tr>
<tr>
<td>Dissociation constant (s⁻¹)</td>
<td>0.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Characteristic binding time (s)</td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>

3.1.4  Comparison between STMS and LTMS

Table 3.2 shows an overview of the main differences between the two model systems. The most important difference between the two systems is the tether length. In the STMS the ratio of the tether length and the particle diameter is only 0.04 and therefore translation and rotation of the particle is almost fully suppressed. Consequently only a small part of the particle is available for binding with the surface. In the LTMS the ratio is 0.41, a tenfold increase compared to the STMS, such that the particle has much more freedom to translate and rotate. A larger part of the particle is able to bind to the surface.

Table 3.2  Overview of the differences between the main properties of the short-tether model system and the long-tether model system.

<table>
<thead>
<tr>
<th></th>
<th>Short-tether model system (STMS)</th>
<th>Long-tether model system (LTMS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tether length (nm)</td>
<td>40</td>
<td>330</td>
</tr>
<tr>
<td>Particle diameter (nm)</td>
<td>1000</td>
<td>800</td>
</tr>
<tr>
<td>Average particle roughness (nm)</td>
<td>150</td>
<td>30</td>
</tr>
<tr>
<td>Relative particle roughness (%)</td>
<td>15</td>
<td>3.75</td>
</tr>
<tr>
<td>Tether length / particle size</td>
<td>0.04</td>
<td>0.41</td>
</tr>
<tr>
<td>Excursion number (N_2)</td>
<td>39</td>
<td>11</td>
</tr>
<tr>
<td>Motion pattern diameter (nm)</td>
<td>408</td>
<td>1221</td>
</tr>
<tr>
<td>Particle functionalization</td>
<td>Streptavidin</td>
<td>Anti-Biotin</td>
</tr>
</tbody>
</table>

The part of the surface that a single tethered particle can probe depends on the length of the tether and the diameter of the particle. Figure 3.5 shows a schematic representation of the particle in three extreme positions. These configurations of the particle correspond to the outermost positions in the motion pattern. With equation 3.2 the diameter \(\phi\) of the motion pattern can be calculated in terms of the contour length of the tether \(l_c\) and the radius of the particle \(R_p\). In the STMS the particle can probe a circular part of the surface with a diameter of 408 nm and in the LTMS the diameter is 1221 nm.
3.2 Sample preparation and dimensions

In this section the preparation of the samples is described. Step-by-step sample preparation protocols of both model systems are given in appendix A. The sample preparation can be divided into three steps. The first step is to clean the bare sample. During the second step the surface and the particles are functionalized. The third step is to incubate the functionalized particles onto the functionalized surface. Each step will be described in a separate subsection.

3.2.1 Bare sample preparation

The first step in the sample preparation is to clean the bare sample. The TPM model systems that have been explained in section 3.1 are prepared on glass cover slips with a size of 26 by 76 by 0.5 mm. These glass substrates are cleaned before being used to minimize non-specific sticking to pollutions on the sample. During cleaning steps of 10 minutes each, the substrates are placed in a sonic bath, first with acetone, subsequently with isopropyl alcohol and finally with ethanol. After each cleaning step the substrates are dried with a gentle flow of compressed nitrogen. Acetone, isopropyl alcohol and ethanol are used to remove greasy stains, dust and other pollutions on the glass substrate. After cleaning the samples, they are stored in a small vacuum chamber until they are used, to prevent dust particles to pollute the samples. The TPM experiments are performed in a plastic fluid well that is attached to the cover slip. Custom 6-well secure seal v2.0 fluid wells from Grace Bio-labs are used so that samples can be flushed between preparation steps in order to remove not-reacted molecules. These fluid wells have a size of about 2 by 18 by 1 mm, as shown in figure 3.6. The active area is the bottom glass surface in the well of $36 \cdot 10^{12}$ nm$^2$. 

\[
\theta = 2 \sqrt{l_c^2 + 2l_c R_b} \tag{3.2}
\]
Figure 3.6 Schematic representation of the layout of the sample. A plastic sticker with fluid wells is attached to a glass substrate.

3.2.2 Surface functionalization

The second step in the sample preparation is the functionalization of the surface. For both model systems, STMS and LTMS, the fluid wells are filled with a sample volume of 25 μL of anti-Texas Red at a concentration of 40 ng/mL. The antibodies are dissolved in a phosphate buffered saline solution (PBS). The incubation time of the antibodies is 60 minutes whereupon each fluid well is flushed with 1 mL of PBS to remove the unbound antibodies. This incubation time of 60 minutes is chosen to make sure that nearly all the antibodies will be physisorped on the surface. In the hypothetical case that all antibodies bind to the lower surface, which is the upper limit, the average free area per antibody is $9 \times 10^3 \text{ nm}^2$. This corresponds to an average antibody-to-antibody distance of 100 nm. In this case there would be on average 15 and 130 Texas Red antibodies within the reach of a tethered particle for the STMS and the LTMS, respectively. In practice this number will be smaller, because not all antibodies will bind. The ideal scenario would be that each particle becomes tethered to a single anti-Texas Red molecule and would not have any other anti-Texas Red molecule in its reach. This situation is possible to create by incubating a lower concentration of anti-Texas Red, however this also leads to a significant drop in the absolute number of particles that will be bound to the surface, decreasing the statistics per experiment. A concentration of 40 ng/mL anti-Texas Red leads to a reasonable number of bound particles and enough of these particles are single tethered. Therefore this concentration is used throughout the experiments.

Subsequently the surface is functionalized with anti-DIG. A sample volume of 25 μL of anti-DIG at a concentration of 5,000 ng/mL is incubated. At this high concentration, if all antibodies bind, each particle would have on average $1.9 \times 10^3$ and $1.6 \times 10^4$ antibodies within its reach for the STMS and the LTMS, respectively. This high concentration is chosen to ensure that the surface is populated densely with DIG antibodies. The number of binding spots for the particle can now be tuned by the concentration of surface-oligos that is incubated. Again an incubation time of 60 minutes was retained. Subsequently a washing step with 1 mL of PBS is performed.

Finally the surface-oligos are incubated on the surface. The DIG tagged oligonucleotides are not dissolved in PBS, but in an ethylenediaminetetraacetic acid buffer (EDTA). All not-DIG containing DNA is stored in Tris-EDTA buffer (TE). The incubation of the surface-oligos is similar to the incubation of the antibodies. The incubation time is 60 minutes and the sample volume is 25 μL. The concentration of surface-oligos during incubation is 100 pM. This concentration corresponds to on average 5 and 50 surface-oligos within the reach of each particle in the STMS and the LTMS, respectively. That is, if all oligos bind to the anti-DIG antibodies. This corresponds to an average oligo-to-oligo distance of 154 nm.
The surface is not fully covered after the incubation with the antibodies and the surface-oligos. To prevent particles from non-specifically sticking to the surface, the surface is blocked with BSA. This protein sticks to the open spots on the surface and in this way blocks the non-specific sticking. Blocking is performed by incubating 25 μL of a 1% (weight/volume) BSA concentration during 5 minutes whereupon a washing step with 1 mL of PBS is performed to wash away unbound BSA.

### 3.2.3 Particle functionalization

Parallel to the functionalization of the surface, the particles are functionalized. Two different functionalization processes need to be distinguished. The functionalization of the 800 nm carboxylic particles with anti-biotin and the functionalization of both types of particles with the dsDNA and the particle-oligos.

The anti-biotin is bound to the 800 nm particles covalently through EDC coupling. Figure 3.7 schematically shows the EDC coupling. The 800 nm particles that are used for the LTMS are provided with a carboxylic coating. The first step in this protocol is to add 1-ethyl-3-[3-diaminopropyl]carbodiimide (EDC) to a solution of the particles. The EDC reacts with the carboxyl groups to activate the particle. After an incubation time of 30 minutes a solution of anti-biotin is added to the solution of particles. The antibodies bind covalently to the particle with one or more amine groups. The EDC groups that are still active in the end are blocked with BSA to prevent the reaction to continue. The details of the full functionalization protocol can be found in appendix A.

![Figure 3.7](image)

**Figure 3.7** Schematic representation of the EDC coupling chemistry between the carboxylic 800 nm particles and the anti-biotin antibodies. The 800 nm particles that are used in the LTMS are functionalized with a carboxylic surface. After adding EDC the OH group of the carboxyl group reacts with the EDC. Subsequently the antibodies are incubated with the particles. The antibodies will bind to the particles with one of their amine groups to form a covalent bond.

Both types of particles are functionalized with double stranded DNA tethers. Each particle requires only one DNA tether to become tethered to the surface. However, there are several reasons why the particles are incubated with a higher concentration of DNA tethers. At first, not all tethers bind to a particle within the incubation time. Secondly, of the tethers that do bind to a particle, some will bind in an indentation on the particle. Since for the STMS the tether is only 40 nm and the average surface roughness is much larger, some tethers will not be able to bind to the surface. Thirdly, if a particle has exactly one tether on its surface, the probability for the particle to become tethered to the surface when incubating the particle in the fluid well is smaller than when the particle has multiple tethers on it. The attachment of multiple tethers per particle has the disadvantage that particles can also be bound to the surface by multiple tethers resulting in confined motion and a non-circular motion pattern.

In the STMS the 40 nm tethers and the 1000 nm particles are incubated in a tube for 60 minutes in a ratio of 150 to 1. This ratio has been optimized so that particles bind to the surface and not too many multiple tethered particles are observed. In the LTMS the 330 nm tethers and the 800 nm particles are...
incubated for 60 minutes in a ratio of 120 to 1. The particles are also functionalized with the particle-oligonucleotides. The number of oligonucleotides per particle is much higher than the number of tethers per particle. The particle-oligonucleotides are incubated with the particles in a ratio of 300,000 to 1.

After the full functionalization of the particles, they are washed magnetically three times to wash away any leftover chemicals. Figure 3.8 shows the process of magnetic washing schematically. Firstly the tube with the solution of functionalized particles is placed in a tube holder. In this tube holder a small permanent magnet is positioned close to the side of each tube. At this point the distribution of particles is still homogeneous. In two minutes the magnetic particles are attracted towards the permanent magnet. The particles accumulate at the side of the tube, but the excess DNA tethers and oligonucleotides does not accumulate. The third step is to pipette away the remaining fluid which contains the excess DNA tethers and oligonucleotides. Subsequently the particles are redispersed in buffer solution. This cycle is repeated three times to ensure that all substances are washed away.

Figure 3.8 Schematic representation of magnetically washing the solution with the functionalized magnetic particles. Step 1: Insert the tubes with the solutions of functionalized particles in the tube holder. Each solution shows a homogeneous distribution of the particles (displayed with a colour). Step 2: Wait for 2 minutes and observe that the particles are attracted to the permanent magnet close to the side of the tube. Step 3: Fill the pipette with the remaining solution and leave the particles in the tube. Subsequently redisperse the particles with buffer solution.

3.2.4 Incubation of particles on the surface
The third and last step in the sample preparation is to incubate the functionalized particles on the functionalized surface. A volume of 25 μL of particle solution with a concentration of about 65 fM is incubated on the functionalized surface. This concentration corresponds to an average particle-to-particle distance of about $6 \cdot 10^3$ nm in the hypothetical case where all particles bind to the surface. This is an average, so there will also be many particles that are too close to each other for the software to analyze them. However, during pipetting steps and magnetic washing steps particles are lost and also only a fraction of the particles that are incubated will actually bind to the surface within the incubation time. Therefore normally not many particles are too close together. As soon as the particles are incubated, they start to sediment to the bottom of the fluid well. The particles contain Fe$_3$O$_3$ grains which have a higher density than water. After a sedimentation time of 60 minutes the sample is turned over. All non-bound particles will sediment away from the upper surface whereas the bound particles
stick to the upper surface. After 30 minutes the non-bound particles have sedimented significantly and the samples are ready to be measured.

3.3 Measurement protocol
In the previous section the preparation of the samples is described. This section deals with all the steps between having prepared the sample and obtaining the motion patterns of the tethered particles. The first subsection deals with the acquisition of the data during the experiments. In the second subsection the analysis of the raw data, to obtain a motion pattern, is described.

3.3.1 Data acquisition
To obtain information about the motion of the micro particles in the two assays, the particles need to be tracked. In this research the particles are imaged with dark field optical microscopy. A Nikon Eclipse Ti microscope with an ANDOR™ iXon3 camera is used with a 20x objective and a numerical aperture of 0.5. Figure 3.9a schematically shows the dark field imaging technique. In this technique the light from the light source is blocked so that all the light that is not scattered by the sample will not be collected by the objective lens. Only the light that is scattered by the sample will be collected.

![Figure 3.9](image)

**Figure 3.9** (a) Schematic representation of the dark field imaging technique. The sample is illuminated with light that will not be collected by the objective lens. Light that is collected by the objective lens has been scattered by the sample. The background in the image therefore appears black while the structure of the sample appears bright. (b) Dark field image of many particles. The bright spot in the red square is magnified and shows what the image of a single particle looks like.

Recordings of the sample are made with an ANDOR™ iXon3 camera. The recordings are made with a frame rate of 30 frames per second and an integration time of 5 ms. Normally measurements of 1800 frames, acquisition time 1 minute, are made, except in case otherwise noted. The next step is to analyze the recordings with TPM analysis software as described in the subsection 3.3.2. Figure 3.9b shows what a typical field of view (FOV) looks like. Many bright spots in a dark background. Most bright spot corresponds to a single particle, but the spots that are significantly brighter correspond to clusters of multiple particles. Due to large scattering of these clusters the background intensity will increase. Also
the presence of air bubbles in the sample leads to an increase in the background signal. Figure 3.9b shows a red square with a magnified image of a scattering profile of a single particle. The intensity of the particle is spread out over multiple pixels. The pixel size is 810 nm. By calculating the centre of intensity, as will be explained in the next subsection, the particle can be localized with a resolution of a few nanometre.

3.3.2 Raw data analysis

A Matlab program to convert the raw data, consisting of the camera images, into the motion patterns of all particles in the field of view has been made by Emiel Visser (PhD student, supervisor). The program can roughly be divided into four steps: In the first step a background correction is performed on each frame. The second step deals with localizing all particles in all frames. The third step deals with correlating the location of each particle from frame to frame. In the fourth step a correction for the sample drift is made. In this subsection each step will be explained in detail.

The first step is to correct for the background signal originating from thermal noise of the camera, out of focus particles and scattering from clusters or air bubbles. To correct for this background signal several wavelet filters\(^27\) are used to suppress the very high frequencies in the signal and the very low frequencies. The background is subtracted from the original signal to correct. In the second step the particles are localized. The detection of each particle in each frame is done by convoluting the frame with the Gaussian fit profile of a single particle. This fit profile is obtained by first manually selecting one particle and fitting the intensity profile of this particle with a two dimensional Gauss function. This profile is used for all frames. Once a particle is detected, and thereby obtaining a rough estimation of the position of the particle, the exact position of the particle is calculated with sub-pixel resolution. The determination of the centre of mass of a particle, that is represented by an intensity profile which is spread out over multiple pixels, is done by first defining a region of interest (ROI) around the rough estimate of the position of the particle. The ROI defines a square of an integer number of pixels around the pixel in which the rough estimate of the position lies in the centre pixel. In this ROI the centre of mass is determined by calculating the centre of intensity. This is performed for each particle in each frame.

Although a background correction is performed, there is still a background signal on top of the signal coming from the particle since the correction is never perfect. The centre of intensity of the signal coming from the particle leads to the correct position of the particle. But the centre of intensity of the background signal, in case of a constant background level, gives the centre of the ROI. The background level is not exactly constant, therefore it will not be exactly in centre of the ROI. An iterative method for determining the position of the particle is created. The ROI is chosen not to be restricted to coincide with whole pixels, but can lie at any point and can therefore contain fractions of pixels. In the calculation of the centre of intensity of the particle, the \(x_{i,j}\) and \(y_{i,j}\) position of the pixels are multiplied by the intensity of that pixel \(I_{i,j}\) which is weighted with the fraction of that pixel \(F_{i,j}\) that is within the ROI. A sum is made over all pixels in the ROI and divided by the total intensity as shown in equations 3.3 and 3.4. Once the centre of intensity is determined, the calculation is repeated with the ROI centred around the calculated centre of mass in an iterative process. In this way the centre of the ROI is iteratively shifted towards the correct position of the centre of intensity of the particle. The iteration is repeated four times for every particle in every frame.

\[
x_{\text{Center of Intensity}} = \frac{\sum_{i,j}^{\text{ROI}} x_{i,j} \cdot F_{i,j} \cdot I_{i,j}}{\sum_{i,j}^{\text{ROI}} F_{i,j} \cdot I_{i,j}} \tag{3.3}
\]
Once the position of each particle in each frame is known, step three is determining the trajectory that a single particle makes from frame to frame. In other words, the location correlation between frames needs to be calculated. If there is a particle at position \( r \) and one frame later there is a particle at position \( r + \Delta r \), where \( \Delta r \) is smaller than some threshold, then this is considered to be the same particle. For high areal density of particles the algorithm cannot tell them apart. Also if particles travel too far from one frame to the other it is impossible to tell which particle corresponds to which particle between frames. Particles that are close to the border of the field of view may in some frames be outside the field of view. These particles can also not be tracked. Once all frames have been correlated, the trajectory that each particle makes in time is known.

The fourth and last step is to correct for the sample drift. Vibrations of the microscope result in movement of the sample as a whole. This results in drift of the particle which needs to be separated from actual motion of the particle with respect to the sample. For this purpose the program identifies particles that exhibit similar movement by setting a threshold on the maximum difference between the motion of two particles. The program calculates the average motion of the particles making similar movement, which corresponds to the drift, and subtracts this motion from the trajectories of all particles.

The other parameter that can be varied is the integration time, which is the exposure time per frame. The integration time that is used influences the signal to noise ratio. For longer integration times, the counts for each particle will be larger and consequently the contrast in the image is larger. However, during the exposure time, the particles are moving and the intensity profile is an average over the integration time. This effect is called motion blur. Due to motion blur the position that is determined is the average over the position of the particle during the integration time. In tethered particle motion experiments motion blur leads to a reduction in the apparent motion pattern\(^{16}\). This can be explained by considering the particle to be at a position close to the border of the motion pattern. Due to the tether the particle will be pulled to the centre of the motion pattern. The average position of the particle is then also somewhat closer to the centre of the motion pattern.

\[
\text{Center of Intensity} = \sum_{i,j}^{\text{ROI}} y_{i,j} \cdot F_{i,j} \cdot I_{i,j} / \sum_{i,j}^{\text{ROI}} F_{i,j} \cdot I_{i,j}
\]
4. Tethered particle motion analysis

Two different types of tethered particles are distinguished, particles with a motion that is time-independent and particles that show time-dependent motion. These two types of motion are both analyzed in two different ways: The analysis of static motion patterns is explained in section 4.1 and the analysis of the particle motion as a function of time is performed in section 4.2. In section 4.3 the algorithm used to distinguish the bound from the unbound state in experimental data is explained.

4.1 Motion pattern analysis

In this section tethered particles are analyzed by looking at their motion pattern. A motion pattern is a graphical representation of the particle positions obtained from a series of consecutive frames and represents a 2D representation of the phase space of the particle. Firstly, an overview is given of the time-independent motion patterns that are observed when performing a TPM experiment. Subsequently the type of motion patterns that is related to binding of the particle with the surface is discussed. The last section discusses how to represent collections of motion patterns systematically.

4.1.1 Time-independent motion

Visser et al.\textsuperscript{28} recognized seven classes of time-independent motion patterns. Figure 4.1 shows one minute recordings of particle motion for six out of seven classes of motion patterns for the STMS. The remaining motion pattern class contains motion patterns that for some reason fall out of the other categories. The simplest tethered particle system are particles bound to the surface by a single tether. Such particles are expected to show a Brownian motion pattern within a spherical cap, due to the confinement by the tether and the surface. The corresponding 2D motion pattern should be circular.

![Motion Pattern Classes](image)

*Figure 4.1 Six out of seven motion pattern classes as defined by Visser et al.\textsuperscript{28} (a) disk-shaped, (b) ring-shaped, (c) bell-shaped, (d) stripe-shaped, (e) triangular-shaped and (f) spot motion pattern.*
An example of a disk-shaped motion pattern is shown in figure 4.1a. The motion pattern is circularly symmetric, which can be understood since there is no a priori reason for an angle dependence. The diameter of this motion pattern is about equal to the calculated value of 408 nm. However, a distribution of the motion pattern sizes is observed with a diameter varying between 300 and 600 nm for the STMS. For the LTMS this size distribution is more narrow, 900-1100 nm. This size distribution is the result of the effective tether length distribution which is primarily due to surface roughness. The ring- and the bell-shaped motion pattern shown in figure 4.1b and 4.1c are disk-shaped motion patterns affected by protrusions on the particle close to the tethering point.

Figure 4.1d shows motion that is restricted to a stripe. This motion pattern corresponds to a particle that is tethered to the surface by two tethers, such that it has a smaller freedom of motion than a single tethered particle. For a triple tethered particle the motion is even more confined. Figure 4.1e shows the triangular motion pattern of a triple tethered particle. One class of motion patterns shows motion that is confined to a spot, as represented in figure 4.1f. The origin of these motion patterns is unknown. The particle could be bound to the surface by many tethers, or non-specifically. The last class (not shown here) contains motion patterns that for some reason fall outside the regular classes. From the seven classes of motion patterns, the particles making a disk-shaped motion pattern as shown in figure 4.1a are the interesting ones for observing binding events. Because these particles probe the largest area on the surface in the most homogeneous way. A typical collection of motion patterns that is obtained when performing a TPM experiment with either of the model systems can be found in appendix B.

4.1.2 Time-dependent motion
A special type of motion patterns are the ones that correspond to time-dependent motion. Time-dependent motion is observed in the motion pattern as a non-homogeneous distribution. Figure 4.2a and 4.2b show an example of a non-homogeneous motion pattern that has been obtained with the STMS and the LTMS, respectively. In the model systems specific oligonucleotide interactions are introduced in addition to the main tether. Temporary bonds of the tethered particle can be formed between an oligonucleotide on the particle and on the surface. Such a temporary bond results in a spot in the motion pattern which has a higher density of data points than the rest of the motion pattern. This spot has a different size and a different geometry for every motion pattern. The position of the spot, in terms of the in-plane radius $r$ and the polar angle $\theta$, differs from motion pattern to motion pattern.

Figure 4.2 Non-homogeneous motion patterns of particles showing time-dependent behaviour of particle motion. Interactions between the particle and the surface are observed as a spot in the motion patterns with a significantly higher density of data points: For (a) STMS and (b) LTMS.
Figure 4.3 shows the motion pattern development of a particle that undergoes time-dependent motion. At first, a particle undergoes Brownian motion as shown in figure 4.3a. The particle is in the free state, only attached to the surface via a single main tether. After a certain time $t_1$ the particle makes an additional oligo-bond with the surface and the system is now in the bound state. Hereby the motion of the particle changes. The particle’s motion is confined to a small part of the original disk-shaped pattern as can be seen in figure 4.3b. Even in the bound state the particle still has a limited amount of freedom of motion. This freedom of motion originates from the anti-DIG-oligo complex having a finite length and working as an effective tether. This spot with a higher density of data points represents the system being temporarily in the bound state. After a time $t_2$ the oligo-bond dissociates and the particle starts to undergo Brownian motion again, as shown in figure 4.3c. The particle is now in the free state again. Note that the motion pattern of this tethered particle may look differently when repeating the measurement. It could be that during the next minute the particle does not make a temporary oligo-bond such that the motion pattern would be disk-shaped.

4.1.3 Motion pattern representation

In this research TPM experiments are performed with different concentrations of oligonucleotides. In order to compare the motion patterns between different experiments, there is the need for a systematic way to represent a collection of motion patterns. For this purpose the motion patterns are ordered based on their size and shape as follows. By calculating the square root of the length of the
eigenvectors of the covariance matrix of the motion pattern, the standard deviation of the data points along minor axis and major axis are determined, as depicted in figure 4.4a. The symmetry of the motion pattern is defined as the ratio of minor amplitude over major amplitude, as shown in equation 4.1. The minor motion amplitude and the symmetry of the motion pattern are used for ordering the motion patterns.

\[
Symmetry = \frac{\text{Minor amplitude}}{\text{Major amplitude}} \tag{4.1}
\]

Figure 4.4b and 4.4c show a motion distribution plot for the STMS and the LTMS, respectively. Each of the motion patterns is placed in the plot according to their minor amplitude and their symmetry as a dot. The local density of dots is indicated by the heat map. In both motion distribution plots roughly two groups of motion patterns can be distinguished. One group has minor amplitude > 50 nm and symmetry > 0.8. This group corresponds to the disk-, ring- and bell-shaped motion patterns. For the STMS this group is located at lower minor amplitude than for the LTMS, because in the LTMS the tether is longer and the motion patterns are therefore larger. The second group is located at minor amplitude < 75 nm and 0 < symmetry < 0.8. This group corresponds to the stripe, triangular and spot patterns. With this representation the single tethered particles are distinguished from multiple tethered particles and spot-patterns. For the analysis of particle motion as a function of the time, the interesting particles are the ones that are single tethered and do not suffer from roughness effects. Ring- and bell-shaped particles are therefore picked out and thrown away manually.

**Figure 4.4** (a) Graph of a stripe pattern in which the minor and major axes are indicated. (b) Motion distribution plot of a collection of motion patterns for the STMS. The patterns are ordered based on the minor motion amplitude and the symmetry of the motion patterns. (c) Motion distribution plot of a collection of motion patterns for the LTMS.

### 4.2 Analysis of particle motion as a function of time

To observe and detect changes in the motion of a particle, for example from the free state to the bound state, the particle motion needs to be analyzed as a function of time. First time-independent particle motion and subsequently time-dependent particle motion will be analyzed. Finally a Matlab algorithm is discussed with which the experimental results will be analyzed in order to discern binding events in the motion of a tethered particle.
4.2.1 Time-independent motion

In this section the motion of single tethered particles that do not show binding events is analyzed as a function of time. This time-independent motion is characteristic of the free state. Figure 4.5a shows the sum of the positions of the particle after 1, 4, 10, 20 and 60 s to show the build-up of a motion pattern in the STMS as a function of time. As time increases the particle has explored a larger part of its phase space. The area that the particle has probed can be represented by the convex hull of the data points. The convex hull is the smallest convex set that contains the data points, as depicted in red in figure 4.5b for 15 data points. The convex hull is calculated as a function of time to represent the fraction of the phase space that the particle has probed after a certain time, shown in figure 4.5c for both the STMS and the LTMS. This graph shows that in both model systems it takes approximately 10 s to probe 75 percent of the phase space. Once the particle has probed the first 75 percent of the motion pattern it takes 50 s to probe the last 25 percent of the motion pattern. During the first seconds, the particle will continuously explore new parts of the motion pattern. At later time points much of the motion pattern has already been visited, and other areas are barely explored. The lines in figure 4.5c for the STMS and the LTMS are almost similar. However, in the first ten seconds the particle in the LTMS probes its phase space faster than the particle in the STMS. The size of the particle is smaller in the LTMS, 800 nm compared to 1000 nm, and therefore the diffusion will be 1.25 times faster. During the last 50 seconds the STMS is faster in probing the remainder of its motion pattern. The total area of the motion pattern in the STMS is much smaller than in the LTMS and although the diffusion constant of the particle in the LTMS is only 1.25 faster, probing the full motion pattern happens faster in the STMS.

Figure 4.5 (a) Snap shots of a motion pattern in the STMS after 1, 4, 10, 20, 60 s showing the build-up of the motion pattern over time. (b) The probed area after 0.5 seconds for the STMS, quantified by the convex hull of the data points. The blue line shows the convex hull of the full motion pattern, which represents the total area that is available. (c) Ratio of probed area over the total area as a function of time for both model systems. The lines represent an average over 25 typical trajectories.
In order to determine how the motion of the particle is affected by the tether, the diffusive motion of the tethered particle is quantified. One method for quantifying the motion of the tethered particle is by calculating the absolute distance that a tethered particle travels in a certain time interval $\Delta t$, the step size. The step size is calculated as shown in equation 4.2. Figure 4.6 shows the step size of the particle for a time interval $\Delta t$ equal to the time between recording two frames, 33 ms, as function of time. The corresponding step size distribution is also shown. The step size shows a significant amount of variation, an intrinsic aspect of Brownian motion. The result is a broad distribution of step sizes. The step size is, on average, larger for the particle in the LTMS which is related to the fact that the particle used for the LTMS is smaller. The amount of variation in the step size can be suppressed by averaging the step size during a certain time window and shifting this window along the time axis.

$$\text{Step size}(t) = |r(t + \Delta t) - r(t)|$$  \quad (4.2)

Figure 4.6 The step size of particles using a time interval $\Delta t$ equal to the time between recording two frames, 33 ms, as function of time and the corresponding histograms (bin size = 10 nm) for the STMS and the LTMS.

A second method for analyzing the random motion of a particle is calculating the mean squared displacement ($MSD$). The mean squared displacement $MSD(\Delta t)$ is defined as the average squared distance travelled by a particle in a time interval $\Delta t$. The $MSD$ is related to the diffusion constant as shown in equation 4.3. Note that this is the same expression as in equation 2.5.

$$MSD(\Delta t) = \langle [r(t + \Delta t) - r(t)]^2 \rangle_t = 4D\Delta t$$  \quad (4.3)

Figure 4.7 shows a graph of the average mean squared displacement for 25 typical trajectories in the STMS and the LTMS including the one sigma bands. It shows that, for a tethered particle, the mean squared displacement increases as a function of the time interval $\Delta t$ over which the $MSD$ is calculated until it reaches a plateau value. This plateau value is larger for the LTMS than for the STMS since it represents the maximum mean squared displacement that a particle can make in a measurement.
system. The size of a motion pattern in the LTMS is larger than the size in the STMS and therefore the maximum mean squared displacement that a particle can make is larger. The dashed red line shows a reference line of the MSD of free diffusion from equation 4.3. The value for the diffusion coefficient that is used for the free diffusion line is the one calculated in section 2.1.1. The free diffusion line is steeper than the measured MSD. This shows that even for small displacements the particle undergoes restricted Brownian motion due to the tether. The experimentally determined standard deviation of the average MSD increases with Δt. For low Δt, diffusion is dominant and in each of the 25 analyzed trajectories, the particle moves about the same distance. However, as the time interval Δt is increased, the regime is entered where the system confinement starts to dominate over the diffusion.

Figure 4.7 Mean squared displacement as a function of the time interval Δt for the STMS and the LTMS. These lines represent an average over 25 typical trajectories and include the one sigma bands. A reference line of the MSD calculated with equation 4.3 is added for both the STMS and the LTMS.

4.2.2 Time-dependent motion
The particles of interest are those that show an inhomogeneous motion pattern and correspond to a changing type of motion in the course of time as shown in figure 4.3. By analyzing the motion of these particles, the time spans where the particle is in the bound state or the free state can be determined. Several methods have been examined for their potential to distinguish the bound from the unbound state. In this section an analysis of time-dependent motion is given in terms of the two methods that showed most potential, namely the step size function and the area function. This analysis is performed for a particle in the STMS. The analysis is similar for a particle in the LTMS, which is shown in appendix C.

Figure 4.8a shows the step size function of a one minute recording of a particle making time-dependent motion. In blue the step size, calculated over a time interval of 33 ms, is shown. The green line shows the step size when an averaging window of 2 s is used. In red the one sigma band of the average of the step size is shown. Several drops in the step size are observed, indicating a binding event. The particle seems to show two binding events: From t ≈ 20 – 40 s and from t ≈ 50 – 60 s. The fluctuations make it difficult to distinguish the bound from the free state. To decrease the amount of fluctuations, the step size...
size is averaged over a window. To quantify the fluctuations, the standard deviation of the step size in each window is calculated. Figure 4.8b shows the average of the standard deviation of the step size, as a function of the window size. When increasing the window size, the amount of fluctuations decreases as expected. However, increasing the window size also leads to a decrease in the time resolution. Therefore a compromise has to be made between amount of statistical fluctuations and time resolution. An averaging-window size of 2 s is chosen since at this point the decrease in fluctuations is becoming less.

![Graph](image)

Figure 4.8 (a) Step size (blue) of a particle making time-dependent motion. The averaged step size (green) is calculated in windows of 2 s and the 1 sigma band is shown. (b) Dependence of the fluctuations in the step size on the window size. (c) Step size (window = 2 s) for different time intervals $\Delta t$. (d) Contrast in the step size defined as the average step size in the free state over the average step size in the bound state.

To increase the contrast between the step size of the particle in the free and the bound state, the time interval over which the step size is calculated is varied. Figure 4.8c shows a graph of the step size averaged in a window of 2 s for different time intervals $\Delta t$. The graph shows that for increasing $\Delta t$ the step size increases. However, when increasing $\Delta t$, the increase in step size is different in time intervals where the particle is in the free state and where the particle is in the bound state. In the bound state the motion is not limited by the time interval over which the step size is calculated, but it is limited by the confinement of the freedom of motion due to the oligo-bond. Figure 4.8d shows the contrast in the step
size, defined as the step size in the free state over the step size in the bound state, as a function of the time interval $\Delta t$. The contrast increases as a function of the $\Delta t$, but the time resolution will decrease with $\Delta t$. A time interval of 0.167 s is chosen since at about this time interval, the increase in contrast becomes lower.

When a particle is in the bound state, the motion of a particle is more strongly confined compared to the motion of a particle is in the free state. Both the step size of the particle and the area that the particle probes decrease temporarily when in the bound state. To represent the area that a particle probes as a function of time, the convex hull is calculated for a shifting window. This is called the area function. Figure 4.9a and shows the area function for a range of window sizes. As the window size is increased from 0.33 s to 4 s, the area is calculated over more data points and the area function in the free state increases about $0.8 \cdot 10^5$ nm$^2$. However, when the system is in the bound state, the area increases only $0.1 \cdot 10^5$ nm$^2$. This is because in the bound state less time is required for the particle to probe its available phase space. So calculating the area over a longer time than the system needs to probe the phase space will not lead to a larger area. For the free state the system is still in the regime where the particle does probe a larger area when increasing the window size. As shown in figure 4.5c the area is still increasing in the regime of 0.33-4 s. The contrast between the free state and the bound state increases as a function of the window size, as shown in figure 4.9b. Again the side effect of increasing the window size is losing time resolution. The same window size as for the step size will be used in the algorithm to distinguish bound and unbound states that is explained in the next section.

The decrease in the step size and the area function when a tethered particle makes an oligo-bond is not always equal. This decrease depends on the position of the oligo relative to the dsDNA tether as will be shown in the next chapter.

![Figure 4.9](image.png)

**Figure 4.9** (a) Area function with a varying window size. (b) The contrast in the area function for different window sizes.

### 4.3 Algorithm to distinguish bound and unbound states

The goal of analyzing the motion of the particles is to discern binding events in the observed motion of the tethered particles. In this section a stepwise description is given of a Matlab algorithm that has been written to analyze the motion of the particles. With this Matlab algorithm the state of the system, bound or free, is determined as a function of time. Binding events that last longer than 3 seconds can be
detected with a 90% success rate. The success rate has been determined by analyzing a large set trajectories with the algorithm and comparing the results to a manual library. The full code of the Matlab algorithm is provided in appendix D.

4.3.1 Step 1: Select interesting motion patterns
After having performed the raw data analysis, described in section 3.3.2, the trajectories of all particles are known. Of these trajectories only the ones that correspond to single tethered particles are of interest. The first step is to filter out all uninteresting motion patterns. By setting thresholds on both the minor amplitude and the symmetry of the motion patterns, a large group of uninteresting motion patterns is filtered out. The used thresholds are: \( \text{symmetry} > 0.75 \) and \( 50 < \text{minor amplitude} < 175 \text{ nm} \) for the STMS and \( \text{symmetry} > 0.85 \) and \( 125 < \text{minor amplitude} < 225 \text{ nm} \) for the LTMS. The ring- and bell-shaped motion patterns that meet the selection criteria and are discarded.

4.3.2 Step 2: Rough calculation of state vector
Of each selected trajectory the state of the tether-particle system as a function of time, the state vector, is calculated. The state vector is defined as a list of zeros and ones where a one corresponds to the system being in the bound state and a zero corresponds to the free state. The algorithm first calculates a rough estimate of the state vector by looking at both the step size function and the area function. With each function the motion is analyzed and the state of the system is determined by setting two thresholds. One threshold to detect a change in state from free to bound, a binding event. This is the lower threshold as schematically shown in figure 4.10a. The other threshold detects a change in state from bound to free, an unbinding event. This is the upper threshold in figure 4.10a. Two different thresholds are used to be less sensitive for statistical fluctuations, because only very large increases or decreases are detected. Only in the frames where both functions regard the system to be in the bound state, the state vector is set to one. Table 4.1 shows the upper and lower thresholds for both functions. The values are chosen such that the lower threshold is at a higher level than the level of the bound state and the upper threshold is about 1 sigma below the level in the free state. The thresholds are different for the two model systems because the dimensions of the systems are different.

![Rough state vector estimation](image)

![Analysis of start and end frame](image)

Figure 4.10 (a) Schematic representation of the use of an upper- and a lower threshold for the determination of the rough estimate of the state of the system. When the function crosses the lower threshold the system enters the bound state. The function needs to cross the upper threshold before the system is considered to be in the free state again. The double threshold serves to reduce the influence of statistical fluctuations. (b) Schematic representation of the method for determining the exact start- and end frame of a binding event. By independently varying the start- and end frame of a binding event and analyzing the area of the binding spot gives the exact start- and end frame of the binding event.
4.3.3 Step 3: Exact calculation of state vector
An estimated state vector has been obtained so far. This state vector consists of many zeros and several groups with ones, which represent binding events. The third and last step is to determine the exact start frame and end frame of each bound period. For this purpose different start- and end frame of the binding event are tested and for each start- and end frame the area that the data points span is calculated. The start- and end frame are varied 20 frames in both directions. It is likely that when the particle makes a first step after unbinding, this step will be out of the area where the particle was bound and this will result in a significant increase of the area that the data points span. This method is schematically represented in figure 4.10b. A threshold for unbinding is defined by an increase in area of more than 20% when increasing the bound period by one frame. Once the exact start- and end frame have been determined for each binding event, the analysis of that trajectory is finished revealing the detectable binding events for further analysis. Step 2 and 3 are subsequently repeated for all interesting trajectories.

Table 4.1 The upper- and lower threshold for the step size function and the area function for the STMS and the LTMS.

<table>
<thead>
<tr>
<th></th>
<th>Short-tether model system (STMS)</th>
<th>Long-tether model system (LTMS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step size function</strong></td>
<td>Lower threshold</td>
<td>60</td>
</tr>
<tr>
<td>(nm)</td>
<td>Upper threshold</td>
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</tr>
<tr>
<td><strong>Area function</strong></td>
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<tr>
<td>(nm$^2$)</td>
<td>Upper threshold</td>
<td>20,000</td>
</tr>
</tbody>
</table>
5. Measuring dissociation kinetics with TPM

This chapter describes the experiments that are performed to explore the potential of time-dependent tethered particle motion for measuring dissociation kinetics on a single-molecule level. Thermal dissociation of short single stranded DNA oligonucleotides is measured in two model systems: The short-tether model system (STMS) and the long-tether model system (LTMS). In each system two different oligonucleotides, 8bp and 10bp, with each a different dissociation constant are measured.

5.1 Experimental results

The experimental results obtained with the STMS and the 8bp oligo are described in this section. To observe repeated binding and unbinding of a tethered particle with the surface via single oligo-bonds, at first, the concentration regime in which mostly single oligo-bonds are formed is determined. Subsequently long time measurements are performed to observe many binding events. At the end of this section control experiments are shown to validate that the oligo-interaction is being measured.

5.1.1 Finding the single oligo-bond regime

The first goal is to find a concentration regime in which many binding events of single bonds can be measured by observing a single particle. To find this regime the particles are functionalized with dsDNA tethers and a large concentration of particle-oligos whereas the concentration of oligos on the surface is varied. For a certain concentration of surface-oligos there will be on average one single surface-oligo available for each particle and the situation shown in figure 2.1 is obtained. In this first experiment the STMS is prepared as explained in section 3.2 and the concentration of surface-oligos is varied from sample to sample. Here the 8bp oligos are used. This experiment has been performed twice, once for concentrations of surface-oligos 0.8-100 pM and once for concentrations 0.5-500 nM. In each experiment a control sample has been prepared in which no surface-oligos are incubated. Of each sample five different field of views are recorded for 60 s at a frame rate of 30 Hz. Figure 5.1 shows the total number of particles bound to the surface as a function of the surface-oligo concentration.

![Figure 5.1](image)

Figure 5.1 Number of particles bound to the surface for samples containing a different concentration of surface-oligos. Three regimes can be distinguished: At low surface-oligo concentrations the number of bound particles does not increase while in the intermediate regime the number of bound particles increases. In the high concentration regime the number of bound particles has reached a plateau value. The error bars represent the standard deviation of five FOVs.
The total number of bound particles are all particles that do not sediment to the bottom when the sample is turned over and thus includes: single- and multiple tethered particles, particles that are bound via multiple oligo bonds and non-specifically bound particles. Three regimes can be distinguished in the graph of figure 5.1. At low concentrations of surface-oligos the number of bound particles does not show any difference from the control sample. The density of surface-oligos is too low to induce many oligo-bonds. Increasing the surface-oligo concentration leads to more bound particles and since the number of dsDNA tethers per particle is constant, these additionally bound particles are bound via oligo-bonds. At high concentrations of surface-oligos the number of bound particles has increased and reaches a plateau.

Subsequently the motion pattern distribution plots of each concentration is analyzed to state something about the motion of the particles. Figure 5.2 shows an overview of the motion pattern distribution plots for different surface-oligo concentrations. Figures 5.2a and 5.2b show motion distribution plots for the low concentration regime. These motion distribution plots resemble the standard TPM motion distribution plot for the STMS which was shown in figure 4.4b. For increasing concentrations of surface-oligos a gradual transition is observed. Increasing the concentration results in a decrease of the intensity of the cloud of single tethered motion patterns and an increase in intensity of the cloud of restricted motion patterns. In the high concentration regime, figure 5.2d and 5.2e, almost all particles show restricted motion. The particles are most likely bound to the surface by multiple oligo bonds, such that their motion is strongly confined.

Figure 5.2 Motion pattern distribution plots of five samples for different concentrations of surface-oligos. A transition is observed from normal TPM for low concentrations of surface-oligos and all particles stuck on the surface for high concentrations of oligos. The concentrations are (a) 0 pM (b) 4 pM (c) 100 pM (d) 500 pM and (e) 50 nM.
Figure 5.2c shows a motion pattern distribution plot for the intermediate concentration regime. In this regime the concentration of surface-oligos is large enough to result in an increase of the number of bound particles, yet not too large that all particles show confined motion due to multiple oligo-bonds. The single bond regime will most probably be found at intermediate concentrations of surface-oligo as a low concentration of oligo bonds will not induce binding events, while a high concentration would immobilize the particles. To quantify the binding behaviour of the particles the number of particles showing binding events is determined with the Matlab algorithm described in section 4.3. The absolute number of particles showing a binding event within the 60 s recording of the particles is shown in figure 5.3 as a function of the surface-oligo concentration. As expected, the particles in the intermediate concentration regime on average show most binding events. However, the fraction of particles showing a binding event is still low: only about 4% at the most optimal surface-oligo concentration of 100 pM. At a zero concentration of surface-oligos there are on average 4 binding events. These binding events cannot be explained by the oligo interaction as the surface-oligos are absent in the system. Non-specific interactions and temporary formation of an additional main tether bonds are most probably responsible for these binding events.

![Figure 5.3](image)

Figure 5.3 The number of particles showing a binding event within the 60 s recording as a function of the concentration of surface-oligos. Three regimes can be distinguished: At low concentration of surface-oligo the particles only show few binding events. At intermediate concentrations the particles show more binding events. At high concentrations the particles show only very occasionally a binding events. The error bars represent the standard deviation of five FOVs.

A reason for observing only few binding events can be a low association rate of the system. The binding rate can be increased by using a higher concentration of surface-oligos. However, incubating more surface-oligos leads to more particles stuck on the surface by multiple oligos. Once a particle is bound to the surface by two oligos, the particle will be bound for a significantly longer time than when bound by a single oligo, such that the particle will often not come loose within the measurement time. For a description of the dissociation of a double oligo-bond see appendix E. All particles that are bound to the surface by multiple oligos are therefore observed as strongly confined motion patterns. In the situation that a tethered particle has a few surface-oligos close to it and two of these surface-oligos are at about the same location, the particle will end up bound with two particle-oligos to the two surface-oligos close
to each other. Binding events of single oligos dissociate within seconds, but once the particle is bound to the two oligos, it will be stuck for a longer time.

At a surface-oligo concentration of 100 pM the system shows most binding events. This concentration corresponds to an average of five surface-oligos available for each particle, assuming all surface-oligos bind homogeneously to the surface. Considering the fact that not all surface-oligos will actually bind, a single surface-oligo available for a particle is reasonable.

5.1.2 Measuring many binding events
Recordings of 60 minutes at a frame rate of 30 Hz are made for the STMS at a concentration of surface-oligos of 100 pM. One out of 231 particles showed repeating binding and unbinding events. Seven particles showed a few binding events and the other particles did not show any binding events. For the particle that showed repeated binding and unbinding the step size is shown in figure 5.4a for a window size of 2 s, and a time interval of 0.17 s. This time traces shows that the step size does not always show the same decrease during each binding event. The origin of this effect will be discussed below. Analyzing the motion of this particle with the Matlab algorithm reveals 53 binding and unbinding events.

![Figure 5.4](image)

Figure 5.4 (a) Step size of a particle showing repeated binding and unbinding events, calculated with a window size of 2 s and a time interval of 0.17 s. (b) Distribution of bond lifetimes. (c) Motion pattern of a part of the data where the binding spots are indicated with a blue contour. (d) Schematic representation of the freedom of motion of the particle when in the bound state for two extreme cases: Small and large distance between the oligo and the tether.
The recordings are analyzed to obtain the distribution of bound times using the Matlab algorithm described in subsection 5.3.2. Figure 5.4b shows the distribution of bond lifetimes for this particle. The bound times range from 3 s to 100 s. The binding events originate from binding of the particle with different surface-oligos on the surface. Because the particle is densely functionalized with particle-oligos, bonds can originate from different particle-oligos. Figure 5.4c shows the motion pattern of a 180 second window in the data where the binding spots are represented by blue circles. For this particle at least four surface-oligos are available. The total of 53 binding events are distributed over these four spots.

The position of an oligo-binding spot relative to the position where the main tether is bound to the surface determines how well a binding event can be detected. When the binding spot is far from the tether-point, the translational motion of the particle is confined more strongly than when the binding spot is close to the tether point. At large distances the tether is stretched and the motion is strongly confined, whereas at small distances there is less stress on the tether and the motion is dominantly determined by the oligonucleotide bond. Since the 3 nm long oligo is bound to an antibody with a maximum length of 15 nm the particle still has some freedom of motion, as illustrated in figure 5.4d. The larger the freedom of motion in the bound state, the smaller the change in the step size and area function will be. This variation in the drop of the step size during a binding event is observed in figure 5.4a. In order to be able to distinguish bound states from free states, the difference in freedom of motion between the two states has to be detectable. In particular for short binding times, 0-5 s, with only a few data points with restricted motion compared to the free state, it is even harder to detect the change. As mentioned in the previous chapter, only binding events with a binding time longer than 3 s can be detected with the algorithm. Obviously, the LTMS gives more contrast in the step size and the area function between the free and the bound state since the freedom of motion in the free state is larger in the LTMS compared to the STMS. The analysis of the determined lifetimes of the binding events will be given in section 5.2.

5.1.3 Control experiments

In the previous subsection particles are observed that show several binding events during the 60 minute recording. However, to be certain that it is the oligonucleotide interaction that is being measured and not some non-specific interaction, several control experiments are performed. In this subsection the control experiments performed on the STMS with the 8bp oligonucleotides are shown. Control experiments are performed to check the influence of the presence of anti-DIG or either of the oligonucleotides on the system. The influence of these components is investigated separately.

First the influence of incubating the anti-DIG on the system is determined by preparing samples with varying concentrations of anti-DIG. In this experiment both oligonucleotides are not present in the system. Of each sample five FOVs are recorded during 60 s at a frame rate of 30 Hz. Figure 5.5a shows the number of bound particles per FOV for different concentrations of anti-DIG. This graph shows that the number of bound particles does not change significantly when incubating a higher concentration of anti-DIG. The motion pattern distribution plots for all anti-DIG concentrations, shown in appendix F, do not show significant changes. Also no binding events were detected in each of these samples. The anti-DIG thus does not affect the time-dependent binding behaviour.

Figures 5.5b and 5.5c show the number of bound particles for concentration series of only surface-oligos and particle-oligos respectively. In the concentration series of the surface-oligos, no particle-oligos are used and vice versa. The number of bound particles does not show large differences when increasing either of the concentrations. The first data point in figure 5.5b does show a difference from the other
data points. However, the motion pattern distribution plots (shown in appendix F) do not show a difference between the one with a zero concentration of surface-oligo and the other concentrations. Most probably due to a pipetting error, there were less particles present in the fluid well with the zero concentration. Also here, the binding-behaviour observed in the model system is not affected by non-specific binding due to the presence of either of the oligos.

The last control experiment is performed to check if binding between the oligos is due to non-specific interaction. For this purpose the experiment described in subsection 5.1.1 is repeated with non-complementary oligonucleotides. On the surface the 10bp surface-oligos are incubated and on the particle the non-complementary 8bp particle-oligos are incubated. The concentration of surface-oligos is varied in the range from 0.8 - 500 pM. Figure 5.5d shows the concentration series of the surface-oligo for the non-complementary system in addition to the 8bp and the 10bp complementary system. As shown in subsection 5.1.1 the complementary system shows a transition from particles tethered by a single main tether to every particle bound to the surface by multiple oligonucleotides when increasing the concentration of surface-oligos. For the non-complementary oligos no increase is observed. The motion pattern motion distribution plots of the non-complementary system do not show significant differences and are shown in appendix F. In conclusion the control experiments show that the binding behaviour observed in subsection 5.1.2 are specific binding events of the oligonucleotides.

Figure 5.5  Number of surface bound particles for several control experiments: (a) Concentration series of anti-DIG. (b) Concentration series of surface-oligo. (c) Concentration series of particle-oligo. (d) Concentration series of non-complementary oligos compared to the concentration series of complementary oligos for the 8bp and the 10bp oligo.
5.2 Analysis of oligo unbinding kinetics

In this section the results of the 60 minute measurements as described in subsection 5.1.2 for both the STMS and LTMS with both the 8bp and the 10bp oligonucleotides are analyzed. In each measurement the motion of all particles has been analyzed with the Matlab algorithm to obtain the distribution of bond lifetimes. The dissociation constant of the oligonucleotide interaction is determined as explained in subsection 2.3.3. Simulations on the dissociation processes, described in subsection 2.5.2, are used to accompany the experimentally obtained data.

5.2.1 Short-tether model system

The results of the experiment with the STMS and the 8bp oligonucleotides have been discussed in subsection 5.1.2. A total of 53 binding events were detected in the motion of a single tethered particle. Figure 5.6a shows the cumulative probability of these binding events. This graph shows the evolution of the number of bound complexes as a function of time. The measured data is fitted with a single exponential decay function to obtain \( k_{off} = 0.095 \text{ s}^{-1} \), see equation 2.17. Some data points show deviations from the exponential fit. These deviations originate mainly from statistical fluctuations as a result of the low number of binding events. Figure 5.6b shows a collection of 10,000 simulations on the dissociation of 53 bonds with a dissociation constant equal to the one obtained from the fit in figure 5.6a, including the measured data. A range of cumulative decay curves is observed. This shows that with only 53 binding events the statistical fluctuations are significant. The measured curve falls within the range of simulations and therefore the deviation of the data from the fit in figure 5.6a can be explained by these statistical variations.

To validate that the observed decay process is single exponential, the cumulative decay function is shown semi-logarithmically in figure 5.6c. The average of the simulations and the 1- and 2-sigma bands are shown together with the measured data. This graph shows a linear behaviour indicating a single exponential decay. However, the tail contains two binding events with a longer life time such that the measured data falls out of the two sigma bands. This kink indicates an additional dissociation process, for example non-specif. But, the chance to find a value just outside the 2 sigma bands is with <0.05 quite possible. To check if there are two dissociation processes additional experiments are needed.

Due to the limited statistics the error in the measured dissociation constant mainly originates from the statistical fluctuations. The error is therefore determined by fitting the 2-sigma bands of the simulations. The dissociation constants obtained from these fits represent an upper and a lower boundary for the measured value which are asymmetrically distributed around the measured value. For the 8bp oligonucleotide in the STMS this results in \( k_{off} = 0.10 (0.08 - 0.15) \text{ s}^{-1} \). The calculated dissociation constant from the semi-empirical relation given by Strunz et al.\(^{26}\), equation 3.1, is equal to \( k_{off} = 0.1 \text{ s}^{-1} \). The calculated value falls within the error of the measured value.

The STMS was also used to measure the dissociation rate constant of 10bp oligonucleotides. A total of 88 binding events are detected by the Matlab algorithm. The binding events do not originate from the motion of a single particle, but they are the result of collecting data from 19 particles. Figure 5.6d shows the semi-logarithmic representation of the cumulative decay of the measured data including the average and the 1- and 2-sigma bands of 10,000 simulations performed on this dissociation process. A single exponential decay with a dissociation constant of \( k_{off} = 0.0039 (0.0031 - 0.0054) \text{ s}^{-1} \) is observed. The dissociation constant calculated with equation 3.2 is higher: \( k_{off} = 0.01 \text{ s}^{-1} \).
Figure 5.6  STMS: (a) Measured cumulative decay for the 8bp oligonucleotide in the STMS including a single exponential fit.
(b) A total of 10,000 simulations on the cumulative decay for 83 bonds of the 8bp oligonucleotides including the measured data. (c) Semi-logarithmic representation of the cumulative decay of the measured curve and average and the 1- and 2-sigma bands of the 10,000 simulated curves for the 8bp oligonucleotide. (d) Semi-logarithmic representation of the cumulative decay for the 10bp oligonucleotide in the STMS.

5.2.2 Long-tether model system
The dissociation constant of the oligonucleotides should not depend on the system in which they are measured. Therefore the same oligonucleotides are also measured with the long-tether model system. The 60 minute measurement of the 8bp oligonucleotides in the LTMS resulted in a total of 79 binding events originating from 7 particles. Figure 5.7a shows the semi-logarithmic representation of the measured data and the simulations. Due to the logarithmic y-axis the data points are not equidistantly in y-direction. Figure 5.7b shows a magnification of the square in figure 5.7a. The measured data shows two slopes, indicating two dissociation processes. In appendix G shows the data fitted with a single and a double exponential function including the residuals of the fits. The average and the 1- and 2-sigma bands are obtained from 10,000 simulations with two dissociation constants: $k_{off,1} = 0.065 (0.051 - 0.083) \text{ s}^{-1}$, $k_{off,2} = 0.0055 (0.0040 - 0.0088) \text{ s}^{-1}$. The first dissociation constant agrees with the dissociation constant obtained for the 8bp oligonucleotide with the STMS, $k_{off} = 0.10 (0.08 - 0.15) \text{ s}^{-1}$. The origin of the second dissociation constant is unknown, two possible mechanisms that can explain this double exponential behaviour will be described.
Two mechanisms can lead to the double exponential in the cumulative decay function. The first mechanism is the occurrence of additional non-specific binding between the particle and the surface. The functionalization of the 800 nm particles in the LTMS is not optimized for suppressing non-specific binding. This hypothesis has been checked by repeating the 60 minutes measurement with the LTMS without the particle-oligos and the surface-oligos to see if non-specific binding occurs. This measurement only showed four binding events for a total of 62 particles. No dissociation constant could be determined from this data. The low amount of binding events suggests that non-specific binding is not the origin of the additional dissociation process. However, more experiments would need to be performed to definite conclusions from this.

The second mechanism is rebinding of the oligonucleotides due to the slow particle diffusion. This mechanism could affect the measured bound times as the distinction between two short-time bonds directly after each other and a single longer bond cannot be made by the detection method. For a
rebinding probability of 0.85 the measured data and the simulations correspond best, as can be seen in figure 5.7c. The measured data falls within the collection of simulations, although the measured curve for short times shows fast dissociation and for long time shows slow dissociation compared to the simulated curves. Also the fact that simulating rebinding was not necessary to reconstruct the measured data for the STMS leads to believe that rebinding is not the origin of this double exponential behaviour. However, a combination of the non-specific binding and rebinding might be the cause.

For the 10bp oligonucleotides a limited number of 24 binding events were observed originating from the motion of 9 particles. Figure 5.7d shows the semi-logarithmic representation of the measured and simulated cumulative decay. A single exponential distribution is observed with a dissociation constant of \( k_{off} = 0.0026 (0.0019 - 0.0047) \text{ s}^{-1} \). This value is in the same range as the value measured for the 10bp oligo in the STMS. However, the statistics are too low to draw definite conclusions about the measured value. More experiments would need to be performed.

### 5.3 Summary

The dissociation constant of an 8bp and a 10bp oligonucleotide with a GC-content of 62.5% and 50%, respectively, is measured with time-dependent tethered particle motion. Table 5.1 shows an overview of the measured dissociation constants and the number of events that have been detected in every measurement, including the calculated values based on equation 3.1. The results show that it is possible to measure dissociation constants with time-dependent tethered particle motion. The dissociation constants obtained with the STMS and the LTMS are equal within the error. The measured values are in the same ballpark as the calculated values, however, only the calculated value for the 8bp oligo in the STMS falls within the error of the measured dissociation constant.

Table 5.1 Overview of the dissociation constants that are measured in this thesis for the STMS and the LTMS, including the number of events that have been detected in each measurement and the calculated value for each oligonucleotide.

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Calculated</th>
<th>STMS</th>
<th>LTMS</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>( k_{off} (\text{s}^{-1}) )</td>
<td>( N )</td>
<td>( k_{off} (\text{s}^{-1}) )</td>
</tr>
<tr>
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<td>0.10 (0.08-0.15)</td>
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<tr>
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<td>88</td>
<td>0.0026 (0.0019-0.0047)</td>
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</tbody>
</table>

For the 8bp oligonucleotide measured with the LTMS a double exponential fit was necessary to properly fit the measured data. The dissociation constant of 0.065 (0.051 – 0.083) s\(^{-1}\) seems to be due to the oligonucleotide interaction. However, the exact origin of the slower dissociation constant, 0.0055 (0.0040 – 0.0088) s\(^{-1}\), is unknown. As shown in the previous subsection, rebinding of the oligos due to the slow diffusion of the particle might be the origin of this double exponential behaviour. Another possible explanation is non-specific binding between the particle and the surface. The surface functionalization of the particles in the LTMS is not optimized to suppress non-specific interaction whereas for the particles in the STMS they are. This hypothesis has been checked by repeating the 60 minutes measurement without both the particle-oligos and the surface-oligos. Only a very low amount of non-specific interaction was observed and no dissociation constant could be determined. More experiments would need to be performed to draw definite conclusions from this.

The values for the off-rate of the oligos determined with the STMS and the LTMS measurements agree with each other. Dissociation in the STMS is slightly faster. The ratio \( k_{off,LTMS} / k_{off,STMS} \) is equal to 0.65 and 0.67 for the 8bp and 10bp oligo, respectively. These ratios are about equal suggesting a dependence on the model system. Although the motion is particle dominated, the faster dissociation
might be explained by the shorter tether putting more stress on the oligonucleotide-bond in the bound state. The stress is due to the tether being stretched more often in the STMS compared to the LTMS. However, this hypothesis is hard to verify with the obtained data. To test this hypothesis the experiments should be repeated with an even higher or lower tether length than the ones used here or another oligonucleotide length should be measured. Also more measurements need to be performed to obtain more statistics leading to more accurate dissociation constants.
6. Conclusion and outlook

In this thesis the potential of tethered particle motion (TPM) as a new method for measuring binding kinetics is explored. As a proof of principle two model systems are exploited: The short-tether model system (STMS) and the long-tether model system (LTMS). In the STMS a 1000 nm particle is tethered to the surface by a 40 nm dsDNA chain. In the LTMS an 800 nm particle is tethered to the surface by a 330 nm dsDNA chain. The specific interaction between the particle and the surface is probed with short complementary single stranded DNA oligonucleotides. The dissociation rate constants of two pairs of complementary oligonucleotides with different lengths, 8 and 10 nucleotides, and a G-C content of 62.5% and 50%, respectively, are measured.

We succeeded to find dissociation constants for both oligonucleotide lengths in both model systems: For the 8bp oligonucleotide a $k_{off,8bp,STMS} = 0.10 \ (0.08 - 0.15) \ s^{-1}$ is found and a $k_{off,8bp,LTMS} = 0.065 \ (0.051 - 0.083) \ s^{-1}$ is found relative to a literature value $k_{off,8bp,litt.} = 0.1 \ s^{-1}$. For the 10bp oligonucleotide a $k_{off,10bp,STMS} = 0.0039 \ (0.0031 - 0.0054) \ s^{-1}$ is found and a $k_{off,10bp,LTMS} = 0.0026 \ (0.0019 - 0.0047) \ s^{-1}$ is found relative to $k_{off,10bp,litt.} = 0.01 \ s^{-1}$. In the measurement of the 8bp oligonucleotide in the LTMS two populations of binding events were obtained. One of which could be explained by the oligonucleotide interaction, but the origin of the other population with remains unknown. Direct rebinding of the oligos after dissociation due to the slow diffusion of the particle might be the origin of this double exponential behaviour. A more probable possible explanation is non-specific binding between the particle and the surface. The surface functionalization of the particles in the LTMS is not optimized to suppress non-specific interaction whereas for the particles in the STMS they are. This hypothesis has been checked by repeating the 60 minutes measurement without both the particle-oligos and the surface-oligos. Only a very low amount of non-specific interaction was observed and no dissociation constant could be determined. More experiments would need to be performed to draw definite conclusions about this hypothesis.

The dissociation constants obtained with the STMS are higher than the ones obtained with the LTMS. Likely there is more stress on the bond due to the shorter tether which can lead to faster dissociation. The ratio $k_{off,LTMS}/k_{off,STMS}$ is equal to 0.65 and 0.67 for the 8bp and 10bp oligo, respectively. These ratios are about equal suggesting a dependence on the model system. However, to test this hypothesis the dissociation constant should be measured in TPM systems with varying tether lengths.

A Matlab algorithm is made that is able to discern binding events in the motion of the particle. This algorithm is based on the confinement of the motion of the tethered particle when bound to the surface by an oligo-bond. Binding events with a lifetime longer than 3 seconds can be detected with a 90% success rate. The success rate has been determined by analyzing a large set of trajectories with the algorithm and comparing the results to a manual library.

Repeated binding of the particle with the surface is observed for 15 particles, which is only a small fraction of the total number of tethered particles. Further optimization of the experimental system, fine-tuning the concentrations, could lead to an improvement in this efficiency. The binding events that have been observed correspond to single-molecule binding events of the same oligonucleotide on the surface binding multiple times with different oligonucleotides on the particle. By lowering the amount of particle-oligos it would be possible to probe binding between the same oligo on the surface and the same oligo on the particle. The kinetics of a single pair of oligonucleotides, or in general two molecules...
with a specific affinity, can be determined. The downside of decreasing the number of binding sites on the particle is a decrease in the association rate of the system.

With the time-dependent tethered particle motion method the kinetics of other interactions can be measured that have a short lifetime. In future research the potential of time-dependent tethered particle motion to be used in a biosensor could be explored. An example of a possible assay could be: Particles functionalized with an antibody against an analyte, tethered to a surface that is also functionalized with the antibodies. The tethered particle can catch the analyte from a solution and bind via the analyte to the surface. After a certain incubation time in which tethered particles can catch analytes the motion of the particles is measured during a short measurement time. From the motion of a single tethered-particle it is possible to tell if the particle has caught an analyte. From the motion of an ensemble of tethered particles the concentration of the analyte can be determined. This biosensor concept would be able to measure low concentrations, no analyte labelling is necessary and a robust signal is obtained with the micrometre sized particles.
7. Literature


8. Appendix

Throughout the thesis several times a reference is made to the appendix for extra information. This supplementary information is provided in this chapter.

8.1 Appendix A: Sample preparation protocols

The protocols for preparing the samples are fully described here. Firstly the short-tether model system, secondly the long-tether model system. Subsequently the anti-biotin functionalization protocol for the 800 nm carboxylic particles is described.

8.1.1 Protocol: Short-tether model system (STMS)

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block buffer</td>
<td>PBS + 1% (w/v) BSA</td>
</tr>
<tr>
<td>Wash buffer:</td>
<td>PBS</td>
</tr>
<tr>
<td>DNA dilution buffer:</td>
<td>TE buffer</td>
</tr>
<tr>
<td>Antibody surface incubation buffer</td>
<td>PBS</td>
</tr>
<tr>
<td>Oligonucleotide (-DIG) dilution buffer</td>
<td>EDTA</td>
</tr>
</tbody>
</table>

**Assay:**

A) Sample preparation

- Clean glass cover slip: 3x 10 minutes in acetone, IPA, methanol in sonic bath
- Attach the fluid cell wells
- DNA and Ab in spin until 5,000 rpm

B) First antibody concentration and incubation

1. Dilute anti-Texas Red (stock: 10 µL of 100 µg/mL)
   - Dilute 10 µL with 990 µL PBS (gives concentration 1 µg/mL)
   - Dilute 200 µL with 800 µL PBS (gives concentration 200 ng/mL)
   - Dilute 200 µL with 800 µL PBS (gives concentration 40 ng/mL)

2. Insert 25 µL on the sample surface in each well

3. Incubate antibodies for 60 minutes at room temperature ___:___ to ___:___

4. Flush 1000 µL PBS through the fluid cell wells after 60 minutes incubation of the antibodies

C) Second antibody concentration and incubation

1. Dilute anti-DIG (2x stock: 10 µL of 50 µg/mL)
   - Stock 1: Dilute 10 µL with 90 µL of PBS (gives concentration 5,000 ng/mL)
   - Stock 2: Dilute 10 µL with 90 µL of PBS (gives concentration 5,000 ng/mL)

2. Insert 25 µL on the sample surface in each well

3. Incubate antibodies for 60 minutes at room temperature ___:___ to ___:___

4. Flush 1000 µL PBS through the fluid cell wells after 60 minutes incubation of the antibodies

D) Sensor oligonucleotide concentration and incubation

1. Dilute surface-oligo 8bp (or 10bp) (stock: 10 µL of 1 µM)
   - Dilute 10 µL with 190 µL of EDTA (gives concentration 50 nM)
   - Dilute 100 µL with 900 µL of EDTA (gives concentration 5 nM)
   - Dilute 100 µL with 900 µL of EDTA (gives concentration 500 pM)
   - Dilute 200 µL with 800 µL of EDTA (gives concentration 100 pM)

2. Insert 25 µL on the sample surface in each well

3. Incubate oligonucleotides for 60 minutes at room temperature ___:___ to ___:___
Flush 1000 µL PBS through the fluid cell wells after 60 minutes incubation of the oligonucleotides

E) Block buffer realization
- Make 300 µL BSA block buffer 1% (w/v), so 3 mg BSA
- Place 25 µL BSA block buffer, in each well
- Incubate for 5 minutes at room temperature ___:___ to ___:___
- Flush with 1000 µL PBS

F) Particle functionalization
- Dilute DNA (stock: 10µL of 100 nM)
  - Dilute 10 µL stock with 390 µL TE buffer (2.5 nM)
  - Mix 10 µL MyOne™ particles (16.6 pM) with 10 µL DNA (2.5 nM) on vortexer (lowest possible level)
- Incubate on the rotating fin for 60 minutes ___:___ to ___:___
- Dilute particle-oligo 8bp (or 10bp) (stock: 10 µL of 100 µM)
  - Dilute 10 µL stock with 190 µL TE buffer (5,000 nM)
  - Add 10 µL particle-oligo (5,000 nM) to particles and DNA.
- Incubate on the rotating fin for 60 minutes ___:___ to ___:___
- Wash particles by magnetic separation 3x with 200 µL of 1% BSA block buffer
- Add 1000 µL of 1% BSA block buffer (keep in rotating fin until you have to use it)

G) Particle incubation
- Add 25 µL functionalized particles to fluid cell
- Incubate 60 minutes (activated surface at bottom) ___:___ to ___:___
- Tape the fluid cell wells shut, turn upside down for 10-30 minutes and clean glass microscope slide in the meantime ___:___ to ___:___
- Perform experiment under microscope
8.1.2 Protocol: Long-tether model system (LTMS)

- **Block buffer (only use on day of production)**: PBS + 1% (w/v) BSA
- **Wash buffer**: PBS
- **DNA dilution buffer**: TE buffer
- **Antibody surface incubation buffer**: PBS
- **Oligonucleotide (-DIG) dilution buffer**: EDTA

**Assay:**

A) **Sample preparation**
- Clean glass cover slip: 3x 10 minutes in acetone, IPA, methanol in sonic bath
- DNA and Ab in spin until 5,000 rpm

B) **First antibody concentration and incubation**
- Dilute anti-Texas Red (stock: 10 µL of 100 µg/mL)
  - Dilute 10 µL with 990 µL PBS (gives concentration 1 µg/mL)
  - Dilute 200 µL with 800 µL PBS (gives concentration 200 ng/mL)
  - Dilute 200 µL with 800 µL PBS (gives concentration 40 ng/mL)
- Insert 25 µL on the sample surface in each well
- Incubate antibodies for 60 minutes at room temperature ___:___ to ___:___
- Flush 1000 µL PBS through the fluid cell wells after 60 minutes incubation of the antibodies

C) **Second antibody concentration and incubation**
- Dilute anti-DIG (2x stock: 10 µL of 50 µg/mL)
  - Stock 1: Dilute 10 µL with 90 µL of PBS (gives concentration 5,000 ng/mL)
  - Stock 2: Dilute 10 µL with 90 µL of PBS (gives concentration 5,000 ng/mL)
- Insert 25 µL on the sample surface in each well
- Incubate antibodies for 60 minutes at room temperature ___:___ to ___:___
- Flush 1000 µL PBS through the fluid cell wells after 60 minutes incubation of the antibodies

D) **Sensor oligonucleotide concentration and incubation**
- Dilute surface-oligo 8bp (or 10bp) (stock: 10 µL of 1 µM)
  - Dilute 10 µL with 190 µL of EDTA (gives concentration 50 nM)
  - Dilute 100 µL with 900 µL of EDTA (gives concentration 5 nM)
  - Dilute 100 µL with 900 µL of EDTA (gives concentration 500 pM)
  - Dilute 200 µL with 800 µL of EDTA (gives concentration 100 pM)
- Insert 25 µL on the sample surface in each well
- Incubate oligonucleotides for 60 minutes at room temperature ___:___ to ___:___
- Flush 1000 µL PBS through the fluid cell wells after 60 minutes incubation of the oligonucleotides

E) **Block buffer realization**
- Make 300 µL BSA block buffer 1% (w/v), so 3 mg BSA
- Place 25 µL BSA block buffer, in each well
- Incubate for 5 minutes at room temperature ___:___ to ___:___
- Flush with 1000 µL PBS

F) **Particle functionalization**
- Sonicate smooth particles (16.6 pM): 5 pulses, 40% duty cycle, 70% amplitude (cold water reservoir)
- Dilute DNA (stock: 3 µL of 4 nM)
  - Dilute 3 µL stock with 3 µL TE buffer (2 nM)
Mix 4 µL particles (16.6 pM) with 4 µL DNA (2 nM) on vortexer (lowest possible level)

Incubate on the rotating fin for 60 minutes ___:___ to ___:___

Dilute particle-oligo 8bp (or 10bp) (stock: 10 µL of 100 µM)
  o Dilute 10 µL stock with 190 µL TE buffer (5,000 nM)

Add 4 µL particle-oligo (5,000 nM) to particles and DNA.

Incubate on the rotating fin for 60 minutes ___:___ to ___:___

Wash particles by magnetic separation 3x with 200 µL of 1% BSA block buffer

Add 1000 µL of 1% BSA block buffer (keep in rotating fin until you have to use it)

G) Particle incubation

Add 25 µL functionalized particles to fluid cell

Incubate 60 minutes (activated surface at bottom) ___:___ to ___:___

Tape the fluid cell wells shut, turn upside down for 10-30 minutes and clean glass microscope slide in the meantime ___:___ to ___:___

Perform experiment under microscope
8.1.3 Protocol: Functionalization of 800 nm carboxylic particles with anti-biotin

| Amount of particles:              | 1 mg          |
| Amount of anti-biotin antibody:   | about 75 μg/mg particles |
| Amount activation reagent (EDC added): | 1 mole equivalent of COOH |
| Coupling volume:                  | 200 μL        |
| Dynaparticles concentration during coupling: | 10 mg/mL |
| Activation/coupling buffer:       | 15 nM MES buffer pH 5.5 |
| Activation reagents:              | EDC (10 mg/mL in dH2O) |
| Activation time:                  | 30 minutes    |
| Coupling time:                    | 3 hours       |
| Coupling temperature:             | Room temperature |
| Wash/storage buffer:              | TBS + 0.1% Tween 20 |
| Block buffer:                     | PBS + 5%(w/v) BSA |
| Centrifuge settings:              | 5000 rpm, 10 minutes, 12 degrees Celsius, 10 pulses |

Method:

1. Pipette stock containing 1 mg PS particles in a DNA epp
2. Suspend particle in 100 μL 15 nM MES pH 5.5
3. Centrifuge particles
4. Repeat twice – totally wash three times
5. Centrifuge again, remove supernatant
6. Resuspend particles in (200 – x) μL 15 nM MES pH 5.5 (calculation of x see below)
7. Add x μL of EDC (10 μL/mL in cold MilliQ), mix by vortex
8. Incubate 30 minutes at room temperature or a roller mixer/rotating fins
9. Transfer to protein epp
10. Centrifuge and remove the supernatant
11. Resuspend particles in 15 nM MES pH 5.5. Total 200 μL = Buffer + antibody
12. Add 75 μg antibody/mg particles and mix by vortex
13. Incubate for 180 minutes at room temperature on a roller mixer/rotating fins
14. Add 200 μL PBS + 5%(w/v) BSA
15. Incubate for 30 minutes (to deactivate EDC groups)
16. Wash 3 times with TBST
17. Resuspend in TBST (10 mg/mL final concentration) for storage
18. Sonicate: sonic finger, 40% duty cycle, 70% amplitude, 10 pulses

Calculation of x:

\[ x = \left( \frac{M_w \text{ of EDC}}{\text{mmol COOH/g particles}} \right) \times \left( \frac{\text{mg of particles}}{\text{Conc. of EDC mg/mL}} \right) \]
8.2 Appendix B: Collection of motion patterns STMS and LTMS
A typical collection of motion patterns that is obtained when performing a TPM experiment with either of the model systems is shown in this section.

8.2.1 Collection of motion patterns STMS

Figure 8.1 Typical collection of motion patterns that is obtained when performing a TPM experiment with the STMS,
8.2.2 Collection of motion patterns LTMS

Figure 8.2 Typical collection of motion patterns that is obtained when performing a TPM experiment with the LTMS,
8.3 Appendix C: Analysis as a function of time in the LTMS

Similar to the analysis as a function of time for the STMS in subsection 4.2.2 here the analysis is shown of the LTMS. The step size function and the area function are calculated for a particle showing time-dependent motion. The window size and the time interval $\Delta t$ are varied here.

Figure 8.3 (a) Step size (blue) of a particle making time-dependent motion. The averaged step size (green) is calculated in windows of 2 s and the 1 sigma band is shown. (b) Dependence of the fluctuations in the step size on the window size. (c) Step size (window = 2 s) for different time interval $\Delta t$. (d) Contrast in the step size defined as the average step size in the free state over the average step size in the bound state. (e) Area function with a varying window size. (f) Contrast in the area function for different window size.
8.4 Appendix D: Matlab algorithm to distinguish bound and unbound states

A Matlab algorithm has been written to discern binding events in the observed motion of the particles. Here the full code of the algorithm is shown. The first subsection shows the main script of the algorithm. Subsequently the script to calculate a rough estimate of the state vector, the script to convert the frame numbers and a script to calculate the exact binding times are shown. Finally the script of the area function and the step size function are shown.

8.4.1 Main script of the algorithm

```matlab
function [StateVector, Bound_events_list, Unbound_events_list] = Determine_binding_kinetics

% Hardcoded variables
N_per_window = 60;

% Ask to select the right files for determining the
[File, PathName] = uigetfile('.mat', 'Selected the files for determining the binding times',
'MultiSelect', 'on');
if iscellstr(File)
    N_Files = numel(File);
elseif ischar(File)
    N_Files = 1;
    File = {File};
else
    error('Not enough files selected')
end

% Ask which trajectory to analyze
prompt = 'Which trajectory needs to be analyzed';
dlg_title = 'Insert trajectory number';
AskTrajectory = inputdlg(prompt, dlg_title, 1, {'1'});

% Initialize the output structure 'States' and
i_Count = 1;
StateVector = [];
Bound_events_list = [];
Unbound_events_list = [];

% Open the data
disp(['Opening file: ' PathName, File{1}]);
FileData = load([PathName, File{1}]);
Dataset = FileData.Dataset;
Result = FileData.Result;

% Determine variables of the file
N_Frames = Dataset.N_Frames;
FrameRate = Dataset.Camera.FPS;
Pixelsize = Dataset.Camera.xPIX;

% Now do this for every trajectory.
i_Trajectory = str2num(AskTrajectory{1});
Result(i_Trajectory).Trajectory = Result(i_Trajectory).Position;
Trajectory = Pixelsize*Result(i_Trajectory).Trajectory;

% Calculate an estimate of the binding times
[Bound_states_rough] = Calculate_states_rough(Dataset, Result, i_Trajectory, N_per_window, 5,
10000, 20000, 60, 80);
% Convert the running mean positions with the actual frame numbers
if sum(Bound_states_rough(1,:)) == 0;
    return
end
[Bound_states_converted] = Convert_mean_coordinates_to_frame_numbers(N_Frames, N_per_window,
Bound_states_Rough);
% Calculate the exact frames in which a bond is formed and
```
% the bond ends in an iterative way (3 times).
[Bound_states_exact, ~] = Calculate_states_exact (Dataset, Result, i_Trajectory,
Bound_states_converted);
[Bound_states_exact, ~] = Calculate_states_exact (Dataset, Result, i_Trajectory,
Bound_states_exact);
[Bound_states_exact, Binding_times] = Calculate_states_exact (Dataset, Result, i_Trajectory,
Bound_states_exact);

% Save the exact binding times
if sum(Bound_states_exact(1,:)) ~= 0;
    StateVector(i_Count).File = File{1};
    StateVector(i_Count).Trajectory = i_Trajectory;
    StateVector(i_Count).BoundOrNot = Bound_states_exact(1,1:N_Frames);
    StateVector(i_Count).BoundTimes = Binding_times;
    Bound_events_list = [Bound_events_list,Binding_times(3,:)];
end

% Show the positions of the particle where the bond is located:
x = Trajectory(1,:);
y = Trajectory(2,:);
axis_settings = [min(x) max(x) min(y) max(y)];
X_bond = zeros(1,N_Frames);
X_bond(1,:) = Bound_states_exact.*Trajectory(1,:);
Y_bond = zeros(1,N_Frames);
Y_bond(1,:) = Bound_states_exact.*Trajectory(2,:);
figure;
plot(x(1:N_Frames), y(1:N_Frames), 'o', X_bond(1:N_Frames),Y_bond(1:N_Frames), 'o');
axis image;
axis(axis_settings);
title('2D trajectory in blue the bond is shows!')
xlabel('x position (nm)')
ylabel('y position (nm)')

% if there is more than one bond, then also save the time between the bonds.
if numel(Binding_times(3,:)) > 1;
    StateVector(i_Count).UnboundTimes = Binding_times(4,:);
    Unbound_events_list = [Unbound_events_list,Binding_times(4,:)];
else
    StateVector(i_Count).UnboundTimes = 0;
end
end

8.4.2 Script for calculating a rough estimate of the state vector

function [Bound_states_rough] = Calculate_states_rough(Dataset, Result, i_Trajectory, N_per_window, N_jump, ThresholdArea, ThresholdAreaTWO, ThresholdStepsize, ThresholdStepsizeTWO)

%ShowTrajectoryAnalysisGUI(Dataset,Result,i_Trajectory);
N_Frames = numel(Result(i_Trajectory).Trajectory(1,:));

% Calculate the area of the running mean
[AreaShiftWindow] = CalculateAreaShiftWindow(Dataset, Result, i_Trajectory, N_per_window);

% Calculate the stepsize and its standard deviation.
[~, ~, StepsizeShiftWindow, ~] = CalculateAverageStepsizeShiftWindowJump(Dataset, Result, i_Trajectory, N_per_window, N_jump);

% Determin the rough binding times
Bound_states_rough = zeros(1,N_Frames-N_jump-N_per_window);
for j_Frame = 1:N_Frames-N_jump-N_per_window
    if StepsizeShiftWindow(1,j_Frame) <= ThresholdStepsize && AreaShiftWindow(1,j_Frame) <= ThresholdArea;
        Bound_states_rough(1,j_Frame) = 1;
    elseif j_Frame-1 > 0 && Bound_states_rough(1,j_Frame-1) == 1 &&
        StepshiftShiftWindow(1,j_Frame) <= ThresholdStepsize && AreaShiftWindow(1,j_Frame) <= ThresholdAreaTWO;
        Bound_states_rough(1,j_Frame) = 1;
    else
        Bound_states_rough(1,j_Frame) = 0;
    end
end

end
### 8.4.3 Script to convert mean coordinates to frame numbers

```matlab
function [Bound_states_converted] = Convert_mean_coordinates_to_frame_numbers(N_Frames,N_per_window,Bound_states_rough)

% Map the rough binding times, which are as a function of the running mean, on to the actual frame numbers.
Dummy = 0;
Bound_states_converted = zeros(1,N_Frames);
for i_Frame_shiftwindow = 1:numel(Bound_states_rough(1,:))
    if Bound_states_rough(1,i_Frame_shiftwindow) == 1;
        Dummy = Dummy + 1;
    end
    if Bound_states_rough(1,i_Frame_shiftwindow) == 0 && Dummy ~=0;
        Bound_states_converted(1,i_Frame_shiftwindow+(N_per_window/2)-Dummy:i_Frame_shiftwindow+N_per_window/2) = 1;
        Dummy = 0;
    end
end
end
```

### 8.4.4 Script to calculate exact start and end frame of binding events

```matlab
function [Bound_states_exact, Binding_times] = Calculate_states_exact(Dataset,Result,i_Trajectory,Bound_states_converted)

% Constants necessary for this calculation.
N_Frames = Dataset.N_Frames;
Pixelsize = Dataset.Camera.xPIX;
Trajectory = Pixelsize*Result(i_Trajectory).Trajectory;

% 1) The first step is to obtain the actual times that the particle is bound to the surface and save them in an array.
j_Count = 0;
k_Count = 0;
for i_Frame = 1:N_Frames;
    % If the particle is bound, start counting the bound time.
    if Bound_states_converted(1,i_Frame) == 1;
        j_Count = j_Count + 1;
    end
    % If the particle is not bound anymore, save the binding time and the start and end frame in the Binding_times array. Only look at binding events larger than 10 frames. Otherwise the bond cannot be detected.
    if Bound_states_converted(1,i_Frame) == 0 && j_Count > 0;
        if j_Count >= 10;
            k_Count = k_Count + 1;
            Binding_times(1,k_Count) = i_Frame - j_Count; % Start Frame of bond
            Binding_times(2,k_Count) = i_Frame - 1; % End Frame of bond
            Binding_times(3,k_Count) = j_Count; % # Frames of bond
        end
        j_Count = 0;
    end
end

% 2) The second step is to analyze each bond and check if the right begin and end frames are listed. If not, change them.
if k_Count > 0;
    for i_Bond = 1:k_Count
        Begin_Frame = Binding_times(1,i_Bond);
        End_Frame = Binding_times(2,i_Bond);
        Total_Frame = Binding_times(3,i_Bond);
        % Calculate the amount of frames that you can go down.
        if Begin_Frame <= 15;
            Delta_frames_down = Begin_Frame - 1;
        else
            Delta_frames_down = 15;
        end
end
```

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% Calculate the amount of frames that you can go up.
if Total_Frame <= 15;
    Delta_frames_up = Total_Frame - 3;
else
    Delta_frames_up = 13;
end
Area = zeros(1,Delta_frames_down+Delta_frames_up+1);
% Check the area of different begin frames.
for i_begin = -Delta_frames_down:1:Delta_frames_up
    [~,Area(i_begin+1+Delta_frames_down)] =
        convhull(Trajectory(1,Begin_Frame+i_begin:End_Frame),Trajectory(2,Begin_Frame+i_begin:End_Frame));
end
% Determine the correct begin frame.
for i_begin = -Delta_frames_up:1:Delta_frames_down
    if Area(i_begin+1+Delta_frames_down) < 1.2*min(Area(1,:));
        Begin_Frame_best = Begin_Frame + i_begin;
    end
end
Binding_times(1,i_Bond) = Begin_Frame_best;
Binding_times(3,i_Bond) = Binding_times(2,k_Count) - Binding_times(1,k_Count) + 1;
% Calculate the amount of frames that you can go up.
if End_Frame > N_Frames - 15;
    Delta_frames_up = N_Frames - End_Frame;
else
    Delta_frames_up = 15;
end
% Calculate the amount of frames that you can go down.
if Total_Frame <= 15;
    Delta_frames_down = Total_Frame - 3;
else
    Delta_frames_down = 13;
end
Area = zeros(1,Delta_frames_down+Delta_frames_up+1);
% Check the area of different end frames.
for i_end = -Delta_frames_down:1:Delta_frames_up
    [~,Area(i_end+1+Delta_frames_down)] =
        convhull(Trajectory(1,Begin_Frame:End_Frame+i_end),Trajectory(2,Begin_Frame:End_Frame+i_end));
end
% Determine the correct end Frame.
for i_end = -Delta_frames_down:1:Delta_frames_up
    if Area(i_end+1+Delta_frames_down) < 1.2*min(Area(1,:));
        End_Frame_best = End_Frame + i_end;
    end
end
Binding_times(2,i_Bond) = End_Frame_best;
Binding_times(3,i_Bond) = End_Frame_best - Begin_Frame_best + 1;
end
% 3) Determine the time between two bonds and save it in the binding_times structure.
N_unbound = numel(Binding_times(1,:))-1;
for i_unbound = 1:N_unbound
    Binding_times(4,i_unbound) = Binding_times(1,i_unbound+1) - Binding_times(2,i_unbound) + 1;
end
% 4) Replace the values in Bound_states_exact with the new correct values that have been calculated in step 2.
Bound_states_exact = zeros(1,N_Frames);
for i_bond = 1:k_Count
    Bound_states_exact(1,Binding_times(1,i_bond):Binding_times(2,i_bond)) = 1;
end
else
    Bound_states_exact = zeros(1,N_Frames);
    Binding_times = zeros(3,1);
end

8.4.5 Script to calculate the area function
function [AreaShiftWindow] = CalculateAreaShiftWindow(Dataset, Result, i_Trace, N_per_window)
% Trajectory information.
N_Frames = Dataset.N_Frames;
Pixelsize = Dataset.Camera.xPIX;
Trajectory = Pixelsize*Result(i_Trace).Trajectory;

% Calculate the convex hull of the particle within each window.
AreaShiftWindow = zeros(1,N_Frames-N_per_window);
for i_Frame = 1:N_Frames-N_per_window
    [~,Area] = convhull(Trajectory(1,i_Frame:i_Frame+N_per_window),Trajectory(2,i_Frame:i_Frame+N_per_window));
    AreaShiftWindow(1,i_Frame) = Area;
end

8.4.6 Script to calculate step size function
function [StepsizePerFrame, StepsizeJump, StepsizeShiftWindow, SD_StepsizeShiftWindow] = CalculateAverageStepsizeShiftWindowJump(Dataset, Result, i_Trace, N_per_window, Jump)

% Obtain the trajectory information.
N_Frames = Dataset.N_Frames;
Pixelsize = Dataset.Camera.xPIX;
Trajectory = Pixelsize*Result(i_Trace).Trajectory;

% The Stepsize from frame to frame and the average stepsize in each window
% are defined here.
StepsizePerFrame = zeros(1,N_Frames-1);
StepsizeShiftWindow = zeros(1,N_Frames-N_per_window-Jump);
StepsizeJump = zeros(1,N_Frames-N_per_window-Jump);

% The Stepsize is calculated for every frame.
for i_Frame = 1:N_Frames-1
    StepsizePerFrame(1,i_Frame) = sqrt((Trajectory(1,i_Frame+1)-Trajectory(1,i_Frame))^2+(Trajectory(2,i_Frame+1)-Trajectory(2,i_Frame))^2);
end

% The Stepsize for a Jump larger than 1 frame.
for i_Frame = 1:N_Frames-Jump
    StepsizeJump(1,i_Frame) = sqrt((Trajectory(1,i_Frame+Jump)-Trajectory(1,i_Frame))^2+(Trajectory(2,i_Frame+Jump)-Trajectory(2,i_Frame))^2);
end

% Calculate the average stepsize per shift window.
for i_Frame = 1:N_Frames-N_per_window-Jump
    StepsizeShiftWindow(1,i_Frame) = mean(StepsizeJump(1,i_Frame:i_Frame+N_per_window));
    SD_StepsizeShiftWindow = std(StepsizeJump(1,i_Frame:i_Frame+N_per_window))/sqrt(N_per_window);
end
Appendix E: Analysis of dissociation of double-oligo bond

In the case that a tethered particle makes a bond with two oligos, as indicated in figure 8.4a, it is expected that the life time of this bond will be longer. The dissociation of a double oligo-bond is a two-step process. First one of the bonds needs to be broken and subsequently the other one. This leads to two pathways for dissociation. When the system is in the transient state, there is a probability to reattach the bond that has already dissociated before the second bond dissociates. This reattachment rate $k_{\text{reatt.}}$ is unknown. Note that in rebinding from the free state to the transient state is not taken into account. The dissociation process of a double oligo-bond can be simulated. However, the reattachment rate first needs to be estimated.

The reattachment rate is estimated from the diffusion limited reaction rate $F$, shown in equation 8.1. The dissociation constant for particles with a radius of $R = 5 \cdot 10^{-7}$ m close to a surface is about $D = 10^{-14}$ m$^2$/s. The concentration $c$ in this system can be approximated by an effective concentration since there is only one oligo. The particle is bound to the surface by a single oligo-bond and the oligo is bound to the surface by a DIG anti-DIG bond. This antibody-oligo complex works as a tether for the particle with a maximum length of 15 nm (antibody) plus 3 nm (oligo). One over the volume in which the two free oligos diffuse is an estimate of the effective concentration. Approximating this volume by a spherical cap with a radius equal to the length of the antibody-oligo complex gives a concentration of about $c \approx 10^{23}$ m$^{-3}$.

$$F = 4\pi \cdot D \cdot R \cdot c \approx 10^3 - 10^4 \text{ s}^{-1}$$  \hspace{1cm} (8.1)

The simulations on dissociation have been performed for a single oligo-bond and a double oligo-bond. The reattachment rate is chosen to be lower than the diffusion limited binding rate, because not every hit will result in a bond: $k_{\text{reatt.}} = 10$ s$^{-1}$. A dissociation constant of $k_{\text{off}} = 0.1$ s$^{-1}$ is used and 100 simulations of 100 binding events are performed. Figure 8.4b shows a logarithmic representation of the cumulative decay. This graph shows that dissociation of a double-oligo bond is much slower than dissociation of a single oligo bond, even for a reattachment rate of $k_{\text{reatt.}} = 10$ s$^{-1}$. On the time scale of the experiment, some double oligo-bonds may dissociate. However, the observed binding events in the experiments mainly originate from single oligo-bonds.

Figure 8.4 (a) Schematic representation of the two-step dissociation process of a double-oligo bond. (b) The logarithm of the number of bound particles as a function of the lifetime for a single and a double oligo-bond using a reattachment rate of $k_{\text{reatt.}} = 10$ s$^{-1}$.
8.6 Appendix F: Motion pattern heat maps of control experiments
Several control experiments have been performed to prove that the observed binding behavior is due to the oligonucleotide interaction. In this supplementary section the motion pattern heat maps obtain in these experiments are shown.

8.6.1 Influence of anti-DIG on non-specific binding in the system

Figure 8.5 Motion pattern heat maps of the control experiment with samples with varying concentrations of anti-DIG: (a) 0 ng/mL, (b) 40 ng/mL, (c) 200 ng/mL, (d) 1,000 ng/mL, (e) 5,000 ng/mL and (f) 20,000 ng/mL. There are differences between the different samples, however, there is no clear trend visible. Only at a concentration of 20,000 ng/mL the motion pattern is quite different. This could be due to an error in the preparation of the sample. In all other experiments an anti-DIG concentration of 5,000 ng/mL is used.
8.6.2 Influence of surface-oligo on non-specific binding in the system

Figure 8.6 Motion pattern heat maps of the control experiment with samples with varying concentrations of surface-oligos: (a) 0 nM, (b) 0.5 nM, (c) 5 nM, (d) 50 nM and (e) 500 nM. There are small differences between the different samples, however, there is no clear trend visible.
8.6.3 Influence of particle-oligo on non-specific binding in the system

Figure 8.7 Motion pattern heat maps of the control experiment with samples with different concentrations of particle-oligos: (a) 0 nM, (b) 0.8 nM, (c) 4 nM, (d) 20 nM, (e) 100 nM and (f) 500 nM. There is some trend with the concentration of particle-oligos. However, there is no large effect and no binding events were detected in these experiments.
Figure 8.8  Motion pattern heat maps of the control experiment with samples with different concentrations of non-complementary oligonucleotides: (a) 0 pM, (b) 4 pM, (c) 20 pM, (d) 100 pM, (e) 500 pM and (f) 2,500 pM. The transition that was observed for the complementary oligonucleotides, shown in figure 6.2, is here not observed.
8.7 Appendix G: Single and double exponential fit of data of 8bp oligo in LTMS

Figure 8.9a shows the cumulative decay of the data obtained for the 8bp oligo in the LTMS fitted with a single exponential decay function. Figure 8.9b shows the residuals of the single exponential fit of figure 8.9a. The data and the fit show major deviations as can be seen in the residuals. The residuals are a factor 3 larger than the residuals for the single exponential fits of the cumulative decay measured for the other oligos and model systems. This leads to believe that the data does not originate from a single dissociation process. Figure 8.9c and 8.9d show a double exponential decay fit and the residuals of the fit, respectively. The double exponential decay function fits the data better, obviously. The amplitude of the residuals is now in the range of the residuals observed for the other measured cumulative decays.

Figure 8.9   (a) Number of bonds as a function of time with a single exponential fit. (b) Residual of the single exponential fit in figure 8.9a. (c) Number of bonds as a function of time with a double exponential fit. (d) Residual of the double exponential fit in figure 8.9c.