Mechanics of the contact interface between cells and functionalized surfaces

Citation for published version (APA):

DOI:
10.6100/IR754366

Document status and date:
Published: 01/01/2013

Document Version:
Publisher’s PDF, also known as Version of Record (includes final page, issue and volume numbers)

Please check the document version of this publication:
• A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
• The final author version and the galley proof are versions of the publication after peer review.
• The final published version features the final layout of the paper including the volume, issue and page numbers.

Link to publication
Mechanics of the contact interface between cells and functionalized surfaces

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Technische Universiteit Eindhoven, op gezag van de rector magnificus, prof.dr.ir. C.J. van Duijn, voor een commissie aangewezen door het College voor Promoties in het openbaar te verdedigen op maandag 17 juni 2013 om 16.00 uur

door

Matthias Irmscher

geboren te Leipzig, Duitsland
# Contents

## Summary

<table>
<thead>
<tr>
<th>Chapter 1</th>
<th>Introduction</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Molecular and cellular diagnostics</td>
<td>2</td>
</tr>
<tr>
<td>1.2</td>
<td>Immune cells and immunological processes</td>
<td>3</td>
</tr>
<tr>
<td>1.2.1</td>
<td>Atherosclerosis</td>
<td>3</td>
</tr>
<tr>
<td>1.2.2</td>
<td>Phagocytosis</td>
<td>5</td>
</tr>
<tr>
<td>1.3</td>
<td>Studying the mechanics of single cells</td>
<td>7</td>
</tr>
<tr>
<td>1.3.1</td>
<td>Overview of techniques</td>
<td>7</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Magnetic manipulation</td>
<td>9</td>
</tr>
<tr>
<td>1.4</td>
<td>Interactions of cells and surfaces</td>
<td>12</td>
</tr>
<tr>
<td>1.4.1</td>
<td>Biological context</td>
<td>12</td>
</tr>
<tr>
<td>1.4.2</td>
<td>Methodological context</td>
<td>12</td>
</tr>
<tr>
<td>1.4.3</td>
<td>Implications for biosensing</td>
<td>13</td>
</tr>
<tr>
<td>1.5</td>
<td>This thesis</td>
<td>13</td>
</tr>
</tbody>
</table>

## Chapter 2 | Experimental and theoretical background | 15 |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Magnetic particles</td>
<td>16</td>
</tr>
<tr>
<td>2.1.1</td>
<td>Magnetic actuation</td>
<td>16</td>
</tr>
<tr>
<td>2.1.2</td>
<td>Ferromagnetic particles</td>
<td>16</td>
</tr>
<tr>
<td>2.1.3</td>
<td>Torque calibration</td>
<td>17</td>
</tr>
<tr>
<td>2.2</td>
<td>Stiffness measurements</td>
<td>19</td>
</tr>
<tr>
<td>2.3</td>
<td>Particle image tracking</td>
<td>22</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Conventional methods</td>
<td>23</td>
</tr>
<tr>
<td>2.3.2</td>
<td>The Hough transform</td>
<td>24</td>
</tr>
<tr>
<td>2.4</td>
<td>The cell membrane</td>
<td>26</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Membrane structure</td>
<td>26</td>
</tr>
<tr>
<td>2.4.2</td>
<td>Membrane mechanics</td>
<td>28</td>
</tr>
<tr>
<td>2.5</td>
<td>Cell-surface interactions</td>
<td>33</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Nonspecific interactions</td>
<td>33</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Specific interactions</td>
<td>37</td>
</tr>
<tr>
<td>2.5.3</td>
<td>Interactions with particles</td>
<td>38</td>
</tr>
<tr>
<td>2.6</td>
<td>Links to this thesis</td>
<td>39</td>
</tr>
</tbody>
</table>

## Chapter 3 | Euler angle particle tracking | 41 |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Introduction</td>
<td>42</td>
</tr>
<tr>
<td>3.1.1</td>
<td>Motivation</td>
<td>42</td>
</tr>
<tr>
<td>3.1.2</td>
<td>Anisotropy of the cell membrane</td>
<td>43</td>
</tr>
<tr>
<td>3.1.3</td>
<td>Membrane receptors</td>
<td>43</td>
</tr>
<tr>
<td>3.1.4</td>
<td>Chapter overview</td>
<td>45</td>
</tr>
<tr>
<td>3.2</td>
<td>Materials and Methods</td>
<td>46</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>3.3</td>
<td>Tracking of Euler angles</td>
<td>49</td>
</tr>
<tr>
<td>3.3.1</td>
<td>General approach</td>
<td>49</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Validation</td>
<td>51</td>
</tr>
<tr>
<td>3.4</td>
<td>Results</td>
<td>56</td>
</tr>
<tr>
<td>3.4.1</td>
<td>Cell stiffness measurements</td>
<td>56</td>
</tr>
<tr>
<td>3.4.2</td>
<td>Application of an isotropic material model</td>
<td>59</td>
</tr>
<tr>
<td>3.4.3</td>
<td>Estimation of embedding angle</td>
<td>60</td>
</tr>
<tr>
<td>3.5</td>
<td>Discussion</td>
<td>63</td>
</tr>
<tr>
<td>3.5.1</td>
<td>Origin of observed anisotropy</td>
<td>63</td>
</tr>
<tr>
<td>3.5.2</td>
<td>Potential improvements and applicability</td>
<td>64</td>
</tr>
<tr>
<td>3.5.3</td>
<td>Outside the box</td>
<td>64</td>
</tr>
<tr>
<td>3.6</td>
<td>Conclusions</td>
<td>66</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>Probing the mechanical stiffness of phagocytic cups</td>
<td>67</td>
</tr>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>68</td>
</tr>
<tr>
<td>4.1.1</td>
<td>Phagocytosis</td>
<td>68</td>
</tr>
<tr>
<td>4.1.2</td>
<td>Mechanical aspects of phagocytosis</td>
<td>68</td>
</tr>
<tr>
<td>4.1.3</td>
<td>Chapter overview</td>
<td>69</td>
</tr>
<tr>
<td>4.2</td>
<td>Materials and Methods</td>
<td>70</td>
</tr>
<tr>
<td>4.3</td>
<td>Results</td>
<td>73</td>
</tr>
<tr>
<td>4.3.1</td>
<td>Successful particle internalization</td>
<td>73</td>
</tr>
<tr>
<td>4.3.2</td>
<td>Interrupted internalization</td>
<td>76</td>
</tr>
<tr>
<td>4.3.3</td>
<td>Modeling the stiffness of a phagocytic cup</td>
<td>77</td>
</tr>
<tr>
<td>4.3.4</td>
<td>Phagocytic activity assays</td>
<td>81</td>
</tr>
<tr>
<td>4.3.5</td>
<td>The viscoelastic environment of internalized particles</td>
<td>82</td>
</tr>
<tr>
<td>4.4</td>
<td>Discussion</td>
<td>83</td>
</tr>
<tr>
<td>4.4.1</td>
<td>Critique of method</td>
<td>83</td>
</tr>
<tr>
<td>4.4.2</td>
<td>Observation of internalization</td>
<td>84</td>
</tr>
<tr>
<td>4.4.3</td>
<td>Interrupted internalization</td>
<td>85</td>
</tr>
<tr>
<td>4.4.4</td>
<td>Linking results and theory</td>
<td>85</td>
</tr>
<tr>
<td>4.4.5</td>
<td>Outlook</td>
<td>90</td>
</tr>
<tr>
<td>4.5</td>
<td>Conclusions</td>
<td>91</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>Nonspecific adhesion and cell detachment</td>
<td>93</td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>94</td>
</tr>
<tr>
<td>5.1.1</td>
<td>Motivation</td>
<td>94</td>
</tr>
<tr>
<td>5.1.2</td>
<td>Chapter overview</td>
<td>94</td>
</tr>
<tr>
<td>5.2</td>
<td>Materials and Methods</td>
<td>94</td>
</tr>
<tr>
<td>5.3</td>
<td>Results</td>
<td>96</td>
</tr>
<tr>
<td>5.3.1</td>
<td>Observations</td>
<td>96</td>
</tr>
<tr>
<td>5.3.2</td>
<td>Modeling</td>
<td>97</td>
</tr>
<tr>
<td>5.4</td>
<td>Discussion</td>
<td>100</td>
</tr>
<tr>
<td>5.4.1</td>
<td>Tether detachment by peeling</td>
<td>100</td>
</tr>
<tr>
<td>5.4.2</td>
<td>Outlook</td>
<td>101</td>
</tr>
<tr>
<td>5.5</td>
<td>Conclusions</td>
<td>105</td>
</tr>
<tr>
<td>Chapter 6</td>
<td>Conclusions and outlook</td>
<td>107</td>
</tr>
<tr>
<td>6.1</td>
<td>Introduction</td>
<td>108</td>
</tr>
</tbody>
</table>
Summary

Mechanics of the contact interface between cells and functionalized surfaces

Current biosensing concepts are largely aimed at measuring the concentration of molecular biomarkers in samples of body fluids. Diseases that are not characterized by pathological molecular concentrations can potentially be diagnosed by determining the presence and size or a functional property of specific cell populations. Since many cell functions have a significant mechanical component, evaluating cell-mechanical properties is a promising direction of research. In this thesis, we describe an investigation of mechanical phenomena that occur at the interfaces between cells and functionalized surfaces, such as the ones that can potentially be integrated into a biosensing device. In particular, we study the dynamics of magnetic particles in contact with monocytic cells and the detachment mechanism of adhering cells from a non-specifically coated surface.

Magnetic particle actuation is a promising tool for cell mechanical research as the applied fields usually do not interact with biological matter. Existing methods, such as magnetic twisting cytometry, have used magnetic actuation and massively parallelized translational particle tracking to extract the dynamic scaling properties of the cytoskeleton. In chapter 3, we focus on single particles with the aim of investigating the details of the cell-particle interaction. By tagging magnetic particles with fluorescent fiduciary nanoparticles, we succeed in tracking not only the translational but also the full rotational dynamics of the magnetic particles. We visualize the particles in simultaneous bright-field and fluorescence mode and track their motion using a custom-written software algorithm. Chapter 3 contains a theoretical and experimental analysis which demonstrates the feasibility of this approach with a translational tracking accuracy of about 30 nm and a angular tracking accuracy of about 2°. We then functionalized the particles and bound them to monocytic THP-1 cells via integrin LFA-1, the scavenger receptor CD14, or via the Fc receptors. By applying magnetic actuation in combination with Euler angle tracking, we found that the probed region of the cell appears isotropic if the particles are bound via LFA-1 or CD14. In contrast, probing with particles that were bound via the Fc receptors revealed an anisotropic stiffness of the contact site with an about ten-fold reduced relative rotational stiffness about one axis. We propose that the observed effect is caused by partial disjoining of the cell membrane from the underlying actin cortex, which is possibly related to the onset of phagocytosis as a result of Fc receptor activation. Generally, the observed receptor-dependent effect underlines that the cell membrane and the actin cortex is a mechanically anisotropic assembly whose degree of interconnectedness determines the measurable mechanical properties.

In chapter 4 we use the Euler angle tracking approach to study phagocytosis as a model process that involves the interaction between particles and cells. Cells internalize particles by actively driving membrane protrusions around them to finally enclose them in vesicles called phagosomes. We measure the mechanical properties of the emerging membrane protrusions, called phagocytic cups, by using functionalized ferromagnetic particles as phagocytic targets and by exposing them to an oscillating magnetic field during their internalization. By simultaneously tracking the transla-
tional and rotational motion of the particle, we infer the stiffness of the dynamically evolving phagocytic cup. Our measurements show that during successful phagocytosis, the measured rotational stiffness initially increases slowly before reaching a peak and thereafter decreases at a faster rate. We capture this process theoretically by treating the phagocytic cup as a deformed membrane in connection with an isotropically elastic actin cortex. In this model description, the peak of stiffness appears when the membrane has engulfed the largest circumference of the particle. The application of this model to our data reveals that the leading edge of the membrane advances at a speed of about 20 nm/s before reaching the equator of the particle. Phagocytosis assays show that the actuation of the particles does not affect the global efficiency of phagocytic uptake. By measuring the viscoelasticity of the particle binding site, we show that internalized particles are embedded in a more viscous environment than externally bound particles. This approach for the first time enables direct and time-resolved measurements of the mechanical properties of emerging phagocytic cups. Our method is suited to study the effect of inhibitors on the mechanics of uptake and it can potentially be used to investigate the reorganization of the cytoskeleton during uptake in micro-rheological terms.

In the last chapter of this thesis we describe an investigation of the detachment kinetics of cells that were allowed to adhere to a positively charged surface. We show that detaching cells do not peel off the substrate but remain transiently attached through 20 to 60 membrane tethers that undergo elongation under the applied fluidic shear stress. We propose that the extrusion of tethers is a consequence of an inhomogeneous adhesive interface with several focal points where the interaction energy of adhesion is maximal. Such a patterned adhesive interface can arise if separation of lipid components leads to local electrostatic charge matching.

The research described in this thesis contributes to the understanding of mechanical processes between cells and the functionalized surfaces of particles and flat substrates. The work on the mechanics of phagocytosis demonstrates how rotational and translational tracking of magnetic particles opens a new direction in cell-mechanical research. Combining the described approach with molecular imaging techniques can yield further insight into the mechanisms that drive macroscopic cellular processes and might reveal novel targets for medical diagnosis and treatment.
The dominant modality in in-vitro diagnostics is to measure concentrations of molecular biomarkers in body fluid as indicators of the presence or severity of a disease. Nowadays, cells and tissue are important targets as well. The white blood cell count, for example, is an established indicator of an infection. Beyond establishing the size of cell populations, measuring their physiological function and its deviation from standard values is expected to expand the capabilities of future in-vitro diagnostic solutions. This chapter sets the scene for this thesis by discussing elements of cellular diagnostics with a particular focus on the mechanical characterization of white blood cells. The second part of this chapter introduces tools that are used in research settings to study cell responses. Finally, the central question of this thesis is formulated and the subsequent chapters are introduced.
1.1. Molecular and cellular diagnostics

The word diagnosis is derived from the Greek language and means to discern or to distinguish. The etymology of the word captures the fact that in order to correctly identify an illness, its symptoms have to be (i) visible and (ii) classifiable.

In the course of history, the field of medicine has spawned an ever-growing range of tools and procedures that enable or facilitate the diagnosis of diseases. These tools and procedures need to be effective and simple enough to be amenable for routine use. Before modern imaging techniques became available, most diagnostic techniques were based on what a doctor could see with bare eyes, which limited the accuracy of the associated diagnostic procedures. The invention of X-ray imaging at the end of the 19th century for the first time enabled the non-invasive diagnosis of conditions with internal symptoms\(^1\). Later developments like computed tomography (CT)\(^2\), sonography\(^3\) and magnetic resonance imaging\(^4,5\) greatly expanded the set of diagnostic tools and procedures at the disposal of physicians.

While modern imaging modalities have made it possible to obtain detailed images of the human body, the acquired level of detail falls short of the molecular and cellular scale. Tumors, for example, are characterized by their diameter\(^6\) and blood vessel occlusion is diagnosed by analyzing millimeter-scale images acquired by angiography. If the resulting treatments are aimed at a macroscopic scale, e.g. a tumor excision or a bypass surgery, macroscopic diagnostic methods are often sufficient. However, the pharmacological treatment of these diseases takes effect at the molecular scale. To improve such treatments and to devise new ones, the diagnosis should ideally be carried out with a corresponding level of detail.

The last decades have seen a steady development of methods that aim to diagnose diseases at the molecular and cellular scale. In a laboratory setting, immunoassays are used to detect protein biomarkers such as troponin\(^7\) and prostate-specific antigen (PSA)\(^8\) to, respectively, diagnose myocardial infarctions and, possibly cancer-related, prostate disorders. Polymerase chain reaction (PCR) enabled the detection of minute amounts of nucleic acids in patient samples and is used in virology\(^9\) and cancer diagnostics\(^10\). Beyond the detection of specific nucleic acid fragments, the field of genomics has given rise to a multitude of genetic sequencing techniques that yield complete maps of the genetic material of an organism. Understanding the function of the genome and its involvement in the pathways that underlie specific diseases is expected to greatly improve personalized treatment\(^11\). Outside laboratories, diagnostic tests for general practitioners and for home use have been introduced to measure e.g. the concentration of glucose\(^*\) or human chorionic gonadotropin\(^†\) (hCG) in a small sample of a body fluid. Owing, amongst others, to the high concentration (μM to mM range) of the target species, glucose and hCG testing are mature fields. Considerable efforts are under way to detect species at lower concentrations, for laboratory as well as for point-of-care settings\(^12-16\).

While certain biological processes are traceable via soluble molecular markers, other processes manifest themselves essentially at the cellular or the tissue level. In pathology, the inspection of tissue from biopsies has been coupled with in-situ genetic analysis\(^17,18\) to locally correlate the morphology of tissue with the genetic profile of its cellular constituents. If the involved cell type can be found in a body fluid, the diagnostic test can be designed to target a specific group of cells. The white blood cell count is routinely measured to determine the severity of an infection. An exam-

---

* Abnormal concentrations of glucose and fluctuations thereof are indicative of diabetes.
† The hormone hCG is produced by the placenta and indicates an ongoing pregnancy.
ple of a test that targets a cell population by a specific molecular signature is the CD4⁺ T-cell count which detects the concentration of CD4-positive T-cells in a blood sample to assess the progression of an HIV infection. Similarly, albeit at considerably lower concentrations, the number of circulating cancer cells is seen as a measure of tumor progression by metastasis.

Nowadays, most molecular or cellular diagnostic tests report the concentration of a target species that is identified by a molecular signature. These approaches can only be effective if the condition in question causes an abnormal concentration of the diagnostic target. Diseases that are caused and/or characterized by the pathological function of a protein or a group of cells, are more difficult to detect. To quantitatively characterize these disease patterns, functional testing at the cell or tissue level may be required. An example of a functional test is platelet aggregometry where the transient formation of clusters of platelets is characterized with the aim of identifying bleeding disorders.

Functional testing of cells can be carried out passively or actively. In passive testing the cell is not disturbed and a specific process is visualized in order to examine and classify its function. However, removing cells from their native in vivo environment frequently creates disturbances that effectively prevent the realization of a real passive test. Future cell functional testing hence critically depends on understanding and controlling the interactions of cells in the artificial environment of an in vitro diagnostic device.

1.2. Immune cells and immunological processes

Circulating cells are promising targets for future cellular diagnostics because patient samples can be obtained more easily than from tissue. White blood cells or leukocytes are particularly intriguing due to their involvement in the immunological processes that are triggered by many diseases. Atherosclerosis and phagocytosis are two examples of processes that are crucially dependent on leukocyte function, as outlined below.

All white blood cells are derived from hematopoietic stem cells that reside in the bone marrow. From this progenitor, two major lineages extend that give rise to myeloid and lymphoid cells (see Fig. 1.1). Neutrophils and monocytes are the two most commonly occurring non-lymphoid leukocytes. While neutrophils are fully differentiated, monocytes are progenitor cells that initially circulate through the bloodstream for 8 to 12 hours and eventually migrate into tissue where they differentiate into macrophages. Monocytes are approximately 8 to 10 μm in diameter and possess kidney-shaped nuclei. Throughout this thesis, cultured THP-1 cells are used as a model system for monocytes because they display many basic immune functions and are readily available. Additionally, THP-1 cells can be differentiated into adherent macrophage-like cells.

1.2.1. Atherosclerosis

Atherosclerosis is an inflammatory condition which is characterized by a thickening of the arterial wall as a result of an accumulation of cells and lipids. At the onset of atherosclerosis, the integrity of the endothelial cell layer is compromised, and as a result, low-density lipoproteins (LDL) can enter sub-endothelial space where they become trapped by interactions with the extracellular matrix (ECM). Oxidation

‡ In passive testing of cells the idea of Richard Feynman resonates that most problems in biology can be solved by 'just looking at the thing'.
of LDL leads to the release of chemokines that increase the expression of adhesion molecules on endothelial cells and circulating monocytes. As a result, monocytes locally adhere to the endothelium and migrate from the blood stream into the tissue where they differentiate into macrophages. Once differentiated, these macrophages begin ingesting locally present LDL particles and turn into so-called foam cells. The continuous release of chemokines gives rise to a positive feedback loop that leads to further growth of the atherosclerotic plaque. Eventually, the lesion can become unstable and release its clotted contents into the blood stream. The thus released clot can locally block the circulation of blood and result in a myocardial infarction or a stroke.

The growth of the atherosclerotic lesion is strongly driven by the extravasation of monocytes as a result of an increased expression of adhesion molecules. Current research is guided by the hypothesis that the severity of atherosclerosis correlates with the emergence of a subset of monocytes with an abnormal level of membrane receptor expression. In view of future applications in functional cell diagnostics, this thesis explores the potential of microparticles to differentiate between subsets of cells by studying the broader framework of cell-surface interactions with quantitative biophysical means.
1.2.2. Phagocytosis

Phagocytosis is the process by which immune cells ingest foreign objects and apoptotic cells that are larger than about 0.5 μm. Smaller objects, such as viruses and macromolecules, are taken up by clathrin-dependent endocytosis while liquids are internalized by macropinocytosis. Many cells are capable of phagocytosis but only macrophages, monocytes and neutrophils are specialized in it and are hence called professional phagocytes.

Phagocytosis can be seen as a process with four stages (see panels A-D of Fig. 1.2 for an illustration of the special case of Fc-receptor-mediated phagocytosis): (A) recognition and binding via specific receptors on the cell, (B) engulfment by actin-driven membrane protrusion, (C) formation of a closed vesicle (phagosome) and (D) degradation of phagosome contents. Successful completion of this sequence is based on the parallel execution of many complex biochemical pathways.

The identification of phagocytic prey occurs upon binding of specific target molecules by receptors on the phagocyte membrane. To increase the robustness of recognition, the molecular targets are highly conserved and generally form integral parts of the invading pathogens. Commonly called pathogen-associated molecular patterns (PAMPs), these targets include mannans in yeast cell walls and lipopolysaccharides in the outer cell wall of Gram-negative bacteria. These patterns are directly recognized by a diverse range of receptors, e.g. the mannose receptor, integrins like CD11b/CD18 and scavenger receptors such as CD14. In some cases, the phagocytic target is identified through a molecular intermediary. Examples of such processes are the binding of complement and the recognition of antibody fragments by the Fc-receptors of the cell. In the latter scheme, an object is first tagged by specific antibodies (a process called opsonization) whose well-conserved tail region in turn serves as a target for cells of the immune system. The initial binding of phagocytic targets has traditionally been regarded as a passive process that is driven by thermal fluctuations of the membrane and the diffusive motion of membrane receptors but recent studies suggest that actively driven protrusions akin to probing tentacles are involved as well. In general, several receptors can be simultaneously engaged in the stage of recognition and it remains challenging to distinguish those that merely bind the target from those that trigger its uptake. Fc-receptors have conclusively been shown to do both and Fc-receptor mediated phagocytosis remains the most commonly studied mechanism of phagocytosis.

Upon binding of the target, its uptake is triggered. In Fc-receptor mediated phagocytosis, the crucial signaling unit is the immunoreceptor tyrosine based activation motif (ITAM) found on the cytoplasmic tail of some Fc-receptors. Fc-receptors that do not possess an ITAM colocalize with membrane-bound subunits that serve as signaling beacons. Once ligated by an Fc region, the receptors are triggered to form clusters that stimulate the phosphorylation of the ITAM by kinases of the Src family. The formation of clusters is thought to be required to generate signals that exceed a threshold beyond which phagocytosis ensues. Single activated receptors hence do not initiate a response and are internalized in a clathrin-dependent way. Upon phosphorylation of ITAM, the kinase SYK binds to the motif and subsequently initiates further signaling pathways that trigger the polymerization of actin which is thought to be the main driver of the protrusion of the membrane. Among the molecules that have been implicated in downstream signaling are calcium, phospholipases, kinases of the PKC family and several phosphoinositide kinases. The local structure of actin is mainly orchestrated by the Arp 2/3 complex. Newly poly-
Figure 1.2 – Stages of Fc-receptor-mediated phagocytosis. (A) A phagocytic target is initially identified via specific bonds between the Fc-receptor and opsonizing antibodies. (B) Subsequently, intracellular signaling triggers a directed deformation of the membrane (phagocytic cup) with the aim of engulfing the external object, which can be an artificial particle or a bacterium. (C) By closing the phagocytic cup, an intracellular vesicle, called a phagosome, is formed. Subsequently, lysosomes begin merging with the phagosome. (D) Acidification and/or enzymatic digestion eventually lead to the neutralization of the phagocytic target.

Merized actin filaments are subsequently connected by cross-linking proteins, such as α-actinin\textsuperscript{41}, into networks that can be contracted by a large family of involved myosin motor proteins\textsuperscript{42,43}.

Eventually, the membrane protrusion closes on itself and the phagocytic target is contained in a vesicle. The phagosome is then coupled to cytoskeletal structures via motor proteins on its external membrane\textsuperscript{44,45}. Enabled by these motor proteins, the phagosomes subsequently undergo a characteristic centripetal motion towards the center of the cell. During this transport phase, the phagosome fuses with intracellular vesicles called endosomes and lysosomes. These vesicles contain proton pumping proteins and specific enzymes which help to lower the pH and to biologically degrade the contents of the phagosome.

Despite the existing comprehensive insight into the biochemical processes at play during internalization of phagocytic targets, little is known about the mechanical determinants of the process, such as deformation energies and forces. A characterization of these 'output variables' will complete our view on how the above molecular processes collude to drive the complex mechanics of phagocytosis. In this thesis, we present a method that enables the mechanical characterization of emerging phagocytic cups in chapter 4 of this thesis. Beyond functional cell diagnostics, this approach can potentially become useful to quantify the mechanical changes that are driven by the biochemical pathways sketched above.
1.3. Studying the mechanics of single cells

The function of single cells can be characterized by properties whose quantitative values correlate with the experimental or medical condition in question. These properties can be static, e.g. fluorescence intensities in flow cytometry, or dynamic, such as excretion profiles or migration velocities.

With functional cell diagnostics in mind, not all of the accessible characteristics make for suitable diagnostic targets because their acquisition necessitates involved biochemical procedures. For this reason, the mechanical properties of cells are a promising basis for functional cell testing. Mechanical properties can usually be determined without the use of labels and most measurement techniques come with an inherent quantitative scale. The deformability of cancer cells, for instance, has been studied as a differentiating property and significant differences have been reported between healthy cells and tumor cells. In other experiments, red blood cells infected by *Plasmodium falciparum*§ were shown to be stiffer than their healthy counterparts. Beyond static properties like stiffness, a wide range of dynamic mechanical processes, e.g. cell spreading, migration or phagocytosis, can potentially serve as a basis for novel diagnostic concepts.

The schematic in Fig. 1.3 depicts a generic pathway that exemplifies how molecular processes, cell mechanics and disease states are linked. At the molecular scale, drug treatments, environmental factors or foreign pathogens interact with cells passively or via specific receptors in the cell membrane. Intracellularly, these interactions lead to the activation of signaling pathways and potentially to increased or decreased gene expression. Resulting changes in structural protein concentrations or turnover rates can affect the mechanical integrity of the cell. At the cellular level, these changes manifest themselves in alterations of passive properties like deformability or shape which in turn affect dynamic processes that are central to many physiological processes. At the tissue or organ level, the molecularly induced stimuli result in pathological patterns that characterize diseases.

The field of biophysics has spawned a number of techniques for cell-mechanical research that enable the investigation of the mechanisms at play during the interaction of cells with artificial surfaces and probes. An overview and discussion of selected available biophysical techniques is given below.

1.3.1. Overview of techniques

During the last three decades, the field of cell mechanics has been driven by the development of a variety of techniques for the mechanical characterization of single cells. Nowadays, many of the techniques are used in parallel as every approach has inherent physical characteristics that enable its use for specific tasks. Fig. 1.4 introduces five commonly used techniques and the length scale at which they operate.

Many early experiments seeking to verify the predictions of elementary concepts in membrane mechanics were carried out with the micropipetting approach. In this setup, a lipid vesicle or a cell is partially aspirated at the orifice of a pipette with an inner diameter of a few micrometers. From the applied pressure difference and the measured deformation of the specimen, properties such as bending stiffness and cortical tension can be determined.

The 1980s saw the development of optical tweezers and atomic force microscopy (AFM), two techniques whose introduction would have a lasting impact.

---

§ *Plasmodium falciparum* is one of the Plasmodium parasites that can cause malaria in humans.
Chapter 1

biochemical factors influenced by ex vivo environment or in vivo processes

drug treatment

altered gene expression, activation or inhibition of signaling pathways

structural changes induced in cell membrane, cytoskeleton and cytosol

changes in cell adherence, deformability, size, shape

changes in cell locomotion, motility

deviation from normal cell function, disease state

Figure 1.3 – Generic biochemical pathways that relate disease states with cell mechanics. External factors trigger biochemical changes that manifest themselves in alterations of the mechanical structure of the cells. In turn, these structural changes affect the function of the cells and lead to physiological conditions that are recognized as diseases. Adapted from ref. 46.

Figure 1.4 – Overview of techniques used in cell mechanics studies. The techniques are depicted in order of the localization of the applied forces. Shear flow assays affect the entire cell while micropipette aspiration targets parts of a cell whose scale is determined by the inner diameter of the pipette. In magnetic twisting cytometry, particles with diameters of 4 to 5 µm are partially embedded into the cell. Optical tweezers offer precise control of the particle position and can hence be used to study initial binding events. Atomic force microscopy (with standard tip radii of a few nanometers) accesses the cell at the molecular scale and has been used to study the conformation of individual membrane proteins in their natural environment. In the depictions, the displacement $d$ is the primary measurable quantity.
on the development of both molecular and cellular biophysics. Optical tweezers take advantage of the force that a refractive particle experiences when interacting with light. With micrometer-sized particles and strongly focused laser beams, the applica-
cable optical forces can reach up to several hundred piconewtons, making optical tweezers suitable for highly localized cell mechanical measurements.

AFM was initially used for dry, solid specimens and became useful for cell mechanics when liquid-phase AFM was introduced\textsuperscript{61,62}. In AFM, a cantilever with a known mechanical stiffness is brought in contact with a sample via a small tip. Upon displacement of the cantilever, the contact point experiences a force whose magnitude is determined by measuring the deflection of the cantilever. The force detection limit of AFM depends on the stiffness of the cantilever. The tip has a radius of a few nanometers and enables highly localized measurements. However, the inhomogeneity of the cell membrane at the nanometer scale usually complicates the interpretation of results in mechanical terms. In this regime, the properties of single membrane constituents dominate the measurements. For this reason, in cell-mechanical studies, the tip of an AFM is frequently replaced by a small sphere.

1.3.2. Magnetic manipulation

Manipulation of biological matter by magnetic forces far precedes the above techniques. First experiments aimed at elucidating the mechanical properties of the cytoplasm have been reported by Crick and Hughes in 1950\textsuperscript{63,64}. In these studies, magnetic particles were injected into cells and exposed to a strong magnetizing field. Subsequent actuation by a much smaller magnetic field then evoked a motion whose time course revealed that the cytoplasm was much more viscous than water and that it had elastic properties. The demonstrated ability to manipulate living matter by application of magnetic fields gave rise to two general approaches that have become standard tools by the end of the 20th century.

Magnetic tweezers take advantage of the force that a particle experiences in an inhomogeneous magnetic field. As indicated by the term 'tweezers', these instruments usually include sharp ferromagnetic tips that locally create strong field gradients in the vicinity of single particles. Due to the high inductance of the iron core, most magnetic tweezers have a small frequency bandwidth\textsuperscript{4} but generally allow for application of high forces\textsuperscript{74} of up to 100 nN on 5 μm particles\textsuperscript{74}. Since the magnetic field gradient around a sharp tip drops off rapidly with increasing distance, magnetic tweezers are inherently unsuitable for multiplexed acquisition of data from multiple particles.

In the past, magnetic tweezers have been used to study the viscoelastic properties of the actin cortex\textsuperscript{75,76} and the cytoplasm\textsuperscript{77,78}. Other work focused on the strength of focal adhesions\textsuperscript{79} and the different roles of integrins in mechanotransduction\textsuperscript{80}.

The original experiments of Crick and Hughes were based on the torque that a magnetic particle experiences when forced into alignment with an externally applied field. This approach requires the particles to carry a fixed magnetic moment that is usually imposed by a strong initial magnetizing pulse. With similar aims as Crick and Hughes, Valberg and coworkers carried out comparable experiments\textsuperscript{81–83} in the 1980’s and measured the viscoelastic properties of the cytoplasm in the presence of

\[ f_c = \frac{R}{(2\pi L)} \]

\( f_c \) is the cutoff frequency of the coil as an inductive low-pass filter. As an example, for the magnetic tweezers setup used by Schmidt et al.\textsuperscript{74} the cutoff frequency is less than 1 Hz. Removing the iron core would increase the bandwidth but decrease the strength of the generated magnetic field.

\[ \tau/R \]

The effective shear force at the surface of a bound particle in magnetic twisting cytometry is taken for comparison.
Figure 1.5 – Relevant energy scales in cellular micromanipulation. The overview compares values of experimental studies in cell mechanics with the work of this thesis (chapters 3-5). Solid diagonal lines represent equal energy according to energy=force×length (blue) or based on the energy characterizing a specific process (red). Typically applied ranges of force/length (solid) and torque/angle (dashed) are indicated by rectangular boxes. For comparison with cell-biophysical work, two single-molecule studies (Janssen11, Rief97) are shown, emphasizing the difference in relevant energy scale. The literature references point to the following studies: Fabry01 65,66 - magnetic twisting cytometry on adherent cells; Vonna03 67 - using magnetic tweezers to probe active retraction of cellular protrusions; Herant05 68 - micropipette-based measurements of cortical tension during phagocytosis of particles; Rosenbluth06 50 - stiffness measurements on non-adherent cells by AFM indentation; Coughlin08 69 - measuring active cellular recoiling in applied shear flow at physiological amplitude; Kress07 70 - measuring filopodial retraction forces using optical tweezers; Janssen11 70 - measuring the torsional stiffness of a pair of proteins; Rief97 71 - AFM-induced unfolding of individual Ig-domains in titin. The processes are as follows: ATP hydrolysis - average free energy gain during conversion of ATP to ADP under physiological conditions; cortex bending - bending rigidity of a bilayer-cortex assembly as $\kappa_B=10^{-18}$ J; particle engulfment - energy required to fully wrap a bilayer-cortex assembly around a particle with diameter 4.5 μm with parameters as in Fig. 2.12; RBC adhesion on PLL - energy of adhesion of a red blood cell adhering to a poly-L-lysine-functionalized surface 72.
various chemical interventions, such as the actin polymerization inhibitor cytochalasin D.

The work of Valberg and others was later extended to particles that were bound to the extracellular face of the cell membrane and whose collective motion was detected by induction coils. In a seminal paper, this technique, now called magnetic twisting cytometry (MTC), was applied to discover that the integrin class of membrane receptors are capable of transmitting mechanical stress from extracellular space to the cytoskeleton. This ground-breaking paper gave a decisive impulse to research aimed at understanding the mechanisms of mechanical interactions between cells and their surroundings. Nowadays summarized under the term mechanotransduction (or mechanobiology), this line of research has spawned intense interdisciplinary research at the intersection between physics and biology.

The introduction of MTC coincided with the adoption of the tensegrity principle, describing the mechanical structure of the cytoskeleton as an ensemble of rods and cables that are under compressive or tensile stress, respectively. In later years, MTC was used to investigate the influence of cell spreading on cell stiffness and the role of different proteins in coupling integrins to the cytoskeleton. MTC proved to be a useful tool to study the differences between differentially treated cell populations as, unlike AFM or optical tweezers, it made it possible to readily acquire data from thousands of cells simultaneously. This point is crucial because the variation in stiffness between cells of the same sample can easily reach two orders of magnitude and many individual measurements need to be taken before a comparison between two samples becomes statistically significant. However, the lack of a single-cell perspective, small dynamic range, complicated instrumentation required for magnetic detection and a susceptibility for misinterpretation of results due to heterogeneous particle binding across a sample eventually led to a new development that retained the principle of actuation but introduced a different means of detection.

To mitigate some of the above shortcomings, magnetic twisting cytometry was combined with microscopic imaging of ensembles of individual particles. With high-speed imaging at hand, this approach enabled dynamic measurements at actuation frequencies of up to 1 kHz. This approach was used to study the dynamic modulus of the cytoskeleton as a function of frequency and led to the discovery of an unexpected power-law dependence, indicating a lack of a dominant time scale of relaxation. Interestingly, this finding was independent of the type of molecular coupling to the cell. The utility of magnetic twisting was further demonstrated by time-dependent measurements which revealed the transient change of the viscoelastic properties of HASM cells upon administration of contractile or relaxing agents.

Through years of application to a wide range of questions, magnetic twisting cytometry has become a standardized tool for research into mechanotransduction and network dynamics. Yet, despite its widespread use, the details of the mechanical interaction between single magnetically actuated particles and cells remain poorly understood. A large array of theoretical studies has sought to simulate the motion of individual particles with the aim of validating the results gained in MTC experiments. On the experimental side, however, the geometry of the binding site which is mainly expressed by the degree of particle embedding, has so far only been studied by reconstruction of confocal microscopy images. As a result, magnetic twisting
Chapter 1

of particles cannot be reliably used to extract absolute material properties. In chapter 3 of this thesis, we introduce an experimental technique that can help to further corroborate or disprove some of the theoretical predictions.

1.4. Interactions of cells and surfaces

1.4.1. Biological context

Cell adhesion is a ubiquitous phenomenon as most cells of the human body adhere to each other or to assemblies of structural proteins to form tissue. Tissue cells are crucially dependent on establishing and retaining these mechanical connections and cannot survive without them. For research purposes, these cells are usually grown on flat substrates that have been coated with proteins, e.g. fibronectin, that mediate adhesion via integrin receptors. While this approach is accepted practice, recent advances in mechanotransduction have raised concerns that the proliferation and migration of cells on two-dimensional substrates differs from behavior that has been observed in more realistic three-dimensional scaffold environments.

Non-adherent (circulating) cells, while not requiring contact for survival, are also regularly involved in adhesive processes. For neutrophils and monocytes, adhesion to the endothelium is an inherent stage of their life cycle as it precedes their migration into the underlying tissue. Adhesion deficiencies caused by a lack or defect of the required molecular receptors has been shown to trigger severe recurring bacterial infections\(^{101}\) while mitigating the impact of inflammatory diseases like atherosclerosis\(^{102}\). Phagocytosis is another example of a process that is tightly regulated by the recognition of specific molecules by the cell, followed by adhesion and engulfment.

1.4.2. Methodological context

Testing the function of cells is typically conducted according to the principle of system identification wherein the stimulus and the response are related to infer a target characteristic. Existing functional testing methods, such as platelet aggregometry or flow cytometry, are often semi-quantitative as they require a calibration scale to assess the acquired results. The field of biophysics can potentially help to overcome this shortcoming and has spawned a number of methods that enable the verification of theoretical models and thereby give rise to a quantitative understanding of biological systems.

In Fig. 1.5, five (cell-)biophysical methods are compared with respect to the scales of force and length (torque and angle) that they employ. The techniques thereby access different energy scales that are in turn related to different physiological processes. In order to study a physiological process, it is hence important that the relevant energy scale and the required degree of localization (see Fig. 1.4) can be reached. Furthermore, the energy scale and the degree of localization determine the appropriate coarseness of applicable quantitative models. In chapter 4 of this thesis, we make use of magnetic microparticle actuation to study phagocytic uptake. In this approach we address the biological context by employing particles are of a similar size as bacteria. The energy scale of the membrane protrusion is matched by actuation of ferromagnetic particles.
1.4.3. Implications for biosensing

In the context of biosensing, it is imperative to understand the processes at play during interaction of cells with the artificial surfaces in a biosensing device. A better insight into the mechanism of these interactions and ways to control them can potentially lead to three future developments.

In a research setting, an improved understanding of cell-surface interactions will help to unravel the coupling between the molecular scale and physiological processes at the level of the whole cell. Understanding these links can potentially reveal new starting points for future treatments. This approach is related to the field of organ-on-a-chip, where molecular and cellular processes are studied in an artificially created tissue environment.

In view of the hypothesis that circulating cells and their classification by subsets carries diagnostically relevant information, future biosensing concepts could be based on the analysis of freshly isolated but fixed blood cells. Fixation prevents that active processes lead to in-vitro degradation of patient material and thereby facilitates the establishment of standard procedures.

Further in the future, live-cell diagnostics aimed at quantifying dynamic cellular processes in patient samples might emerge in an effort to diagnose complex disease patterns. This development brings with it the challenge of establishing standards for diagnostic work with living cells as opposed to fixed cells.

1.5. This thesis

The objective of this thesis is to investigate static and dynamic mechanical interactions that cells can potentially experience in the artificial environment of a biosensing device. Some of these interactions cannot be avoided and their consequences need to be understood better than is currently the case. Other interactions can be initiated deliberately and offer insight into the physiological workings of the cell.

In chapter 2 the experimental and theoretical foundation of this thesis is laid out. The first two sections of chapter 2 cover the primary methodological tools of magnetic particles and image processing. The latter sections introduce elementary concepts of membrane mechanics and interfacial forces.

Chapter 3 introduces Euler angle tracking as an extension of conventional particle tracking methods. The chapter introduces the general approach of using fluorescent markers to measure the rotational and translational movement of a particle with 5 degrees of freedom. Using this approach in combination with magnetic actuation to probe the mechanics of the cell cortex, we reveal previously unreported patterns of motion. Our measurements are indicative of receptor-induced changes in the mechanical structure of the contact region between the particle and the cell.

In chapter 4 of this thesis, we used Euler angle tracking to study the cellular uptake of particles by phagocytosis. To this end, we functionalized magnetic particles such that they would be recognized as phagocytic prey upon making contact with a cell. By dynamically measuring translational and rotational stiffness during the uptake phase, we detected characteristic mechanical patterns that are representative of phagocytosis. Furthermore, we provide a theoretical model of a phagocytic cup that can explain the observed characteristics.

Chapter 5 of this thesis touches upon the interaction of cells with non-specifically coated substrates. We applied a fluid shear flow to detach cells from their substrate. By using differential interference contrast (DIC) imaging and measuring the trajec-
tory of single detaching cells, we found that the cells do not simply peel off the substrate but remain transiently attached by multiple membrane tethers that subsequently fail under the applied load. We suggest that the formation of tethers is intricately linked to the topology of the adhesion zone.
Experimental and theoretical background

The primary objective of cell mechanical research is to further the understanding of mechanisms that determine the fate of cells in their native environment. However, understanding how cells interact with an artificial environment is equally important in view of an ever growing interest in diagnostic tools targeting whole cells. In this chapter, the theoretical foundations for this thesis are laid. In 2.1, the properties of magnetic particles are explained. Section 2.3 gives an introduction to particle image tracking with a particular focus on the Hough transform. Section 2.4 gives an overview of the structure of cell membranes and provides a framework for a mathematical description of their mechanical properties. The last part of this chapter discusses the interactions between cell membranes and surfaces.
2.1. Magnetic particles

Due to their size and a large number of available surface modifications, micrometer-sized particles provide an ideal handle for investigations at the single-cell level. In chapter 3 and 4 of this thesis, we use ferromagnetic particles to exert forces on cells. In this section, their most important characteristics are given.

2.1.1. Magnetic actuation

In ferromagnetic materials, the magnetic spins of unpaired electrons give rise to a net magnetization of the sample, even if no external magnetic field is applied. Magnetic materials are organized in domains whose boundaries delineate volumes that contain elementary magnetic moments with the same orientation. Domains arise due to the balance between quantum-mechanical exchange interaction, classical magnetostatic forces and randomizing thermal forces. Unlike in paramagnetic materials, the exchange interaction between neighboring spins is dominant in ferromagnetic materials, hence allowing for stable domains to persist at room temperature. At high temperature, thermal forces become dominant and as consequence, ferromagnetic materials lose their domain structure when heated above the critical Curie temperature.

Macroscopically, a magnetic particle can to first order be approximated as a magnetic dipole. If exposed to an external magnetic field, the dipole moment will experience a force and a torque that aim to minimize the interaction energy between the dipole and the field. If the interaction with an external magnetic field is assumed not to alter either the dipole or the field, the force $F$ and torque $\tau$ are given by

\[
F = \nabla (m \cdot B) \quad \text{and} \quad \tau = m \times B.
\]

Here, $m$ denotes the magnetic moment vector of the particle and $B$ is the flux density vector of the external magnetic field. Equation 2.1 expresses that, given a constant magnetic moment, a particle only experiences a force if the surrounding field is inhomogeneous, i.e. its gradient is non-zero. Equation 2.2 shows that the resulting torque will drive the magnetic dipole moment into alignment with the magnetic field lines.

2.1.2. Ferromagnetic particles

In absence of an external magnetic field and without prior exposure to one, ferromagnetic materials have a disordered domain structure. As a result, the net magnetization of the material vanishes. To magnetize the material, it has to be exposed to an external magnetic field whose interaction energy with individual domains is strong enough to enforce reordering. The magnetic field at which domains reorient is called the coercive field. It is material-dependent and is usually not an isotropic property of the object. Depending on the crystalline structure of the material, the shape of the object and the structure of its surface, the coercive field can be lowest along certain axes. These axes are called easy axes because the magnetic field required to magnetize the object in these directions is minimal.
Experimental and theoretical background

Compared to superparamagnetic particles*, the coercivity of ferromagnetic particles is often much higher. As a result, strong magnetic fields are necessary to fully magnetize ferromagnetic particles. After removal of the magnetizing field, the particles retain a remanent magnetic moment. In the experiments described in this thesis, we will use this remanent moment to apply forces and torques. Since the magnetic fields that are used to actuate the particles are below the coercive threshold, no significant remagnetization occurs on the time scale of an experiment.

The particles we used in chapters 3 and 4 were ferromagnetic with a diameter of about 4.4 μm (Spherotech, Inc., Lake Forest, IL, see Fig. 2.2). They are made by coating polystyrene spheres with a layer of chromium dioxide. To render the surface bio-compatible and amenable to chemical modification, a final polystyrene shell is applied (see103 for details of the manufacturing process). The surface morphology of the particles is shown in SEM images in Fig. 2.1. In all our experiments, we made use of particles with a carboxyl termination and covalently bound proteins via free amine groups, using EDC/NHS chemistry.

2.1.3. Torque calibration

To determine the torque on the particles upon application of a magnetic field, the magnetic moment of the particles had to be measured. To this end, we fluorescently tagged our particles as described in the methods section of chapter 3 and magnetized them by applying a magnetic field pulse of at least 100 mT. We then suspended them in an aqueous glycerin solution with a water content of about 6 w% and applied a rotating homogeneous magnetic field by powering all four coils of a quadrupole setup (previously described by Janssen et al.104). At a fixed rotation frequency of 1 Hz and magnetic flux densities of more than 5 mT, all particles rotated at the same frequency as the field because the magnetic torque was large enough to overcome the hydrody-

* Superparamagnetic particles consist of ferromagnetic grains embedded in a polystyrene matrix. The spins in individual grains are rapidly randomized due to dominant thermal forces. As a result, the particle is non-magnetic in absence of an external field. In presence of a magnetic field, the individual ferromagnetic domains align and give rise to a larger magnetic moment than commonly observed in paramagnetic materials.
namic drag of the viscous fluid. By decreasing the magnetic flux density in steps of 0.1 mT, we determined the minimum field strength (from hereon called the critical field strength) at which the particles could still follow the field. Below this limit, the applied magnetic torque is lower than the hydrodynamic drag and the particles enter a characteristic ”wiggling” regime of motion \(^{105}\). At the critical field strength \(B_{\text{crit}}\), the magnetic torque equals the hydrodynamic drag:

\[
mB_{\text{crit}} = 8\pi \eta R^3 \frac{d\varphi}{dt}
\]

Here, \(\eta\) is the viscosity of the medium, \(R\) is the radius of the particle and \(d\varphi/dt\) is the rotation frequency of the particle (equal to the field frequency in equilibrium).

We determined the critical field strength for 7 particles from the same sample. We used a value of \(\eta = (0.40\pm0.06)\) Pa·s\(^{106}\) and assumed an uncertainty of 15% due to the strong dependence of the viscosity of aqueous glycerin solutions on temperature and water content. The presence of the surface presents another source of uncertainty as the assumption of a bulk liquid fails for small distances. Simulations have shown that the resulting increase in effective viscosity is less than 20%\(^{107}\). The error resulting from the uncertainty of the applied current and magnetic field was on the order of 1-2% and could be neglected in the face of the uncertainty of the viscosity. The resulting mean magnetic moment in a sample of 7 particles was \(m=2.2\cdot10^{-13}\) Am\(^2\) with a coefficient of variation of 15%. The observed spread of values can be mainly attributed to the imperfect monodispersity of the size of the particles which was approximately 10% for the batch we used. The average remanent magnetic moment we determined is in good agreement with the value of \((2.3\pm0.23)\cdot10^{-13}\) Am\(^2\) that Laurent et al.\(^{100}\) obtained from bulk measurements using a modified vibrating sample magnetometer (VSM) and the Foner method\(^{108}\). De los Santos et al. used a SQUID\(^{1}\) sensor to measure the bulk magnetization of dried particle samples and found a remanent magnetic moment of about \(4\cdot10^{-13}\) Am\(^2\).\(^{109}\)

\(^{1}\) Abbreviation for superconducting quantum interference device.
Experimental and theoretical background

Figure 2.3 – Simulation of a magnetic particle in a rotating magnetic field. At low magnetic field frequencies, magnetic torque and fluid drag are always in balance and the particle can follow the imposed field. Above the critical frequency, the particle movement is intermittently asynchronous with the field and periods of retrograde angular motion (‘wiggles’) begin to appear (see inset). As a result, the average rotation frequency drops. The data was obtained from a Brownian dynamics simulation with parameters that represent the experiment carried out to calibrate the magnetic moment of ferromagnetic Spherotech particles: \( m = 2.2 \times 10^{-13} \text{ Am}^2 \), \( B = 3 \text{ mT} \), \( \eta = 0.4 \text{ Pa s} \), \( R = 2.25 \mu \text{m} \).

The resulting peak magnetic torque on a particle is given by the product of the magnetic moment and the maximum magnetic flux density of the actuating field. At a typical actuating field of 8 mT, the applied magnetic torque is hence about \( 1.8 \times 10^{-15} \text{ Nm} \), or 38 Pa, if normalized by the volume of the particle. This value is comparable to torque amplitudes that have commonly been applied in magnetic twisting cytometry.

2.2. Stiffness measurements

Due to their size and the applicable range of forces and torques, magnetic particles are frequently used to study the local mechanical stiffness of cells. To this end a magnetic field of a well-defined magnitude and gradient is applied to exert a torque on a particle that is in contact with a cell. The displacement of the particle in response to the field can then be measured by a variety of means and be related to the applied torque or force. The quotient of both quantities is a stiffness. To obtain the inherent material property that governs the resistance to deformation, e.g. the shear modulus \( G \), the geometry of the contact region needs to be known. Given the usual complexity of the contact interface between a particle and a cell, the formulation of a suitable model is formidable task. Fortunately, the measured stiffness values can often be processed in alternative ways to extract time-resolved behavior or scaling laws that can yield insight into the structure of the material.

Cells derive their structural stability from an inhomogeneous meshwork of entangled filamentous proteins, called the cytoskeleton. While individual filaments are mainly elastic and can be modeled as bendable rods, the networks they form exhibit viscoelastic properties. This gives rise to rheological phenomena such as stress-strain hysteresis, creep and stress relaxation. These properties are most easily observed in the time-domain. Many micromanipulation studies characterized the viscoelastic properties of cells by analyzing the deformation in response to a constant applied force or (somewhat more difficult to measure) the gradual decrease of stress under
an imposed constant strain. These approaches are suitable if the main focus is on the static material properties. If the probed region of the cell is expected to change dynamically, the applied stress can be modulated with an oscillation of known frequency. The stiffness of the material is then obtained by evaluating the Fourier spectra of applied torque and measured displacement at the applied frequency of actuation. This approach enables measurements in the presence of noise while offering bandwidths of up to 1 kHz that are mainly limited by the maximum frequency of the applied magnetic field.

Figure 2.4 – Oscillatory actuation. (A) A particle in contact with a viscoelastic medium is magnetized to assume a magnetic moment in the $xy$-plane. In a continuously rotating magnetic field in the same plane, the particle experiences a time-varying magnetic torque that is opposed by the complex stiffness of the material. Note: the zero-frequency component of the angle measurement has been removed for clarity. (B) The resulting displacement of the particle assumes a sinusoidal shape and is phase-shifted by $\Delta$ with respect to the applied torque. (C) The Fourier spectrum of the amplitude of $\phi$ exhibits a pronounced peak at the driving frequency (here, $f_a=0.5$ Hz). The absence of higher harmonics indicates that the relationship between torque and angle is linear in the range of applied torques.

In oscillatory actuation, the magnetic field is modulated with a frequency $f_a$ such that the torque on the particle takes on a sinusoidal shape:

$$\tau(t) = \tau_0 \sin (2\pi f_a t). \quad (2.4)$$

In response to the induced torque, the material of the cell will locally deform. This deformation is measured as the phase-delayed angular excursion of the particle (see Fig. 2.4)

$$\varphi(t) = \varphi_0 \sin (2\pi f_a t + \Delta). \quad (2.5)$$

The value of the phase shift $\Delta$ varies between 0° for an ideally elastic material and 90° for a purely viscous environment. Torque and displacement are related to each other by the dynamic modulus $G^*$ which in Fourier space is given by

$$G^*(f) = \frac{\tau^*(f)}{\Phi(f)}. \quad (2.6)$$

If the probed material is linearly viscoelastic, the measured deformation is phase-shifted with respect to the oscillatory actuation, but not distorted. In this case, the Fourier spectrum of $\Phi$ does not contain higher harmonics and the dynamic modulus can be determined by evaluating $G^*$ at the driving frequency $f_a$. The dynamic modulus can be decomposed into its components as follows:

$$G^* = G' + iG''. \quad (2.7)$$
Here, $G'$ and $G''$ denote the storage (elastic) modulus and loss (viscous) modulus, respectively. The quotient $\delta = G''/G'$ is called the loss tangent and provides a scale-free parameter of the general nature of the probed region, $\delta = 0$ being perfectly elastic and $\delta \to \infty$ indicating a purely viscous fluid.

Within the framework of this thesis, we carried out two types of oscillatory experiments. In frequency sweeps, a series of actuation sets at different frequencies is applied to obtain the frequency dependence of the dynamic modulus. In time sweeps, the sample is continuously actuated by a magnetic field at a fixed frequency. In both types of experiments, the magnetic actuation was phase-locked to the recording frequency of the camera, typically such that 15 frames were recorded per actuation cycle. To obtain acceptable signal-to-noise (S/N) ratios, the stiffness was calculated on intervals that comprised several actuation cycles. In a time sweep experiment, the recorded time trace was thus evaluated inside a rectangular window that included integer multiples of one actuation cycle (see Fig. 2.5).

![Figure 2.5 – Extraction of stiffness values from oscillatory actuation. In a typical time-sweep experiment, the measured displacement is evaluated on intervals that each contain cycles. The example depicts a measurement where the character of the probed region gradually became more solid-like. This is indicated by the increase of the storage modulus $G'$ in relation to the loss modulus $G''$ (or the decrease of the loss tangent $\delta$). The absolute increase in stiffness (expressed as $|G^*|$) is relatively small as the dynamic modulus of the material is dominated by its viscous component.](image)

The acquired stiffness values are subject to uncertainty due to measurement noise. To estimate the propagated error, we decompose the dynamic modulus into its components:

$$G^* = \frac{\Re\{\tau^*\Re\{\phi^*\} + \Im\{\tau^*\Im\{\phi^*\}\}}{\Re\{\phi^*\}^2 + \Im\{\phi^*\}^2} + \frac{\Im\{\tau^*\Re\{\phi^*\} + \Re\{\tau^*\Im\{\phi^*\}\}}{\Re\{\phi^*\}^2 + \Im\{\phi^*\}^2}.$$  (2.8)

Here, $\Re\{\cdot\}$ and $\Im\{\cdot\}$ denote taking the real and imaginary part, respectively. We carried out most of our experiments at an actuation frequency of 0.5 Hz where the driving current (and the magnetic field) is not distorted by the self-inductance of the coils. Hence, we assume that the applied torque is perfectly sinusoidal such that the real part of $\tau^*$ vanishes. The absolute value of $G^*$ (as evaluated in chapter 4) can now
be written as

$$|G^*| = \sqrt{\mathcal{I}\{\tau^*\}\mathcal{I}\{\phi^*\})^2 + \mathcal{I}\{\tau^*\}\mathcal{R}\{\phi^*\})^2 \over \mathcal{R}\{\phi^*\})^2 + \mathcal{I}\{\phi^*\})^2}. \quad (2.9)$$

The uncertainty of $|G^*|$ can then be approximated by the total differential

$$\Delta |G^*| = \left| \frac{\partial |G^*|}{\partial \mathcal{I}\{\tau^*\}} \right| \Delta \mathcal{I}\{\tau^*\} + \left| \frac{\partial |G^*|}{\partial \mathcal{I}\{\phi^*\}} \right| \Delta \mathcal{I}\{\phi^*\} + \left| \frac{\partial |G^*|}{\partial \mathcal{R}\{\phi^*\}} \right| \Delta \mathcal{R}\{\phi^*\}. \quad (2.10)$$

The uncertainty $\Delta \mathcal{I}\{\tau^*\}$ has to be estimated for the experimental setting and depends on the alignment of the magnetized particles with the applied field and the variability of the magnetic moment in a sample of particles. To obtain estimates of the uncertainties $\Delta \mathcal{R}\{\phi^*\}$ and $\Delta \mathcal{I}\{\phi^*\}$, we consider the measurement noise in the Fourier spectrum of the angular displacement. We assume that the measured time course is a superposition of the real angular displacement of the particles, $\phi_a$, with measurement noise $e$

$$\varphi(t) = \varphi_a(t) + e(t), \quad (2.11)$$

where we assume that $\varphi_a$ is a harmonic function that lags the applied torque and that $e$ is normally distributed white noise with zero mean and variance $\sigma^2$. We calculate $e(t)$ on the examined interval by subtracting spectra in Fourier space and transforming back into the time domain:

$$e(t) = \mathcal{F}^{-1}\{\Phi - \Phi\delta(f_a)\}. \quad (2.12)$$

Here, $\delta$ is the Dirac delta function. The variance of the noise can now be estimated from a Gaussian fit of the histogram of amplitudes in $e(t)$. Finally, we write the uncertainties of the determined Fourier components at the driving frequency $f_a$ as

$$\Delta \mathcal{R}\{\phi^*\} = \Delta \mathcal{I}\{\phi^*\} = \sqrt{2\sigma^2 \over L}. \quad (2.13)$$

Here, $L$ denotes the number of samples on the considered interval. To decrease the uncertainty of the determined stiffness values, it is hence advisable to strike a balance between sampling length and desired time resolution.

### 2.3. Particle image tracking

With the rapid rise of digital image acquisition methods came the need to rapidly process large amounts of visual data. A common task is the position detection of point-like light sources in an image, often with the aim of reconstructing their trajectories. Within an appropriate theoretical framework, these trajectories can then be analyzed to reveal the properties of the underlying physical system. A plethora of algorithms has been created to track point-like objects under a variety of conditions. This section gives a short introduction to particle tracking in general and the circular Hough transform in particular.
2.3.1. Conventional methods

Particles have been used to manipulate and tag molecular and cellular targets. Due to the scaling relationship between applicable force and particle size, the manipulation of cellular matter often requires micron-sized particles whose diameter is clearly above the diffraction limit of typical light microscopes. Particles that serve as tags, however, are commonly selected to be as small as possible to avoid disturbing the native diffusion behavior of the molecule they are attached to. Due to their small size, these particles can no longer be resolved by conventional light microscopy and appear as diffraction-limited spots. In this size range, the tracking of particles is similar to the tracking of individual fluorescent molecules.

2.3.1.1. Centroid tracking

In centroid tracking a particle is considered a two-dimensional set of pixels whose center of mass is equivalent to the position of the particle. This algorithm is time-efficient, it does not impose restrictions on the shape of the particle and it can be used at different length scales. Depending on the image quality, the performance of the algorithm can be substantially reduced by additional filter operations that are required to suppress noise and background. To determine the pixels that represent the particle, the image is typically thresholded. To decrease the dependence on a fixed threshold value, the coordinates of each pixel are weighed by their respective intensity. For large targets like the magnetic particles used in this thesis, centroid tracking is more susceptible for position bias than cross-correlation tracking at signal-to-noise ratios of less than 6. This algorithm has been used for real-time analysis of magnetic twisting cytometry data, interactions between colloidal particles, passive microrheology and for tracking the Brownian motion of individual membrane constituents.

2.3.1.2. Gaussian fitting

The image of a single spherical (approximated as a point source) particle with a diameter below the diffraction limit (≈ 200 nm) arises as the convolution of the light source with the point-spread function (PSF) of the imaging system. For circular apertures, the resulting intensity distribution is an Airy pattern with a pronounced zero-order that is commonly approximated by a Gaussian distribution. The positions of single particles can hence be found by directly fitting two-dimensional Gaussians to the image data. In the diffraction-limited regime, this method has been shown to operate better than centroid tracking or cross-correlation tracking, both in terms of position bias and position variance. Gaussian fitting should not be used for particles that are larger than approximately 500 nm since their intensity pattern is no longer Gaussian. In the past, Gaussian fit tracking has been frequently used in fluorescence microscopy studies of single molecules, an approach often called fluorescence speckle microscopy (FSM). More recently, Gaussian fits were employed to accurately determine the positions of single fluorophores in images recorded with super-resolution techniques such as STORM and PALM.

---

This list is by far not exhaustive in its breadth but focuses on references that explicitly mention centroid tracking as the used tracking routine.
2.3.1.3. Cross-correlation

Particles that are larger than the diffraction limit are often tracked by cross-correlating a stationary image of the particle (called a kernel) with the recorded images. The position of the particle is then equivalent to the position of the maximum in the cross-correlation map. At S/N ratios below 4, cross-correlation tracking exhibits very low bias, but has a higher position uncertainty than centroid tracking. At higher S/N ratios, both algorithms perform equally well\textsuperscript{111}.

2.3.2. The Hough transform

The magnetic particles we used here are much larger than the diffraction limit of the imaging system. As a result, the particles appear as circular areas with relatively sharp edges that separate them from the background. If no fluorescent markers are present on the particles and the depth of field is larger than one particle diameter, their positions can be determined by centroid tracking or by cross-correlation tracking (see above). While these algorithms are time-efficient, they are prone to errors if the intensity distribution of the visible part of the particle is not stationary and the particles are free to rotate. This is the case if fluorescent markers are present on the particle or if as a result of a small depth of field, parts of the particle are not in focus and imperfections are visible. In those situations, the intensity pattern would change during rotation of the particle and a conventional tracking algorithm would recognize translation where there is none. However, the circular circumference of the particle would remain stationary and can hence serve as a feature for detection.

The Hough transform has originally been invented by Paul Hough\textsuperscript{120} to automate the detection of lines in bubble chamber images. It was later extended to detect other parametric objects like circles and ellipses\textsuperscript{121}. Nowadays, the Hough transform is routinely used in computer vision applications.

The general idea of the Hough transform is to find a set of pixels that are part of the same geometric object. To this end, candidate pixels are selected and their locations are mapped into a space (called the accumulator) whose dimensions represent the parameters that are necessary to describe the object to be found. The location of the object in the image space then emerges as a maximum in the parameter space.

To find the outline of a circular particle, the pixels at its edge are the candidate pixels that serve as input for the Hough transform. The determination of edge pixels \((x, y)\) can be carried out by a number of image segmentation and filtering techniques. The simplest approach is to calculate the gradient matrix of the image and selecting the locations of its local maxima as candidates. More sophisticated methods of finding edges include the Canny filter, the Sobel filter or morphological operations like the watershed transformation.

The acquired set of candidate pixels ideally lie on the same circle. In Cartesian coordinates, a circle is represented by the expression

\[
R^2 = (x - a)^2 + (y - b)^2 ,
\]

where \(R\) is the radius of the circle and \((a, b)\) the location of its center. The parameter space for circles is three-dimensional and is spanned by \(a, b\) and \(R\). Assuming that a pixel \((x, y)\) in the image space is part of a circle with unknown size and location, the locus of possible parameters \((a, b, R)\) is represented by a circular cone in the accumulator space (see Fig. 2.6). Each point on the wall of the cone describes a circle that the pixel \((x, y)\) could be a part of. If for every pixel on a circle, the respective locus in
Experimental and theoretical background

Figure 2.6 – Overview of the circular Hough transform. (A) A set of candidate pixels that form the edge of at least one circle is acquired by filtering or image segmentation. (B) For each candidate pixel, the locus of potential parameters is constructed. The resulting cones overlap in parameter space at points \((a, b, R)\). For clarity, only two cones corresponding to the candidate pixels \((12, 23)\) (blue cone) and \((21, 15)\) (red cone) are depicted. (C) For each radius, the overlapping cones give rise to an intensity distribution. In the depicted case, the local maxima of these distributions lie in the planes \(R=8\) and \(R=15\). The locations of these maxima correspond to the center positions of the circles in the original image. The centers of the circles in the original image are thus located at \((20, 23)\) and \((34, 17)\) and have the radii 8 and 15, respectively.

the parameter space is drawn, the point where a maximum number of cones intersect represents the location and size of the circle in the image space. The detection of a circle in image space is thus transformed into the search for a maximum in parameter space.

Finding the correct maxima in the accumulator space poses additional difficulties as every plane has at least one local maximum. Especially if the number of circles in the image space is unknown, it is not sufficient to simply select the most pronounced maxima. Since the intensities of maxima in the planes with constant \(R\) represent the number of intersecting parameter loci, they are proportional to the number of pixels on a circle. Given a certain image resolution, the values of the maxima in the parameter space should thus be normalized by their respective radii to formulate a general selection criterion.
2.4. The cell membrane

The interaction of a cell with its environment is crucially determined by the functionality of the cell membrane. Separating intra- and extracellular space, the membrane serves as a semipermeable barrier and enables tight control of the plethora of chemical reactions that drive every process in the cellular life cycle. Nowadays, the view of the cell as a mere reaction vessel has long been abandoned and substantial research efforts are directed at the mechanical aspects of cell biology. Since the research in this thesis is concerned with the mechanical interactions between cells and surfaces, we here give an overview of the structure of the membrane and provide the mathematical basis for the modeling in chapters 4 and 5.

2.4.1. Membrane structure

The cell membrane principally consists of lipid molecules and proteins. Under physiological conditions, the membrane is typically associated with a thin sheet of filamentous proteins, called the cortical cytoskeleton.

2.4.1.1. The lipid bilayer

Lipids constitute the vast majority of molecules in a typical cell membrane and are present at a density of about $10^6$ per $\mu m^2$. The main components of all cell membranes are phospholipids, cholesterol and glycolipids. All membrane lipids have a hydrophobic (nonpolar) and a hydrophilic (polar) region. Phospholipids have a total length of about 3 nm and consist of a polar head group and two hydrocarbon tails. There is a large variety of phospholipids which differ with respect to their head groups and the length and shape of their tail groups. Cholesterol intercalates with phospholipids and serves to modulate the fluidity of the membrane. Glycolipids allocate to the extracellular side of membranes and provide the cell with a protective coating of sugar residues. In addition, some glycolipids are thought to mediate cell-cell adhesion by performing as ligands for a group of carbohydrate-binding membrane proteins, called lectins.

Membrane lipids spontaneously self-assemble to minimize the exposure of nonpolar regions to polar water molecules. Depending on the shape (intrinsic curvature) and the polarity of individual molecules, the resulting structures resemble flat sheets, spheres, or cylinders. Given the right concentration, some lipids form spherical vesicles that enclose a hydrophilic core inside a curved bilayer. Such minimal model systems can be reconstituted in a laboratory and are frequently used to study the interaction of membranes with surfaces and with other vesicles. The spherical shape of a freely suspended cell is hence a consequence of the minimal energy state of an ensemble of lipid molecules.

Due to the hydrophobic interactions that shape a bilayer, individual molecules rarely switch sides between the two monolayers. However, they are free to rotate about their long axis and to diffuse throughout the monolayer by swapping positions with neighboring molecules. To determine the mobility of lipids in bilayers fluorescent dyes and small gold particles have been attached to their head groups. By either following individual lipids or studying ensemble dynamics, the associated diffusion constant of lipid molecules was determined to be on the order of 1 $\mu m^2/s$. As a consequence, a lipid bilayer cannot withstand shear forces and is frequently termed a 'two-dimensional fluid'.
Experimental and theoretical background

Figure 2.7 – Overview of the structure of the cell membrane. The cell membrane comprises a bilayer of lipid molecules that self-assemble to minimize the exposure of their hydrophobic sections. Specialized membrane proteins are lodged into the bilayer and perform a variety of tasks. Integrins span the bilayer and establish mechanical connections between the extracellular space and the intracellular cytoskeleton (via adaptor proteins). Other membrane proteins, such as glycoprophatidylinositol(GPI)-anchored ones like CD14 (see chapter 3) do not have an intracellular domain.

Lipid bilayers minimize exposure of their hydrophobic regions to the surrounding aqueous solvent. As a result, bilayers exhibit a finite resistance to stretching and bending. Similar to macroscopic thin sheets (e.g. sheets of metal), lipid bilayers can undergo dramatic bending fluctuations without failing while intraplanar strains of more than 5% will lead to rupture.

2.4.1.2. Membrane proteins

A pure lipid bilayer as a cell membrane fulfills the purpose of isolating the intracellular space from its environment but does not provide a means of communication. This task is performed by dedicated proteins that are inserted into the membrane such that their hydrophilic regions face the extracellular space or the cytoplasm and their hydrophobic regions are in contact with the non-polar parts of the surrounding lipid molecules. The principle of hydrophobic matching in combination with the specific protein structure give rise to a variety of ways by which proteins associate with the lipid bilayer.

A large group of membrane proteins possess both intracellular and extracellular domains and span the entire lipid bilayer (see Fig. 2.7). Examples of such proteins are ion channels and integrins. By changing their conformation, some of these proteins can switch between an inactive and an active state to become selectively permeable for ions (in case of channel proteins) or to transmit force (in case of integrins). Similar
to lipid molecules, membrane proteins can freely diffuse in the membrane although the cortical cytoskeleton sets limits to this motion (see section 2.4.1.3).

A lot of membrane proteins perform a signaling function by binding extracellular molecules (ligands) and triggering a chemical pathway inside the cell. Important examples are the epidermal growth factor (EGF) receptor and the Fc receptor. The former binds the polypeptide EGF which triggers cell growth, differentiation and proliferation\(^{127}\). The Fc receptor binds the crystallizable fragment of immunoglobulins and is strongly involved in the recognition and neutralization of pathogens\(^{128}\).

### 2.4.1.3. The cortical cytoskeleton

Essential processes such as cell crawling, division and phagocytosis require the cell to exert forces on their environment. This is achieved by the cytoskeleton, a heterogeneous network of filamentous structural proteins. The most important cytoskeletal proteins are actin, intermediate filaments and microtubules.

The organization of the network differs between cell types and depends on the task a cell has to perform. In animal cells most actin filaments are located in a meshwork lining the intracellular side of the plasma membrane, called the actin cortex. This meshwork is approximately 0.5 to 1 μm thick and has a mesh size of about 0.1 μm\(^{129,130}\). The cortex is linked to the cell membrane by adaptor proteins such as ezrin, radixin and moesin, collectively known as ERM proteins\(^{131}\). Via specific tail regions, these proteins simultaneously bind to membrane proteins and actin filaments. Other actin-binding proteins such as vinculin and talin possess positively charged groups that enable the aspecific adsorption to the cell membrane.

By association with membrane proteins, the cortex does not only impart stability but also contributes to the spatial organization of receptors. As specific proteins in the membrane form links with the underlying cytoskeletal meshwork, their diffusivity is greatly reduced and they effectively become immobile obstacles for other proteins. This mechanism gives rise to a compartmentalization of the membrane that has first been recognized and described within the framework of the fluid-mosaic model by Singer and Nicolson in 1972\(^{132}\).

### 2.4.2. Membrane mechanics

#### 2.4.2.1. Membrane stretching

In equilibrium, the molecules in a lipid bilayer have a mean spacing \(a_0\) that is determined by their attractive and repulsive energies. Stretching the membrane increases the average spacing and exposes the hydrophobic tail regions of the lipids to the surrounding water. This increase in interfacial energy generates a restoring force and bestows elasticity on the membrane. To arrive at an expression that relates the energy of a lipid-water interface to a mechanical modulus, we consider the interfacial energy of a single molecule.

The energy required to fully expose one lipid molecule is given by the interfacial tension of the lipid-water interface \(\gamma\), divided by the density of lipids in the membrane \(a_0^{-2}\). At typical values of \(\gamma=50\ \text{mN/m}\) and \(a_0=1\ \text{nm}\), the resulting energy per lipid is between 10 and 20 kT. This energy is comparable to the binding energies of non-covalent bonds and underlines that a lipid membrane is structurally stable at typical laboratory time scales and temperatures.

In a first approximation, the energy of repulsion scales with the intermolecular spacing as \(a^{-2}\).\(^{133}\) The interfacial energy of a single lipid as a function of the mean
area $A = a^2$ it covers is then given by\textsuperscript{134}

\[ E = \frac{\alpha}{A} + \gamma A, \tag{2.15} \]

where $\alpha$ is an arbitrary scaling constant. From $\partial E/\partial A=0$ follows the covered area at equilibrium as $A_0 = \sqrt{\alpha/\gamma}$. By Taylor expansion the interfacial energy can now be written as (see Fig. 2.8)

\[ E = 2\gamma A_0 + \frac{\gamma}{A_0} (A - A_0)^2. \tag{2.16} \]

Dividing $E$ by $A_0$ gives the energy density for elastic deformation which for small deformations can also be expressed as\textsuperscript{134}

\[ \frac{E}{A_0} = 2\gamma + \frac{K_A}{2} (u_{xx} + u_{yy})^2, \tag{2.17} \]

where $K_A$ is the area expansion modulus and $u_{xx}$ and $u_{yy}$ are the diagonal components of the strain tensor during biaxial stretching of the membrane. Comparison of eqs. 2.16 and 2.17 readily yields an estimate of the expansion modulus in terms of the interfacial energy:

\[ K_A = 2\gamma. \tag{2.18} \]

This value of $K_A$ is valid for a monolayer of lipid molecules and twice as large for a bilayer.

The area expansion modulus can be experimentally determined by recording the stress-strain relationship of a membrane. This is typically done by partially aspirating a cell or a lipid vesicle into the orifice of a micropipette with an inner diameter of a few micrometers. As a lipid bilayer forms a vesicle, a surface tension $\sigma$ appears in the membrane to maintain the curvature. The Young-Laplace equation states that
the pressure difference $\Delta p$ across a thin-walled spherical vesicle of radius $R$ is then given by

$$\Delta p_{\text{ves}} = 2 \frac{\sigma}{R}. \tag{2.19}$$

In an experiment, the pressure difference $\Delta p_{\text{int}}$ between the interior of the micropipette and the inside of the vesicle results in partial aspiration and the formation of a spherical cap with radius $R_1$:

$$\Delta p_{\text{int}} = 2 \frac{\sigma}{R_1}. \tag{2.20}$$

By controlling the pressure difference $\Delta p = \Delta p_{\text{int}} - \Delta p_{\text{ves}}$ and measuring the protrusion of the vesicle into the pipette, the tension in the membrane can be determined from $^{135,136}$

$$\sigma = \frac{\Delta p}{2} \frac{R_1}{1 - \frac{R_1}{R}}. \tag{2.21}$$

Aspirating the vesicle with sufficient pressure results in an extension of its surface area. As a result, the membrane tension increases proportionally to the area expansion modulus:

$$\sigma = K_A \frac{\Delta A}{A_0}. \tag{2.22}$$

The change in surface area can be determined from micrographs under the assumption of rotational symmetry about the long axis of the pipette. By then equating eqs. 2.21 and 2.22, an expression is found that allows to extract the area expansion modulus from experiments as the slope of the curve $\Delta A/A_0$ vs. $\Delta p$.

The derivations above establish a link between the molecular composition of a lipid membrane and its macroscopically accessible properties. Importantly, the above equations have been derived for single-component lipid vesicles. The structure of cell membranes is considerably more complex as membrane proteins, mixtures of lipids and, perhaps most importantly, the underlying cytoskeleton can be expected to influence the tension in the membrane. If active cell locomotion is avoided, one can still measure an effective cortical tension but the link to the molecular scale is less clear. The micropipetting approach has first been used in 1954$^{137}$ to determine the elastic membrane properties of red blood cells and the method and its analysis have undergone multiple refinements$^{55,56}$. In later experiments with migrating cells such as granulocytes$^{68,138,139}$ and macrophages$^{140}$, it was essential to inhibit active cell motion into or out of the micropipette by tuning the ionic composition of the buffer solution and the surface treatment of the micropipette.

2.4.2.2. Membrane bending

As mentioned above, membranes exhibit considerable resistance against an increase of their surface area. Owing to their thickness of just a few nanometers (for pure lipid bilayers), comparatively little energy is required to produce large bending deformations. The energy required to bend a membrane depends on the curvature and the intrinsic resistance to bending, which in turn is determined by the molecular composition. It is expressed as the Helfrich-Canham free energy$^{141,142}$

$$E = \int \left( \frac{K_b}{2} (C_1 + C_2 - C_0)^2 + \kappa_G C_1 C_2 \right) dA. \tag{2.23}$$
The shape of a bent membrane is described as a surface $h(x,y)$. If the function $h(x,y)$ is known, its curvature can be calculated from the local tangents to the surface. The mean curvature is the arithmetic mean of the minimum and the maximum (principal) curvatures in a point. Their directions in $P$ are delineated by blue lines.

Here, $C_1$ and $C_2$ are the principal curvatures of the membrane surface, $C_0$ is the spontaneous curvature, $\kappa_b$ denotes the bending energy and $\kappa_G$ is the Gaussian bending rigidity. The contribution of the Gaussian curvature $C_1 C_2$ is commonly neglected as it is invariant under deformation of the membrane and only contributes to the free energy if the topological configuration of the membrane changes, e.g. during transition from a sphere to a torus. To compute the free energy of a deformed membrane, one hence needs to find an expression for the mean curvature $(C_1 + C_2)/2$.

For simple geometries such as spheres or cylinders, the curvature can be easily determined but more complex shapes require a generalized approach. The membrane is commonly described as a suspended sheet whose local deformations are captured by the height function $h(x,y)$ in an external Cartesian coordinate system (see Fig. 2.9). Within the Monge representation, the function $h(x,y)$ is uniquely defined for each $(x,y)$, i.e. membrane overhangs are not permitted. Analogous to the description of a filamentous polymer, the surface is locally described by the unit normal vector

$$n = \frac{r_x \times r_y}{|r_x \times r_y|} = \frac{1}{\sqrt{1 + h_x^2 + h_y^2}} \begin{pmatrix} -h_x \\ -h_y \\ 1 \end{pmatrix},$$

(2.24)

where $r_x$ and $r_y$ are the local tangent vectors of $h$ in $x$- and $y$-direction, represented by $(1, 0, h_x)$ and $(0, 1, h_y)$, respectively. $h_x = \partial h/\partial x$ and $h_y = \partial h/\partial y$ denote the local slopes in direction $x$ and $y$.

The local curvature of a surface is defined as

$$C = n \frac{\partial^2 r}{\partial s^2},$$

(2.25)

where $s$ is a step in an arbitrary direction. This implies that the curvature in a point $h(x,y)$ depends on the direction of the tangent. The curvature in a point is uniquely described by two principal curvatures, i.e. the maximum and minimum values of $C$. In Cartesian coordinates, a lengthy derivation leads to the following expression for the mean curvature:

$$\frac{C_1 + C_2}{2} = \frac{1}{2} \left( \frac{1 + h_x^2}{h_{yy}} + \left(1 + h_y^2\right) h_{xx} - 2h_x h_y h_{xy} \right) \frac{1}{(1 + h_x^2 + h_y^2)^2}$$

(2.26)
In chapter 4 we calculate the energy of a deformed membrane in an axisymmetric model where the height of the membrane becomes a function of the radius $r$ and the mean curvature is given by:

$$
\frac{C_1 + C_2}{2} = \frac{1}{2} \left( \frac{h_{rr}}{(1 + h_r^2)^{3/2}} + \frac{h_r}{r (1 + h_r^2)^{3/2}} \right).
$$

(2.27)

Expressions 2.26 and 2.27 can only be evaluated if the shape function $h$ is known. In equilibrium, the shape of the membrane can be found by imposing appropriate boundary conditions and minimizing $E$.

2.4.2.3. Energy minimization

The results from the preceding sections can be combined by writing the energy of deformation as

$$
E = \int \left( \frac{K_b}{2} (C_1 + C_2 - C_0)^2 + \sigma \right) dA.
$$

(2.28)

In this equation the two main contributions to the membrane energy are taken into account. The surface tension acts to reduce the surface area of the membrane and the bending energy minimizes the mean curvature.

The functional in eq. 2.28 allows to compute the energy of a membrane configuration given by the function $h$. The exact shape of the membrane depends on the parameters of eq. 2.28 and is generally only known at its boundaries. In equilibrium, $h$ is a solution of eq. 2.26 (or 2.27) that minimizes $E$ and can be found by invoking the appropriate Euler-Lagrange equation. In chapter 4 the shape of the membrane is described in a one-dimensional axisymmetric system. In this case, the energy functional has the form

$$
E = \int L(r, h_r, h_{rr}) dr
$$

and its stationary solution can be found by solving

$$
0 = \frac{\partial^2}{\partial r^2} \left( \frac{\partial L}{\partial (h_{rr})} \right) - \frac{\partial}{\partial r} \left( \frac{\partial L}{\partial (h_r)} \right).
$$

(2.29)

This fourth-order non-linear differential equation can generally only be solved numerically. Only under assumption of small deformations, an analytical solution of eq. 2.29 can be found.

The energy functional in eq. 2.28 can be extended to include the following additional factors that influence the free energy of the membrane:

(i) Differential stretching - The above description of bending assumes that the membrane is a thin sheet with negligible thickness. If a lipid bilayer is bent, the inner monolayer is stretched while the outer monolayer is compressed. This effect is captured in the model of area-difference elasticity.

(ii) Suppression of membrane undulations - At low tension, the membrane is undergoing thermally driven bending fluctuations, similar to an unstretched polymer in solution. Work needs to be done to suppress these undulations, for example by applying tension on the membrane through aspiration in a micropipette. The unstretched surface area $A_0$ hence refers to the macroscopic area of the cell, after suppression of the thermally induced wrinkles. This effect has first been described by Helfrich and Servuss and was later refined by Evans and Rawicz.

(iii) Volume constraints - An increase in volume at constant pressure difference across the membrane reduces the surface tension. In most experiments, the total change in volume is negligible.
(iv) Constant surface tension - The surface tension is assumed to be constant. This approximation is valid for small areal strains and if the cell can provide additional membrane to buffer an increase in tension\(^{68,149,150}\).

2.5. Cell-surface interactions

2.5.1. Nonspecific interactions

The non-specific adhesion of biological membranes to surfaces is mediated by the fundamental interactions that are commonly found in colloidal systems. Primarily, these are electrostatic interactions, van der Waals (vdW) interactions and hydrophobic interactions. Electrostatic and vdW interactions have been summarized in the DLVO theory and are often sufficient to describe the stability of colloidal suspensions and the adhesion of particles to charged surfaces\(^ {151}\). The interaction of biological membranes with surfaces is more complicated since biological membranes and their constituents (e.g. lipid molecules and membrane proteins) exhibit substantial thermally induced deformations. These fluctuations give rise to additional interactions that are often repulsive. In the following sections elementary aspects of membrane adhesion are discussed.

2.5.1.1. Van der Waals interaction

Van der Waals forces arise as the electric dipoles of neighboring atoms interact and align under a minimization of free energy. vdW forces are usually attractive but can be repulsive for certain pairings of interacting surfaces. The vdW interaction energy per area between two flat surfaces is given by\(^ {151}\)

\[
E_{vdW} = -\frac{H}{12\pi h^2}.
\]  

(2.30)

Here, \(H\) is the Hamaker constant and \(h\) is the separation between the two surfaces. This equation is valid if the spacing between the surfaces is small compared to the thickness of the lipid bilayer (typically on the order of 5 nm) and has to be corrected for larger separations\(^ {52,153}\). With the force density between the two surfaces given by \(F = -\frac{1}{h}\frac{\partial E}{\partial h}\), the Hamaker constant is positive for attractive vdW interactions and negative for repulsive interactions. The Hamaker constant is typically on the order of \(10^{-20}\) J\(^ {151}\). At a separation of \(h = 0.4\) nm which assumes that each surface has a monolayer hydration shell, the specific interaction energy is approximately \(1.7\) mJ/m\(^2\). For a circular interaction area of radius 10 nm, the resulting interaction energy is approximately 120 kT. This energy could arise from the interaction of about 80 lipid molecules (spaced 1 nm apart) with a surface and is several times higher than that of strong specific ligand-receptor pairings. However, it clearly cannot be the only interaction potential at play because cells usually do not spontaneously adhere to uncoated surfaces or to each other.

2.5.1.2. Electrostatic interaction

The outer face of most cell membranes is negatively charged as a result of dissociation of membrane-bound species. The main contribution stems from gangliosides, a group of glycolipids, and membrane-associated glycoproteins. Both contain the monosaccharide sialic acid whose negative charge provides the cell membrane with a hydration shell and prevents the nonspecific clustering of cells.
Figure 2.10 – The electrostatic interaction of a cell membrane with a substrate. (A) When in contact with an aqueous solution, dissociation of head groups creates negative charges on the surface of the cell membrane that are partially neutralized by counterions from solution. Shown here is a substrate that has been chemically modified to acquire a positive charge. (B) If $\sigma_1/\sigma_2 > 0$, the cell membrane will always be repelled by the surface but if $\sigma_1/\sigma_2 < 0$, the membrane can approach the surface and reach an equilibrium position that only vanishes if $\sigma_1 = |\sigma_2|$. Panel 2.10B has been adapted from ref. 154.

In aqueous solutions that contain ions, the dissociation of head groups and the absorption of (co-)ions from solution gives rise to an electric double layer where the attraction between positive surface charges and the negative ions is balanced by the entropic pressure that drives ions away from the surface. This competition is captured by the Poisson-Boltzmann (PB) equation. The linearization of the PB equation proposed by Debye and Hückel results in the characteristic length scale with which the electric potential decays due to ionic screening:

$$\kappa^{-1} = \sqrt{\frac{\varepsilon_0 \varepsilon_r kT}{2 N_A e^2 I_c}}.$$  \hspace{1cm} (2.31)

Here, $\kappa^{-1}$ is the so-called Debye length, $\varepsilon_0$ is the vacuum permittivity, $\varepsilon_r$ is the relative permittivity of the solvent ($\varepsilon_r = 80$ for water), $N_A$ is the Avogadro constant and $I_c$ is the ionic strength of the solvent. The Debye length only depends on the conditions of the solvent and is about 1 nm in typical physiological buffer solutions with ionic concentrations on the order of 100 mM.

If two surfaces with surface charge densities $\sigma_1$ and $\sigma_2$ interact with each other, their electric double layers overlap. The resulting pressure between two plates at spacing $h$ and the corresponding free energy per unit area are given by\textsuperscript{154–157}

$$P(h) = \frac{8 \pi \sigma_2^2}{\varepsilon_0 \varepsilon_r} \left[ 1 + \frac{2 \sigma_1}{\sigma_2} \cosh \kappa h + \left( \frac{\sigma_1}{\sigma_2} \right)^2 \right] \frac{(\sinh \kappa h)^2}{(\sinh \kappa h)^2}$$ \hspace{1cm} (2.32)

$$E_{el}(h) = -\int_{\infty}^{h} Pdh = \frac{2 \pi}{\varepsilon_0 \varepsilon_r \kappa} \left( \sigma_1^2 + \sigma_2^2 \right) \exp (-\kappa h) + 2 \sigma_1 \sigma_2.$$ \hspace{1cm} (2.33)

If the charges on both surfaces have equal sign, the interaction will always be repulsive. For charge densities with different signs, the surfaces will be attracted to each other at large separations but will generally not be able to make contact unless $|\sigma_1| = |\sigma_2|$. This effect is caused by the entropic pressure of increasingly confined counterions and is illustrated in Fig. 2.10. A similar equation exists for the assumption of constant surface potentials. The validity of eqs. 2.32 and 2.33 is limited to
Experimental and theoretical background

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Charge density [Cm$^{-2}$]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cell</td>
<td>-0.038</td>
<td>Georgieva et al.$^{158}$</td>
</tr>
<tr>
<td>Liver cell</td>
<td>-0.022</td>
<td>Dobrzynska et al.$^{159}$</td>
</tr>
<tr>
<td>Thromobocyte</td>
<td>-0.01</td>
<td>Kotynska et al.$^{160}$</td>
</tr>
</tbody>
</table>

Table 2.1 – Charge densities on cell membranes. Charge densities on most cells range between 0.01 and 0.1 Cm$^{-2}$ and are negative at physiological pH.

small surface potentials ($\psi < 25$ mV) or to large separations ($\kappa h > 1$)$^{154}$. At small separations, the assumption of constant surface charge density fails as counterions from solution will recombine under the increasing electrostatic pressure$^{152}$.

Estimates of the surface charge density can be obtained by measuring the electrophoretic mobility of the cells in an electric field. At physiological pH, the surface charge density of most eukaryotic cells is on the order of -0.01 Cm$^{-2}$, equivalent to about one negative charge per 16 nm$^2$. Table 2.1 gives an overview of typical surface charge densities.

The above equations treat the cell membrane as a surface with a uniform charge density. This assumption only holds for single-component lipid vesicles. In multi-component vesicles the lateral diffusion of lipids in the outer monolayer can readily lead to non-uniform charge distributions as non-ionic lipid molecules are driven away from the contact interface. Nardi et al.$^{157,161}$ studied this unmixing process theoretically and experimentally and found that the adhesion zone between a charged lipid membrane and an oppositely charged surface takes on a patchy appearance as islands of strong adhesion emerge. In these zones, the equilibrium density of surface charges on the membrane is locally matched by the fixed density on the surface, i.e. $\sigma_1 = |\sigma_2|$. Those parts of the membrane that are not in close contact with the surface exhibit a lower charge density and form blister-like structures that bulge under the osmotic pressure inside the compartments that they form. These effects have been observed in red blood cells in contact with positively charged polylysine$^{72,162}$.

The surface charge density on a functionalized surface can be estimated from the number density of functional groups. Microparticles and other polymeric surfaces are often terminated by carboxyl groups to enable convenient conjugation of proteins. These groups are typically spaced apart to occupy an area of 1 to 12.5 nm$^2$$^{163}$, giving rise to a theoretical maximum charge density of -0.16 Cm$^{-2}$. However, at typical pK$a$ values of about 3 to 5, not more than 0.1% of these groups will deprotonate in a neutral aqueous solution and the expected surface charge density will hence assume a value much closer to zero. Once a particle is functionalized with proteins, the resulting overall charge density is mainly determined by the structure and orientation of the protein. The number density can be estimated from the theoretical packing limit of proteins on the surface of the particle which, owing to the size of a protein, is typically much lower than for carboxyl groups. Assuming that one protein has lateral dimensions of about 5 nm, the maximum number density is expected to be about 0.04 nm$^{-2}$. If each protein displays one effective charge, the surface charge density should be on the order of 0.005 Cm$^{-2}$. Measurements on nearly complete monolayers of streptavidin indeed revealed an effective surface charge density of about -0.019 Cm$^{-2}$$^{164}$ at neutral pH and a comparatively low ionic concentration.
2.5.1.3. Steric interactions

Cells and functionalized substrates usually do not possess smooth surfaces but are covered by flexible hydrophilic macromolecules with dimensions of the same order of magnitude as other relevant length scales, such as the Debye length. Bringing two such surfaces into close proximity results in confinement of the molecules on both surfaces. The ensuing reduction of entropy gives rise to a repulsive force between the surfaces. This effect bestows a protective function on the dense layer of glyco-proteins (glyocalyx) on the outer membrane of most eukaryotic cells as it prevents the adhesion of bacteria. To stabilize colloidal systems entropic repulsion is often leveraged by grafting polymers onto the surfaces of particles.

The structure of polymers is characterized by the contour length $l$ of the chain and its persistence length $l_p$. The latter is an intrinsic property of the polymer and depends on its bending rigidity. If the polymer is shorter than its persistence length, it acts like a stiff rod. If $l > l_p$, the polymer curls up to maximize its configurational entropy. If the polymer is solvated by a solvent that minimizes interaction of the chain with itself, it will assume a compact conformation that is characterized by the radius of gyration, $R_g$ (see Fig. 2.11A). Not surprisingly, the steric repulsion between two surfaces of which at least one is decorated with polymers is dependent on the size of the polymer and on the solvent. Additionally, the steric interaction is influenced by the surface concentration of the polymers and by the way the molecules are coupled to the surface.

Depending on the functionalization of the surface, different interaction potentials have to be considered. If both surfaces are covered by low concentrations $c_0$ of polymers, the interaction energy between the surfaces is approximately given by

$$E_{st} = 36kTc_0 \exp \left( -\frac{h}{R_g} \right).$$  

(2.34)

---

$^5$ At low concentrations polymers of the same surface do not overlap with each other and acquire a globular shape. This concentration regime is called the mushroom regime. At high concentrations, interactions between the polymers straighten them out, giving rise to the term brush regime.
This expression is valid for separations down to $h = 2R_g$. If only one of the surfaces carries a polymeric coat, the following expression can be used:

$$E_{st} = \frac{\pi^2}{6} kT c_0 \left( \frac{R_g}{h} \right)^2 \exp \left[ -1.5 \left( \frac{h}{R_g} \right)^2 \right]. \quad (2.35)$$

Both equations are valid for constant surface concentrations of the repelling polymer chains. See Fig. 2.11B for a depiction of the interaction energy landscape that arises as a consequence of the confinement of surface-bound polymer chains. However, as for electrostatic interactions, phase separations in multi-component membranes can readily invalidate the assumption of constant surface concentration. To capture this effect, Bruinsma et al. assumed the polymer groups to be at fixed chemical potential and went on to show that the resulting interaction energy exhibits two minima that are representative of strong and weak adhesion sites.

It remains to determine which type of surface group dominates the entropic repulsion of a cell from a surface. While glycolipids and glycoproteins have rather small head groups that protrude only a few nanometers, the extracellular domains of integrins have a size of about 20 nm. Other, even larger, molecules can absorb on the outer face of the membrane which further complicates the definition of a predominantly interacting part of the membrane.

### 2.5.2. Specific interactions

The functioning of many biological processes rests on the association of proteins that uniquely interact with each other. These bonds are called specific bonds if they exhibit low cross-reactivity. At the atomic level, these bonds are an ensemble of physicochemical interactions of different nature (e.g. electrostatic, van der Waals, steric, etc.) that are spatially organized to allow for the binding of a fitting counterpart. For this reason, specific interactions are often said to work according to the key-lock principle. In addition to the interactions that are discussed in this chapter, hydrogen bonds and hydrophobic interactions play an important role in the formation of specific bonds. Hydrogen bonds are formed between electronegative atoms and positively polarized hydrogen and typically have a energy of 5 to 10 kT per bond, which makes them stronger than typical vdW bonds, but much weaker than covalent or ionic bonds. Hydrophobic interactions are forces that arise when water molecules are expelled from the contact zone between two hydrophobic surfaces, such as between self-assembling lipids. Experimental measurements and theoretical treatments of hydrophobic interactions are difficult due to the coexistence with other interactions and due to the inherent complexity. Examples of specific interactions are the integrin-fibrinogen bond that leads to the formation of focal adhesions and the bond between an antibody and the Fc receptor which triggers phagocytosis.

The strength of a specific bond is characterized by an effective interaction potential whose shape is determined by the type and spatial distribution of the interactions that constitute the bond. Owing to the close fit between two binding molecules, this energy landscape usually has a pronounced minimum that is separated from the unbound state by a barrier. Under thermal activation, there is a finite probability for the bond to spontaneously dissociate. This probability is determined chiefly by the depth of the minimum and the shape of the barrier. If a force is applied on the bond, the energy landscape is deformed such that the probability of bond failure increases.

Early concepts of thermally induced failure of chemical bonds have been discussed by
Kramers\textsuperscript{167} and have been extended to include the application of force by Zhurkov\textsuperscript{168} and Bell\textsuperscript{169}. Later refinements by Evans\textsuperscript{170–172} gave rise to the so-called Bell-Evans model of force-induced bond rupture where the rate at which bonds break is given by:

$$k = k_0 g(F) \exp \frac{\Delta E_{bd}(F)}{kT}. \quad (2.36)$$

Here, $k$ and $k_0$ denote the failure rates in the stressed and unstressed case, respectively. The functions $g(F)$ and $E_{bd}(F)$ describe the deformation of the energy landscape under an applied force $F$. For small deformations of the potential well, the following simplified expression is frequently used:

$$k = k_0 \exp \frac{Fx_{\beta}}{kT}. \quad (2.37)$$

The value $x_{\beta}$ is the distance of the energy minimum from the cusp of the adjoining barrier and is often assumed to be constant. While harboring serious simplifications, this equation has been used to describe a variety of force-dependent dissociation processes, mainly for its ease of implementation and because detailed information about the shape of the energy landscape are difficult to acquire. Recently, alternative approaches\textsuperscript{173–176} were developed to acquire the free energy landscape of a bond and to analyze bond breaking events such that some of the severe limitations of the model based on equation 2.37 could be circumvented.

### 2.5.3. Interactions with particles

Above discussions of cell-surface interactions have assumed that both surfaces are planar and that the cell membrane does not deform as a result of contact. However, if the cell membrane interacts with objects such as the micron-sized particles used in chapters 3 and 4, it may accommodate the shape of the rigid particle and assume a curved conformation. In absence of active processes, stable large-area binding between a particle and a cell membrane is hence only possible if the energy gained by adhesion is larger than the energy required for deformation of the membrane.

The adhesion of a particle results in a decrease of the free surface energy. The gained energy $E_{adh}$ can originate from any of the specific and nonspecific interactions discussed above and can be described as a surface energy $\gamma$ (energy per unit area). As the interface is created, the membrane (partially) wraps around the particle with radius $R$ and is bent in the process. The energy $E_b$ for bending the membrane is given by eq. 2.28. The binding of a particle to a cell membrane (beyond the theoretical limit of a point contact) hence requires that

$$E_{adh} \geq E_b \quad (2.38)$$

$$\int \gamma dA \geq \int \left( \frac{\kappa_b}{2} (C_1 + C_2)^2 + \sigma \right) dA \quad (2.39)$$

$$\gamma \geq \frac{\kappa_b}{2} (C_1 + C_2)^2 + \sigma. \quad (2.40)$$

Assuming that the particle ‘sinks’ into the cell and that the membrane is otherwise flat (see Fig. 2.12A), the mean curvature is equal to $R^{-1}$. The requirement for particle binding is then given by

$$\gamma \geq \frac{2\kappa_b}{R^2} + \sigma. \quad (2.41)$$
Figure 2.12 – The energetic requirement for particle binding by a flexible membrane. (A) In the simplest case of binding, the particle ‘sinks’ into the membrane and does not cause membrane deformations beyond its radius. The particle is bound by a host of interactions that have an effective interfacial energy of $\gamma$. The energy gained by forming bonds drives the deformation of the membrane, represented by the bending energy $E_b$. (B) The minimum interfacial energy required to bind the particle depends on the radius of the particle and the bending rigidity and surface tension of the membrane, including the subjacent cortex. For small particles, the membrane deformation is dominated by the bending term of eq. 2.41 while for larger radii the surface tension determines whether the particle is bound or not. The depicted values of $\kappa_b$ and $\sigma$ were taken from ref. 143.

This inequality readily reveals the important insight that the binding of particle depends on the mechanical properties of the cell and that larger particles can be more easily bound. For large particles, the minimum interfacial energy converges towards the value of the cortical tension of the membrane (see Fig. 2.12B). The example in Fig. 2.12B yields a minimum interfacial energy of $0.025 \text{ mJ/m}^2$ or about $6000 \text{ kT/\mu m}^2$. Assuming that only specific bonds contribute to the binding of the particle and each bond contributes with $10 \text{ kT}$, the required density of receptors on the surface of the cell has to be $600/\text{\mu m}^2$. If the wrapping process is to be seen in the context of Fc-receptor mediated phagocytosis, this value is reasonable as Fc receptor densities on the order of $100/\text{\mu m}^2$ to $1000/\text{\mu m}^2$ are typical for phagocytic cells 177.

The above discussion has been vastly extended by computing the equilibrium shape of the membrane during phagocytosis (instead of imposing it) and by assuming that the particle is soft enough to deform under the stress that the protrusion of the membrane exerts 178. In other approaches, the diffusion of receptors in the membrane was taken into account to model the time course of the wrapping process analytically 179–181 or numerically 143, 182. Some of these studies also treated the engulfment of non-spherical particles 180, 182.

2.6. Links to this thesis

Many of the topics presented in this chapter pertain to at least two of the topical chapters. The section on magnetic particles will help to understand the actuation protocols used in chapters 3 and 4 and underpin the arguments concerning misalignment of the magnetic moment that are brought forward in chapter 3. The discussion of available particle tracking routines with particular focus on the Hough transform rationalize the choices made to realize the Euler angle tracking described in chapter 3. The structure and mechanics of the cell membrane are covered in detail because all three chapters feature membrane-related effects. In chapter 3, a qualitative argument
is presented to explain the observed effects while chapters 4 and 5 in part rely on the quantitative description in terms of the Helfrich free energy. Lastly, the treatment of physicochemical cell-surface interactions serves to aid the arguments in chapter 5 and it gives an understanding of the link between the binding energy of a particle and the mechanical reorganization of the membrane.
3 Euler angle particle tracking

The mechanical properties of the cell membrane and the subjacent actin cortex are determinants of a variety of processes in immunity and cell division. The lipid bilayer itself and its connection to the actin cortex are anisotropic. An accurate description of the mechanical structure of the cell membrane and the involved dynamics therefore necessitates a measurement technique that can capture the inherent anisotropy of the system. Here, we combine magnetic particle actuation with rotational and translational particle tracking to simultaneously measure the mechanical stiffness of monocytic cells in three rotational and two translational directions. When using particles that bind via integrins to the cell membrane and the subjacent cortex, we measured an isotropic stiffness and a characteristic power-law dependence of the shear modulus on the applied frequency. When using particles functionalized with immunoglobulin G, we measured an anisotropic stiffness with a ten-fold reduced value in one dimension. We suggest that the observed reduced stiffness in the plane of the cell membrane is caused by a local detachment of the lipid bilayer from the subjacent cytoskeletal cortex. We expect that our technique will enable new insights into the mechanical properties of the cell membrane that will help to better understand membrane processes such as phagocytosis and blebbing.*

* Parts of this chapter have been published in M. Irmscher, A.M. de Jong, H. Kress† & M.W.J. Prins† Probing the cell membrane by magnetic particle actuation and Euler angle tracking. *Biophysical Journal* 102, 698-708 (2012). The dagger indicates joint corresponding authorship.
3.1. Introduction

3.1.1. Motivation

Cells receive mechanical and biochemical cues from a highly inhomogeneous and anisotropic environment through a variety of interactions. They are capable of reacting to these cues by generating mechanical stress. Examples of physiological processes that involve mechanical responses are the migration of cells in a chemical gradient, cytokinesis, cell rolling and the ingestion of extracellular objects. Each of these processes is enabled on the molecular scale where complex pathways regulate the microscopic mechanical response.

The mechanical properties of cells have been studied by a variety of means that span a broad spectrum of applicable forces, frequencies and degrees of localization. The measurement methods can be classified as active or passive. Active techniques measure the deformation of the cell in response to a known externally generated force while passive techniques analyze the fluctuations due to thermal forces that generate appreciable displacements in soft-matter systems. In the past, cells have been actively deformed by applying suction pressure via a micropipette, by indenting the cell surface with a solid tip and by applying forces and torques on micro-particles that have been brought in contact with the cell. Micro-particles have been found to be particularly useful for cell studies since they enable the highly localized application of forces in the relevant force regime between 1 pN and 1 nN. Importantly, particles can interact with cells on a molecular scale and thus enable the study of cellular responses under the spatially inhomogeneous stimulating conditions that are typically found in nature. In the past, micro-particles have been manipulated by optical and magnetic traps. Optical trapping allows for precise positional control over single and multiple particles but the applicable forces are typically limited to a few hundred piconewtons, not the least by the damaging effect of high laser powers. In contrast, the position of magnetic particles is more difficult to control but forces of up to several nanonewtons can be achieved without causing damage to biological matter.

One method that has been used for the study of the mechanical properties of cells is magnetic twisting cytometry (MTC) where the movement of a magnetic particle under torque is measured. Originally devised for the study of ensembles of cells, application of the technique yielded the insight that integrin receptors transmit mechanical stress into the cytoskeleton. To avoid population averaging and to enable dynamic measurements, Fabry and coworkers measured the translation of individual particles microscopically and found that the stiffness of cells scales as a power law of actuation frequency. Measuring translation of particles instead of rotation was based on the assumption that the lateral displacement of a particle held the same information as the rotation it simultaneously undergoes. Mijailovich et al. confirmed this assumption by simulating the stresses and strains that develop when a torque is applied to a particle that is partially immersed in a cell with isotropic mechanical properties. The authors modeled the cell as a slab with an isotropic shear modulus. This assumption can hold for a small region around the particle wherein the introduced stresses decay towards zero. However, it is in general not true for larger regions or the cell as a whole. The relevant length scale of stress decay was estimated to be on the order of 1 μm for a particle with a diameter of 4.5 μm. As applications of MTC have focused on the study of the network mechanics of the cytoskeleton, great care has been taken to ensure a direct link between the magnetic...
particle and the filamentous protein networks within the cell, e.g. by formation of focal adhesions. Considering the dimensions of the cytoskeleton, the assumption of an isotropic material within the probed region of about 1 μm around the particle probe was valid in the systems considered. However, when no tight connection between the particle and the cytoskeleton can be guaranteed, the region adjoining the cell-particle interface is highly anisotropic.

3.1.2. Anisotropy of the cell membrane

The particle binds to proteins lodged in an approximately 10 nm thick lipid bilayer which has been modeled as a two-dimensional fluid\textsuperscript{192}. Discrete distributed bonds connect the bilayer with the cortical part of the cytoskeleton whose network structure as a whole has viscoelastic properties\textsuperscript{193}. The bending modulus $\kappa_b$ of an intact bilayer-cortex assembly ($\kappa_b \approx 10^{-18}$ J)\textsuperscript{194} and an unsupported bilayer ($\kappa_b \approx 10^{-19}$ J)\textsuperscript{195} differ by approximately one order of magnitude. From this description it becomes clear that the region probed by the magnetic particle cannot be considered isotropic unless the link between the lipid bilayer and the cell cortex is intact and the applied torque is coupled into the cytoskeleton. Previous research into the mechanics of cell membranes has shown that there are several processes during which the link between membrane and cytoskeleton is severed. This fact has been demonstrated by pulling of long membrane tethers from blebbing cells\textsuperscript{196} and is thought to enable the engulfment of foreign objects during phagocytosis\textsuperscript{149,182}. Once the link between the lipid bilayer and cell cortex is altered, the assumption of isotropic material properties fails. For example, the response of a magnetic particle under torque can be expected to change as depicted in Fig. 3.1B. Hence, under those conditions, the analysis of particle translation renders an incomplete picture of the processes at play at the interface between a magnetic particle and a cell.

![Figure 3.1](image)

**Figure 3.1** – Independence of translation and rotation of bound particles. Applying a torque to a magnetic particle bound to a cell membrane induces a movement that can be characterized as a superposition of translation and rotation. (A) Under the assumption of isotropic material properties within the probed region, the translation and rotation of the particle are coupled and one can be inferred from the other. (B) In a general case, the particle can undergo movements that are indistinguishable by translational measurements and can only be quantified by additional rotational tracking.

3.1.3. Membrane receptors

This chapter repeatedly refers to specific molecular bonds that are established between particles and cells. The following sections give a short overview of the receptors that are targeted in this work.
3.1.3.1. Integrins

Integrins are a group of membrane receptors that allow cells to transmit mechanical and chemical stimuli across the cell membrane. They enable the adhesion of cells to each other and the surrounding extracellular matrix and, by way of two-way signaling, receive signals that control cell growth and differentiation. Given the general importance of cell adhesion, integrins are crucially involved in tissue development, hemostasis and leukocyte traffic. Conditions such as bleeding disorders\textsuperscript{197}, tumor metastasis\textsuperscript{198}, and auto-immune diseases such as atherosclerosis\textsuperscript{199} or inflammatory bowel disease\textsuperscript{200} can all be traced back to integrin (mal)function.

All integrins are heterodimers of an α-chain and a β-chain that both span the lipid bilayer. In total, 18 α-subunits and 8 β-subunits have been identified and found to form 24 different dimers\textsuperscript{201}. Integrins have an active and an inactive conformation which allows them to be permanently present on the membrane. Given the right trigger, the receptor can be switched to its active conformation. Once activated, the integrin can bind extracellular ligands and, by way of several adaptor proteins, can establish a mechanical connection with the actin cytoskeleton\textsuperscript{122}.

THP-1 cells express a variety of integrins such as very late antigen (VLA-4)\textsuperscript{202} and lymphocyte function-associated antigen (LFA-1)\textsuperscript{203}. LFA-1 has been identified in 1981\textsuperscript{204} and consists of the subunits β\textsubscript{2} (also called CD18) and α\textsubscript{L} (CD11a). The native ligand of LFA-1 is intracellular adhesion molecule 1 (ICAM-1) which is constitutively expressed on endothelial cells. Under resting conditions, both LFA-1 and ICAM-1 are expressed at low levels and adhesion between leukocytes and the endothelium is rare. In diseases such as atherosclerosis\textsuperscript{23}, inflammatory cytokines trigger increased expression and enable tight binding of leukocytes to the endothelium and, ultimately, extravasation, the migration of immune cells from the blood stream into the subjacent tissue.

In previous work, magnetic particles have been coated with peptide sequences containing the RGD motif that is known to bind to a distinct subgroup of integrins and to induce the formation of focal adhesions. In this way, the magnetically induced forces were transmitted to the cytoskeleton\textsuperscript{65,84}. Since THP-1 cells do not express RGD-binding integrins, we instead used monoclonal antibodies against the α\textsubscript{L}-subunit of LFA-1 (CD11a) to establish a mechanical connection to the underlying cytoskeleton.

3.1.3.2. CD14

THP-1 cells are frequently used as a model cell line for primary human monocytes. They have been frequently used for research into the role of monocytes in the response of the innate immune system to bacterial infections. The protein CD14 has been identified as a pattern recognition receptor for lipopolysaccharide (LPS) that can be found in the cell wall of gram-negative bacteria. Since CD14 lacks a cytoplasmic tail, it cannot signal on its own and has been found to associate with other proteins such as Toll-like receptor 4 (TLR-4), that do have signaling capabilities. It is unclear how CD14 is connected to the cytoskeleton. In this work, we used particles functionalized with antibodies against CD14 to test whether the lack of an apparent association with the cytoskeleton alone leads to different results compared to integrin-mediated attachment. In a similar approach by Fabry et al., the protein CD45 on neutrophils was targeted for magnetic twisting cytometry\textsuperscript{66}. Despite having a cytoplasmic tail, the connection of CD45 with the cytoskeleton is not defined.
3.1.3.3. Fc receptors

As a first line of defense, the immune system produces specific antibodies to single out pathogens for removal by specialized cells, called phagocytes. If an antibody against a specific molecular marker on the surface of the invading object exists, the antibody will bind the pathogen in a process called opsonization. The highly conserved tail region of the antibody, also called the crystallizable fragment (Fc), is then recognized by the eponymous family of Fc receptors on phagocytic cells. The ligation of the Fc receptor triggers phagocytosis, a process during which the cell internalizes the pathogen and proceeds to destroy it.

Fc receptors (FcR) are classified by the group of immunoglobulins they recognize, e.g. FcγR binds IgG and FcαR binds IgA. Each group has several subgroups. THP-1 cells express FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). Due to an about 100× higher affinity for IgG, FcγRI can be expected to be the main mediator of particle binding. FcγRII serves to modulate the downstream signaling of FcγRI. FcγRIII has a low affinity for IgG and is expressed at about 10 times lower densities than the other Fc receptors (see Table 3.1 for an overview of receptor densities). Fc receptors are primarily involved in signaling and do not make a direct connection with the cytoskeleton. However, ligation of FcγRI triggers the localized polymerization of actin with the aim of forming a phagocytic cup.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Receptors per cell</th>
<th>Average density [μm⁻²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>10800 ± 2600</td>
<td>11</td>
</tr>
<tr>
<td>FcγRI</td>
<td>27900 ± 7600</td>
<td>28</td>
</tr>
<tr>
<td>FcγRII</td>
<td>53500 ± 16100</td>
<td>53</td>
</tr>
<tr>
<td>FcγRIII</td>
<td>2200 ± 1000</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3.1 – The density of surface receptors on THP-1 cells, as measured by Antal-Szalmas et al.177. The number of CD14 receptors has been determined by different antibodies, yielding differing total numbers of receptors. Here, the average value is given. The resulting average densities have been calculated under assumption of a spherical cell with a diameter of 18 μm.

3.1.4. Chapter overview

In this chapter, we present a novel method that combines magnetic particle actuation with rotational and translational particle tracking to study the stiffness of the interface between a particle and a cell (see Fig. 3.2). To verify proper functioning of the algorithm and to systematically explore its limits, we first apply our method to computersimulated images of particles. We then demonstrate the general applicability of the tracking technique by quantifying the translational and rotational motion of a freely moving sphere in a laminar flow. Independently measuring translation and rotation allows us to determine whether the sphere is simply rolling or moving in a more complex way, e.g. by skidding. In the main part of this chapter, we measure the orientationally resolved stiffness of the contact region between a functionalized particle and a cell. We achieve this by tracking the motion of the particle with five degrees of freedom in an oscillating magnetic field. When using particles functionalized with antibodies against CD11a, a part of integrin LFA-1, or against CD14, a membrane protein without an intracellular domain, the probed regions of the cells had isotropic properties and we could reproduce the previously demonstrated power-law dependence of the shear modulus on the applied frequency of actuation. However, when employing particles with a nonspecific IgG coating, we found an unexpected anisotropy of...
the mechanical stiffness. The observed anisotropy can possibly be explained by a local detachment of the plasma membrane from the underlying cytoskeleton.

3.2. Materials and Methods

Particle preparation

Carboxyl-coated ferromagnetic particles with a diameter of about 4.5 μm (Spherotech, Lake Forest, IL) were used in the experiments. The particles were functionalized with monoclonal antibodies against membrane proteins CD11a (Acris Antibodies, Herford, Germany), CD14 (Acris Antibodies, Herford, Germany) or with mouse immunoglobulin from serum (Merck Chemicals, Darmstadt, Germany).

In all cases we covalently coupled the antibodies to the particles in a two-step reaction. Particles (500 μg) were first washed with coupling buffer (100 mM 2-N-morpholinoethanesulfonic acid (MES) at pH 5). To activate carboxyl groups, the particles were suspended in 25 μl 25 mM MES buffer containing 3 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 3 mg N-hydroxysuccinimide (NHS). Upon incubation, excess EDC and NHS were removed by washing the particles twice in MES buffer. Then the particles were allowed to react with the antibodies in the coupling buffer. Typically, 10 μg of monoclonal antibodies or 200 μg of mouse IgG were used to functionalize 500 μg of magnetic particles. After 2 hours of incubation the remaining carboxyl groups were quenched by spinning down the particles and incubating them in 100 μl 50 mM ethanolamine for 1 hour. Finally, the particles were washed in 0.1 M phosphate buffered saline (PBS) with 0.5% BSA (wash buffer) three times and stored in the same buffer.

To verify successful coupling, the particles were incubated with fluorescent secondary anti-mouse antibodies (Invitrogen, Life Technologies, Carlsbad, CA). The mean fluorescence intensity of Alexa488-tagged secondary anti-mouse IgG (Invitrogen) per particle was measured by flow cytometry and taken as an indicator of the number of immobilized antibodies (see Fig. 3.3A).

Upon successful functionalization, 50 μl of a 1:50 dilution of magnetic particles were mixed with about 25 ng of biotinylated polyclonal goat anti-mouse antibody (Thermo Fisher Scientific, Rockford, IL) and incubated for 20 minutes at room temperature on a shaker. Subsequently, unbound antibodies were removed by washing the particles twice with 100 μl of wash buffer. The original concentration was reconstituted by suspending the particles in 50 μl of wash buffer. Then, the magnetic particles were labeled with fluorescent streptavidin-coated nano-particles. A volume of 1 μl of a 1:100 dilution of streptavidin-coated fluorescent spheres (0.2 μm diameter, Bangs Labs) was added to the magnetic particles and allowed to bind under agitation for 20 minutes at room temperature. If microscopic observation showed undesired aggregation of particles, the vial containing the particle mixture was dipped into an ultrasonic bath for 5 seconds to break up the largest clusters. Under the chosen experimental conditions, 1 to 5 markers were visible per particle (see Fig. 3.3B).

Cell culture and preparation

Human acute monocytic leukemia cells (THP-1) were used as a model for primary human monocytes due to their comparable morphology and functionality. As such, they express Fc receptors that specifically bind the crystallizable region of immunoglobulins (Fc region). It has been shown that THP-1 cells express the IgG-specific receptors FcγRI and FcγRII at relatively high levels compared to neutrophils
Euler angle particle tracking

IgG

Figure 3.2 – Overview of the experiment. (A,B) Monocytic cells were immobilized on glass cover slips coated with poly-l-lysine. Magnetic particles were functionalized with antibodies and labeled with fluorescent fiduciary markers. The particles were then bound to the cells and magnetized in vertical direction. An in-plane unidirectional sinusoidal magnetic field was then used to actuate the particles. (C) A micrograph of a typical interaction between a cell and a tagged particle under simultaneous bright-field and epifluorescence illumination. (D) To target Fc receptors, particles functionalized with nonspecific mouse IgG were used. In experiments with monoclonal antibodies against CD14 or CD11a, cross-reactivity of exposed Fc regions with Fc receptors was prevented by application of a blocking reagent. Schematics are not to scale.

Figure 3.3 – Antibody loading and marker visibility. (A) The amount of IgG binding sites per particle is saturated under the parameters we used for the functionalization of our magnetic particles (indicated by arrow). The loading was measured by flow cytometry after labeling available IgG by secondary anti-mouse antibodies tagged with fluorescent Alexa 488. The solid curve is a fit of the data with a second-order rate equation that describes the reaction of IgG on the particles with secondary antibodies in solution under the assumption of gradual depletion of IgG binding sites. (B) Under the experimental conditions described in the Materials and Methods section, between 1 and 5 markers are visible (on the top hemisphere) on 80% of all magnetic particles.
and primary monocytes. THP-1 cells were cultured at 37°C in Roswell Park Memorial Institute medium (RPMI-1640, Life Technologies, Carlsbad, CA) supplemented with 25 mM HEPES buffer, GlutaMax-I and 10% fetal calf serum (Gibco, Life Technologies, Carlsbad, CA). The cells were kept at concentrations between $10^5$ cells/ml and $10^6$ cells/ml. Before an experiment approximately 1 ml of cell solution at a concentration of $8 \cdot 10^5$ cells/ml to $10^6$ cells/ml was taken from culture for sample preparation. For experiments where ligation of Fc receptors was undesirable, 0.5 ml of cells were incubated with 5 μl of Fc-blocking reagent (Miltenyi Biotech, Germany) under slight agitation for 20 minutes at 37°C.

Fluid chambers

Fluid chambers were made from two glass cover slips (Menzel-Gläser, Braunschweig, Germany) and two stretched Parafilm (Pechiney Plastic Packaging, Chicago, IL) strips that served to create a clearance of approximately 100 μm between the glass surfaces. Upon assembly, the fluid chambers were filled with poly-L-lysine (PLL) solution (0.1% w/v in deionized water, Sigma Aldrich, St. Louis, MO) and left to incubate overnight at room temperature in a moist Petri dish. Prior to each experiment, the fluid chambers were washed several times with RPMI-1640 medium.

Sample preparation

Cells were introduced into the fluid chambers by dispensing about 50 μl of cell solution onto the area of the cover slip next to the inlet of the chamber. If passive displacement of the medium inside the chamber was not sufficient, dry tissue paper was used to create a (small) capillary drag force at the outlet and thereby pull the cell solution into the chamber. Subsequently, the cells were allowed to settle onto the PLL-coated glass surfaces for two to three minutes before tagged magnetic particles at an approximate concentration of $5 \cdot 10^6$ particles/ml were brought into the chamber in the same way.

The successful binding of particles to the cells was verified by observation of immobile particles in a shear flow of approximately 10 to 50 μm/s. The resulting shear stress was physiological at about 0.1 pNμm$^{-2}$ (1 dyn⋅cm$^{-2}$) and was considered negligible compared to the stress applied during magnetic twisting. Once an appreciable number of particle-cell bonds had formed, the sample was magnetized perpendicularly to the coverslip (along the $z$-axis) by shortly placing it onto a permanent magnet with an almost perfectly vertical magnetic field of at least 150 mT. Upon exposure to the magnetizing field, the particles retained a remanent magnetic moment of about $2.2 \cdot 10^{-13}$ Am$^2$ in $z$-direction (see section 2.1.3 for details on the calibration of the magnetic moment). After magnetization, the sample was transferred onto the magnetic setup to start the measurements. About 5 minutes passed between magnetization of the sample and the beginning of an experiment.

Magnetic field generation

Experiments were carried out on a custom-made magnetic quadrupole setup as previously described by Janssen et al. Two opposite coils were used to generate a sinusoidally varying magnetic field in horizontal direction (see Fig. 3.2) with equal components in $x$- and $y$-direction. In a typical setting the coils were operated between 0.1 and 20 Hz and at a peak current of 250 mA which corresponded to a magnetic flux density of 8 mT. The applied specific torques ranged from 15 to 80 Pa and
are within the range of values applied in previous studies. The homogeneity of the magnetic field has previously been verified by finite element simulations. Within 2 mm of the central vertical symmetry axis the lateral field gradient is 0.25 Tm\(^{-1}\) and the vertical field gradient is 1.5 Tm\(^{-1}\). At a remanent magnetic moment of about \(2 \times 10^{-13}\) Am\(^2\), the lateral and vertical forces on a ferromagnetic particle thus become 0.05 pN and 0.3 pN, respectively. The gravitational force on a particle is about 0.7 pN which renders the influence of the field gradient negligible.

**Image acquisition**

Imaging was performed with a water immersion lens with a numerical aperture of NA = 0.9 and a magnification of 63\(\times\) on an upright light microscope (Leica DM6000M, Leica Microsystems, Wetzlar, Germany). Fluorescence imaging of the tracer spheres was supplemented by bright-field imaging which was sufficient to render the outlines of the magnetic particles detectable for further image processing. Images were acquired with a camera equipped with an electron-multiplying charge-coupled device (EMCCD, Luca S, Andor Technology, Belfast, Northern Ireland). In experiments without fluorescent markers, images were acquired with a high-speed camera (Redlake X3, IDT, San Jose, CA). The acquisition of images was locked to the driving frequency of the magnetic coils to record 15 frames per actuation cycle. The relatively low maximum frame rate of the EMCCD (about 30 frames per second at typical illumination) effectively restricted the actuation frequencies to below 2 Hz when acquiring fluorescence signals.

### 3.3. Tracking of Euler angles

#### 3.3.1. General approach

We bound ferromagnetic particles to cell membranes and actuated them in an oscillating magnetic field. To enable tracking of the rotational motion of the particles, we coupled small fluorescent tracer spheres to their surfaces. Imaging was performed under simultaneous bright-field and fluorescent imaging. Details about particle functionalization and imaging can be found in section 3.2.

Our algorithm for tracking the position and the orientation of the magnetic particle first determines the position of the particle in every frame. Subsequently, the locations of the fluorescent markers with respect to the center of the magnetic particle are tracked. Finally, the rotational motion of the particle around the three major axes is calculated from the obtained trajectories.

We determine the location of the magnetic carrier particle by applying the Hough transform for the detection of circular features. In order to minimize computation time, the approximate radius of the magnetic particle is used as a constraint for the detection. We also tested algorithms that are based on determining the intensity-weighted centroid of the magnetic particle. However we found that these algorithms were less robust due to the non-uniform intensity distribution on the visible surface of the carrier particle. Furthermore, centroid-based methods are in general prone to errors when the particle is in proximity of the edges of a cell.

We subsequently use the found locations to center a region of interest (ROI) of fixed size around the particle in each frame. Within this ROI, we then apply a centroid-based algorithm to locate the fluorescent fiduciary markers which appear as peaks of light intensity. To ensure the effectiveness of the localization of the fluorescent markers, the background illumination of the simultaneously acquired bright-
field images needs to be sufficiently dim to avoid the confounding influence of light reflections on the surface of the magnetic particle.

The positions of the fiduciary markers in each frame are finally fed into a tracking algorithm that reconstructs the trajectories of individual markers by analyzing the spatial and temporal evolution of the determined coordinates. Between each two successive frames the algorithm attempts to correlate the positions of the fluorescent markers to construct their trajectories. This places a limit on the maximum density of fiduciary markers that can be used at a given camera frame rate. The spacing of different markers on the carrier particle should be well above the distance covered by each individual marker within one frame interval.

Upon locating and identifying the fiduciary markers for each frame, we express their positions in a three-dimensional Cartesian coordinate system (see Fig. 3.8). Although we do not measure the $z$-position of the markers directly, we can infer it from the $x$- and $y$-position by assuming that a fluorescent marker is at a constant distance from the center of the magnetic particle such that

$$z = \sqrt{(R + r)^2 - y^2 - x^2}, \quad (3.1)$$

where $R$ and $r$ are the radii of the magnetic carrier particle and the fluorescent marker, respectively. Finally, we write the locations of $k$ markers present in frame $i$ in matrix format:

$$X_i = \begin{pmatrix} x_i^1 & \cdots & x_i^k \\ y_i^1 & \cdots & y_i^k \\ z_i^1 & \cdots & z_i^k \end{pmatrix} \quad (3.2)$$

The locations of the fiduciary markers in each frame are fixed with respect to each other and represent a set of points in a particle-fixed coordinate system. According to the Euler rotation theorem, the rotation of the sphere can now be described by three successive rotations that map a given set of marker locations into that found for a successive frame.

$$X_{i+1} = R_3 R_2 R_1 X_i \quad (3.3)$$

The parameters of the required matrices are three angles which we chose according to the pitch-roll-yaw convention\textsuperscript{206}. Using this notation, the angles $\psi$, $\theta$ and $\varphi$ describe rotations about the axes $x$, $y$ and $z$, respectively. The Euler matrix $R$ can now be written as the product of three successive rotations:

$$R = R_\psi R_\theta R_\varphi \quad (3.4)$$

Since the point sets that represent the locations of markers are noisy, the Euler matrix cannot be directly calculated by inversion. Instead, we determine $R$ by solving the least-squares problem

$$E = ||R \cdot X_i - X_{i+1}||^2 \quad (3.5)$$

If only one marker is visible, the solution found by minimizing $E$ is not necessarily unique since there are often several sets of rotations that can map $X_i$ onto $X_{i+1}$. If a main rotational axis can be identified by inspection, boundaries can be imposed on the minimization routine to suppress alternative solutions. For more complex motions, at least two fiduciary markers are necessary to yield meaningful tracking results.
The accuracy of the angular tracking algorithm largely depends on the precision of the circular Hough transform used for finding the center of the magnetic carrier particle. Since in our movies the contrast between particle and background is low compared to traditional bright-field imaging, the edge detection is mainly hampered by camera noise. To estimate the impact of noise on the center tracking, we imposed additive Gaussian white noise at different amplitudes on a test image and recorded the results of repeated attempts to track the center of a simulated particle. At noise levels comparable to actual values we estimated a tracking accuracy of about 30 nm. This value is high compared to other particle tracking routines and can be explained by the low contrast caused by the trade-off between simultaneous bright-field and fluorescence imaging.

The error in the tracking of the position of the magnetic particle influences all subsequent processing steps since the determined location serves to define the reference frame. Hence, the precision of tracking the fiducial markers is limited by the error of the circular Hough transform. The uncertainty in the rotational angle derived from the location of the fiducial markers can thus be expressed by $\Delta \theta = \Delta x / R$. With a particle radius of about 2.3 $\mu$m, the accuracy of the angular tracking routine is therefore on the order of 1 to 3°. The error in the calculation of the $z$ position of a marker given by Eq. 3.1 can be estimated by

$$\Delta z = \sqrt{(R - \Delta R)^2 - (r + \Delta r)^2 - \sqrt{R^2 - r^2}}$$

(3.6)

where $r^2 = x^2 + y^2$ and, correspondingly, $\Delta r = 42$ nm. The algorithm of the Hough transform currently only yields a pixel-accurate estimate of the radius of a tracked particle. In our experiments, we assume a corresponding uncertainty of $\Delta R = 100$ nm. At a typical distance of $x = 1$ $\mu$m from the center of the particle, the estimated absolute error in $z$ is thus about 130 nm (relative error 0.07). Conclusively, the most precise measurements can be made with magnetic particles that carry multiple nanoparticle markers close to their zenith position at $(x = 0, y = 0, z = R + r)$.

3.3.2. Validation

To test the validity and robustness of our tracking algorithm, we tested it both on real samples and on simulated images of particles.

3.3.2.1. Translational tracking accuracy

As detailed above, the overall tracking performance hinges strongly on the accuracy with which the outline of the magnetic particle can be detected by the Hough transform. So far, the Hough transform has mainly been used in applications that do not share the stringent requirements of particle tracking at the microscale. In conventional magnetic twisting cytometry, the intensity-weighed centroid of the particles is tracked. This is a valid approach if the surface of a particle has an intensity pattern that does not change if the particle moves. At low magnifications, this is often true and most magnetic particles will appear as circular black spots. However, at the higher magnifications we typically used in our experiments, irregularities on the surfaces of the particles created intensity distributions that changed as the particles rotated. Under those circumstances, a centroid tracking algorithm would detect translation on a purely rotating particle. This situation is aggravated if fluorescent markers are present on the particle.
Here, we tested the performance of the Hough transform in tracking stationary particles that were immobilized on a glass substrate by drying. We used a piezoelectric stage actuator (Nano LPS, Mad City Labs, Madison, WI) to move the substrate to predefined positions with high accuracy (0.6 nm, according to the manufacturer). We then used both the Hough transform and the centroid tracking method to find the positions of the particles in every frame. As shown in Fig. 3.4, there were strong position fluctuations in absence of actuation (FWHM ≈ 55 nm, data not shown). We attribute these fluctuations to vibrations of the microscope table and to Brownian motion of the particle. Since we dried the particles on their substrate, the latter contribution was expected to be small. At a translation amplitude of 50 nm, the instantaneous position of the particle hence was subject to substantial noise. Nonetheless, the Fourier transform of the data shows that the average amplitude of the particle oscillations could be extracted with less than 3% deviation from the real value. We did not perform experiments at smaller actuation amplitudes because we could not fully eliminate oscillations from the system.

![Tracking untagged particles. Untagged particles were affixed to a coverslip and placed onto a piezoelectrically controlled microscope stage. The stage underwent defined sinusoidal translations in one dimension with amplitudes 50 nm (top) and 1 µm (bottom). The observed positional fluctuations at t < 2 s arise from imperfect damping of the microscope table and a small amount of Brownian motion. While measuring the instantaneous position of the particle is subject to substantial noise, the Fourier transform of 10 cycles reveals less than 3% deviation from the set amplitude.](image)

Conclusively, we found that both algorithms are capable of resolving linear displacements at amplitudes down to 50 nm. This indicates that using the Hough transform to determine the position of a particle does not carry a penalty in comparison to the more commonly used centroid tracking.

### 3.3.2.2. Tracking accuracy in the presence of noise

Noise is a recurring obstacle in image analysis. As qualitatively outlined above, the accuracy of our particle tracking method hinges on the contrast between background, magnetic particle and fluorescent markers. Achieving near-optimum contrast in this setting requires an inefficient use of the dynamic range of the camera and results in noise.

To quantitatively investigate the susceptibility of the Hough tracking method to the presence of noise, we simulated a noise-free image and superposed it with in-
increasing levels of Gaussian white noise. At each noise level, the noise pattern was generated 50 times and the position of the particle was determined. The results are shown in Fig. 3.5. The simulation demonstrates that at typical fluctuation widths of the image noise, the standard deviation of the determined position is approximately 20 nm. At a normalized noise variance of $10^{-3}$, the standard deviation of the noise in an 8-bit image is $255\sqrt{10^{-3}} \approx 8$. Since the intensity contrast between the particle and the background is approximately 30, the algorithm fails due to ambiguous results as the Hough transform returns several locations that are all equally likely. To determine the position of a particle in this regime, the parameters of the Hough transform have to be adjusted or constraints on allowed locations have to be introduced.

3.3.2.3. Rotational tracking of simulated particles

To further test the performance of the Hough transform and to characterize the performance of the rotational tracking method, we simulated images of fluorescently tagged particles. In this way, we could investigate the performance of the Hough transform under different levels of contrast and noise. More importantly, creating time-lapse movies of particles undergoing defined rotational movements made it possible to also characterize the performance of our rotational particle tracking method.

Using simulated images to validate the performance of the rotational tracking algorithm is advantageous as it allows to model motion patterns that could not be achieved in practice. While well-defined translations can be realized by placing the sample on a piezoelectric stage, inducing particle rotation is more involved. The setup at our disposal allowed the accurate application of a magnetic field in the $xy$-plane containing the sample and could hence only be used to induce known angular
displacements about the $z$-axis. Since the ability to track rotations about the $x$ and $y$ axes is a central feature of our method, we therefore had to resort to using simulated time-lapse sequences for calibration.

![Simulated images of particles](image)

**Figure 3.6 – Simulating images of particles.** Particle images were simulated by superposition of two boxcar functions with a truncated Gaussian. The fluorescent markers were simulated as small Gaussians with high intensity. The relative intensities were adjusted to approximate an exemplary image from an actual experiment. The magnitude of camera noise was estimated from the intensity fluctuations in an image region without features. Gaussian noise was then added to the simulated image such that the noise variances were comparable.

To create particle images that represent the actual appearance of the particles we used in experiments, we analyzed the cross-section of a still image. The edge of the particle is marked by a ring of increased intensity that represents the highest order of the diffractive pattern. At the magnification we used in our experiments, the focal depth of the objective was too small to acquire a sharp image of the particle along the optical axis. As a result, if the focus plane was coplanar with the hemisection of the particle, the central area of the particle image would appear slightly brighter than the rim region. The fluorescent particles appeared as blurred spots since their diameter was below the diffraction limit.

We simulated particles as a superposition of several intensity profiles:

$$I(x,y) = I_d \Pi(d_c, w_d) - I_{int} \Pi(d_c, w_{int}) +$$

$$I_{def} \exp \left[ -(x-x_c)^2 \right] \left[ -(y-y_c)^2 \right] \Pi(d_c, w_{int}) +$$

$$\sum_{k=1}^{n} I_{fl} \exp \left[ -(x-x_k)^2 \right] \left[ -(y-y_k)^2 \right] \Pi(d_c, w_{fl}) + I_{bkg} + I_n$$

with $d_c = \sqrt{(x-x_c)^2 + (y-y_c)^2}$ and $\Pi(d, w)$ is a two-dimensional boxcar function with $\Pi=1$ for $d < w/2$ and $\Pi = 0$ for $d > w/2$. The intensity amplitudes

(3.7)
were chosen to achieve approximate agreement between the cross-sectional intensity profiles of a simulated image and the image of an actual particle. We assumed Gaussian white noise and adjusted the variance such that the intensity distributions of background pixels matched. To simulate blur, the images were finally subjected to a 2-dimensional Gaussian filter. An example of a simulated image is shown in Fig. 3.6.

The rotations of the particle were simulated by consecutive mapping the three-dimensional locations of the markers with respect to an initial reference set. Their projections onto the $xy$-plane were then used to draw the markers in individual frames. Since we used our algorithm mainly for the tracking of small angular displacements below $20^\circ$, we did not take into account that the rotation of the magnetic particle leads to defocusing of the fluorescent markers (out-of-focus blur). If desired, this effect could be integrated by modeling the dependence of the point-spread function on the axial distance from the focus plane. In our example, we linked the translation of the particle to its rotation by assuming a no-slip rolling motion, i.e. $\Delta \theta R = \Delta x$.

![Figure 3.7 – Tracking simulated trajectories. A time-lapse sequence of a particle undergoing simultaneous translation and rotation was simulated to calibrate the accuracy of the rotational tracking algorithm in the presence of 1 (red circles in time domain and red lines in frequency domain) and 2 (blue squares and lines) markers. The deviation from the set motion (black line in time domain) for the case of 1 marker shows that at least two markers are necessary to accurately resolve the rotations of the particle. While the motion in $\theta$ and $\varphi$ is resolved with less than 1% error, the tracking result in $\psi$ overestimates the actual motion by about 70% (see Fourier spectra in the right column). This error is caused by the limited accuracy of the translational tracking as the associated maximum displacement in $y$ is about 0.4 pixels.](image-url)
As an example, we simulated 5 cycles of sinusoidal particle oscillations at a frequency of 0.5 Hz and a sampling rate of 7.5 Hz. The angular displacement amplitudes were $\psi = 1^\circ$, $\varphi = 20^\circ$ and $\theta = 10^\circ$. The results are shown in Fig. 3.7. If only one marker is present on the particle, the trajectory of the particle cannot be reproduced. At least two visible markers are necessary to accurately track the three-dimensional rotation of a magnetic particle. This can be understood as two markers uniquely define the orientation of the magnetic particle if its center position is known and the $z$-coordinates of the markers can be deduced from their position in the $xy$-plane. In extension of this argument, if the location of the particle is unknown, its rotation can also be reconstructed from the motions of three markers that are all subject to the constraint of being at a constant radial distance from the center of the magnetic particle.

3.3.2.4. Tracking particle motion in a laminar flow

We have characterized the performance of our algorithm under experimental conditions by tracking the motion of a particle moving over a surface in a laminar flow (see Fig. 3.8A,B). By analyzing the translation and rotation of the particle independently, we reveal the quantitative superposition of both fundamental modes of movement and demonstrate the robustness of the rotational tracking. By relating the translational and rotational velocities to each other, the particle is shown to undergo a motion that we describe as skidding (see Fig. 3.8C,D). This test also indicates that outside cell mechanical research, the Euler angle tracking approach can potentially be useful for the analysis of near-wall flow phenomena in microfluidic systems, such as the ones investigated by Derks et al.

3.4. Results

3.4.1. Cell stiffness measurements

We applied rotational particle tracking to measure the local mechanical stiffness of the cells. In our experiments, we used particles coated with anti-CD11a, anti-CD14 and nonspecific mouse IgG. The particles were tagged with fluorescent markers for Euler angle tracking. We immobilized the cells on a PLL-coated substrate and then bound particles to the cells (see Fig. 3.2). Thereafter we magnetized the samples in a strong vertical magnetic field and subsequently applied a harmonically oscillating field in horizontal direction. A similar method has previously been applied for magnetic twisting cytometry studies. During the experiments, we monitored the motion of individual particles to infer qualitative and quantitative information about the coupling of the particle with the cell.

3.4.1.1. Particle binding via CD11a or CD14

Particles that carried an antibody against CD11a or CD14 underwent a sinusoidal rocking motion along the axis imposed by the direction of the horizontal magnetic field. The displacement of the particle center was on the order of 20 to 200 nm and clearly discernible. The simultaneous rotation of the particle was about 1 to 10 degrees. An example of the motion pattern of a particle in contact with an isotropic contact region is given in Fig. 3.9. The idealized motion of a particle bound to a cell with an isotropic contact region has previously been simulated in detail. To determine the dynamic moduli of the contact region between particle and cell, we used a
Figure 3.8 – Extracting the mode of motion from translational and rotational trajectories. (A) In every frame, the region of interest was centered on the magnetic particle. The location of each fiduciary marker in a carrier particle-fixed three-dimensional Cartesian coordinate system was then calculated under the assumption that the radial distance to the center of the magnetic particle remained constant. (B) The exemplary movement of a particle in a laminar flow was tracked in x, y and three rotational angles. After about 3 seconds, the motion of the particle is dominated by rotation around the y-axis, denoted by the angle $\theta$. The Euler rotation angles were calculated with respect to the initial orientation of the particle. Micrographs show the particle at four time points. (C) The unidirectional motion of a sphere above a surface can take several forms. If the sphere does not rotate, it is purely translating. If the length of the perimeter section that is covered by the rotation angle is equal to the displacement of the center, the sphere is said to undergo pure rolling. If the displacement of the center is larger or smaller than the perimeter section, the sphere is skidding or slipping, respectively. The motion of the particle can be described as a superposition of pure translation and pure rolling. (D) From the tracked rotational motion the displacement of the particle under ideal rolling is calculated by $(\psi, \theta) \times R$. By adding translational components with constant velocities of $v_x = 1.4 \, \mu m/s$ and $v_y = 0.5 \, \mu m/s$, the originally traced displacements of the particle, $x(t)$ and $y(t)$, are approximated very well. This indicates that the particle is skidding.
method previously described by Fabry et al.\textsuperscript{65}. Our data follow the structural damping law with a power law exponent of $x=1.24$ (Fig. 3.10A) which is in good agreement with the value found for primary blood neutrophils\textsuperscript{66}.

![Figure 3.9](image)

**Figure 3.9 – Typical motion of a particle bound to a cell with nearly isotropic stiffness.** Anti-CD11a Particles bound via integrin LFA-1 typically undergo a characteristic rocking motion in the direction of the externally applied field. The particle mainly translates in $x$- and $y$-direction and concomitantly rotates about these axes (respective angles $\theta, \psi$).

### 3.4.1.2. Particle binding via nonspecific IgG

Particles with a non-specific IgG coating showed a markedly different motion when subjected to the same magnetic actuation protocol. The translational displacements of the particles (see Fig. 3.11D) were smaller than expected from the rotational motion and they were not restricted to the main axis of the external magnetic field. In contrast to the experiments with coatings against CD14 and CD11a, rotation of particles about the $z$-axis (angle $\varphi$) of the imaging plane was observed (Fig. 3.11B). Even without fiducial markers, the rotational motion was clearly visible by eye. However, for precise quantification of the rotation the fiducial markers were necessary.

We attribute the observed rotation around the $z$-axis to elastic compliance of the cell in the $xy$-plane and to the presence of an in-plane component $m_{xy}$ of the magnetic moment of the particle. An $xy$-component of the magnetic moment occurs when the magnetizing field is not ideally vertical or the particle is reoriented after the initial magnetization step, e.g. by active movements induced by the cell. The resulting in-plane magnetic moment is likely to be much smaller than the one imposed
in vertical direction. The applicable torque, in turn, is determined not only by the in-plane magnitude of the magnetic moment but also by its orientation with respect to the axis along which the magnetic field lines run. In our experiments the magnitude and direction of the in-plane component of the magnetic moment can be estimated. To this end we tracked the orientation of an unactuated particle during a time interval of 10 minutes and measured reorientations in $\theta$ and $\psi$ of not more than 10°. Hence, we estimated the mean in-plane component to be 10% of the absolute torque. The uncertainty of this approximation was assumed to be 10% of the absolute torque as well.

3.4.2. Application of an isotropic material model

Considering the small amplitude of the in-plane torque, the angular excursions in $\phi$ (see Fig. 3.11B) are surprisingly large compared to the excursions in $\theta$ and $\psi$ (see Fig. 3.11) and indicate a strongly reduced stiffness in the plane of the particle-cell interface. The anisotropy of the probed region becomes apparent when we apply an isotropic model description. For this purpose, we assume that half of the particle is embedded in a homogenous isotropic linear elastic medium characterized by Young’s modulus $E$ and Poisson’s ratio $\nu$. Under these conditions, the shear modulus $G$ is given by $G = E/(2(1 + \nu))$. The elastic torque exerted on a particle rotated by an angle $\theta$ now becomes

$$\tau = \frac{4}{3} \pi R^3 E \theta = 2V_p (1 + \nu) G \theta$$

(3.8)

$$T = \frac{\tau}{V_p} = 2(1 + \nu) G \theta$$

(3.9)

Here, $V_p$ is the volume of the particle which is subsequently used to normalize the torque to introduce the torque density $T$. We obtain the shear modulus by adapting a method proposed by Fabry et al. which yields the complex dynamic modulus $G^*$ as the quotient of the Fourier transforms of the normalized torque $T^*$ and the rotational displacement at the frequency of actuation $f_a$.

We also calculated the shear modulus from the dimensionless normalized translation $\tilde{d} = d/R$ of the particle by using the results of Mjailovich et. al. Here, the quotient $G_d = T/\tilde{d}$ is called the apparent shear modulus because it does not take into account the geometry of the contact interface between particle and cell. The actual shear modulus of the cell material can then be obtained by using the scaling factor $\beta$ which depends on the degree of particle embedding and the thickness of the cell:

$$G = \frac{G_d}{\beta}$$

(3.10)

In line with the above assumptions, we use $\beta = 1$ for a semi-embedded particle and a cell thickness of 5 μm. If the contact region were indeed an isotropic medium, the values of G obtained from the rotation and the translation of the particle should be equal. Indeed, the shear moduli calculated from the translation of the particle and the rotations $\theta$ and $\psi$ agree reasonably well (Fig. 3.11). However, the shear modulus ascribed to the rotation $\phi$ is significantly lower than that for the other coordinates (Fig. 3.11E, F). In total, we recorded this behavior in 8 different cells from 4 independent experiments. This is in contrast to experiments wherein antiCD11a-coated
particles were used to establish a strong link to the cytoskeleton. In that case, we predominantly observed an isotropic stiffness across all probed dimensions ($x$, $y$), $\theta$, $\varphi$ and $\psi$ (see Fig. 3.10).

In conclusion, we have shown that the measured stiffness of the probed region of the cell membrane is not uniform in all orientations and that the assumption of material isotropy fails when particles are used that are coated with nonspecific IgG. To quantitatively understand the origin of the observed anisotropy, we expect that a model description of the interface comprising the particle surface, the lipid bilayer and the cytoskeleton is needed.

3.4.3. Estimation of embedding angle

In magnetic twisting cytometry, it is generally not possible to determine the absolute value of the shear modulus directly since the measured particle translations depend not only on the material properties of the cell but also on the embedding angle of the particle and the distance between particle and substrate. Neglecting the weak influence of the cell thickness, the shear modulus could hence only be determined by using an estimate of the embedding angle, e.g. from SEM images, to transform the apparent shear modulus \(^97\). As mentioned above, a particle that is partially embedded in a cell will undergo both translation and rotation upon the application of torque. While the individual amplitudes of translation and rotation are determined by the elasticity of the material, their ratio is given by the structure of the probed region. If the particle is strongly coupled to the cytoskeleton and the probed region is thereby isotropic, measuring the ratio of translation to rotation hence makes it possible to directly determine the embedding angle of the probing particle.

To illustrate this procedure, we used a generalized abacus (see Fig. 3.12) that has previously been published by Ohayon et al. \(^98\). They modeled the cell as a slab with an isotropic neo-Hookean elasticity and a partially embedded particle on its apical surface. The authors then explored the motion of the particle in response to an applied
Figure 3.11 – Tracking motion and time course of measured cellular stiffness for a particle with a non-specific IgG coating. (A-C, left column) As shown by the respective Fourier transforms (right column), the rotational displacement of the particle is on the order of 2°. The motion in \( \varphi \) is unexpectedly large since it is generated by a substantially lower torque than that in \( \psi \) and \( \theta \). Since tracking in \( \varphi \) does not require the \( z \) coordinate, the obtained motion is subject to considerably less noise. (D) The translation of the particle is restricted to amplitudes of about 50 nm. (E) Elastic moduli calculated from the particle motion depicted in panels A-D. The moduli calculated from translation and rotation in \( \psi \) and \( \theta \) are similar while the modulus in \( \varphi \) is about one order of magnitude lower. (F) Similar results obtained in a different experiment. The shaded areas depict the standard error of the mean.
torque while varying its embedding angle \( \alpha \). As expected, for \( \alpha \to 0 \), the particle is rolling with \( \Delta \theta R = \Delta x \). At the maximum embedding angle of 180°, the particle was found to mainly rotate.

From Fig. 3.9, the displacement in \( y \), normalized by the particle radius \( R=2.1 \mu m \), determined from micrographs), is approximately \( \delta_y=0.023 \) with the associated rotation \( \psi=0.054 \) rad. The relevant ratio thus becomes

\[
\frac{\psi}{\delta_y} = 2.35. \tag{3.11}
\]

To determine the corresponding embedding angle from the abacus, the distance of the particle from the substrate needs to be determined. Given that the cells in our experiments did not spread significantly, we estimated that the thickness of the cell is at least equal to the diameter of the particle, such that \( \frac{2R}{h_u} \leq 1 \). In this regime, the strain field induced by actuation of the particle decays entirely within the cell body and the stiffness of the substrate has little influence. Under this assumption, we can readily determine that the embedding angle is approximately 80°. This is a comparatively high value as previous research has reported values on the order of 40°. However, we found a value of about 25° for another sample and it is very likely that the embedding angle is a function of time.

Besides being time-dependent, the embedding angle presumably depends on the material properties of the cell membrane and the density and type of available binding sites on the particle. The equilibrium embedding angle is reached if the deformation energy of the membrane is equal to the energy gained by the formation of bonds. In our experiments, the antibody density on the particles was nearly saturated such that most variability in the embedding angle is expected to originate from cell-to-cell variations of the bending stiffness and cortical tension of the membrane. This aspect underlines why it is crucial to ensure that the ligand density on the particles is close
Euler angle particle tracking

63

to saturation. In the linear regime of the loading curve in Fig. 3.3A, the antibody density will greatly differ between particles. As a result, the embedding angles will also vary, even if the relevant material properties of the respective cells are the same. The resulting spread in measured apparent shear moduli could thus be misinterpreted as a distribution of the elastic properties of the cells.

3.5. Discussion

3.5.1. Origin of observed anisotropy

Magnetic particle microrheology methods have so far relied on the assumption that the mechanical properties of the probed region are isotropic. As mentioned above, this assumption is valid under the conditions of experiments aimed at studying cytoskeletal dynamics where the membrane is constantly and strongly coupled to the cytoskeleton. In the general case, however, the system of the plasma membrane and the subjacent cytoskeletal cortex cannot be seen as an isotropic entity.

Firstly, the cytoskeleton itself is not isotropically organized. By applying a magnetic twisting field along two perpendicular axes, it has previously been shown that adherent cells display anisotropic stiffness due to a preferential orientation of stress fibers. Directional particle tracking microrheology later revealed that unidirectional shear flow triggers a reorganization of the cytoskeleton to effectuate an increase in compliance parallel to the flow direction.

Secondly, as mentioned above, tight coupling to the cytoskeleton is often taken as an argument to neglect the influence of the coupling between the lipid bilayer and the underlying cortex. Unexpected motion of actuated particles such as rolling on the membrane or no apparent translation at all have been observed before but could not be quantitatively interpreted. It has been speculated that improper linkage of a membrane receptor to the cytoskeleton could lead to reduced movement due to lack of a mechanical support structure.

Our experiments with particles coated with CD11a or CD14 yielded the expected power-law rheology that has been measured for a wide range of different cell types. This result is evidence of a strong link between the targeted membrane receptor and the cytoskeleton. While the antibody against CD11a is expected to bind to integrins directly, the nature of the cytoskeletal link when using particles that bind to CD14 is undefined. CD14 itself does not possess an intracellular domain and thus should not be able to establish a mechanical connection. However, it has been shown to colocalize with a variety of other membrane receptors that might provide an indirect link.

In our experiments with nonspecific IgG-coated particles, we assume that binding is mainly established through Fc receptors. These receptors are known to play a major role in the phagocytosis of opsonized targets and specifically have been shown to diffuse freely in the lipid bilayer. This finding indicates that on the time scale of an experiment Fc receptors do not necessarily have a link with the cytoskeleton. Elasticity measurements on a particle bound to free receptors would then produce values that are representative of the mechanical properties of the lipid bilayer itself and its degree of connectivity with the underlying cortex. Topological factors such as excess membrane or membrane folds are likely to play a role as well. The measured anisotropy of the mechanical stiffness thus can be understood as the result of membrane processes that increase the rotational freedom of the probing particle. This does not necessarily imply an inherently anisotropic stiffness of the cell membrane.
itself. When combined with a suitable model description, we expect that our method can thus be used to study processes such as membrane ruffling and partial dissociation of the plasma membrane from the cortex.

3.5.2. Potential improvements and applicability

Our method makes it possible to simultaneously measure the local stiffness of a cell in five coordinates which represent two translational and three rotational degrees of freedom. This constitutes an improvement over previous single-cell techniques that only measured translation and thus never acquired more than three coordinates at the same time. Hence, our technique is inherently suited for studies of the heterogeneous assembly of lipid bilayer and cortex in live cells.

In contrast to methods that make use of magnetic tweezers with sharp tips, our method is inherently suited for multiplexing because the force on the particles can be considered homogeneous within the field of view which has a size of about $150 \times 100 \, \mu m^2$. Multiplexing is very useful for studies of cell-to-cell differences in large populations\textsuperscript{66}.

The current implementation of our angular tracking technique has a limited resolution caused by the need to simultaneously acquire bright-field and fluorescent images. Switching between bright-field and fluorescence modes for each frame can break this trade-off but complicates the temporal synchronization with the magnetic actuation. A better solution is the use of two cameras and a dichroic mirror to achieve spatial separation of the bright-field and fluorescent signals. Our technique can be further improved by more stringently controlling the orientation of the magnetic moments of the particles, for example with three independent orthogonal coils as presented by Hu et al.\textsuperscript{208}. By unifying the magnetization and actuation of particles in situ, undesired changes of the applicable torques due to cell-induced motion of the particle can be minimized.

3.5.3. Outside the box

The use of micro-particles is not limited to studies of cell membrane mechanics. There are other applications that would benefit from simultaneous rotational and translational tracking. To further illustrate the usefulness of our approach, one potential application is highlighted here.

One of the central goals of biophysics is the accurate characterization of the elementary building blocks of biological systems with respect to both structure and physiologically relevant properties. The use of protein crystallography and electron microscopy revealed the atomic structure of many molecules but left more complex behavior concealed. With the development of techniques such as optical tweezers, magnetic tweezers and atomic force microscopy, it became possible to manipulate single molecules.

In a typical experiment, a molecule is sandwiched between a substrate and a micro-particle (see Fig. 3.13). By then exerting a known torque or a force on the particle and measuring the concomitant movement of the particle, one can determine the mechanical properties of the molecule and study the structural changes that it undergoes. Such approaches have revealed the effects of restriction enzymes on the conformation of DNA molecules\textsuperscript{213,214}, the behavior of super-coiled sections of DNA\textsuperscript{215}, and the torsional stiffness of proteins complexes\textsuperscript{70}.

A common complication in these experiments is the frequently ill-defined geometry of the molecular attachment sites. In experiments wherein torque is applied to
deform a molecule, it is essential to decouple torque and force to achieve a purely unimodal deformation. In optical traps, the application of force is independent of torque because a spherical particle is free to rotate inside an ideally symmetric focal spot. In magnetic tweezers, the decoupling of applied force and torque requires substantial efforts in shaping suitable magnetic fields by superposition. Nonetheless, even if a pure torque is exerted on a tethered particle, the variability of the location where the molecule is attached to the particle can lead to particle translations and, consequently, to stretching. Vice versa, exerting a stretching force on a molecule that has an off-center attachment point will also induce an elastic torque that leads to rotation of the particle and, unless corrected for, will result in inaccurate force-extension curves.

Given the difficulties of experimentally achieving pure stretching or pure twisting of a molecule, one can alternatively attempt to measure the associated material properties simultaneously. To this end, it is necessary to separate the effect of applied torque and induced torque. This can be achieved by measuring both translation and rotation of the particle simultaneously in response to suitably designed actuation protocols. If in addition to our method the $z$-position is acquired as well, the motion of the particle can be fully resolved in all six degrees of freedom. The motion of the particle is theoretically described by a set of six Langevin equations. The parameters in this set of equations are the linear and torsional elastic constants of the molecule and the locations of its attachment points on the surface of the particle (expressed by two angles in spherical coordinates) and the substrate. Given the motion of the particle in six degrees of freedom, these parameters can then be extracted from one experiment.
3.6. Conclusions

We have combined rotational particle tracking and magnetic particle actuation to investigate the mechanical properties of the interface between a functionalized particle and a cell. Previous investigations have mostly rested on the assumption that the probed region is mechanically isotropic and could therefore be characterized by measuring one mode of deformation at a time.

Here, we have demonstrated that mechanical anisotropy can arise as a result of binding via specific molecular ligands. By applying three-dimensional rotational particle tracking, we showed that ligation of Fc receptors leads to a strongly reduced stiffness in the plane of the cell membrane. We attribute the observed behavior to the loosening of links between the lipid bilayer and the cytoskeletal cortex. As of now, we do not have a quantitative description of such a local detachment process and the resulting loss in mechanical stability. A suitable model will have to determine the effective stiffness as a function of the size of the detached region of the membrane.

We envision that our combination of Euler angle particle tracking and magnetic particle microrheology will become a powerful tool for studies of the mechanical properties of the cell membrane and for the quantification of time-dependent interactions between the lipid bilayer and the cytoskeleton. Beyond research in cell mechanics, the application of Euler angle particle tracking is potentially useful for particle-based single-molecule studies where the tracking of additional degrees of freedom will improve the control of the experiment and the accuracy of extracted parameters.
Probing the mechanical stiffness of phagocytic cups

The uptake of matter by phagocytosis is a highly conserved function of the mammalian innate immune system. It is of key importance in the defense against bacterial pathogens, in the removal of apoptotic cells, and in the control of cancerous tumor growth. Substantial work has been directed at the elucidation of the pathways that drive the engulfment of objects, but despite the fact that phagocytosis is an inherently mechanical process, little is known about the forces and energies that the cell generates during internalization. Here, we use functionalized magnetic particles as phagocytic targets and track their motion while actuating them in an oscillating magnetic field, in order to measure the translational and rotational stiffnesses of the phagocytic cup as a function of time. The measured evolution of stiffness reveals a characteristic pattern with a pronounced peak preceding the finalization of uptake. The measured stiffness values and their time-dependence are in agreement with a model that describes the phagocytic cup as a pre-stressed membrane connected to an elastically deformable actin cortex. In the context of this model, the stiffness peak is the direct manifestation of a previously described mechanical bottleneck that can result in stalled phagocytic uptake. A comparison of model and data suggests that the membrane advances around the particle at a speed of about 20 nm/s. This approach is a novel way of measuring the mechanical properties of emerging phagocytic cups in situ and in real-time, without the need for fluorescent labeling of the cells. We expect that the method will be useful for research into the influence of uptake inhibitors, such as CD47.

* Parts of this chapter have been published in M. Irmscher, A.M. de Jong, H. Kress† & M.W.J. Prins† A method for time-resolved measurements of the mechanics of phagocytic cups. Journal of the Royal Society Interface 10, 20121048 (2013). The dagger indicates joint corresponding authorship.
4.1. Introduction

4.1.1. Phagocytosis

The ability of cells to bind and ingest objects by a process called phagocytosis is a highly conserved mechanism and can be found in a variety of different systems\(^{218}\). While unicellular organisms perform phagocytosis to take up nutrients\(^ {219}\), the process is mostly known as a key feature of the innate immune system of higher-order organisms. Several types of immune cells are capable of carrying out phagocytosis, but specialized white blood cells called phagocytes are most effective.

In the course of phagocytosis potentially harmful objects such as bacteria, apoptotic cells or foreign particles larger than about 0.5 μm\(^ {32,36}\) are first identified via native or acquired molecular patterns on their surfaces that allow immune cells to bind them. Depending on the ligand-receptor interaction, a specific signaling cascade then triggers a directed and localized deformation of the membrane with the aim of internalizing the bound object. A variety of molecules have been shown to function as phagocytic markers\(^ {32}\), most prominently complement and crystalline fragments (Fc regions) of immunoglobulins\(^ {220}\). The latter are bound by Fc receptors (FcR) whose structure is conserved across the different cell types that express it. Interestingly, genetic transfection of Fc receptors has been shown to enable phagocytosis even in cell lines that normally lack the receptor\(^ {221}\). Due to its robust and unambiguous response, Fc receptor-mediated phagocytosis remains the most frequently studied mechanism by which cells recognize and internalize external objects.

During the engulfment phase, the cell membrane is deformed such that the interaction area with the phagocytic target increases and a so-called phagocytic cup is formed. In the case of FcR-mediated phagocytosis, the membrane deformations are made permanent by new bonds that are formed in a zipper-like fashion\(^ {222}\). As the membrane is protruding, the ligation of additional receptors triggers the localized polymerization of actin filaments which provide a stabilizing scaffold. The engulfment of the particle is finalized when the membrane closes in on itself at which point the phagocytic target is entirely surrounded by an envelope comprising a lipid double layer and the previously formed actin shell\(^ {223}\). This object, called a phagosome, subsequently undergoes a sequence of steps that are aimed at destroying its contents by acidification and enzymatic digestion.

4.1.2. Mechanical aspects of phagocytosis

The actin-dependent reorganization of the membrane during the formation of a phagocytic cup is an inherently mechanical process\(^ {143,182}\). The deformation of the membrane is governed by mechanical parameters such as the cortical tension and the bending stiffness of the cell membrane\(^ {68}\). Depending on the size of the object, engulfing the phagocytic target may require the mobilization of excess membrane that can be provided through the unwrapping of membrane folds\(^ {68,149}\). The shape and orientation of the phagocytic target\(^ {143,182}\) as well as its elastic stiffness\(^ {224}\) have been shown to have an influence on the effectiveness of phagocytosis. All of these parameters are defined on a larger scale than the molecular processes that drive phagocytosis and many questions regarding the bridging of these scales remain unanswered.

In a classic experiment, the cortical tension of a phagocyte is measured dynamically by holding a non-adherent neutrophil in a micropipette at constant suction pressure while presenting it with a suitable phagocytic target\(^ {225}\). These experiments have shown that the engulfment of a medium-sized target coincides with an increase
in cortical tension as the cell membrane stretches and excess membrane is released from wrinkles. The trajectory of the target during ingestion has been found to depend on its coating, indicating that molecular recognition triggers distinct modes of force generation. Despite the insights the micropipetting approach has provided, it has the disadvantage that a connection is made between a globally measured quantity and a highly localized cellular process.

Given the complexity of the phagocytic process and the related multitude of experimental variables - some of which are difficult to determine independently by experiment - numerical simulations have been used to model the formation of the phagocytic cup. Herant et al. used the interpenetrating flow formalism to reveal the sequence of mechanisms required for internalization of a particle. Tollis et al. simulated the thermal fluctuations of the membrane and proposed actin-independent 'passive' uptake as an alternative mechanism.

Micro-particles are uniquely suited to measure forces at the site of phagocytosis. Particles in a weak optical trap have been used as phagocytic bait to reveal that protruding filopodia pull targets closer in discrete steps. During this initial stage of phagocytosis, optical tweezers are most useful due to their force resolution on the order of piconewtons. At a later stage, when the particle is in the process of being wrapped by the protruding membrane, the energy gained by the formation of multiple bonds supersedes the interaction energy between the particle and the optical trap. In this regime, discernible displacements can only be produced by methods such as magnetic tweezers or atomic force microscopes. While both the initial transport of particles by retracting filopodia and the viscoelastic environment of phagosomes have been characterized in mechanical terms, measurements of the forces that drive the formation of the phagocytic cup remain elusive.

4.1.3. Chapter overview

We developed a novel experimental approach that uses an oscillating magnetic field to actively deform the contact region between a particle and a macrophage that is in the process of internalizing the particle by phagocytosis. By measuring the induced excursions of the particle, we determine the stiffness of the phagocytic cup quantitatively and with high temporal resolution. Our approach extends the general idea of optical magnetic twisting cytometry, a method that has previously been used to study the viscoelasticity of cytoskeletal networks, by adding rotational tracking at the single-cell level. Furthermore, we developed a mechanical model to allow for a quantitative interpretation of our measurements within the framework of phagocytic cup formation.

In this chapter, we present our method and the data we acquired on the evolution of translational and rotational stiffness during both successful and interrupted internalizations of particles. We found that during successful phagocytosis, the stiffness of the contact region between particle and cell first increases before peaking and converging to plateau values. During the uptake, the particle is accelerated and pulled towards the cell center. This cell-induced displacement does not necessarily coincide with the peak in stiffness. In cases of interrupted internalization, the stiffness initially increases steeply, but then drops until the particle eventually detaches from the cell. Prior to detachment, the cell membrane undergoes strong bending deformations, indicating that the lipid bilayer has lost its association with the actin cortex. We interpret this effect as the forced breakdown of stalled phagocytic cups. Finally, to understand the obtained measurements quantitatively, we present a model of the
Chapter 4

phagocytic cup that describes the stiffness as a function of particle engulfment and takes into account the deformation of the membrane and the elasticity of the underlying actin cortex. In the framework of this model, the peak in stiffness arises as the direct manifestation of the mechanical bottleneck that has been suggested as the origin of stalled phagocytic cups and the slope of the increasing stiffness can be interpreted as the characteristic speed of membrane advancement.

4.2. Materials and Methods

Particle preparation

Ferromagnetic particles with a diameter of 4.5 μm (Spherotech, Lake Forest, IL) were prepared as described in chapter 3 and in ref. Briefly, mouse immunoglobulin (IgG) from serum was covalently bound to the carboxyl-terminated particle surface by way of a two-step reaction based on 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-Hydroxysuccinimide (NHS). We used flow cytometry to verify that the particles were loaded with mouse IgG at saturating levels. The magnetic particles were subsequently labeled with fluorescent spheres (diameter 0.2 μm) as described in chapter 3.

For experiments with a mixed functionalization with IgG and CD47, mouse IgG and protein G were bound to the particle in two successive steps. We made particles with a functionalization that comprised both IgG and a small fraction of protein G. First, protein G was incubated with a stock solution (ca. particles/ml) of particles at a concentration of 50 μg/ml for 30 minutes. Then, the same volume of EDC dissolved in 100 mM MES buffer at 100 mg/ml was added to the solution and incubation proceeded for another hour. The particles were finally washed and resuspended in 100 mM MES buffer. The volume was again doubled by addition of 100 mg/ml NHS. Mouse IgG was added such that the effective concentration per stock volume of particles was 1 mg/ml. The sample was then incubated for 1 hour under a vortexer. The sample was washed once and the coupling reaction was stopped by suspension in 50 mM ethanolamine. Finally, the particles were washed three times and resuspended in PBS with an addition of 0.5% (w/v) BSA. Prior to experiments, the particle stock solution was diluted 1:50 in Dulbecco’s Modified Eagle Medium (DMEM) and 200 ng of recombinant human Fc-CD47 (R&D Systems, Minneapolis, MN) was added to the solution. After one hour of incubation, the particles were washed twice and resuspended in DMEM. The resulting functionalization made it possible to use the particles for IgG-mediated experiments (with protein G unbound) as well as for experiments with partial inhibition by CD47 (with Fc-CD47 bound to the available protein G).

Upon incubation of the cells with an Fc receptor blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany), the probability of binding was strongly reduced. This observation indicated that the cell-particle interaction is Fc receptor-mediated. It is unclear which specific interaction between the available receptors (FcγRI, FcγRII and FcγRIII, see chapter 3) and the antibody isotypes present in mouse serum mediated the binding. It is known that the affinities of human Fc receptors for the different isotypes of mouse IgG differ by orders of magnitude.

Fluid chambers

Fluid chambers were assembled from a 18×24 mm² bottom glass slide and a 15×15 mm² top cover slip. Between the two, a clearance was created by two narrow, ap-
Probing the mechanical stiffness of phagocytic cups

Figure 4.1 – Experimental setup and protocol. (A) Particles were functionalized with mouse IgG and allowed to bind to human macrophages adherent to a glass slide. The particles were magnetized in the $xy$-plane and subsequently actuated by a continuously rotating field in the same plane. In this way, particle drift around the $z$-axis could not lead to a change in torque. (B) The stiffness was calculated by evaluating the Fourier spectra of the angular displacements inside a moving window and by relating the amplitude to the applied magnetic torque. (C) A confocal image (top view and slice from $z$ stack) of a differentiated THP-1 cell whose actin cytoskeleton has been fluorescently labelled by LifeAct. Upon differentiation, the cells spread on the glass substrate and adhere firmly. The dashed lines indicate the position of the slice. The scale bar is 10 μm.

proximately 100 μm thick strips of double-sided tape. The final volume of a fluid chamber was thus about 20 μl. The bottom glass slides were first cleaned in ethanol, then rinsed in deionized water and blown dry. After the tape was attached, the bottom substrates were sterilized under UV illumination for 1 hour. Cells were seeded onto the glass substrates on the day of preparation.

Cell culture and sample preparation

Human acute monocytic leukemia cells (THP-1) were cultured at 37°C in Roswell Park Memorial Institute medium (RPMI-1640, Life Technologies, Carlsbad, CA) supplemented with 25mM HEPES buffer, GlutaMax-I, 10% fetal calf serum (Gibco, Life Technologies, Carlsbad, CA) and 1% penicillin and streptomycin. Two days before an experiment, the concentration and viability of the cells was verified using a two-component stain (ViaCount, Millipore). If necessary, the concentration was adjusted to about $5 \times 10^6$/ml. To trigger differentiation into macrophages, phorbol-12-myristate-13-acetate (PMA) dissolved in dimethylsulfoxide (DMSO) was added to the medium to reach a final concentration of 160 nM. The dilution of DMSO in the culture medium was 1:1250. Approximately 500 μl of cell solution were then dispensed onto each prepared substrate and the samples were incubated in sealed Petri dishes at 37°C. After two days, the cells adhered to the glass surface and some cells had spread significantly. One hour before an experiment, the fluid chambers were sealed with the top cover slip and the cell medium was replaced by serum-free DMEM with 25 mM HEPES buffer.
Magnetic actuation

Experiments were carried out on a magnetic quadropole setup as previously described by Janssen et al. Here, we used four pair-wise perpendicular coils to generate a rotating magnetic field in the plane of the sample (see Fig. 4.1). We conducted all of our experiments at a rotation frequency of 0.5 Hz and at magnetic flux densities ranging from 3.7 to 37 mT. As explained in chapter 3 and in ref. 70, the generated field was homogeneous with only minor lateral field gradients within the dimensions of the sample. In a typical experiment, the particles were initially bound to the membrane. The sample was then transferred to a Helmholtz coil that produced a homogeneous magnetic field in a 300 μs pulse of about 120 mT. The vector of the remanent magnetic moment was in the $xy$-plane and the actuating field was continuously rotating in the same plane. Under such an actuation scheme, the particle rotated about its $z$-axis in an approximately sinusoidal fashion. Unlike in other experiments where the actuating field and the magnetic moment of the particle are ideally perpendicular to each other, a reorientation of the particle in $\phi$ (see Fig. 4.1) did not change the torque it experienced in interaction with the rotating field. To facilitate the quantitative interpretation of experiments, particles that were mainly rotating about their $z$-axes were chosen. We then only evaluated the rotational stiffness about $z$ but used the available markers to detect possible reorientations of the particle.

Sample heating

To keep the samples at the physiological temperature of 37°C, a 180 μm thick transparent indium tin oxide heater (Cell Micro Controls, Norfolk, VA) was placed underneath the glass slide carrying the cells. This approach did not appreciably affect the homogeneity or horizontal orientation of the magnetic field in the plane of the sample and hardly decreased the image quality. The temperature was measured by a thermistor probe in contact with the heated surface. A separate experiment verified that in equilibrium the temperature inside the fluid chamber was not more than 4°C below the set temperature. To avoid convection currents due to the temperature gradient between the sample and the body of the microscope, an additional heater (Cell Micro Controls, Norfolk, VA) was wrapped around the immersion objective to keep it at a constant temperature of 40°C.

Image acquisition

Imaging was performed on an upright microscope using a 63× water immersion lens with an NA of 0.9. Samples were imaged in fluorescence mode with simultaneous weak bright-field illumination. Under these conditions, both the fluorescent tracer particles and the outline of the magnetic particles were visible. Images were acquired using a CMOS camera (Andor Neo, Andor, Belfast, UK) at a frame rate of 7.5 fps and an exposure time of 10 ms. The image acquisition was synchronized with the magnet to record 15 frames per cycle at an actuation frequency of 0.5 Hz. Given the long time scale of the experiment, image acquisition occasionally had to be interrupted to save data to the hard drive of a computer. Images were processed offline using the approach outlined in chapter 3. Briefly, the Hough transform was used to correct for the translation of the magnetic particles while the fluorescent spheres were detected by conventional centroid tracking.
Phagocytosis activity assays

To determine the global rate of particle uptake by the cells in a specific sample, we determined the ratio of internalized particles versus the total number of cell-particle contacts. After 45 minutes of contact between particles and cells, 5 μl of 2 mg/ml goat anti-mouse antibody with a fluorescent Alexa488 label (Life Technologies, Carlsbad, CA) were added to the chamber. To prevent further phagocytic activity, the sample was then incubated on ice for 30 minutes. After incubation, the sample was washed with copious amounts of cold DMEM. We then scored at least 80 cells by counting the number of cell-particle contacts in bright-field mode and determining the fraction of internalized (i.e. non-fluorescent) particles.

Life-Act transfection

For transfection, undifferentiated THP-1 cells were diluted in medium to a concentration of about $3 \times 10^5$ /ml. In a 24-well plate, 1 ml of cell solution were added to each well. To induce differentiation, 125 μM PMA dissolved in DMSO was added to the wells such that the final concentration was 160 nM. The cells were then incubated at 37°C and at 5% CO$_2$ for 72 hours. For each well, the transfection reagents were prepared as follows: 20 μl Opti-MEM I reduced serum medium (Life Technologies, Carlsbad, CA) was added to a tube together with 100 ng pCMVLifeAct-TagGFP2 plasmid (Ibidi, Martinsried, Germany) at 997 ng/μl and 100 nl Plus reagents (Life Technologies), mixed, and subsequently incubated for 10 minutes at room temperature. Finally, 5 μl Lipofectamine LTX (Life Technologies) were added to the tube and the sample was incubated for 25 minutes. To start the transfection, 25 μl of the DNA-lipid mixture were added to each well containing differentiated THP-1 cells. The well plate was gently rocked to achieve mixing and was subsequently incubated for 24 hours at 37°C and at 5% CO$_2$.

Confocal imaging

Imaging was carried out on a Nikon Ti Eclipse laser scanning confocal microscope using an argon laser at 488 nm as an excitation source. Z-stacks were acquired with a resolution of 0.6 μm per $xy$-slice. Acquisition of a full stack took approximately 10 minutes.

4.3. Results

We introduced fluorescently tagged magnetic particles to fluid chambers containing adherent THP-1 macrophages and allowed the particles to bind. By imaging single particles in simultaneous bright-field and fluorescence mode, we then measured the translational and rotational stiffness of the dynamically changing contact interface between particles and cells. Here, we describe typical results and suggest a mechanical model to explain the observed data.

4.3.1. Successful particle internalization

At the beginning of an experiment, a majority of particles was initially bound to the membranes of the cells, which can be attributed to the avid binding of the cellular Fc receptors to the Fc regions of the immunoglobulins on the magnetic particles. In each sample several phagocytosis events occurred during an experiment and a limited
number of internalization events could be recorded. We specifically picked particles that were marked by at least two clearly visible fluorescent markers.

The rotating magnetic field caused the particles to undergo rotational as well as translational oscillations due to the partial embedding of the particles in the cells. The obtained rotational and translational trajectories were processed by analyzing the Fourier spectra inside a sweeping window function (see Fig. 4.1). As explained in chapter 3, the separate evaluation of rotational and translational oscillations is essential because the contact region between the particle and the cell is anisotropic. In the theory section of this article we will present a model of a phagocytic cup that indeed predicts an anisotropic stiffness.

We monitored the stiffness during the internalization of particles in real-time and found a characteristic evolution of the stiffness (see Fig. 4.2A,C). The rotational stiffness initially increased slowly and intermittently reached local maxima during phases...
of increased velocity. Eventually, the rotational stiffness peaked and dropped off at a higher rate than it previously increased. The translational stiffness qualitatively followed a similar sequence but its peak did not necessarily coincide with the peak of the rotational stiffness. At the end of the process, both the rotational and the translational stabilities converged to plateaus below their respective maximum values.

The rate at which the rotational stiffness increased before reaching the peak was well-preserved across different cells. Fig. 4.3 shows a comparison between four data sets that were recorded on different days. To extract the involved time scale, we normalized the data to the absolute stiffness at the peak of each curve and translated the curve such that the peak occurred at \( t=0 \) s. Three curves were recorded at 37°C, with remarkable agreement of the slopes; the fourth curve was recorded at a temperature of about 26°C and showed a significantly lower slope.

Simultaneously, we monitored the overall trajectory of the phagocytic targets. Figure 4.2B shows the movement of the particle in the \( xy \)-plane with the color-coded absolute velocity averaged over 20-second periods. The velocities but not the depicted positions have been corrected for motion of the cell itself. Clearly, the particle is drawn into the cell in almost a straight line at a non-constant velocity. The maximum particle velocity of about 35 nm/s is reached during the peak phase of the rotational stiffness. Note that this is the \( xy \)-component of the velocity and that the three-dimensional velocity can be larger. In other phagocytosis cases, the phase of increased velocity preceded the peak in stiffness (see Fig. 4.2D for an example).

We considered a particle successfully internalized if the translational and rotational stiffness stabilized and did no longer change appreciably after a period that included the dynamic evolution shown in Fig. 4.2. In some cases we observed that particles that had undergone this transition started rotating about the secondary axes after the particle reached its final position (see Fig. 4.4).
4.3.2. Interrupted internalization

In parallel to successful internalizations, we encountered particles whose uptake had begun but ultimately did not finish. We have recorded 9 of such cases where measurements show a sudden increase in stiffness, followed by a short plateau and, eventually, the detachment of the particle (see Fig. 4.5). In most of these cases, the particles had been bound to the apical membrane of the cells for periods of up to 30 minutes. Upon the beginning of a measurement, the rotational stiffness of the contact region first remained constant. We observed nearly constant stiffness values for up to 200 seconds. Surprisingly, and initially without visible changes in the morphology of the cell, the stiffness then increased 3 to 5-fold before peaking and proceeding to decrease. We found that the duration of the slope leading to the peak is about 30 to 50 seconds. This time was consistent across different cells and did not depend on the absolute stiffness which varied by one order of magnitude. Compared to cases of successful internalization, the stiffness increased at least twice as fast.

Typically, the stiffness peak was followed by a period wherein the stiffness decreased slowly with intermittent fluctuations. At the end of this intermediate phase the oscillations of the particle rapidly increased. Interestingly, we found that this drop in stiffness was frequently accompanied by the gradual advent of conspicuous periodic deformations of distal parts of the cell body (see Fig. 4.6). When the par-
Figure 4.5 – Evolution of stiffness during interrupted phagocytosis. (A) Initially the rotational stiffness remains relatively unchanged, then suddenly rises and proceeds to fall off. At \( t = 600 \) s of the example, the particle was still loosely attached but underwent large excursions that prohibited rotational tracking. Shortly thereafter, the particle detached from the cell. (B) During the initial spike, the stiffness increases 3 to 5-fold. The process takes about 30 to 50 seconds and is well preserved across cells \((N = 9)\). The average rate at which the stiffness increases before the peak (solid red line, slope \( \approx 0.015 \) s\(^{-1}\)) is clearly higher than in cases of successful internalization.

Figure 4.6 – Emerging membrane flickering during failed particle uptake. Before particles detach in cases of interrupted phagocytosis, strong oscillations of the cell membrane emerge. The magnitude of those oscillations is demonstrated in this example where the in-plane translation of fiduciary spot on the membrane was tracked during actuation of the loosely attached magnetic particle, directly before it detached. The displayed motion coincides with times \( t > 600 \) s in Fig. 4.5A of this article.

tence finally detached, the edge of the cell often looked rugged and occasionally we observed pieces of membrane material on the freely spinning particle.

4.3.3. Modeling the stiffness of a phagocytic cup

To understand our data quantitatively, we drew upon a model of a phagocytic cup developed by van Zon et al.\(^\text{143}\) that describes the interaction potential between a particle and a cell membrane based on the elastic energy stored in the protrusion of the membrane. We extended this model to extract the apparent rotational stiffness of the phagocytic cup. Additionally, we had to account for the elastic potential of the contact region that we dynamically probed by actuation of the particles.

The formation of new bonds leads to an increase in the membrane area adherent to the particle. This wrapping process effectively restricts the motion of the particle inside a continuously steepening elastic potential that is dominated by the mechanical properties of the membrane and the underlying actin cortex. In this context, the bonds between the cell and the particle are considered infinitely stiff connections.
Given the large number of available Fc receptors and IgG molecules on the particles, several hundreds to thousands of bonds are expected to form during the engulfment of the particle. Magnetic actuation creates maximum shear forces on the order of 1 nN which, if evenly shared, results in an effective force per bond on the orders of a few piconewtons. This is not enough to break the bonds inside the phagocytic cup, especially in the face of rapid rebinding as a result of the actively driven protrusion of the membrane.

The model of van Zon et al. describes the phagocytic cup as a radially symmetric protrusion of the membrane. The energy required to form the cup comes from the formation of bonds along the cell-particle interface and is described by the Helfrich free energy functional:

$$E = \int \left( \frac{\kappa_b}{2} (C_1 + C_2 - C_0)^2 + \sigma \right) dA.$$  \hspace{1cm} (4.1)

Here, \((C_1 + C_2)/2\) is the mean curvature, \(C_0\) is the spontaneous curvature of the membrane which we assume to be zero due to the spreading of the cells, \(dA\) is a surface element and \(\kappa_b\) and \(\sigma\) are the bending energy and surface tension of the cell membrane, respectively. The equilibrium shape \(h(r)\) of the membrane between an attachment point \((r_0, h_0)\) (see Fig. 4.8A) at the circumference of the particle and an arbitrary cut-off radius is determined by minimizing the energy \(E\). We followed the approach by van Zon et al. and used the shooting method to numerically solve the fourth-order differential equation (see eq. 2.29) that yields the shape of the membrane \(h(r)\) as its solution. The resulting membrane shapes are exemplified in Fig. 4.7.

In accordance with experimentally determined values, we chose a value of \(1 \times 10^{-18}\) J for the bending energy of the assembly of lipid bilayer and actin cortex. This value is about one order of magnitude higher than for unsupported lipid bilayers and mainly originates from the bending stiffness of the thin sheet of actin filaments in the cortical cytoskeleton. Since we are relating the calculated energies to the undeformed reference state of the membrane and because the bending energy is dominated by the actin cortex, we do not take the microstructure of the membrane into account. If one were to integrate the absolute Helfrich free energy of bending energy by integration over the highly curved ruffles of a typical leukocyte membrane, the resulting values would far surpass our results. The energy stored in the membrane fold close to the transition from bound to unbound membrane is small compared to the overall free energy of the phagocytic cup and can hence be neglected. Using above value for the bending energy of the membrane and typical values for the cortical tension in the range of \(10^{-5}\) to \(10^{-3}\), it becomes apparent that the deformation of the cell during phagocytosis is governed chiefly by the cortical tension, i.e. the tendency of the cell to minimize its surface. This is concurrent with computationally backed studies of particle phagocytosis.

Once the equilibrium shape for a specific position of the cell-particle contact line is known, the associated energy can be calculated using eq. 4.1. Van Zon et al. calculated the resistance against further extension of the membrane in terms of the force

$$F_S = -\frac{\partial E}{\partial S}.$$  \hspace{1cm} (4.2)

\(^\dagger\) With \(C_0=0\), the reference energy of the undeformed membrane becomes \(E_{ud} = \pi \sigma r_1^2\). \(^\ddagger\) The Supporting Material of ref. 143 has a detailed explanation of the mathematical procedure.
Here, $S = \alpha R$ is the arc length along the engulfed portion of the particle (see Fig. 4.8A). Since the membrane is attached to the particle, this force also acts on the particle and effectively draws it into the phagocytic cup. As the membrane advances, the force increases because an increasingly large circumference has to be engulfed.

Starting from this model description, we derive the associated stiffness of the phagocytic cup. We consider the force $F_S$ a distributed force acting along the leading edge of the membrane. If during actuation of the particle the phagocytic cup is twisted around its $z$-axis by a small angle $\varphi$ and no bonds are broken, the size of the cup and the force associated with it remain constant but a restoring force will counteract the rotation of the particle (see Fig. 4.8A). The resulting torque on the particle is approximately

$$\tau_z = F_S R \sin \alpha \sin \varphi.$$  \hspace{1cm} (4.3)

Under a small-angle approximation for $\varphi$, the stiffness around $z$ hence becomes

$$k_{m\varphi} = \frac{\tau_z}{\varphi} \approx F_S R \sin \alpha.$$  \hspace{1cm} (4.4)

This component of the stiffness has the character of a prestress as the force $F_S$ does not depend on the deformation induced by the rotation of the particle.

In addition to this torque, the material surrounding the particle creates an elastic potential whose associated stiffness is determined by the degree of particle internalization. The problem of the apparent stiffness experienced by a partially embedded particle has been comprehensively treated by analytical and numerical methods\textsuperscript{98,99}. Due to the complexity of the cell structure, the probed region is mostly regarded as a homogeneous isotropic medium with shear modulus $G$. We have previously pointed out the pitfalls of this assumption (see chapter 3 and ref.\textsuperscript{230}) but we assume here that the connection between lipid bilayer and actin cortex is preserved throughout the internalization of the particle since (partial) disjoining would diminish the essential ability to exert force on the phagocytic target. Under this assumption, we based our calculation of the isotropic stiffness on the results of an FEM simulation carried out by Ohayon et al.\textsuperscript{98}. In their model, the particle is partially embedded in a hyperelastic (nonlinearly elastic; commonly used for biological materials) medium and undergoes both rotation and translation in response to an applied torque. The effective
Figure 4.8 – Modeling the stiffness of a phagocytic cup. (A) The protrusion of the membrane exerts a force $F_S$ on the particle, similar to an extruded membrane tube. Twisting the phagocytic cup by a small angle $\varphi$ results in a restoring force and a stiffness $k_{m\varphi}$ that oppose the externally applied magnetic torque. The magnitude of the torque depends on the force $F_S$ and the position of the leading edge of the cup, indicated by the arc length $S = \alpha R$. (B) We model the total stiffness experienced by the particle as the sum of the membrane protrusion stiffness $k_{m\varphi}$ and an isotropic elastic stiffness $k_{iso}$ that depends on the shear modulus of the actin cortex. (B, inset) The velocity first remains constant at about 20 nm/s and rises to about 120 nm/s at the point of half-engulfment.

Shear modulus can then be found by multiplying the material shear modulus $G$ with an expression that depends on the embedding angle $\alpha$ and the distance of the particle from the surface of the underlying substrate, $h_u$:

$$G_{iso} = \left( A(\alpha) + B(\alpha) \frac{2R}{h_u} \right) G.$$  \hspace{1cm} (4.5)

Here, $A$ and $B$ are power series of the embedding angle. This effective shear modulus is then used to calculate the associated stiffness

$$k_{iso} = 6V G_{iso},$$ \hspace{1cm} (4.6)

where $V$ is the volume of the particle. The total stiffness experienced by a rotating particle is finally given by the linear superposition of the prestress component and the elastic stiffness of the emerging actin envelope:

$$k_\varphi = k_{m\varphi} + k_{iso}.$$ \hspace{1cm} (4.7)

Our model does not include the dynamics of the growth of the phagocytic cup. However, the calculated values can be linked to our measurements by mapping the size parameter $S$ onto a time scale and by adjusting the parameters of the model such that the characteristic maxima and the rising slopes overlap. For this region of the dataset, we can then extract the approximate speed of cup advancement from the local value of $dS/dt$ under the assumption that the cortical tension and the shear modulus are constant.

We applied our model to the data set from Fig. 4.2A by assuming a cortical tension of $\sigma = 0.2$ mN/m and a shear modulus of $G = 100$ Pa (see Discussion section for rationale). Using these values, the peak stiffness is about $5 \times 10^{-14}$ Nm/rad. The slope
leading to the peak overlaps with the measurements at a velocity of \( \frac{dS}{dt} = 20 \text{ nm/s} \). Upon reaching the peak, the rotational stiffness drops off at an approximately 7 times higher rate than it increased before the peak. A comparison of the model with our data (see 4.8B) showed that the velocity increased to about 120 nm/s. In the framework of this model description, the membrane hence first advances at a nearly constant speed from an initial embedding angle of about 50° at \( t = 50 \text{ s} \) until it reaches the point of half-engulfment and proceeds to rapidly close the phagocytic cup. The normalized graph in Fig. 4.3 shows that throughout different cells the speed of cup progression is remarkably well conserved at about 20 nm/s. The measurement we recorded at 26°C shows a reduced speed of approximately 10 nm/s.

### 4.3.4. Phagocytic activity assays

In microparticle experiments that involve the active induction of deformation it is essential to study the potential interference of the particle actuation with the physiological processes. For example, the pulling velocity of filopodia has been shown to depend on the counteracting force exerted on the tip by an optically trapped particle. In the context of our method, we decided to verify whether the oscillatory particle actuation has an influence on the internalization process.

We performed a series of assays designed to determine the binary outcome of phagocytosis at the single-cell level. We used secondary anti-mouse antibodies with an Alexa-488 label to distinguish internalized from non-internalized particles. The antibodies bind to external particles but do not bind to particles inside cells. After incubation, we counted the number of internalized particles and related it to the total number of particle-cell contacts. We conducted this assay both for samples that had been incubated in absence of magnetic actuation and for samples that had been subjected to magnetic particle actuation. The results show that under both condi-
tions, about 60% of all particles are internalized within a time window of 45 minutes (see Fig. 4.9). We also performed assays wherein the cells were given only 30 minutes for internalization and we found that the efficiency was close to 60% (results not shown). The degree of particle internalization was thus close to saturation and the remaining 40% are possibly cases of stalled phagocytosis. In fact, some of the particles scored as ‘external’ had a crescent shape in fluorescence which indicates that the cell did not manage to fully engulf the respective particle.

To test the validity of the assay itself, we performed additional tests at low temperature and in the presence of inhibitors cytochalasin D and CD47. Both conditions are known to decrease phagocytic activity, the former through a general reduction of cellular activity, the latter by specific inhibition of actin polymerization. Our assay showed that at 24 to 27°C and under actuation, particle internalization was reduced to about 40% while the presence of 10 μM cytochalasin D at 37°C almost completely abolished phagocytic uptake (see Fig. 4.9).

The presence of protein G on our particles allowed us to couple additional specific antibodies or recombinant Fc-chimera versions of proteins to our particles without affecting the overall concentration of IgG on the particles. We made use of this option by adding Fc-CD47 to the particles. As previously reported by Tsai et al., the presence of CD47 at the phagocytic synapse abrogates the activity of myosin-IIA and hence diminishes the efficiency of Fc receptor mediated phagocytosis\(^{233}\). In line with their findings, we found that IgG particles with an added CD47 fraction were internalized significantly less efficiently.

4.3.5. The viscoelastic environment of internalized particles

The stiffness measurements presented above have all been carried out at a constant amplitude and frequency with the aim of capturing the time evolution of the particle engulfment. As pointed out in section 2.2, the use of frequency-varying actuation can potentially yield additional information about the viscoelastic properties of the probed region. Given the difficulties associated with determining whether a particle has been phagocytosed, we investigated the viscoelastic environment of internalized particles and compared it to that of externally bound ones. To this end, we carried out experiments wherein we picked particles of which we knew whether they were internalized and subjected them to actuation protocols with an actuation frequency varying from 0.1 to 20 Hz. To avoid the rotational tracking as a source of noise, we tracked the particles in bright-field mode and evaluated their translational motion pattern. We magnetized the particles in the z-direction and subsequently actuated one pair of coils, as described in section 3.2. To achieve higher temporal resolution than in usual experiments, the exposure of the camera was phase-locked to the magnetic actuation via a shared trigger line.

The graph in Fig. 4.10A shows the frequency dependence of the absolute dynamic modulus. Five traces have been recorded for external and internalized particles each. While the variation between individual samples is substantial, the averaged curves for each group of particles reveal that the stiffness depends on the applied actuation frequency as a power law \(G \propto f^x\) with exponents \(x = 0.2\) and \(x = 0.44\) for external and internalized particles, respectively. It has to be noted that the absolute stiffness of the probed region can change during the recording of a frequency sweep. As a result, the recorded slopes are distorted which potentially leads to a misinterpretation of the viscoelastic character of the probed region.
To circumvent the influence of time-dependent changes of the stiffness, the viscoelasticity of the material can be characterized by recording the loss tangent during short actuation bursts. We hence actuated the particles (same samples as in Fig. 4.10A) with 5 cycles at 0.5 Hz and constant amplitude and determined δ. The results in Fig. 4.10B reveal that externally bound particles exhibit a narrow distribution of loss tangents with a characteristic average value of δ = 0.31 ± 0.06. In contrast, the loss tangents of internalized particles varied greatly with an average of δ = 0.76 ± 0.40. This difference indicates that the movement of internalized particles is more dissipative than that of externally bound particles. In an alternative formulation, internalized particles experience a more fluid-like environment.

4.4. Discussion

4.4.1. Critique of method

In the previous sections we have presented a method to study the dynamic evolution of stiffness of a phagocytic cup during the uptake of a spherical magnetic particle. To measure the stiffness, we employ simultaneous bright-field and fluorescence imaging and track the motion of the particle in an oscillating magnetic field. The stiffness and its changes are a consequence of the increasingly protruding membrane which restricts the motion of the particle. Our method allows us to detect the internalization of a particle based on its motion and the associated stiffness, without the need for fluorescent labeling of the cell membrane.

Previous research into the mechanical aspects of phagocytosis has focused on the extracellular transport by extended filopodia, on the viscoelasticity of the cytoplasm, or on the global change in surface tension during particle internalization. To our knowledge, our approach for the first time allows localized quan-
quantitative measurements of the mechanics of the phagocytic cup progression, thereby filling a gap in the temporal sequence of events during internalization.

In the context of phagocytosis, magnetic tweezers have been used to exert pulling forces on externally bound particles. While such approaches are suitable for studying the viscoelastic properties of the actin cortex, they are prone to disturb the formation of the phagocytic cup because the vector of the applied force is nearly anti-parallel to the preferred pull-in direction of the particle. By applying a continuously rotating 'tickling field', we apply a shear force that is perpendicular to the symmetry axis of the phagocytic cup (if the particle is not taken up 'sideways') and hence less likely to disturb the physiological activity of the cell.

To confirm that the application of a magnetic tickling field indeed does not affect the efficiency of phagocytosis, we performed assays to measure the binary outcome of particle binding at the single-particle level. These assays showed no significant difference in phagocytic efficiency between samples that had been incubated under control conditions and those that had been exposed to magnetic actuation. While this result proves that our method does not significantly hinder phagocytosis, it also indicates that phagocytosis is not facilitated by the application of force. While less intuitive than a detrimental effect, the application of force at the micro-scale has been shown to elicit strengthening of the underlying cytoskeletal structures. In the context of phagocytic uptake, such behavior has (to our knowledge) not been reported.

An important advantage of continuous magnetic actuation is the high temporal resolution. To limit the amount of data, we restricted our frame rate to 7.5 frames per second. However, at an exposure time of 10 ms, we could have acquired as much as 90 frames per second, thereby effectively decreasing measurement noise by a factor of $\sqrt{12}$ at unchanged temporal resolution.

4.4.2. Observation of internalization

During internalization, an initially bound particle moves to a position within the perimeter of the cell. The fact that the outline of the particle is fully enclosed by the cell does not necessarily indicate finalized uptake since we have observed that a particle reached a final position before the rotational stiffness peaked. Extracellular transport of the particle and its engulfment can thus be independent events that can proceed simultaneously or sequentially. Presumably, the transport of the particle towards the cell center is a process by which the contact interface is increased in an effort to facilitate engulfment. This pull-in motion can be accomplished by extending filopodia or by contractive processes at the binding site between the particle and the cell. Given the comparatively long time of contact between particles and cells in our experiments, the latter mechanism is more likely.

Since the extent of the phagocytic cup could not be visualized directly, finalized uptake of the particle had to be determined from the associated secondary measurements. As mentioned above, one criterion was the cell-induced displacement of the particle. This movement could be less pronounced in cases where the measurement was started after the particle had already been pulled closer. A second criterion was the observation of a peak in rotational stiffness, followed by a drop and stabilization at a lower level. Whether both rotational and translational stiffness decreased by an equal factor presumably depended on the way the nascent phagosome latched onto the cytoskeletal support structures. The emergence of strong rotations about secondary axes at nearly constant translational stiffness was seen as a clear sign of fin-
ished phagocytosis. We attribute this observation to the fact that the phagosome has an increased rotational freedom within the cytoplasm when the connection to the cell membrane is lost (see Fig. 4.4 for an example).

4.4.3. Interrupted internalization

At first glance, the evolutions of stiffness in cases of interrupted and successful internalization seem similar, as the stiffness first peaks before dropping precipitously. However, in cases of interrupted internalization, both the translational and rotational stiffness do not reach a plateau after peaking. Instead, they concomitantly proceed to drop until the particle detaches. This is in contrast to successful internalizations where the translational and rotational stiffnesses converge to new levels, reflecting the geometry of phagosomal attachment inside the cell. The sequence of events during interrupted internalizations can be likened to a catastrophic failure during which the connection between the cell and the particle is severed. In this process, bonds between the particle and the cell membrane are broken and, as indicated by the onset of strong membrane fluctuations, the link between lipid bilayer and actin cortex is severed.

At the start of experiments that culminated in particle detachment, the rotational and translational stiffness often remained comparatively constant for periods of up to 4 minutes. Notably, this is the time of observation, not the total time of contact between particle and cell, which can be longer. Given that during phagocytosis the membrane continuously wraps around the particle, one would normally not expect extended periods without any changes in stiffness. However, the internalization of particles and bacteria is known to stall even under physiological conditions. This has been explained by invoking the idea of a mechanical bottleneck that sets a limit to the extrusion of the membrane and is determined by the size and orientation of the phagocytic target and the potential of the cell to generate force by the directed polymerization of actin. In the context of our experiments, the extended period of nearly constant stiffness at the start of an experiment hence likely indicates that the phagocytic cup has stalled.

The dramatic increase in stiffness is in these cases presumably not caused by a rapid advance of the membrane. While the onset of phagocytosis immediately after formation of the first bonds is known to be occasionally delayed, the process itself proceeds continuously until it either finishes or stalls. Judging from the measured stiffness the particles had already been partially engulfed.

The unfolding of membrane wrinkles is one process by which cells increase their available surface area during the late stages of the uptake phase. Since this process involves the irreversible breaking of bonds and a change in surface area, it has previously been modeled as a viscous contribution to the cortical tension. The unfolding of wrinkles and the subsequent increase in surface area would hence lead to a transient rise of the effective cortical tension and the associated stiffness. As in our experiments the membrane eventually appears to detach under continuous actuation, we cannot readily determine if the presumed unfolding of wrinkles is a spontaneous process initiated by the cell or whether it is induced by the actuation.

4.4.4. Linking results and theory

Our measurements show that the rotational stiffness peaks during the internalization of a particle. This is not obvious as conventional continuum mechanics models predict that an increasingly embedded particle experiences a monotonously rising stiff-
Chapter 4

prestress \( F \) + elastic deformation

torsion angle \( \phi \)

restoring force \((F_{\text{pre}} + F_{\text{el}})\sin\phi\)

restoring torque \((F_{\text{pre}} + F_{\text{el}})RS\sin\phi\)

driving torque \( \tau \)

Figure 4.11 – A macroscopic analog of a phagocytic cup. The architecture of a prestressed phagocytic cup is comparable to the model of a hyperboloid. Such a model consists of two disks that are connected by flexible ropes and kept at a constant distance by a central axis. The more tension exists in the ropes, the higher the resistance against rotating one disk while keeping the other one fixed.

However, in Fc receptor mediated phagocytosis a protrusion of the membrane grows around the target. Since this protrusion is stabilized by bonds, the particle experiences a growing force in vertical direction. As pointed out by van Zon et al., this force becomes maximal when the leading edge of the membrane extends to the equator of the particle. We treat this force as a prestress that is independent of the deformation induced by actuation. Under small torsional deformations, the shearing of the force field then gives rise to a restoring torque on the particle. Within this framework, the measured peak in stiffness could thus be seen as direct evidence of a mechanical bottleneck. It is also instructive to consider the model of a hyperboloid for comparison (see Fig. 4.11).

The prestress in the membrane depends on the bending energy and the effective cortical tension of the composite bilayer-cortex assembly. In line with previous findings, we assumed a value of \( 1 \times 10^{-18} \) J for the bending stiffness of the bilayer-cortex assembly. The resting cortical tension of macrophages has been shown to be almost an order of magnitude higher than in neutrophils. We used the resting tension of suspended J774 macrophages (0.14 mN/m) as a point of reference for our model. Since we conducted our experiments on adherent macrophages, this value is presumably a lower limit as we expect that the deviation from a spherical shape induces an increase in cortical tension. Measurements have revealed that macrophages can increase their surface area by about 10% at nearly constant cortical tension. The engulfment of a particle with a diameter of 5 μm by a 15-18 μm macrophage requires an increase in surface area by about 30% and the concomitant cortical tension can rise up to 1.5 mN/m. We conclude that the value of the cortical tension we use in our model is in fact only constant on short time segments, such as the characteristic region around the peak of stiffness.

In addition to the torque from the prestress in the membrane, the particle experiences an elastic restoring torque as the material of the phagocytic cup is deformed by actuation. The main contribution to this elasticity comes from the actin meshwork that lines the interior side of the lipid bilayer. Its elasticity is strongly determined by the concentration of actin and the degree to which it is cross-linked. To find appropriate model parameters, we refer to magnetic microrheology studies that have found that the shear modulus of the actin cortex of endothelial cells is on the order...
of 100 Pa under baseline conditions. The addition of the contractile agent histamine resulted in rapid stiffening by more than 1 order of magnitude, presumably by an increase in cross-linking density. During phagocytosis, actin is enriched in the rim of the phagocytic cup and as a result, the stiffness of the contact region increases. It is thus unlikely that the shear modulus remains constant throughout the experiment. As mentioned above, in the later stages of the internalization, the effective shear modulus is thought to be increasingly determined by the mechanical linkage between the phagosome and the cytoskeleton.

Driving the protrusion of the membrane is an actin-dependent process (see Fig. 4.9, also ref. 234) and one can generally expect that a higher concentration of actin results in a higher driving force at the leading edge of the phagocytic cup. However, as the concentration of actin in the advancing edge of the phagocytic cup increases, more energy is required to further push out the membrane because the associated bending stiffness of the actin cortex increases as well. This balance between elasticity of the membrane and the available driving force is one possible explanation why successful phagocytosis can be observed at stiffness values that differ by one order of magnitude between cells. The model we adapted from van Zon et al. takes the increase of actin concentration into account by assuming that the energy of the bound membrane is increased by a factor of 5 with respect to that of the unbound membrane.

Our description of the stiffness of a phagocytic cup leads to an interesting prediction about the internalization of particles coated with complement. Previously, those particles have been said to 'sink' into the cell, i.e. without the formation of a membrane protrusion. Considering our two-component model, the evolution of stiffness in those cases should thus display a less pronounced maximum but instead mainly reflect the change in effective shear modulus of the surrounding medium as the particle is internalized.

The mechanical model of a phagocytic cup that we propose here also implies that the stiffness of the contact interface is anisotropic because the membrane deformation creates a state of uniaxial prestress that is superposed onto the elasticity of the material (which in itself is not necessarily isotropic). This mechanism is depicted in Fig. 4.12. As the particle is twisted about its z-axis, the restoring torque reads as...
Figure 4.13 – Kinetics of actin polymerization at the leading edge of a growing phagocytic cup. At the leading edge of the phagocytic cup monomers with a length of $\delta$ bind to the barbed end of growing filaments at a rate $k_{on}$ and unbind at rate $k_{off}$. The effective rate of filament extension is determined by the local monomer concentration $c_m$ and strongly depends on the force $F_S$ that acts on the tip of the filament. The counteracting force is determined by the size of the phagocytic cup and reaches its maximum at the point of half-engulfment.

follows:

$$\tau_z = F_S R \sin \varphi \sin \alpha + k_{iso}(\alpha) \varphi.$$  \hspace{1cm} (4.8)

If twisted about the $y$-axis (or, equivalently, about the $x$-axis), the restoring torque is

$$\tau_y = \int_{\varphi=0}^{\varphi=\pi} F_S R \sin \alpha \sin \varphi' d\varphi' + k_{iso}(\alpha) \theta$$ \hspace{1cm} (4.9)

$$\tau_y = \frac{2}{\pi} F_S R \sin \alpha + k_{iso}(\alpha) \theta.$$ \hspace{1cm} (4.10)

For rotations about $x$ and $y$ at a fixed size of the phagocytic cup, the restoring torque hence has a constant offset as a result of the anisotropic deformation of the membrane. The restoring torque for rotations about the $z$-axis (angle $\varphi$) is, in contrast, proportional to the sine of the angular deformation. As a result, equal applied torques will lead to larger excursions in $\varphi$ than in $\psi$ and $\theta$. The emergence of a phagocytic cup might hence provide an explanation for the observed stiffness anisotropy during probing with IgG-functionalized particles (see 3.4.2). In reality, phagocytic cups do not follow the idealized geometry that our model assumes. The effect on the effective stiffness can thus be more or less pronounced and, in extreme cases wherein the particle is taken up by a sideways growing cup, even reversed.

Our measurements are in agreement with the calculated model values, when the cup grows at a constant velocity before the stiffness reaches its peak and accelerates after passing the maximum. Importantly, the velocity $dS/dt$ at which the membrane advances is not (directly) related to the instantaneous velocity of the particle itself because contractive forces at the base of the phagocytic cup can lead to its retraction, even if the membrane does not advance. From the superposition of size and time scales, we could extract the effective speed at which the phagocytic cup grows around the particle. As the protrusion is driven by the directed polymerization of actin, the speed at which the cup advances is directly related to the counteracting force that the membrane exerts on the growing ends of the filaments.

Application of our model resulted in growth velocities of the phagocytic cup of about 20 nm/s for the phase preceding the stiffness peak. To verify the plausibility of this value, we refer to theoretical considerations about the extension speed of actin-driven membrane protrusions. Given the parameters shown in Fig. 4.8, the
maximum force resisting further growth of the membrane is approximately 17 nN or 4000 kT/nm. To put this value into perspective, we consider the polymerization of actin. The reduction of free energy by addition of an actin monomer to the barbed end of a growing filament depends on the concentration of monomers. Lamellipodia are comparable in structure to phagocytic cups and have been reported to contain monomeric actin at concentrations of about $c_m = 150 \mu M$. With a critical concentration of $c_0 = 0.1 \mu M$, about 7 kT are gained by addition of a monomer that extends the filament by about $\delta = 2.8$ nm. To overcome the maximum resistance at the equator of the particle, the membrane hence has to be pushed outwards by simultaneous elongation of at least 1600 filaments. Distributed along the largest perimeter of the particle, the resulting mean filament density would be at least $\approx 110/\mu m$, which is on the same order of magnitude as values reported for lamellipodia. To obtain an estimate of the speed at which the membrane is pushed outwards, we make use of the Brownian ratchet model which assumes that force-dependent thermal fluctuations in the membrane are stabilized by the addition of monomers. The growth velocity $v$ of the filament then becomes \(^{(4.11)}\)

$$v = \delta \left( k_{on} c_m \exp \frac{F \delta}{kT} - k_{off} \right).$$

Here, $F$ is the counteracting force each filament experiences and $k_{on} = 10 \mu M^{-1}s^{-1}$ and $k_{off} = 0.1 s^{-1}$ are the rates of monomer addition and removal, respectively. If we assume a force of 10 pN per filament, the resulting growth speed is about 3 nm/s. In view of the strong nonlinearity of the force-velocity relationship, this is a reasonable estimate (a force of 8 pN per filament results in a speed of 20 nm/s). The exponential increase of velocity at low forces also explains the sharp drop in stiffness beyond its maximum. If a fixed number of filaments have pushed the membrane past the point of half-engulfment, their growth rate will increase exponentially as the retention force from the membrane drops. The temperature dependence of actin polymerization could also explain the reduced rate of stiffening we observed at 26°C as the polymerization velocity increases by 25% (at 8 pN, by 70% at 10 pN) between $T = 26^\circ C$ and $T = 37^\circ C$.

These considerations serve to illustrate the plausibility of the mechanical model we have applied. Approximating the dynamics of particle engulfment in this way requires several simplifications. Importantly, we do not take into account (i) network effects \(^{239}\), and (ii) changes in the concentration of monomeric actin. Including these aspects in future refinements of the model will likely require the acquisition of additional measurements to avoid overfitting the obtained data.

### 4.4.4.1. Viscoelastic environment of internalized particles

The approach we are presenting in this chapter is based on an indirect observation of phagocytosis events. Internalization is detected by way of the associated changes in effective stiffness that a magnetic particle experiences during its engulfment. We have noticed that a large majority of particles that are known to have been internalized\(^5\) undergoes a characteristic motion that differs from that of externally bound particles. Qualitatively, the motion appears to be more viscously dominated than that of externally bound particles. A similar effect appears in motion patterns of objects moving in liquids at different Reynolds numbers.

\(^5\) We have carried out phagocytosis assays and actuated particles after fluorescent tagging. This made it possible to clearly determine which particles had been internalized.
To test whether this observation is quantifiable, we have measured the frequency dependence of the dynamic modulus and the loss tangents of both externally bound and internalized particles. Our results show that internalized particles indeed experience a more viscous environment. Due to the variability of dynamic moduli across a cell population, we cannot readily determine how storage and loss moduli differ in absolute terms. From the ratio, we can conclude that internalized particles experience a more fluid-like environment, but not whether the probed environment is more viscous (and equally elastic) or less elastic (and equally viscous). However, since the connection of the particle with the actin cortex as the main support structure is severed as the phagosome is formed, the latter option is more likely.

The insight that internalized particles are embedded in a more fluid-like environment can potentially be useful to determine the outcome of phagocytosis experiments without having to fluorescently tag the particles as described in section 4.3.4.

Measuring the loss tangent during a phagocytosis experiment did not yield conclusive results which we attribute to the fact that the synchronization between camera and magnetic actuation was achieved by simultaneous triggering of the first frame via the software of the camera. This approach is more prone to phase delays than the rigorous frame-by-frame coupling through a common trigger line that we used for recording the results shown in Fig. 4.10.

4.4.5. Outlook

Future work should be directed at verifying some of the model assumptions experimentally. Importantly, the extends of the phagocytic cup could be imaged by live-cell confocal microscopy. The data from such experiments could be used to investigate the correlation between measured increases in stiffness and advances of the rim of the phagocytic cup. The apparent concentration of proteins at the phagocytic cup can usually not be evaluated in absolute terms but might give away the relative increase, e.g. of actin, and thereby allow estimates of the change in shear modulus. To obtain better estimates of the cortical tension and/or its changes, the integration of our technique with the micropipetting approach or with optical tweezers is thinkable. The former approach would yield absolute values for \( \sigma \) while the latter approach needs additional knowledge about the membrane composition to be fully quantitative.

The purpose of our analytical model is to provide a theoretical framework with a minimal number of parameters that capture the essential mechanics of the system, in order to explain (i) semi-quantitatively the magnitude of the stiffnesses we observed in our measurements and (ii) qualitatively the time course, i.e. the appearance of a sharp maximum. The model is not meant to comprehensively describe the process of phagocytosis with all its complexities.

When additional experiments yield better estimates of the relevant parameters, we may find that the minimal-parameter model is valid in certain regimes and invalid in other regimes. Based on these insights, we may proceed with further extensions of the model. For example, the contractile movements of the actin-myosin cortex could be included by modeling a deformed mesh whose individual nodes are modeled as force dipoles. Once the time-dependent progression of the phagocytic cup can be measured, it would also become possible to relate the term \( dS/dt \), which we now have to assume, to the speed of actin polymerization at the rim of the protruding leading edge of the cup. In this way, the model could possibly be extended to include molecular kinetics.
Beyond studying the transient growth of the phagocytic cup, our method could be used to investigate the dynamics of receptor disengagement and actin depolymerization upon successful internalization of a particle. Such measurements might provide novel insight into the mechanisms by which phagosomes are prepared for intracellular transport.

Actively probing the phagocytic cup also enables systematic studies into the viscoelastic properties of the contact region between the phagocytic target and the cell. As bilayer and actin cortex are dynamically reorganizing, measuring the elastic and viscous moduli (and their ratio) might reveal the network dynamics at play and help to better understand the mechanical aspects of failed uptake.

We think that our method is suitable to study the influence of uptake inhibitors on the dynamics of phagocytic cup formation. Recently, CD47 has been identified as a membrane marker whose presence on phagocytic targets decreases the efficiency of uptake by suppression of myosin activity\(^{233,240}\). This effect is thought to shield tumor cells from phagocytes and blocking CD47 was suggested as a therapeutic approach\(^{241,242}\). However, while the inhibiting effect of CD47 has been studied, it is not clear how it affects the mechanics of phagocytic cup formation. Phagocytosis assays such as the ones that Tsai and Discher\(^{233}\) used to demonstrate the inhibiting effect of CD47, only capture the static outcome of a phagocytic challenge after a fixed period of time. Using our method, it would become possible to determine whether a reduction of uptake is caused by slower engulfment speed or by an increased occurrence of stalled cups with otherwise unchanged dynamics. The answer to this question would contribute to the understanding of the role of myosin motors in phagocytic cup formation and closure.

4.5. Conclusions

In this work, we have used magnetic particles as phagocytic targets to actively probe the stiffness inside an evolving phagocytic cup. By actuating the particles and tracking their rotational and translational motion, we were able to characterize the mechanics of the internalization of a particle. Our measurements revealed characteristic patterns of particle trajectories and simultaneous evolutions of mechanical stiff-
nesses. To understand the results quantitatively, we established a composite model that treats the probed region as a prestressed membrane in connection with an elastically deformable actin cortex. We show that the main determinants of the model, the cortical tension of the cell membrane and the shear modulus of the actin cortex, are within the expected range of values. Our method, in combination with the theoretical framework we present, constitutes a novel approach to study the mechanics of phagocytic cup formation at the single-cell level and in real-time.
5 Nonspecific adhesion and cell detachment

The adhesion of cells to surfaces plays a crucial role in processes related to motility and tissue growth. Nonspecific interactions with a surface, e.g. by electrostatic or van der Waals forces, can complement specific molecular interactions and can themselves support strong adhesion. In order to understand the mechanism by which cells establish an adhesive interface in absence of specific proteins, we have studied the detachment kinetics of monocytic cells from glass surfaces coated with poly-L-lysine. We exposed adhering cells to a shear flow and studied their deformation and detachment trajectories. Our experiments reveal that between 20 and 60 parallel membrane tethers form prior to detachment from the surface. We propose that the extraction of tethers is the consequence of an inhomogeneous adhesion interface and model the detachment mechanism as the dynamic extrusion of cooperatively loaded tethers. In our model, individual tethers detach by a peeling process wherein a zone of a few nanometers is loaded by the externally applied force. Our findings suggest that the formation of an inhomogeneous non-specific adhesion interface between a cell and its substrate gives rise to more complex dynamics of detachment than previously discussed.*

5.1. Introduction

5.1.1. Motivation

Cells attach to surfaces via specific and non-specific interactions. While molecular attachments have been widely studied, largely with the aim of elucidating the specific mechanisms of mechanotransduction, purely nonspecific cell adhesion has received less attention. Colloidal and entropic interactions accompany every contact between a cell and a surface and their influence can facilitate or inhibit the formation of specific bonds. Nonspecific interactions of cells with their substrates play an important role in tissue culture and the early stages of the formation of biofilms. A better understanding of non-specific cellular adhesion can thus potentially be leveraged to improve the design of implantable surfaces and scaffolds for tissue engineering purposes.

The interaction between a cell and a substrate is commonly characterized by the work required to remove the interface between the two. This adhesion energy of a cell and the underlying substrate has been quantified by applying a defined fluid shear stress while imaging the deformation of the contact interface or measuring the stochastic detachment of the cells. The latter method typically yields desorption curves that can be interpreted as a transition between the bound and the unbound state. The relationship between the detachment of the cell and parameters at the molecular level is then established by application of a model that describes the mechanism of detachment.

To obtain statistical significance, desorption assays are typically carried out on large numbers of individual cells and on time scales of minutes. As a consequence, the spatial and temporal resolution of such experiments is limited and the details of the detachment process itself go unnoticed.

5.1.2. Chapter overview

In this chapter, we present an investigation of the detachment mechanism of cells from a positively charged surface. For this purpose, we used a shear flow assay and measured the displacement of single cells as a function of time. Under modest shear stresses, the cells deform in a viscoelastic manner and relax backwards upon cessation of the shear flow. Under high shear stress, the cells remain transiently attached to the surface by several membrane tether before ultimately detaching. In section 5.3.2.1 we use a simplified model of tether elongation to relate the measured displacement velocity to the number of tethers. In section 5.3.2.2 we argue that in absence of molecular attachment sites, the non-uniform apposition of the electrostatically adhering area accommodates the formation of strongly adhering patches from which multiple parallel membrane tethers can be drawn. Finally, we suggest that single tethers are peeled off the substrate and that the force required to simultaneously initiate an ensemble of lipid tethers constitutes a threshold force beyond which the detachment of individual cells ensues.

5.2. Materials and Methods

Cells

Human monocytic THP-1 cells were cultured in RPMI-1640 medium, supplemented with 10% FCS and 1% Penicillin/Streptomycin and kept at concentrations between
2×10^5 and 1×10^6 1/ml. THP-1 cells were chosen for their ability to culture them in suspension and their almost ideally spheroid shape which facilitated the calculation of the applied shear force.

Sample preparation

A 4mm wide, 75 mm long clearance was cut into double-sided tape with a thickness of 200 um. Glass slides with dimensions 24x76 mm (Menzel-Gläser, Braunschweig, Germany) were cleaned in isopropanol and deionized water and the tape was attached. Subsequently, the channel floor was incubated with a solution of poly-L-lysine (PLL, Mw=150000 to 300000 g/mol, 1150 to 2300 monomer units, Sigma Aldrich) for 1 hour. Upon incubation, the microfluidic channel was washed with DI water. The microfluidic assembly was finalized by attaching a 125 μm polymethylmethacrylate (PMMA) strip to close off the channel and two inlet ports to accommodate the attachment of fluidic tubing.

Calculation of shear force

To calculate the approximate force acting on an individual cell in a laminar shear flow, we modeled the cells as spheres in contact with a flat surface. This is motivated by previous work\cite{248} and the observation that within the time scale of a typical experiment undifferentiated THP-1 cells do not spread significantly upon contact with PLL-coated glass slides but retain a round shape with a diameter of about 18 μm. The expression for the drag force acting on a sphere in contact with a wall have been derived by Goldman et al.\cite{249} and are given by

\[ F_d = F_d^* \frac{6 \pi \eta R^2 S}{S} \]  

(5.1)

Here, \( \eta \) is the dynamic viscosity which we assume as \( \eta = 10^{-3} \text{Pa}\cdot\text{s} \), \( R \) is the radius of the cell and \( F_d^* \) is a dimensionless parameter whose value depends on the distance between the sphere and the wall. In our case we assumed a distance of \( R = 9 \mu m \) between the center of the cell and the substrate in which case \( F_d^* = 1.7 \).

To calculate \( S \), we assume that the flow is laminar and fully developed. In this case, the velocity profile in the rectangular channel is given by\cite{250}

\[ \frac{v(x, y)}{v_{avg}} = \left( \frac{m+1}{m} \right) \left( \frac{n+1}{n} \right) \left( 1 - \left( \frac{2y}{h} \right)^n \right) \left( 1 - \left( \frac{2x}{w} \right)^m \right). \]  

(5.2)

Here, \( v_{avg} \) is the mean axial velocity, \( h \) and \( w \) are the thickness and width of the channel, respectively, and \( m \) and \( n \) are constants that depend on the aspect ratio \( h/w \) of the channel cross-section. The shear rate close to the channel surface is then given by

\[ S = \left. \frac{\partial v(0, y)}{\partial y} \right|_{y=\frac{h}{2}} = \frac{6Q}{wh^2} \frac{m+1}{m}. \]  

(5.3)

Experimental protocol

Before each experiment, the microfluidic chamber was flushed with PBS. Subsequently, THP-1 cells suspended in culture medium were pumped into the channel at a flow speed of 10 μl/min and allowed to adhere under no-flow conditions for 3 minutes. Each sample was subjected to 8 consecutive actuation periods lasting 30 s each at flow speeds increasing from 100 μl/min to 800 μl/min. Each actuation period was followed by a pause of 2 min to allow the cells to relax.
Figure 5.1 – Application of force on adherent cells in a shear flow assay. (A) When cells detach from electrostatically charged surfaces the adhesive interface can be gradually peeled away (solid line) or cooperatively loaded through multiple focal patches (dashed line). We supplement a regular shear-flow assay by adding trajectory tracking to determine the mode of detachment for individual cells. (B) DIC images of the stretching process reveal that the main body of the cell undergoes little deformation while a thin adhesive patch at the upstream side of the cell seems to support the load. (C) Under constant shear stress, loosely adhering cells first deform (or displace) elastically before undergoing viscoelastic creep. When the shear flow is turned off, they relax back toward their original position.

Imaging

Movies of cells were recorded on an inverted microscope (Leica DMI5000, Wetzlar, Germany) under bright-field illumination with a 20× objective (NA = 0.4) using a charge-coupled device (CCD) camera (IDT X3) at a frame rate of 5 Hz. The DIC images were recorded on an inverted microscope (Nikon Ti-E/B, Tokyo, Japan) with a 60× water immersion objective (NA = 1.2). The movies were processed by a custom-made image segmentation algorithm written in Matlab (Mathworks, Natick, MA). The algorithm invokes the Canny filter to detect the outer edges of each cell and calculates the centroid of all internal pixels to obtain the location of the cell.

5.3. Results

5.3.1. Observations

Upon initiation of a fluid flow at low shear rates, the cells displaced in direction of the flow. The movement typically included an immediate displacement $\Delta x_0$, followed by a gradual deformation wherein the position of the cells approached an equilibrium position. After cessation of the flow, the position of the cells relaxed back to their original values (see Fig. 5.1).

The trajectory of a cell terminates at the moment of detachment since the flow speeds were too high to image free cells in suspension. Before each instance of detachment, the displacement of the cell deviated from the curve of viscoelastic creep and became a linear function of time. At this point of transition, the instantaneous slope of the curve increased and progressively steepened, occasionally at clearly visible kinks in the displacement curves. During this linear phase of displacement, the aspect ratio of the cell changed only slightly while the total distance traversed reached several cell diameters. If the flow was turned off during this phase the cell returned to its resting position (see Fig. 5.2), which indicated that a physical link with its original location persisted throughout the duration of the shear flow.
Since no apparent link was visible under bright-field illumination and we could exclude fluidic effects such as backflows, we speculated that thin lipid tethers were drawn from the cell membrane and thus provided a link with its resting position. Using DIC imaging at 60 × magnification, we were able to visualize these tethers and found that multiple parallel tethers extended from the dorsal sides of the cells. The tethers were stretched under flow conditions and partially retracted and curled up upon discontinuation of the shear flow. Since the width of the tethers is close to the diffraction limit, we estimate their diameter to be on the order of 200 nm.

5.3.2. Modeling

5.3.2.1. Tether elongation

Our data show that the measured displacement of the cells becomes a linear function of time when lipid tethers are formed. In that situation the constant shear force is in dynamic equilibrium with the sum of dissipative forces acting on each tether. The equation of motion of a cell that is attached to the channel floor via \( N \) tethers that share the applied load is given by a simple extension of the general single-tether equation:

\[
\frac{dx(t)}{dt} = \zeta \left( \frac{F_d}{N(t)} - F_0 \right)
\]  

(5.4)

Here, \( \zeta \) is the effective inverse viscosity of the tether, \( F_0 \) is the force that is necessary to retain a tether at constant length and \( F_d \) is the drag force acting on the cell body. \( N \) is a function of time because the connections break sequentially during the detachment phase and as a consequence the effective force on each remaining tether increases. The effective viscosity \( \zeta^{-1} \) summarizes the friction between lipids in the same monolayer, between layers and between the inner monolayer and the cytoskeleton\(^{192}\). We assume a value of \( \zeta = 5 \times 10^5 \text{ (Ns/m)}^{-1} \) for the inverse effective viscosity. This value is slightly higher than previously reported values\(^{192,253}\) since we expect additional friction to arise from the interaction of the cell body with the surface. Our model does not include the effects of membrane depletion or tether coalescence\(^{254}\) as the currently implemented experimental method does not allow a verification of their influence.

The force \( F_0 \) that is required to retain a static tether can be estimated from the surface tension \( \sigma \) and the bending energy \( \kappa_b \) of the membrane as\(^{192,255}\)

\[
F_0 = 2\pi \sqrt{2\kappa_b \sigma}
\]

(5.5)

In this work, we have assumed \( \sigma = 50 \text{ pN\mu m}^{-1} \) and \( \kappa_b = 1 \text{ pN\mu m} \) to arrive at an equilibrium force of \( F_0 = 63 \text{ pN} \). The comparatively high value of the bending energy\(^{232}\) could indicate that the tethers are not entirely void of cytoskeletal filaments. The expression in eq. 5.5 can only give an approximation as it contains several serious simplifications. Most significantly, the membrane is assumed to be a thin monolayer with zero thickness and adhesion between cytoskeleton and the bilayer\(^{256}\) is neglected. Both effects give rise to additional terms in eq. 5.5 and their influence on the dynamics of tether extraction have been studied extensively.

Within the framework of our model description, a threshold force arises from the energy barrier that opposes the formation of \( N_0 \) cylindrical tethers. The value of the threshold force is higher than the force that is necessary to maintain the cylindrical tethers at a constant length\(^{257}\) and is known to depend linearly on the size of the spot on which the force is exerted\(^{213}\).
5.3.2.2. Electrostatic adhesion of membrane patches

In our experiments, the attachment site of each tether is an area wherein the cell membrane is in close contact with the glass substrate. Before tether elongation, the interaction energy landscape as a function of cell-surface distance arises from the superposition of attractive van der Waals forces and attractive electrostatic interaction between the PLL-coated surface and the cell membrane. The interaction of negatively charged cell membranes with PLL-coated substrates is typically dominated by electrostatic interactions and can be described by the following interaction potential as a function of the distance $h$ (see also section 2.5.1.2):\(^{154,155}\)

$$E_{el} = \frac{2\pi}{\epsilon_0 \epsilon_r \kappa} \left( \sigma_1^2 + \sigma_2^2 \right) \exp (-\kappa h) + 2 \sigma_1 \sigma_2 \sinh \kappa h. \quad (5.6)$$

Here, $\sigma_1$ and $\sigma_2$ are the surface charge densities of the surface and the cell membrane, respectively, $\epsilon = \epsilon_0 \epsilon_r$ is the permittivity of water ($\epsilon_r = 80$) and $\kappa$ is the inverse Debye length ($\kappa^{-1} \approx 1$ nm for a 0.15 M salt solution). We assumed that the surface charge density of the cell membrane is $-0.04$ Cm$^{-2}$ and that an excess of positive charges by the surface treatment results in a value of approximately $0.5$ Cm$^{-2}$ for $\sigma_1$. The latter value has a high uncertainty as the effective number of charges that are free to interact with the cell membrane is unknown. However, for a large range of values, if $|\sigma_1| > |\sigma_2|$, varying $\sigma_1$ only results in a shift of the equilibrium distance without affecting the depth of the resulting energy minimum. With the above parameters, the equilibrium spacing between cell membrane and surface is 2.5 nm at an energy density of 27 mJm$^{-2}$. 
As argued by Nardi et al., equation 5.6 is strictly speaking only valid for single-component lipid vesicles. In multi-component vesicles and in red blood cells, segregation of membrane components was shown to result in local charge matching between the interacting surfaces. As a result, the adhesion interface took on an inhomogeneous appearance with tightly adhering spots separated by blister-like cavities at increased osmotic pressure by confined counterions from the buffer solution.

In comparison with Hategan et al., we used a comparatively low concentration of poly-L-lysine of 0.1 mg/ml to functionalize our glass substrates but due to the high molecular weight of more than 150 kDa, the resulting surface charge density should be sufficient to evoke the formation of adhesion pattern as previously reported.

5.3.2.3. Peeling of attachment sites

In the context of our experiment, the tethers are hence likely supported by patches of strong electrostatic adherence. Under a sustained shear force, these tethers fail successively until the cell finally detaches. We describe each adhesive spot as a circular area with the diameter of one tether. From eq. 5.6, the adhesion energy of one tether attachment site with a diameter of 100 nm is on the order of $10^5$ kT. With a force per tether of about 100 pN and characteristic distances of a few nanometers, this energy barrier can only be lowered by a few tens of kT. Therefore the complete tether attachment site cannot be removed at once. Instead, each tether is likely to fail by peeling whereby the applied force is focused in a small adhesion zone (see Fig. 5.3). For such a small area, the adhesive energy is much lower and the applied force can distort the energy landscape sufficiently to enable rapid detachment.

The Bell-Evans model has been frequently used to describe the thermally activated dissociation of specific bonds under an applied force. In our experiments, the interaction of the cell membrane with the substrate is electrostatic and no discrete bonds can be identified. Instead, we perceive the adhesion energy of the peeling
zone as the equivalent of the binding energy. A similar discretization approach has been used to describe the detachment of absorbed proteins as the sequential failure of multiple bonds\textsuperscript{258,259}. In the framework of this model, the average lifetime $\tau$ of a bond is given by\textsuperscript{170,171}

$$\tau = \frac{2\pi \gamma}{\sqrt{\kappa_{\text{min}} \kappa_{\text{max}}}} \exp \frac{\Delta E - Fh}{kT}. \quad (5.7)$$

Here, $\kappa_{\text{min}}$ and $\kappa_{\text{max}}$ are the curvatures of the energy landscape at the minimum and the cusp of the barrier, respectively, $\gamma$ is the friction coefficient and $F$ is the applied force (per tether). The prefactor in eq. 5.7 is the so-called attempt frequency. We used $\gamma = 2 \times 10^{-8}$ pN·s·nm$^{-1}$ and obtained $\kappa_{\text{min}}$ and $\kappa_{\text{max}}$ from the deformed energy landscape $E(h) - Fh$ (see inset of Fig. 5.3B). The resulting attempt frequency was approximately $10^{-6}$ s$^{-1}$. We assume that the entire force $F$ is acting to propagate the peeling zone at the rear end of the tether attachment site (see Fig. 5.3A). We divided the adhesion area of a single tether into segments of the size of the peeling zone and found the lifetime of the tether by obtaining the sum of the segment lifetimes. We obtain an upper limit of the lifetime of a tethered cell by assuming that the tethers detach one after the other. The lower limit is given by the scenario wherein all tethers fail simultaneously and the total lifetime is equal to the lifetime of the first tether to fail. However, the calculated intervals constitute average lifetimes and are subject to stochastic fluctuations. For a single tether, we had to assume a peeling zone of about 7 nm$^2$ to arrive at lifetimes on the order of seconds. With a mean spacing of 1 nm between lipid molecules in the membrane, effectively less than about 10 molecules are thus simultaneously pulled off by the applied force.

The simulation shows that the lifetime of a tethered cell is a strongly non-linear function of the number of tethers. Beyond a pronounced tipping point at $N \approx 0.9N_0$ the failure rate of single tethers rises steeply as the applied force is distributed across a rapidly diminishing number of tethers (see Fig. 5.3B). This tipping point explains why the detachment of cells occurs at seemingly high numbers of remaining tethers, as exemplified in Fig. 5.2. Finally, in our experiment we cannot determine whether the failure of a tether is related to the detachment of its tip from the substrate or to the rupture of the membrane. Lysis tensions of cell membranes are on the order of several mN/m\textsuperscript{260} and are only reached in the final stages of our experiment when few tethers bear the applied shear force. The desorption of the tether tip is hence likely the dominant mechanism during the detachment of the cells.

### 5.4. Discussion

#### 5.4.1. Tether detachment by peeling

The initial stage of adhesion of cells to electrostatically charged surfaces has been shown to be a dynamic process wherein the adhesive surface area increases on a time scale of minutes until a steady state is reached\textsuperscript{248}. Owing to the mobility of different membrane constituents, interaction with the surface can result in a departure from the initial mixed state of the membrane and lead to inhomogeneous adhesion zones\textsuperscript{153,157}. During this process, the distance between membrane and surface is not uniform across the contact interface and as a result, the mechanical support of the cell hinges on several patches wherein tight contact has been made. Upon application of a shear force, the loosely adhering regions will rapidly detach and the stress will be focused on the remaining adhesive patches. If the patch size is sufficiently small, i.e.
close to the limit of a point force, the local normal stresses on the membrane can result in the extraction of lipid tethers.

In order to form a stable cylindrical tether, the membrane has to be extruded locally and will initially take on the shape of a hyperboloid. The force required to initiate the extraction of a tether is higher than the force needed to hold it at a constant length. This implies that, given a sufficiently focused application of force at one spot of the adhesion interface, a tether will be extruded and immediately proceed to extend as the applied load per tether is always larger than the holding force for a single tether, i.e. \( F_d/N_0 > F_0 \). In our experiments, cells that have been incubated for longer than 10 minutes no longer detached under the applied shear stresses (results not shown). This can be explained by the growth of individual adhesive sites as the cell membrane adapts to the charged surface. Once a critical size is reached, the force required to extract a tether becomes higher than the available shear force.

The existence of a force threshold for the detachment of cells is in line with previous findings. The origin of the threshold has been attributed to the successive peeling of discrete receptors. Here, we suggest that the energetic barrier for the initiation of multiple tethers gives rise to a similar threshold force. Under the experimental conditions we chose here, this force barrier seems to be lower than that for receptor peeling. On other substrates, such as polystyrene, the evolution of the adhesion site might lead to different detachment kinetics.

The extraction of low numbers of multiple tethers has been implicated in cell rolling. Here, we report the formation of a large number of multiple tethers as a consequence of an inhomogeneous adhesive interface. During the observed formation of multiple membrane tethers the binding spots are cooperatively, rather than successively, loaded. The applied shear force is thus distributed across all available binding sites. At a low density of attachment sites, this tether mechanism (see dashed line sketch in Fig. 5.1A) leads to a smaller force per attachment point than a peeling process wherein a small number of bonds at a time are sharing the load.

Even with conservative estimates of the electrostatic adhesion energy, the applied force is not sufficient to remove the adhesive spot at the end of a single tether at once. We estimated that an adhesive front with an effective size of 7 nm\(^2\) could give rise to the lifetimes we typically observed in experiments. Due to the exponential dependence of the lifetime on the absolute adhesion energy, slightly larger peeling areas result in tether lifetimes beyond the laboratory scale. The lower limit of the peeling area is given by the dimensions of the membrane and its bending rigidity. In the extreme case of folding back on itself, the length of the peeling zone, \( L_p \), becomes equal to the thickness of the membrane which is on the order of 5 to 6 nm. For a single row of lipid molecules, the effective area would hence be 5 to 6 nm\(^2\). These considerations indicate that the peeling likely occurs on the level of single molecules where the applied shear force is sufficient to tilt the landscape of the interaction energy such that the membrane progressively delaminates and detachment ensues.

5.4.2. Outlook

The research presented in this chapter establishes a link between the two disparate fields of colloidal adhesion and cell membrane mechanics. As a result, the modeling approach we chose includes a number of parameters that are not readily available and have to be estimated. Future research in this area should thus focus on obtaining independent measurements of relevant parameters. Two general lines can be identified and we will discuss both of them below.
5.4.2.1. Visualizing the adhesive interface

The force balance during the extrusion of multiple tethers hinges on the instantaneous number and size of the tethers. The number and, to lesser extent, the size of the tethers can be obtained from images of the contact interface during the extrusion process. For this purpose, surface-sensitive techniques such as total internal reflection (fluorescence) microscopy (TIRFM) or interference reflection microscopy (IRM) are well suited due to their exclusive sensitivity for a thin plane at the interface between glass and buffer solution. At sufficiently fine sectioning in $z$-direction, confocal microscopy is a potentially viable alternative.

Before TIRF microscopy became common, IRM was the primary surface-sensitive technique and it has been used extensively to study processes such as exocytosis\textsuperscript{265,266} and adhesion\textsuperscript{267,268}. Briefly, IRM is based on the interference of light that has been reflected at different interfaces in the vicinity of the substrate. In the simplest case, light is reflected at the interfaces between glass and membrane (in locations of direct contact), between glass and medium and between medium and membrane. In absence of reflections inside the optical system and given an objective with a high NA\textsuperscript{268} to suppress higher-order fringes, the brightness of the resulting interference regions becomes proportional to the distance of the membrane from the substrate.

TIRF microscopy has been developed later than IRM and in many fields replaced it due to its higher sensitivity to changes in distance between membrane and substrate and due to the ability to image specific fluorescently labeled molecules inside the imaging plane\textsuperscript{269,270}. If no fluorescent labels are applied, the objects in the imaging plane can still be visualized by the light they scatter. This approach is usually not applicable for biological membranes due to their small scattering cross-section but it has been successfully used to study the dynamics of colloidal particles in the vicinity of a surface\textsuperscript{271,272}. In TIRFM, a light beam is totally internally reflected at the interface between glass and liquid. As a result, a standing evanescent wave is generated whose intensity decays exponentially as a function of distance from the reflecting interface. The surface sensitivity of the technique arises as only fluorophores within a typical decay length of about 100 nm are excited by the evanescent field.

Either TIRF or IRM provides a possibility to determine the number of adhesive tether attachment. However, since it is likely that the size of the adhesive sites is close to the diffractive limit, TIRF is supposedly the better option as diffraction-limited spots would be easier to locate if fluorescently labeled. However, stable staining of the membrane (without rapid endocytosis of the dye molecules) or transfection of a fluorescently labeled reporter molecule that inserts into or binds to the membrane at a sufficiently high concentration are both non-trivial tasks. In a comparable approach, TIRF has been used to study the dynamic tethering of rolling cells\textsuperscript{273,274} at selectin-coated surfaces.

Measuring the number of adhesive sites contributes to a more precise determination of the holding force $F_0$ if one assumes that the effective viscosity $\zeta^{-1}$ remains constant during intermittent failure of single tethers. Additionally, the assumption that tether detach rather than breaking can be verified as well since abrupt changes in cell velocity should be accompanied by a decrease of the number of attachment sites. Depending on the size of the sites and the available time resolution, direct imaging might also reveal the peeling process of single tethers.
5.4.2.2. Studying adhesion strength

In this work, we based our calculations of the electrostatic double-layer interaction between the cell membrane and the PLL-coated substrate on assumptions and literature values of the surface charge densities. To further refine our model parameters and to derive general statements about the electrostatic interactions, independent measurements are necessary.

The interaction of surfaces at the sub-micron scale can be described in terms of energy or in terms of forces or pressure. As the nonspecific peeling model that we proposed in this chapter underlines, the strength of an adhesion is primarily given by the interaction potential. The force required to remove the interface can vary by orders of magnitude and is crucially dependent on the geometry of the induced separation and the mechanical compliance of the involved materials. Only if these additional determinants are known, can the force that leads to detachment be related to the adhesion energy.

Alternatively, the interaction energy of two surfaces can be determined by measuring the attractive or repulsive force between them in equilibrium. For this purpose, several generations of surface force apparati (SFA) have been designed\textsuperscript{275–277}. Commonly, these instruments allow for precise control of the separation between two surfaces. In the instrument designed by Israelachvili et al.\textsuperscript{276}, the distance is measured interferometrically and the force is determined from the deflection of a calibrated spring. The development of the atomic force microscope (AFM)\textsuperscript{60} spawned a second generation of instruments that could sensitively measure the interaction forces between charged surfaces across aqueous solutions. Typically, the sharp tip of a conventional AFM would be replaced by a colloidal particle with radius \( R \) and the force per unit area is obtained as \( F/R \) from the Derjaguin approximation\textsuperscript{278,279}.

Besides potentially yielding better approximations of the parameters that govern the cell-substrate interactions, the insights from experiments as sketched above can be used to minimize nonspecific interactions between cells and colloidal particles while still allowing for specific receptor-ligand binding to occur. In such a scenario the application of a controlled force on the particle can be expected to yield detachment kinetics that can be analyzed in the framework of the Bell-Evans model (see section 2.5.2). Due to the stochastic nature of bond failure, many bond rupture events have to be recorded before the underlying parameters can be reliably extracted. In practice, the critical level of significance can be reached by repeated measurements on the same specimen or by highly parallel acquisition of data.

Given the high spatial and temporal resolution of an AFM, it seems the primary choice for probing the adhesion strength between cellular receptors and their ligands. The cantilevers of early AFM were too stiff to generate discernible displacements in the low pN regime that is relevant when interfacing with soft-matter systems, such as cell membranes or single molecules. Only when advances in micromachining enabled the fabrication of thin cantilevers, AFM became a suitable tool for studies on biological membranes. In the meantime, the biomembrane force probe (BFM) found widespread use. Originally developed by Evans et al.\textsuperscript{280}, it consists of a ligand-coated sphere that is glued to a pressurized membrane sphere which in turn is held at the orifice of a micropipette. Under an applied axial load, the membrane capsule deforms, thereby fulfilling the function of a force transducer. In an experiment, the coated sphere is brought in contact with a cell and subsequently retracted. In a similar way as in AFM experiments, the acquired force-distance curve then reveals the molecular properties of the established contact interface. To achieve the
desired single-molecule resolution, the density of the particle functionalization and the contact time between particle and cell are adjusted such that approximately 3 of 10 repeated approachments lead to the formation of a bond. Under the assumption that the number of bonds formed during contact follows Poisson statistics, a 30% success rate translates into an 85% chance that a recorded rupture event is indeed a single bond breaking \(^{281–283}\).

In view of future biosensing applications, magnetically enforced dissociation is a viable approach due to its inherent potential for multiplexing \(^{284–286}\). So far, magnetic tweezers have been used to dissociate pairs of purified proteins wherein one binding partner was bound to a solid substrate. To explore the kinetics of ligands and receptors in their native environment, the magnetic particles have to be functionalized such that nonspecific binding to the constituents of the cell membrane is abrogated without affecting the ability to form specific bonds. This aspect requires considerably more attention than in single-cell experiments with repeated apposition because the time and pressure of contact cannot be controlled conveniently across a population of particles. The functionalization of the particles hence has to be adjusted to allow for single-molecule binding under equilibrium conditions where the rate of bond formation is zero, thereby approximating the idealized stable point-contact between a particle and a cell. In this regime, the mechanics of the contact site are negligible\(^{†}\) and magnetic particle probing is expected to become a viable tool to access molecular characteristics of membrane receptors.

5.4.2.3. Methods of reducing nonspecific interactions

The occurrence of nonspecific interactions is a recurring topic in studies that involve interfacing biological systems with artificial probes. For colloidal solutions, these interactions can be described within the framework of the DLVO theory that includes both electrostatic and van der Waals forces. Typically, aggregation of particles is not a concern at low salt concentrations due to the large screening length. However, at physiological ionic concentrations, the Debye length is on the order of 1 nm and nonspecific interactions become more likely. For this reason, surfaces are often passivated with proteins such as bovine serum albumin (BSA) or casein to prevent nonspecific adsorption. In a more sophisticated approach, molecular spacers such as polyethylene glycol (PEG) have been used to increase the hydrophilicity of proteins and particles and to introduce a steric repulsion force. Both effects are not captured by traditional DLVO theory.

To prepare micro-particles for use in force spectroscopy as outlined above, heterobifunctional PEG molecules can be used as linkers that passivate the surface and minimize nonspecific interactions while their end groups support the attachment of specific protein ligands. At sufficiently high surface coverage, the linker molecules will form a brush-like structure whose thickness determines the equilibrium distance between the particle and the cell membrane. The challenge in this approach is then to strike a balance between the entropic and hydrophilic repulsion and the required proximity of the proteins and their receptors in the cell membrane.

\(^{†}\) If the receptor is coupled to the cytoskeleton, the loss of this connection under force can lead to the extraction of a membrane tether. As the particle moves out of focus, this situation can be mistaken as a dissociation event and hence has to be distinguished from the actual detachment of the particle from the membrane.
5.5. Conclusions

In this chapter, we studied the nonspecific adhesion of cells and established a connection between an inhomogeneous adhesive interface and the kinetics of detachment. Our modeling approach is dependent on several ad-hoc estimates for crucial parameters but nonetheless is sufficient to qualitatively explain our experimental observations. From microscopy images, the number of membrane tubes and, given sufficient resolution, the diameter of the tethers can be estimated. The approach we demonstrate here can be extended to acquire more data about the detachment process. Internal reflection microscopy (IRM) or total internal reflection microscopy (TIRM) are microscopy methods that are suitable for imaging of the adhesion area and could potentially be leveraged to determine the number, size and distribution of the sites where the lipid tethers make contact with the substrate (see section 5.4.2.1).

In summary, we have studied the detachment behavior of non-specifically adhering cells with an inhomogeneous adhesive interface. In particular, we have found that these cells do not simply peel off under an applied shear stress but detach whilst transiently being held by multiple lipid tethers that share the load. These tethers are sufficiently long-lived to extend by several micrometers and are likely to detach individually by peeling of their contact sites. Our work demonstrates that previously proposed models for the detachment of cells have to be extended to account for more complex mechanisms of detachment. Such models can then be used to quantitatively analyze the results from cell detachment assays and distinguish cells with a homogeneous adhesion interface from those with an inhomogeneous interface.
The research described in this thesis embodies two possible approaches to study interfacial mechanical effects between live cells and artificial surfaces. During the course of these studies, new knowledge was generated and the boundaries to related research and obstacles for future investigations were sharpened. In this concluding chapter, we summarize the main findings and provide an outlook towards future work.
6.1. Introduction

The aim of the work summarized in this thesis was to investigate the interactions between cells and surfaces in the perspective of future biosensing solutions that use cells as diagnostic targets. To this end, we employed biophysical measurement techniques to study the mechanical properties and processes that occur as cell membranes interact with functionalized surfaces.

In chapters 3 and 4, the use of magnetic particles as mechanical probes at the single cell level was investigated. These studies showed that enhanced motion tracking of single particles leads to an increased resolution for mechanical effects that are likely related to the connectivity between the lipid bilayer and the actin cortex of a cell. In chapter 4, we demonstrated the usefulness of advanced particle tracking and a suitably chosen actuation scheme for dynamic measurements at the site of evolving phagocytic cups. In chapter 5, we investigated the interaction of cells with a flat substrate and the resulting desorption behavior under application of a shear flow. The obtained results indicate that the inhomogeneity of the contact interface between a cell and a substrate can lead to detachment kinetics that go beyond the currently used model of peeling detachment. In this chapter, the conclusions from the previous chapters are used to synthesize general insight in the perspective of both future research and biosensing applications.

6.2. Accessing the molecular scale

Biophysical research is conducted at different length scales, ranging from molecules to cells, tissues and even whole organisms. In most of its subdisciplines, there is a 'drive towards the bottom' that is motivated by the notion that in order to fully understand a system, one needs to understand its elementary architecture.

In the present thesis, we measured mechanical processes as the integrated behavior of multiple heterogeneous molecular constituents. The magnetic particles we used to apply forces and torques (see overview in Fig. 1.5) typically establish a contact area on the order of 1 μm². In chapter 5, cells remain transiently attached via patches with a diameter of about 100 nm. At these length scales, the heterogeneity of the cell membrane (and the subjacent cortex) prohibits the identification of discrete molecular effects. To nevertheless obtain molecular resolution, three possible avenues exist:

(i) Simplification of the biological system - If the mechanical properties of the cell membrane are known to strongly depend on a few molecular components, a model system can be reconstituted from these components. Such approaches are common in studies of the dynamics of vesicles in contact with surfaces and each other. Using appropriate numerical and analytical models of the interactions between lipid molecules, the interfacial energy and the bending energy of the vesicle membrane can be predicted and experimentally verified by measuring, e.g. the bending fluctuations of the vesicle.

(ii) Controlling multivalent interactions - The equilibrium interfacial contact area between a micrometer-sized particle and a cell membrane can potentially be minimized to the limit of single molecule binding by tightly controlling the molecular architecture on the particle surface. Commonly used techniques from colloidal chemistry, e.g. the use of PEG brushes, in combination with theoretical approaches to valency-limited interactions between surfaces, can help to systematically iden-
Conclusions and outlook

109

tify suitable conditions. Once molecular contact can be established in equilibrium, the magnetic particle can serve as a transducer for force spectroscopy methods.

(iii) Using nanometer-sized particles - Single molecules can be targeted directly by reducing the size of the particles themselves to the molecular scale. This approach has limited use for force spectroscopy applications since the scaling of the magnetic energy reduces the applicable forces below values that would noticeably affect most non-covalent bonds. Owing to the fluidity of the cell membrane, the forces are, however, sufficient to influence the diffusive dynamics of single molecules.

6.3. The biosensing perspective

At its current level of maturity, the magnetic probing technique presented in chapters 3 and 4 can be used in research environments. In such settings, phagocytosis should be regarded more broadly as a behavior in cell motility that shares many characteristics with cell migration. With this perspective, the presented method enables systematic quantitative studies on the effect of molecular agents on the mechanical machinery of the cell. In the context of disease mechanisms, such studies can lead to the discovery of novel pharmacological substances for more effective treatments.

Measuring the mechanical properties of cells is an intriguing topic within the confines of biophysical research and in the perspective of diagnostic biosensing devices this thesis provides an exploratory basis for further studies. The research we conducted has shown that absolute measurements of mechanical properties are subject to strong variations across populations and, depending on the localization of the measurement, on a single cell. These variations are the result of multiple effects that cannot easily be distinguished in experiments, e.g. bilayer-cortex connectivity, cortex density and degree of cross-linking, membrane tension and motor activity. This fact is reflected in the conspicuous lack of comprehensive quantitative models that can be rigorously verified in whole-cell mechanical experiments.

Some molecular processes lead to scale-free emergent phenomena that can potentially be addressed by future biosensing applications because they are subject to less variation. For example, magnetic twisting cytometry has served as a useful tool to study the frequency scaling behavior of cytoskeletal mechanics and in chapter 4 we have used a similar approach to distinguish internalized from non-internalized particles. Besides scale-free emergent phenomena, parameters that are related to the efficiency of functional processes, e.g. the time required for phagocytosis or the time for a platelet to fully spread, are likely to exhibit less variation due to evolutionary selection pressure. This reflects the ability of a cell to compensate non-optimal variations in some of its subsystems in order to achieve a goal that might be essential to the survival of the organism that contains the cell.

In the perspective of future biosensing devices, the ability of cells to actively adapt to a change of their environment is a formidable obstacle since it can lead to uncontrolled, and possibly unrecognized, degradation of patient samples. To enable the acquisition of specific parameters, most cell-diagnostic applications, such as flow cytometry, hence incorporate fixated cells whose morphology has been 'frozen' by chemical cross-linking. To aid standardization and to increase robustness, it is thus likely that future generations of cell biosensing devices will initially be based on fixated samples as well.

Since cells die during fixation, actively driven processes that might hold information about the state of the cells cannot be measured on fixated samples. Nowadays, platelet aggregometry is the main group of diagnostic tests that record the function
of living cells. The development of functional tests for eukaryotic cells will require
the discovery of processes that occur in a similarly robust manner as the irreversible
in-vitro activation of platelets. The methods of cell biophysics can contribute to the
discovery process by generating mechanistic insight into the fundamental processes
that drive the function of cells. With growing insight into these fundamental mech-
anisms, we can eventually expect the emergence of application-oriented research
aimed at identifying robust parameters that are subject to disease-related deviations
and are amenable for measurements in biosensing devices.

6.4. Future work

The research presented in this thesis proves the feasibility of the proposed methods.
To obtain a better understanding of the biophysics of cellular adhesion processes,
the developed techniques can be improved and combined with other methods to
broaden the acquired picture.

As demonstrated in chapter 4, magnetic probing of phagocytosis has the potential
to aid our understanding of the processes that drive the active and directed reorgani-
zation of the membrane and the underlying actin cortex. In a next step, the existing
measurement setup should be extended to include fluorescence imaging of relevant
molecules such as actin, myosin or specific membrane lipids. With the dynamics of
those molecules in hand, the measured stiffness can then be related to the geometry
and molecular composition of the phagocytic cup. These correlations can poten-
tially unravel the individual molecular contributions to the concerted macroscopic
effort of particle internalization and might have generalizable bearing on other pro-
cesses in cell motility. Additionally, the measurement setup would benefit from a
system that allows for controlled initiation of contact between single magnetic parti-
cles and cells. This enhancement is expected to increase the throughput beyond the
level where relative differences between controlled populations become appreciable
and statistically significant.

Pending additional experimental data, the theoretical model we used to rational-
ize our measurements of the stiffness of phagocytic cups can be extended. At the
moment it is based on a minimal set of parameters that are thought to be the main
determinants of stiffness. Using data from time-lapse images, the kinetics of actin
polymerization or the diffusive dynamics of the Fc receptors could be integrated to
arrive at a fully dynamic model description. At any time, further refinements should
be understood as a support of stiffness measurements on live cells. In order to ren-
der a complete image of the morphological rearrangements during the phagocytosis
of particles, complex computational models based on finite element methods
are more suitable.

The investigation of non-specific adhesion and flow-induced detachment dynam-
ics of cells should be extended by inclusion of surface-sensitive microscopy. Once
the morphology of the contact interface can be imaged directly, many of the current
assumptions about the morphology of the interface can be verified. As in the work
with magnetic particles, the acquisition of qualitatively new data enables verification,
and possibly the extension, of the currently used theoretical model.
Bibliography


Nomenclature

Abbreviations

*FWHM* Full width at half-maximum

(EM)CCD (Electron-multiplying) charge-coupled device

AFM Atomic force microscope

BSA Bovine serum albumin

CD Cluster of differentiation

CD11a α-subunit of integrin LFA-1

CD14 Co-receptor of lipopolysaccharide

CD18 β-subunit of integrin LFA-1

DLVO Derjaguin-Landau-Verwey-Overbeek

EDC 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

Fc Crystallizable fragment of an immunoglobulin

Fc(γ)R Fc receptor (for immunoglobulin G)

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IgG Immunoglobulin

IRM Interference reflection microscopy

LFA-1 Lymphocyte function-associated antigen, consists of subunits α (CD11a) and β (CD18)

LPS Lipopolysaccharide

MES 2-N-morpholinoethanesulfonic acid

NHS N-hydroxysuccinimide

PBS Phosphate buffered saline

PEG Polyethylene glycol

PLL Poly-L-lysine

PMMA Polymethylmethacrylate

RPMI Roswell Park Memorial Institute (medium)

SFA Surface forces apparatus

TIRFM Total internal reflection fluorescence microscopy

Constants

\( \epsilon_0 \) Vacuum permittivity [F m\(^{-1}\)]

\( e \) Elementary charge [C]

\( k \) Boltzmann constant [J K\(^{-1}\)]

\( N_A \) Avogadro constant [mol\(^{-1}\)]

Variables

\( \alpha \) Half-angle of embedded particle section [deg]

\( \epsilon_r \) Relative permittivity [-]
\( \eta \) Dynamic viscosity \([\text{Nm}^{-2}\text{s}]\)
\( \kappa^{-1} \) Debye length \([\text{m}]\)
\( \kappa_b \) Bending energy \([\text{J}]\)
\( \nu \) Poisson's ratio \([-\text{-}]\)
\( \psi \) Rotation angle about \(x\)-axis \([\text{deg}]\)
\( \sigma \) Cortical tension \([\text{Nm}^{-1}]\)
\( \tau \) Magnetic torque \([\text{Nm}]\)
\( \theta \) Rotation angle about \(y\)-axis \([\text{deg}]\)
\( \varphi \) Rotation angle about \(z\)-axis \([\text{deg}]\)
\( A \) Surface area \([\text{m}^2]\)
\( B \) Magnetic flux density \([\text{T}]\)
\( C_1, C_2 \) Principal curvatures \([\text{m}^{-1}]\)
\( E \) Young's modulus \([\text{Pa}]\)
\( G \) Shear modulus \([\text{Pa}]\)
\( G^* \) Complex dynamic modulus with \(G^* = G' + iG''\) \([\text{Pa}]\)
\( G' \) Storage modulus \([\text{Pa}]\)
\( G'' \) Loss modulus \([\text{Pa}]\)
\( H \) Hamaker constant \([\text{J}]\)
\( h \) Distance between interacting surfaces \([\text{m}]\)
\( I \) Image intensity \([-\text{-}]\)
\( I_c \) Ionic strength \([\text{mol m}^{-3}]\)
\( k \) Rotational stiffness \([\text{Nm rad}^{-1}]\)
\( K_A \) Area expansion modulus \([\text{Nm}^{-1}]\)
\( m \) Magnetic moment \([\text{Am}^2]\)
\( p \) Pressure \([\text{Nm}^{-2}]\)
\( R \) Particle radius \([\text{m}]\)
\( S \) Length of internalized particle perimeter \([\text{m}]\)
\( T \) Temperature \([\text{K}]\)
\( T \) Torque, normalized by particle volume \(\tau/V\) \([\text{N m}^{-2}]\) or \([\text{Pa}]\)
\( u \) Strain \([-\text{-}]\)
\( V \) Volume \([\text{m}^3]\)
\( \text{NA} \) Numerical aperture \([-\text{-}]\)
Publications

Journal articles


Oral presentations


Poster presentations (selection)


† Joint corresponding authorship.
Matthias Irmscher was born in Leipzig, Germany on June 18, 1983. He grew up in Berlin and graduated from secondary school at Johann-Gottfried-Herder-Gymnasium in 2002. Following one year of alternative civilian service in the IT department of a hospital, Matthias went on to study Mechatronics at Ilmenau University of Technology. In 2006, he was awarded a Fulbright Scholarship which allowed him to spend one year specializing in MEMS technology at the University of Maryland at College Park, MD, USA. In April 2008, Matthias moved to Eindhoven, The Netherlands to carry out an internship at Philips Research. His project dealt with the improvement of a magnetic actuator for the Magnotech biosensor platform. He stayed at Philips to carry out his Master’s thesis project on the development of a magnetic actuation system for rapid sample enrichment. Matthias graduated from Ilmenau University of Technology in March 2009 with a Dipl.-Ing. degree in Mechatronics.

In May 2009, Matthias started working as a PhD student in the group Molecular Biosensors for Molecular Diagnostics (MBx) at the Applied Physics department of Eindhoven University of Technology. Under the supervision of prof. Menno Prins, he carried out four years of research to advance the understanding of mechanical interactions at the interface between live cells and functionalized surfaces in the context of future cellular biosensing concepts. This thesis summarizes the main results of his work.
Acknowledgements

First of all I would like to thank my advisor Menno Prins for his unwavering support and his encouragements to keep the big picture in view. Thank you for giving me the opportunity to explore my interests beyond engineering and for giving me the freedom to interpret my project in a liberal way. I greatly appreciate your ability to critically examine the details of my work within the limited time at your disposal.

I am no less grateful to my supervisors Arthur de Jong and Holger Kress for their daily support. Arthur, your door was always open for impromptu discussions and your critical feedback helped me to distinguish practical from non-practical ideas. Holger joined our group when my project was already well underway but he nonetheless helped me to mold it into its current shape. Your experience with cell biophysics and phagocytosis helped me to put my work into perspective and your ever-constructive critique made for great sparring that contributed to my development as a scientist. I wish you the best of luck in building your own productive research group in Bayreuth.

I greatly appreciate the work of the members of my thesis committee Marileen Dogterom, Volkmar Heinrich, Luc Brunsveld and Kees Storm. Thank you for taking the time to read my thesis and for providing your feedback.

The work presented in this thesis would not have materialized without the help from the students whose efforts I supervised. I would like to thank Karel van Laarhoven for doing all the experimental work in chapter 5, including the finicky assembly of our custom-made fluid chambers. It was good fun discussing not only the research, but also a wide range of other topics outside science. I would also like to thank Marco Fiorito and Nicoleta Ploscariu for helping me to explore alternative routes of research and I hope that you will remember your time in Eindhoven mainly as a great experience that can impossibly be marred by frustrating lab work. Finally, I would like to thank Loek Verhees and Bert Verhoeven for helping me kick-start my project in the very beginning. To all of you: much success in your future careers!

Outside our group a number of collaborators helped me with the non-physical aspects of my work. Jeroen, you initially taught me how to isolate primary cells from blood until I realized that, given the rate of failed experiments, I would either make the Eindhoven blood bank run dry or take 10 years to graduate. When I switched to using THP-1 cells, you periodically defrosted and supplied me with a new batch of those critters. Thank you and good luck in finishing your own PhD thesis! Micha, we spent almost a year trying to wrap our minds around the results of Marco’s experiments. There was a lot of shared frustration but not enough to derail two exiled Germans from good old Mahlsdorf. Thanks for your support and all the best for finishing your own project! Gerard, it was great fun working with you. Thank you for making me realize that inexplicable results are quite normal in cell biology, even if you are a properly educated biologist and not an ingenuous physicist. Finally, I would like to thank the long list of people who participated in the CTMM meetings and commented on my work.

I would like to extend my gratitude to my colleagues from the MBx group who contributed to an enjoyable atmosphere. Leo, thank you for maintaining an open-door policy and for the occasional discussions about the theoretical aspects of my
work or about the latest electronic gadgets. Peter, thank you for the enjoyable discussions about 'cooking'. I wish you lots of success in pursuing your career as a young professor. Loes, thank you for sharing the pain of large sample-to-sample variations, the shared time at conferences and, finally, for leading the way in finishing your PhD. Claudia, thank you for keeping the lab organized and for your efforts in 'illuminating' my cells. Xander, Francis, Marijn, Alexander, Kelly, Konrad, Fabiola, Stefano, Roland, Denis: thank you for the countless hours we spent chatting while having lunch or coffee. Specifically for the aborigines among you: without our well-regulated lunch break, I probably would never have learnt to speak proper Dutch - thank you for being patient!

Outside the workplace, there were many people who I spent the good and the not-so-good hours with. Francis, thank you for some early hints on how to navigate a PhD project and thanks for the time we spent riding bikes and tasting beers. Irma, thank you for shared musings on all non-physical things and the critical eye on the design of my thesis cover. Many other friends are spread across countries and I hope that we will have the time to see each other more frequently in the future.

I am eternally grateful to my parents for teaching me the benefits of hard work and commitment. Thank you and the rest of the family for your steadfast support and your love. Finally, Grace: thank you for your unconditional love, your understanding, your composure and your smiles. We have quite a journey behind us and I don't regret a single step. One of the first sentences you taught me still holds: 我很喜歡和你在一起.