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Dit proefschrift is goedgekeurd door de promotor:

prof.dr.ir. M.W.J. Prins

Copromotor:
dr. L.J. van IJzendoorn

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1 Introduction

A point-of-care biosensor is a compact device to detect biomarkers in small amounts of complex fluids like blood or saliva. Sensors for clinically relevant biomarkers have to meet high standards on specificity and sensitivity and, furthermore, the detection should be rapid. An important class of sensor concepts involves the use of protein coated particles. In this thesis we will address one of the key processes that determine the specificity and sensitivity of particle based biosensors: the non-specific interactions of proteins and protein coated particles with the substrate materials that are employed.

In this chapter a general introduction will be given on existing biosensors and new developments in this field, with a focus on magnetic particle based biosensors. Biomarkers that are detected using biosensors are often proteins and, furthermore, bioreceptor molecules that are used to bind these biomarkers in the sensor are often antibody proteins. Therefore, the general structure of protein molecules is introduced and a short overview is given of cardiac biomarkers that are employed as targets in biosensor design. To detect biomarkers, immunoassays are employed in the design of biosensors. We describe the working principle and a number of existing commercial biosensors as well as new developments based on magnetic particle labels. We introduce our model system that is used in experiments throughout this thesis to study non-specific interactions. The physicochemical interactions underlying the energy potentials of particle–surface interactions are explained. Techniques are introduced to study protein–surface interactions in the context of particles as well as on the single molecule level. An overview is given of literature related to non-specific protein–surface interactions with emphasis on systems related to our model. Our research approach is introduced to get a better understanding of non-specific interactions between proteins and surfaces, in order to be able to control these in future biosensor designs. Finally, the structure of this thesis is explained.

1.1 Biomarkers

Biomarkers are molecules in the human body that can give information on the condition of a patient, about processes in the body or about diseases, like cardiovascular diseases. The concentrations in which biomarkers are present in the human body can be very low, into the sub-picomolar (pM) regime [1].

Since cardiovascular diseases are a major cause of death, biosensors that are able
Chapter 1: Introduction

Figure 1.1: The structure of proteins shown at different levels. The primary structure is formed by the chain of amino acids, the secondary structure consists of elements like the β sheet and α helix, the tertiary structure is the total three dimensional structure of the polypeptide and the quaternary structure is formed when multiple polypeptide chains come together to form a larger structure (the tertiary structure of the P13 protein is shown and the quaternary structure of hemoglobin). Figure adapted from Wikimedia Commons.

Biomarkers can present an advance in healthcare. For example, chest pain can be related to acute coronary syndrome as well as to non-cardiac diseases. A fast and easy cardiac biomarker test would help to rule in or rule out certain cardiac causes for the chest pain. Point-of-care biosensors are being developed for cardiac biomarkers, which can be individually assessed or combined in a panel to increase reliability [2–4].

Amongst others, B-type natriuretic peptide, cardiac Troponin I and myoglobin are important biomarkers for cardiovascular diseases. B-type natriuretic peptide is a peptide released by the heart in response to myocardial tension and increased intravascular volume. It is an indicator of heart failure and can be used as a prognostic indicator for survival of patients with a relevant detection range that goes into the sub-pM regime [5]. Cardiac Troponin I is a regulatory protein that controls interaction between actin and myosin in heart muscle and is released in pM concentrations upon damage. It is detectable in the blood a few hours after the onset of an acute coronary event [6]. Myoglobin is a protein located in muscle tissue that stores oxygen. It is released into the blood stream one to two hours after the onset of acute myocardial infarction [7]. Levels above ~10 nM are considered abnormal [4].

1.2 Proteins

Most biomarkers are proteins, like the cardiac proteins mentioned above. Furthermore, specific bioreceptor molecules used in biosensors are often antibody proteins. To better understand non-specific protein interactions, we introduce the general structure of protein molecules.

Proteins are polypeptides that are built from a set of 20 different amino acids linked
by peptide bonds. The sequence in which the amino acids are linked is encoded in the cell’s DNA. The different levels of the structure of proteins are illustrated in figure 1.1. The polypeptide chain formed by the linked amino acids is called the primary structure. By hydrogen bonding between the amino acid’s side chains secondary structures are formed, like β sheets and α helices. The resulting three-dimensional structure of the polypeptide chain is called the protein’s tertiary structure and this is formed by interactions amongst the amino acid side chains. For example disulfide bonds can be formed, but the most important driver in water-soluble proteins is the hydrophobic effect: in a watery environment hydrophobic amino acids cluster in the interior of the protein while hydrophilic amino acids form the protein outer surface [8]. Some proteins, e.g. antibodies, are formed from an assembly of a number of polypeptide chains, resulting in the quaternary structure.

Parts of antibody protein molecules, the so-called paratopes, are able to specifically recognize and bind to other molecules by non-covalent interactions. In the human body, the main task of antibodies is to bind to molecules that are exogenous to the body and in this way signal the rest of the immune system to react. Antibodies can be used in a biosensor to specifically recognize a known biomarker. To produce antibodies of a single type for this purpose, hybridoma technology is employed in which specific antibody producing cells are fused with cancer cells and grown in tissue culture [9].

1.3 Biosensors

The detection of biomarkers at the point-of-care can significantly improve the efficiency of our healthcare system and this goal can be attained by developing point-of-care biosensors, which can be used at the bedside of the patient or in the doctors office, ideally without a specialist being needed to obtain a reliable result. Immunoassays are regularly employed to detect biomarkers in laboratories and can also be scaled down to be used in biosensors. In this section, an overview is given of existing commercial biosensors, most of which are based on immunoassays. Furthermore, details are given on the development of biosensors based on magnetic particle labels.

1.3.1 Immunoassays

In an immunoassay, very specific receptors to the biomarker (often antibody proteins) are used to bind the biomarker to a surface and to label the biomarker for detection. The signal from the labels is read out to give an output related to the biomarker concentration in the sample. In figure 1.2 (a) a schematic representation is shown of the specific binding of a biomarker molecule in an immunoassay on a surface functionalized with antibody molecules, the biomarker is labeled by a particle for detection.

In an immunoassay both the sensitivity and the specificity of detection are important. The sensitivity should be high enough to measure the low biomarker concentration, typically in the pM regime. Especially when an immunoassay is used in the design of a biosensor, the specificity is critical to its performance. In the complex fluids that are analyzed in a biosensor (e.g. blood and saliva) very many different proteins are present apart from the biomarker that can influence the detection by interfering with the immunoassay. In blood some proteins are present in mM concentrations. At the typical biomarker concentration in the pM regime, very many different proteins
are present [10]. To reach the goal of good specificity for the desired biomarker, non-specific interactions with other molecules should be suppressed.

1.3.2 Existing commercial biosensors

To make biosensors suitable for use at the point-of-care, the biosensor should enable a specific and sensitive detection of the biomarker with a quantitative output of the biomarker concentration. An existing commercial biosensing device that gives a quantitative output is the blood glucose test. Diabetics use this test to measure their blood glucose level in a droplet of blood from a finger prick. It uses a.o. a redox enzyme which enables electrochemical detection [11]. Glucose is present in human blood in mM concentrations and this relatively high concentration is detectable by electrochemical methods.

A commercial biosensing device for lower biomarker concentrations is the lateral flow test for human chorionic gonadotropin (hCG, a pregnancy biomarker), which is based on an immunoassay. Concentrations of about 100 pM can be detected in urine and changes in concentration of about an order of magnitude can be distinguished to estimate the time since conception [12]. This technology is, however, not accurate enough to be generally applicable for protein biomarker testing.

An extensive overview of biosensing devices based on immunoassays (as well as on chemistries and nucleic acid testing) is given by Chin et al. [11]; both existing products and products in development are discussed. An important challenge for the development of point-of-care biosensors is the integration of all the assay steps from sample collection to reporting the result in an automated fashion [11].

1.3.3 Biosensors based on magnetic particle labels

In an immunoassay, labels are often used to facilitate detection of captured biomarker molecules. Different types of labels can be used, like fluorophores, enzymes or particles. By using magnetic particles as labels, the signal resulting from the assay can be read out using a magnetic or an optical measurement. For example a complementary metal-oxide-semiconductor chip [13] or giant magnetoresistance detection elements can be used [14]. The magnetic immuno-chromatographic test system that is being developed by MagnaBioSciences uses thin film induction coils to measure the magnetic
Figure 1.3: Actuation steps in the sandwich immunoassay employed in the Magnotech system based on magnetic particles: (a) capture of the biomarkers by the particles, (b) magnetic forces bring the particles close to the surface to allow binding via the biomarkers, (c) removal of unbound and weakly bound particles by magnetic forces, (d) detection of remaining particles by frustrated total internal reflection [18].

particles [15]. Magnisense aims at developing point-of-care tests for cardiac markers, also by measuring the magnetic response of magnetic particle labels [16].

An advantage of the use of magnetic particles as labels in an immunoassay is that they can be actuated and thereby the assay can be accelerated. This property is used in the Magnotech biosensing system that is being developed by Philips. A point-of-care biosensor is being developed for the detection of the cardiac biomarker Troponin I [17]. The particles used in the biosensor as labels are superparamagnetic and are actuated to accelerate their transport. The particles consist of a polymer matrix filled with magnetic grains. The absence of a permanent magnetic moment prevents magnetic clustering of the particles in the absence of an applied field, while the magnetic moment induced by an external magnetic field allows actuation.

In figure 1.3 the actuation steps in the immunoassay are illustrated. The first step is to capture the target molecules. In the second step the particles are pulled down toward the surface. Here, specific binding occurs with the bioreceptor molecules. In the third step, unbound and weakly bound particles are removed from the sensor surface by applying a force. Finally, the surface coverage of particles is read out by frustrated total internal reflection, which is representative for the biomarker concentration in the sample [18].

To be able to optimally use the possibilities of magnetic particles as labels in an assay, a thorough understanding of the non-specific interactions between such particles and surfaces is needed. In this thesis we will statistically address both the association and the dissociation of non-specifically interacting protein-coated particles with a biosensor surface. Furthermore, a study on the interaction between single protein molecules and biosensor surfaces will be presented.

1.4 Model system

For the experiments performed in this thesis we chose a model system which consists of the cardiac biomarker protein myoglobin and an (oxidized) polystyrene surface, as shown in figure 1.4. This model system is not based on a specific sensor design
but rather it is intended to develop the techniques to characterize particle–surface and protein–surface interactions. Furthermore, myoglobin has been chosen as a model protein because it can be used in molecular dynamics simulations (see section 1.4.3) due to its small size and well characterized structure.

### 1.4.1 Myoglobin

To study the non-specific interactions between protein coated particles and polymer surfaces, we chose myoglobin as the model protein. Myoglobin is a relevant cardiac biomarker (as introduced in section 1.1) with a well studied structure. It consists of 153 amino acids and has a weight of 17184 Da [20]. About 70% of the main chain is folded into α-helices, while the rest of the chain forms turns and loops [8]. This gives the protein a globular structure and a size of $\sim 4.5 \times 3.5 \times 2.5 \text{ nm}^3$. Myoglobin contains a heme group to bind oxygen. A schematic picture of the tertiary structure of myoglobin is shown in figure 1.4 (a).

Myoglobin is a water soluble protein. Charged residues are absent from its inside. Clustered hydrophobic groups, buried inside the molecule, give the molecule a high thermodynamic stability in an aqueous medium [8]. On its outside both polar and non-polar residues are present. The isoelectric point of myoglobin is 6.6 [21].

### 1.4.2 Polymer biosensing substrates

Polymer materials are often used as a substrate material for immunoassays and point-of-care devices. For a substrate material to be suitable for use in a biosensor, a number of requirements have to be met. As can be seen in figure 1.2 (a) an immunoassay requires functionalization of the surface with bioreceptor molecules, often antibody proteins, to be able to specifically bind biomarkers. The antibody molecules should be present in a high surface density to get a high sensitivity. The sensitivity is also influenced by the conformation of the antibodies: the interaction between the
molecules and the surface can lead to conformational changes that alter their affinity to the biomarkers.

On the one hand, non-specific interactions are used to functionalize the surface with antibody molecules; on the other hand, non-specific interactions between the biomarkers and the surface should be prevented to increase specificity. Modification of the surface allows one to control the properties of the surface and thereby the non-specific interactions can be influenced to reach the desired sensitivity and specificity.

In most laboratory scale solid-phase immunoassays, proteins are bound to a polymer surface. Especially the polymer polystyrene is a frequently used material for immunoassays because of its easy processing and cost-effective mass production by e.g. injection molding. Polystyrene can be produced in many forms like petri dishes, wellplates, uniform latex particles or custom designed cartridges. Proteins easily adsorb to the material and the polystyrene binding capacity can be modified by changing the surface oxygen content [1]. A commonly used method to make polystyrene more hydrophilic is by oxidation, for example by UV/ozone treatment [22].

We chose polystyrene as model surface material. Atactic polystyrene was used, which means that the benzene rings of the styrene monomers are randomly distributed along the polymer chain and therefore no crystallization can take place. The random orientation of the styrene monomers is illustrated in figure 1.4 (b). The polystyrene surfaces were oxidatively modified to change their hydrophilicity. A schematic representation of a myoglobin coated particle interacting with a polystyrene surface is shown in figure 1.4 (c).

1.4.3 Molecular dynamics simulations
The experimental work described in this thesis is part of a larger project in which also molecular dynamics simulations have been carried out of amorphous polystyrene films as well as of the myoglobin protein. The roughness of the polystyrene and its interactions with water were studied in detail by simulations. The simulated polystyrene was also oxidized like the real polystyrene in experiments [23]. Furthermore, the initial stages of myoglobin binding to the surfaces have been characterized [24].

1.5 Non-specific physicochemical interactions
Non-specific interactions are important for the performance of biosensors. Non-specific protein–surface interactions are characterized by the non-covalent binding of proteins to surfaces without specific recognition being present like in biomarker–antibody binding. In some cases, non-specific interactions in biosensors are desired, for example to coat substrates with antibodies. In other cases non-specific interactions limit the performance of biosensors, for example in the case where background signals are increased due to binding without the presence of a target molecule [10]. Also, biomarkers that non-specifically bind on a surface that is not the detecting surface, are lost for detection. These types of non-specific interactions are illustrated in figure 1.2 (b).

In the case of particle based biosensors, non-specific interactions can bind particles to the surface without a biomarker molecule being present, leading to false positive signals that have a negative influence on the sensor sensitivity. False positive signals can be caused by the binding of a coated particle on a coated substrate, by for example by antibody–antibody interactions, and also by the binding of a coated particle on a
bare part of the substrate. Both cases are illustrated in figure 1.2 (b). It is important to have a good understanding of these interactions so that they can be minimized in the design of biosensors.

Non-covalent interactions that can occur amongst protein molecules and between proteins and surfaces are determined by properties of the amino acids that make up the protein as well as by the properties of the surface. Depending on for example the charge and hydrophobicity of the components in the system, the interactions can be even stronger than the interactions that determine the protein tertiary structure. When for example a water-soluble protein is brought close to a hydrophobic surface, the hydrophobic effect might favor an interaction of the hydrophobic amino acids with the surface instead of with amino acids inside the protein. This will cause such a protein to lose its tertiary structure upon surface contact.

When the interactions between particles and surfaces are studied, the properties of the particles as well as the surface are important. Surface modification can be used to change the properties and thereby influence the non-specific particle–surface interactions. The non-specific and non-covalent binding of protein molecules or particles to surfaces occurs by an interplay of a number of different physicochemical interactions. We will briefly discuss the relevant forces in this system: van der Waals interaction, which is present between all bodies and is usually attractive; electrostatic interaction that is present between charged bodies; and hydrophobic interaction which is present between hydrophobic (patches on) surfaces. Furthermore, at molecular scales also steric interaction (that can be used to quantify polymer entropic interaction forces), ion correlation (which is an attractive electrostatic interaction that enhances the Hamaker constant), acid-base interactions (that account for polar interactions) and hydrogen bonding may play a role, as is explained in [25] and [26], but these will not be quantified in our system.

The van der Waals interaction is almost always attractive and it is dominant on short distances between the interacting bodies. To calculate the magnitude of the van der Waals interaction, the Hamaker constant is used [27]. This material property is experimentally determined by measuring Lifshitz-van der Waals constants for the individual interacting materials and combining them with the interacting medium. It has been shown that an error in Lifshitz-van der Waals constants in vacuum of 20% can lead to errors of 500% in constants in a third medium (like water) [28]. Therefore, caution is recommended in using van der Waals energy calculations to get quantitative results on interactions. For the macroscopic system of a sphere on a flat surface the van der Waals energy can be calculated from:

\[ E = -\frac{AR}{6D}, \]  

(1.1)

with A the Hamaker constant, R the radius of the sphere and D the distance between the sphere and the surface [25].

The electrostatic interaction is longer ranged and it is repulsive for bodies with equal-signed charge. When a solution is present between the interacting bodies, the charges are screened by ions in the solution and the interaction decays over a characteristic length, which is called the Debye length \( \lambda_D \). The Debye length depends on
Figure 1.5: Typical shape of the interaction potential between a particle and a surface. The primary and secondary minimum are indicated and separated by an energy barrier. The shape results from a strong short-range attractive interaction (e.g. van der Waals, hydrophobic and/or hydrogen bonding), a medium-range repulsive interaction (e.g. electrostatic or steric) and a long-range attractive interaction (e.g. magnetic, gravitational). The ranges of the interactions are indicated in the figure.

The properties of the solution, an important determinant being the ionic strength \( I_c \), expressed in mole/m\(^3\), according to:

\[
\lambda_D = \sqrt{\frac{\epsilon_0 \epsilon_r k_B T}{2 N_A e^2 I_c}}, \tag{1.2}
\]

with \( \epsilon_0 \) the permittivity of free space, \( \epsilon_r \) the relative dielectric constant of the solution, \( k_B \) the Boltzmann constant, \( T \) the temperature, \( N_A \) Avogadro’s number and \( e \) the elementary charge. The electrostatic interaction energy can be calculated from the Poisson-Boltzmann equation, a continuum theory. This equation has been adapted to calculate the electrostatic interaction energy for a sphere on a flat surface:

\[
E = -RZ \exp \left( -\frac{D}{\lambda_D} \right), \tag{1.3}
\]

with \( Z \) dependent on the surface potentials of the interacting bodies [25].

The quantitative descriptions of the van der Waals and electrostatic interactions together are called the DLVO theory (developed by Derjaguin, Landau, Verwey and Overbeek). Although DLVO theory has been developed to describe interactions in colloidal systems, it can be used more generally, so we will apply DLVO theory to describe the interactions between protein coated particles and surfaces in our experiments.

Hydrophobic interactions cause an attractive interaction between hydrophobic surfaces in water. Both long-range and short-range hydrophobic interactions have been
observed; at distances below 10 nm the interaction can be even stronger than the van der Waals interaction [29]. Although the hydrophobicity of the surfaces is known to play a role in the magnitude of the interaction, the exact origin is still debated and no well-defined quantitative description is present that is usable at all distances [25]. Also the influence of the type and amount of ions in the solution on the hydrophobic interaction has not been unambiguously determined [29].

To be able to explain trends observed when studying particle–surface interactions, we use a parameterized interaction potential based on standard DLVO theory consisting of van der Waals and electrostatic interactions, and we include gravitational and magnetic forces as present in our experiments. Taken all together, the energies form a curve of potential energy with a deep minimum close to the surface, which is caused by the Van der Waals attraction. If the electrostatic repulsive energy is larger than the Van der Waals attraction, an energy maximum is present and a secondary minimum further away from the surface is formed. The difference between the maximum and secondary minimum determines an energy barrier that particles have to cross to bind to the surface, as illustrated in figure 1.5.

In figure 1.6 it is shown that at high ionic strengths, the electrostatic interactions are shielded at very short distance, so Van der Waals interactions take over and the barrier disappears. The influence of gravity and magnetic energy on the shape of the energy landscape at short distances is negligible, but their longer range action causes the particles to sediment onto the surface. Inhomogeneities in the surface and particle properties influence the energy barrier height, as will be discussed in the experimental chapters 3 and 4.

1.6 State of the art in studying non-specific interactions

In this section a review is given of relevant literature concerning non-specific interactions between proteins, particles and surfaces. Many studies have been performed on the non-specific binding of proteins to surfaces, both to increase understanding of the appearing phenomena as well as in the context of the development of non-fouling
surfaces for medical implants or surface-capture assays, like they are used in biosensors. In this review we focus on literature relevant for our model system of myoglobin interacting with an oxidized polystyrene surface.

1.6.1 Surface modifications affect protein adsorption

The adsorption of protein molecules has been studied on many different surface materials. Surface hydrophobicity has been chosen as a central surface descriptor in a Biomolecular Adsorption Database [30], since it is known in general that the hydrophobicity of the surface is an important determinant for protein adsorption: for increasing hydrophobicity the amount of adsorbing protein increases. This can be understood by the fact that a hydrophilic surface will be hydrated and in that way the interfacial energy between the surface and the solution in which the protein is present will be low, leading to a low free energy change for adsorption [31]. The influence of surface hydrophobicity on protein adsorption has for example been shown for albumin, which is the main protein in blood [32–35], and also for myoglobin [36].

Since a high hydrophobicity of a surface increases the protein absorption, reduction of the protein adsorption can be attained by making surfaces more hydrophilic. A different material with a different hydrophobicity can be chosen, but it is also possible to change the hydrophobicity of the existing surface. There is a large range of options for making a surface more hydrophilic. For example, proteins like bovine serum albumin or detergents can be adsorbed on to the surface and thereby prevent other proteins from binding [2, 37].

A standard strategy to make polymer materials more hydrophilic is by oxidizing them. Surface oxidation can be carried out by for example oxygen plasma exposure and UV/ozone treatment [22, 38]. It has been shown that UV/ozone treatment is suited to produce modified polystyrene surfaces in a controlled way [34, 38]. In the oxidation process, grains of low molecular weight molecules are formed that make the surface rougher. The grains also have an influence on aging of the polymer: after one month the oxygen content is decreased by ~10%. The grains can be removed by washing of the surface in water [34, 39, 40]. Polystyrene surfaces modified by UV/ozone and washed in water were shown to be stable for at least 8 months [38].

A different approach is to functionalize the surface with other molecules with desired properties. To prevent protein adsorption by steric effects, surface functionalization can be performed using for example polyethylene glycol and polyethylene oxide polymers (the only difference lies in their lengths), which are interesting options since they are relatively easily grafted to or from surfaces. An extra advantage is that their end groups can be modified. Polyethylene glycol surface modification has been shown to influence the adsorption of myoglobin on polystyrene surfaces: depending on the end groups, adsorption can be reduced [41]. The modification of end groups is also possible for self assembled monolayers, which can be applied onto metal surfaces [31].

1.6.2 Measurement of protein association

To study the influence of surface modification on the binding of proteins, different techniques are available. When the protein is radioactively labeled (e.g. by $^{125}$I), the amount of radioactivity of the surface is a direct measure for the amount of protein
adsorbed [33]. X-ray photoelectron spectroscopy measurements can be used to quantify the proteinaceous nitrogen level of the surface with adsorbed protein [34]. Mass balance measurements also allow to quantify the amount of bound protein [35]. All these techniques give endpoint data since the quantification of the amount of bound protein is carried out after the surface with adsorbed protein has been removed from the solution and dried.

To study the adsorption of proteins on surfaces in real time without labeling, ellipsometry and surface plasmon resonance can be used. Ellipsometry is based on the change in polarisation of a laser beam that is reflected on the sample surface. The polarisation change is caused by the difference in refractive index between the surface itself and the protein layer adsorbed to it and the layer thickness can be calculated. The surfaces used in ellipsometry experiments must be flat and reflective with a refractive index that is not too close to the refractive index of the protein layer. Polymers have a refractive index close to proteins and therefore, they can only be used when they are spincoated on a reflective surface with a high refractive index [42]. In surface plasmon resonance (SPR) a surface plasmon polariton is formed, dependent on the properties of the surface–liquid interface, which is thereby sensitive to the adsorption of proteins. Such surface plasmon polaritons can only be created at metal surfaces, like gold or silver. However, also polymer surfaces can be studied using SPR when a thin layer is coated onto the metal surface. The association rate that is measured using ellipsometry and SPR is influenced by the diffusion process of the proteins toward the surface and therefore the diffusive properties of the coatings are important.

1.6.3 Structure and interactions of proteins on surfaces

To study the interaction of protein coated particles on surfaces, a manipulation force microscope can be used that laterally moves a particle over a surface. In this device, a protein coated particle is pushed over a surface while at the same time the adhesion force is measured [43, 44]. Protein molecules on a surface can be studied by force spectroscopy using an atomic force microscope (AFM): an AFM tip is functionalized with a layer of protein molecules to measure the interactions on a surface. Using AFM tips coated with bovine serum albumin, different regions on polystyrene surfaces partly coated with BSA [45] and different self assembled monolayer surface coatings [46] have been discriminated based on the adhesion force (upto 20 nN). When examining in more detail the force–distance curves that are obtained when the tip is retracted from the surface, multiple adhesive jumps have been observed, particularly on hydrophobic flat surfaces, that signify multiple steps in the unfolding of proteins before the tip is released from the surface. These steps can be a few hundred pN in size [47–49].

The functionalization of the AFM tips can be performed in such a way that interactions between a single molecule and a surface are studied. This has for example been done for polysaccharides and denatured polypeptides. For the polysaccharide xanthan, stretching of the molecule was observed, visible in the force–distance curves by increasing force for increasing distance followed by a step to a lower force. Dependent on the molecular structure, also plateaus at a constant force of 400 pN were observed [50]. Force plateaus at forces up to ~100 pN have been observed for single peptides that were pulled from a surface [51, 52]. In a system with a positively charged...
polymer on the AFM tip and a negatively charged polymer on the surface, plateaus at \(\sim 100 \text{ pN}\) were attributed to zipper-like disruption of single bonds. Also stretching of the polymers was observed by spring-like events and these were attributed to multiple ionic bonds that share the applied force [53].

Besides the non-specific interactions mentioned above, also specific interactions of proteins have been studied using AFM force spectroscopy. Interactions between different molecules have been studied as well as the internal structure of a single protein. For measuring specific interactions between two molecules, the surface is functionalized with molecules specifically binding to the protein on the tip [54, 55]. To investigate the internal structure of a single protein, the protein is bound to the tip as well as the surface (possibly via linkers). Information on the protein’s internal structure can be used to get a better understanding of protein folding, since numerous theories are proposed, but there is no consensus on which (if any) is correct [56]. The unfolding of for example the proteins titin and green fluorescent protein has been shown to occur at forces up to a few hundred pN after a spring-like extension from which spring constants could be determined [57, 58]. An extensive overview of the study of intermolecular and intramolecular interactions up to 2006 can be found in [59] and [60].

To summarize, AFM force–distance measurements with multiple proteins have shown that the extension interaction between proteins and surfaces is characterized by multiple steps. Since the observed interaction curves are the result of the interplay of the interactions of multiple proteins with the surface, no correlation with the protein structure has been made. When the internal structure of single proteins is studied, the observed steps are preceded by a spring-like extension of the protein that can be interpreted in terms of elastic properties of the protein molecule. For unstructured molecules interacting with a surface, also force plateaus are observed that can be attributed to a steady unbinding of the molecule from the surface.

Furthermore, a large range of optical spectroscopic techniques is available to study the amount and orientation of proteins adsorbing at an interface. For example optical waveguide lightmode spectroscopy, dual polarization interferometry and Fourier transform infrared spectroscopy can be used for this purpose [61]. All these techniques probe a surface that is much larger than the size of a protein molecule and, therefore, they give average properties of a layer of protein molecules and are not suited for studying single protein molecules.

### 1.6.4 Dissociation of particles from surfaces

To measure the dissociation of particles from surfaces, AFM force spectroscopy has been extended to colloid probe AFM in which instead of a normal AFM tip now a colloid particle is fixed to the AFM cantilever. Colloid probe AFM has for example been used to measure interactions between a polystyrene particle and hydrophilic and hydrophobic glass surfaces [62] and to measure interactions between a polysaccharide coated particle and surface [63]. Also the interaction of polystyrene colloids on protein coated surfaces has been studied under different conditions, like variation of the ionic strength [64–66]. The dissociation of particles from a surface can also be studied without applying a force, e.g. by evanescent-field microscopy or by SPR.

Options to study force-induced dissociation of particles from surfaces without a
physical contact between the measurement device and the particle are laminar flow, optical tweezers and magnetic tweezers. In a laminar flow chamber, particles that are bound to the surface are exposed to a hydrodynamic drag to study their dissociation [67]. In optical tweezers transparent particles are trapped in the focus of a laser beam and in this way they are displaced and the forces exerted on them are measured. Magnetic tweezers use magnetic particles and a magnetic field gradient to apply a force to them [68]. Therefore, the superparamagnetic particles that are used in biosensors can also be used as a tool to probe interactions by dissociation measurements.

Magnetic tweezers are a flexible tool to study dissociation of bound particles by applying a pulling force perpendicular to the surface on which the particles are located. The direction of the force applied by magnetic tweezers is dependent on the geometry of the magnets. The force is induced by a magnetic field gradient that is created by either a permanent magnet or an electromagnet. Application of the force by an electromagnet offers the possibility to vary the force by simply changing the current through the electromagnet coil. The pole pieces of an electromagnet can be designed in such a way that the magnetic field is uniform over a large area, thereby allowing hundreds of particles to be studied at the same time and under identical conditions [69–71]. Magnetic tweezers are widely applicable, since biological material in general is not influenced by magnetic fields and a large range of forces from $10^{-3}$ pN up to $10^4$ pN can be accessed [68]. Magnetic tweezers can also be used to probe single molecular bonds between functionalized particles and a functionalized surface [71].

1.7 Question and research approach
The central research question that is addressed in this thesis is how we can better understand non-specific interactions in the context of particle based biosensors. To be able to improve immunoassay biosensor performance, non-specific protein–surface and particle–surface interactions should be controlled. For example, knowledge of the force to pull non-specifically bound protein coated particles from the sensor surface can help improve the performance of particle based biosensors. To achieve control over non-specific interactions, we have developed a number of methods to characterize the interactions between protein coated particles and surfaces.

As a first tool, the rotating particles probe has been developed to study the association of particles to a surface. Secondly, the dissociation of particles that are non-specifically bound to surfaces has been studied by applying a force using magnetic tweezers. Thirdly, to zoom in on the interactions at the single-protein level, AFM force spectroscopy has been applied. Here we discuss the techniques that were used in experiments in this thesis. Our choices are compared with existing state-of-the-art techniques in table 1.1.

1.7.1 Rotating particles probe to quantify association
Existing methods to measure the association of proteins to surfaces without labeling are a.o. ellipsometry and SPR. However, both techniques determine association rates that are influenced by the diffusion of the protein. Therefore, to characterize the non-specific binding of particles to surfaces, we worked on a new technique, named the rotating particles probe, as will be explained in more detail in chapter 3. In this measurement technique proteins are brought close to the surface by the particles to which
Table 1.1: Measurement techniques that are state-of-the-art for the different research topics (particle association, protein structure when it is bound on the surface and particle dissociation) and the methods as used in this thesis.

<table>
<thead>
<tr>
<th>research topic</th>
<th>state-of-the-art techniques</th>
<th>this thesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>association</td>
<td>ellipsometry, SPR</td>
<td>rotating particles probe</td>
</tr>
<tr>
<td>structure on surface</td>
<td>AFM force spectroscopy, optical spectroscopic techniques</td>
<td>AFM force spectroscopy (single molecule, non-specific interactions)</td>
</tr>
<tr>
<td>when bound</td>
<td></td>
<td></td>
</tr>
<tr>
<td>association</td>
<td>AFM force spectroscopy, optical spectroscopic techniques</td>
<td>magnetic tweezers</td>
</tr>
<tr>
<td>dissociation</td>
<td>AFM force spectroscopy, optical tweezers, SPR, magnetic tweezers</td>
<td></td>
</tr>
</tbody>
</table>

they are bound and in this way diffusion does not play a role. Gravity has a negligible influence on the energy barrier for association in the potential energy landscape and therefore the rotating particles probe allows to probe this energy barrier.

The rotating particles probe technique is based on the fact that the magnetic particles employed in biosensors rotate when a rotating magnetic field is applied. When a particle becomes bound to a surface, it stops rotating. This property allows to discriminate between bound and unbound particles by applying a rotating magnetic field [72]. Magnetic particles in rotating magnetic fields have been used before to study the coiling of DNA [73] and the torsion stiffness of a protein pair [74]. These applications were based on the investigation of the movement of single particles. The rotating particles probe has been designed to probe the physicochemical interactions between an ensemble of particles (with single particle resolution) and a substrate in a fluid to give good statistics in a short time [72].

1.7.2 Magnetic force induced dissociation

Dissociation of particles from a surface is studied by magnetic tweezers experiments, as will be presented in chapter 4. The magnet is designed to study hundreds of particles at the same time and an electromagnet is used to allow to change the applied force. To be able to quantitatively study interactions, the force applied to the particles should be calibrated. A calibration method is desired that determines the force on the particles without detailed knowledge about the magnetic properties of the setup.

Forces applied to particles in a magnetic tweezers setup have been calculated from the detailed magnetic properties of the particles and the magnetic setup [75, 76]. However, in general this information is not available with high accuracy. Another method that has been used is to bind the particle to a surface via a tether whose mechanical properties are exactly known and then calculate the force from the observed movement of the particle [73]. This method is dependent on the suitability of the particle to bind a tether to it in a well defined way as well as on the knowledge of the exact properties of this tether. Both are not obvious for new systems.

The force acting on the particles can also be determined by magnetophoresis. When the particles have reached an equilibrium, their velocity will be constant and the magnetic force will be counterbalanced by the drag force: \( F_M = -F_D \). The gravi-
tational forces on microparticles are very small and can be neglected. Since the movement of the particles is dominated by viscous forces (Re $\ll 1$), the particles experience Stokes drag:

$$F_D = -6\pi \eta r v \lambda,$$

(1.4)

with $\eta$ the dynamic viscosity of water and $\lambda$ a correction factor for the drag close to a wall. In bulk, $\lambda = 1$ and Comsol Multiphysics simulations for a particle moving away from a (no-slip) wall showed that this regime is reached at a distance of a few particle radii from the surface [70]. In this thesis the velocity of the particles was only measured at distances more than a few particle radii away from the surface, so $\lambda = 1$.

1.7.3 AFM force spectroscopy

To zoom in on the non-specific interactions occurring between single protein molecules and surfaces, force spectroscopy can be employed. For example an AFM can be used to apply a force to a molecule while at the same time the displacement is measured. In this way, in chapter 5 force–distance curves are recorded of the interaction between a protein functionalized tip and a surface. These measurements can be performed in fluid to allow to study interactions in their native environment. If the AFM tip only has one molecule bound to it, single-molecule interactions on the surface can be studied. To the best of our knowledge, non-specific interactions between a single protein and a surface have not yet been studied before using AFM spectroscopy.

1.8 This thesis

To be able to optimize biosensor performance, it is crucial to get a better insight in the interactions occurring between protein coated particles and polymer surfaces. Since it is known that protein–surface interactions are determined by chemical composition, hydrophilicity and roughness of the surface, we chose to develop a model surface of spincoated polystyrene with well characterized properties, which is described in chapter 2. Association of protein coated particles on polystyrene surfaces is measured using the rotating particles probe and is described with a model including a distributed energy barrier, in chapter 3. To distinguish between specifically and non-specifically bound particles on a surface by magnetic actuation, the dissociation forces should be known in detail. In chapter 4, we describe magnetic tweezers measurements as well as a model which takes into account a distribution of energy barrier values to characterize the dissociation. Finally, single-molecule AFM force spectroscopy was performed and the unfolding of the myoglobin protein molecule is interpreted in chapter 5. In chapter 6, we end with an outlook on the application of the findings in this thesis to the design of biosensors.
This chapter gives an overview of the preparation and characterization of polystyrene surfaces which are used as a model system in the experiments in this thesis to study the interaction of proteins and protein coated particles with polymer surfaces. Spincoated polystyrene was oxidized by UV/ozone to change its hydrophilicity and thereby change the interaction with protein molecules. Low protein binding is desired in parts of the biosensor device to prevent loss of biomarker molecules. The surface hydrophilicity was quantified by water contact angle measurements, and XPS measurements showed the chemical composition of the surface. The surface roughness at the scale of proteins was quantified by AFM topography measurements. Most results shown in this chapter have been published in [23].

2.1 Preparation

To be able to study interactions of proteins with the polystyrene surface it is required that it has well defined properties: the roughness should be low on the scale of protein dimensions and the chemical properties should be reproducible. AFM roughness measurements on commercial polystyrene surfaces like wellplates and coverslips showed that their roughness is large on the scale of proteins, typically tens of nanometers. Furthermore, XPS measurements showed that elements are present like chlorine, most likely due to additives. Therefore, we chose to prepare polystyrene surfaces ourselves.

To control roughness, the polystyrene surfaces were prepared by spincoating from a toluene solution. UV/ozone oxidation was used to change the polystyrene hydrophilicity [22] which in turn influences protein adsorption [34]. This section describes the standard protocol that we used for preparing polystyrene surfaces. In some experiments part of the protocol was adjusted, these deviations are mentioned in the text of those experiments.

2.1.1 Polystyrene surface

Polystyrene surfaces were prepared on glass coverslips of \( \sim 150 \mu \text{m} \) thickness. The coverslips were cleaned by immersion in solvents in an ultrasonic bath for 5–10 minutes (subsequently acetone, isopropanol and ethanol); in between the steps, excess solvent was removed and after the last step the surfaces were blown dry with nitrogen.

After cleaning, a polyimide attachment layer was applied in order to later prevent the polystyrene layer from detaching from the glass surface when washed in water or
treated in solution. The polyimide (Sigma Aldrich, poly(3,3’,4,4’-benzophenonetetra-
carboxylic dianhydride-co-4,4’-oxydianiline/1,3-phenylenediamine), amic acid solu-
tion) was dissolved at 0.33 g/mL or 0.033 g/mL in DMSO (Merck, dimethyl sulfoxide
for synthesis). The entire glass surface was covered with this solution after which
the spincoater was turned on (Electronic Micro Systems Ltd. Photo Resist Spincoater
Model 4000, 5 s 1000 rpm (ramp 1) and 40 s 5000 rpm (ramp 5)). Then, the surfaces
were placed on a hotplate for at least 15 minutes at 90°C to evaporate solvent and
subsequently at least 1 hour at 200°C to crosslink the polymers.

The used polystyrene (Alfa Aesar, Polystyrene, atactic, formula weight 125 000–
250 000) consists of 1000–1440 monomers, resulting in 300–361 nm contour length
for each chain, using the assumption that two C–C bonds per monomer are present
with each a length of \( d = 0.154 \text{ nm} \) and an angle of \( \theta = 109^\circ \) in between \( (l_0 = Nd \sin \frac{\theta}{2}) \). The polystyrene was dissolved at 2 wt% in toluene (Merck, for analysis).
Spincoating was performed at 1000 rpm, which leads to a film thickness of \( \sim 200 \text{ nm} \),
as shown by Hall et al. on silicon wafers [77]. The surfaces were left overnight in the
fumehood to let all toluene evaporate.

### 2.1.2 Ultraviolet–ozone oxidation

The polystyrene surfaces were oxidized in a UV/ozone chamber filled with ambient
air (Novascan, PSD-UV ultraviolet/ozone probe and surface decontamination unit) in
which they were placed at \( \sim 3 \text{ cm} \) from the mercury vapor lamp [38, 39]. UV/ozone
treatment allows to controllably oxidize hydrocarbon-based polymers without the need
for complex equipment like a vacuum chamber [38]. The polymer molecules can be
excited and/or dissociated by the short wavelength UV light produced by the lamp.
Furthermore, atomic oxygen and ozone are produced which in turn also react with the
polymer molecules [78].

Oxidation was carried out for times up to 10 minutes. The mercury lamp could
only be turned on when the chamber was closed, so it was not warmed up before
placing the samples. After irradiation the chamber was immediately opened to aid
in reproducibility. The oxidized surfaces were washed in Millipore water (resistivity
18.2 M\( \Omega \)-cm) to remove grains of low-molecular-weight oxidized material [39]. Wash-
ing in water increases the stability of UV/ozone oxidized polystyrene and it has been
shown that it can be stored for at least 8 months before changes in surface chemistry
are measurable by XPS and contact angle measurements [38]. However, we prepared
the oxidized surfaces at most one day in advance. The depth of UV/ozone modification
of the polystyrene is known to be deeper than the probing depth of XPS (\( \sim 10 \text{ nm} \)) [39].

### 2.2 Roughness measurements

Atomic force microscopy (AFM) is routinely used to measure surface roughness. The
principal components of an AFM system are illustrated in figure 2.1. AFM operation
is based on a very sharp tip (radius order of nm) which is integrated into the free end
of a cantilever, to probe the sample surface. The tip material typically is silicon ni-
tride or silicon. A laser beam reflects off the cantilever onto a quadrant photodetector,
which is used to observe the movement of the lasercle when the cantilever is bent
by the interaction force between tip and surface. In this way, sub-nm deflections can
be observed. The sensitivity of the detector (nm/V) is calibrated by moving the can-
tilever for a known distance on to a hard surface. The sample is placed on a x,y-piezo scanner to allow accurate movement and a z-piezo is employed to precisely control the distance between the probe and the sample [79].

There are multiple modes to operate an AFM. The classic mode is contact mode, in which the tip is scanned over the surface while it is in contact and the z-piezo is actuated to keep the force constant. Thereby, the z-movement represents the surface roughness. A disadvantage of the contact mode is the continuous application of force on to the sample, especially lateral forces can damage the sample surface.

In tapping mode the cantilever is vibrated near its resonant frequency while scanning the surface. There is contact between tip and sample for only a small fraction of the time, low forces are applied and especially the lateral forces are negligible. In tapping mode, the z-piezo is actuated to maintain a constant oscillation amplitude, which means a constant height above the surface and therefore the z-movement represents the surface roughness.

A relatively new AFM operation mode is PeakForce QNM (quantitative nanomechanical mapping) which combines scanning with force spectroscopy. The cantilever is moved up and down at 1 kHz (far away from resonance); the downward movement proceeds until a certain force (the trigger force that can be set by the user) is reached, then it is retracted again. In this way, the surface height as well as many other material properties can be extracted, like adhesion, deformation and modulus [81].

Surface topography can be conveniently described using amplitude parameters, which measure vertical surface deviations. The ‘root mean square roughness’ $R_q$ represents the standard deviation of the distribution of surface heights $z_i$. $R_q$ is generally used and is more sensitive to large deviations from the mean $\bar{z}$ than the also commonly used arithmetic average [82].

$$R_q = \sqrt{\frac{1}{n} \sum_{i=1}^{n} (z_i - \bar{z})^2}.$$ (2.1)
First, surface roughness measurements have been performed in air. A Veeco MultiMode instrument was used in tapping mode with a tip with a radius of 10 nm. 0.5×0.5 μm² scans were made of polystyrene surfaces that were prepared as described in section 2.1, UV/ozone oxidized for 0, 30, 180 and 600 s. Two scans were made on each sample. The obtained data was analyzed using Nanoscope Analysis software and background corrected by a second order plane fit over the complete scanned image. Typical scans are shown in figure 2.2. The measured root mean square roughness \( R_q \) was calculated from the distribution of surface heights as in equation 2.1 and found to be in the range of atomic dimensions. The measured roughnesses for each oxidation time are shown in table 2.1. The roughness increases from 0.17 nm to 0.35 nm for an oxidation time that increases from 0 to 600 s, which is similar to the results of Browne et al. for polystyrene spincoated on to a silicon wafer: for non-oxidized surfaces \( R_q < 0.3 \) nm and upon oxidation up to 600 s, all \( R_q \) fall in the range 0.2–0.5 nm [34].

Roughness measurements have also been performed in fluid (Millipore water). A Bruker BioScope Catalyst system was used, operated in Peak Force QNM mode using
Table 2.1: Roughness measured on spincoated polystyrene samples UV/ozone oxidized for different times; typical scans are shown in figure 2.2.

<table>
<thead>
<tr>
<th>oxidation time (s)</th>
<th>$R_q$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>30</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>180</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>600</td>
<td>0.35 ± 0.01</td>
</tr>
</tbody>
</table>

A tip with a radius of 20 nm. The surfaces were prepared as described in section 2.1, with an extra cleaning step before application of the polyimide consisting of 10 minutes UV/ozone treatment. Non-oxidized polystyrene surfaces and surfaces oxidized for 600 s were measured, three samples of each were used. Nanoscope Analysis software was used to analyze the data using a second order plane fit background correction of the scanned images. $0.5 \times 0.5 \mu m^2$ scans showed the roughness $R_q$ (equation 2.1) again to be on the scale of atomic dimensions and to increase from $0.36 \pm 0.11$ nm to $0.55 \pm 0.18$ nm by oxidation. A relatively large sample-to-sample variation of more than 50% was observed, which is probably also the cause of the difference between the measured roughnesses in air and in fluid.

All observed roughnesses are well below 1 nm, which means that the polystyrene surfaces are very flat on the scale of the dimensions of proteins, which are typically a few nanometers in size. Nanostructures that are smaller than the size of the tip can not be detected, but these structures are not expected due to the surface preparation by spincoating of a relatively thick layer of polystyrene. Interestingly, the calculated roughness of amorphous polystyrene films in molecular dynamics simulations is reported to be about 0.4 nm [23]. The intrinsic polystyrene roughness observed in the simulations is created by the conformation of the main chains (important determinant is the radius of gyration) and the orientation of the phenyl rings. This means that the experimental surface roughness measured with a tip radius of 20 nm is very similar to the intrinsic polystyrene roughness, so it is probably not caused by the method of sample preparation.

### 2.3 Contact angle measurements

Water contact angle measurements can be used to quantify the hydrophilic character of polystyrene (after UV/ozone treatment) [22]. Using an automated dispense unit (DataPhysics OCA 30 Instrument Gm, Germany, model TBU 90E, with working software OCA 20), a droplet of 10 µL Millipore water was formed, hanging on the needle. Then, the substrate was moved up until it touched the droplet, then moved down until the droplet was released. Using the camera of the instrument, an image of the droplet on the surface was captured and analysed with ellipse fitting (in the software). In this way, left and right static contact angles on the surface were determined for each droplet.

The measured water contact angles are shown in figure 2.3 for surfaces with different oxidation times. Non-oxidized polystyrene has a water contact angle of ~90°, making it hydrophobic. With increasing oxidation time the contact angle decreases
Figure 2.3: Measured water contact angles on UV/ozone oxidized polystyrene, each point shows the average and standard deviation of at least 50 measurements (2 angles per droplet, typically 12 droplets per sample). A line is included to guide the eye.

down to a plateau value of ∼50°, demonstrating increased hydrophilicity. A comparable trend was observed by Klein et al. who spincoated atactic polystyrene from toluene onto silicon wafers, then exposed the surfaces to UV/ozone for specified times and washed in isopropanol. In their experiments the water contact angle starts at ∼90°, then decreases sharply after short UV/ozone exposure times and plateaus at longer times (∼2 min) at ∼75° [22]. Comparing the absolute values of measured static contact angles is however difficult because of the lack of an independent standard [83].

2.4 Oxygen content

The surface chemical composition of the (oxidized) polystyrene surfaces was quantified using X-ray photoelectron spectroscopy (XPS) [84]. This measurement technique is based on the photoelectric effect (photons can knock out electrons from materials):

\[ E_{\text{photon}} = E_{\text{binding}} + E_{\text{kinetic}} \]  

So, if photons with a known energy are used and the kinetic energy of the released electrons is measured, the binding energy of the elements in the material can be determined. The binding energy is unique for each element and its local chemical structure. The ejected electrons have a well defined energy representative for the chemical environment when released from within their escape depth, so at most ∼5–10 nm from the top of the sample. From within the escape depth electrons are ejected without scattering. Measurements need to be performed in ultra-high vacuum, since also elements present in air will scatter photons and will interfere with analysis of the intended material.

In our experiments photons were emitted from an aluminum anode Al Kα (operating at 150 W), with a photon energy of \( E_{\text{photon}} = 1486.6 \text{ eV} \). The kinetic energy of the released electrons was measured using a VG CLAM II hemispherical analyzer with a channeltron detector. The elemental composition was obtained from survey scans.
Table 2.2: Contributions to the carbon 1s peak used for fitting the XPS region scans based on the convention of Browne et al. (see examples in figure 2.4 (b)) [34].

<table>
<thead>
<tr>
<th>energy (eV)</th>
<th>species</th>
</tr>
</thead>
<tbody>
<tr>
<td>285</td>
<td>C–C/C–H</td>
</tr>
<tr>
<td>284.7</td>
<td>aromatic C</td>
</tr>
<tr>
<td>291.5 ± 0.3</td>
<td>π → π* shakeup (aromatic)</td>
</tr>
<tr>
<td>286.4 ± 0.3</td>
<td>C–OH</td>
</tr>
<tr>
<td>287.6 ± 0.3</td>
<td>C=O</td>
</tr>
<tr>
<td>289.9 ± 0.3</td>
<td>O–C=O</td>
</tr>
</tbody>
</table>

Figure 2.4: (a) XPS survey scans for three UV/ozone oxidation times, showing the presence of carbon and oxygen in the sample. The peak at 285 eV originates from electrons of the carbon 1s orbital and the peak at 530 eV from electrons of the oxygen 1s orbital. The spectra are offset along the y-axis for clarity. (b) Detailed region scans of the carbon 1s peak of a non-oxidized sample (0 s) and a 600 s oxidized sample with decomposition in individual contributions as explained in the text.

(constant pass energy 100 eV) and the local chemical structure of the elements from region scans (constant pass energy of 50 eV). The background pressure was 2·10⁻⁹ mbar.

To process the data, the CasaXPS program was used to fit the measured curves with a Tougaard background correction [85]. Since the polystyrene surfaces are insulating, charging of the surfaces occurs which changes the observed binding energy. Therefore, all spectra were aligned using the carbon 1s peak at 285 eV as is commonly done [34, 38, 84]. The obtained peak areas are a measure for the amounts of the elements present (after correction with the appropriate sensitivity factors). The detailed region scans of the carbon 1s peak reveal different types of chemical bonds between carbon and oxygen. The peak positions used to quantify the species present in these region scans are based on the convention used by Browne et al., as shown in table 2.2 [34].

For the XPS measurements, the polystyrene surfaces were prepared on glass microscope slides cut in 13 × 13 mm² pieces, following the protocol as described in
Table 2.3: Overview of observed oxygen content after UV/ozone oxidation of polystyrene and washing in water.

<table>
<thead>
<tr>
<th>surface</th>
<th>time to saturation</th>
<th>plateau oxygen content (at.%)</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>spincoated on glass</td>
<td>~5 min</td>
<td>~24</td>
<td>this thesis, [23]</td>
</tr>
<tr>
<td>petri dish</td>
<td>~3 min.</td>
<td>~27</td>
<td>[40]</td>
</tr>
<tr>
<td>spincoated on silicon wafer</td>
<td>~3 min.</td>
<td>~22</td>
<td>[34]</td>
</tr>
<tr>
<td>petri dish</td>
<td>~2-3 min.</td>
<td>~20-25</td>
<td>[39]</td>
</tr>
<tr>
<td>petri dish</td>
<td>~2 min.</td>
<td>~15</td>
<td>[38]</td>
</tr>
</tbody>
</table>

The observed oxidation is very similar to what others observed for comparable UV/ozone treatments of polystyrene surfaces, as illustrated in table 2.3. In all cases at short treatment times a fast increase of the oxygen content was observed and at longer times a plateau. All mentioned results were obtained for samples that were washed in water after oxidation.

From figure 2.5 (b) it can also be seen that the main part of the oxygen atoms in the sample has a double bond to carbon in the form of C=O and O–C=O. This differs from the observation by other groups that C–OH is the main bond [34, 39, 40].

It is important to note that the shape of the curve of water contact angle as a function of UV/ozone treatment time (figure 2.3) shows an inverted trend compared to the curve of oxygen content as a function of time (figure 2.5 (a)). This signifies the importance of oxygen in the top of the surface for its hydrophilicity. Oxygen at the surface increases the possibility to form hydrogen bonds, which is a good measure for...
Figure 2.5: (a) Elemental composition of samples as a function of oxidation time, derived from peak areas of the different elements in XPS survey spectra. (b) Quantification of oxygen species derived from fits of detailed region scans of the carbon 1s peak, using the contributions from table 2.2; the lines are included to guide the eye.

2.5 Comparison with molecular dynamics simulations

As introduced in section 1.4, the experiments in this thesis are part of a larger project in which the interaction between polystyrene and proteins was also studied with molecular dynamics simulations [24]. From this perspective, the hydrophilicity of real samples measured in experiments was compared to properties of the simulated surfaces: the water contact angle and the number of hydrogen bonds were compared as a function of oxidation time. The hydrophilicity of the surfaces in the molecular dynamics simulations was tuned by the amount of oxygen atoms attached to the phenyl rings of the polystyrene (in ortho and meta position). This is motivated by the fact that according to Saito et al. one of the main processes in the ozonization of aromatic compounds is normal ozonolysis which gives αβ-dicarbonyls, which means that the oxygen has been attached to the phenyl rings [86]. Also, experiments of Klein et al. using Near-Edge X-ray Absorption Spectroscopy show strong preference for ozone to react with the double bonds of the phenyl rings rather than with the single bonds in the backbone [22].

Different amounts of oxygen were included to obtain systems with 0, 6, 12, 18, and 24 % oxygen content at the surface. These values are consistent with values measured by XPS after UV/ozone oxidation of spincoated polystyrene, as shown in section 2.4. For each degree of oxidation four statistically independent systems were prepared and simulations were performed for them; final results are averages of these four systems. Figure 2.6 shows the simulated number of hydrogen bonds that has a linear dependence on the degree of oxidation and the measured water contact angles that show this linear trend as well [23]. This demonstrates that oxygen at the polystyrene surface accounts for the formation of hydrogen bonds and thereby surface hydrophilicity, leading to a change in water contact angle.
2.6 Conclusions

To allow measurement of interactions of proteins and protein coated particles on polystyrene surfaces, we investigated a procedure for preparing surfaces with well characterized properties. We developed a series of UV/ozone oxidized spincoated surfaces that have a roughness not exceeding \( \sim 0.5 \) nm, which is very flat on the scale of protein dimensions. The measured roughness is comparable to the roughness of amorphous polystyrene films calculated with molecular dynamics simulations.

UV/ozone treatment increases the oxygen content of the surface in a well-controlled manner, as shown by XPS measurements. After UV/ozone treatment the surfaces were washed in water to remove low molecular weight oxidized species. Survey scans show a saturation in the oxygen content at \( \sim 24\% \) from \( \sim 300 \) s and this is similar to what other groups have observed for UV/ozone oxidized polystyrene surfaces. A detailed analysis of the chemical structure of the carbon 1s peak shows a saturation of the total oxygen content at \( \sim 30\% \). The water contact angle of the surfaces decreases with increasing oxidation time demonstrating an increasing hydrophilicity and the contact angle shows an inverted trend compared to the oxygen content of the surfaces.

In summary, we have developed a well defined polystyrene surface with the possibility to modify its oxygen content and thereby its hydrophilicity. This model polystyrene surface will be used in the experiments throughout this thesis to investigate the influence of surface modification on the non-specific interaction with proteins and particles to optimize biosensor design.
Probing particle–surface association

Non-specific interactions between the magnetic particles employed in biosensors and the biosensor surface are important for the assay sensitivity, since binding of particles without the presence of a biomarker will cause false positive signals. To get insight into the origin of these interactions, we study the association of particles to the surface. State-of-the-art techniques like ellipsometry and SPR can only measure bound particles after a washing step to remove unbound particles. Since we did not want to disturb the system by a washing step, we used the rotating particles probe which allows to quantify the association of particles on a surface. As a model for the biosensor surface, we used oxidatively modified polystyrene as characterized in chapter 2.

First, we describe the setup for performing rotating particles probe experiments to assess particle binding and the method to prepare protein coated particles and control particles. Then, we introduce the model that we have developed to interpret the measurements. This model is based on the assumption that the energy barrier that the particles have to cross to bind to the surface does not have one unique value, but rather has a distribution of values caused by the inhomogeneity of the particle and polystyrene surface. Most of the results presented in this chapter were published in [87].

3.1 Materials and methods

Rotating particles have been used by Janssen et al. to measure the interaction of streptavidin coated particles on a glass surface as a function of the ionic strength of the solution [72]. The data reveal a clear increase of binding for increasing ionic strength. To explain the observations, the distance between the particle and the surface was calculated at which a minimum in interaction energy occurs, determined by electrostatic, van der Waals, gravitational and magnetic energies. The experimental conditions at which particles were binding to the surface yielded a calculated distance of the energy minimum that was comparable to the surface roughness of the used magnetic particles.

We now introduce the setup of the rotating particles probe and the preparation method of the magnetic particles. We also explain how rotation of the particles can be used to probe binding.

3.1.1 Rotating particles probe

We used the rotating particles probe setup as introduced by Janssen et al., which consists of four electromagnet coils with soft iron poles connected by a soft-iron yoke.
Figure 3.1: (a) Magnet of the rotating particles probe setup (top view). (b) Schematic of bound and unbound particles on a surface.

The poles are slanted to direct the magnetic field in the plane of the sample. A photo of the setup is shown in figure 3.1 (a). The current through the coils is individually controlled and the current through each of the coils is 90° out of phase with respect to its neighboring coil to create the rotating field [72]. Throughout all experiments a magnetic field of 20 mT and a rotation frequency of 1 Hz were used.

To assess the rotation of the particles, the rotating particles probe setup was placed on a microscope (Leica DM6000M, upright microscope). Particles were observed using a 63× water immersion objective without additional magnification. Movies were recorded using a high speed camera (Redlake MotionPro HS-3) operated at 50 Hz. The movies were analyzed by counting the single particles, discriminating between ‘unbound’ (rotating synchronously with the magnetic field) and ‘bound’ particles (not following the rotating field). Since all particles were sedimented on the surface, they stayed in focus during the measurements. A schematic of particles rotating on a surface is shown in figure 3.1 (b).

Oxidized polystyrene surfaces were prepared and characterized as described in chapter 2. For measurements with the control particles which were not coated with myoglobin, in the cleaning procedure of the coverslips also 10 minutes UV/ozone exposure was included before applying polyimide on the surface. The properties of the polystyrene were varied by UV/ozone oxidation times between 0 and 120 seconds, leading to oxygen contents up to ~24%.

Fluid cells were created on the polystyrene surfaces using Secure-Seal imaging spacers (9 mm diameter, 120 µm depth). The cells were filled with prepared particle solution with specified pH and ionic strength and closed with a glass coverslip. The particles in the fluid cell were incubated for 5 minutes to be able to measure in a steady-state situation. It has been shown that for ionic strengths above 2.5 mM within 3 minutes a constant fraction of rotating particles is obtained and for increasing ionic strength the time to reach a steady state is decreased [72]. By careful inspection of a selection of 10 of the recorded movies (10 s each, total ~300 particles), no particles were found to bind or dissociate during the observation time, putting an upper limit to the association and dissociation rates of ~3·10^{-5} s^{-1}.
The aqueous solution was phosphate buffered saline (Sigma Aldrich, pH 7.4) that was diluted with deionized water to the desired ionic strength. In the system with myoglobin coated particles, for pH values 3.3 and 10, hydrogen chloride or sodium hydroxide were added. In the system with control particles, for pH 3.7 a MES buffer (2-(N-morpholino)ethanesulfonic acid, Sigma Aldrich) was used, adjusted by adding hydrogen chloride and for pH 10 a sodium bicarbonate/sodium hydroxide buffer.

### 3.1.2 Protein coated particles

To be able to image magnetic particles with a normal far-field microscope, their radius should be at least 1 µm. To minimize the influence of surface roughness on the observed interactions, smooth particles are desired. Carboxylic acid groups on the particles’ surface allow to covalently bind proteins to them. We chose to use Invitrogen Dynabeads M-270 Carboxylic Acid particles with a radius of 1.4 µm, consisting of magnetite grains in a highly crosslinked polystyrene matrix with a hydrophilic glycidyl ether surface coating and carboxyl groups introduced on the surface [88]. A scanning electron microscope image of the M-270 particles can be seen in figure 3.2.

In ideal superparamagnetic particles no permanent magnetic moment is present. However, it turns out that in practice there is a small permanent moment that allows the particles to rotate in the applied rotating field. The smoothness of these M-270 particles makes it impossible to observe the rotation of naked particles. This is in contrast to the M-280 particles that were used before [72], which have an irregular surface structure that distorts symmetry and thereby allows rotational tracking. Therefore, small magnetic particles were attached to the M-270 particles which served as optical tags.

The M-270 particles were functionalized using an EDC–NHS binding scheme. EDC (Pierce, 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) and NHS (Merck, N-Hydroxysuccinimide) in MES-buffer (Sigma Aldrich) were used to activate the carboxyl groups on the particles, both at 100 mg/mL at pH 5. Then, biotin molecules were introduced on the surface via a linker with a primary amine (Biotin...
Figure 3.3: (a) Microscope image of an M-270 particle labeled with a small MagSense particle to visualize rotation. (b) A schematic of the binding between particle and tag (not to scale).

EZ-link, 10 mM, Pierce). Unbound biotin EZ-link was washed away and subsequently myoglobin (CalBiochem; Human, Recombinant E. coli) was incubated at 0.02 mg/mL in MES-buffer and covalent peptide bonds were formed between the amines of the protein and the activated carboxyl groups on the surface. For the control particles the incubation was carried out only in MES-buffer, without myoglobin being added. Finally, leftover activated carboxyl groups were quenched by incubation with ethanolamine (Sigma Aldrich).

Flow cytometry measurements were performed using fluorescently labeled primary antibodies as well as labeled secondary antibodies on the myoglobin coated particles to study the influence of the concentration of protein on the final surface coverage of the particles. It was shown that for concentrations above $7 \cdot 10^{-3}$ mg/mL the surface coverage is saturated. Therefore, the myoglobin concentration of 0.02 mg/mL used for coating the particles will create particles with a saturated myoglobin surface.

To determine the surface potential of the particles, electrophoresis measurements have been carried out using both myoglobin coated particles and control particles. Although the error in the measured values was too large to be able to discriminate between the different particles, for both types of particles the zeta potential was found to range between $-30$ mV and $-60$ mV.

As tag particles, magnetic MagSense 500 nm polystyrene particles functionalized with streptavidin were used [89]. These small particle labels were bound to the M-270 particles via biotin–streptavidin coupling. The concentration of tag particles was adjusted so most of the M-270 particles had at least one tag and still minimal loading; this was confirmed by visual inspection in the microscope. Figure 3.3 shows a schematic picture of the system together with a microscope image of an M-270 particle labeled with a single tag particle. In figure 3.3 (a), diffraction rings can be seen around the particle itself and its tag. The tag distorts the symmetry of the system so rotational motion can be tracked.

To minimize the effect of the presence of the tag particles on non-specific interactions, minimal loading was applied. When it is assumed that three tags are bound to the particle, about 20% of the particle can not touch the surface because the approach is sterically hindered by the presence of a tag. Differently stated, in 20% of the possible orientations of the particle a streptavidin coated tag will contact the surface instead of the particle itself. Since the magnetic tags induce magnetic anisotropy in the
system, they can be aligned in the plane of the magnetic field. This has been employed to prevent the tags from being underneath the particle by performing the incubation in a small, homogeneous magnetic field of 1 mT in a Helmholtz coil pair. The magnetic field incubation has only been applied to the measurements of the control particles presented in this chapter, but an extra measurement series of myoglobin coated particles with incubation in a magnetic field (not shown) did not give significant differences with the data presented here.

3.1.3 Using rotation to probe binding

Recorded movies of the particles on the surface in the rotating magnetic field were analyzed by counting single particles. By eye, bound and unbound particles were discriminated based on their rotation. If rotation was invisible because the particle was not tagged, it was disregarded. Also particles that were part of a cluster were ignored. Typically, in the field of view of a single measurement a few tens of particles could be counted. Each movie lasted for 10 s.

It is important to verify that application of torque does not change the fraction of bound particles. A first support was given by the observation that in the performed measurements bound particles do not start rotating: no transitions from ‘bound’ to ‘unbound’ were observed. To verify that the energy barrier for rotation exceeds the maximum energy that can be supplied to the system by the application of the torque, we will now make some quantitative estimations.

The presence of a barrier for rotation implies that the system is rotationally asymmetric. The asymmetry can be caused by a distribution of proteins over the particles’ surface, by the roughness of the particles themselves or by non-uniformities of the polystyrene surface. The height of the energy barrier for an interacting patch with a radius of 100 nm can be estimated. Using the surface free energy of polystyrene (42.5 mJ/m$^2$) [90], the interaction energy is found to be about $3 \cdot 10^5 k_B T$. In the experimental system multiple patches will be interacting. This estimation of the rotational energy barrier should be compared to the energy applied by the torque to see whether the torque can overcome the barrier for rotation.

The energy supplied to the particles in the rotating magnetic field can be estimated as the product of the maximum torque and the angle of rotation required to overcome the energy barrier. The maximum torque on M-270 particles in a field of 20 mT is $10^3 k_B T$/rad [91]. The angle of rotation to overcome the energy barrier depends on the exact layout of the patchy interactions, but it is unlikely to be more than 1 rad, corresponding to a maximum torque of $10^3 k_B T$. Consequently, the applied torque is unlikely to be able to break the bond between the particles and the surface.

The applied torque determines the rotational energy barriers that can be studied in the rotating particles probe. Since the applied field can be lowered and in this way the applied torque will be lowered proportionally, the probe is also suited for systems with lower rotational energy barriers. With the right choice of magnetic field for the system under study, the rotating particles act as a valid probe that does not influence the fraction of bound particles.
3.2 Experimental results

The fraction of rotating particles was recorded on differently oxidized polystyrene surfaces in solutions with different pH values. In all our experiments of the myoglobin coated particles as well as the control particles, we observe that particles which become bound to a surface do not unbind during the timescale of the experiments (the unbinding rate is at most ∼3·10⁻⁵ s⁻¹, see section 3.1.1). Measurement results are shown in figure 3.4. All data points are averages over typically 6 fields of view, recorded on two or three samples, of typically tens of particles each. Error bars represent the standard deviations. The sometimes large deviations are caused by occasional variations in sample homogeneity and by sample-to-sample variations. The error bars in some cases even extend below 0 or above 1, but fractions outside the range 0 to 1 of course did not occur in the measurements. In spite of those variations, in most cases the curves corresponding to differently oxidized samples can be clearly distinguished.

Figures 3.4 (a1), (b1) and (c1) show the fraction of unbound myoglobin coated particles for differently oxidized surfaces in solutions with a pH of 3.3, 7.4 and 10. All curves have a characteristic s-like shape starting at an unbound particle ratio of 1 for low ionic strengths and decreasing towards 0 for increasing ionic strength. With increasing oxidation time, a higher ionic strength is needed to bind the same fraction of particles. For example, at pH 7.4 (figure 3.4 (b1)) the concentration at which 50% of the particles is bound shifts from ∼10 mM to ∼100 mM for a change in oxidation time from 0 s to 72 s. With increasing pH, a clear shift of the curves to higher ionic strengths is observed. For example, the concentration at which 50% of the particles is bound for 60 s oxidized polystyrene is ∼2 mM for pH 3.3, ∼25 mM for pH 7.4 and ∼200 mM for pH 10, as can be seen in figure 3.4. It is possible that the particle surface or the polystyrene surface induces unfolding of the myoglobin molecules e.g. by hydrophobic interactions that are stronger than those inside the protein. The rotating particles probe measurements quantify the effect of the ionic strength on binding for the total system of myoglobin coated particle and the (oxidized) polystyrene surface, so possible unfolding is included.

Figures 3.4 (a2), (b2) and (c2) show the fraction of unbound control particles (without myoglobin) for differently oxidized surfaces in solutions with a pH of 3.7, 7.4 and 10. At pH 3.7 and 10, the order of the curves is identical to the order observed for the myoglobin coated particles: for increasing oxidation time, a higher ionic strength is needed to bind the same fraction of particles. For pH 7.4 no clear curves can be distinguished, which will be discussed in section 3.4. Compared to the myoglobin coated particles, the binding of the control particles starts at higher ionic strengths for every pH, which means that at the same ionic strength, less interaction occurs. For pH 7.4 and pH 10, the shape of the curves is different from the shape of the curves of the myoglobin coated particles: for the control particles, at high ionic strengths not all particles bind.

3.3 Modeling of particle–surface interactions

Using rotating particles probe measurements, it is found that binding of particles to oxidized polystyrene surfaces as a function of ionic strength gives curves with a characteristic s-like shape. The properties of the curves are dependent on the oxidation
Figure 3.4: Fraction of rotating magnetic particles as a function of ionic strength on differently oxidized polystyrene surfaces (0 s to 120 s) and pH values (3.3 to 10). Myoglobin coated particles ((a1), (b1) and (c1)) as well as control particles without myoglobin ((a2), (b2) and (c2)) were used. Data was fitted with equation 3.7 using the restriction $D > \sqrt{2}$ for (a1)–(c1), (a2) and the dashed lines in (c2); no restriction was used for the continuous line in (c2). Parameters are shown in figure 3.6. Note the different ionic strength scales.
time of the polystyrene surface, the pH of the solution and the type of particles (myoglobin coated or control).

We introduce a model to interpret the experimental data of the rotating particles probe. The description is based on the energy barriers that the particles have to cross in order to bind to the surface, as introduced in section 1.5. In all our experiments, we did not observe unbinding of particles. This single-direction kinetics argues against a statistical model description that uses a dynamic equilibrium of binding and unbinding. Apparently, a model is required which explains that only a fraction of the particles is able to overcome an energy barrier and binds during incubation.

In practice, we may expect that not all particles experience the exact same energy barrier due to small variations in particle or surface treatment. Therefore, the key assumption of the model is that the particles experience a distribution of energy barrier heights, so that the particle binding data reflect an average barrier height as well as a variability of barrier heights.

At first, we show that the energy barrier can be parameterized as a function of the ionic strength of the solution. Subsequently, a model is introduced that describes the binding of particles by including a normal distribution of energy barrier values. We validate the model by fitting the experimental data shown in figure 3.4 and extract two characteristic barrier parameters for the non-specific interactions between particles and surface. Finally, we interpret the derived parameters in terms of the underlying physicochemical interactions.

### 3.3.1 Energy barrier height

The interactions occurring between particles and surfaces were introduced in section 1.5. We explained how DLVO theory (electrostatic and van der Waals interactions) combined with gravitational and magnetic interactions can be used to calculate interaction potentials. From the interaction potential the energy barrier height can be extracted as illustrated in figure 1.5 and reproduced in the inset in figure 3.5.

The energy expressions of the DLVO interactions (equations 1.1 and 1.3) do not allow an analytical solution for the energy barrier height. Therefore, we calculate the barrier height numerically for the range of ionic strengths used in the experiments. For the electrostatic interactions, the surface potential was chosen to be $-30 \text{ mV}$, which is a typical value for polystyrene; for oxidized polystyrene the potential will be more negative than for non-oxidized polystyrene [92]. Particle surface potentials were chosen to be $-20 \text{ mV}$, $-25 \text{ mV}$ and $-30 \text{ mV}$. For the van der Waals interactions the typical Hamaker constant of non-conducting solids or proteins in water or salt solutions, $10^{-20} \text{ J}$, was used [25]. Variation of the Hamaker constant from $0.5 \cdot 10^{-20} \text{ J}$ to $1.5 \cdot 10^{-20} \text{ J}$ did not have any influence on the general shape of the curves. The gravitational energy for the microparticles is $E_g = 6.8 \cdot 10^{-14} h \text{ J}$ with $h$ the height in meters of the center of mass of the particle above the surface. The magnetic energy is $E_m = 1.3 \cdot 10^{-16} h \text{ J}$, caused by a small vertical magnetic gradient in the setup [70]. Calculated energy barrier values are shown in figure 3.5.

To allow for an analytical derivation of the rate of the binding of particles to the surface, an analytical expression for the barrier height is needed. The expression should cover the conditions in which particles can cross the barrier and bind to the surface. On the time scale of the experiment, energy barriers in excess of $25 k_B T$ are not
Figure 3.5: Symbols show calculated energy barrier values (the difference between the maximum and the secondary minimum of the potential energy) for a surface potential of $-30\,\text{mV}$ and particle potentials of $-20\,\text{mV}$, $-25\,\text{mV}$ and $-30\,\text{mV}$ with Hamaker constant $10^{-20}\,\text{J}$. The lines show the parametrization of the barrier height as a function of ionic strength as given in equation 3.1. The inset shows how the energy barrier height was calculated from the energy potential.

likely to be crossed by thermal energy (this takes hours to days) [25]. Therefore, we parameterize the function for barrier heights up to a few hundred $k_BT$ as function of ionic strength $I_c$ and a convenient description of the energy barrier height is given by equation 3.1, which includes a constant $C'$ to describe the ionic strength dependence of the energy barrier and a constant $D'$ which is independent of ionic strength.

$$E_b = \frac{C'}{I_c} + D'.$$

The calculated energy barrier values and the corresponding parameterized curves are shown as a function of ionic strength in figure 3.5. The height of the energy barrier that particles have to cross to bind to the surface is very sensitive to variations in the exact particle, surface and environmental properties. A difference in potential of only 1 mV (for either particle or surface) already causes a change in barrier height of $30\, k_BT$ in physiological buffer conditions (150 mM). Note that although no other interactions than van der Waals, electrostatic, gravitational and magnetic are included in the barrier height parametrization, parameters obtained from fitting the data will also include the other interactions. For example, hydrophobic interaction will increase the attractive interactions and thereby lower the energy barrier while steric interactions might raise the barrier.

### 3.3.2 Distributed energy barrier model

When studying the energy barriers of an ensemble of particles interacting with a surface, it should be taken into account that properties of the surface as well as the particles might vary. Not every location on the surface will have the exact same properties, between particles the properties can vary and variation might even occur over the surface of individual particles. When particles approach the surface, they will find local
energy minima determined by their position on the surface and by their orientation. To describe these variations we implement a distribution of energy barriers.

The rate of change of the number of unbound particles \(n\) with a given energy barrier \(E_b > 0\) can be expressed using the Arrhenius equation:

\[
\frac{dn}{dt} = -nk_0 \exp\left(-\frac{E_b}{k_BT}\right),
\]

with \(k_0\) the Arrhenius pre-exponential (attempt frequency), \(k_B\) the Boltzmann constant and \(T\) temperature. The number of unbound particles with barrier \(E_b\) after an incubation time \(t\) is given by:

\[
n = n_0 \exp\left(-tk_0 \exp\left(-\frac{E_b}{k_BT}\right)\right),
\]

with \(n_0\) the initial number of unbound particles with unique barrier \(E_b\).

In order to model the inhomogeneity of the particle and surface, a distribution of energy barrier values, \(f(E_b)\), is introduced. The total number of particles is represented by \(N_0\). We assume that particles are initially randomly distributed over all possible states, so \(n_0(E_b) = N_0 f(E_b) dE_b\). The number of unbound particles \(N\) can be found by integrating \(n\) (equation 3.3) over all energy barrier values:

\[
N = N_0 \int_{-\infty}^{\infty} f(E_b) \exp\left(-tk_0 \exp\left(-\frac{E_b}{k_BT}\right)\right) dE_b.
\]

If we assume for \(f(E_b)\) a gaussian energy distribution with mean energy barrier height \(E_{b,0}\) and width \(\sigma\), we get:

\[
\frac{N}{N_0} = \frac{1}{\sigma \sqrt{2\pi}} \int_{-\infty}^{\infty} \exp\left(-\frac{(E_b - E_{b,0})^2}{2\sigma^2}\right) \exp\left(-tk_0 \exp\left(-\frac{E_b}{k_BT}\right)\right) dE_b.
\]

The second exponent in the integral can be approximated with a step function at critical energy \(E_c = k_BT \ln (2tk_0)\). This assumption is valid if the spread in energy barrier values is larger than the thermal energy spread (\(\sqrt{2}\sigma/k_BT > 1\), as shown by Suuberg [93]), which is reasonable since a 1 mV variation in surface potential causes already \(30k_BT\) change in barrier height. Thus, the integral can be expressed as an error function:

\[
\frac{N}{N_0} = \frac{1}{2} \left[ 1 - \text{erf}\left(\frac{E_c - E_{b,0}}{\sqrt{2} \sigma}\right) \right].
\]

By filling in the parameterized energy barrier height from equation 3.1, an expression is obtained that describes the fraction of unbound particles as a function of ionic strength.

\[
\frac{N}{N_0} = \frac{1}{2} \left[ 1 - \text{erf}\left(\frac{C}{I_c + D}\right) \right],
\]

with

\[
C = \frac{C'}{\sqrt{2} \sigma} \quad \text{and} \quad D = \frac{-D' + k_BT \ln (2tk_0)}{\sqrt{2} \sigma}.
\]
Basically, $C$ [mM] expresses the position of the curve along the concentration axis, while $D$ determines the bound fraction at infinite ionic strength as well as the slope of the curve. Two different regimes can be distinguished based on the binding of the particles at infinite ionic strength. We can distinguish single particles in an ensemble of a few tens of particles, say 1 in 40, causing $N/N_0 < 0.025$ to be the lowest observable value. In the first regime not all particles bind at infinite ionic strength ($N/N_0 > 0.025$), then $D < \sqrt{2}$; second, if all particles do bind at infinite ionic strength ($N/N_0 < 0.025$), then $D > \sqrt{2}$. In view of the data in figure 3.4, we are in the second regime for the myoglobin coated particles. For the control particles at high pH we are in the first regime.

### 3.4 Discussion

The measurements of the control particles at pH 7.4 did not show the typical s-shaped curves as did the other measurements and therefore the data could not be fitted with equation 3.7. Probably, the scatter in the data is caused by instability in the system. This could be caused by the fact that the pH of the solution is close to the isoelectric point of the particles. This condition gives a small electrostatic interaction and thereby a minor influence of the ionic strength on the fraction of binding particles. The trend of the data leveling off at high ionic strength suggests that not all particles bind at infinite ionic strength and therefore $D < \sqrt{2}$. Also differences in variability of the other measurements might be explained by the stability of the system caused by the difference between the pH of the solution and the isoelectric point of the particles.

The fraction of rotating particles as a function of ionic strength, as shown in figure 3.4, was fitted with equation 3.7 by chi-square minimization using the Levenberg-Marquardt algorithm in Origin 8.5 and the typical dependency between $C$ and $D$ was $\sim 0.9$. The error bars were not included in the fits. The restriction $D > \sqrt{2}$ was applied for the data of the myoglobin coated particles, corresponding to the regime wherein all particles bind at infinite ionic strength. For the control particles at pH 3.7 also $D > \sqrt{2}$ was found. At pH 7.4 the measurements of the control particles did not show clear curves that could be fitted, but the trend in the data suggests that even at very high ionic strength not all particles will bind, so $D < \sqrt{2}$. This value of $D < \sqrt{2}$ was also found for the measurements of the control particles at pH 10: the dashed lines show fits with restriction $D > \sqrt{2}$ and these clearly do not match the shape of the curves. The fitted curves in figure 3.4 are in agreement with the measured data as a function of ionic strength and a clear distinction can be made between measurements on differently oxidized surfaces. Interaction parameters $C$ and $D$ were extracted for each measurement series and are shown in figure 3.6.

For the myoglobin coated particles, interaction parameter $D$ (figure 3.6 (b1)) is found to vary between $\sqrt{2}$ and 3 without a trend correlated to oxidation time or pH. Interaction parameter $C$ (figure 3.6 (a1)) varies in the range $\sim 2–1000$ mM and shows a clear trend of increasing values for increasing surface oxidation time as well as for increasing pH. This reflects the shift of the curves to higher ionic strengths as shown in figure 3.4.

The control particles do not show any trend in the fitted $D$-values either (figure 3.6 (b2)). A remarkable difference with the myoglobin coated particles is observed at pH 10, where $D < 1$ shows that even at high ionic strengths not all particles bind to
particles with myoglobin control particles (no myoglobin)

\[ a1) \]

\[ \textit{C (mM)} \]

\[ 1000 \]

\[ 100 \]

\[ 10 \]

\[ 1 \]

\[ \text{oxidation time (s)} \]

\[ 0 \]

\[ 20 \]

\[ 40 \]

\[ 60 \]

\[ 80 \]

\[ 100 \]

\[ a2) \]

\[ \textit{C (mM)} \]

\[ 1000 \]

\[ 100 \]

\[ 10 \]

\[ 1 \]

\[ \text{oxidation time (s)} \]

\[ 0 \]

\[ 20 \]

\[ 40 \]

\[ 60 \]

\[ 80 \]

\[ 100 \]

\[ b1) \]

\[ \textit{D} \]

\[ 0 \]

\[ 1 \]

\[ 2 \]

\[ 3 \]

\[ 4 \]

\[ 5 \]

\[ \text{oxidation time (s)} \]

\[ 0 \]

\[ 20 \]

\[ 40 \]

\[ 60 \]

\[ 80 \]

\[ 100 \]

\[ b2) \]

\[ \textit{D} \]

\[ 0 \]

\[ 1 \]

\[ 2 \]

\[ 3 \]

\[ 4 \]

\[ 5 \]

\[ \text{oxidation time (s)} \]

\[ 0 \]

\[ 20 \]

\[ 40 \]

\[ 60 \]

\[ 80 \]

\[ 100 \]

Figure 3.6: Interaction parameters \( C \) and \( D \) as a function of polystyrene UV/ozone oxidation time, extracted from the data in figure 3.4 using equation 3.7. For the myoglobin coated particles (panels (a1) and (b1)), \( D \) was restricted to \( D > \sqrt{2} \). The parameters for the control particles are shown in panels (a2) and (b2). Dashed lines are drawn to guide the eye.

the surface. The restricted fit with \( D > \sqrt{2} \) is much less accurate in reproducing the data, so the shape of these curves can only be understood when assuming that not all particles bind at infinite ionic strength. For pH 3.7, \( D > \sqrt{2} \) so for high ionic strength all particles bind to the surface like the myoglobin coated particles. The values of \( C \) again show a trend of increasing values for increasing surface oxidation time and pH. The differences in the interactions observed for myoglobin coated particles and control particles are caused by an interplay of all interactions taking place, like van der Waals, electrostatic and hydrophobic interactions.

### 3.4.1 Trends in extracted parameters

Fitting parameter \( C \) [mM] quantifies the influence of the ionic strength on the energy barrier height. Together with parameter \( D \) the energy barrier height can be calculated if it is assumed that the critical energy \( E_c \) (defined after equation 3.5, dependent on incubation time and attempt frequency) and \( \sigma \) are constant. A fixed incubation time was used in the experiments and it is likely that the attempt frequency and \( \sigma \) will not vary between the measurements. This allows the trends observed in \( C \) to be translated to trends in the energy barrier height. For increasing oxidation time and pH, the increasing value of \( C \) as observed in the experiments describes an increasing energy barrier height.
Electrostatic interactions are determined by the particle and surface potentials: higher potentials cause more repulsion and thereby a higher energy barrier. The ionic strength influences electrostatic interactions by changing the shielding. Since shielding changes the position of the energy barrier, the contribution of the van der Waals energy to the barrier height is influenced as well. Van der Waals interactions are determined by the Hamaker constant: a higher constant indicates more attraction, thus a lower energy barrier. Also other interactions, like hydrophobic interactions, will contribute to \( C \), and since these interactions can not be independently varied in the experiment, \( C \) includes all contributions to the energy barrier that are dependent on the ionic strength.

The first trend observed in the measurements is increasing \( C \), thus increasing energy barrier height, for increasing oxidation time, which was observed at all pH values. This can be explained by the fact that oxidized polystyrene has a more negative surface potential than non-oxidized polystyrene and therefore the electrostatic repulsion will be increased, raising the energy barrier [92]. Also the decrease of Hamaker constant that has been reported for oxidation of polystyrene [94] increases the energy barrier height by lowering the attractive van der Waals interaction. On top of that, oxidation lowers the water contact angle on polystyrene, reducing its hydrophobicity and thereby the hydrophobic interactions, which are attractive [23]. Lowering attractive interactions will increase the energy barrier height which is reflected in an increasing value of \( C \).

The second trend shows increasing \( C \) for increasing pH values. For (oxidized) polystyrene surfaces an increasing pH gives rise to increasing negative surface potential [92]. Since both the surface and the particles were negatively charged, a high pH causes high zeta potentials and thereby high electrostatic repulsion, which directly increases the barrier height. At low pH, the opposite holds: both surface and particle will have a low (absolute) charge, so there is weak electrostatic repulsion, leading to a lower energy barrier.

3.4.2 Estimation of energy barrier heights

Although the different contributions to the energy barrier can not be distinguished in the experiments, the total height of the energy barrier can be calculated from the fitted values. Using equation 3.7, we can relate the found parameters to actual energy barrier heights (using the parameterization of equation 3.1). The used incubation time of 5 minutes gives a critical energy \( E_c = k_B T \ln(2t k_0) \approx (6.4 + \ln k_0) k_B T \). Due to this logarithmic dependence, the critical energy is very insensitive to variations in \( k_0 \) and a very broad range of \( 10 < k_0 < 10^9 \) only gives rise to 8.7 \( k_B T < E_c < 27 k_B T \). As an estimate of the attempt frequency, we use the diffusion of a particle with radius 1.4 \( \mu \)m in water at room temperature over 0.1 nm: the diffusion constant is \( D = k_B T / 6 \pi \eta r = 1.5 \cdot 10^{-13} \) m\(^2\)/s (with the dynamic viscosity of water \( \eta = 10^{-3} \) N s/m\(^2\)) and diffusion time \( t = (3)^2 / (2D) = 3 \cdot 10^{-8} \) s, thus \( k_0 = 1/t = 3 \cdot 10^7 \) s\(^{-1}\), giving \( E_c = 24 k_B T \).

Further, to make an estimate of \( C' \) and \( D' \), a value of the energy barrier spread \( \sigma \) is needed. The energy barrier spread will be caused by variation in substrate properties, particle-to-particle variation and variations over each particle. The material with the largest variation will determine the resulting value of \( \sigma \). If the variation in surface properties determines \( \sigma \), the difference in properties between myoglobin coated
particles and control particles will be insignificant for the measured $\sigma$. However, if the particle properties determine $\sigma$, a possible difference in the variation over each particle and between myoglobin coated particles and control particles will be relevant to take into account. Inhomogeneities of the surface and particle treatment could for example cause variations in the surface potential. The difference in surface potential between oxidized and non-oxidized polystyrene has been measured by streaming potential measurements in a parallel plate configuration to be about 20 mV [92].

From particle dissociation measurements of myoglobin coated particles from oxidized polystyrene surfaces (see chapter 4) an energy barrier spread is found of $\sigma \approx 7k_B T$. This spread is measured on the energy barrier for dissociation, which was determined by pulling particles out of the deep (primary) energy minimum close to the surface. For association the height of the energy barrier is defined from the shallow secondary minimum. So, the same barrier is probed from different sides by dissociation and association and therefore the same spread is expected to be present in both cases. We use this same value of energy barrier spread for the myoglobin coated particles in the rotating particles probe experiments. Since no dissociation experiments have been performed on control particles, we will use the value of $\sigma = 7k_B T$ for the control particles in the rotating particles probe experiments as well.

If more accurate estimations of the energy barrier spread are desired, it can be determined from measurements at different incubation times for the system with associating particles. This can be performed for different types of particles to compare myoglobin coated and control particles. Each incubation time gives a unique critical energy and using the values of $D$ fitted in the measurements, $\sigma$ can be calculated (see equation 3.7): $\sigma = (E_{c_1} - E_{c_2})/(\sqrt{2}(D_{12} - D_{11}))$.

To determine the observed energy barriers, $C'$ and $D'$ are calculated using $E_c = 24k_BT$, $\sigma = 7k_BT$ and the values found for $C$ and $D$ for myoglobin coated particles ($2 < C < 10^3$ and $D \approx 2$):

$$20 < C' < 10^4 [k_BT \text{ mM}]$$
$$D' \approx 4 [k_BT].$$

(3.8)

For the control particles, $C$ falls in the same range as for the myoglobin coated particles as does $D$ for pH 3.7. For pH 10 $D$ is markedly different: $D < 0.5$ instead of $D > \sqrt{2}$, leading to:

$$D' > 19 [k_BT].$$

(3.9)

Theoretically, at infinite ionic strength, $E_b = D'$. The fact that $D'$ is much smaller for the myoglobin coated particles than for the control particles at pH 10 corresponds to the fact that for the control particles at high ionic strength not all particles bind, but the coated particles do.

We can compare the experimentally derived values for $C'$ and $D'$ (given in equation 3.8 for myoglobin and in equation 3.9 for the control particles) with the energy barrier calculations, as in figure 3.5 taking into account electrostatic, van der Waals, gravitational and magnetic energies and neglecting possible hydrophobic and steric interactions. The calculated values found for $C'$ are typically $2 \cdot 10^4$ to $3 \cdot 10^4 k_BT$ mM, a few times larger than the experimentally estimated maximum value of $10^4 k_BT$ mM. The values calculated for $D'$ are in the range $-60$ to $160 k_BT$, so the experimentally
observed values fall in this range. Deviations between calculated and observed energy barrier values may be explained by the fact that in our calculations hydrophobic interactions have been neglected. Hydrophobic interactions tend to lower the energy barrier which is what we observe when comparing calculated and measured energy barrier heights.

To visualize the energy barrier heights in the different systems, these have been calculated from the fitted values of $C$ and $D$ at two different ionic strengths (30 mM and 150 mM) and plotted in figure 3.7 as a function of oxidation time. In some cases negative values for the energy barrier are calculated, but since the model is only defined for positive energy barriers (see equation 3.2), these are left out. The trends of increasing energy barriers for increasing pH and increasing oxidation time are again visible. It should be noted that experimentally only a few tens of particles are probed at the same time, and therefore it is not possible to discriminate the energy barrier at which 2.5% of the particles is unbound ($22k_BT$) from higher energy barriers or at which 2.5% is bound ($27k_BT$) from lower barriers. By fitting the complete curves as a function of ionic strength, higher and lower values can be determined, but errors increase for energy barrier values at ionic strengths that are far away from the transition of unbound to bound (e.g. 30 mM, pH 10, 90 s oxidation and 150 mM, pH 3.3 and pH 3.7). Effectively, the model can only be reliably applied in a certain valid range around the transition and this range is dependent on the energy barrier model as well as the statistics of the data.

3.4.3 Different energy barrier parameterization

The energy barrier parameterization, given in equation 3.1, was proposed to describe the energy barrier calculated from DLVO theory. The decrease of the barrier for increasing ionic strength shows the dominance of electrostatic interactions for the energy barrier height. Knowing that the ionic strength dependence is determined by the Debye length of the solution (equation 1.2), also the parameterization of equation 3.10 can be proposed.

$$E_b = \frac{C'}{\sqrt{I_c}} + D'$$

(3.10)

This parameterization is illustrated in figure 3.8 together with energy barrier val-
ues calculated from DLVO theory. This parameterization does not perfectly follow the calculated DLVO energy barrier values, similar to the parameterization of equation 3.1. But we have to realize that in the measurements more contributions to the energy barrier will be present than just the DLVO energy, like for example hydrophobic interactions, and this might change the dependence of the barrier height on ionic strength. If the parameterization of the energy barrier inserted in equation 3.6 reproduces the shape of the measured curves ($N/N_0$ versus $I_c$), the parameterization is valid to describe the energy barrier. In fact, the shape of the curves and the parameterization that can fit this shape give information on the real energy barrier properties.

The parameterizations in equation 3.10 reproduces the measured data in a way that looks comparable to the fits with the parameterization used before (equation 3.1). For the new parameterization, the definitions of $C$ and $D$ as given in equation 3.7 are still valid, but the units of $C$ change to [√mM]. The same trends are found for $C$ and $D$ as shown in figure 3.6. In this case the values of $C$ vary in the range 3–100 √mM and $D$ up to ~6. Only the measurements of the control beads at pH 10 show values of $D < 1$, which is related to the fact that at high ionic strength the fraction of rotating particles does not go to zero. Figure 3.9 shows the energy barriers calculated from the fitted $C$ and $D$ values for two different ionic strengths (as in figure 3.7 for the parameterization of equation 3.1).

The energy barrier values for the parameterization in equation 3.10 are found to be negative in more cases than the standard parameterization of equation 3.1, which indicates that the standard parameterization is more suited to obtain physically relevant energy barrier values. The new parameterization apparently has a smaller valid range around the transition from unbound to bound particles. In the ranges close to the transition, however, the energy barrier values found with the parameterization of equations 3.1 and 3.10 are comparable, so in this regime they can both be used to describe the measured data. Probably, the best parameterization will have a dependence on $I_c^x$ with $0.5 < x < 1$ to incorporate not only the ionic strength dependence of the electrostatic interactions, but also of the van der Waals, hydrophobic and possible
other interactions.

3.5 Conclusions

We have measured the interaction of myoglobin coated particles and particles without myoglobin on oxidized polystyrene surfaces using the rotating particles probe, which quantifies interactions by recording the fraction of rotating particles. Since no particles are observed to unbind during the measurements, we have developed a distributed energy barrier model to explain the shape of the measured curves as a function of the ionic strength of the solution.

The interaction barrier is parameterized to be inversely dependent on the ionic strength of the solution, characterized by two parameters, one describing the ionic strength dependence of the barrier ($C$) and one describing the barrier height at very high ionic strength ($D$). The high ionic strength term is found to vary in a small range ($\sqrt{2} \lesssim D \lesssim 3$ for the coated particles, $D < 1$ for the control particles at high ionic strength), independent of oxidation time and pH. In contrast, the ionic strength dependence term clearly varies with oxidation time of the surface and pH of the solution.

Increasing oxidation and increasing pH both lead to increasing $C$ values, which corresponds to a shift of the curve and binding of the particles at higher ionic strengths. The shift is caused by the increase of barrier height by the increase of the electrostatic interaction, by the decrease of the Hamaker constant or by change of the hydrophobic interactions. Thus, a high pH and long surface oxidation are favorable in order to minimize non-specific binding of particles to polystyrene surfaces while in the same conditions control particles have less tendency to bind than myoglobin coated particles. The energy barrier heights can be quantified by the $C$ and $D$ values together with constants that should be independently determined: the attempt frequency for binding of the particles, the incubation time and the spread in the energy barrier values.

In figure 3.10 a schematic energy landscape is shown, in which the energy barrier for association is indicated, as well as the spread on the barrier. For the spread in the energy barrier values the spread found from dissociation experiments (chapter 4) was used. In the employed incubation time, particles will cross energy barriers up to $\sim 22 k_B T$, allowing to extract the fraction of bound particles from the energy barrier.
distribution, as illustrated in figure 3.10 for a single curve.

In conclusion, we have presented a novel method to analyze interactions between particles and a surface, using the rotating particles probe with a distributed energy barrier model. Our measurements on myoglobin coated particles, control particles and polystyrene surfaces reveal a consistent picture for a wide range of fluid conditions (ionic strength, pH) and surface conditions (oxidation state). This method paves the way for further studies to quantify the mechanisms underlying non-specific interactions between protein coated particles and polymer surfaces in different matrices and can be applied to characterize materials systems that are used in biosensors. The particle and surface properties can be varied as well as the fluid conditions.

The rotating particles probe technique together with the distributed energy barrier model might be used in future experiments to quantify the influence of various interactions between particle and surface on the energy barrier height. To study the influence of hydrophobic interactions, ideally these should be independently varied. An option might be to use various concentrations of surfactants to change the hydrophobic interactions between the particles and the surface. Surfactants are regularly used in immunoassays and biosensor design but it is still difficult to quantify their effect. To study how steric interactions influence the energy barrier, linkers could be bound to the particles and/or the surface. By varying their length and again looking for a good energy barrier parameterization, the influence of the steric interaction on the energy barrier may be determined. Also linkers are regularly applied in the surface functionalization of biosensors. Observation of higher numbers of particles will allow to discriminate energy barrier values further away from the transition and thereby a parameterization can be found that has a larger valid regime and can be used to calculate the energy barriers in a biosensor system for a large range of properties.

3.6 Acknowledgement

I would like to thank Delia Sprijdon for the rotating particles probe measurements on myoglobin coated particles that she performed during her stay in our group as part of her PhD research. I would like to thank Anne van Gorkom for the measurements on control particles (as well as on myoglobin coated particles) during her Bachelor end project.
The sensitivity and specificity of particle based biosensors depend amongst others on the non-specific interactions between the protein coated particles and surfaces. When magnetic particles are used, a force can be applied on them by a magnetic field gradient. This force can be used to pull particles off the surface and thereby provides a means to separate particles with different bond strengths. The so-called magnetic tweezers technique directly measures the dissociation kinetics of the bond between the particle and the surface.

To quantitatively interpret magnetic tweezers experiments, a calibration of the force on the particles should be performed. In section 4.1 we introduce time-of-flight measurements as a new method to determine the force on the particles. The magnitude of the force on the particles can be determined independent of their magnetic microstructure by measuring the velocity of the particles while a constant magnetic force is applied to them. A schematic of the setup used in the calibration and dissociation measurements is shown in figure 4.1.

Dissociation measurements of myoglobin coated particles from oxidized polystyrene surfaces were performed at different forces as described in section 4.2. Models to describe the kinetics of the dissociation of bonds already exist for specific interactions. To be able to describe also non-specific interactions, we extend the existing models to include a distribution of energy barriers for dissociation in section 4.3. Based on the measured dissociation curves, the dissociation of non-specific bonds can now be characterized by a gaussian distribution of energy barrier values. A manuscript presenting the results of this chapter is in preparation.

### 4.1 Calibration measurements

Calibration of the force applied to particles in a magnetic tweezers setup is needed to quantify the interactions between particles and surfaces. The force on the magnetic particles is determined by the magnetic moment of the particles $\vec{m}$ and the gradient of the magnetic field $\vec{B}$:

$$ F = \nabla (\vec{m} \cdot \vec{B}). $$

(4.1)

In our experiments superparamagnetic particles were used. The magnetic moment of superparamagnetic particles is induced by an external magnetic field that aligns the individual moments of the small ferromagnetic grains inside the particle. The de-
dependence of the total magnetic moment of the particle on the applied magnetic field is described by its susceptibility. Generally, the susceptibility of the particles is not known because it depends on details of the grain size distribution that can vary from particle to particle. Furthermore, there will be variability in the size of the particles, leading to spread in the velocity and thereby in the calculated applied force in a magnetic tweezers experiment. Since dissociation measurements are performed on a large ensemble of particles at the same time, a calibration technique is desired that allows to calibrate the force for the average properties of an ensemble of particles.

When the detailed magnetic properties of the particles and the setup are known, it is possible to calculate the applied force, as has been done before [75, 76]. Often, not all magnetic properties are known in detail. For example the magnetic field gradient applied by the magnetic tweezers is difficult to measure or predict exactly at the location of the particles. Furthermore, the magnetic properties of the particles are dependent on the grain size distribution, which is not known for each individual particle. Therefore, it is advantageous to calibrate directly the force applied by the magnet on the particles.

Force calibration has been performed on single particles in a number of different ways. One option to calibrate the force on a particle in a magnetic tweezers is to study the Brownian movement of that particle when it has been bound to the surface via a tether with well-known mechanical properties [73]. Also the Brownian motion of a particle that has been trapped above a microscopic current wire in the surface can be used to calibrate the force [95]. These methods based on the Brownian motion of particles require extra knowledge about the properties of the tether or the current wire. Magnetophoresis-based methods offer the possibility to calibrate the force by measuring the velocity. The force is calculated via the Stokes drag (equation 1.4), which relates the velocity to the force when equilibrium has been reached and viscous forces dominate the movement.
Force calibration via Stokes drag has for example been applied to individual particles in a glass cuvette filled with glycerol at different distances to a permanent magnet. At each distance 5–10 particles (2.25 µm radius) were studied and the observed force varied up to ∼20% [96]. Also the force on a single M-270 particle (1.4 µm radius, suspended in a CsCl solution to prevent settling) has been calibrated using an electromagnet. At low currents (< 0.5 A), a quadratic dependence of the force on the current was found (up to ∼2 pN) and at high currents (up to 3 A) the force increases linearly with current (up to ∼10 pN). The error in the measurement in the linear regime is ∼25% (except for the last measurement point where it is > 50%) [97]. Also current wires next to a microfluidic channel can be used to apply a force to superparamagnetic particles (radius 3 µm). In this case a population of 20 particles was studied simultaneously and when 90% had passed the channel this was defined as the capture time, which was in turn used to calculate the velocity. Typical errors of ∼30% were observed [98, 99].

When dissociation measurements are performed, particles are pulled away from the substrate and their velocity can not be observed directly in the microscope. One option to measure the velocity in this case is to relate the observed diffraction pattern of the particles to their height, which requires the production of a lookup table for each particle. This has been done for particles (radius 2.25 µm) in an electromagnetic tweezers, resulting in a quadratic dependence of the force on the current for low currents and a linear dependence for higher currents. Measurements of at least five particles show errors of > 30% in the linear regime [100].

The calibration methods mentioned above require studying the movements of single particles and therefore, a lot of measurements are required to find the average properties of a large ensemble of particles. To study a large number of particles at the same time, we propose to use time-of-flight measurements. By measuring the time-of-flight of the particles at a number of different focal planes the particle velocity is calculated in a straightforward way. Using the Stokes equation, the force can be calculated from the velocity and no detailed knowledge on the magnetic field gradient and the magnetic properties of the particles is needed.

4.1.1 Materials and methods

The magnetic tweezers setup consists of an electromagnet with a soft iron core, as described in [70]. The current through the electromagnet can be controlled up to 1 A and determines the produced magnetic field and gradient. For a constant distance between the tip and a Hall sensor, the magnetic field was measured to be linearly dependent on the current. The core extends outside the coil and tapers off to a flat tip with a radius of 1 mm. This tip was designed to produce a homogeneous magnetic field gradient over the field of view of the microscope (which is smaller than 1 × 1 mm²). In simulations a maximum deviation of ∼10% was found over this area at a distance of 500 µm between the tip and the surface for a current of 0.4 A. For comparison, the spatial uniformity of force may also be extracted from the time-of-flight movies, but this comparison has not yet been done. The design of the electromagnet tip allows a lot of particles to be studied at the same time and under identical conditions [70].

A schematic setup of the calibration setup is shown in figure 4.1 (b). The calibration measurements were performed by placing superparamagnetic particles in a fluid
cell, made from two glass coverslips and two SecureSeal Imaging Spacers to create a well of 9 mm diameter and ∼240 µm height. After some waiting time to let all particles sediment, the fluid cell was placed on an inverted microscope (Leica DMI5000M, used with 20× objective), equipped with a high speed camera (Redlake MotionPro X3), typically operated at 1000 Hz. The electromagnet was placed on top of the fluid cell, at a well defined tip–sample distance (see figure 4.1 (b)). The tip–sample distance as well as the distance between the bottom of the sample and the focal plane were determined by moving the objective and focussing on each plane, giving an accuracy of ∼5 µm for the tip–sample distance and ∼2 µm for the focal plane. The magnet as well as the camera were controlled via a National Instruments Data Acquisition Card and LabVIEW software to synchronize the switching on of the electromagnet and the camera at the same trigger.

M-270 particles (Invitrogen Dynabeads M-270 Carboxylic Acid, radius 1.4 µm, coefficient of variance <3% [88]) and MagSense particles (MagSense Carboxyl, radius 500 nm, coefficient of variance <20% [101]) were used in calibration measurements. The M-270 Carboxylic Acid particles were also used in the rotating particles probe measurements (chapter 3) and will be used in the dissociation measurements (section 4.2; for the calibration they were not functionalized). The MagSense particles were used in order to further study the calibration technique. Both types of particles were suspended in deionized water and did not bind to the glass surface of the fluid cell.

A calibration measurement series consisted of time-of-flight measurements at a number of different focal planes. For each measurement at a single focal plane a new sample was prepared in which the particles were sedimented on the bottom of the fluid cell. The sample was placed on the microscope (focused at the desired focal plane) with the electromagnet on top (figure 4.1 (b)) and on the same trigger the magnet and the camera were turned on. The time for the particles to reach the focal plane was determined by analyzing the recorded movie: in each frame the number of particles was counted. A background correction was performed on each frame of the movie either by subtracting an average image of the complete movie (M-270 particles, using home-written software as used by Janssen et al. [70]) or by bandpass filtering (MagSense particles, using ImageJ). Then, the image was transformed to a black-and-white image and the particles were identified based on their size and circularity. In this way, clusters of particles were excluded from the measurements. Curves were obtained showing the number of particles in a certain focal plane as a function of time.

The mean time-of-flight was extracted from the curves as in figure 4.2 (a). The measured data appear to fit a log-normal distribution and the mean time-of-flight can be calculated. Although the mean of the distribution can be very accurately determined, an error is introduced by the image analysis, which we estimate to be ∼10% of the mean time-of-flight from our experience in sensitivity to image analysis settings. The particle velocity was given by a linear fit of time-of-flight versus focal plane data, as in figure 4.2 (b). Measurement series were recorded for a number of different distances between the magnet tip and the sample and for different currents through the electromagnet coil.

The focal planes were chosen to be at least a few particle radii away from the surface, which excludes the influence of drag close to the surface of the fluid cell.
and allows the use of equation 1.4 with $\lambda = 1$. The magnetic force can be calculated directly from the velocity of the particles:

$$F_M = 6\pi\eta rv.$$  (4.2)

The dynamic viscosity of water is $\eta = 10^{-3}$ N s/m$^2$ and the radius $r$ is used as given by the manufacturer of the particles. Often, together with the mean radius a coefficient of variance is given by the supplier. A variation in the radii of the particles leads to a spread in the force, as will a variation in the magnetic content of the particles. Recently, it has become recognized that instead of single parameter values, distributions of properties like size and magnetization play an important role in the behavior of micrometer sized superparamagnetic particles [102].

4.1.2 Results

Calibration measurements on MagSense Carboxyl particles were performed for different currents through the electromagnet coil. Figure 4.2 (a) shows some example curves of the number of particles extracted from the movies as a function of time, together with log-normal fits at three different focal planes. For each combination of focal plane and pulling force a single sample was prepared and measured. The fitting was performed by reducing chi-square using Origin 8.5. Figure 4.2 (b) shows the linear fits to extract the velocities from the obtained times-of-flight. The fits do not pass through the origin, since close to the surface equation 4.2 is not valid (equation 1.4 should be applied with $\lambda > 1$). The velocities are used together with equation 4.2 to calculate the magnetic force (figure 4.2 (c)). An average particle radius of 500 nm was used, determined by the manufacturer.

Also the force on M-270 particles was calibrated. The mean values of the log-normal fits of the number of particles are plotted in figure 4.3 (a1) and (b1). In figure 4.3 (b1) it can be seen that the switching of the camera and magnet were not at the same trigger. As for the MagSense particles, a linear relation was found between the current and the magnetic force on the particles, shown in figure 4.3 (a2). For the M-270 particles the maximum force that could be applied at 1 A was a bit higher than for the MagSense particles ($\sim 70$ pN instead of $\sim 60$ pN). The dependence of the force on the distance between the sample and the magnet tip was also studied at a constant current of 1 A, and the results are shown in figure 4.3 (b2). The force decreases for increasing tip–sample distance, similar to the results shown in [96]. The decreasing force is caused by a decrease of the magnetic gradient with increasing distance. The exact dependence of the magnetic gradient as a function of distance is determined by the shape of the magnet tip.

For the MagSense particles the error in the magnetic force was a few pN while for the M-270 particles errors of $\sim 10$ pN were found. The accuracy of the force calibration is determined by the time-of-flight precision extracted from the movies. The polydispersity of the size and magnetic content of the particles and the accuracy of the image processing to detect the particles have an influence on the width of the time-of-flight curves as in figure 4.2 (a). Both for the M-270 particles and the MagSense particles the width of the curves amounts to $\sim 50\%$ of their mean value.

Polydispersity in the size or magnetic content of the particles will lead to a spread in the particle velocities. This is recorded as the width of the curve of the number of
Figure 4.2: Steps in the analysis of the calibration data, illustrated for MagSense particles. (a) Number of particles as function of time for three different focal planes. A log-normal fit was used to extract the time-of-flight. (b) Time-of-flight measurements for a number of focal planes allow to extract particle velocity by a linear fit. This is done for a number of different currents through the electromagnet coil. The error bars indicate an error of 10% introduced by the image analysis. (c) Force applied to particles, calculated from their velocity determined in time-of-flight measurements. The magnet tip was positioned at 390 μm from the bottom of the sample.

particles at a focal plane as function of time (figure 4.2 (a)). From the information of the manufacturers it is known that the coefficient of variance for the M-270 particles is much lower than for the MagSense particles (size variability of 3% compared to 20%). Only for the M-270 particles information was found on the variability in the magnetic content: 28% particle-to-particle variation was observed for the maximum torque [91]. The maximum torque is dependent on the size of the particles ($R^3$), so about 19% of the variation is caused by other properties of the system, like the magnetic content. Since the particle types have similar relative widths of the time-of-flight curves, we conclude that a velocity variation due to particle polydispersity is not dominantly causing the large curve widths.

Another important determinant for the width of the curves is the image processing to detect the particles. The contrast in the image as well as the background correction determine how well the particles can be distinguished. To reduce the width, it is important to detect particles only at the moment when they are in focus. When the particles are out of focus, they appear as a larger blur in the microscope image. Detecting the particles only in the focal plane therefore could be achieved by a strict size limit. The
threshold to transform the image to black-and-white also influences the width of the curves: a lower threshold causes a broader curve, since also particles that are out of focus are detected. Probably, the accuracy of detecting the average time-of-flight can be improved for the M-270 particles by a different background correction than subtracting an average image, for example based on bandpass filtering as for the MagSense particles.

In the measurements the curve width is predominantly caused by optical variability of the system: the background and contrast in the movie frames is not uniform. If the detection procedure is improved such that the particles are only detected when they are in focus, the curve width will be determined by the variation of the force on the particles and thereby yield information on the variability in size and magnetic content of the particles.

In summary, we have described a time-of-flight calibration technique to determine the applied force to a large ensemble of particles without detailed knowledge of magnetic properties of the particles and the setup, which is identical to the setup that will be used for dissociation measurements.

### 4.2 Dissociation measurements

The dissociation of myoglobin coated particles bound to oxidized polystyrene surfaces was studied in a magnetic tweezers setup. Dissociation curves were recorded that show the number of particles that remain bound to the surface upon application
of a certain force, as a function of time. The forces were quantified by the calibration measurements described in section 4.1.

### 4.2.1 Materials and methods

The magnetic tweezers setup consisted of the same electromagnet as described for the calibration measurements in section 4.1.1 and illustrated in figure 4.1 (a). An important property is the design of the magnet core with a flat tip that allows to study hundreds of particles under identical force conditions. The magnetic tweezers setup was described in detail in [71]. The magnet was placed on a microscope (Leica DM6000M, upright microscope) and an objective of 20× was used, combined with 1.5× additional magnification. The fluid cell was placed on top of the magnet and the microscope was focused on the surface with bound particles. A high-speed camera (Redlake Motion-Pro HS-3) was used to record movies of the particles on the surface. Before turning on the magnet, a picture was taken for reference. The magnet was turned on at a certain current (corresponding to a certain magnetic force) and simultaneously the recording of the movie was started at a frame rate of 20 Hz. Finally, the movie was analyzed by counting the number of particles in each frame.

The particles used in the dissociation experiments were superparamagnetic M-270 Carboxylic Acid particles (Invitrogen Dynabeads, radius 1.4 \( \mu \)m [88]), functionalized with myoglobin using EDC–NHS coupling as described in section 3.1.2. Unreacted surface groups were passivated by quenching with ethanolamine. The particles were used at a concentration of \( \sim 10^7 \) particles/mL in phosphate buffered saline at a standard ionic strength of 150 mM (8 \( \mu \)L per fluid cell). The surfaces were spincoated polystyrene on glass coverslips, prepared and UV/ozone oxidized as described in section 2.1. A fluid cell was produced from these polystyrene surfaces with a SecureSeal Imaging Spacer and a glass coverslip.

It is known that the M-270 particles are not ideally superparamagnetic, but have a small permanent magnetic moment [74]. This property is employed in the rotating particles probe as described in chapter 3. To be able to study the dissociation of the particles, it is desired that only an out-of-plane pulling force is applied and application of a torque to the bond is prevented. A torque can be excluded by aligning the permanent moments in the direction of the applied field, which is perpendicular to the surface. Alignment of the permanent moments can be achieved by applying a homogeneous vertical magnetic field to the particles prior to the binding to the surface. Only a small field should be used to prevent clustering of the particles by the induced magnetic moments. We chose to use a Helmholtz coil pair to apply a homogeneous vertical magnetic field of 1 mT during the incubation.

At the start of an experiment, a fluid cell was filled with particle solution and incubated for 90 s. During this time, all particles sedimented onto the surface under the influence of gravity while at the same moment their permanent magnetic moments were aligned perpendicular to the surface. After the incubation, the cell was removed from the Helmholtz coil setup, the cell was turned around and placed on the magnetic tweezers setup. Thereafter, a waiting time of 60 s was employed to allow unbound particles to fall off the surface before turning on the pulling magnet.
4.2.2 Results

Dissociation curves have been measured for myoglobin coated particles that were non-specifically bound to polystyrene surfaces. For each combination of oxidation time and force three individual samples were studied. A range of oxidized surfaces (90 s, 180 s and 300 s) was used in the experiments. The initial number of bound particles $N_0$ was counted on the reference picture which was taken just before switching on the pulling magnet. Figure 4.4 shows the dependence of $N_0$ on the polystyrene oxidation time. The number of bound particles and the standard deviation were extracted from three measurements. For a larger oxidation time, less particles were bound to the surface.

The number of particles binding to the substrate is determined by the association rate as well as the number of particles present. As has been shown in chapter 3 the energy barrier for association of myoglobin coated particles to a polystyrene surface increases for increasing oxidation time. Since the same number of particles was incubated on each surface, the higher energy barrier for oxidized surfaces explains the lower number of bound particles.

The forces exerted with the magnetic tweezers setup to dissociate the particles from the surface were 30 pN, 50 pN and 70 pN, as determined in the calibration (section 4.1). In figure 4.5 typical measured curves show the number of particles on the polystyrene surface as a function of time; each curve is an average of three measurements on individually prepared samples.

Increasing the oxidation time of the polystyrene surface causes a larger fraction of the particles to get dissociated: the fraction of particles remaining on the surface at the end of the experiment decreases. In the next section we will discuss the time and force dependence of the dissociation curves.

Figure 4.4: Number of particles binding to substrates that were oxidized for different times. The average values and standard deviation of three individual, identically prepared, samples are shown.
Figure 4.5: Dissociation curves of myoglobin coated particles from an oxidized polystyrene surface. Every curve is an average of three individual, identically prepared, samples. The surfaces were oxidized for (a) 90 s, (b) 180 s and (c) 300 s. Applied pulling forces were 30 pN, 50 pN and 70 pN. The continuous line shows the fit of the data using equations 4.9 and 4.11. The dashed line shows a double exponential fit as in [71].

### 4.3 Dissociation of bonds

In 1978, Bell postulated an equation to describe the lifetime of receptor–ligand bonds under an applied force [103] based on the equation found by Zhurkov (in 1965) to describe the strength of solids [104]. In 1997, Evans reformulated Bell’s formula for the off rate $\nu$ for increased bond dissociation under external force as:

$$\nu = \omega_0 \exp \left( -\frac{E_b - F \cdot x\beta}{k_B T} \right), \quad (4.3)$$

with $\omega_0$ the natural vibration frequency of the bond in vacuum, $E_b$ the energy barrier of the transition state and mechanical energy $F \cdot x\beta$. $F$ is the applied force that acts along a reaction coordinate $x$ to reach $x\beta$ at the transition state, as indicated in figure 4.6. The shape and location of the energy barrier are assumed to be unchanged by the applied force, only its height is lowered [105]. In figure 4.6 an interaction potential of a bond is sketched with its deformation when a force is applied. Also the energy barrier height
Figure 4.6: (a) Free energy as a function of distance along reaction coordinate $x$ for a bond between receptor and ligand, with $E_b$ the energy barrier for unbinding and $x_β$ the range of the interaction. (b) An applied force $F$ decreases the free energy linearly with distance along the reaction coordinate.

$E_b$ is indicated. The spontaneous unbinding rate constant $k_-$ is given by:

$$k_- = \omega_0 \exp \left( \frac{-E_b}{k_B T} \right)$$  \hfill (4.4)

Using the off rate defined in equation 4.3, it is possible to calculate the change of the number of bonds for a single energy barrier value ($E_b$) for an applied force $F$:

$$\frac{dn}{dt} = -n\omega_0 \exp \left[ -\frac{E_b - F \cdot x_β}{k_B T} \right].$$  \hfill (4.5)

with $n$ the number of bonds with the energy barrier $E_b$, $\omega_0$ the attempt frequency and $x_β$ the bond length. The number of remaining bonds is obtained by integrating over the pulling time $t_p$ (the time that a force is applied):

$$\frac{n}{n_0} = \exp \int_0^{t_p} -\omega_0 \exp \left[ -\frac{E_b - F \cdot x_β}{k_B T} \right] dt$$

$$= \exp \left( -t_p \omega_0 \exp \left[ -\frac{E_b - F \cdot x_β}{k_B T} \right] \right),$$  \hfill (4.6)

with $n_0$ the number of bonds at time 0. The force and barrier parameters were assumed to be constant over time.

Implementing equation 4.4 for the unbinding rate constant, equation 4.6 can be rewritten to a single exponential decay that can be used to fit dissociation curves (number of bonds as a function of time) and find the off-rate $k_{off}(F)$ as a function of the applied force:

$$\frac{n}{n_0} = \exp \left( -t_p k_- \exp \left[ \frac{F \cdot x_β}{k_B T} \right] \right) = \exp \left( -t_p k_{off}(F) \right).$$  \hfill (4.7)
When two different types of bonds are present in the system, the number of remaining bonds is the sum of two exponential decays:

\[
\frac{n}{n_0} = A \exp\left(-t \cdot k_{-A} \exp\left(\frac{F \cdot x_{pA}}{k_B T}\right)\right) + B \exp\left(-t \cdot k_{-B} \exp\left(\frac{F \cdot x_{pB}}{k_B T}\right)\right),
\]

(4.8)

with \(A\) and \(B\) the initial fractions of the different bonds.

### 4.3.1 Dissociation of particle–surface bonds

The Bell–Evans model as discussed above has been developed for specific bonds between two molecules. However, its use has been extended and it has been applied to numerous particle based magnetic force assays of which we mention a few. The single exponential of equation 4.7 has been applied to the interaction between streptavidin or avidin and biotin to extract \(k_{\text{off}}\) at a number of different forces. Extrapolation to \(F = 0\) allows to extract \(k_{\text{off}}(0)\) and \(x_{p}\) [96]. Dissociation curves of protein A and its antibody were interpreted by the double exponential decay of equation 4.8 whereby a weak bond and a strong bond were hypothesized [69]. The approach employing populations of different bonds has been extended even further in assays incorporating biotin, anti-biotin and streptavidin. In these experiments three exponential decays were found that were identified as non-specific weak interactions (fast dissociation), specific single molecular interactions (intermediate dissociation) and non-specific strong interactions (slow dissociation). Non-specific interactions between a blocked surface and biotin coupled particles could be described by the two non-specific contributions [71].

The specific bond between two molecules, like an antibody and its ligand, is a very well defined interaction that is characterized by an energy barrier that will be identical for all individual interacting pairs. In the case of non-specific interactions of a protein coated particle on a surface, the energy barrier is less strictly defined, since not all proteins on the particle surface will interact in the exact same way with the surface, e.g. because proteins can have different orientations on the particle and therefore, it is impossible for them to have the exact same interaction with the surface. The fact that the dissociation curves in the semilogarithmic plots in figure 4.5 do not have straight segments, shows that a single or double exponential decay does not suffice to describe the non-specific interactions between myoglobin coated particles and oxidized polystyrene surfaces.

We hypothesize that the non-specific interactions can be described by the simultaneous action of a distribution of energy barrier values. Berberan-Santos et al. have described different mathematical approaches that could be taken to extract a distribution [106]. In systems where it is known beforehand which interactions are occurring, a model for the energy barrier distribution can be formulated. This approach is used when dissociation curves are fitted with two or three different populations as in equation 4.8. If the system is less well defined, it may be possible to extract a distribution from the measurement data. However, this approach requires accurate data with very clear features. Another option is to use an a priori mathematical function to describe the distribution.

To describe non-specific interactions, we expect a continuous distribution of energy barrier values. One function to describe the decay resulting from a continuous distribution is the stretched exponential function [106]. A function to describe a con-
tinuous distribution is the gaussian distribution. It has for example been used to modify the Bell model to interpret measurements on the unfolding of a single protein that was pulled on with an AFM tip. The dissociation rate, which includes the attempt frequency as well as the energy barrier, was supposed to be inherent to this system. Therefore, only fluctuations in the applied force $F$ and $x_\beta$ were included in this so-called random Bell model (and no fluctuations in $\omega_0$ and $E_b$). Fluctuations in $x_\beta$ were assumed to arise from configurational disorder of the protein, thereby representing the physical quantity of interest while fluctuations in $F$ arose from the experimental conditions [107]. Since our system of myoglobin coated particles on an oxidized polystyrene surface is expected to generate variable barriers we hypothesize that the non-specific interactions can be described by a gaussian distribution of energy barrier values $E_b$.

4.3.2 Dissociation in case of a gaussian barrier distribution

As explained in the previous section, the curves in figure 4.5 are not characteristic for single energy barriers and we hypothesize that the non-specific interactions may be described by a gaussian distribution of energy barrier values. In this section we extend Bell’s model to include a distribution of energy barriers instead of a number of individual barriers. Starting from equation 4.6, a normalized distribution of occupied energy barrier states $f(E_b)$, allows to express the total number of remaining bonds as:

$$\frac{N}{N_0} = \int_{-\infty}^{\infty} \exp\left(-t_p \omega_0 \exp\left[-\frac{E_b - F \cdot x_\beta}{k_B T}\right]\right) f(E_b) dE_b, \quad (4.9)$$

with $N_0$ the total number of bonds at $t_p = 0$ for all possible energy barriers. $N$ represents all bonds present in the system, while $n$ represents a specific population of bonds with energy barrier $E_b$. The dissociation process modifies the state occupation, favoring the rupture of low barrier states versus high barrier states. The equation does not take into account any possible state exchange (e.g. high barrier to low barrier) during the time of the pulling experiment.

In the limit of a single energy barrier, the energy barrier distribution is a delta function and equation 4.9 is simplified to a single exponential decay as in equation 4.7:

$$f(E_b) = \delta(E_0) \rightarrow \frac{N}{N_0} = \exp\left(-t_p \omega_0 \exp\left[-\frac{E_0 - F \cdot x_\beta}{k_B T}\right]\right). \quad (4.10)$$

The shape of the dissociation curves for a single energy barrier is illustrated in figure 4.7. In case the bonds are distributed over two distinct energy barrier values, a double exponential decay results, as is also shown in figure 4.7. This can for example be the case if two populations of bonds are present with their own characteristics. This approach can be extended to include multiple populations.

Equation 4.9 can now be used with a gaussian distribution of energy barrier values around a central value $E_0$:

$$f(E_b) = \frac{1}{\sigma \sqrt{2\pi}} \exp\left(-\frac{(E_b - E_0)^2}{2\sigma^2}\right), \quad (4.11)$$

with $\sigma$ the width of the distribution.
Figure 4.7: Fraction of remaining bonds \( \frac{N}{N_0} \) as a function of pulling time \( t_p \) for a single energy barrier at \( E_0 = 22 k_B T \) (\( f(E_0) = \delta(22 k_B T) \)) and for bonds evenly distributed over two energy barrier values of \( 20 k_B T \) and \( 25 k_B T \) (\( f(E_0) = 1/2 \delta(20 k_B T) + 1/2 \delta(25 k_B T) \)), calculated using equation 4.9 with \( x_β = 0.5 \) nm, \( ω_0 = 3 \cdot 10^7 \) s\(^{-1} \) and a pulling force \( F = 20 \) pN.

In the plots in figure 4.8 the central energy barrier value was varied; the dissociation curves as well as the fraction of bonds remaining after a fixed time are shown. Obviously, figure 4.8 (a) shows that a higher energy barrier causes less bonds to be broken. The range of \( E_0 \)-values resulting in a fraction of bonds between 0 and 1 becomes broader for higher \( σ \).

Figure 4.9 shows the influence of the distribution width \( σ \) on the shape of the curve and the fraction of bonds remaining after a fixed time. A broader distribution gives a steeper reduction at short times while for longer times less bonds are broken compared to a more narrow distribution. The behavior at longer times determines the fraction of remaining bonds, that therefore will increase with increasing \( σ \).

The influence of the applied force on the remaining fraction of bonds after a fixed time can also be studied. In figure 4.10 the fraction of bonds that remains in the case of a single energy barrier as well as for a distribution of energy barriers is plotted.
Figure 4.9: (a) Dissociation curves for a gaussian distribution of energy barrier values ($E_0 = 22k_B T$), showing the fraction of remaining bonds ($N/N_0$) as a function of time ($t_p$) for $\sigma$ varied between $1k_B T$ and $7k_B T$ with $x_0 = 0.5\text{ nm}$, $\omega_0 = 3 \cdot 10^7 \text{s}^{-1}$ and $F = 20\text{ pN}$. (b) The fraction of remaining bonds after a constant time (50 s), dependent on $\sigma$.

![Dissociation curves for a gaussian distribution of energy barrier values](image)

Figure 4.10: Fraction of remaining bonds ($N/N_0$) as a function of pulling force for a single energy barrier between $20k_B T$ and $24k_B T$ and for a gaussian distribution of energy barrier values centered between $20k_B T$ and $24k_B T$, $\sigma = 2k_B T$. The pulling time $t_p = 50\text{ s}$, $x_0 = 0.5\text{ nm}$ and $\omega_0 = 3 \cdot 10^7 \text{s}^{-1}$.

![Fraction of remaining bonds as a function of pulling force](image)

In all cases the thermal energy of the system is observed to dissociate a fraction of bonds even without a force being applied. It can be seen that for a distribution the dependence of the final fraction on the force is less pronounced than for the single energy barrier.

### 4.3.3 Parameters and sensitivity analysis

To test the hypothesis that the non-specific bonds between protein coated particles and surfaces have a gaussian distribution of energy barrier values, equation 4.9 together with equation 4.11 can be compared with the measured dissociation curves. The variable $t_p$ is the time of the recording of the dissociation curve and $F$ is the force applied to the particles as determined by the calibration. The curves can be fitted by the equation to deliver both $E_0$ and $\sigma$, provided that values are available for $\omega_0$ and $x_0$. We assume a constant value for the pulling force.

The force $F$ used in the fitting is determined from the calibration as described in section 4.1. The error in the determined force can be $\sim10\%$ of its value. An error in the chosen value of $F$ will lead to an error in the determined $E_0$. For a force of 50 pN, a 10% force error will lead to an error in $E_0$ of $\sim0.7k_B T$, which can not be distinguished.
from thermal fluctuations. From the calibration and single bead measurements it is known that in general a distribution of forces is present, caused by a spread in size and magnetic properties of the particles. The values for the spread in force could not be independently determined from the measurements, but because of the small coefficient of variance for the M-270 particles (<3% for the size) it is unlikely that this has a significant influence on the distribution of the energy barrier values. In single particle measurements variations in applied force up to 25% have been observed for M-270 particles, but these were caused by the detection and not by the properties of the particles themselves [97].

Since the kinetic process of unbinding is governed by viscous friction, Evans et al. argue that the vibration frequency of the bond should not be the only determinant of the prefactor of the dissociation rate ($\omega_0$, see equation 4.3) [105]. Instead, the prefactor should be the attempt frequency for diffusive escape of the bond. As an estimation for this attempt frequency, we follow the approach of Evans and calculate the diffusion of a particle with a radius of 1.4 $\mu$m in water over a distance of 0.1 nm at room temperature and use this in equation 4.9: $\omega_0 = 3 \cdot 10^7$ s$^{-1}$. The same value has been used as the attempt frequency for particle association in section 3.4.2, where also the calculation can be found.

A possible inaccuracy in $\omega_0$ translates in an inaccuracy in the energy barrier value. Inspection of equations 4.9 and 4.11 shows that an increase in the attempt frequency lowers the fraction of remaining bonds; $\omega_0$ scales the dissociation curve along the time-axis. When fitting one particular dissociation curve, a change in $\omega_0$ can only be compensated by a change in $E_0$. Since $\omega_0$ is placed outside the exponent in which $E_b$ (turning into $E_0$ after integration) is located, $E_0$ will change logarithmically with change of $\omega_0$: for example a hundredfold increase in $\omega_0$ leads to an increase in $E_0$ by $(\ln 100) k_B T \approx 4.6 k_B T$.

Bell estimates the value of $x_\beta$ to be between the range for individual interactions that together cause the binding (0.1–0.2 nm) and the dimension of an antibody binding cleft (1 nm). We use the same estimation: $x_\beta = 0.5$ nm [103]. A higher bond length lowers the fraction of remaining bonds; furthermore, a higher bond length increases the importance of the applied force relative to the energy barrier. When fitting one particular dissociation curve, a change in $x_\beta$ can only be compensated by a change in $E_0$. In this case, both $x_\beta$ and $E_0$ are located inside the same exponent, so there will be a linear relationship between them. For example, an increase in $x_\beta$ of 0.25 nm leads to an increase in $E_0$ by $F \times 0.25 \cdot 10^{-9}$ J. For a force of 50 pN this amounts to 3.1 $k_B T$.

When fitting the data, we assumed that $\omega_0 = 3 \cdot 10^7$ s$^{-1}$ and $x_\beta = 0.5$ nm were constant and $E_0$ and $\sigma$ were determined for these values of $\omega_0$ and $x_\beta$. If more reliable values of $\omega_0$ and $x_\beta$ come available at a later stage, the $E_0$ values found from the fits can be easily corrected as described in the previous paragraphs.

For fitting the measured dissociation curves with equations 4.9 and 4.11, a function was used to find the minimum variance within a constrained set of $E_0$ and $\sigma$ values, based on the sequential quadratic programming method and implemented in Matlab R2012b. The variance was calculated as the mean of the squared deviations between the datapoints and the fit. A typical variance profile found for a large range of $E_0$ and $\sigma$ values is shown in figure 4.11 (a). It can be seen that a trough of low variance values is present in the data which illustrates the interdependence between
the $E_0$ and $\sigma$ parameters. To quantify this interdependence, we determined the range of $E_0$ and $\sigma$ representing variances up to 1.1 times the minimum variance. This range will be represented by error bars on the fitted values. Figure 4.11 (b) shows a zoom in of the variance around the minimum values of $E_0$ and $\sigma$, in which the error region is indicated. In the fits the parameters $E_0$ and $\sigma$ were independently varied and their correlation has not been explicitly studied. However, the model is based on the assumption that the position and width of the energy barrier are independent.

### 4.3.4 Bond strength distributions

In section 4.2 dissociation curves were shown of non-specific interactions of myoglobin coated particles on oxidized polystyrene surfaces. Figure 4.5 shows that the model based on a gaussian distribution of energy barrier values (solid line) closely agrees with the data, while a double exponential decay (dashed line, based on two distinct energy barriers) does not suffice to describe the data. Apparently, the energy barrier distribution is a better description of the observed system than the regularly used description by distinct populations. In figure 4.12 the fitted values of $E_0$ and $\sigma$ from the distributed energy barrier model are shown together with the error ranges defined by 1.1 times the minimum variance. These fits were performed on the average dissociation curves of three measurements on individual samples. When the fitting is performed on individual measurements before averaging the results, the found parameters differ at most $\sim$10% from the values presented here.

The validity of the model is supported by the independence of the obtained intrinsic energy barrier of the system $E_0$ on the applied force. For experiments carried out with a different force on a similarly prepared sample, the same $E_0$ is found: the applied force modifies the total barrier that the particles experience by an amount $F \cdot x_B$, which is included in the fits. Also the values of $\sigma$ do not show a dependence on the applied force for the same reason. The values found for $\sigma$ are in the range $\sim$5–10 $k_B T$, which is higher than just the thermal energy $k_B T$, showing that a real spread on the barrier is present. Furthermore, $\sigma$ is a significant part of $E_0$, but not too close to it, which means...
that the energy barrier distribution is still well defined: if $\sigma$ would be larger than $E_0$, the barrier would not be well defined anymore.

A clear trend can be observed showing a decreasing energy barrier height $E_0$ for increasing oxidation time, which means that particles are more easily pulled off an oxidized surface. The lowering of the energy barrier upon oxidation thereby gives a quantitative measure of the influence of oxidation on the non-specific interaction between protein-coated particles and a polystyrene surface: by increasing the oxidation time from 90 s to 300 s, the energy barrier decreases from $\sim 38 k_B T$ to $\sim 24 k_B T$. The specific biological bond that has one of the highest known affinities is the bond between avidin and biotin, which has a binding free energy of $\sim 35 k_B T$ at room temperature [25]. The specific bond between antibodies and their antigens is less strong and for one particular antibody the interaction energy with a number of antigens is found in the range $7 - 13 k_B T$ [108]. So we conclude that the non-specific interaction energy of the protein coated particles on the oxidized polystyrene surface fall in the same range as the combination of a small number of specific bonds between biological molecules.

The error ranges found for $\sigma$ are relatively large which shows that the pulling experiment and analysis method are not very sensitive to a change in $\sigma$. The width of the distribution $\sigma$ does not show a trend related to the oxidation, so apparently the distribution of the energy barrier values is not dependent on the surface treatment. The inhomogeneities causing the width of the distribution can be related to the surface as well as to the particles. Surface roughness is not expected to cause inhomogeneous interactions, since it has been shown in chapter 2 that the polystyrene surfaces are very flat and the influence of oxidation on surface roughness is limited. It is more likely that particle inhomogeneities cause the spread in the distribution by for example their surface roughness or an inhomogeneous distribution of proteins over the particle surface, in position and/or orientation.
4.3.5 Specific interactions

The applicability of the model presented here can also be investigated for specific bonds. In immunoassays the biosensor surface is often functionalized by adsorbing antibodies. This implies that the orientation of the antibody molecules is not well defined, leading to different reaction coordinates in pulling experiments and thereby to different effective energy barriers. Furthermore, adsorption of antibodies may also change their conformation, which is expected to influence the energy barrier for unbinding of antigens as well. Therefore, we study a number of dissociation curves of specific bonds as measured by Pérez Ruiz et al. [109] and characterize these by a distribution of energy barrier values.

The system consisted of M-270 particles, functionalized by EDC–NHS coupling with an antibody against Arah1 protein, which is a peanut allergen (similar to the functionalization with myoglobin described in section 3.1.2). The Arah1 protein was adsorbed onto polystyrene surfaces. For measuring dissociation curves, a protocol similar to the one described in section 4.2.1 was employed. Dissociation curves for a number of different forces are shown in figure 4.13. For each curve, 2 to 4 individual samples were measured. Fits with the distributed energy barrier model are included and the extracted parameters are shown in figure 4.14.

First, it is observed that some of the dissociation curves show discrete steps. This can be attributed to single particles coming off the surface. The low statistics due to the low number of particles in these curves leads to relatively large errors in the fits. The dissociation curves of the specific interaction of Arah1 protein and its antibody seem to be explained reasonably well by assuming a distributed energy barrier. The fitted parameters $E_0 \approx 20–25 \, k_B T$ and $\sigma \approx 4–8 \, k_B T$ are independent of the applied force, which confirms the validity of the model. At low forces the dynamic range of the measurement is relatively small, which probably causes the fit to be less unique and
therefore a large error for the $\sigma$-values is found. The specific interaction between the Aral1 protein, which is a trimer, and its antibody might have a different attempt frequency than the non-specific interaction between myoglobin and polystyrene. Therefore, choosing a different value for $\omega_0$ might improve the fits. It is also possible that $\omega_0$ or $x_\beta$ in this system have a spread in their values, which is reflected in a spread in $E_0$ in our fitting procedure.

The common method to explain dissociation curves of specific interactions is by assuming a single energy barrier or two bond populations with distinct energy barriers. This is a good method if these different bond populations can be confirmed in control experiments. However, when the populations cannot be demonstrated independently, a continuous distribution of energy barrier values may be present. To test whether a single energy barrier with accompanying spread $\sigma$ is a valid model for a biosensor surface that is functionalized with antibodies, an assay could be performed in which antibodies are bound to the surface in a well controlled specific orientation. It is expected that orientation of the antibodies leads to a small spread in their affinity and thereby a small $\sigma$ for the energy barrier distribution. The same assay with randomly oriented (adsorbed) antibodies is expected to give the same central energy barrier $E_0$, but a larger spread in affinities and thereby a larger $\sigma$.

### 4.4 Conclusions

We have studied the energy barriers for dissociation of myoglobin coated particles from oxidized polystyrene surfaces by applying a pulling force using magnetic tweezers. We first introduced a new calibration technique for the magnetic tweezers setup. Previous force calibrations required a detailed knowledge about the magnetic field gradients and magnetic particle properties or the methods could only be applied to single particles or a small number. Our time-of-flight calibration technique allows to measure the average magnetic force on a large ensemble of particles. Unbound magnetic particles were pulled away from a substrate and movies were recorded at focal planes at well defined heights above the sample bottom. From analysis of the number of particles in each frame of the movie the time-of-flight of the particles was deter-
mined, which was used to calculate the average particle velocity. Using Stokes law the velocity was translated into the magnetic force that was applied to the particles.

The magnetic force is linearly dependent on the current through the electromagnet coil and decreases for increasing distance between magnet tip and sample. Two different types of particles were used in calibration measurements and since both have different size and magnetic content, they have different maximum magnetic forces that could be applied to them (at minimum tip–sample distance and a maximum current of 1 A for the electromagnet): \( \sim 60 \text{ pN} \) for MagSense particles \((r = 500 \text{ nm})\) and \( \sim 70 \text{ pN} \) for M-270 particles \((r = 1.4 \mu\text{m})\). This calibration technique is generally applicable to determine forces on superparamagnetic particles in an electromagnetic tweezers setup as long as the particles are large enough to be detected in movie frames to determine their time-of-flight.

In the procedure to extract the time-of-flight from the movies, the influence of the spread in the particle properties is obscured by the optical analysis, for example the light intensity, threshold settings and the particle recognition algorithm. When all these parameters are carefully characterized and controlled, it might be possible to extract the spread in the particle properties (e.g. size or magnetic content) from the width of the curves. The coefficient of variance of the diameter of the M-270 particles used in the dissociation experiments is \(< 3\%\). Measurements on the paramagnetic moment of M-270 particles show a standard deviation of \((7 \pm 1)\%\), including both the variation in size and in magnetic content [110]. The variation in size together with a spread in the magnetic properties of the particles therefore will cause a variation in the force applied on the particles of not more than \(\sim 10\%\), which has only a minor influence on the height of the energy barrier for dissociation.

Dissociation measurements were performed on myoglobin coated particles bound to oxidized polystyrene surfaces. It was observed that for longer oxidation times, less particles bind before the pulling is started, which can be explained by the higher energy barrier for association that is found for increasing oxidation in chapter 3.

Based on the Bell–Evans formula describing the behavior of bonds under stress, we derived an equation to calculate the number of bonds as a function of pulling time for an arbitrary distribution of energy barrier values. Equations were derived for a gaussian distribution of energy barrier values and these were found to match the shape of the measured curves much better than the regularly used description based on distinct populations with unique energy barriers. Measured dissociation curves were fitted and the mean energy barrier height \(E_0\) as well as the width of the distribution \(\sigma\) were extracted from the data. The energy barrier height and spread were found to be independent of the applied force, which confirms the assumption in the model that the applied force only deforms the energy barrier.

A clear trend of decreasing energy barrier height with increasing oxidation was observed: for oxidation times from 90 s to 300 s the energy barrier decreases from \(\sim 38k_B T\) to \(\sim 24k_B T\). \(\sigma\) is found in the range \((\sim 7 \pm 4)k_B T\) for all measurements. Apparently oxidation of the surface decreases the non-specific interactions, which might be electrostatic or hydrophobic. The energy barrier for dissociation of the non-specific interaction of myoglobin coated particles on oxidized polystyrene is in the same range as the combination of a small number of single strong specific biological interactions (the strongest known being avidin–biotin with an energy barrier of \(35k_B T\)).
Figure 4.15: (a) Illustration of the energy barrier for dissociation $E_{h,\text{dissoc}}$ and its spread $\sigma$. (b) Energy barrier distribution calculated from the measurement for an oxidation time of 300 s at a force of 50 pN.

Figure 4.15 a schematic energy landscape is shown, in which the energy barrier for dissociation is indicated, as well as the spread on the barrier. In the time that the pulling force is applied, particles will cross energy barriers up to $\sim 32 k_B T$, allowing to extract the fraction of dissociated particles from the energy barrier distribution, as illustrated in figure 4.15 for a single curve.

Magnetic tweezers pulling experiments together with the distributed energy barrier model can be applied to get a better understanding of non-specific interactions between protein coated particles and polymer surfaces. When a gaussian distribution of energy barriers does not suffice to explain the measured data, also other energy barrier distributions can be included in the model. Furthermore, we propose that the distributed energy barrier model might be suited to also describe specific interactions between protein coated particles and surfaces. In our experiments the specific interaction between Arah1 protein and its antibody showed a lower energy barrier than the non-specific interaction between myoglobin coated particles and a polystyrene surface, but for applications in a biosensor these values should be compared in the same system. It is expected that in general specific and non-specific interactions in the same system will be distinguishable.

Magnetic tweezers experiments can be used to optimize the design of biosensors that are based on the use of magnetic particles. By varying the surface treatment, particle coating or solution properties (pH, ionic strength), the resulting energy barriers for dissociation and the width of the distribution can be determined and components with the desired properties can be implemented in the sensor design. Furthermore, dissociation by magnetic pulling might be applicable to discriminate specific and non-specific bonds to increase the biosensor specificity.

### 4.5 Acknowledgement

I would like to thank Asha Jacob for the calibration measurements of the M-270 particles. I would like to thank Elena Pérez Ruiz for the measurements on the specific interaction between Arah1 coated particles and antibody functionalized surfaces.
5 Single molecule force spectroscopy

In this chapter we investigate the non-specific interaction of a single protein and an (oxidized) polystyrene model surface. The work is relevant for biosensing because it can give insight into mechanisms associated to the loss of biomarkers that bind directly to the substrate. By detailed knowledge of protein–surface interactions, it might also be possible to provide insight in the non-specific binding of protein coated particles to the biosensor surface. In this chapter we investigate the interaction of a single myoglobin molecule to the polystyrene model surface with atomic force microscope (AFM) based force spectroscopy.

We first discuss the details of the method to measure force–distance curves with an AFM system and give a literature review on the characteristic events that are typically observed in force–distance curves. Then, we explain the details of the measurements we performed, together with the normalization of the force–distance curves that is required to quantitatively characterize the measured events. Finally, the observed events are discussed. Force–distance curves of control tips (without myoglobin) show mainly plateau events that can be attributed to the pulling of polystyrene chains out of the polystyrene substrate by the AFM tip. Myoglobin functionalized tips show much more spring-like events. These spring-like events can be attributed to the stretching and unfolding of single myoglobin molecules between the tip and the surface as well as to the spring-like behavior of polymer segments from the polystyrene surface. A manuscript is in preparation to publish these results.

5.1 Force-distance curves

AFM measurements are regularly used to scan surface topography, as described in section 2.2. An alternative purpose to use the AFM for is force spectroscopy. In force spectroscopy, the forces between the tip and the sample are measured at a given location on the sample surface [79]. In force–extension mode, the sample is moved away from the cantilever at a constant velocity while at the same time the restoring force of the molecule under study bends the cantilever [111]. The resulting force–distance curves can be analyzed to identify properties of the molecule under study. AFM has a height control and force control which are accurate enough to make the measurements limited by thermal fluctuations.

Force–distance curves are recorded by changing the distance between AFM probe and sample while at the same time measuring the applied force, as sketched in fig-
Figure 5.1: (a) Method to measure force–distance curves. (b) Retraction curve illustrating plateaus, the plateau forces are indicated. (c) Retraction curve illustrating a spring-like event, the length of the spring-like event is indicated and its associated unfolding force.

A measurement is started by first approaching the tip and the sample. The approach is continued until the force applied on the surface reaches a specified value, the so-called trigger force. The recording of the force as a function of approach distance gives the approach curve. After an optional waiting time on the surface, the cantilever is retracted while again recording force and height; this gives the retraction curve. In the first part of the retraction curve contact between the tip and surface is visible, followed by possible interactions and finally the tip is free from the surface.

When an AFM tip touches a surface and thereafter pulls away from the surface, it regularly occurs that the tip is not released from the surface at once but rather multiple steps occur in the interaction before the tip unbinds from the surface. Different characteristics of the events between the steps in the retraction curves are illustrated in figure 5.1. A plateau occurs if the force during the retraction between two steps stays constant (b). When the force increases with increasing distance, a spring-like event is detected (c). Both types of events are discussed in detail below.

5.1.1 Plateaus

Plateaus have been observed in force–distance retraction curves in different systems with peptides and polymers. In table 5.1 an overview is given of a number of these systems and the observed plateau forces. The materials system is described by the molecule that was bound to the tip and the material on the surface. Plateau forces were measured relative to the free state of the cantilever, which is the baseline of the curve (as in figure 5.1). Single plateaus are attributed to continuous desorption of single chains, which is also described as zipper-like disruption of single bonds or
Table 5.1: Overview of different systems in which plateaus have been observed in force–distance retraction curves.

<table>
<thead>
<tr>
<th>tip or colloid coating</th>
<th>surface coating</th>
<th>plateau force (pN)</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>xanthan (polysaccharide)</td>
<td>400</td>
<td>[50]</td>
</tr>
<tr>
<td>–</td>
<td>polyvinylamine</td>
<td>50–100</td>
<td>[113]</td>
</tr>
<tr>
<td>polyvinylamine</td>
<td>mica and calcite</td>
<td>50–100</td>
<td>[114]</td>
</tr>
<tr>
<td>spider silk peptide</td>
<td>hydrophobic diamond</td>
<td>58 and 116</td>
<td>[51]</td>
</tr>
<tr>
<td>synthetic peptide</td>
<td>SAM, PMMA, PTFE, Ti, glass</td>
<td>up to 120</td>
<td>[52]</td>
</tr>
<tr>
<td>positively charged</td>
<td>polymer</td>
<td>100</td>
<td>[53]</td>
</tr>
<tr>
<td>colloid, dermatan</td>
<td>sulfate</td>
<td>multiples of 15 and 50</td>
<td>[63]</td>
</tr>
<tr>
<td>sulfate</td>
<td>carboxymethylcellulose</td>
<td>75–300</td>
<td>[115]</td>
</tr>
<tr>
<td>(1 µm, 4.5 µm) silica</td>
<td>hydrophilic and hydrophobic</td>
<td>multiples of 52</td>
<td>[62]</td>
</tr>
</tbody>
</table>

peeling of the chains. Where multiple plateaus appear, these are ascribed to multiple chains that one after another detach in the same way as the single chains. In some cases interactions between the multiple chains was observed. The plateau forces are found to be dependent on the combination of materials on tip and surface.

The observed plateau forces in the case where polymers are pulled into a poor solvent can be quantitatively explained by the surface energy $\gamma$ and taking the stretched polymer chain to be a cylinder with radius $r$ [112]:

$$F_{plateau} = 2\pi r \gamma.$$  \hspace{1cm} (5.1)

Calculating the chain radius from the observed plateau force gives values of tenths of nanometers, which is on the order of molecular dimensions. Thormann et al. used this same formula to calculate the radius of polystyrene chains pulled from a colloid probe into a water environment to be 0.28 nm, using a surface energy of $\gamma = 30 \text{ mJ/m}^2$ (the macroscopic surface energy of the polystyrene–water interface) [62].

### 5.1.2 Spring-like events

Spring-like events have been observed in force–distance curves in systems where the interaction between single molecules and surfaces was studied. These spring-like events are usually attributed to the stretching of a molecule between the tip and the surface. Stretching of a molecule can only occur if it is bound both to the tip and to the surface. Stretching of polymers has for example been observed for polyvinylamine that was covalently bound to the AFM tip and the surface [113]. Also non-covalent attachment of polymers to silicon nitride tips and surfaces shows stretching of individual chains in a good solvent with unfolding forces up to a few hundred pN [112].

The extension of polystyrene molecules from a colloid probe into a poor solvent is also observed to have spring-like events on top of the plateaus, again with unfolding forces up to a few hundred pN [62].
Chapter 5: Single molecule force spectroscopy

The internal structure of protein molecules can be studied by binding single protein molecules between the tip and the surface and studying the observed unfolding events. Unfolding of protein molecules has been shown to lead to spring-like events in force–distance retraction curves with typical unfolding forces of a few hundred pN. This research field has been extensively reviewed, for example in [60] and [59]. Measurements have been mainly applied to polyproteins which consist of a large number of identical proteins in a chain that will unfold in an identical way one after the other [111] or to proteins with many identical subunits. The spring-like extension events can be characterized by relating the peak unfolding force of the event to the distance between two consecutive events. For these repetitive systems the combination of peak unfolding force and event length allows to identify the segment of the protein that unfolds.

To describe single spring-like extension events in more detail, different models can be used. The most used models are the freely jointed chain model and the wormlike chain model which are both limits of the Kratky–Porod model [116]. In the Kratky–Porod model, the polymer chain is described as consisting of \( N \) segments of length \( b \) with orientation \( \vec{t}_i \), leading to a total length \( l_0 = Nb \), which is often referred to as the contour length of the polymer. Angular correlation between the segments is assumed to decay exponentially along the chain on a typical length scale, called the persistence length \( \xi \).

In the freely jointed chain model, the polymer is treated as a chain of rigid subunits of length \( b = 2\xi \) that do not have any angular correlation. This gives rise to entropic elastic behavior, which is expressed in the force–extension relation:

\[
\frac{\langle z \rangle}{l_0} = \coth \left( \frac{Fb}{k_BT} \right) - \frac{k_BT}{Fb},
\]

(5.2)

with \( \langle z \rangle \) the mean extension of the chain and \( F \) the applied force.

In the wormlike chain model the polymer is described as an inextensible continuum elastic body, which only includes the bending energy. It is a continuous version of the Kratky–Porod model \((b \to 0)\) and can not be analytically solved. An approximate interpolation function is given in [117]:

\[
\frac{F \xi}{k_BT} = \frac{\langle z \rangle}{l_0} - \frac{1}{4} + \frac{1}{4 \left(1 - \frac{\omega}{\bar{\omega}}\right)^2}.
\]

(5.3)

When a protein is not (covalently) bound between the tip and the surface, but only to the tip, apart from the internal structure of the protein, also interactions with the surface can be studied by force–distance curve analysis. It has been observed that multiple proteins on an AFM tip that non-specifically interact with a surface also show spring-like events, particularly on hydrophobic surfaces [48], but the events could not be quantitatively interpreted.

In this chapter we study the non-specific interaction of single myoglobin molecules on a polystyrene surface. To the best of our knowledge, this is the first time that detailed studies on the structure of myoglobin and its non-specific interactions were performed by force–distance curve analysis. The combination of unfolding force and the length of the spring-like events will be studied (defined as indicated in figure 5.1).
The myoglobin molecule does not have a repetitive structure and the way the molecule binds to the surface might not be identical for each approach. However, it can be investigated whether in the analysis of a large number of retraction curves some features do recur that could be related to a certain orientation or interaction (e.g. related to the length of the α helices in the molecule). Also wormlike chain fitting of the spring-like events will be performed.

5.2 Materials and methods

The interaction between myoglobin located on an AFM tip and (oxidized) polystyrene was studied by recording force–distance curves on polystyrene surfaces. We first introduce the details of the preparation of the model surfaces and the functionalized AFM tips. Then, the AFM system used for recording the force–distance curves is explained, followed by the method to normalize the measured force–distance curves to be able to quantitatively interpret the observed events.

5.2.1 Surface and tip functionalization

The polystyrene model surfaces were spincoated from toluene onto a glass coverslip as described in section 2.1. By placing an aluminum mask on the surfaces and exposing them to UV/ozone, the uncovered part was oxidized for 600 seconds. The surfaces obtained in this way could be used to measure with a single tip the interactions on non-oxidized as well as on oxidized polystyrene without the need to replace the sample.

Silanization is commonly used to improve adhesion of polystyrene films to glass, for example by Bevan et al. [118] who use silanization with trimethylchlorosilane before they spincoat polystyrene from toluene. We also used a silanization treatment to promote the attachment of the polystyrene on the glass substrate. After cleaning, the glass coverslips were immersed in 0.5 wt% dichlorodimethylsilane (Sigma Aldrich) in dichloromethane (Merck, analytical grade) for one minute. Then, they were washed in acetone (and optionally ethanol). Finally, the coverslips were glued onto a glass microscope slide for ease of use.

The AFM tip was cleaned by washing in chloroform. Subsequently, it was immersed in ethanolamine (Sigma Aldrich, ReagentPlus grade) dissolved in DMSO (Merck, for synthesis, volume ethanolamine:DMSO=3.1:10) overnight to create amine groups on the silicon nitride surface. Afterwards, it was washed in DMSO and ethanol, and dried with N₂. To allow binding of the protein, the tip was incubated in glutaraldehyde (Calbiochem, 25% aqueous solution) in Millipore water, by placing a droplet on top for 15 minutes. Subsequently, the tip was washed in Millipore water. Incubation of the protein was carried out by placing a droplet on the tip containing 25 µL 200–300 ng/µL myoglobin (Calbiochem; myoglobin, human, recombinant, E. Coli) in PBS for 15 minutes. Alternatively, the tip was terminated with ethanolamine to serve as control.

The number of proteins per tip was controlled by choosing a procedure that gives a low density of amino groups on the tip surface. Esterification with ethanolamine has been shown to give rise to 2021±205 active sites/µm² [119]. As illustrated in figure 5.2 the nominal tip radius of the used tips was 20 nm, so assuming possible interaction up to 2.5 nm from the surface gives an interacting surface area of 314 nm². Assuming that one amine group binds one protein molecule gives 0.63 ± 0.06 molecules per tip.
In most experiments therefore 0 or 1 molecule will be present on the tip, so single molecule experiments can be performed.

5.2.2 AFM system

The components of an AFM system are illustrated in figure 2.1 and the most important distances in the system are indicated in figure 5.3 (a). When force is applied on the tip, the cantilever bends and therefore the force can be calculated from the deflection of the cantilever. The cantilever deflection is deduced from the position of the laser spot on the photodetector and the deflection sensitivity of the detector. The deflection of the cantilever together with its spring constant allows to calculate the applied force from Hooke’s law \( F = -k \cdot x \), with \( k \) the spring constant), which is valid for small deflections of the cantilever.

The cantilever spring constant is calibrated by measuring the cantilever’s mechanical response to thermal noise in ambient air. The resonance frequency of the cantilever is dependent on the spring constant. By assuming a single degree of freedom and using the energy equipartition theorem, the spring constant can be calculated. In our measurements, the cantilever’s spring constants were typically 0.01–1 N/m.

Before using a new tip, its deflection sensitivity and spring constant were calibrated. The force–distance curves were recorded at a speed of 100 nm/s and the probe was moved down and up for 200–250 nm. The trigger force was typically 500 pN. Away from the surface as well as on the surface, the waiting time was chosen to be 1 s and typically 1000 force–distance curves were recorded in a single measurement series. When analyzing the data, it turned out that in some cases the deflection sensitivity changed throughout the measurements. This can for example be caused by slight movement of the probe in the course of the measurements. Therefore, a fit of the first 5% of the retraction curve data was used to determine the deflection sensitivity for each individual curve.

All measurements were carried out with a Bruker BioScope Catalyst AFM system on a Leica DMI4000B inverted microscope. This combination allows to combine AFM measurements with optical detection. Using optical detection the position of the tips was determined above the partly oxidized samples and they were moved to the desired location, either non-oxidized or oxidized for 600 s. Furthermore, the system is designed to work with liquid so as to allow to study proteins in their native environment.
5.2.3 Retraction curve normalization

Information about the bond formed between the tip and the surface can be deduced from the dissociation process, which is measured by the force–distance retraction curve. In all retraction curves two different interaction regimes can be identified. First, at short distances the tip is pushed onto the surface and since the surface is rigid, the force is determined by the movement of the piezo and the cantilever spring constant. Second, at large distances the tip is free from the surface, so no force is applied.

An example of a retraction curve is shown in figure 5.3 (b). The measured force is plotted as a function of the height of the piezo actuator. The second interaction regime where the tip is free from the surface is used to define the force baseline at 0 pN. The force is defined to be positive when the tip is pushed onto the surface and thereby bends upward. Binding of the tip to the surface causes the cantilever to bend down when the sample is moved away, which results in a negative force in the force-extension curves. The first interaction regime where the tip is in contact with the surface can be seen in the first part of the curve, up to ~370 nm. This surface contact region is used to define the surface at 0 nm height: the intersection of the 0 pN baseline and a linear fit of the surface contact region determine the origin, resulting in the curve shown in figure 5.3 (c).

The processes occurring during the unfolding of a molecule positioned between the tip and the surface can be characterized by measuring the tip–sample separation, which gives more insight than the probe height. The actual distance between the AFM tip and the sample can be calculated from the probe height and the cantilever deflection $d_{\text{cantilever}}$:

$$\text{separation} = \text{probe height} + d_{\text{cantilever}}, \quad (5.4)$$

as shown figure 5.3 (a). The cantilever deflection is calculated from the cantilever spring constant $k$:

$$d_{\text{cantilever}} = \frac{\text{deflection force}}{k}. \quad (5.5)$$

The transformed retraction curve as a function of tip–sample separation is shown in figure 5.3 (d).

A parameter that can be directly extracted from the retraction curve is the adhesion force, which is defined as the absolute maximum observed force between tip and sample. In figure 5.3 (c) and (d) it thus is the deepest point of the curve. The adhesion force can be used to quantify the interaction between the tip and the sample. For example, the interaction between an AFM tip coated with a number of different blood plasma proteins has been studied on differently functionalized surfaces and the adhesion force was found to depend on both the choice of protein and surface [47]. The adhesion force can only be a measure of the protein–surface interactions when the tip is completely covered by the protein to shield the tip–surface interactions. In our measurements the average protein coverage of the tip is of the order of one, so the adhesion force will be a combination of protein–surface and tip–surface interactions. Therefore the adhesion force can not be directly used to quantify the protein–surface interaction. As we will see, other features like plateaus and spring-like events (as illustrated in figure 5.1) will bear information on the protein–surface interaction.
5.3 Measurement results

The interaction of myoglobin functionalized tips with polystyrene surfaces has been studied in PBS buffer. Two different treatments of the polystyrene were employed: the surfaces were partly non-oxidized and partly oxidized for 600 s. In addition to the myoglobin functionalized tips, also control tips were used: the first type of control tip was a bare SiN-tip, not treated before use and the second type of control tip was terminated with ethanolamine instead of binding myoglobin. It is important to note that even in the case where myoglobin was incubated on the tip, it is not guaranteed that indeed a myoglobin molecule was present on the part of the tip interacting with the surface.

Different types of interactions can be observed in the measured force–distance retraction curves. When there is no attractive interaction between the tip and the surface, the retraction curve does not show a negative force as in figure 5.3, but rather immediately returns to the free state (baseline) at the moment the tip is no longer pushed down onto the surface. The most straightforward attractive interaction between tip and surface can be seen in the retraction curve in figure 5.3: a single step in which the tip unbinds. When multiple steps occur before unbinding of the tip from the surface, the
Figure 5.4: Overview of all measurement series performed with control tips and myoglobin functionalized tips (Mb) on polystyrene surfaces that were not oxidized (PS) or oxidized by 600 s UV/ozone treatment (PS 600 s). The bare tip was made of silicon nitride and control tips were terminated by ethanolamine. The fraction of the total measured curves that shows interaction is indicated by the total length of the bars, while the occurrence of plateaus and spring-like events is indicated by the colors as shown in the legend. ‘both’ indicates that in the same curve at least one plateau and one spring-like event occur. The gray parts of the bars signify curves with interaction, but without events, so they show just a single step in unbinding.

Events that happen in between can be classified to be ‘plateau’ or ‘spring-like’ events as has been discussed in section 5.1.

In figure 5.4 an overview is given of all measurements that will be referred to in the rest of this chapter. All measured curves (typically ∼1000 per tip) were baseline corrected and the surface location was determined as described in section 5.2.3. Per curve it was determined whether any adhesive interaction was present by identifying steps in the retraction curves. For the curves that did show interaction, it was determined in what fraction of the curves, plateaus or spring-like events were observed. For this purpose, the data between two consecutive steps (if present) was analyzed. An event was only counted when a minimum event length of 1 nm between the steps was met. The standard deviation of the event data was compared to the standard deviation of the data of the baseline: an event was designated to be a plateau if its standard deviation was lower than 1.2 times the standard deviation of the baseline. When the standard deviation was larger, the event was designated to be spring-like. No dependence of the number of detected events in a single curve on the position of the measurement in the series was observed.

The fraction of curves with events that do show both at least one plateau and one spring-like event are indicated in figure 5.4 by ‘both’. It can be observed that only in few cases both types of events occur in the same curves; typically only a few % (with a maximum of 18 %) of the curves with events shows both at least one plateau and one spring-like event. Probably, when the retraction starts, one type of interaction mechanism is dominant. Apparently, plateaus and spring-like events belong to differ-
ent interaction mechanisms that are not likely to occur in a single retraction curve.

5.3.1 Control tips: polystyrene interaction

Since on the control tips no myoglobin is present, all interactions observed in these measurements should be attributed to the interaction between the ethanolamine terminated tip and the polystyrene surface. Comparing the interactions on non-oxidized polystyrene with the interactions on oxidized polystyrene, it can be seen that oxidation reduces the interactions by a large amount: only in 3–4% of the curves recorded on oxidized polystyrene interactions were observed, compared to 74–99% of the curves on non-oxidized polystyrene. Clearly, very little adhesive interaction is present between control tips and oxidized surfaces. For the bare silicon nitride tip on non-oxidized polystyrene the amount of observed interaction is comparable to the control tips. However, almost all interaction occurs without events, indicating that the interaction between a silicon nitride tip and polystyrene has a different character compared to an ethanolamine terminated tip.

When only the curves recorded on non-oxidized polystyrene are considered, for the control tips ∼35–63% of the curves which show events upon interaction, display plateaus. The observation of events indicates that the interaction between the tip and the polystyrene chain can be stronger than the mutual interaction between polystyrene chains possibly caused by a difference in the van der Waals interactions. The plateaus can be characterized by their mean forces. A typical curve with two plateaus is shown in figure 5.1. For all observed plateaus for the two control tips the forces were combined in the histograms in figure 5.5. The spring-like events that were observed for the control tips on non-oxidized polystyrene result from the stretching of polystyrene chains and will be discussed in the next section.

In our measurements of the control tips on non-oxidized polystyrene, most of the mean plateau forces are found to be around 100 pN and 200 pN. The interactions occurring in our system can probably be best compared to the polystyrene colloid probe

Figure 5.5: Histograms showing the mean forces of the force plateaus in the force–distance retraction curves of (a) control tip 1 with trigger force 450 pN and (b) control tip 2 with trigger force 300 pN. The insets show the complete force range.
on a silica surface [62]. In that report, the unbinding force was attributed to single polystyrene chains being pulled from the particle into the water environment. In our case polystyrene chains are being pulled from the surface by the AFM tip. Since water is a poor solvent for polystyrene, the observed forces will be given by the surface energy of the polystyrene–water interface (γ = 30 mJ/m²). When applying equation 5.1 and the plateau force of 100 pN found in our system, the radius of the polystyrene chains is calculated to be 0.53 nm, which corresponds to ~3–4 C–C bonds. For polystyrene colloid probe interactions on a surface a radius of the extended polystyrene chains of 0.28 nm was found [62], which is about half of our value. This difference might indicate that in our system the polystyrene chains are picked up not at their ends but somewhere in the middle, as is illustrated in figure 5.6. This will cause double chains of polystyrene to be pulled from the surface and the surface energy for two chains will be twice the surface energy for a single chain, resulting in an effective radius that is twice the radius of a single chain.

At maximum two clearly distinguishable mean plateau forces (at 100 pN and 200 pN) were observed between the control tips and the polystyrene surface. For the polystyrene colloid probe and the silica surface up to 5 distinguishable plateaus were observed. This difference can be understood from the fact that the colloid probe will have a much larger interacting area on the surface compared to the AFM tip which has a radius of 20 nm, so in an AFM experiment it is less likely that multiple polystyrene chains bind at the same time.

### 5.3.2 Myoglobin functionalized tips

Myoglobin functionalized tips show tip–surface interaction in 1–12% of the curves on oxidized polystyrene and in at least 44% of the curves on non-oxidized polystyrene. Oxidation thus reduces the interactions between the tip and the surface. This is in agreement with the fact that proteins in general show less interaction with hydrophilic surfaces, as discussed in section 1.6. It is also consistent with the observation for the control tips that oxidation of the surface reduces the interactions.

The types of events occurring for the myoglobin tips on non-oxidized polystyrene are mostly spring-like (48–94% of all curves with events). When comparing the fraction of curves with plateau events, it is noticed that 3 out of 4 myoglobin tips show plateaus in 6–16% of the curves with events, which is much lower than for the control tips (35–63%). The myoglobin tip denoted by ‘Mb 3’ however, shows plateaus in 52% of the curves with events. Probably this indicates that after the functionalization no
myoglobin is present at a position on the tip where it can interact with the surface and therefore characteristics similar to those of the control tips were found.

Plateaus observed in retraction curves of myoglobin tips are found to have forces around 100 pN, which is identical to the plateau force for the control tips. This indicates that the plateaus observed for the myoglobin functionalized tips are probably of the same origin as the plateaus observed for the control tips. Since plateau events occur much less for the myoglobin functionalized tips than for the control tips, the presence of a myoglobin molecule probably inhibits the binding of polystyrene chains to the tip, thereby preventing the regular occurrence of polystyrene chains being pulled out of the surface.

Spring-like events occur more frequently for myoglobin tips than for control tips (84–94% compared to 37–65%). This indicates that a large part of the spring-like events will be related to the unfolding and stretching of myoglobin. Considering the possible stretching of the myoglobin molecule, its total length determines the maximum tip–surface separation over which interactions can be expected. The total length of the myoglobin molecule is $\sim 61$ nm (0.4 nm per amino acid [120]), which means that interactions with a single myoglobin molecule can not extend further than 61 nm. The maximum interaction distance can only be reached if the myoglobin is bound exactly at its end to the AFM tip and the other end to the surface. Note that the covalent bond between the tip and the myoglobin is formed between one of the 32 amine groups distributed over the molecule and the glutaraldehyde on the tip. Therefore, a distribution is expected of maximum interaction distances with a maximum of 61 nm.

For spring-like events with a maximum length of 60 nm a scatter plot of the event length and the peak unfolding force is shown in figure 5.7 for all tips. It can be seen that for this maximum interaction length most unfolding events occur at forces of 200–400 pN, which is the typical range of forces associated with intraprotein interactions [59].

The majority of the events is found in the first bin of the histograms in figure 5.7, counting events with spring lengths up to 10 nm. We attribute these events to interactions of the myoglobin molecule. Two different types of unfolding of the myoglobin molecule can be envisaged as illustrated in figure 5.8: internal segments of the molecule can unfold or protein segments may be ruptured from the surface. In both cases, upon release of the segment (indicated by the jump in the force–distance retraction curve) the released part will be stretched. At the end of the spring-like event (where the maximum force is reached), the tension is released and a next part of the myoglobin molecule can unfold or be released from the surface. The observation of spring-like events in subsequent retraction curves shows that the myoglobin molecule in each curve has internal structure or structure on the surface. However, it is not clear whether this is its native structure.

As introduced in section 1.4, the myoglobin molecule has a size of $\sim 4.5 \times 3.5 \times 2.5$ nm$^3$. About 70% of the myoglobin molecule has a secondary structure of $\alpha$ helices with turns and loops in between the helices. A spring length of more than a few nm therefore must correspond to a number of $\alpha$ helices that are stretched at the same time. Also for the events observed for the control tips the majority is found for spring lengths up to 10 nm, so the spring length as a parameter can not be used to discriminate between myoglobin spring-like events and polystyrene spring-like events.
Figure 5.7: Scatter plots of the event length and peak unfolding force of spring-like events. The insets show histograms of the event lengths up to 100 nm (bar width 10 nm).
The shapes of the spring-like events observed for the control tips show a noticeable difference when compared to spring-like events for a myoglobin functionalized tip. A few typical example events are shown in figure 5.9. Wormlike chain fitting allows to describe spring-like events in protein force–distance curves in terms of the total length of the spring that is stretched, as introduced in section 5.1, by using equation 5.3. We chose the persistence length $\xi = 1$ nm, which is a value found for many non-globular proteins [121]. This value is an indication for the rigidity of a chain of amino acids, and is on the order of a few amino acids. Therefore, we also apply it to the globular myoglobin. 1 nm is also the persistence length of atactic polystyrene [122]. The contour length is the asymptote of the length of the spring and therefore its value is restricted to be larger than the maximum separation observed in the event.

The wormlike chain fits to the spring-like events in the example curves are included in figure 5.9. The myoglobin spring-like events can be much better described by the wormlike chain model than the polystyrene-related events, which strengthens the interpretation that the observed events are caused by stretching of the myoglobin molecule. The almost linear behavior of the polystyrene spring-like events suggests that the polystyrene chains behave more like Hookean springs. Now we focus on further interpretation of the myoglobin events.

It is interesting to note that the measured shape of the myoglobin spring-like events approach a force plateau for shorter distances, after which the extension starts. Force plateaus have also been observed for unstructured peptides that are pulled from a surface into a solvent [51, 52]. Two effects take place when myoglobin unfolds: first, the internal (hydrophobic) structure is exposed to water, and second, a mechanical spring-like extension occurs. We could model the force plateau resulting from the exposure of hydrophobic parts of the molecule by including a baseline in the wormlike chain fit:

$$F = \frac{k_B T}{\xi} \left( \frac{\langle z \rangle}{l_0} - \frac{1}{4} + \frac{1}{4 \left( 1 - \frac{\langle z \rangle}{l_0} \right)^2} \right) + \text{baseline.}$$

(5.6)

In figure 5.10 myoglobin related spring-like events are fitted by the wormlike chain model with a free baseline. Inclusion of a baseline only has a minor effect on the fitted contour lengths, but for the baseline itself typical values are found below 100 pN, for the curves in figure 5.10 around −25 pN. This plateau force is lower than the 100 pN plateau force for polystyrene extension (section 5.3.1), which can be understood from
the fact that the protein chain will be less hydrophobic than the polystyrene chain, leading to a lower surface energy.

To be able to further characterize the myoglobin-related spring-like events, the interfering spring-like events caused by the polystyrene should be prevented. Since the polystyrene chains are pulled out of the surface by the AFM tip, an option to reach this goal in the future may be to include a linker between the myoglobin molecule and the tip which is longer than the range over which the polystyrene–tip interaction occurs.

## 5.4 Conclusions

The interactions between myoglobin functionalized AFM tips and polystyrene surfaces were studied by analyzing force–distance retraction curves. The polystyrene surfaces were partly oxidized and also control tips (without myoglobin) were studied. For the control tips as well as for the functionalized tips on 600 s oxidized polystyrene much less interaction was observed than on the non-oxidized polystyrene. The interactions that occur on the non-oxidized polystyrene can have different manifestations in the force distance curves: we have observed simple attractions with a single step in which the cantilever comes loose from the surface, and we have observed multiple
steps occurring in the unbinding process of the tip from the surface. The events occurring between the steps can have two different shapes: plateau or spring-like. In the plateaus the force is constant while the distance between tip and surface increases and for the spring-like events the force increases for increasing distance between the tip and the sample.

In the measurements of the control tips many more plateaus are observed than for the myoglobin functionalized tips. We attribute the plateaus to polystyrene chains that are pulled from the surface into the water environment. In the measurements of the myoglobin tips more spring-like events were observed than for the control tips. Most spring-like events have a length and peak force that match the values typically found for protein unfolding or protein dissociation measurements. However, the length and peak force found for the events measured for polystyrene extension using control tips fall in the same range.

The shape of the spring-like events induced by myoglobin is different from the shape of the spring-like polystyrene events. The myoglobin events can be better described by the wormlike chain model. Typical contour lengths of a few tens of nm’s are observed, which corresponds to the total length of the myoglobin molecule of 60 nm. To be able to independently study the interactions and unfolding of myoglobin on the polystyrene surface the materials system could be changed, for example by including a linker between the AFM tip and the myoglobin molecule which is longer than the maximum interaction length of the polystyrene.

The force–distance measurements presented in this chapter have revealed that unfolding events can occur in the non-specific interaction between a protein molecule and a polymer surface. This finding has impact for follow-up research on particle based biosensors. We expect that protein unfolding may play a role when protein coated particles interact with a polymer surface, thereby influencing the non-specific association and dissociation of the particles. In future work, it will be interesting to perform force–distance studies of particle-surface interactions, to see if we can quantify the role of protein unfolding in these interactions. In a biosensor, protein unfolding can probably also occur during the surface biofunctionalization process, when antibodies are bound to the biosensor surface. Unfolding may reduce the antibody activity and generate a surface with a spread of antibody activities. Thus it will be very

Figure 5.10: Spring-like events measured with tip Mb 4, identical to figure 5.9, now fitted with a free baseline. Fitted contour lengths and baselines are indicated.
interesting to map the activity of antibodies by probing the biosensor surface with a biomarker-functionalized AFM tip while recording force–distance curves at different positions.

5.5 Acknowledgement

I would like to thank Emiel Visser for the numerous force–distance measurements that he performed during his graduation project.
The work in this thesis focussed on non-specific protein-surface interactions in the context of particle based biosensors. Biosensors are devices to measure biomarkers in small amounts of complex fluids and the generally low concentration of biomarkers requires sensitive and specific detection. An immunoassay with antibody coated particle labels can be used as a basis to design such a biosensor. The use of magnetic particles as labels allows actuation of the particles by magnetic fields, to accelerate the transport of the biomarker molecules to increase the speed of the assay and to separate bound and unbound particles by magnetic forces to increase the specificity.

Both the sensitivity and the specificity of the biosensor are to a large part determined by non-specific interactions. Three processes governed by non-specific interactions can be identified that are crucial for the functioning of the biosensor.

- A first process is the functionalization of the biosensor surface and the particles with specific bioreceptor molecules, like antibody proteins. Antibodies can be immobilized (in part or in whole) by physicochemical adsorption or bound by for example EDC–NHS coupling. The sensitivity of the biosensor is amongst others determined by the amount of the specific bioreceptor molecules, by their orientation on the surface and by their conformation.

- A second non-specific interaction that can affect the sensitivity of the biosensor is the binding of biomarker molecules on surfaces in the biosensing device that are not the biosensor surface itself, like channels. These biomarkers will be lost for detection.

- A third process that limits the specificity of the biosensor is the background signal caused by non-specific binding of particle labels on the biosensor surface without the presence of a biomarker.

In this chapter we combine the results of the different experimental approaches employed in this thesis to give an outlook on future experiments and the implementation in the design of biosensors.

6.1 Conclusions

In this thesis, a model biosensor surface consisting of spincoated polystyrene was investigated. In chapter 2 we described the procedure and we characterized the properties of these surfaces. The surfaces were found to be flat on the scale of protein
dimensions and UV/ozone treatment was used to increase the oxygen content of the surfaces in a well-controlled manner. An increasing oxidation time was demonstrated to give an increasing hydrophilicity of the surface, which is known to have a distinct influence on the interaction between proteins and the surface.

To study the association and dissociation of non-specifically bound magnetic particles on the model biosensor surfaces, we used rotating particles probe measurements (chapters 3) and magnetic tweezers dissociation measurements (chapter 4). The magnetic particles were functionalized with the biomarker protein myoglobin. The total energy barriers for association and dissociation are formed by the combined action of electrostatic interactions, van der Waals interactions, magnetic interactions, gravity, hydrophobic interactions and possibly other interactions, like steric interactions. An important finding was that for association as well as for dissociation the measurements could be explained by a distribution of energy barrier values. In figure 6.1 we illustrate the energy barrier diagram with spread $\sigma$.

In the rotating particles probe measurements bound particles were discriminated from unbound particles by a rotating magnetic field. The fraction of rotating particles was recorded as a function of ionic strength and the shape of these curves could be explained by a distributed energy barrier model. The energy barrier was parameterized to be inversely dependent on the ionic strength of the solution. A consistent picture was obtained for a wide range of fluid conditions and surface conditions: increasing ionic strength decreases the energy barrier, while the energy barrier increases for increasing pH and oxidation time of the polystyrene surface.

Dissociation was studied using magnetic tweezers to pull protein coated particles from oxidized polystyrene surfaces. A time-of-flight calibration technique was developed that allows to measure the average magnetic force on a large ensemble of particles. This calibration technique is generally applicable for magnetic tweezers experiments on particles that are large enough to be observed in a microscope. Dissociation curves were recorded that show the number of bound particles as a function of time under a constant applied force. The dissociation curves could be conveniently described by extending the Bell–Evans formula for specific interactions by including a (gaussian) distribution of energy barrier values to describe the non-specific interactions. For increasing oxidation of the polystyrene surface, a decreasing energy barrier
height was present for the dissociation of the myoglobin coated particles from the surface. The distributed energy barrier model was also shown to be applicable to dissociation measurements of a specific protein-antibody interaction.

In chapter 5 AFM force spectroscopy measurements were used to study the interaction of single myoglobin molecules on the polystyrene model biosensor surface. Much more interaction was observed on non-oxidized polystyrene than on 600 s oxidized polystyrene, which is in agreement with the higher energy barrier for association that has been observed for increasing oxidation in the rotating particles probe measurements. Interactions between the polystyrene and the myoglobin on the AFM tip as well as the tip itself were observed. In many cases, the dissociation occurred in steps, which means that macromolecular structures need to be included in the interactions of the surface and the protein, going beyond a continuum description such as in DLVO calculations. Plateaus in the measured force–distance retraction curves were attributed to polystyrene chains that were pulled from the surface into the solution. Spring-like events were observed for extension of polystyrene chains as well as for the myoglobin molecules and these events could be discriminated by their shape. The single myoglobin molecule stretching events could be conveniently described by the wormlike chain model.

6.2 Functionalization of the biosensor surface

To get more insight in the functionalization of the biosensor surface with specific binding molecules, we employed magnetic tweezers dissociation measurements. It was shown that dissociation of specific interactions can be described by a distributed energy barrier, which explains the curves much better than a single exponential decay from a single energy barrier in the Bell–Evans model. If distinct populations of bonds can not be identified, we demonstrated that a distribution of energy barriers offers an interpretable and testable view on the system. The approach is suitable to address further questions, e.g. on molecular orientation. For example, if a functionalization leads to orientation of the molecules on the surface, a high affinity to the biomarker and a narrow energy barrier distribution may be measured. Other functionalizations, like physical adsorption, give a random orientation and therefore a broad distribution can be expected. Differently functionalized surfaces with a controlled variation of the orientation of the molecules can now be characterized by their spread in energy barriers and in this way, the homogeneity of the surface functionalization can be investigated.

As a variation to changing the surface functionalization, also the treatment of the particle labels can be changed. In this thesis we use EDC–NHS coupling of the protein to the particles, which causes a random orientation of the protein molecules. If a particle functionalization would be used that orients the molecules on the surface, it is expected that the dissociation energy barrier distribution of specific interactions will be narrow, just like for the surface functionalization. By changing both the surface and particle functionalizations, it can be discriminated whether the observed spread in the energy barrier is caused by inhomogeneities on the particle or on the surface.
Chapter 6: Outlook

6.3 Binding of biomarker molecules

The non-specific binding of biomarker molecules to channels and other surfaces in the biosensing device can be prevented by oxidation of the polymer surface, as has been demonstrated by the high barrier for association, the low barrier for dissociation and the low interaction in AFM force spectroscopy for the binding of myoglobin and myoglobin coated particles on oxidized polystyrene. Upon oxidation, also the interaction with control particles has been shown to be reduced by a high barrier for association and the oxidation also prevents the pulling of polymer chains from the surface, which might be explained by an increase of repulsive electrostatic interactions.

In AFM measurements, polymer extension and myoglobin extension are observed in the same experiment. Although the data suggests that polystyrene only binds to the AFM tip where no myoglobin is present, it can not be excluded that the polystyrene can also bind via the myoglobin. To investigate whether the pulling of polymer chains from the surface also plays a role in a biosensor, colloid probe AFM could be applied. Analysis of the spring-like events in the force–distance retraction curves allows to discriminate polystyrene extension from myoglobin extension and thereby possible polystyrene extension can be identified.

6.4 Binding of particle labels

Most information in this thesis was obtained on the non-specific binding of particle labels on the biosensor surface. We found that these interactions are governed by distributions of energy barriers and the energy barriers are found to shift as a function of the surface treatment and solution properties. This has allowed us to quantify and characterize variations of the system in terms of the energy barriers for association and dissociation.

To determine the energy barrier for association of particle labels, in the rotating particles probe measurements a variation of ionic strength of the solution was employed. A parameterization of the energy barrier as a function of ionic strength is needed to extract the energy barrier value for a certain system. When different systems are used than those in this thesis, it might be appropriate to change the energy barrier parameterization. The parameterization should include the dominant interactions between the particle and the surface that occur in the measurements and should describe the dependence on ionic strength of the resulting energy barrier.

As mentioned, the distributed energy barrier model for dissociation can be used to quantify the energy barrier and the accompanying spread for both non-specific and specific interactions. The model can be extended to study both interactions in the same experimental system. When two different bonds are present in the system, the energy barrier distribution for dissociation can be chosen as the combination of two gaussian distributions. We then expect that specific interactions will give a narrower gaussian curve while non-specific interactions will give a broader gaussian curve. Identification of these contributions may allow one to independently study the non-specific and specific interactions in different experimental systems. For application in a biosensor, knowledge of the energy barrier distributions in the chosen materials system allows to calculate the effect of actuation on the populations of specifically and non-specifically bound particles. Ideally, an actuation force can be chosen to minimize
the non-specifically bound particles while keeping as many as possible of the specific bonds intact.

Optimization of the surface and particle functionalization to give narrow distributions might help to generate a large difference between specific and non-specific interactions. Sharp energy barrier distributions might also be used to separate different types of specific bonds, e.g. in multiplexed assays in which a number of different antibodies are coated on the surface.

The polymer surfaces that are used in commercial biosensors may in some cases be quite rough in view of our measurements on commercially produced polystyrene in the form of cover slips, petri dishes and wellplates. Therefore it may be interesting to study the dependence of non-specific interactions on the surface roughness. It is possible to systematically investigate the influence of roughness on the non-specific binding of particle labels by their energy barriers in association and dissociation measurements. Colloid probe AFM measurements on surfaces with controlled nanoscale roughness (in an idealized system with identical asperities) have shown that the asperity density relative to the particle size determines the adhesion [123]. Also calculations have been performed on the energy barrier for association as a function of asperity radius, asperity density and particle radius. From the energy barriers deposition rates were calculated and compared to experiments [124]. We think that it will be very interesting to apply the rotating particles probe measurements also in non-ideal systems, in order to directly measure the energy barrier for association.

Although polymer oxidation can effectively prevent protein and particle binding, this is not applicable in all biosensor designs. As an alternative to oxidation, for example polyethylene glycol surface chemistries have been studied that cover the entire surface with polymer chains [31]. Non-biofouling surface coatings for use in biosensors are also commercially being developed, like VitroStealth [125]. To combine the non-biofouling properties with specific binding on the biosensor surface, PolyAn developed a three-dimensional matrix to which functional groups are coupled [126]. The non-specific interaction of particle labels on these surface coatings can be quantified by association and dissociation measurements. By using the proposed double gaussian distribution for the functionalized surfaces, the specific and non-specific interactions might be characterized simultaneously.

As mentioned before, the biosensor surface is usually covered with molecules like antibody proteins to allow specific binding of the particle label via biomarkers. Non-specific interactions will also occur between the protein coated surface and the protein coated particles. To increase the specificity of immunoassays, non-ionic surfactants are routinely added to the sample fluid [1]. Non-ionic surfactants are known to influence hydrophobic interactions and thereby they influence the non-specific interactions between the surface and particle labels, but surfactants can also affect the specific interactions. It will be interesting to apply magnetic tweezers pulling experiments combined with a distributed energy barrier model to study and quantify these influences. When the double gaussian distribution of energy barriers in introduced, the specific and non-specific bonds can be simultaneously studied in the same system provided that their characteristics are distinctive.

An important characteristic of the complex fluids that are analyzed in biosensors is the presence of many different proteins and other molecules at higher concentrations
than the biomarker itself. These might interfere with the specific as well as with the non-specific interactions. To quantify the influence of molecules in the complex fluid, it will be interesting to measure association and dissociation barriers with the rotating particles probe and with magnetic tweezers dissociation measurements. The energy barriers themselves combined with their spreads give information on the interactions in the system.

In conclusion, we expect that the experimental tools and model description developed in this thesis will help to further improve our understanding of non-specific and specific interactions in a variety of materials systems, leading to better defined surfaces and particles for biosensing in complex matrices.
Non-specific protein–surface interactions in the context of particle based biosensors

Biosensors are compact devices that can be used at the point-of-care to measure low quantities of biomarkers in a complex body fluid like blood or saliva. Many biomarkers in the human body are protein molecules, for example the biomarkers that are used for the diagnosis of myocardial infarction. Sensitive detection of protein biomarkers in a biosensor is enabled by the use of molecules with a high specific affinity for the biomarker; typically these are antibodies. The basic concept of a particle-based biosensor is that the specific binding of antibodies to the protein biomarkers causes particle labels to become attached to a sensor surface, and thereby the amount of bound particles becomes a measure for the biomarker concentration in solution. The sensitivity and specificity of the biosensor are determined by the specific as well as the non-specific interactions in the assay. Non-specific interactions can for example induce particle binding without biomarkers being present, leading to false positive signals. Therefore it is important to reduce the non-specific interactions as much as possible and to try to understand the underlying mechanisms.

Biosensor surfaces are often made of a polymeric material like polystyrene because of its excellent manufacturing and modification properties. There are a number of strategies to reduce non-specific binding on polymers, for example blocking of the surface with a protein like BSA or increasing the surface hydrophilicity by an oxidative treatment. Within the non-specific binding processes several physicochemical interactions play a role like electrostatic, van der Waals, hydrogen bonding, hydrophobic and steric or roughness related interactions; and on top of that protein conformations can change. However, the effects of the surface treatments on non-specific interactions with particles and single proteins have not been characterized and understood in detail.

The first topic addressed in this work was to develop a model polystyrene surface with well controlled properties that could be varied to influence the non-specific interactions. We chose a spincoated polystyrene surface that was flat on the scale of proteins (as shown by AFM measurements, $R_q < 0.55$ nm) to minimize the role of roughness in the interactions. The polymer surfaces were treated with UV/ozone in order to control the hydrophilicity of the surface. A direct relation was observed between treatment time and hydrophilicity, with an increase of surface oxygen content (measured by XPS, up to ~24\% from ~300 s oxidation) causing a reduction of water contact angle. These UV/ozone treated polystyrene surfaces with a range of different hydrophilicities yield a platform for studying protein interactions.
The first interaction we quantified was the non-specific association of protein-coated particles to the polystyrene surface. We described the interaction with an energy barrier for association that depends on the properties of both the surface and the solution. The experiments were performed with superparamagnetic particles; such particles are known to be suitable labels in integrated high-sensitivity biosensors due to the fact that the particles can be manipulated by magnetic fields. The particles consist of a polystyrene matrix filled with magnetic nanoparticles. In our experiments the particles were coated with myoglobin, a well-established cardiac biomarker. A new technique, the rotating particles probe, was used to quantify the fraction of unbound particles by measuring their response to a rotating magnetic field. To describe the non-specific binding process we propose a model with a distribution of energy barrier values and this model was shown to accurately fit the measured data. The extracted parameters signified high energy barrier values for binding on surfaces with long oxidation times and for solutions with high pH or low ionic strength. Both hydrophilicity and electrostatic interactions play an important role in the observed non-specific association. The energy barrier for association could be quantified by using the energy barrier spread from dissociation measurements: at physiological buffer conditions (150 mM) the energy barriers were found in the range $0–60 \, k_B T$.

Next, we studied the dissociation of non-specifically bound particles. For this purpose we used magnetic tweezers and the same protein-coated particles as for the association measurements. Forces applied by the magnetic field gradient were calibrated by time-of-flight measurements. The force-induced dissociation measurements were performed by recording the number of bound particles as a function of time during the application of a constant force of 30 pN, 50 pN or 70 pN. The data appear not to obey the dissociation kinetics of bonds with a single energy barrier as in the standard Bell model. We show that the dissociation of the non-specific bonds can be modeled with a distribution of energy barrier values. The fits reveal that the energy barrier for unbinding decreases for increasing oxidation time (from 90 s to 300 s) from $38 \, k_B T$ to $25 \, k_B T$ with a constant spread of $(7 \pm 4) \, k_B T$. So the association as well as the dissociation experiments show that the hydrophilicity of the surface is an important determinant for non-specific interactions; and both processes reveal a distribution of energy barriers rather than a single energy barrier.

Finally, we zoomed in on the non-specific interaction between single proteins and a polystyrene surface. AFM tips were functionalized with single myoglobin molecules and force–distance curves were recorded. In the retraction curves clear steps were observed. These steps entail that the tip does not detach from the surface at once. The events appearing in between the steps have two different characteristics: either the force stays constant during retraction or the force increases like the stretching of a spring. There are two processes to which these events can be attributed: first, the pulling of polymer chains from the polystyrene surface and, second, disruption associated with the protein structure. The data reveal that the non-specific interaction between a single protein and a polymer surface can be stronger than the internal structure of the protein.

These results provide new experimental approaches to study non-specific interactions between protein-coated particles and biosensor surfaces. We have learned that the interactions can be described by a generalized interaction potential that is char-
acteristic for the properties of the surface and the composition of the solution. An important finding is that the energy barriers for association as well as for dissociation are given by a distribution rather than a single value or a set of values. Furthermore, first experiments on the non-specific binding of a single myoglobin molecule showed that the non-specific forces can be stronger than the internal protein structure. Overall, the experiments form a first step and foundation for the study of non-specific interactions between polymer surfaces and protein-coated particles as well as single protein molecules. Further research should focus on extensions to different proteins, surfaces and solution compositions, in order to study the validity range of the model descriptions; and on a stepwise increase of the complexity of the materials system with the aim to develop a complete understanding of the specific and non-specific interactions in biosensor assays.
Curriculum vitae

08-01-1984 Born in Leiderdorp, The Netherlands

1996-2008 vwo, Christiaan Huygens College, Eindhoven, The Netherlands

2002-2006 Bachelor’s degree Applied Physics, Eindhoven University of Technology

’04-’06: teaching assistant at department of Applied Physics

Sep ’04-Sep ’05: board member of Applied Physics study association ‘Johannes Diderik van der Waals’

May-Aug ’06: Bachelor’s project at group Molecular Biosensors for Medical Diagnostics (quantification of binding sites on an activated biosensor surface)

2007 pre-Master’s program Biomolecular Sciences, Utrecht University

2006-2008 Master’s degree Applied Physics, Eindhoven University of Technology

Sep-Nov ’07: internship, Holst Centre (TNO), System-in-foil (investigation of the influence of solution processing of organic semiconductors on their use for thin film transistors)

Master’s project at group Physics of Nanostructures (investigation of the influence of spin injection on organic magnetoresistance)

2009-2013 PhD research, group Molecular Biosensors for Medical Diagnostics, department of Applied Physics, Eindhoven University of Technology, financed by the Dutch Polymer Institute
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Journal articles

Interactions between Protein Coated Particles and Polymer Surfaces Studied with the Rotating Particles Probe
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Roughness and Ordering at the Interface of Oxidized Polystyrene and Water
Stela Andrea Muntean, Marijn Kemper, Leo J. van IJzendoorn, and Alexey V. Lyulin
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M. Kemper, E. Pérez Ruiz, L.J van IJzendoorn, and M.W.J. Prins
in preparation

Non-specific myoglobin–polystyrene interactions characterized by force–distance measurements in an atomic force microscope
M. Kemper, E.W.A. Visser, L.J van IJzendoorn, and M.W.J. Prins
in preparation

Not related to this work:
Spin–spin interactions in organic magnetoresistance probed by angle-dependent measurements
W. Wagemans, A.J. Schellekens, M. Kemper, F.L. Bloom, P.A. Bobbert, and B. Koopmans

Conference talks

Energy barrier distributions for the interaction of protein-coated particles with polymer surfaces
22 January 2013: Physics@FOM, Veldhoven

Orientation of adsorbed myoglobin on (oxidized) polystyrene
Posters, international

Non-specific interactions between single proteins and polystyrene surfaces
M. Kemper, E.W.A. Visser, L.J. van IJzendoorn, M.W.J. Prins

Rotating particles probe to study protein–surface interactions (Best Poster Award)
M. Kemper, D. Spridon, L.J. van IJzendoorn, M.W.J. Prins
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Orientation of adsorbed myoglobin on (oxidized) polystyrene
M. Kemper, L.J. van IJzendoorn, M.W.J. Prins

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Orientation of adsorbed myoglobin on (oxidized) polystyrene
M. Kemper, L.J. van IJzendoorn, M.W.J. Prins
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Polysensor: Interactions between polymer surfaces and proteins
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13-14 October 2010: DPI FPS mini-symposium, Noordwijkhout
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Molecular Dynamics Simulations of Polystyrene Surfaces for Biomedical Applications
S.A. Muntean, A.V. Lyulin, M. Kemper, L.J. van IJzendoorn, M.W.J. Prins
28-29 September 2009: FOM annual Dutch meeting on Molecular and Cellular Biophysics, Veldhoven

Characterizing Polystyrene for Studying Protein Interactions
M. Kemper, L.J. van IJzendoorn, S.A. Muntean, A. Lyulin, M.W.J. Prins
28-29 September 2009: FOM annual Dutch meeting on Molecular and Cellular Biophysics, Veldhoven
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