A New Strategy for the Analysis of Biospecific Interactions, Exemplified for Calmodulin and Two of its Targets
A NEW STRATEGY FOR THE ANALYSIS OF BIOSPECIFIC INTERACTIONS, EXEMPLIFIED FOR CALMODULIN AND TWO OF ITS TARGETS

LUKAS TRAXLER

PhD Thesis

Institute of Biophysics
Johannes Kepler University Linz
STATUTORY DECLARATION

I hereby declare that the thesis submitted is my own unaided work, that I have not used other than the sources indicated, and that all direct and indirect sources are acknowledged as references. This printed thesis is identical with the electronic version submitted.

Linz, Austria, November 20, 2017

______________________________
Lukas Traxler
ACKNOWLEDGMENTS

It would not have been possible to perform the work for this thesis without the help and support of people around me, to only some of whom it is possible to give particular mention here.

Many thanks go to . . .

. . . my supervisor Hermann Gruber for his guidance, expertise, motivation, and his continuous support in all matters.

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. . . Tatsiana Charnavets at BIOCEV for providing me access to the Microscale Thermophoresis setup.

. . . my parents, brother, and my partner in life for all the things that cannot be described in a single sentence.
ABSTRACT

Calcium entry into non-excitable cells is mainly carried by store-operated channels, whereby Orai acts as the calcium channel in the plasma membrane and the stromal interaction molecule (STIM) functions as the calcium sensor in the endoplasmic reticulum (ER), as well as the activator of Orai channels. Compared with activation of Orai, much less is known about the mechanism of Ca\(^{2+}\)-dependent inactivation (CDI) processes. It has been proposed that binding of calmodulin (CaM) to a highly conserved N-terminal segment of Orai1 is important for CDI, although its exact role remains unclear. Typically, CaM binds most of its targets by wrapping around an amphipathic \(\alpha\)-helix. The N-terminus of Orai proteins contains a conserved CaM-binding segment adjacent to the first transmembrane helix, but the binding mechanism is only partially characterized. Here, a new concerted strategy for the analysis of biospecific interactions using microscale thermophoresis (MST), surface plasmon resonance (SPR), and atomic force microscopy (AFM) was employed to study the binding equilibria, the kinetics, and the single-molecular interaction forces involved in the binding of CaM to the conserved helical segments of Orai1 and Orai3. The results consistently indicated step-wise binding of two separate target peptides to the two lobes of CaM. An unparalleled high affinity was found when two Orai peptides were dimerized or immobilized at high lateral density, thereby mimicking the close proximity of the N-termini in native Orai oligomers. The analogous experiments with smooth muscle myosin light chain kinase (smMLCK) showed only the expected 1:1 binding, confirming the validity of our methods.
ZUSAMMENFASSUNG

The underlying research project of this thesis was embedded in the interdis-
ciplinary PhD college “NanoCell”, a joint program between different institu-
tions. The research described in the following thesis was conducted at the 
Institute of Biophysics at Johannes Kepler University Linz between October, 
2014 and October, 2017. The financial support for this work came from the 
State of Upper Austria, which is gratefully acknowledged.

The major results presented in this thesis have been published in the fol-
lowing article:

- L. Traxler et al. Detailed evidence for an unparalleled interaction mode 
press. [210]

Furthermore, parts of my PhD work were contributions to other articles, book 
chapters, and conference proceedings, as listed hereafter:

- D. Zauner et al. Regenerative biosensor chips based on switchable mu-
tants of avidin - a systematic study. *Sens Actuator B Chem*, 229:646–654, 
2016. [232]

- C. Knoglinger et al. Regenerative biosensor for use with biotinylated 

- L. Traxler et al. Reversible immobilization of biotinylated baits on re-
generative sensor chips: comparison of switchable avidin mutants with 
wild-type streptavidin. In: *Advances in Sensors: Reviews*. IFSA Publish-
ing, in press. [211]

- L. Traxler et al. Characterization of the Orai-calmodulin interaction 

- L. Traxler et al. Convenient biological interaction analysis with a regen-

One article has been submitted and is currently under review:

- M. Fahrner et al. Communication between N-terminus and Loop2 tunes 

As the first three of the above listed publications (Zauner et al. [232], Knog-
linger et al. [114], Traxler et al. [211]) were essential for many experiments 
within this thesis, they are enclosed in the appendix along with an explicit 
description of my respective contribution.
CONTENTS

1 MOTIVATION, OBJECTIVES AND OUTLINE OF THIS THESIS 1

I INTRODUCTION 5

2 BIOLOGICAL BACKGROUND 7
  2.1 Introduction to Calcium Signaling and Ca$^{2+}$-Binding Proteins 7
  2.2 Calmodulin 8
    2.2.1 Target Recognition and Binding 11
  2.3 Calcium Entry Into Cells 12
    2.3.1 The Two Key Players: STIM and Orai 12

3 METHODS FOR BIOSPECIFIC INTERACTION ANALYSIS 17
  3.1 Microscale Thermophoresis 17
    3.1.1 Theoretical Background of Thermophoresis 18
    3.1.2 Experimental Implementation 20
    3.1.3 MST Timetrace 20
    3.1.4 Signal Evaluation 22
  3.2 Surface Plasmon Resonance 25
    3.2.1 Principle of Operation: Overview 26
    3.2.2 Theory of Surface Plasmon Resonance 27
    3.2.3 Sensorgram and Signal Analysis 30
  3.3 Atomic Force Microscopy 33
    3.3.1 Functional Principle 33
    3.3.2 AFM Force Spectroscopy 35
    3.3.3 Theory of Force Spectroscopy 38

II EXPERIMENTAL SECTION 47

4 EXPERIMENTAL SECTION 49
  4.1 Materials 49
    4.1.1 Buffers and Reagent Solutions 49
    4.1.2 Peptides and Proteins 50
  4.2 Methods 52
    4.2.1 Microscale Thermophoresis 52
    4.2.2 Surface Plasmon Resonance 53
    4.2.3 AFM Single Molecule Recognition Force Spectroscopy 56
    4.2.4 Dimerization of Orai1 and smMLCK CMBDs 63

III RESULTS AND DISCUSSION 65

5 RESULTS AND DISCUSSION 67
  5.1 Interaction between Calmodulin and Orai 67
    5.1.1 Microscale Thermophoresis 67
    5.1.2 Surface Plasmon Resonance 75
    5.1.3 AFM Single Molecule Recognition Force Spectroscopy 84
  5.2 Homomeric Interaction between the Orai1 N-Terminus and Loop2 92

6 CONCLUSIONS AND OUTLOOK 97
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Crystal structure of the CaM-Orai1 CMBD complex.</td>
<td>2</td>
</tr>
<tr>
<td>1.2</td>
<td>Affinities of the interaction between isolated Orai1 CMBD and either full-length CaM, truncated CaM lacking the C-terminus (CaM-N), or truncated CaM lacking the N-terminus (CaM-C).</td>
<td>3</td>
</tr>
<tr>
<td>2.1</td>
<td>Crystal structure of a typical EF-hand.</td>
<td>8</td>
</tr>
<tr>
<td>2.2</td>
<td>Different CaM-binding proteins with their cellular localization and function.</td>
<td>9</td>
</tr>
<tr>
<td>2.3</td>
<td>Ribbon presentations of CaM.</td>
<td>10</td>
</tr>
<tr>
<td>2.4</td>
<td>Structures of CaM in complex with different targets.</td>
<td>11</td>
</tr>
<tr>
<td>2.5</td>
<td>Scheme of the STIM1 protein with regions essential for the STIM1/Orai1 signaling cascade.</td>
<td>13</td>
</tr>
<tr>
<td>2.6</td>
<td>Schematic illustration of the Orai1 protein.</td>
<td>14</td>
</tr>
<tr>
<td>2.7</td>
<td>Schematic illustration of the channel formation by six dOrai monomers based on the crystal structure of drosophila melanogaster.</td>
<td>15</td>
</tr>
<tr>
<td>2.8</td>
<td>Schematic depiction of the activation process of Orai channels.</td>
<td>16</td>
</tr>
<tr>
<td>3.1</td>
<td>Microscale thermophoresis setup and measurement principle.</td>
<td>18</td>
</tr>
<tr>
<td>3.2</td>
<td>Simulation of temperature and concentration changes in MST.</td>
<td>21</td>
</tr>
<tr>
<td>3.3</td>
<td>Full presentation of a typical MST experiment.</td>
<td>22</td>
</tr>
<tr>
<td>3.4</td>
<td>Typical setup of an SPR biosensor.</td>
<td>25</td>
</tr>
<tr>
<td>3.5</td>
<td>Schematic illustration of the SPR effect at the interface between the glass prism and the sensor chip.</td>
<td>26</td>
</tr>
<tr>
<td>3.6</td>
<td>Typical sensorgram of an SPR measurement.</td>
<td>31</td>
</tr>
<tr>
<td>3.7</td>
<td>Scanning electron microscopy images of a (used) AFM cantilever.</td>
<td>33</td>
</tr>
<tr>
<td>3.8</td>
<td>Schematic illustration of a typical AFM setup with optical readout in its two main operating modes.</td>
<td>34</td>
</tr>
<tr>
<td>3.9</td>
<td>AFM force spectroscopy mode.</td>
<td>35</td>
</tr>
<tr>
<td>3.10</td>
<td>Schematic illustration of a typical force distance cycle.</td>
<td>36</td>
</tr>
<tr>
<td>3.11</td>
<td>Schematic illustration of the energy landscape for a dissociation process of a receptor-ligand complex.</td>
<td>40</td>
</tr>
<tr>
<td>3.12</td>
<td>Exemplary force distribution and resulting loading rate dependence plot.</td>
<td>40</td>
</tr>
<tr>
<td>3.13</td>
<td>Energy landscapes of the dissociation process along a reaction coordinate of a biomolecular complex with the corresponding loading rate dependence.</td>
<td>43</td>
</tr>
<tr>
<td>3.14</td>
<td>Conceptual free energy landscape with two barriers.</td>
<td>44</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>3.15</td>
<td>Three-step protocol for coupling a sensor molecule to a silicon (nitride) AFM tip.</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>Scheme of the immobilization strategy for coupling CaM or CMBD peptides to the chip surface via a pre-immobilized streptavidin monolayer.</td>
<td></td>
</tr>
<tr>
<td>4.2</td>
<td>Protocol for the removal of streptavidin and a biotinylated bait from a mixed desthiobiotin-SAM.</td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>AFM cantilever tip and surface functionalization with CaM and Orai CMBD peptides.</td>
<td></td>
</tr>
<tr>
<td>4.4</td>
<td>AFM cantilever tip and surface functionalization with Orai loop2 and N-terminal fragments of Orai.</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>Example of a force-distance curve and the extraction of unbinding forces.</td>
<td></td>
</tr>
<tr>
<td>4.6</td>
<td>Overview of the extracted parameters of an exemplary force spectroscopy dataset containing 1026 individual FDCs at a given tip retraction velocity.</td>
<td></td>
</tr>
<tr>
<td>4.7</td>
<td>Dimerization of Orai1 and smMLCK CMBDs and separation from non-dimerized monomers by size exclusion chromatography.</td>
<td></td>
</tr>
<tr>
<td>5.1</td>
<td>Schematic illustration of the Orai protein and the aa sequences of different Orai CMBDs.</td>
<td></td>
</tr>
<tr>
<td>5.2</td>
<td>MST equilibrium measurements of the interaction between CaM and two different Orai1 CMBDs.</td>
<td></td>
</tr>
<tr>
<td>5.3</td>
<td>MST measurements of the interaction between CaM and two different versions of Orai3 CMBD.</td>
<td></td>
</tr>
<tr>
<td>5.4</td>
<td>MST measurements of the interaction between CaM and the CMBD of dOrai.</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>MST measurements of the interaction between CaM and the CMBD of smMLCK.</td>
<td></td>
</tr>
<tr>
<td>5.6</td>
<td>Overview of MST measurements with various different Orai CMBDs.</td>
<td></td>
</tr>
<tr>
<td>5.7</td>
<td>Schematic illustration of the binding mechanism of Orai1 or smMLCK monomers and dimers.</td>
<td></td>
</tr>
<tr>
<td>5.8</td>
<td>MST measurement of the interaction between CaM and the dimerized CMBDs of Orai1 and smMLCK.</td>
<td></td>
</tr>
<tr>
<td>5.9</td>
<td>MST measurement of the interaction between CaM and the dimerized CMBDs of Orai1 and smMLCK in the presence of 50 μM TCEP.</td>
<td></td>
</tr>
<tr>
<td>5.10</td>
<td>Typical SPR experiment with either CaM or the respective CMBD peptide in solution.</td>
<td></td>
</tr>
<tr>
<td>5.11</td>
<td>Proof of the Ca$^{2+}$-dependency of the interaction between Orai1 CMBD and CaM.</td>
<td></td>
</tr>
<tr>
<td>5.12</td>
<td>SPR data analysis of the bivalent interaction between soluble CaM and surface-bound Orai1 CMBD.</td>
<td></td>
</tr>
<tr>
<td>5.13</td>
<td>Interaction of soluble CaM with the CMBD of Orai1$<em>{70-91}$ or Orai1$</em>{69-88}$ measured by SPR.</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.14 Interaction of soluble CaM with the CMBD of Orai3_{46–66} or Orai3_{52–66} measured by SPR.  
Figure 5.15 Interaction of soluble CaM with the CMBD of smMLCK measured by SPR.  
Figure 5.16 SPR data of the interaction between soluble Orai1 or soluble smMLCK CMBD and immobilized CaM.  
Figure 5.17 SPR data of the interaction between soluble Orai1 or soluble Orai3 with immobilized CaM.  
Figure 5.18 SPR data analysis of the time course of monovalent and bivalent bond formation.  
Figure 5.19 Forced dissociation of bound CaM by removal of Ca^{2+} ions.  
Figure 5.20 SPR data of the interaction between dimerized soluble Orai1 CMBD or dimerized soluble smMLCK CMBD and immobilized CaM.  
Figure 5.21 Detection and extraction of unbinding forces using single molecule force spectroscopy.  
Figure 5.22 Binding probability for the interaction between tip-tethered CaM and immobilized Orai1/Orai3 CMBD.  
Figure 5.23 Interaction between tip-tethered CaM and Orai1 or Orai3 CMBD on the substrate.  
Figure 5.24 Distribution of unbinding forces for Orai1 and Orai3 as a function of tip-surface contact time.  
Figure 5.25 Loading rate dependence plot of the interaction between tip-tethered CaM and surface-immobilized Orai1/Orai3 CMBD.  
Figure 5.26 Force distribution of the interaction between CaM and Orai3 CMBD.  
Figure 5.27 Loading rate dependence of the reversed functionalization scheme with CaM on the substrate and Orai1 or Orai3 on the tip.  
Figure 5.28 Unbinding forces and Bell-Evans fit for support-bound smMLCK peptide and tip-tethered CaM.  
Figure 5.29 Binding probability between immobilized Orai1_{70–91} or Orai1_{79–91} and tip-tethered Orai1-L2 or Orai3-L2, respectively.  
Figure 5.30 Box plot of the prevailing interaction forces at a loading rate of ~5000 pN s^{-1}.  
Figure 5.31 Overlay of the force distributions (shown as pdfs) of the interactions between tip-tethered Orai1-L2 and surface-immobilized Orai1_{79–91} or Orai1_{70–91}.  
Figure 5.32 Loading rate dependence plots of the interaction between tip-tethered Orai1-L2 or Orai3-L2 and surface-immobilized Orai1_{79–91} or Orai1_{70–91}.  
Figure 6.1  Schematic illustration of the combined methodological approach to determine the equilibrium constants, kinetic rate constants, and single-molecular interaction forces between different Orai segments and CaM. 97

LIST OF TABLES

Table 5.1  Overview of the determined kinetic rates and equilibrium constants of the interaction between CaM and different CMBDs. 91
**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>alternating current</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>APTES</td>
<td>(3-aminopropyl)triethoxysilane</td>
</tr>
<tr>
<td>Biotin-LC-NHS</td>
<td>succinimidyl 6-(biotinamido)hexanoate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>CRAC activation domain</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CC</td>
<td>coiled-coil</td>
</tr>
<tr>
<td>CDI</td>
<td>Ca(^{2+})-dependent inactivation</td>
</tr>
<tr>
<td>CMBD</td>
<td>calmodulin binding domain</td>
</tr>
<tr>
<td>CRAC</td>
<td>Ca(^{2+}) release-activated Ca(^{2+})</td>
</tr>
<tr>
<td>CTID</td>
<td>C-terminal inhibitory domain</td>
</tr>
<tr>
<td>DC</td>
<td>direct current</td>
</tr>
<tr>
<td>DFS</td>
<td>dynamic force spectroscopy</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ETON</td>
<td>extended transmembrane Orai1 N-terminal</td>
</tr>
<tr>
<td>FCDI</td>
<td>fast Ca(^{2+})-dependent inactivation</td>
</tr>
<tr>
<td>FDC</td>
<td>force-distance cycle</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamate decarboxylase</td>
</tr>
<tr>
<td>GTC</td>
<td>guanidinium thiocyanate</td>
</tr>
<tr>
<td>HBS</td>
<td>HEPES-buffered saline</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IP(_3)</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
</tr>
<tr>
<td>LED</td>
<td>light-emitting diode</td>
</tr>
<tr>
<td>LRD</td>
<td>loading rate dependence</td>
</tr>
<tr>
<td>MAC</td>
<td>magnetic alternating current</td>
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<tr>
<td>MD</td>
<td>molecular dynamics</td>
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<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
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<td>MST</td>
<td>microscale thermophoresis</td>
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<td>MT</td>
<td>mass transport</td>
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<td>NHS</td>
<td>N-hydroxysuccinimide</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>NTA</td>
<td>nitritoltriacetic acid</td>
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<tr>
<td>OBD</td>
<td>optical beam deflection</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PDB</td>
<td>protein data bank</td>
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<tr>
<td>pdf</td>
<td>probability density function</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>RU</td>
<td>resonance unit</td>
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<tr>
<td>SAM</td>
<td>self-assembled monolayer</td>
</tr>
<tr>
<td></td>
<td>(when concerning gold surfaces)</td>
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<tr>
<td>SAM</td>
<td>sterile alpha motif</td>
</tr>
<tr>
<td></td>
<td>(when concerning the structure of STIM)</td>
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<tr>
<td>SARAF</td>
<td>SOCE-activated regulatory factor</td>
</tr>
<tr>
<td>SCDI</td>
<td>slow Ca(^{2+})-dependent inactivation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>smMLCK</td>
<td>smooth muscle myosin light chain kinase</td>
</tr>
<tr>
<td>SMRFS</td>
<td>single molecule recognition force spectroscopy</td>
</tr>
<tr>
<td>SOAR</td>
<td>STIM-Orai activation region</td>
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<tr>
<td>SOC</td>
<td>store-operated channel</td>
</tr>
<tr>
<td>SOCE</td>
<td>store-operated Ca(^{2+}) entry</td>
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<tr>
<td>SP</td>
<td>surface plasmon</td>
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<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
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<tr>
<td>STIM</td>
<td>stromal interaction molecule</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TCEP</td>
<td>tris(carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
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<tr>
<td>UV/VIS</td>
<td>ultraviolet-visible spectroscopy</td>
</tr>
</tbody>
</table>
### SYMBOLS

<table>
<thead>
<tr>
<th>SYMBOL</th>
<th>DESCRIPTION</th>
<th>UNIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$</td>
<td>surface</td>
<td>m²</td>
</tr>
<tr>
<td>$A_0$</td>
<td>zero frequency amplitude</td>
<td></td>
</tr>
<tr>
<td>$A_{white}$</td>
<td>white noise amplitude</td>
<td></td>
</tr>
<tr>
<td>$c$</td>
<td>molar concentration</td>
<td>mol l$^{-1}$, M</td>
</tr>
<tr>
<td>$c$</td>
<td>speed of light in vacuum</td>
<td>$\sim 3.00 \cdot 10^8$ m s$^{-1}$</td>
</tr>
<tr>
<td>$c_T$</td>
<td>steady-state molar concentration</td>
<td>mol l$^{-1}$, M</td>
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<tr>
<td>$D$</td>
<td>diffusion coefficient</td>
<td>m$^2$ s$^{-1}$</td>
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<td>$D_T$</td>
<td>thermal diffusion coefficient</td>
<td>m$^2$ s$^{-1}$K$^{-1}$</td>
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<tr>
<td>$E$</td>
<td>electric field</td>
<td>N C$^{-1}$</td>
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<td>$G^*$</td>
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<tr>
<td>$\lambda_{DH}$</td>
<td>Debye-Hueckel screening length</td>
<td>m</td>
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</table>
\( N \) number of unbinding events

\( N_B \) number of bonds

\( n \) refractive index

\( \omega \) radial frequency \( \text{rad s}^{-1} \)

\( \omega_0 \) radial resonance frequency \( \text{rad s}^{-1} \)

\( P \) area under power spectrum curve \( \text{m}^2 \)

\( p(F) \) probability distribution of forces at rupture

\( Q \) quality factor

\( R \) SPR response \( \text{RU } (= 0.0001^\circ) \)

\( r \) loading rate \( \text{N s}^{-1} \)

\( R_{eq} \) SPR response at equilibrium \( \text{RU} \)

\( R_{max} \) maximum SPR response \( \text{RU} \)

\( \vec{r} \) respective position vector

\( S(t) \) time-dependent bond survival probability

\( s_{\text{hyd}} \) hydration entropy per surface \( \text{J mol}^{-1}\text{K}^{-1}\text{m}^{-2} \)

\( S_T \) Soret coefficient \( \text{K}^{-1} \)

\( \sigma \) standard deviation

\( \sigma_{eff} \) effective surface charge density \( \text{e m}^{-2} \)

\( T \) temperature \( \text{K} \)

\( t \) time \( \text{s} \)

\( \tau_0 \) lifetime \( \text{s} \)

\( \Theta_i \) angle of incidence \( ^\circ \)

\( \Theta_r \) angle of refraction \( ^\circ \)

\( \Theta_c \) critical angle \( ^\circ \)

\( v \) (pulling) velocity \( \text{m s}^{-1} \)

\( x_\beta \) width of energy barrier \( \text{m} \)

\( z \) cantilever displacement \( \text{m} \)
Calcium is an important second messenger involved in many cellular processes such as cell proliferation, T-cell activation, muscle contraction, egg fertilization, or apoptosis.\[207, 214\] Calcium entry into non-excitable cells is mainly carried by so-called Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels, whereby Orai acts as calcium channel in the plasma membrane, while the stromal interaction molecule (STIM) functions as a calcium sensor in the endoplasmic reticulum (ER) and as an activator of Orai channels. A reduction of Ca\(^{2+}\) in the ER causes STIM to oligomerize and to accumulate at the ER-plasma membrane where it binds directly to Orai and activates its channel function to induce Ca\(^{2+}\) influx into the cell.\[173, 52\]

As described in the next section (Sec. 2.3), the underlying structural and functional molecular mechanisms for proper channel activation have been studied extensively by many groups.\[52\] However, compared with activation, much less information is available regarding the Ca\(^{2+}\)-dependent inactivation (CDI) of Orai channels, which is an important negative feedback mechanism and thus an indispensable requirement for cell homeostasis which keeps the intracellular Ca\(^{2+}\) level within the tolerable range from \(10^{-7}\) to \(10^{-6}\) M. In general, the CDI of Orai channels is separated into fast (FCDI) and slow (SCDI) CDI.\[165, 172\] FCDI was shown to occur within tens of milliseconds after channel activation, but to be significantly slower for Orai1 than for Orai2 and Orai3.\[129, 122, 188\] SCDI is supposed to take several minutes for completion.\[52\]

Within the C-terminal part of STIM1, various domains have been identified to influence the inactivation of Orai channels, most prominently the acidic CRAC modulatory domain (CMD) region.\[50, 122, 148\] Additionally, the extent and the rate of CDI was found to steeply increase with the STIM/Orai expression ratio,\[190\] and the isolated Orai-binding domain (CRAC activation domain, CAD) of STIM1 was not able to induce CDI[166],[173] However, it is assumed that - besides the two major players Orai1 and STIM1 themselves - CDI of Orai channels is also influenced by the proteins SARAF (SOCE-associated regulatory factor) and calmodulin (CaM), as well as by additional regulatory proteins.\[52\] SARAF, an ER-resident protein, has been observed to be regulated by the C-terminal inhibitory domain (CTID) of STIM1 and to interact with the CRAC activation domain (CAD) of STIM1 only in the presence of Orai1.\[107, 162, 29\]

Calmodulin, a small ubiquitous eukaryotic protein (cf. Sec. 2.2), has been implicated to play a crucial role by acting as Ca\(^{2+}\) sensor for CDI. Besides its well-known function as mediator of various Ca\(^{2+}\)-dependent cellular processes, CaM is used as constitutive or dissociable Ca\(^{2+}\)-sensing subunit by
different classes of ion channels, including voltage-gated \( \text{Ca}^{2+} \) channels, various \( \text{Ca}^{2+} \)- or ligand-gated channels, or transient receptor potential (TRP) family channels.\cite{186} Upon binding of \( \text{Ca}^{2+} \), CaM undergoes conformational changes and exposes a hydrophobic patch in both the N- and C-terminal lobe, which is assumed to be essential for target recognition.

Despite a lacking consensus sequence for CaM-binding sites, known primary target peptides are usually basic amphiphilic helices with both a hydrophobic and cationic side.\cite{41, 47, 65, 158}

![Figure 1.1: Crystal structure of the CaM-Orai1 CMBD complex.\cite{131} (A) Ribbon representation of Orai1 CMBD (green) interacting with CaM (blue). Only the C-terminal lobe of CaM binds one CMBD peptide. \( \text{Ca}^{2+} \) ions are illustrated as red spheres. (B) Surface and stick representation. The atoms are color-coded as: C: green, N: blue, O: red for Orai1 CMBD; and C: yellow, N: blue; O: red; and S: orange for CaM.](image)

It has been shown that binding of CaM to a highly conserved N-terminal domain of Orai1 (calmodulin binding domain (CMBD)) with similar properties is \( \text{Ca}^{2+} \)-dependent and important for CDI.\cite{148} Mutagenesis studies indicated a strong correlation between the elimination of CaM-binding and a loss of FCDI.\cite{148} More recently, however, the role of CaM for FCDI \textit{in vivo} was questioned and a model for possible channel inactivation due to conformational changes of the Orai1 pore itself was proposed.\cite{149} Although the exact role and contribution of CaM to CDI \textit{in vivo} remains unclear, \textit{in vitro} experiments evidenced specific binding between the isolated N-terminal CMBD Orai1\textsubscript{69–88} and CaM.\cite{131} The crystal structure of this complex revealed an unusual extended conformation of CaM with only its C-terminal lobe being associated with one Orai1 CMBD (Fig. 1.1).\cite{131} Parallel pull-down and isothermal titration calorimetry (ITC) experiments, however, suggested a bivalent interaction mode with both the C- and N-terminal lobe of CaM binding one Orai1 CMBD peptide each with different affinities of \( K_{d,C} = 1.1 \ \mu\text{M} \) and \( K_{d,N} = 4.6 \ \mu\text{M} \) (Fig. 1.2).\cite{131}

Motivated by these findings, we aimed at a full characterization of the interaction mechanism between CaM and different Orai CMBDs, for the fol-
Figure 1.2: Affinities of the interaction between isolated Orai1 CMBD and either full-length CaM (left panel), truncated CaM lacking the C-terminus (CaM-N, center), or truncated CaM lacking the N-terminus (CaM-C, right). ITC data indicate that full-length CaM interacts with two Orai1 CMBDs, whereas the truncated CaM versions only bind one CMBD peptide. The affinities of full-length CaM and CaM-C were found to be similar (1.3 \(\mu\)M and 1.1 \(\mu\)M, respectively), the one of CaM-N was found to be slightly lower (4.6 \(\mu\)M).

following reasons: (i) the published affinity values are much higher (~1 and ~5 \(\mu\)M) than usually reported for Ca\(^{2+}\)-induced CaM binding (typically between \(10^{-7}\) to \(10^{-11}\) M[42]) and were probably influenced by the high CaM concentration used in ITC, (ii) no comparable experimental data for the homologue Orai3, that is known to have distinct differences in FCDI, is available, (iii) the transitions between monovalent and bivalent bond formation have not yet been addressed, and, most prominently, (iv) quantitative information about the kinetics of stepwise association and dissociation is still missing.

To address those items, we used a new strategy for the analysis of biospecific interactions by a combination of complementary \textit{in vitro} methods. Equilibrium measurements by microscale thermophoresis (MST) allowed the determination of the distinct affinities of both binding steps at equilibrium, surface plasmon resonance (SPR) provided information about the kinetic rate constants, and single molecule recognition force spectroscopy (SMRFS) revealed the single molecular interaction forces of monovalent and bivalent binding, as well as the time dependence of the transition between the two states.

For comparison and validation of our measurement approach, we used the CaM target sequence of smooth muscle myosin light chain kinase (smMLCK), as CaM interacts with this domain in the typical and well characterized collapsed 1:1 binding mode with one CMBD bound to one CaM molecule.[142]
OUTLINE OF THE THESIS

The thesis at hand is separated into four main parts:

In the INTRODUCTION (Part i on the facing page), the biological background of the investigated system with emphasis on the molecular protagonists and their interaction is described (Chapter 2 on page 7), followed by an explanation of the physical mechanisms and the functional principles of the employed techniques for biospecific interaction analysis (Chapter 3 on page 17).

The EXPERIMENTAL SECTION (Part ii on page 47) contains the used materials and reagents together with the respective protocols for the preparatory procedures required for conducting the experiments.

In the RESULTS AND DISCUSSION section (Part iii on page 65), the main results of the PhD work are presented, accompanied by a discussion of the respective findings (Chapter 5 on page 67). Finally, the obtained results are shortly summarized and concluded (Chapter 6 on page 97).

Please, note that other contributions to self-contained studies are not presented in the Results Section, but the respective studies are enclosed as a whole in the APPENDIX (Part iv on page 101) to allow for a clearer understanding.
Part I

INTRODUCTION

This part presents an overview about the investigated biological system with emphasis on the two major keyplayers calmodulin and Orai. Additionally, a description of the used methods for biochemical and biophysical interaction analysis and their main functional principle is given.
BIOLICAL BACKGROUND

The focus of this thesis lies primarily on the in-depth characterization of the interaction mechanism between calmodulin and N-terminal Orai fragments. In addition, the molecular interplay between the Orai N-terminus and the cytosolic loop connecting the second and third transmembrane region was investigated at the single-molecule level. In this chapter, the molecular protagonists for these studies will be presented with respect to their biological function and their role in cellular processes. The aim of this chapter is to provide a profound overview about the respective processes with the biological components involved, with no intention to be exhaustive. For a detailed description of the aspects mentioned in the following, comprehensive reviews are suggested within all sections.

2.1 INTRODUCTION TO CALCIUM SIGNALING AND CA\(^{2+}\)-BINDING PROTEINS

Calcium (Ca\(^{2+}\)) is essential for various biological functions and is involved in the regulation of many cellular processes ranging from gene transcription to cell apoptosis.\[^{214}\] Inside the cell, the concentration of free Ca\(^{2+}\) is typically held constant at a level of \(~10^{-7}\) M, whereas the extracellular Ca\(^{2+}\) concentration is \(~10^{4}\) times higher in the range of \(~10^{-3}\) M.\[^{214, 36}\] This concentration gradient provides the potential for an import of Ca\(^{2+}\) into the cell, where it can act as a second messenger. Typically, an extracellular stimulus triggers either a Ca\(^{2+}\) influx from outside the cell via certain channels in the plasma membrane, or a Ca\(^{2+}\) release from intracellular stores such as the endoplasmic reticulum (ER) into the cytosol, resulting in an approximately 100-fold increase in free Ca\(^{2+}\).\[^{36, 214}\]

The free Ca\(^{2+}\) is released in elemental aliquots (called sparks, puffs, or waves) and is only shortly available to act as a cellular signal before it gets transported to intracellular storage sites or outside the cell. The transient rise in the cytosolic Ca\(^{2+}\) concentration is decoded by different intracellular Ca\(^{2+}\)-binding proteins that translate the signal into biochemical changes, either by direct Ca\(^{2+}\)-dependent regulation (such as protein kinase C) or via different mediator proteins that couple the Ca\(^{2+}\) signals to biochemical and cellular changes.\[^{36}\] In general, most of the several hundred Ca\(^{2+}\)-binding proteins that have been identified share a common Ca\(^{2+}\)-binding motif\[^{151, 117, 100}\] that comprises about 30 amino acids and consists of a helix-loop-helix (two \(\alpha\)-helices that are bridged by a Ca\(^{2+}\)-chelation loop), commonly referred to as EF-HAND MOTIF (as it was first observed formed by helices E and F of parvalbumin\[^{118}\]).\[^{214}\]
8 biological background

Figure 2.1: Crystal structure of a typical EF-hand. (a) The single EF-hand motif (total length of 29 amino acids) from the N-terminal lobe of CaM binds one Ca\(^{2+}\) ion. (b) A pair of EF-hand motifs from the N-terminal lobe of CaM forms a globular Ca\(^{2+}\)-binding domain with two complexed Ca\(^{2+}\) ions (PDB code 1EXR[224]). Bound Ca\(^{2+}\) ions are shown as yellow spheres. Image section from [79].

In most Ca\(^{2+}\)-binding proteins, two EF-hand motifs are in close proximity to each other so that the resulting EF-hand pair forms a stable four helix bundle[31, 117] (globular domain) that presumably stabilizes the protein conformation and increases the Ca\(^{2+}\) affinity (Fig. 2.1).[213]

EF-hand proteins are typically responding to binding of Ca\(^{2+}\) in two different ways: either they are undergoing no significant conformational changes and just serve as Ca\(^{2+}\) buffers or Ca\(^{2+}\) transporters (e.g., parvalbumin), or they undergo Ca\(^{2+}\)-dependent conformational changes and are serving as Ca\(^{2+}\) sensors such as troponin C or calmodulin.[36, 79]

2.2 CALMODULIN

The discovery of CaM goes back to the early 1970s,[86] as two laboratories independently discovered a protein factor that activated enzymatic activity.[33, 110] This factor was found to be present in several tissues[34] and its action was shown to be dependent on the presence of Ca\(^{2+}\).[109, 111] Soon after these first findings, the first documentations of CaM purification to chemical homogeneity were published.[201, 220] The term CALMODULIN (CALCIUM-MODULATED PROTEIN) was finally proposed by Cheung in 1978.[35]

CaM is a small (148 amino acids), ubiquitous eukaryotic protein that interacts with and regulates numerous different target proteins that play a role in cellular processes where Ca\(^{2+}\) serves as second messenger, such as muscle contraction, metabolism, inflammation, apoptosis, or the immune response (Fig. 2.2).[91, 207] CaM is exceptionally well conserved across eukaryotes[213, 36, 214]; between human and plants it is almost 90% identical.[207] It represents the most extensively studied protein in its class of eukaryotic proteins and can be called a prototype for Ca\(^{2+}\)-signal transduction and homeostasis in eukaryotic cells.[213] Binding of CaM typically induces structural or con-
formational changes in the respective target protein which lacks the ability to bind Ca\textsuperscript{2+} with the affinity and kinetics that are required for the biological response.\textsuperscript{213} The major function of CaM can therefore be stated as the translation of the Ca\textsuperscript{2+} signal into different cellular processes.\textsuperscript{42}

CaM is found in essentially all eukaryotic cell types,\textsuperscript{42, 217} ranging from yeast to plants to humans.\textsuperscript{216, 100, 235} Its structure reveals a dumbbell shape with two EF-hand pairs forming two globular domains\textsuperscript{6, 119, 7, 30} that are typically referred to as N- and C-terminal lobe\textsuperscript{207} (Fig. 2.3). The N- and C-terminal lobe are connected via a long \( \alpha \)-helical linker of approximately eight turns, that has been shown to be rather flexible in solution.\textsuperscript{9, 101, 192, 87} In solution, the two lobes therefore do not adopt a defined orientation relative to each other but display more a tumbling motion.\textsuperscript{214} The two EF-hand pairs on the N- and C-terminal lobe are rather similar and share 48\% sequence identity and 75\% sequence homology.\textsuperscript{214} but, however, the C-terminal lobe has a \( \sim \)10-fold higher Ca\textsuperscript{2+}-binding affinity (\( K_d \approx 0.2 \) \( \mu \text{M} \)) than the N-terminal lobe (\( K_d \approx 2 \) \( \mu \text{M} \)).\textsuperscript{171, 157} These affinities fall perfectly within the range of intracellular Ca\textsuperscript{2+} concentrations exhibited by most cells, which explains the important role of CaM as highly sensitive Ca\textsuperscript{2+} sensor. CaM also binds Mg\textsuperscript{2+} and K\textsuperscript{+} ions, but with \( 10^3 \)- \( 10^4 \) lower affinity than Ca\textsuperscript{2+}.\textsuperscript{84}

In response to an increased cellular Ca\textsuperscript{2+} concentration as it is typical during cell activation where [Ca\textsuperscript{2+}] changes from \( \sim 10^{-7} - 10^{-8} \) \( \text{M} \) in the resting state to \( \sim 10^{-6} \) \( \text{M} \) in the activated state, CaM binds four Ca\textsuperscript{2+} ions with micromolar affinity, two on both lobes (Fig. 2.3).
Upon binding of Ca$^{2+}$ ions, CaM typically undergoes conformational changes (Fig. 2.3) and is able to recognize and bind to a variety of different target proteins with high affinities on the order of $10^{-7}$ to $10^{-11}$ M.\cite{42} Interestingly, the equilibrium dissociation constant $K_d$ for Ca$^{2+}$-binding decreases significantly upon binding to a target peptide,\cite{214} which is explained by a lower dissociation rate $k_d$ of the Ca$^{2+}$ ions from the EF-hands.\cite{23, 167, 145} The overall structure of CaM with and without complexed Ca$^{2+}$ ions is rather similar,\cite{70, 120, 236} the major change in conformation due to Ca$^{2+}$-binding is an alternation of the relative orientation (i.e., a change of the interhelical angles within the EF-hands of the helices) in the two lobes.\cite{171, 100, 37} This rearrangement of the lobes lead to a more open conformation and to the exposure of several hydrophobic residues, that play an essential role in target binding.\cite{36, 214}

Dissociation of the CaM-target peptide complex is thought to be initiated by dissociation of the N-terminally bound Ca$^{2+}$ ions from CaM, followed by the loss of Ca$^{2+}$ from the C-terminal lobe and the final dissociation of the target peptide.\cite{23}

As the majority of CaM-dependent systems are enzymatic, the typical general mechanism of enzyme (E) activation by CaM can be described as follows:\cite{86}

\[
nCa^{2+} + CaM \rightleftharpoons Ca_n^{2+} \cdot CaM^* + E \rightleftharpoons Ca_n^{2+} \cdot CaM^* \cdot E \quad (2.1)
\]

After binding of $n$ Ca$^{2+}$ ions, CaM undergoes a conformational change (indicated by the asterisk) and is then able to recognize and bind to the inactive enzyme, forming the active ternary complex. If the intracellular Ca$^{2+}$ level decreases, Ca$^{2+}$ dissociates from CaM and the following dissociation of the ternary complex finally results in a loss of enzymatic activity.

However, CaM has also been shown to bind in its apo-form (without complexed Ca$^{2+}$ ions) to some targets (so-called “IQ-motifs”), or to change its affinity towards the target site after binding of Ca$^{2+}$. 

Figure 2.3: Ribbon presentations of CaM. (a) CaM without complexed Ca$^{2+}$ (apo-CaM). (b) CaM with two complexed Ca$^{2+}$ ions at both the N-terminal and the C-terminal lobe. Bound Ca$^{2+}$ ions are shown as yellow spheres. PDB codes 1CFD[120] and 1CLLL[30], respectively. Image section from [214].
2.2 CALMODULIN

2.2.1 Target Recognition and Binding

In general, the way of target binding and target regulation by CaM is highly diverse and offers a versatile system to link the Ca\(^{2+}\) concentration to the regulation of the respective target protein.\(^{91, 207}\) Most physiological relevant CaM targets are proteins or small peptides, but CaM has also been shown to bind to hormones, toxins, or small drug-like molecules.\(^{214}\) Analysis of CaM-binding peptides revealed no classical sequence homology between different targets, but, however, it has been shown that minimal structural characteristics have to be fulfilled. Due to its exceptional conformational flexibility, CaM interacts with its target helices by various different interaction mechanisms and binding stoichiometries; for some of the most extensively described complexes see Fig. 2.4.

Typical CaM targets have a high propensity to fold into a basic, amphiphilic \(\alpha\)-helix,\(^{41, 47, 65, 46, 158}\) they possess a net positive charge of the binding region, and have large hydrophobic residues in conserved positions, which are used for motif classification (e.g., 1-10, 1-14 or 1-16 motif\(^{91}\)).\(^{207, 214}\) Based on those structural features, several CaM-binding targets have been identified or synthetically engineered by means of computational predictions.\(^{230, 48}\) Ikura et al.\(^{230}\) established a comprehensive CaM target database with >300 CaM binding sequences (published as of 2004).

In the most common binding mode as shown for the interaction of CaM and segments from myosin light chain kinase (MLCK)\(^{102, 142}\), CaM shows a similar structure as in its apo-form\(^{6, 7}\) with its N- and C-terminal lobe.
wrapping around the target peptide, but the interconnecting central helix being disrupted and forming a flexible linker (Fig. 2.4, top left). The peptide sequences show a characteristic 1-14 spacing of hydrophobic anchor residues, that directly dock into the hydrophobic pockets on the two lobes of CaM.\[207\]

A recent comprehensive review about the binding mechanisms by which CaM interacts with different targets is given by Tidow and Nissen\[207\], who conclude their review with the following statement:

“There is no doubt that CaM, a uniquely adapted calcium sensor and effector in eukaryotic cell biology, will continue to reveal unexpected surprises.”\[207\]

Other reviews about the role of calmodulin and aspects about its target recognition can be found in Refs. [42], [36], [91] and [214].

2.3 Calcium Entry into Cells

The main pathway for Ca\(^{2+}\) entry into cells is constituted by store-operated calcium channels (SOCs) which occur in almost all cell types.\[16, 17, 22, 52\] SOCs respond to a reduction of Ca\(^{2+}\) inside the ER due to inositol 1,4,5-trisphosphate (IP\(_3\))-induced Ca\(^{2+}\) release through IP\(_3\) receptors in the ER membrane and therefore play a homeostatic role in refilling the ER stores after Ca\(^{2+}\) has been released and pumped out of the cell.\[165, 173\] The most prominent representative among SOCs is the calcium release-activated calcium (CRAC) channel.\[164, 52\] While the idea of SOCs has been already proposed in the 1980s,\[174\] the underlying molecular basis remained unknown for more than 20 years.\[69, 173, 169, 165\] About a decade ago, two proteins were found to fully reconstitute CRAC channel function: the stromal interaction molecule (STIM) as the Ca\(^{2+}\) sensor in the ER membrane,\[127, 182\] and Orai (or CRACM1) as the SOC subunit in the plasma membrane\[69, 215, 238\]. Upon the discovery of those two molecular keyplayers that accomplish store-operated Ca\(^{2+}\) entry, various groups focused on elucidating their functional characteristics and the major cellular events that are involved in the activation of SOC channels. More recently, first structural and mechanistic models for STIM\[199, 240, 229, 200\] and Orai\[96\] and their interplay were published.

2.3.1 The Two Key Players: STIM and Orai

STIM proteins The family of STIM proteins includes STIM1 and STIM2,\[196\] which are both expressed in the ER,\[127, 136, 237, 195\] are highly conserved,\[38\] and are closely related with ~61% sequence identity\[26\]. STIM proteins are single transmembrane (TM) proteins with a well conserved N-terminal domain that is formed by the EF-hand and the sterile-\(\alpha\)-motif (SAM) domain\[199, 240\] that senses the Ca\(^{2+}\) concentration in the ER and a long, less conserved cytosolic strand that is needed for coupling to Orai proteins in the plasma membrane.\[27\] The cytosolic C-terminal part of STIM1 contains the essential binding sites for coupling to Orai proteins (Fig. 2.5);\[97, 147\] three
conserved coiled-coil domains (CC1, CC2, CC3), the CRAC modulatory domain, the C-terminal inhibitory domain (CTID), the serine/proline- (Ser/Pro) rich region, a Thr-Arg-Ile-Pro (TRIP) sequence and a lysine-rich region at the end of the C-terminus.

Figure 2.5: Scheme of the full-length, human STIM1 protein with regions essential for the STIM1/Orai1 signaling cascade. The insets show the structures of the N-terminal EF-sterile-α-motif (SAM) domain, an α-helical domain of CC1 (CC1α3) and CC2, and the SOAR region.

It has been shown, that the minimal domain for successful Orai channel activation (termed CRAC activation domain (CAD) or STIM-Orai activation region (SOAR))[173]) includes only CC2 and CC3 (Fig. 2.5).[229] However, the structure of full-length STIM1 in its resting state is still elusive.[52] Upon depletion of the ER, Ca\(^{2+}\) dissociates from the N-terminal EF-hand and initiates the CRAC signaling cascade, which transforms STIM1 into its active conformation.[52] STIM1 further multimerizes and redistributes into puncta close to the plasma membrane,[10, 28, 26, 166, 228] where it binds to Orai proteins to initiate pronounced CRAC currents.[146, 147].[52]

**Orai Proteins** Orai proteins are representing the pore-forming Ca\(^{2+}\)-selective ion channels in the plasma membrane and include three homologues (Orai1, Orai2, Orai3).[69, 215, 21] Each Orai monomer is a four TM-spanning protein with one cytosolic and two extracellular loops and the cytosolic N- and C-termini (Fig. 2.6).[108, 144, 168] While the TM regions share high pairwise sequence identity (TM1 is fully conserved among all homologues), the terminal strands and the TM-connecting loops are less conserved, with the exception of the domains which are involved in direct STIM1 binding.[93]

Major structural differences between the homologues are found in the extracellular loops and in the N- and C-terminal segments[194] (e.g., 34% and 46% sequence homology, respectively between Orai1 and Orai3).[52] Most importantly, both the N- and the C-terminal strands are needed for functional coupling to STIM1.[123, 146, 166, 51, 141, 161, 239] For the calcium-dependent inactivation (CDI) of Orai channels it has been shown that both the N-terminus as well as the intracellular loop (loop II-III) are playing an
Figure 2.6: Schematic illustration of the Orai1 protein.[173] The sequences for Orai1 and Orai3 are compared in the N-terminal region, in the I–II and II–III loop, and in the C-terminus. Selected functional domains are indicated by colored bars or circles (thick orange bars in the N- and C-termini: putative STIM1 binding sites on Orai1; yellow and red lines: locations of mutations causing gain-of-function or loss-of-function phenotypes, respectively; black lines: mutations affecting ion selectivity; purple letters: residues important for CDI; red letters: residues important for STIM binding and gating; blue letters: charged residues in the I–II loop (black spheres); blue spheres: target of redox inhibition (C195) and the glycosylation site of Orai1 (N223)).

important role, with the exact underlying molecular mechanism remaining unclear.[148, 15, 149] Recently, the idea of a hexameric channel formation by oligomerization of six Orai monomers (Fig. 2.7) has been put forward based of the crystal structure of Orai from *drosophila melanogaster* (dOrai),[96] while in the years before the Orai channel was thought to be composed of four Orai monomers.[168, 144, 139, 135, 49]

Three dOrai dimers are assumed to form the channel, with the C-termini of adjacent dOrai monomers coupling to each other in an antiparallel manner.[96, 146, 147, 152] The resulting overall threefold symmetry shows that the pore (with a diameter of ~6 Å, Fig. 2.7) is surrounded by three rings of TM domains: the TM1 region of each monomer that together form the pore, parts of TM2 and TM3, and TM4.[96] The cytosolic loop that connects TM2 and TM3 has not been resolved in the crystal structure of dOrai and the structural resolution of the extracellular loops is not available, too. However, based on molecular dynamics simulations it is supposed that loop III-IV shows the highest flexibility.[76]

**Overview about the STIM-Orai coupling machinery** In its resting state, STIM1 is homogeneously distributed and forms dimers, mainly
via C-terminal interactions within the CC1 and CAD/SOAR regions (“in-tramolecular clamp”).\[147, 241, 134\] After ER store depletion, STIM1 proteins oligomerize,\[40, 128, 146\] and redistribute into distinct puncta at ER-PM junctions.\[228, 182, 127, 133\] Oligomerization of STIM1 was shown to occur before coupling to Orai and the lysine-rich region at the very C-terminal end of STIM1 is assumed to control puncta formation but seems to have no effect on the oligomerization.\[128\] Dissociation of Ca\(^{2+}\) from its N-terminal EF hand (\(K_d\) of \(\sim 200\) and \(\sim 500\) µM for STIM1 and STIM2, respectively)\[239\]) induces a conformational change that gets transmitted via the TM domain to the C-terminus and switches STIM1 in its activated state. In this state, the intramolecular clamp is released and STIM1 changes its conformation to an extended state for the exposure of the CAD/SOAR region to interact with Orai1.\[173, 52\] It has been shown that CAD directly interacts with both the C- and the N-terminus of Orai1, but no interaction has been observed with the intracellular loop.\[166, 51\]

The interaction mechanism between the C-terminus of STIM1 and the C-terminus of Orai1 is structurally rather well resolved, while the (weaker) one between STIM1 C-terminus and Orai1 N-terminus is only poorly defined.\[52\] Single point mutations in the C-terminal part (L273 and L276) of Orai1 have been shown to be sufficient to fully abrogate communication with STIM1.\[75, 146\] Based on the crystal structure of dOrai, the same residues were found to be also involved in the C-terminal coupling of Orai dimers to form the hexameric channel,\[96\] as well as in channel gating.\[163\] The major STIM1-interacting region in the N-terminal part of Orai1 is comprised by the conserved, elongated extension of TM1 into the cytosol (often referred to as ETON (extended TM Orai1 N-terminal) region, aa73-90).\[166, 51\] Several
point mutations within this region resulted in the complete loss of STIM1 coupling and activation of the Orai1 channel.\[51, 130, 218\]

![Figure 2.8: Schematic depiction of the activation process of Orai channels.][219] Upon depletion of the ER, STIM1 oligomerizes and switches into its activated, elongated conformation. By forming puncta close to the PM, STIM1 then interacts via its C-terminus with the pore forming Orai protein in the PM, leading to an activation of the Orai channel and influx of Ca\(^{2+}\).

Although the exact binding mechanism between STIM1 and the cytosolic strands of Orai1 is not fully resolved, it is clear that both the N- and C-termini are needed for successful activation, as deletion or mutation of one of the termini led to a loss of Orai1 function.\[51, 141, 161, 163\] However, it is assumed that the C-terminal region of Orai1 poses the stronger binding site for the interaction with STIM1.\[166\] When the C-terminal binding site was deleted, no interaction with STIM1 was observed, neither with the full-length protein nor with fragments thereof.\[123, 146\] On the contrary, when the putative N-terminal binding site of Orai1 was deleted, Orai1 was able to partially interact with STIM1 (most probably via its C-terminal part) but no Ca\(^{2+}\) currents were generated, indicating a loss in functionality.\[50, 146\]

Interestingly, several studies have reported a dependence of Orai activation on the amount of interacting STIM1 proteins, with a minimum of one or two molecules needed for successful coupling.\[95, 124, 190, 191\] In patch-clamp experiments, the maximum Ca\(^{2+}\) current for activation and inactivation was found to occur when eight STIM1 proteins were involved,\[124\] indicating that Orai channel activation is not following an “all-or-none” pattern but shows gradual variations. Structural information about the possible alignment and coupling of multiple STIM1 proteins to the proposed hexameric dOrai channel, however, is still lacking.\[52, 173\]

For a detailed description of the STIM-Orai coupling machinery, please see two recent reviews given in Refs. \[173\] and \[52\].
As outlined in Chap. 1, the research questions within this thesis were addressed by three complementary in vitro methods. Equilibrium measurements by microscale thermophoresis (MST) were performed to yield the equilibrium constants (affinities), surface plasmon resonance (SPR) provided information about the distinct kinetic rate constants during association and dissociation, and atomic force microscopy-based single molecule recognition force spectroscopy (AFM SMRFS) revealed the interaction forces and the underlying energy landscape at the single-molecule level. In the following sections, these three techniques will be described in more detail, allowing for a thorough understanding of the underlying theoretical and experimental principles.

3.1 Microscale Thermophoresis

Microscale thermophoresis is a rather new technique, based on an effect called thermophoresis or thermodiffusion. The technology of MST is complementary to surface-based biosensors like SPR or calorimetric methods like isothermal titration calorimetry (ITC), but has distinct advantages. In comparison to SPR, no labor-intensive assay preparation and immobilization strategies to suppress non-specific adhesion are required and as both binding partners are soluble, no surface artifacts like mass transport limitations can influence the binding process. Nevertheless, as MST only allows to determine equilibrium constants, SPR is the method of choice if kinetic rate constants are of special interest.

The technique of ITC, one of the few methods that is truly label-free, gives an insight into the thermodynamic properties of an interaction and additionally provides information about the binding stoichiometry. As the dissipated or adsorbed heat of a reaction is measured directly, a rather high concentration (in the range of milligrams per milliliter) of at least one of the binding partners is required to gain a detectable amount of heat.

MST uses a different approach for detecting the equilibrium constants of an interaction: it detects changes in size, charge, and the hydration shell of molecules and is therefore applicable for a wide range of molecules, ranging from small peptides to multi-subunit complexes and liposomes. Among other advantages, MST requires very little sample amounts (few microliters), is performed with both binding partners fully in solution and in any buffer, and has a broad range of detectable affinities (from pM to mM). The only prerequisite for MST measurements is the need for labeling one binding partner with a fluorophore, as the readout mechanism is based on the detection of changes in fluorescence intensity.
3.1.1 Theoretical Background of Thermophoresis

The functional principle of MST is based on the effect of thermophoresis, which states that a local temperature gradient in an aqueous solution induces not only a heat flow but also a flow of the molecules that are present in this solution. This effect, also known as Ludwig-Soret effect, was first described in 1856 by Carl Ludwig.\cite{132} While thermophoresis in gaseous mixtures is well explained by theory and has been proven experimentally, thermophoresis in aqueous solutions was studied later\cite{197} and remained less well understood, as its theoretical explanation is still under debate.\cite{58}

![Microscale thermophoresis setup and measurement principle](image)

Figure 3.1: Microscale thermophoresis setup and measurement principle (NanoTemper Technologies, Germany). Measurements are performed by sequentially scanning up to 16 capillaries containing a constant (low) concentration of the labeled molecule and different concentrations of the respective ligand. The fluorophore in the capillary is excited and the emitted fluorescence intensity is detected and monitored over time through the same objective (left). By locally heating a defined sample volume with a focused IR-Laser, the timetrace of a typical experiment with distinct phases is obtained: (1) The IR-laser is off and the molecules are homogeneously distributed, yielding a constant fluorescence intensity. (2) Fluorophore-inherent temperature-dependent change in fluorescence (temperature jump or T-jump). (3) Decrease in fluorescence intensity due to thermophoresis. (4) Steady state phase in which thermodiffusion is counterbalanced by mass diffusion. (5) The IR-laser is switched off and an inverse T-jump occurs, followed by backdiffusion into the previously heated area due to pure mass diffusion. The illustration is based on \cite{105} and the time scan was recorded from labeled calmodulin in absence of ligand.

In general, the velocity $v$ of a molecule is linearly dependent on the temperature gradient $\nabla T$\cite{105}

$$v = -D_T \cdot \nabla T$$

(3.1)
with $D_T$ as thermal diffusion coefficient. As thermal diffusion can be depicted as a flow of molecules $j$ which is dependent on the molecule concentration $c$, it follows that[193]

$$j = -cD_T \cdot \nabla T$$

(3.2)

In steady state, thermal diffusion is counterbalanced by mass diffusion[193]

$$j = D \cdot \nabla c$$

(3.3)

with $D$ being the diffusion coefficient. For low molecule concentrations, the resulting molecular flow therefore is given as[105]

$$j = -cD_T \cdot \nabla T - D \cdot \nabla c$$

(3.4)

The induced change in concentration due to the thermophoretic molecule movements is determined by the ratio of $D_T$ and $D$, typically referred to as Soret coefficient $S_T$

$$S_T = \frac{D_T}{D}$$

(3.5)

which thus determines the extent of thermodiffusion in the steady state.[58]

The Soret coefficient is hereby depending on the size of the molecule, on its charge as well as on its hydration shell (cf. Eq. 3.8).[58, 105]

Considering $D_T$ and $D$ as temperature-independent and integrating Eq. 3.4 would yield a steady state concentration $c_T$ of[105]

$$c_T = c \cdot \exp(-S_TdT)$$

(3.6)

when the temperature is increased by $dT$ above ambient temperature.[105]

In other words, the steady state concentration change (i.e., the ratio of the apparent molecule concentrations in the heated ($c_{hot}$) and non-heated area ($c_{cold}$) due to a change in temperature $\Delta T$ is proportional by

$$\frac{c_{hot}}{c_{cold}} = \exp(-S_T\Delta T)$$

(3.7)

Typically, the Soret coefficient is positive for most molecules, resulting in a depletion of the heated area. However, as the Soret coefficient is strongly depending on the molecule’s properties, also the opposite effect (i.e., an increase in $c_{hot}$) can be observed. Duhr et al.[58] have experimentally shown that the Soret coefficient can be expressed as

$$S_T = \frac{A}{k_BT} \left(-s_{hyd} + \frac{\beta \sigma_{eff}^2}{4\varepsilon\varepsilon_0 T} \times \lambda_{DH}\right)$$

(3.8)

with $A$ being the molecule’s surface area, $k_B$ the Boltzmann constant, $T$ the temperature, $s_{hyd}$ the hydration entropy of the molecule-solution interface, $\sigma_{eff}$ the effective surface charge density, $\varepsilon$ the dielectric constant (and $\beta$ its temperature derivative) and $\lambda_{DH}$ the Debye-Hueckel screening length.
3.1.2 Experimental Implementation

A typical commercially available MST device (NanoTemper Technologies, Germany) consists of an infrared (IR)-laser, that is coupled directly into the light path for fluorescence excitation and detection via a dichroic mirror, using the same optics (cf. Fig. 3.1). A very small volume (in the nanoliter range) in each of up to 16 capillaries is heated sequentially by typically 2 - 6 K by the IR-laser. The capillaries hereby contain different concentrations of unlabeled ligand (serially diluted by a constant factor) while the concentration of the labeled molecule is held constant. As the IR-laser is focused into the sample, a local temperature increase with mK reproducibility within the range of a few hundred micrometers can be achieved.[105] The emitted fluorescence intensity in the focus of the IR-laser is continuously monitored, and the intensity before the laser is switched on (“cold” state) as well as the intensity before the laser is switched off (“hot” state) are related and are used to characterize the thermophoretic molecule movements in each capillary.

3.1.3 MST Timetrace

A typical MST timetrace is mainly dependent on the IR-laser on- and off-times and can be separated into the following phases:[105]

- Initial fluorescence (IR-laser off)
- Temperature jump (IR-laser on)
- Thermophoresis (IR-laser on)
- Inverse temperature jump (IR-laser off)
- Backdiffusion (IR-laser off)

Jerabek-Willemsen et al.[105] simulated the dominant physical effects during an MST experiment for a molecule with a diffusion coefficient $D = 150 \ \mu m^2/s$ and a thermal diffusion coefficient $D_T = 5.6 \ \mu m^2/(sK)$. In less than one second after the IR-laser had been switched on, the temperature was found to be in equilibrium (for a change in temperature $\Delta T = 2.5 \ \text{K}$). On the contrary, the process of thermophoresis that builds up a concentration gradient in the capillary is rather slow and takes about 1 - 30 s (Fig. 3.2).

In the beginning of the measurement, the IR-laser is switched off, therefore a constant fluorescence intensity is detected (Fig. 3.1, phase 1). Ideally, the measured fluorescence should be stable and not decrease over time which would indicate a bleaching of the used fluorophore. In addition, the fluorescence intensity in all capillaries should be almost identical and not deviate by more than 5 - 10%. Higher deviations are typically related to aggregation processes and/or adsorption to the capillary walls. However, the emitted fluorescence intensity can also be directly influenced by the interaction of the two binding partners, most likely due to changes in the electrostatic surrounding of the fluorophore during complex formation. In these cases, the
dissociation constant might be directly derived from the changes in initial fluorescence.\[105\]

After a few seconds of recording the initial fluorescence (typically 5 s), the IR-laser is switched on, which results in an increase in temperature (typically 2 - 6 K) and an abrupt change (<1 s) in fluorescence intensity (Fig. 3.1, phase 2). This so called temperature jump (T-jump) corresponds to the temperature-dependent change of fluorescence yield which is an inherent property of most fluorophores and thus strongly depends on the used fluorophore.\[193\] The changes in fluorescence yield might be related to a change in absorption, quantum yield, fluorescence lifetime, or a spectral shift of the emitted fluorescence.\[233, 105\] Moreover, these properties are also dependent on the conformation and the local amino acid composition of the protein to which the fluorophore is coupled to. If the unlabeled ligand binds close to the fluorophore or if the binding induces a conformational change in the labeled protein, the temperature dependence of fluorescence might also be changed and therefore can be detected in the T-jump.\[24, 105\]

As the T-jump is occurring almost instantaneously when the IR-laser is switched on, it can be easily separated from the following phase, in which the thermophoretic movement of the molecules sets in (Fig. 3.1, phase 3). Thermophoresis is a diffusion-limited process and becomes significant after the T-jump, typically more than one second after the IR-laser had been switched on. Any effects, that have an influence on the Soret coefficient (i.e., changes in size, charge, or in the hydration shell; cf. Eq. 3.8) will influence the process.
of thermophoresis. MST therefore allows also the detection of binding of low-weight molecules (such as peptides or even ions) to big labeled molecules, as not only changes in size have an effect on the measurement signal.[221] After typically 30 s, the IR-laser is switched off, resulting in an inverse T-jump due to cooling of the sample. As the temperature gradient as driving force for thermodiffusion disappears, backdiffusion of the molecules, driven by pure mass diffusion, leads to a compensation of the concentration gradient (Fig. 3.1, phase 5). Since the process of backdiffusion is dependent on the diffusion velocity of the labeled molecules (and therefore on their size), information about the hydrodynamic radius can be extracted.[198]

Figure 3.3: Full presentation of a typical MST experiment as performed for this thesis. (a) Changes in the fluorescence intensity of the labeled molecules (receptors) due to thermophoresis-dependent depletion of the heated spot are monitored over time. Upon binding to the unlabeled ligand, the depletion behavior is gradually changing with respect to the ligand concentration and results in different time courses for each concentration. Shown are the normalized fluorescence changes of three independent measurements, the traces of unbound receptors are shown in red, the ones of bound receptors are shown in green. The change in thermophoresis is expressed as normalized fluorescence $F_{\text{norm}}$, determined by the ratio of the average fluorescence intensity in the marked red time span $F_{\text{hot}}$ and blue time span $F_{\text{cold}}$, respectively. (b) By plotting the change in normalized fluorescence $\Delta F_{\text{norm}}$ versus the logarithmic ligand concentrations, a typical binding curve is obtained. Fitting this binding curve to Eq. 3.19 yields the equilibrium dissociation constant $K_d$ (see main text).

### 3.1.4 Signal Evaluation

For a proper quantification of the changes in fluorescence intensity due to thermophoresis, the ratio of fluorescence intensity in the steady state and after the T-jump (~1 s after laser heating) is calculated in each capillary. In most cases, including of the fast fluorescence change in the T-jump phase (ratio of fluorescence in the steady state and fluorescence before laser heating) enhances the obtained signal amplitude and improves the signal-to-noise ratio.[105] The normalized fluorescence $F_{\text{norm}}$ is determined as
with $F_1$ being the fluorescence after thermodiffusion ("$F_{\text{hot}}$") and $F_0$ the initial fluorescence or the fluorescence after the T-jump ("$F_{\text{cold}}$"). It should be noted, that for determination of $F_1$ the steady state does not necessarily has to be reached if the traces of bound and unbound states can be sufficiently discriminated.\[221\]

In case of small changes in temperature and concentrations as typical in MST experiments ($\Delta T \sim 2 - 6 \text{ K}$), the steady state concentration change (Eq. 3.7) can be approximated by linearization as\[193\]

$$
\frac{c_{\text{hot}}}{c_{\text{cold}}} = \exp(-S_T \Delta T) \approx 1 - S_T \Delta T
$$

(3.10)

$F_{\text{norm}}$ depicts the changes in fluorescence intensity due to thermophoresis and is thereby dependent on the Soret coefficient $S_T$, leading to\[193\]

$$
F_{\text{norm}} = 1 - S_T \Delta T
$$

(3.11)

In case the change in fluorescence due to the fluorophore’s temperature dependency $\frac{\delta F}{\delta T}$ is considered, $F_{\text{norm}}$ follows\[193\]

$$
F_{\text{norm}} = 1 + \left(\frac{\delta F}{\delta T} - S_T\right) \Delta T
$$

(3.12)

The equilibrium dissociation constant is determined by analyzing the change in $F_{\text{norm}}$ as a function of the concentration of the unlabeled binding partner. The thermophoretic movements of unbound and bound fluorescently-labeled molecules superpose linearly and the bound fraction $FB$ can be described by\[193\]

$$
F_{\text{norm}} = (1 - FB) \cdot F_{\text{norm,UB}} + FB \cdot F_{\text{norm,B}}
$$

(3.13)

with $F_{\text{norm,UB}}$ and $F_{\text{norm,B}}$ being the normalized fluorescence of the unbound and bound fraction, respectively. For many cases, the complex formation can be described by a simple model according to the law of mass action.$[105]$ Assuming two interacting binding partners $A$ and $B$ forming a complex $AB$

$$
A + B \rightleftharpoons AB
$$

(3.14)

the equilibrium dissociation constant is defined as

$$
K_d = \frac{[A]_{\text{free}} [B]_{\text{free}}}{[AB]}
$$

(3.15)

with $[A]_{\text{free}}$ and $[B]_{\text{free}}$ being the concentrations of the free (unbound) molecules $A$ and $B$, and $[AB]$ the concentration of the complex $AB$. The free concentrations are not known but can be expressed by the total concentrations $[A]$ and $[B]$ as

$$
[A] = [A]_{\text{free}} + [AB]
$$

(3.16)
and

\[ [B] = [B]_{\text{free}} + [AB] \]  

(3.17)

yielding

\[
K_d = \frac{[A]_{\text{free}} [B]_{\text{free}}}{[AB]} = \frac{([A] - [AB]) ([B] - [AB])}{[AB]} 
\]

(3.18)

Considering \( A \) as the unlabeled binding partner and \( B \) as the labeled one whose concentration is held constant, Eq. 3.18 can be solved for the bound fraction of \( B \), \( FB \):

\[
FB = \frac{[AB]}{[B]} \frac{[A] + [B] + K_d - \sqrt{([A] + [B] + K_d)^2 - 4 [A] [B]}}{2 [B]} 
\]

(3.19)

As \( F_{\text{norm}} \) linearly reports \( FB \) and \( K_d \) is the only free parameter, \( F_{\text{norm}} \) can be directly fitted to this equation to obtain \( K_d \) from the MST data.

Typically, \( F_{\text{norm}} \) is plotted in per mil (‰) against the logarithm of the total concentration of the unlabeled binding partner \( A \). The resulting sigmoidal-shaped curve allows an by-eye inspection of the two plateaus corresponding to the unbound and bound state as well as a first estimation of the affinity (equilibrium dissociation constant \( K_d \)) of the interaction (cf. Fig. 3.3b). After fitting the binding curve to Eq. 3.19, the fitted \( F_{\text{norm}} \) value of the unbound state is typically subtracted as a baseline value to yield the relative change in normalized fluorescence \( \Delta F_{\text{norm}} \).[193, 105]
3.2 SURFACE PLASMON RESONANCE

Since the first observation of SPR in 1902 by Wood[226, 227] and since its potential for biosensing applications was discovered in the early 1980s,[125, 126] the technique of surface plasmon resonance (SPR) has emerged to one of the most powerful techniques for determining the specificity, affinity and kinetic parameters of interactions between a variety of different macromolecules.[153] In contrast to other biosensing techniques like fluorescence-based methods, SPR is a label-free technique where none of the interaction partners of interest has to be labeled and therefore no elaborative labeling protocols or special tags are needed. The use of molecular labels can introduce potential steric hindrance or influence the structural configuration of the molecule which might affect the labeled molecule’s affinity for its binding partner. Besides this significant advantage, SPR biosensors offer real-time data acquisition and are relatively easy to use, allowing for a high measurement throughput and a fast switching between different (biological) systems.[44]

Figure 3.4: Typical setup of an SPR biosensor on the basis of a BIAcore® instrument.[137] A sensor chip is sandwiched between a prism and a fluidic chamber with the gold layer facing downwards and being in contact with the sample. Polarized light is totally reflected at the glass/metal interface and the reflected light is detected. At a certain angle (resonance angle), an intensity minimum in the reflected light occurs (position I in the sensorgram, top right) that is dependent on the refractive index in the immediate vicinity of the gold layer. Binding of molecules to the sensor surface leads to a change in the refractive index and therefore to a shift of the resonance angle (from position I to II), which is plotted over time in a typical sensorgram (bottom right).

The technique of SPR is based on measuring the binding of (bio)molecules to a surface via an optical readout system. In typical biosensing SPR experiments, sensor chips with a gold surface are used which is derivatized in order to immobilize the respective receptor molecules on the substrate. Having the receptor molecules on the surface, a ligand-containing solution is injected into the fluidic system and allowed to flow over the chip surface for a certain time period. If the soluble ligand interacts with the receptor on the surface, the measured SPR signal is increasing, whereby both the absolute increase as well as the time-dependent rate are used to characterize the interaction (Fig. 3.4).
26 METHODS FOR BIOSPECIFIC INTERACTION ANALYSIS

Figure 3.5: Schematic illustration of the SPR effect at the interface between the glass prism and the sensor chip.[137] An evanescent field $E$ is generated by totally internally reflected $p$-polarized light at the glass/metal interface if the incident light’s momentum in the plane of the surface $k_x$ matches the one of the surface plasmon $k_{SP}$ in the gold layer. SPR is observed as an intensity minimum (dip) in the reflected light at a certain incident angle $\Theta$.

3.2.1 Principle of Operation: Overview

The functional principle of SPR is based on the formation of so-called surface plasmons (SP), which are collective electromagnetic oscillations arising from the interaction of light with metals like gold or silver.[160] Those oscillations propagate along the interface between materials with negative and positive permittivities such as metal/dielectric layers, thereby forming an electric (evanescent) field that exponentially decays into the surrounding medium with a penetration depth of some hundred nanometers (depending on the metal, the wavelength of the incident light and the refractive index of the dielectricum).[234, 94]

Although the evanescent field decays into both the metal and dielectric medium, the majority of the field is present in the dielectric medium due to higher damping in the metal layer.[44] If the incident light’s wavevector component parallel to the metal surface matches the one of the SP (occurs at a certain angle, called “resonance angle”), the intensity of the reflected light will drop (often referred to as “dip”).[44] Thus, as the evanescent field is strongly depending on a change in refractive index of the dielectric sensing surface, any change in this layer (e.g., due to binding of proteins) will cause a shift in the resonance angle (Fig. 3.5).

In most SPR applications, the so-called Kretschmann-configuration[116] is used. Here, the metal layer (typically gold, evaporated on a glass chip) is placed between a reflecting interface (prism) and a second medium (usually a fluidic chamber, either filled with air or liquid), which maximizes the efficiency of SP generation in comparison to the configuration described first by Otto[160], where the metal layer is separated by a short distance from the reflecting interface.[150]. The incident light wave passes first through the prism and is totally reflected at the prism/metal interface, generating an evanescent
3.2.2 Theory of Surface Plasmon Resonance

The description of the underlying physical principles of SPR requires a thorough consideration of the processes that take place at the interface between two materials with different refractive indices. In the following, the most important phenomena of SPR beyond the above given phenomenological explanation are shortly described, without the intention of giving an exhaustive overview.

**Total Internal Reflection** When light passes through a boundary between two different isotropic media (e.g., water, air, glass), the relation between the angle of incidence $\Theta_i$ and refraction $\Theta_r$ is described by Snell’s law as

$$\frac{\sin \Theta_i}{\sin \Theta_r} = \frac{v_1}{v_2} = \frac{\lambda_1}{\lambda_2} = \frac{n_2}{n_1}$$

with $v$ and $\lambda$ being the phase velocity and wavelength of light in the respective medium, respectively, and $n$ being the refractive index in the respective medium.

If light travels from one medium with higher refractive index $n_1$ to a medium with lower refractive index $n_2$ with an incident angle $\Theta_i$ that is greater than the so-called critical angle $\Theta_c$, total internal reflection will occur. The critical angle is thereby the incidence angle for which the angle of refraction is $90^\circ$. According to Eq. \ref{eq:snell}, the angle of incidence follows

$$\sin \Theta_i = \frac{n_2}{n_1} \sin \Theta_r$$

and under the assumption that $\Theta_r = 90^\circ$ the critical angle $\Theta_c$ is given as

$$\Theta_c = \arcsin \left( \frac{n_2}{n_1} \right)$$

**The Evanescent Wave** The physical measurement principle of SPR is based on the generation of an evanescent wave under the conditions of total internal reflection, that is dependent on the refractive index of the material in which it is propagating. An evanescent wave can be mathematically described by an electric field $E$ \cite{187}

$$E = E_0 \exp(j \omega t - j \vec{k} \cdot \vec{r})$$

with $E_0$ being the amplitude of the electric field, $j = \sqrt{-1}$, $\omega$ the angular frequency, $\vec{k}$ the wavevector and $\vec{r}$ the position vector. Considering the three-dimensional case with $r = (x, y, z)$, Eq. \ref{eq:evanescent} can be written as\cite{187}
The magnitude of the wavevector \( \vec{k} \), whose direction is parallel to the direction of wave propagation, is given as\[187\]

\[
k = \sqrt{k_x^2 + k_y^2 + k_z^2} = n \frac{2\pi}{\lambda} = n \frac{\omega}{c}
\]  

(3.25)

where \( n \) is the refractive index of the medium, and \( \lambda \) and \( c \) are the wavelength and propagation velocity in vacuum, respectively.\[187\]

If such a wave is refracted at the interface between two media 1 and 2 with different refractive indices \( n_1 \) and \( n_2 \) and the direction of the incident light beam is chosen such that \( k_z = 0 \), Snell’s law describes the relation between the angle of incidence \( \Theta_i \) and refraction \( \Theta_r \) according to

\[
n_1 \sin \Theta_i = n_2 \sin \Theta_r
\]

(3.26)
or, equivalently,

\[
k_{z1} = k_{z2} \equiv k_z
\]

(3.27)

Based on Eq. 3.25 and Eq. 3.27, an expression for the wavevector’s component perpendicular to the surface, \( k_y \), can be found\[160\]

\[
k_{y2}^2 = n_1^2 \left( \frac{2\pi}{\lambda} \right)^2 \left( \frac{n_2^2}{n_1^2} - \sin^2 \Theta_i \right)
\]

(3.28)

Assuming that the refractive index \( n_1 \) is higher than \( n_2 \) and \( \sin \alpha > n_2/n_1 \), \( k_y \) gets purely imaginary. In this case, there is only a traveling wave parallel to the interface with an electric field\[187\]

\[
E_2 = E_0 e^{\kappa_2 y} \exp(j \omega t - j k_x x)
\]

(3.29)

and a characteristic distance \( 1/k_{y2} \equiv 1/jk_{y2} \). This electric field is usually referred to as evanescent field, as it is exponentially decaying in y-direction (i.e., the direction perpendicular to the surface). Its penetration depth can be calculated according to Eq. 3.28 and is typically of the order of \( \sim \lambda/2 \), which accordingly explains why only changes in close vicinity to the sensor surface can be detected by SPR.\[187\]

The magnitude of the parallel component of the wavevector of the evanescent wave \( k_x \) is accordingly expressed as

\[
k_x = \frac{2\pi}{\lambda} n_1 \sin \Theta_i
\]

(3.30)

**Surface Plasmons** If a nonmagnetic gold layer with suitable thickness (typically \( \sim 50 \) nm) is placed at the interface between the two media, the evanescent wave is enhanced because at a specific angle (greater than the critical angle \( \Theta_c \)), delocalized electrons of the gold layer (surface plasmons) are excited. Surface plasmons are coherent delocalized electron oscillations that propagate within the metal surface at the interface between a metal and
a dielectric (medium 2). The magnitude of the wave vector of the surface plasmon $k_{SP}$ is dependent on the dielectric constants of both the gold layer $\varepsilon_g$ and the dielectric medium $\varepsilon_2$, given as [206]

$$k_{SP} = \frac{2\pi}{\lambda} \sqrt{\frac{\varepsilon_2\varepsilon_g}{\varepsilon_2 + \varepsilon_g}}$$

(3.31)

For nonabsorbing media, the dielectric constant $\varepsilon$ equals the square of the refractive index $n$ [187, 206]

$$\varepsilon \equiv n^2$$

(3.32)

so that $k_{SP}$ can be determined as [206]

$$k_{SP} = \frac{2\pi}{\lambda} \sqrt{\frac{n_2^2n_g^2}{n_2^2 + n_g^2}}$$

(3.33)

with $n_g$ and $n_2$ being the refractive indices of gold and the second medium in the vicinity of the interface. Please, note that the incoming light has to be p-polarized, as an electric field component perpendicular to the interface is required for the excitation of surface plasmons.

**Surface Plasmon Resonance**  If the magnitude of the wavevector of the evanescent wave $k_x$ matches the one of the wave vector of the surface plasmon $k_{SP}$, surface plasmons are excited (which is typically referred to as surface plasmon resonance (SPR)). Under these conditions, the energy of the incident light is transferred to plasmons and the intensity of the reflected light is reduced (dip in intensity, cf. Fig. 3.5). [137] By combining Eq. 3.30 and Eq. 3.31, the resonance angle at which SPR occurs $\Theta_{SPR}$ can be calculated as [206]

$$\Theta_{SPR} = \arcsin \left( \frac{1}{n_1} \sqrt{\frac{n_2^2n_g^2}{n_2^2 + n_g^2}} \right)$$

(3.34)

As the refractive indices of the prism/sensor chip combination $n_1$ and of the gold layer $n_g$ are solely material-related parameters and not changed during an experiment, the only free parameter that influences $\Theta_{SPR}$ is the refractive index of the medium in close vicinity to the gold layer $n_2$. In case of SPR biosensors, binding of biomolecules to the gold surface changes this refractive index and results in a shift of the resonance angle $\Theta_{SPR}$, which is continuously monitored over time.

**Surface Plasmon Properties and Dependencies** Besides the refractive index of the medium close to the gold surface $n_2$ (and the wavelength of incident light which is typically constant), several factors contribute to the shape of the dip in the reflected light’s intensity. With incident light in the visible and near IR region and considering an interface between glass and an
aqueous solution as it is typical for SPR biosensors, the only materials that are practically suitable for generating SPR are gold, silver, and aluminum.[137]

Theoretically, many other metals including copper, sodium, indium, titanium, and chromium, have been shown to exhibit the effect of SPR in the visible region.[1] However, certain critical limitations such as inertness of the metal, sharpness of the resonance dip, chemical resistance and compatibility with the sample, and the cost factor, have to be considered. For biosensing applications, gold is most suitable as it produces a rather sharp SPR dip in the near IR region, it is inert, and can easily be chemically modified.[1]

In addition to the used metal layer, its thickness strongly influences the shape of the resonance dip, as only a certain thickness (dependent on the applied wavelength, for gold typically ~40 - 50 nm) produces a distinct, sharp dip and enhances the generated electric field.[137, 187]

3.2.3 Sensorgram and Signal Analysis

The measurement signal of a typical SPR device is plotted as the change in resonance angle over time (sensorgram). If the refractive index of the solution in close vicinity to the sensor surface is changing, the resonance angle will change accordingly as explained in Sec. 3.2.2. Typically, the shift in the resonance angle is depicted as resonance unit (RU), whereby 1000 RU represent a shift of 0.1° in resonance angle. As a rule of thumb, a signal increase of 1000 RU corresponds to a change in surface protein concentration of about 1 ng/mm², almost independent on the type of protein.[137] Importantly, it has to be noted that the measured signal always includes any change in refractive index, regardless if this change is caused by a change in the buffer composition (usually referred to as bulk effect) or due to association processes to the sensor surface. As a consequence, the obtained response due to binding of an injected analyte has to be corrected for the bulk effect which is typically done by subtracting the signal measured in a reference cell without receptor molecules.

As shown in Fig. 3.6, a typical sensorgram of an interaction between a soluble analyte A and a surface-immobilized ligand B

\[ A + B \rightleftharpoons AB \]  (3.35)

consists of three phases: In the association phase, the complex formation begins, which is governed by the association rate constant \( k_a \) (l mol\(^{-1}\)s\(^{-1}\) or M\(^{-1}\)s\(^{-1}\)) and the dissociation rate constant \( k_d \) (s\(^{-1}\)). For a simple bimolecular binding model that is assumed to follow a pseudo first order kinetics, the following differential equation for the association process can be derived:

\[ \frac{d[AB]}{dt} = k_a[A][B] - k_d[AB] \]  (3.36)

Please, note that the concentrations of the surface-immobilized ligand \([B]\) and the formed complex \([AB]\) are indirectly determined by the shift in resonance angle and are not approached as concentrations in solution as for \([A]\).
However, for sensor chips with a three-dimensional dextran coating with defined length, a conversion factor can be introduced to estimate the molar concentrations.\[112\]

Figure 3.6: Typical sensorgram of an SPR measurement.\[137\] After continuous buffer flow over the sensor surface with immobilized ligand, the analyte is injected into the flow chamber. In the beginning of the association phase, a sharp signal increase is observed (bulk effect) due to the different refractive indices of the running buffer and the sample solution. When changing to running buffer after the injection, the same effect is seen in the opposite direction, followed by dissociation of the bound analyte molecules. Typically, any remaining analyte at the end of the dissociation phase is removed by a regeneration solution (especially for interactions with low dissociation rates).

If equilibrium (steady state phase) is reached as illustrated by the plateau at the end of the association phase in Fig. 3.6, the concentration of the complex \([AB]\) is directly proportional to the response \(R_{eq}\). If the total surface binding capacity \(R_{max}\) is known, the concentration of free ligand \([B]\) can be derived from \(R_{max} - R_{eq}\) and the association equation Eq. 3.36 for complex formation \(d[AB]/dt\) (corresponds to the measured change in response \(dR(t)/dt\)) can be written as

\[
\frac{d[AB]}{dt} = \frac{dR(t)}{dt} = k_a[A](R_{max} - R_{eq}) - k_dR_{eq} = 0
\]

Solving for \(R_{eq}\) yields

\[
R_{eq} = \frac{k_a[A]R_{max}}{k_a[A] + k_d} = \left(\frac{[A]}{[A] + K_d}\right) R_{max} = \left(\frac{[A]k_a}{[A]k_a + 1}\right) R_{max}
\]

with \(R_{max}\) being the maximum binding capacity in RU where all binding sites on the sensor surface are occupied. By determining \(R_{eq}\) for different analyte
concentrations \([A]\) and plotting \(R_{eq}\) against \([A]\), the equilibrium dissociation constant \(K_d\) (mol l\(^{-1}\) (M)) (and the equilibrium association constant \(K_a\) (l mol\(^{-1}\) (M\(^{-1}\))))

\[
K_d = K_a^{-1} = \frac{[A][B]}{[AB]} = \frac{k_d}{k_a}
\] (3.39)

can be determined by using a Langmuir isotherm. The advantage of this equilibrium method is that it is independent of putative mass transport limitations or rebinding effects and therefore also high protein concentrations and low flow rates can be used.\(^{[212]}\)

The analytical solution for the association equation (Eq. 3.36) is given by the integrated rate equation\(^{[159]}\)

\[
R(t) = \frac{k_a[A]R_{max}}{k_d[A] + K_d} \cdot (1 - e^{-(k_a[A]+k_d)t})
\] (3.40)

whereby the left term represents \(R_{eq}\), thus

\[
R(t) = R_{eq} \cdot (1 - e^{-(k_a[A]+k_d)t})
\] (3.41)

At the end of the analyte injection, the sample is replaced with running buffer and bound molecules dissociate from the sensor surface, resulting in a decrease in resonance angle. Disregarding rebinding effects, complex dissociation in the dissociation phase follows according to

\[
-\frac{d[AB]}{dt} = k_d[AB]
\] (3.42)

As the measured SPR signal \(R(t)\) directly corresponds to the complex concentration \([AB]\), Eq. 3.42 can be written directly as

\[
-\frac{dR(t)}{dt} = k_dR(t)
\] (3.43)

The analytical solution for the dissociation equation Eq. 3.42 is described by a first-order exponential decay

\[
R(t) = R_0e^{-k_dt}
\] (3.44)

where \(R_0\) is the SPR signal at the end of the association phase before dissociation sets in.

As a robust determination of the affinity or the kinetics of an interaction requires the injection of various analyte concentrations, the remaining analyte after one injection is typically removed by a regeneration solution with a low or high pH and/or salt concentration. Special care has to be taken to obtain a complete regeneration while leaving the immobilized ligand in place and its activity intact.
3.3 Atomic Force Microscopy

Since its invention in 1986,[19] the Atomic Force Microscope (AFM) has developed into a powerful tool for not only topographical but also functional characterization of various specimens. Besides its application in the field of electronics, semi-conductors or material sciences, the technique of AFM paved the way for the investigation of biological samples at (sub-)nanometer resolution. Its unique flexibility to image, probe and manipulate different (bio-)materials under close-to-native conditions made it the most versatile instrument in nanoscience and nanotechnology.[78, 57]

3.3.1 Functional Principle

The functional principle of an Atomic Force Microscope is based on the interaction of an extremely sharp tip with the substrate. The tip (with a tip radius of typically few nm)[20] is mounted at the end of a cantilever that is usually micro-fabricated from silicon or silicon nitride (Fig. 3.7).

Today, numerous cantilever designs are available (triangular, rectangular, magnetic, tipless, etc.) and have to be chosen according to the respective mode of operation and research question. Cantilevers are classified by their spring constant and their resonance frequency, two parameters that play an important role in both force sensing and topography imaging.

Figure 3.7: Scanning electron microscopy images of a (used) AFM cantilever.[103]
Left: Illustration of the cantilever with the tip with 1000x magnification, the scale bar corresponds to 20 µm. Right: Illustration of the tip with 3000x magnification, the scale bar corresponds to 10 µm.

By bringing the tip in close contact with the sample surface, any forces between tip and surface (e.g., electrostatic, van der Waals, capillary forces, etc.) are registered through a deflection of the cantilever that is usually determined by an optical readout system. For this purpose, a laser beam is focused on the backside of the cantilever and reflected onto a position-sensitive photodetector (usually referred to as optical beam deflection (OBD)[21]). As the photodetector typically consists of a four-segment photodiode, both the vertical deflection of the cantilever due to changes in sample height as well as the lateral deflection (torsion) due to friction forces are registered. Using a piezoelectric actuator, the tip can be positioned and moved with nanometer precision in three dimensions. The photodiode difference signal is fed into a
feedback loop and serves as command variable for the piezoelectric actuator, hereby enabling the tip to maintain a constant force (or constant height) in relation to the sample surface. By linewise scanning the sample surface, a height image is obtained that resembles the sample topography with a resolution depending on the tip radius, the sample corrugation, the physical properties of the sample and the feedback control parameters.[57]

Two main operating modes can be used to generate an image by AFM: static or DC-mode and dynamic or AC-mode (Fig. 3.8).

In the **static mode**, the tip is brought into contact with the surface and moved across it while keeping the cantilever deflection (i.e., the force that is applied to the sample) constant via a feedback loop. The image contrast mainly depends on the applied force and the spring constant of the used cantilever, but, however, even at low forces considerable shear forces can be generated that can damage the sample, especially if very soft biological specimen like cells are imaged.[25]

In the **dynamic mode** (or tapping mode), the cantilever is oscillating at or near its resonance frequency (either acoustically driven by an additional piezo or driven by an oscillating magnetic field) while its oscillation amplitude is held constant. The tip just intermittently interacts with the sample surface, which thus minimizes the tip-surface contact time and the possibility of potential sample damages and makes the dynamic mode ideally suited for studying soft biological samples like cells.[92]

It should be mentioned, that over the past decades various subtypes of the respective main AFM operating modes - often in combination with specially designed tips - have been developed (e.g., magnetic AC mode (MAC mode®, Keysight Technologies, USA), PeakForce Tapping® (Bruker, USA), Multifrequency Imaging[77], etc.). Moreover, technological advances have allowed the imaging speed to be increased dramatically, so that high-speed AFM can be used to study dynamic molecular processes on a timescale of
milliseconds.\cite{3, 4, 5} For a recent review and discussion of different available AFM modes and their applications see Ref. \cite{57}.

### 3.3.2 AFM Force Spectroscopy

Besides the operating modes for topographical mapping of different sample surfaces as described above, the AFM can be used for a quantitative determination of the prevailing interaction forces between two (bio-)molecules of interest. AFM force spectroscopy allows for the measurement of pico-Newton ($10^{-12}$ N) forces of single molecular interactions, thereby providing fundamental insights into the molecular basis of numerous biological processes\cite{88} such as protein-(un)folding\cite{178, 155}, DNA mechanics\cite{179}, cell adhesion\cite{14}, or molecular recognition\cite{71, 121, 89}. In the force spectroscopy mode, the tip is held at a constant position in the x-y plane and is only moved in the z-direction at a constant velocity (Fig. 3.9).

![AFM force spectroscopy mode](image)

Figure 3.9: AFM force spectroscopy mode. The tip-functionalized cantilever is continuously approached to and withdrawn from the functionalized surface. Upon interaction between the ligand on the tip and the receptor on the surface, the resulting cantilever deflection during retraction allows for determination of the unbinding force.

To specifically probe the interaction of two binding partners, they have to be immobilized to the substrate and to the tip itself, respectively. As ideally just one ligand should be tethered to the AFM tip to ensure the detection of single-molecule interaction forces, the chemical functionalization of the tip is one of the most crucial parts in performing force spectroscopy experiments (cf. Sec. 3.3.3.1).

#### 3.3.2.1 The Force-Distance Cycle

In order to determine the prevailing interaction forces between a receptor-ligand pair, the cantilever with the tip-tethered ligand has to be repeatedly brought in contact to the surface with the immobilized receptor molecule. For
this purpose, the cantilever is moved only in the z-direction at a fixed lateral position towards the surface and is subsequently retracted. The deflection of the cantilever is continuously monitored and plotted over the vertical distance of the tip in relation to the sample surface (corresponds to the vertical piezo displacement). The resulting plot is typically called force-distance cycle (FDC), as the acting force can get directly determined from the cantilever deflection (Fig. 3-10).
the cantilever, and an increasing force is exerted on the receptor-ligand bond until it fails at a certain critical force value (i.e., rupture force or unbinding force, \( f_u \) in Fig. 3.10). As the ligand molecule is typically tethered via a flexible PEG-linker to the tip, stretching of the linker prior the unbinding event results in a typical nonlinear, parabolic force profile[113] if the origin of the attraction force is not a specific receptor-ligand interaction (e.g., due to tip adhesion), the observed force profile is linear. When no interaction occurred, the cantilever rests in its equilibrium position during further retraction and the retrace curve looks similar to the approach curve.

In any case, the presence of unbinding events due to putative receptor-ligand interactions has to be carefully proven by so-called blocking experiments. For this purpose, either the receptors on the surface are blocked by addition of free ligand to the measurement buffer, or the ligand on the tip gets inactivated by injection of free receptor molecules. In both cases, specific unbinding events are typically disappearing to a large extent and are only observed occasionally.

In most AFM setups, the cantilever bending is usually detected optically by measuring the position of a laser beam that is reflected from the backside of the cantilever via a split photodiode. The resulting photodiode voltage then can get correlated to the cantilever deflection by recording a force-distance curve on a stiff support. Assuming that the cantilever deflection is proportional to the travel distance of the piezo in z-direction, the slope of the increasing deflection signal during surface contact allows for determination of the sensitivity (= 1/slope). Multiplying the output of the photodiode with the sensitivity finally gives the displacement \( \Delta z \) of the cantilever. Considering the fact that the cantilever acts as a small spring, the cantilever displacement \( \Delta z \) can get converted to force values by applying Hooke’s law

\[
F = k_c \cdot \Delta z
\]  

(3.45)

with \( k_c \) being the spring constant of the cantilever.

### 3.3.2.2 Spring Constant Determination

A cantilever behaves like a Hookian spring with a certain spring (force) constant \( k_c \). According to Hooke’s law, the force that is acting on the cantilever can be calculated by multiplying its spring constant with its displacement (in one dimension). Thus, the spring constant of the respective cantilever has to be determined in order to obtain reliable force values. To date, several methods for spring constant determination are available, with each of them having certain advantages and drawbacks.[63] Most methods can be grouped in four categories:[43] (i) **THEORETICAL METHODS** are based on the calculation of the spring constant from its geometry and material properties, (ii) **DYNAMIC METHODS** are based on measuring dynamic properties of the cantilever (e.g., its resonance frequency or quality factor) and combining them with a theoretical model (e.g., Sader-method[183, 184, 185]), (iii) **THERMAL METHODS** link the spring constant to the thermal vibrations of the cantilever.
at thermal equilibrium\cite{99}, and (iv) methods that use *force standards* for the determination of the spring constant.

Due to its accuracy and usability, the thermal noise method is commonly used. Here, the fluctuations of the cantilever (modeled as a simple harmonic oscillator at thermal equilibrium) are monitored in air and the spring constant is determined based on the equipartition theorem. The spring constant can be expressed as\cite{63}

\[ k_c = \frac{k_B T}{\langle z^2 \rangle} \]  

(3.46)

where \( k_B \) is the Boltzmann constant (\( 1.38 \cdot 10^{-23} \text{ J K}^{-1} \)), \( T \) the temperature and \( \langle z^2 \rangle \) the mean square displacement of the cantilever. The latter is determined by recording the power spectrum under cantilever resonance, fitting a simple harmonic oscillator model\cite{63}

\[ A = A_{\text{white}} + \frac{A_0 \omega_0^4}{(\omega^2 - \omega_0^2)^2 + (\omega \omega_0 Q)^2} \]

with \( A_{\text{white}} \) being the white noise, \( A_0 \) the zero frequency amplitude, \( \omega_0 \) the radial resonance frequency, and \( Q \) the quality factor, and integrating without taking \( A_{\text{white}} \) into account. The spring constant is then given as\cite{99}

\[ k_c = \frac{k_B T}{P} \]

where \( P \) is the area under the power spectrum curve of the thermal fluctuations alone.

### 3.3.3 Theory of Force Spectroscopy

The interaction between two molecules can be approximated by a configuration consisting of two energetic states, bound and unbound, that are separated by a single energy barrier. Association of the two molecules will occur if the free energy of the bound state is lower than the sum of the free energies of the two separated molecules. As most biological interactions such as van der Waals or electrostatic interactions are of reversible nature, the lifetime of the bond \( \tau_0 \) without any applied external force is inversely dependent on the dissociation rate \( k_d \)

\[ \tau_0 = \frac{1}{k_d} \]  

(3.47)

The survival probability \( S(t) \) (i.e., the probability to be in the bound state) for the system under a linearly increasing force can be described with a first order rate equation by solving the master equation\cite{66, 203}:

\[ \frac{dS(t)}{dt} = -k(t) \cdot S(t) \]  

(3.48)

yielding\cite{63}
\[ S(t) = \exp \left[ -\int_0^t k(t')dt' \right] \] (3.49)

At rupture, the probability distribution \( p(F) \) of forces \( F \) is related to \( S(t) \) by\[63\]

\[ p(F) dF = -\dot{S}(t) dt \] (3.50)

resulting in\[63\]

\[ p(F) = \frac{k(F)}{dF/dt} \exp \left( -\int_0^F \frac{k(F')}{dF'/dt} dF' \right) \] (3.51)

Based on the framework of Bell\[13\], an applied force \( F \) will decrease the activation free energy for a reaction without external force, \( \Delta G^*(0) \) to

\[ \Delta G^*(F) = \Delta G^*(0) - Fx_\beta \] (3.52)

which makes dissociation by thermal fluctuations more likely (Fig. 3.11). As in AFM experiments both binding partners are fixed to either the tip or the substrate and pulled apart from each other, the force-induced dissociation process can be considered as a quasi-irreversible process where rebinding can be neglected. During this irreversible molecular transition from the bound to the unbound state, the molecule is thought to move along a combined free energy surface \( E(x) = E(x) - F(x) \) in the direction of pulling, \( x \).[63] The intrinsic free energy surface \( E_0(x) \) is assumed to have a single minimum at \( x = 0 \) and a maximum at \( x = x_\beta \) with a barrier of height \( \Delta G^* \). As the surrounding heat bath causes energy fluctuations and the system can be assumed as small, those energy fluctuations will result in a stochastic escape process.[63] The applied external force lowers the height of the energy barrier and therefore facilitates dissociation caused by the energy fluctuations (Fig. 3.11). According to the single energy barrier model\[13][66\], the increased rate of bond dissociation under external force \( k(F) \) can be described as

\[ k(F) = k_d \cdot e^{(Fx_\beta/k_B T)} \] (3.53)

with \( k_d \) being the dissociation rate at zero external force and assuming that \( x_\beta \) is not affected by the external force.

In force spectroscopy, the vertical cantilever movements (in z-direction) are typically performed at a constant velocity \( v \), which results in a linear force ramp

\[ F(t) = k_c \cdot v \cdot t \] (3.54)

with \( k_c \) being the cantilever spring constant. Importantly, as for most force spectroscopy measurements the ligand is tethered flexibly to the tip, the spring constant of the bare cantilever \( k_c \) has to be corrected for contributions from the linker molecule, resulting in the effective spring constant \( k_{eff} \).
Figure 3.11: Schematic illustration of the energy landscape for a dissociation process of a receptor-ligand complex, either without (solid line) or with applied external force \( F \) (dashed line).\(^{20}\) \( k_{\text{off}} \) depicts the dissociation rate (in the text \( k_d \)), and \( x_\beta \) the distance of the energy barrier from the energy minimum to the energy maximum along the projection of the applied force. In case of an applied force \( F \), the height of the energy barrier \( \Delta G^* \) (activation free energy) gets lowered by \( Fx_\beta \) to \( \Delta G^*(F) \).

\[
k_{\text{eff}}^{-1} = k_c^{-1} + k_{\text{linker}}^{-1}
\]

(3.55)

As the spring constant of the linker molecule \( k_{\text{linker}} \) is depending on the applied force, a simple but powerful approximation\(^{63}\) is to determine \( k_{\text{linker}} \) by fitting a worm-like chain model\(^{138}\) to the force profile at the point of rupture,\(^{67,74}\) yielding a linear force ramp with

\[
F(t) = k_{\text{eff}} \cdot v \cdot t
\]

(3.56)

The product of the effective spring constant \( k_{\text{eff}} \) and the pulling velocity \( v \) here represents the force rate (with the unit N/s) with which the bond is loaded and is typically referred to as the so-called loading rate \( r \).

By substituting Eq. 3.53 and Eq. 3.56 into Eq. 3.51, an analytical expression for the observed rupture force distribution is obtained\(^{63}\)

\[
p(F) = \frac{k_{\text{eff}}}{r} \exp \left( \frac{Fx_\beta}{k_B T} - \frac{k_d k_B T}{rx_\beta} \cdot \left( e^{\frac{r x_\beta}{k_B T}} - 1 \right) \right)
\]

(3.57)

where \( k_d \) is the dissociation rate in absence of an applied external force. The most probable rupture force \( F^* \) is hereby found to be linearly dependent on the logarithm of the loading rate \( r \) with

\[
F^* = \frac{k_B T}{x_\beta} \cdot \ln \left( \frac{x_\beta r}{k_B T k_d} \right)
\]

(3.58)

The above described model based on the framework of Bell\(^{13}\) and adapted from Evans\(^{66}\) to the requirements of single molecule force spectroscopy (often referred to as Bell-Evans model) represents a simple phenomenological model for the force-induced escape over a single, sharp energy barrier in
the thermally activated regime. According to the model, the most probable unbinding force is scaling linearly with the loading rate.

As the force needed to induce dissociation within a certain time $\tau$ is critically depending on the time scale the experiments are performed (in case of AFM experiments in the range of milliseconds),[66][104][8] shorter times (i.e., faster loading rates) will result in larger rupture forces because thermal activation will be too slow to facilitate dissociation. On the other hand, if $\tau$ is chosen longer than the natural dissociation time of a receptor-ligand complex, no external force will be needed and thus a zero rupture force would be measured.[8] As the unbinding process is of stochastic nature, many unbinding events have to be evaluated in order to obtain a sound and statistically robust estimate for the most probable unbinding force. In a typical force spectroscopy experiment, more than $10^4$ force-distance cycles are performed, that - depending on the receptor-ligand system - usually result in $>100$ unbinding events. The force distribution of such a data set can be determined by a classical intuitive approach using histograms, or by calculating the empirical probability density function (pdf) to overcome the disadvantages of histograms:[12] (i) The choice of classes (bins) in histograms is subjective, (ii) a histogram represents a discontinuous data depiction with distinct jumps at the class boundaries, and (iii) single data points cannot be weighted according to their reliability.

Accordingly, probability density functions of all unbinding events (and unbinding lengths) can be determined by summing up Gaussian distributions with unitary area that are calculated from the mean and variance of each unbinding event:[12]

$$pdf(F) = \frac{N}{\sum_{i=1}^{N}} \frac{1}{\sqrt{2\pi \sigma_i^2}} \exp\left(\frac{(F - F_i)^2}{2\sigma_i^2}\right)$$

(3.59)

with $N$ being the number of all unbinding events, $F_i$ the measured unbinding forces and $\sigma_i$ the corresponding standard deviation.[12, 63] For characterizing the energy landscape of a receptor-ligand interaction, the most probable unbinding force $F^*$ per loading rate $r$ is determined by fitting a Gaussian function to the calculated pdf. Plotting $F^*$ over $\ln(r)$ (so called loading rate dependence, LRD) and fitting a linear fit based on the Bell-Evans model[13, 66] (Eq. 3.58) results in a straight line, whereby the slope carries information about the width of the energy barrier $\beta$ and by extrapolation to zero force, the dissociation rate constant without applied force $k_d$ can get determined (Fig. 3.12).

**Multiple energy barriers** As complex formation between two (bio-) molecules typically involves many intermolecular bonds (multiple hydrogen bonds, van der Waals interactions), dissociation does not necessarily have to occur as a single-step process involving only one single energy barrier, but can proceed via intermediate states. Therefore, the energy landscape might be of more complex nature with two or more energy barriers.[143]
According to the Bell-Evans model, the presence of more energy barriers that have to be overcome during the dissociation process along the pulling coordinate will result in multiple linear regimes in the loading rate dependence plot. Each of the regimes yields a distinct dissociation rate $k_d$ and width of the energy barrier $x_{\beta}$ that characterize the escape over the respective energy barrier (Fig. 3.13). Multiple energy barriers along the dissociation pathway lead to a complex energy landscape (Fig. 3.13C) with a highly discontinuous LRD plot.

In the (simple) case of two energy barriers, the bound and the unbound state are separated by two barriers along the pulling direction, with an intermediate state in between (energy landscape $E(x)$ along the pulling direction $x$, Fig. 3.14). If a small force ($F_1$) is applied to the complex, the potential gets linearly decreased by $F_1 \cdot x$, resulting in a new combined potential with $E(x) - F_1 \cdot x$, where the outer energy barrier is still the rate limiting one. If the applied force is increased to $F_2$ (resulting combined potential $E(x) - F_2 \cdot x$), the transition state will change from the outer barrier to the inner one. Further increasing the external applied force to $F_3$ (resulting combined potential $E(x) - F_3 \cdot x$), will lead to lowering of the energy barrier to the level of the initial minimum and would allow unhindered dissociation of the complex without any applied force.

Despite its simplicity, it has been shown that the unbinding process of many biological interactions can be explained and modeled by the Bell-Evans model. Nevertheless, different approaches to better characterize the energy landscape of dissociation under external force have emerged in the last years. Dudko, Hummer and Szabo adapted and extended the Bell-Evans model, particularly by using a harmonic potential with either a cusp-like or a linear-cubic energy surface and applying Kramers’ theory of diffusive barrier
Figure 3.13: Energy landscapes of the dissociation process along a reaction coordinate of a biomolecular complex (left) with the corresponding loading rate dependence (right).[20] In case of one energy barrier (A), the LRD plot results in only one straight line with one slope. The presence of more energy barriers leads to multiple slopes with each of them corresponding to one energy barrier (B). If the energy landscape is complex, the resulting LRD plot will get highly discontinuous (C).

crossing,[115], resulting in a better approximation of the kinetic parameters and extraction of thermodynamic parameters.[54, 55, 56, 98]

Furthermore, Friddle, De Yoreo and coworkers[73] addressed one of the main simplifications made in the Bell-Evans model, that no rebinding is occurring during force spectroscopy experiments. According to their model, the dissociation of a receptor-ligand complex passes through two phases: in the first phase (equilibrium phase) at lower pulling velocities (loading rates), the molecules are able to rebind, whereas in the kinetic phase at higher loading rates, the molecules unbind irreversibly.[73]

A comprehensive overview about the analysis of experimental force spectroscopy data with different models can be found in Refs. [20] and [154], as well as in Hane et al.[85], who compared the three prevalent models (Bell-Evans, Dudko-Hummer-Szabo and Friddle-De Yoreo) to study the binding forces of peptide interactions. Most importantly, the choice of the appropriate theoretical model to describe experimentally determined unbinding forces has to be based on the obtained force spectrum. Non-linear models should only be used if the unbinding forces are not scaling linearly with $\ln(r)$, otherwise the resulting parameters are not meaningful.

3.3.3.1 AFM Tip Chemistry

For performing force spectroscopy experiments, the inert AFM tip (typically made of silicon nitride) has to be converted into a biosensor by immobilizing a sensing molecule (ligand) to the apex of the tip; in parallel, the cognate
Figure 3.14: Conceptual free energy landscape with two barriers. For the unper-
turbed system without applied force \((E(x))\) and for a small applied external force \((E(x) - F_1 \cdot x)\), the transition state is located at the outer barrier. For higher forces \((E(x) - F_2 \cdot x)\), the transition state moves from the outer barrier to the inner barrier. By further increasing the applied force \((E(x) - F_3 \cdot x)\), the energy barrier is lowered to the level of the initial energy minimum, resulting in unhindered dissociation.

receptor molecule has to be immobilized on a flat surface. Most importantly, a firm attachment of both binding partners is required in order to ensure that rupture only occurs between the receptor and ligand molecule and not at their anchoring points on the substrate or on the tip.

Immobilization can be performed by either covalent attachment, via biospe-
cific interactions (e.g., avidin and biotin) or via simple physisorption. Al-
though the latter two methods were shown to be sufficient under many cir-
mstances, covalent chemistry is preferred in most cases as covalent bonds are at least ten times stronger (1-2 nN) than typical receptor-ligand bonds. While the immobilization of the receptor to the substrate is strongly de-
pendent on the used substrate material (e.g., mica, glass, gold, etc.) as well as on the receptor molecule itself, the functionalization of the tip with the cognate ligand molecule is of utmost significance as ideally just one molecule should be tethered to the apex of the tip. Typically, the respective ligand molecule is tethered to the tip via a flexible polymer chain, most promi-
nently for the following reasons: (i) the flexible attachment allows the tip-tethered molecule to retain sufficient mobility to interact with its binding partner, (ii) the length of the linker defines a certain volume in which the molecule can freely diffuse and an interaction is possible, and (iii) in case of an interaction, the non-linear stretching of the elastic linker allows for discrimination between unspecific tip adhesion and specific receptor-ligand interactions.

Polyethyleneglycol (PEG) linkers proved to be ideally suited as molecular tethers as they have two defined end groups while the rest of the chain is chemically and physically inert, thus preventing a possible interaction be-
tween the linker itself and the substrate. In addition, the use of heterobifunc-
tional linkers (i.e., linkers with two different end groups) allows for step-wise coupling to avoid loop formations by first conjugating the linker onto the
Figure 3.15: Three-step protocol for coupling a sensor molecule to a silicon (nitride) AFM tip.\cite{81} 1) Aminofunctionalization of the AFM tip to generate reactive amino groups on the tip surface. Shown is the gas phase silanization with APTES. 2) Attachment of the PEG-crosslinker via its NHS-ester. 3) Coupling of the sensor molecule to the free end of the crosslinker.

In general, the functionalization of the most commonly used silicon (nitride) tips is usually performed in three steps (cf. Fig. 3.15):

1. **Aminofunctionalization to generate reactive groups on the tip surface:**

   As a first step, the surface of the chemically rather inert silicon nitride tip has to be derivatized to introduce reactive amino (NH$_2$) groups. Typically, aminofunctionalization with ethanolamine-hydrochloride\cite{181, 60, 202} or aminosilanization protocols\cite{181, 60, 11, 59} are used for this purpose, with the latter relying either on vapour-phase deposition\cite{45, 32} or liquid-phase modification under dry conditions\cite{60}. Importantly, a proper adjustment of the density of generated amino groups on the tip surface has to be ensured in order to result in statistically one to two reactive amino groups on the tip apex for successful subsequent coupling of ideally only one PEG-linker.\cite{60} For the studies conducted in this work, a vapour-phase aminosilanization protocol using Amino-
propyltriethoxysilane (APTES, 3-(triethoxysilyl)propylamine) with triethylamine as catalyst[60] was used.

2. **UNILATERAL ATTACHMENT OF A HETEROBIFUNCTIONAL CROSSLINER:**
   After aminofunctionalization, the PEG-linker is conjugated onto the tip via reaction of its amino-reactive end (usually an N-hydroxysuccinimide (NHS) ester) with the amino groups on the tip surface. Depending on the ligand, linkers with different reactive groups can be used (e.g. with an aldehyde group for coupling of proteins, with a maleimide group for coupling of thiolated molecules, or with nitrilotriacetic acid (NTA) for coupling of His-tagged proteins). If the used PEG-linker already carries the ligand molecule (e.g., biotin) on the other end, tip functionalization is finished after this step.

3. **COUPLING OF THE LIGAND MOLECULE:**
   Once the PEG-linker is attached to the AFM tip, the ligand molecule is coupled to the free end of the linker molecule.

In summary, it has to be pointed out that although there is no “perfect” linker, almost any kind of ligand molecule can be successfully tethered to the tip.[63] Additionally, besides the above described functionalization procedures for silicon or silicon nitride tips, corresponding protocols are also available for tips made out of other materials such as gold.[62]
Part II

EXPERIMENTAL SECTION

This part comprises the used materials (e.g., reagents, buffers or proteins) and detailed protocols of the preparatory procedures required for conducting the experiments within this thesis. For reasons of clarity and comprehensibility, the descriptions are structured in accordance to the respective experimental methods.
EXPERIMENTAL SECTION

4.1 MATERIALS

4.1.1 Buffers and Reagent Solutions

**Biotin** (200 mM) was prepared by weighing 244.3 mg biotin (1 mmol) and 157.4 mg Tris base (1.3 mmol) into a tube and adding of water to a final volume of 4 ml. The mixture was shaken to dissolve all components. The pH was adjusted to 8 - 8.5 and water was added to a final volume of 5 ml. Aliquots of 1 ml and 100 µl were prepared and immediately frozen at -25 °C to prevent degradation. Frozen aliquots were thawed and gently mixed directly before injection.

**Calcium-Chloride** (CaCl$_2$) solution was prepared by dissolving CaCl$_2$ dihydrate (147.01 g/mol) at a concentration of 14.70 mg/ml (100 mM) in water. Aliquots were stored at -25 °C.

**EDTA** (Ethlenediaminetetraacetic acid) solution was prepared by dissolving EDTA-Na$_2$ (372.24 g/mol) at a concentration of 37.22 mg/ml (100 mM) in water and adjusting the pH to 7.5 by adding of NaOH. Aliquots were stored at -25 °C.

**EGTA** (Ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid) solution was prepared by dissolving EGTA (380.35 g/mol) at a concentration of 38.04 mg/ml (100 mM) in water. Aliquots were stored at -25 °C.

**Glycine** (100 mM, pH 2.5) solution was prepared by dissolving 7.0 g glycine in 90 ml water, adjusting the pH to 2.5 with HCl, and adding water to a final volume of 100 ml. Aliquots were stored at -25 °C.

**GTC** (6 M) was prepared by dissolving 7.09 g guanidinium thiocyanate in 4.28 ml water. The vial was capped and the mixture was shaken until everything was dissolved. The vial was wrapped in aluminum foil to prevent exposure to daylight and stored at room temperature.

**HBS** 7.3 contained 150 mM NaCl, 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), adjusted to pH 7.3 with NaOH. When stated, either 2 mM CaCl$_2$ (for calmodulin interaction studies) or 2 mM EGTA (for control experiments without Ca$^{2+}$) was added.

**HEPES** (nominally 1 M, pH 9.6) was prepared by first dissolving 7.150 g
HEPES acid in exactly 30 ml, yielding an exactly 1 M solution of HEPES acid. After adjusting the pH to 9.6 with 20% NaOH (w/v, 5 M), the real HEPES concentration was well below 1 M, only the nominal concentration value was stated as "1 M". Aliquots were stored at -25 °C.

PBS 7.3 (phosphate-buffered saline, pH 7.3) contained 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, yielding pH 7.3.

Pepsin (2 mg/ml, pH 2.5) was prepared by dissolving pepsin from porcine gastric mucosa (Sigma P7012) in 1 M glycine buffer (pH 2.5). Typically, 5 mg pepsin was dissolved in 2.5 ml glycine buffer and stored at 4 °C for up to 2 weeks.

TCEP hydrochloride (100 mM) stock solution was prepared by dissolving tris(2-carboxyethyl)phosphine hydrochloride (286.65 g/mol) in water at a final concentration of 28.7 mg/ml. Aliquots were stored at -25 °C and thawed immediately before usage.

All reagents were purchased from Sigma-Aldrich (Vienna, Austria) unless otherwise specified.

4.1.2 Peptides and Proteins

Biotinylated BSA was prepared by reacting BSA (10 mg/ml) in buffer 8.3 (100 mM NaCl, 35 mM boric acid, adjusted to pH 8.3 with NaOH) with a 10-fold molar excess of Biotin-LC-NHS (Thermo Scientific, USA), followed by gel filtration in PBS 7.3 on Superdex 200 (10 x 300 mm) at a flow rate of 0.5 ml/min. The monomer peak was harvested, the concentration was calculated from the absorbance A₂₈₀, and the sample was diluted to 1 mg/ml. Aliquots were stored at -25 °C.

Biotinylated calmodulin was prepared by dissolving 1.0 mg CaM (59.5 nmol) in 500 μl PBS (pH 7.5) and adding 12 μl DMSO containing 10 mM (120 nmol) Biotin-LC-NHS (Thermo Scientific, USA). After 1 h, biotinylated CaM was separated from unreacted Biotin-LC-NHS by loading the reaction mixture on a PD-10 size exclusion column (GE Healthcare, USA) and eluting with HBS 7.3 as described.[82] The concentration was calculated from the absorbance A₂₈₀, and the sample was diluted to 30 μM with HBS 7.3. 10 μl aliquots were stored at -25 °C and thawed immediately before usage.

BSA (bovine serum albumin, fatty acid-free, fraction V, Roche, USA) was dissolved in PBS 7.3 at a concentration of 20 mg/ml and gel filtered on Superdex 200 (10 x 300 mm) at a flow rate of 0.5 ml/min. The monomer peak was harvested, the concentration was calculated from the absorbance A₂₈₀, and the sample was diluted with PBS 7.3 to a BSA concentration of 1 mg/ml. Aliquots were stored at -25 °C.
**Materials**

**Calmodulin** (human CaM, recombinantly produced) was purchased from Enzo Life Sciences, USA (BML-SE325-0001), dissolved in HBS 7.3 without Ca$^{2+}$ and stored in 40 μM aliquots at -25 °C. Before usage, one portion was thawed and diluted to the desired concentration with HBS 7.3 (with 2 mM Ca$^{2+}$ or 2 mM EGTA as stated).

**Cam-Alexa647** was prepared by dissolving 1.0 mg CaM (59.5 nmol) in 500 μl PBS (pH 7.5) and adding 7.5 μl DMSO containing 24.66 mM (185 nmol) Alexa647-NHS (Thermo Scientific, USA). After reacting for 1 h at RT in the dark, labeled CaM was separated from unreacted dye by loading the reaction mixture on a PD-10 size exclusion column (GE Healthcare, USA) and eluting with HBS 7.3 as described.[82] The concentration was calculated from the absorbance $A_{280}$, and the sample was diluted to 30 μM with HBS 7.3. 30 μM aliquots were stored at -25 °C and thawed immediately before usage.

**Orai loop 2 peptides:**
Orai1 loop2 (aa136-186 with mutation C143G; FALMISTGILPNIEAVSNHNLNSVKEPHERMHRIELAWAFSTVIGTL) and Orai3 loop2 (aa111-161 with mutation C118G; FALMVSTGILPNIEAVSNHNLNSVHQSPHQRHLRYVELAWGFLSTGTGFL) were custom-synthesized with >95% purity by Synpeptide ([http://www.synpeptide.com](http://www.synpeptide.com)).

**Orai N-terminal calmodulin-binding domain (CMBD) peptides:**
The N-terminal CMBDs of human Orai170−91 (SMQALSWRKLYLSRAKLKA-SSR), Orai169−88 (HSMQALSWRKLYLSRAKLKA) and Orai346−66 (LRALSWRLYLSRAKLKASSR) were custom-synthesized with a C-terminal cysteine for site-specific coupling by DgPeptides ([http://www.dgpeptides.com](http://www.dgpeptides.com)) with >95% purity. For the AFM experiments with Orai loop2, a shorter N-terminal Orai segment (Orai179−91) was custom-synthesized with a C-terminal cysteine by Synpeptide.

The CMBD of smMLCK from *gallus gallus* ([142]) (RKWQKTGHAVRAIGRLSS) was custom-synthesized with a C-terminal cysteine for site-specific coupling by Synpeptide with >95% purity.

Additionally, Orai170−91, Orai169−88, Orai346−66, Orai352−66 and smMLCK CMBD were custom-synthesized without the C-terminal cysteine by Synpeptide with >95% purity.

For dimerization experiments, CMBD Orai170−91 and smMLCK CMBD were custom-synthesized with a C-terminal GGGGC-spacer by Synpeptide with >95% purity.

All peptides were dissolved in DMSO at a concentration of 20 mg/ml and stored in aliquots at -25 °C. Exact concentrations were determined from an aliquot which had been diluted with HBS 7.3 by absorption at 280 nm, using UV/VIS spectroscopy.

**Streptavidin** (Sigma S4762) was dissolved in 150 mM NaCl solution at a concentration of 1.20 mg/ml (20 μM) and stored at -25 °C. Before use, one
portion was thawed and diluted to 2 μM streptavidin and 60 μl was injected in the SPR device at a flow of 20 μl/min.

4.2 METHODS

4.2.1 Microscale Thermophoresis

Binding of CaM to different CMBDs was studied in solution using microscale thermophoresis.[105] Alexa647-labeled CaM (final concentration of 1 nM) was mixed with different peptide concentrations ranging from 0.305 nM to 10 μM (16 serial dilutions by a factor of two) in HBS 7.3 including 1 mg/ml BSA and either 2 mM Ca²⁺ or 2 mM EGTA. The respective mixtures were transferred into standard treated capillaries (NanoTemper Technologies, Germany) and measured using 80% LED and 40% IR-laser power with 30 s laser-on times and an excitation wavelength region of 605 – 645 nm. For the interaction of CaM with dimerized Orai1 CMBD (or with dimerized smMLCK peptide), the peptide concentrations were varied between 0.7 pM and 10 μM (16 serial dilutions by a factor of three) and CaM was kept constant at a concentration of 0.5 nM while using 95% LED power. All experiments were performed using a MST Monolith NT.115 instrument (NanoTemper Technologies, Germany) with the blue/red LED filter combination.

4.2.1.1 Microscale Thermophoresis Data Analysis

Data analysis was performed as explained in Sec. 3.1.4 and previously described in detail.[193] Both the temperature jump, which is determined by inherent properties of the fluorophore, as well as the thermophoretic motion of the molecules due to increasing temperature were included in signal analysis. The change in normalized fluorescence \( F_{\text{norm}} \) (ratio between initial fluorescence and fluorescence after thermodiffusion) linearly reports the fraction of bound molecules and was thus analyzed as a function of the concentration of the titrated binding partner. According to the law of mass action (Eq. 3.18), the equilibrium dissociation constant \( K_d \) was determined by solving for the bound fraction of fluorescently-labeled CaM, \( \text{CaM}_{\text{bound}} \) (based on Eq. 3.19)

\[
\text{CaM}_{\text{bound}} = \frac{[\text{CaM}\cdot\text{CMBD}]}{[\text{CaM}]} = \frac{[\text{CMBD}] + [\text{CaM}] + K_d - \sqrt{([\text{CMBD}] + [\text{CaM}] + K_d)^2 - 4[\text{CMBD}][\text{CaM}]}}{2[\text{CaM}]}
\]

and fitting \( F_{\text{norm}} \) to this equation. \( F_{\text{norm}} \) was plotted in per mil (‰) against the concentration of the titrated binding partner \( A \) (i.e., the respective CMBD) on a semi-logarithmic scale, whereby \( F_{\text{norm}} \) of the unbound state was subtracted as a baseline value to yield \( \Delta F_{\text{norm}} \) as depicted throughout this thesis.
4.2.2 Surface Plasmon Resonance

Cleaning of bare glass chips and evaporation of a 3 nm chromium and 41 nm gold layer, as well as cleaning of the gold surface was performed as described.[83] All experiments were performed using a mixed self-assembled monolayer (SAM) of alkanethiol derivatives as previously described,[170] except that 20% of the SAM components carried a desthiobiotin group instead of biotin. This exchange allowed for repeated chip functionalization with streptavidin and different biotinylated bait molecules (cf. Sec. 4.2.2.2).[114] The sensor chips were mounted on the chip holders with non-permanent double-sided adhesive tape and inserted in a BIAcore® X device (BIAcore, GE Healthcare, USA) for SPR measurements. Freshly degassed and filtered (0.45 μm) buffer (HBS 7.3) was constantly run over the chip surface at a typical flow rate of 20 μl/min, unless stated otherwise. The resonance angle was recorded at a 1 Hz sampling rate in both flow cells and expressed in resonance units (1 RU = 0.0001°).

4.2.2.1 Immobilization of CaM and CMBDs

Sensors chips with desthiobiotin-terminated SAM[114] were mounted in the SPR device and pretreated with two alternating injections of BSA (1 mg/ml) and 0.5% SDS. After injection of streptavidin (2 μM, 3 min), biotinylated CaM (2 μM) was injected for 3 min to result in a dense CaM layer (Fig. 4.1a).

![Figure 4.1: Scheme of the immobilization strategy for coupling (a) CaM or (b) CMBD peptides to the chip surface via a pre-immobilized streptavidin monolayer.](image)

Alternatively, the respective CMBD peptides were immobilized on a streptavidin monolayer by first injecting biotin-PEG11-maleimide to generate free maleimide groups for subsequent site-specific coupling of cysteine-terminated CMBD peptides (Fig. 4.1b). Coupling of CMBDs (0.5 mM) was performed at a low flow rate of 2 μl/min to obtain a long contact time of typically 30 min.

4.2.2.2 Regeneration of Desthiobiotin Chips

After one set of measurement series, the SPR sensor chip with a mixed desthiobiotin-SAM was regenerated by four consecutive injections (3 min...
each) of biotin (200 mM), guanidinium thiocyanate (GTC, 6 M), pepsin (2 mg/ml), and SDS (0.5%) (Fig. 4.2). Subsequently, streptavidin and either biotinylated CaM or biotin-PEG11-maleimide and CMBD peptides were injected for a new measurement series. A comprehensive description and background information about the regenerative chip surface used for the SPR measurements in this thesis is given in Traxler et al. (enclosed in the Appendix on page 125).

![Figure 4.2: Protocol for the removal of streptavidin and a biotinylated bait from a mixed desthiobiotin-SAM as described in Ref. [114].] Biotin (200 mM) was dissolved in water, using Tris base for the adjustment of pH 8 - 8.5. Guanidinium thiocyanate (GTC) was dissolved in water at a concentration of 6 M (62.4%, w/w). Pepsin was dissolved in 1 M glycine (pH 2.5). All injections were performed for 3 min.

### 4.2.2.3 Surface Plasmon Resonance Data Analysis

For determination of kinetic rates and equilibrium constants, the experimentally determined binding curves were fitted with appropriate models, taking into account mass transport limitations. The respective association and dissociation periods were evaluated using double referencing. For binding of CaM to surface-immobilized Orai CMBDs, the “bivalent analyte model”, which assumes a successive two-step binding mechanism between CaM and two adjacent Orai peptides, was employed. For all other interactions, the simple “1:1 Langmuir binding model” was used. The corresponding reaction equation for bivalent CaM binding according to the bivalent analyte model is given as

\[
A + 2B \xrightleftharpoons[k_{d,1}]{k_{a,1}} AB + B \xrightleftharpoons[k_{a,2}]{k_{d,2}} ABB
\]  

(4.2)
For the sake of clarity, $A$ stands for calmodulin and $B$ represents Orai CMBDs on the sensor surface. The kinetic rate constants are described by the following differential equations:

\[
\frac{d[B]}{dt} = -(2k_{a,1} \cdot [A][B] - k_{d,1} \cdot [AB]) - (k_{a,2} \cdot [AB][B] - 2k_{d,2} \cdot [ABB]) \quad (4.3)
\]

\[
\frac{d[AB]}{dt} = (2k_{a,1} \cdot [A][B] - k_{d,1} \cdot [AB]) - (k_{a,2} \cdot [AB][B] - 2k_{d,2} \cdot [ABB]) \quad (4.4)
\]

\[
\frac{d[ABB]}{dt} = k_{a,2} \cdot [AB][B] - 2k_{d,2} \cdot [ABB] \quad (4.5)
\]

The factor of two in Eqs. 4.3 - 4.5 is introduced because the first binding step can occur to either of the two binding sites of CaM, while the second one can only occur to the remaining unoccupied one.\[39\] In the case of mass transfer limitation, the concentration of injected CaM [A]_{solution} is different from the concentration at the surface [A] as diffusion of CaM from the bulk solution to the surface is governed by the mass transfer coefficient $k_t$, yielding

\[
\frac{d[A]}{dt} = k_t \cdot ([A]_{solution} - [A]) - (2k_{a,1} \cdot [A][B] - k_{d,1} \cdot [AB]) \quad (4.6)
\]

All evaluations were done using BIAevaluation 3.2 software (BIAcore, GE Healthcare, USA).
4.2.3 **AFM Single Molecule Recognition Force Spectroscopy**

### 4.2.3.1 Aminofunctionalization of Silicon Substrates and Cantilever Tips

Substrate surfaces and cantilever tips were modified prior the biological functionalization to generate free amino groups. Polished silicon wafers were cut into pieces of ~20 x 20 mm and cleaned twice by ultrasonication in ethanol p.a. for 10 min. After pre-oxidizing the silicon chips using air plasma (80 W, 4 min), the chips and cantilever tips were aminofunctionalized using (3-aminopropyl)triethoxysilane (APTES) in gas phase as described (cf. Sec. 3.3.3.1).[61] Briefly, the chips and tips were washed 3 x 10 min in chloroform and placed in a desiccator containing one tray with 60 µl APTES and one tray with 20 µl triethylamine under argon atmosphere. After 2 h of incubation, the trays were removed and the desiccator was flooded with argon for 5 min. After the following “curing” phase (48 - 72 h), the silicon chips and cantilever tips were immediately processed further (see below), or stored under argon for maximum one week.

### 4.2.3.2 Tip Functionalization with CaM

A polyethylene glycol (PEG) linker was attached to aminofunctionalized AFM cantilever tips (Si₃N₄/MSCT, Bruker, USA) by incubating the cantilevers for 2 h in chloroform containing 1 mg/ml NHS-PEG₁₈-acetal and 0.5% (v/v) triethylamine as base.[222] After washing 3 times in chloroform (10 min each) to remove the excess of unbound linker molecules, the acetal group of the linker was converted into an aldehyde residue by incubation in 1% v/v citric acid (10 min), followed by washing in water (3 x 5 min). CaM was coupled to the aldehyde residue of the free end of the PEG linker (Fig. 4.3a) by incubating the tips for 1 h in 0.2 mg/ml CaM solution containing 20 mM NaCNBH₃,[222] 10 min before the reaction was stopped, 5 mM ethanolamine was added to saturate the remaining aldehyde with amino groups.[222] Subsequently, the tips were washed in HBS 7.3 (3 x 5 min) and stored under HBS 7.3 at 4 °C for maximum 5 days.

### 4.2.3.3 Tip Functionalization with Orai CMBDs

APTES-functionalized cantilever tips were incubated for 2 h in chloroform containing 1 mg/ml NHS-PEG₂₇-maleimide (Polypure, Norway) and 0.5% (v/v) triethylamine as catalyst. After washing three times in chloroform (10 min each), the tips were incubated overnight under argon in HBS 7.3 containing ~0.4 mM Orai1 CMBD or Orai3 CMBD, 1 mM EDTA, 5 mM TCEP and 5 mM HEPES (Fig. 4.3b). Subsequently, the tips were washed in HBS 7.3 (3 x 5 min) and stored under HBS 7.3 at 4 °C for maximum 5 days.

### 4.2.3.4 Tip Functionalization with Orai Loop2

A polyethylene glycol (PEG) linker was attached to aminofunctionalized AFM cantilever tips (Si₃N₄/MSCT, Bruker, USA) by incubating the cantilevers for
4.2 METHODS

Surface Functionalization with CaM or Orai CMBDs

CaM, the Orai1 or Orai3 CMBD peptides, or the shorter Orai179-91 peptide were coupled to the amino-groups on the surface of aminofunctionalized silicon chips via either NHS-PEG18-acetal or NHS-PEG27-maleimide linkers as described for AFM tips above (Fig. 4.3 or Fig. 4.4, respectively). Functionalized silicon surfaces were stored under HBS 7.3 at 4 °C for maximum 5 days.

Figure 4.3: AFM cantilever tip and surface functionalization with CaM and Orai CMBD peptides. Si₃N₄ cantilevers and silicon chips were first aminofunctionalized by gas-phase reaction with APTES. (a) A heterobifunctional polyethylene glycol (PEG) linker (~6 nm) was conjugated to tip-bound amino groups, followed by attachment of CaM to the free terminal aldehyde group. CMBD peptides with a C-terminal cysteine were attached similarly to the silicon chips using a heterobifunctional crosslinker with a maleimide group (~9 nm). (b) Reversed coupling scheme for tethering of CMBDs to the cantilever tip and of CaM to the silicon chips.

2 h in chloroform containing 1 mg/ml NHS-PEG₁₈-acetal and 0.5% (v/v) triethylamine as base.[222] After washing 3 times in chloroform (10 min each) to remove the excess of unbound linker molecules, the acetal group of the linker was converted into an aldehyde residue by incubation in 1% v/v citric acid (10 min), followed by washing in water (3 x 5 min). Orai1 loop2 or Orai3 loop2 was dissolved in DMSO, 20x diluted with HBS 7.3, and then coupled to the aldehyde residue of the free end of the PEG-linker by incubating the tips for 1 h in 5 mg/ml peptide solution containing 20 mM NaCNBH₃ (Fig. 4.4).[222] 10 min before the reaction was stopped, 5 mM ethanolamine was added to saturate the remaining aldehyde with amino groups.[222] Subsequently, the tips were washed in HBS 7.3 (3 x 5 min) and stored under HBS 7.3 at 4 °C for maximum 5 days.
4.2.3.6 Force Spectroscopy Experiments

All force measurements were carried out at room temperature by using a PicoPlus 5500 AFM (Keysight Technologies, USA) and cantilevers with a nominal spring constant of 0.03 N/m. Exact spring constants were determined using the thermal noise method.[99] The cantilever with functionalized tip was moved towards the surface with the respective receptor molecules until a certain preset deflection (i.e., force limit) of the cantilever was reached. The cantilever deflection was continuously recorded by a laser beam focused on the cantilever’s back side and plotted over the tip-surface distance. The deflection was converted into corresponding force values ($F$) according to Hooke’s law ($F = k_{c} \cdot \Delta z$, with $k_{c}$ being the cantilever spring constant and $\Delta z$ the cantilever displacement). Upon binding of the tip-tethered molecule to the surface-immobilized one, a pulling force was developed during the upward movement of the cantilever. At a critical force (i.e., unbinding force), the formed receptor-ligand complex dissociated and the cantilever jumped back into its neutral position.

More than 1000 force-distance curves with at least three functionalized tips were performed and the average binding probability was calculated. The binding probability was defined as the fraction of force curves showing a specific unbinding event; e.g., if 100 from 1000 measured curves show an unbinding event, then the binding probability equals 10%. The x-y position of the tip was varied every ~50 to 100 FDCs, to avoid position-related artifacts. The specificity of the interaction was proofed by comparing the binding probability in presence of 2 mM Ca$^{2+}$ with the one when measuring in 2 mM EGTA-containing buffer. An unpaired t-test was used for evaluating the statistical significance between changes in binding probability.

Dynamic force spectroscopy (DFS) measurements were performed by varying the force rate with which the molecular bond was loaded (i.e., loading rate $r$, product of pulling velocity $v$ and effective spring constant $k_{eff}$). For
this, the pulling velocity was varied between 50 and 8000 nm/s, resulting in loading rates from \( \sim 10^2 \) to \( \sim 10^5 \) pN/s. >1000 force-distance curves per pulling velocity were recorded.

### 4.2.3.7 Cantilever Spring Constant Determination

Due to variations during the manufacturing process of cantilever chips, the nominal values for both the spring constant and the resonance frequency can vary significantly between single cantilevers, even between these from the same batch or wafer. Therefore, the individual spring constant of each cantilever was determined experimentally after the experiments have been performed. All spring constants were determined using the “Thermal K” option from Keysight Technologies, USA, which is based on the thermal noise method.[99]

Cantilever chips were transferred from HBS 7.3 to water, air-dried on a layer of parafilm® and mounted onto the AFM scanner. To determine the sensitivity of the system, the tip was approached to a silicon chip and ~20 FDCs were recorded at a rate of 1 Hz and a deflection limit of 1 V. After determining the sensitivity, the thermal cantilever oscillations in air were recorded and transformed to the frequency domain using Fourier transformation to yield a power spectral density plot from which the spring constant could be determined.[99]

### 4.2.3.8 Force Spectroscopy Data Analysis

Data analysis was done using MATLAB (The MathWorks, USA). Force curves were analyzed as described by Baumgartner et al.[12]. Unbinding events were identified during cantilever retraction by a typical non-linear increase in force due to stretching of the elastic PEG-linker, followed by an abrupt return (jump) to the resting position of the cantilever (baseline). The height of this jump is directly reflecting the unbinding (rupture) force and the slope at time of rupture represents the spring constant of the PEG-linker \( k_{\text{linker}} \) (Fig. 4.5a).

The effective spring constant of the system \( k_{\text{eff}} \) was calculated according to \( k_{\text{eff}} = (k_{\text{linker}}^{-1} + k_{c}^{-1})^{-1} \) with \( k_{c} \) being the cantilever spring constant as determined by the thermal noise method.[99] The loading rate of each individual force-distance curve was calculated by multiplying the effective spring constant with the pulling velocity. As an empirical estimate for the force distribution, the probability density function (pdf) of all unbinding events per pulling velocity was calculated (cf. Eq. 3.59 on page 41). For this purpose, single Gaussian functions of unitary area were calculated from the mean values and the standard deviations of each unbinding force value. The Gaussians were added up and then normalized to yield the empirical pdf. The pdf can be seen as the equivalent of “continuous” histograms that takes into account the uncertainties in determining the unbinding force, and therefore it has the advantage that the values are weighted by their reliability. Furthermore, by use of the continuous pdfs instead of the discontinuous histograms the problem of data binning is avoided (cf. Sec. 3.3.3 on page 38).
Figure 4.5: Example of a force-distance curve and the extraction of unbinding forces.
(a) The functionalized AFM cantilever tip gets approached to the surface with the immobilized binding partner (trace, black line). When the tip comes in contact with the surface (contact point), the tip gets bent upwards, resulting in an increase in force until a certain force value (force limit) is reached. When subsequently withdrawing the cantilever tip from the surface (retrace, green line), an interaction between the tip-tethered molecule and the surface-bound molecule will cause a stretching of the PEG-linker and downward bending of the cantilever. The force, at which the complex dissociates (bond rupture) and the cantilever jumps back to its resting position directly reflects the unbinding force of the respective interaction. Force-distance curves where no interaction occurs show very similar trace and retrace curves with no characteristic nonlinear stretching of the PEG-linker (inset). (b) As an empirical estimate, the probability density function (pdf) of all unbinding forces is calculated and allows for determination of the most probable unbinding force without the problem of data binning as in conventional histograms (gray bars).

FDCs were analyzed using in-house developed MATLAB routines, which allowed to select single unbinding events and extract the unbinding forces, unbinding lengths and the effective spring constant. The binding probability, distributions of unbinding force, unbinding length and the effective spring constant $k_{\text{eff}}$, as well as an overlay of all FDCs and a plot of unbinding force vs. unbinding length were generated (Fig. 4.6) and used for further data analysis as described.

For determination of the parameters that characterize the molecular transition during the dissociation process (i.e., the dissociation rate constant $k_d$ and the distance from the energy minimum to the maximum $x_\beta$, cf. Sec. 3.3.3 on page 38), the most probable unbinding forces per loading rate were plotted over the logarithm of the loading rate, to obtain the so-called loading rate-dependence plot (cf. Fig. 3.12 on page 42).

The most probable unbinding force $F^*$ was determined by fitting a Gaussian function to the force pdf, and the loading rate $r$ was calculated by multiplying the effective spring constant $k_{\text{eff}}$ with the tip retraction velocity $v$. Based on the theory that a single sharp energy barrier is crossed in the thermally activated regime and that rebinding can be neglected, the most probable unbinding force is expected to rise linearly with logarithmically increasing loading rate.[13, 66]
Figure 4.6: Overview of the extracted parameters of an exemplary force spectroscopy dataset containing 1026 individual FDCs at a given tip retraction velocity. The panels (from left to right) show the distribution of unbinding forces, the distribution of unbinding lengths (both as pdf), a scatter plot of unbinding forces vs. unbinding lengths, the distribution of the effective spring constant \( k_{\text{eff}} \) (as pdf), the distribution of unbinding events (i.e., the number of unbinding events per curve), and an overlay of all FDCs.

### 4.2.3.9 Rupture of Two Parallel Bonds

For the interaction between tip-tethered CaM and surface-immobilized Orai CMBDs, two distinct peaks in the force pdfs were observed, most likely arising from bivalent binding of both CaM lobes to two adjacent Orai CMBDs. Due to this parallel bond architecture, the force load is shared between the two bonds. As each lobe can bind independently to one Orai CMBD, the association, as well as dissociation process, happen in an uncorrelated manner without mechanical coupling between the two bonds, meaning that each bond can fail independently. Upon failure of one bond, the force load gets redistributed to the surviving bond. As this redistribution leads to an abrupt increase in force load for the surviving bond and therefore reduces its lifetime to nanosecond levels,[175] the rupture of the second bond occurs very shortly after the first one. Due to the finite bandwidth of the AFM, the sequential bond breakages are registered as a single rupture event with almost double unbinding force.[175] As shown by Williams[223] and experimentally proved by other groups,[205, 204, 175], the force-induced rupture of multiple identical and uncorrelated bonds can be described as a Markovian sequence. The most probable unbinding force \( F^* \) and measured loading rate \( r \) hereby scale with the number of involved bonds \( N_B \) (=2) as

\[
\begin{align*}
    r &= k_d \frac{k_B T}{x_\beta} \left[ \sum_{l=1}^{N_B} \frac{1}{l^2 e^{-\frac{r^*}{k_B T}}} \right]^{-1}
\end{align*}
\]  

(4.7)
where \( k_d \) and \( x_\beta \) are the respective parameters derived from the single bond analysis. Accordingly, the dissociation rate \( k_{d,N_\beta} \) for contemporaneous rupture of two bonds is calculated as

\[
k_{d,N_\beta} = k_d \left[ \sum_{i=1}^{N_\beta} \frac{1}{I_i} \right]^{-1}
\]  

(4.8)
Dimerization of Orai\textsubscript{1} and smMLCK CMBDs

For dimerization of Orai\textsubscript{1} or of smMLCK, both CMBDs were synthesized with an additional C-terminal GGGGC-spacer (Orai\textsubscript{1}: SMQALSWRKLYL-SRAKLASSRGGGGC, smMLCK: RKWQKTGHAVRAIGRLSSGGGC). The C-terminal cysteines allowed for linkage via disulfide bond formation (Fig. 4.7). The four glycine residues were introduced to mimic the distance between two Orai N-termini in the hexameric channel formation \textit{in vivo} (cf. Fig. 2.7 on page 15).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure47.png}
\caption{Dimerization of Orai\textsubscript{1} and smMLCK CMBDs and separation from non-dimerized monomers by size exclusion chromatography. (a) Orai\textsubscript{1} and smMLCK CMBD peptides with an additional GGGGC spacer were stirred for 48 h at RT to allow linkage by formation of disulfide bonds. (b) Illustration of the dimerized GGGGC linker with a resulting extended length of approx. 30 Å. (c) Chromatogram of the reaction mixture with detection of absorbance at 280 nm. The gray area shows the fraction of dimerized Orai\textsubscript{1} CMBDs which was collected. When 50 mM 2-mercaptoethanol was added to the reaction mixture and the latter was loaded on the column, the first shoulder disappeared, proving the successful reduction of disulfide bonds. Aqueous 0.1\% acetic acid was used as running buffer.}
\end{figure}

0.5 mg of Orai\textsubscript{1} or of smMLCK was dissolved in 50 μl DMSO each (final concentrations of 3.4 mM and 4.1 mM, respectively) and the solution was
gently stirred for 48 h at RT to achieve disulfide bond formation through air oxidation. Afterwards, the respective solution was diluted with HBS 7.3 to a final volume of 500 μl. Dimers of Orai1 and smMLCK were separated from monomers by size exclusion chromatography on a Superdex Peptide HR 10/300 column (GE Healthcare, USA) with aqueous 0.1% acetic acid (pH 3.2) as running buffer. The exact peptide concentrations in the respective fractions were determined by absorption at 280 nm using UV/VIS spectroscopy. To verify the supposed absorption peak of the dimer fraction in the chromatogram, 0.5 mg of Orai1 or smMLCK was dissolved in 50 μl DMSO and 2-mercaptoethanol was added at a final concentration of 50 mM to ensure a complete reduction of disulfide bonds. After 1 h of incubation time and performing the purification run as before, the first absorption peak disappeared, proofing the successful cleavage of the dimers and thus the applicability of the air oxidation method for linking of two cysteine residues (Fig. 4.7c).
Part III

RESULTS AND DISCUSSION

This part comprises the main results obtained within the experimental work for this thesis, along with a discussion and the main drawn conclusions. A concluding discussion and an outlook are presented separately in the last chapter of this part. Contributed work to various self-contained studies is enclosed in the appendix.
RESULTS AND DISCUSSION

The focus of this thesis lies primarily on the in-depth characterization of the interaction mechanism between calmodulin (CaM) and N-terminal Orai fragments, which is supposed to play a role for the Ca$^{2+}$-dependent inactivation (CDI) of Orai channels. The results of this study have been recently published.$^{[210]}$

In addition, the intramolecular interplay between the Orai N-terminus and the cytosolic loop connecting its second and third transmembrane region was investigated at the single-molecule level. In the following, the results of those studies will be presented. As outlined in the Preface and implemented in the Introduction and the Experimental Section, also the structure of this chapter will be based on the experimental methods used (MST, SPR, and AFM).

5.1 INTERACTION BETWEEN CALMODULIN AND ORAI

Although the exact role and contribution of CaM to CDI of Orai channels in vivo remains unclear (cf. Chap. 1 on page 1), in vitro experiments evidenced specific binding between an isolated N-terminal CMBD of Orai1 and CaM.$^{[131]}$ The crystal structure of this complex revealed an unusual extended conformation of CaM with only its C-terminal lobe being associated with one Orai1 CMBD (Fig. 1.1 on page 2).$^{[131]}$ Parallel pull-down and isothermal titration calorimetry (Fig. 1.2 on page 3) experiments, however, suggested a bivalent interaction mode with both the C- and N-terminal lobe of CaM binding one Orai1 CMBD peptide.$^{[131]}$ Based on the motivation as stated in Chap. 1 on page 1, we examined the interaction between CaM and the CMBDs of human Orai1 and Orai3 (including fragments with slightly varying lengths), the putative CMBD of dOrai from drosophila melanogaster, as well as the CMBD of smMLCK (for an overview of the used fragments see Fig. 5.1).

5.1.1 Microscale Thermophoresis

For equilibrium measurements by MST, a constant concentration of 1 nM CaM (labeled with Alexa647) was titrated with the respective CMBD in different capillaries, ranging from concentrations between 0.305 nM to 10 µM.

$^{*}$ The following chapter 5.1 is to a great extent identical to the published article in Angew. Chem. Int. Ed.$^{[210]}$. Only minor adaptions and additions were introduced to facilitate the implementation of the Supporting Information of the article into the continuous flow of the chapter. As the original version of the article$^{[210]}$ was written by myself and all experimental work reported therein was performed by myself, it would have been neither meaningful nor possible to rewrite the below chapter 5.1 with completely different wording.
The fluorescence intensity of one spot in each capillary was measured before, during, and after this spot was heated with an IR-laser by 3 - 4 K (cf. Fig. 3.1 on page 18). During heating (typically 30 sec), the initially homogenously distributed molecules diffused out of the heated spot. As the heat-induced depletion of fluorophores is altered by binding of a non-labeled ligand (i.e., the respective CMBD; cf. Fig. 3.3 on page 22), the normalized fluorescence changes $\Delta F_{\text{norm}}$ were correlated with the CMBD concentration, and thus a typical binding curve was obtained.

Figure 5.1: Schematic illustration of the Orai protein and the aa sequences of different Orai calmodulin-binding domains (CMBD). The CMBDs are located in the highly conserved N-terminal cytosolic part directly before the first transmembrane region. Shown are the different fragments used in this study: hOrai170−91 and hOrai346−66 are the CMBDs of the two human isoforms Orai1 and Orai3, respectively (referred to as Orai1 and Orai3 CMBD). Both sequences just differ in 3 aa as highlighted in red. For comparison with previously published affinity values, a shorter version of hOrai1 CMBD consisting of aa69−88 (hOrai69−88) was examined, too. Additionally, the interaction of CaM with the putative CMBD of drosophila melanogaster (dOrai, aa142−163) was characterized, as well as the interaction with a truncated version of hOrai3 lacking the first six amino acids (hOrai352−66). Empty yellow circles in the sequences of hOrai69−88 and hOrai352−66 indicate the lacking (or additional) aa in relation to the full sequence of Orai1 or Orai3 CMBD, respectively. Green marked aa indicate the difference in sequence between the putative CMBD of dOrai from hOrai70−91.

For the interaction between CaM and Orai1, two distinct binding steps were identified (Fig. 5.2a): one with a high affinity ($K_{d,1} \approx 4 \text{ nM}$), and
a second one with a significantly lower affinity ($K_{d,2} \approx 1.1 \mu M$). The appearance of a fluorescence decrease in the first step and an increase in the second binding step are well compatible with the complex mechanism of thermophoresis.[105] In line with the study of Liu et al.[131], these two binding steps probably arise from the sequential binding of two Orai peptides to the two lobes of CaM (see pictogram in Fig. 5.2a). As expected, the $K_d$ value of the first step was in the same range as typical CaM targets, rather than $\approx 1 \mu M$ as previously reported for Orai1.[131] Our Orai1\textsubscript{70–91} segment was slightly different, but similar results as in Fig. 5.2a were also obtained with Orai1\textsubscript{69–88} that had been used in Ref. [131] (Fig. 5.2b).

Figure 5.2: MST equilibrium measurements of the interaction between CaM and two different versions of Orai1 CMBD. Alexa647-labeled CaM (1 nM) was titrated with the CMBD peptides of (a) Orai1\textsubscript{70–91} or (b) Orai1\textsubscript{69–88} from 0.3 nM to 10 \mu M peptide concentration. The change in the thermophoretic signal (plotted as change in normalized fluorescence $\Delta F_{\text{norm}}$) in presence (2 mM Ca\textsuperscript{2+}, filled squares) and absence of Ca\textsuperscript{2+} (2 mM EGTA, open squares) was monitored and fitted to Eq. 4.1 to yield the $K_d$ value of each binding step. Error bars represent the SD of each data point calculated from three independent thermophoresis measurements. The respective CMBD sequence is illustrated beside the graphs. The yellow spheres in (b) indicate differences in length (i.e., added or omitted amino acids) in relation to Orai1\textsubscript{70–91} (cf. Fig. 5.1). Figure adapted from [210].

In parallel, we performed the same experiments with the putative N-terminal CMBD of Orai3, which differs only in 3 aa positions from Orai1 as illustrated by the red marked aa residues in Fig. 5.1. Here, a very similar two-step behavior with two discrete affinities was found ($K_{d,1} \approx 9$ nM, $K_{d,2} \approx 0.5 \mu M$; Fig. 5.3a). When we used a truncated mutant of Orai3 CMBD lacking aa46-51, no binding of CaM was observed (Fig. 5.3b), in line with the reported loss of CDI as previously shown.[15] Given the fact that the sequence of Orai3 CMBD is only differing from the one of Orai1 CMBD by three aa at positions 46, 47 and 53 (cf. Fig. 5.1), the arginine at position 53 is the only difference between the deletion mutant Orai3\textsubscript{52–66} and the corresponding truncated version Orai1\textsubscript{77–91} (which has a lysine instead of the arginine at position 53). As both Orai1 and Orai3 show a two-step binding behavior with similar affinities, the loss of binding upon deletion of the first six aa is also likely to induce the same effect and abolish CaM-binding for Orai1 CMBD.
Figure 5.3: MST measurements of the interaction between CaM and two different versions of Orai3 CMBD. Alexa647-labeled CaM (1 nM) was titrated with the CMBD peptides of (a) Orai336–66 or (b) a truncated version of Orai3 CMBD lacking the first six aa, Orai352–66. A two-step binding behavior similar to that of Orai1 was observed for Orai3 CMBD. No binding to CaM was observed for Orai352–66. The red marked aa in (a) indicate the differences in relation to Orai1 CMBD, the yellow spheres in (b) indicate the N-terminal deletion of six aa. Figure adapted from [210].

The N-terminal segment directly before the first transmembrane region is not only very similar among the human isoforms Orai1, Orai2, and Orai3 (cf. Fig. 2.7 on page 15), but is also highly conserved from drosophila melanogaster to human Orai proteins (~77% sequence identity between dOrai142–163 and hOrai170–91, Fig. 5.1). Accordingly, performing the same MST experiments with the putative CMBD of dOrai revealed a two-step binding mechanism as seen for Orai1 and Orai3 CMBD, with very similar $K_d$ values (Fig. 5.4).

Figure 5.4: MST measurements of the interaction between CaM and the CMBD of dOrai. A two-step binding behavior as for Orai1 and Orai3 CMBD was observed, with affinities similar to those of Orai1. The green marked aa indicate the differences in relation to Orai1 CMBD. Figure adapted from [210].

For MST experiments, we statistically labeled CaM with the amino-reactive Alexa 647-NHS fluorophore (see Sec. 4.1.2 on page 50) with only 3-fold excess of dye to maximize the fraction of mono-labeled CaM while minimizing the
number of non-labeled molecules, which would bias the MST results. In order to rule out that the two-step binding pattern seen for all Orai CMBDs was induced by the statistical labeling of CaM, we measured the interaction between labeled CaM and the CMBD of smooth muscle myosin light chain kinase (smMLCK). Here, the crystal structure and biochemical measurements unambiguously showed a 1:1 binding mode. Accordingly, our data also indicated only one binding event, with an affinity of ~2.5 nM (Fig. 5.5), which is in good agreement with published values and similar to the $K_{d,1}$ values measured for the interactions between CaM and different CMBDs of Orai.

![Figure 5.5: MST measurements of the interaction between CaM and the CMBD of smMLCK. Only one binding step with a similar affinity as the high affinity binding step for Orai CMBDs was observed, indicating a 1:1 interaction as illustrated by the pictogram. Figure adapted from [210].](image)

It has to be pointed out, that in the absence of Ca$^{2+}$ (2 mM EGTA) no interaction of CaM was detected with any Orai CMBD (and smMLCK) peptide (open squares in Figs. 5.2 - 5.5), indicating specific Ca$^{2+}$-dependent binding. The results of the MST equilibrium measurements in solution clearly showed that one CaM molecule seems to interact with two separate Orai CMBD peptides (cf. Fig. 5.6). Based on the available crystal structure of dOrai[96], Orai1 and Orai3 are supposed to form hexamers in vivo, with the N-terminal strands forming parallel bundles of $\alpha$-helices (cf. Fig. 2.7 on page 15). For mimicking this configuration, we extended the CMBD peptides of Orai1 and smMLCK with a C-terminal GGGGC linker and induced dimerization by a disulfide bond between the C-terminal cysteines (cf. Sec. 4.2.4 on page 63). The resulting linker (~30 Å, Fig. 4.7 on page 63) is long enough to mimic the maximum distance between two opposite N-terminal helices in an Orai hexamer. In case of Orai, the two arms of the peptide dimer are expected to bind almost simultaneously to the two lobes of one CaM molecule, which should result in a single high-affinity binding step in MST (Fig. 5.7a). For smMLCK, the crystal structure indicates that CaM binds only a single target helix in a “wrap-around” fashion with 1:1 binding stoichiometry.[142] There-
Figure 5.6: Overview of MST measurements with various different Orai CMBDs as illustrated in Fig. 5.1. The two-binding step behavior was observed for all Orai CMBDs and the determined affinities were similar, with one high affinity binding step at \( \sim 10^{-9} \) M and a second step with significantly lower affinity at \( \sim 10^{-6} \) M. Truncated Orai1352–66 showed no interaction with CaM.

Therefore, dimerization of one smMLCK CMBD should hardly alter the binding mode and the affinity for CaM, as the second arm of the peptide dimer cannot interact with the same CaM molecule (Fig. 5.7b).

Indeed, our experiments revealed a single binding step (Fig. 5.8a), with a 10^2-fold lower \( K_d \) than any of the \( K_d \) values in two-step binding (Fig. 5.2a). In case of smMLCK, where CaM is known to wrap around a single target helix, no increase in affinity is expected and was indeed confirmed by the MST data (Fig. 5.8b); the moderate increase in \( K_d \) from \( \sim 3 \) nM to \( \sim 5 \) nM showed that dimerization caused even some perturbation of the normal 1:1 binding mode, in contrast to Orai1, where dimerization had decreased the \( K_d \) by a factor of 10^2.
5.1 Interaction between Calmodulin and Orai

Figure 5.7: Schematic illustration of the binding mechanism of Orai1 or smMLCK monomers and dimers.  
(a) Binding of Orai1 CMBD to CaM. One CMBD is able to bind to each of the two lobes of CaM, resulting in the association of two Orai1 CMBDs per CaM molecule. When dimerizing Orai1 CMBD, binding of one CMBD to the first lobe of CaM significantly enhanced the association of the second CMBD to the second lobe of CaM.  
(b) No increase in affinity was observed for smMLCK, as CaM binds only one smMLCK peptide.

Figure 5.8: MST measurement of the interaction between CaM and the dimerized CMBDs of Orai1 and smMLCK.  
(a) Only one binding step with an affinity of ~20 pM was observed when titrating CaM (0.5 nM) with the dimerized Orai1 CMBD from 0.7 pM to 10 μM peptide concentration.  
(b) In the case of dimerized smMLCK, no increase in affinity was observed. The respective sequences of the peptide dimer with the additional linker (purple letters) are shown below the binding curve. Image section from [210].
Figure 5.9: MST measurement of the interaction between CaM and the dimerized CMBDs of Orai1 and smMLCK in the presence of 50 μM TCEP. Reduction of the disulfide bond resulted in the cleavage of the peptide dimers and restored the two step-binding behavior for Orai1 CMBD (a), whereas it had very little effect in the case of smMLCK CMBD (b). The respective sequences of the peptide monomer with the additional linker (purple letters) are shown below the binding curve. Image section from [210].

As a control, the dimers of Orai1 and smMLCK were cleaved into monomers by disulfide reduction using 50 μM TCEP (cf. Sec. 4.2.4 on page 63) and thereby the behavior of the monomeric peptides was restored as expected (Fig. 5.9): The reduced Orai1 CMBD dimer was found to exhibit the same two-step binding behavior with similar affinities as observed with the monomer (Fig. 5.2a), and the binding curve of reduced smMLCK CMBD dimer did not change significantly and showed a very similar affinity as the monomer (Fig. 5.5).

Together, the MST data indicate that simultaneous bivalent binding of a linked pair or Orai1 CMBD segments to one CaM molecule results in a dramatic affinity increase (Fig. 5.8a) which far exceeds the affinity seen in the conventional 1:1 binding mode (Fig. 5.5).
5.1.2 Surface Plasmon Resonance

As the two lobes of CaM were found to have remarkably different affinities, we further tried to characterize the time-dependent process of bond formation and the transition from monovalent to bivalent interaction. For this purpose, we used the technique of SPR and measured the interaction between surface-immobilized CMBD peptides and soluble CaM that was injected into the flow cell. In the homogeneous MST assay, dimerization was the only option to mimic the oligomeric state of the Orai N-termini in vivo, where a bundle of parallel N-terminal segments protrudes into the cytosol.

![Figure 5.10: Typical SPR experiment with either CaM or the respective CMBD peptide in solution.](image)

(a) Orai1 CMBD was covalently immobilized via coupling of its C-terminal cysteine (cf. Sec. 4.2.2.1 on page 53) to the free maleimide group of biotinylated PEG-chains on top of a streptavidin monolayer. CaM was injected at different concentrations followed by a 1 min pulse of 0.5% SDS, which caused instantaneous detachment of CaM while leaving the peptide layer fully intact. In the second flow cell, the bare SAM-surface was used as reference, where no binding of CaM was detected. (b) Biotinylated CaM was immobilized directly on top of a streptavidin monolayer, followed by injections of Orai1 CMBD at different concentrations. Glycine (pH 2.5) was used as regeneration solution. Buffer blank injections were done in all measurements for double referencing.
For better reconstitution of the native situation, the CMBD segments were tethered at high lateral density to the flat surface of a sensor chip via flexible polyethylene glycol (PEG\textsubscript{11}) linkers, using streptavidin as mediator (pictogram in Fig. 5.10 and Fig. 4.1). In the reversed configuration, biotinylated CaM was immobilized similarly on top of a streptavidin layer. For all measurements a newly developed regenerative desthiobiotin-surface\textsuperscript{114} was used, which allowed for easy switching between different CMBDs and for many measurement cycles with the same sensor chip (for details see Appendix C on page 125).

As shown in Fig. 5.10, a typical SPR experiment included the immobilization of the ligand molecule (either the respective CMBD peptide or CaM), followed by multiple injections of the corresponding analyte at different concentrations. In between the injections, bound analytes were always removed with 0.5% sodium dodecyl sulfate (SDS) or 100 mM glycine (pH 2.5). Prior to kinetic experiments, the suitability of the respective regeneration method was extensively tested, in order to guarantee complete regeneration while leaving the ligand layer fully intact.

![Figure 5.11: Proof of the \(Ca^{2+}\)-dependency of the interaction between Orai\textsubscript{1} CMBD and CaM.\textsuperscript{210} Orai\textsubscript{1} CMBD was covalently immobilized as shown in Fig. 4.1 and CaM was injected at a concentration of 16 nM in presence of either 2 mM \(Ca^{2+}\) (black line) or 2 mM EGTA (gray line). Negligible binding was observed without any \(Ca^{2+}\) present, indicating that CaM is not able to associate with Orai\textsubscript{1} CMBD in its apo-conformation without complexed \(Ca^{2+}\) ions. Shown are always three averaged curves together with the standard deviation as error bars. The results for the other studied Orai CMBDs as well as for smMLCK were analogous to the curves shown here.](image)

Similarly as observed in solution by MST measurements, binding of CaM to CMBDs only occurred in the presence of \(Ca^{2+}\). In \(Ca^{2+}\)-free environment, even injections of CaM at high micromolar concentrations resulted in only negligible (unspecific) binding. Moreover, when CaM was allowed to bind to immobilized Orai or smMLCK CMBDs in \(Ca^{2+}\)-containing buffer, the pre-bound CaM layer was dissociated instantaneously upon chelation of \(Ca^{2+}\) by an injection of 2 mM EGTA (Fig. 5.11, red line).
For determination of kinetic and equilibrium constants, CaM was injected at different concentrations and bound CaM was always removed with 0.5% SDS (Fig. 5.10a).

When we immobilized Orai1 CMBD on the sensor surface and injected CaM at different concentrations (with regeneration steps in between, as shown...
78 results and discussion

Figure 5.13: Interaction of soluble CaM with the CMBD of (a) Orai1\textsubscript{70–91} or (b) Orai1\textsubscript{69–88} measured by SPR. Shown are the experimentally determined binding curves (colored lines) together with a global fit based on the bivalent analyte model (solid black lines), which accounts for a a bivalent interaction of CaM with two adjacent Orai1 CMBDs (see pictograms). Figure adapted from [210].

As the best fit was obtained with the bivalent analyte model, which assumes sequential association of the soluble analyte to two adjacent immobilized ligand molecules (and therefore results in two association and dissociation rate constants and two equilibrium constants for both binding steps), also the SPR data serve as indication for bivalent binding of CaM to two Orai CMBDs. As an advantage over MST, SPR experiments allowed for the determination of kinetic rate constants and therefore provided additional information about time-dependent bond formation and dissociation.

The first binding step gave a $K_d$ value of 4.7 nM, which was in good agreement with MST data (3.8 nM, Fig. 5.2a). Due to the SPR experimental setup with one binding partner being immobilized on a two-dimensional surface, the exact molar concentration of immobilized Orai1 CMBDs is difficult to determine. As the association of CaM to the second Orai peptide is dependent on the amount of immobilized Orai CMBDs (expressed in RU), the second association rate constant $k_{a,2}$ has the unit (RU$^{-1}$s$^{-1}$) instead of the conventional...
unit (M$^{-1}$s$^{-1}$), which makes a quantitative comparison of $K_{d,1}$ (unit M) and $K_{d,2}$ (unit RU) impossible.

As in solution, SPR experiments with the shorter Orai1 CMBD (Orai$^{169–88}$) yielded very similar $K_d$ values with only slightly lower affinities for both binding steps (Fig. 5.13b). Similarly, when we immobilized the CMBD of Orai3 on the chip surface, the same pattern of binding curves was observed and similar affinities as for Orai1 were found (Fig. 5.14a). Injection of CaM upon immobilization of truncated Orai$^{352–66}$ did not result in a detectable change in refractive index, even at a very high concentration of 10 µM CaM no binding occurred (Fig. 5.14b).

The bivalent binding mode was unique for Orai peptides and not seen in the parallel control with immobilized smMLCK peptides. Here, the binding curves were readily fitted by the simple Langmuir 1:1 binding model (Fig. 5.15) with the same $K_d$ as seen in MST (Fig. 5.5).
tides with distinct kinetics and affinities. When we reversed the immobilization scheme by tethering CaM to the chip surface and injecting soluble Orai CMBD peptides, the resulting binding curves could always be fitted by the simple 1:1 Langmuir binding model.

The resulting single affinity for Orai, however, represents the apparent $K_d$ value and results from the fact that SPR cannot discriminate between the different affinities of the two CaM lobes (for Orai $K_{d,1} = 3.8$ nM, $K_{d,2} = 1.1$ μM, according to Fig. 5.2a); therefore, the Langmuir model interpreted the binding of both peptides as one binding step with an averaged $K_d$ value of 38 nM (Fig. 5.16a). Very similar results were also obtained for the shorter Orai169–88 peptide, as well as for Orai146–66 (Fig. 5.17).

For the CMBD of smMLCK, the binding curves could be well fitted by the simple 1:1 model as in Fig. 5.15, with no significant change in affinity (Fig. 5.16b, the moderately lower $K_d$ is most probably the effect of conformational preference).

Figure 5.16: SPR data of the interaction between (a) soluble Orai1 CMBD or (b) soluble smMLCK CMBD and immobilized CaM. In both cases, the binding curves were well fitted by a simple Langmuir 1:1 binding model. The $K_d$ value in (a) represents an averaged affinity over both binding steps. Image section from [210].

Figure 5.17: SPR data of the interaction between (a) soluble Orai169–88 or (b) soluble Orai146–66 with immobilized CaM. In both cases, the binding curves were fitted by a simple Langmuir 1:1 binding model. Figure adapted from [210].
The bivalent analyte model assumes step-wise binding of soluble CaM to two separate CMBD peptides, and therefore yields two association and dissociation rate constants and two equilibrium constants for both binding steps. The overall binding response in Fig. 5.13 and Fig. 5.14a, which comprises both the response from the first binding step (CaM bound to one Orai CMBD) and from the second binding step (CaM bound to two Orai CMBDs), can be split in two components, which correspond to the monovalent (AB) and bivalent complex (ABB) (Fig. 5.18).

The deconvolutions of the binding response for soluble CaM and immobilized Orai CMBDs (Fig. 5.13a) at low and high CaM concentrations gave a very different picture: At low CaM concentration (4 nM, Fig. 5.18a), the vast majority of CaM molecules rapidly formed a 2:1 complex (ABB) and the monovalent complex (AB) just played a minor role. At high CaM concentration (64 nM, Fig. 5.18b), bivalent binding predominated only at the very beginning of the injection and then remained at a low but stable level. In any case, the bivalent interaction (ABB) dominated during the subsequent buffer washing period and greatly slowed the overall dissociation process due to its higher stability. This effect can also be clearly seen by comparison of the dissociation phases in Fig. 5.13a (CaM in solution, Orai1 immobilized) and Fig. 5.16a (Orai1 in solution, CaM immobilized). When Orai1 CMBD was immobilized and CaM was injected (Fig. 5.13a), the dissociation rate was ~10 times lower than in the reversed configuration with immobilized CaM. A sim-
ilar scenario possibly also occurs *in vivo*, where bundles of CMBD segments protrude from the membrane into the cytosol where soluble CaM is present.

The stabilizing effect from bivalent CaM binding was also observed when pre-bound CaM was dissociated by 2 mM EGTA (Fig. 5.19a). Injection of 1 µM CaM (cyan trace) resulted in a high level of monovalently bound CaM, but it was instantaneously lost when injecting EGTA. In contrast, 16 nM CaM (red trace) gave a lower level of binding, with a significant bivalent fraction that was difficult to remove by EGTA. The results for Orai3 and Orai169−88 were very similar to the curves shown in Fig. 5.19a. With immobilized smMLCK CMBD, injection of EGTA always resulted in instantaneous and complete dissociation at all concentrations (Fig. 5.19b), as expected for strictly monovalent binding.

Figure 5.19: Forced dissociation of bound CaM by removal of Ca$^{2+}$ ions.[210] Orai1 or smMLCK CMBD was covalently immobilized as shown in Fig. 4.1 on page 53. CaM was injected at a concentration of 16 nM (red line) or 1 µM (cyan line) in presence of 2 mM Ca$^{2+}$, followed by injection of 2 mM EGTA after 3 minutes of association (arrow). For Orai1 CMBD (a), immediate dissociation was observed for both CaM populations, but, however, the removal of CaM that had been injected at a concentration of 16 nM was found to be incomplete, while complete dissociation was observed for the other case. Most probably, high concentrations of CaM result in predominantly monovalent interactions with only one lobe of CaM being able to bind one Orai CMBD due to steric hindrance. For smMLCK (b), no difference between the two injected CaM concentrations was observed, CaM was completely and instantaneously dissociated in both cases. Shown are always three averaged curves together with the standard deviation as error bars.

In the MST experiments, dimerization of the Orai1 peptide had resulted in an over 100-fold affinity increase (Fig. 5.2a and Fig. 5.8a). In the analogous SPR experiments with CaM at the surface and Orai1 dimers in solution, we also observed a ~70-fold increase in affinity (Fig. 5.20a). Comparison of the kinetics showed that this increase in affinity was caused by a ~100-fold decrease of the dissociation rate constant $k_d$, while the association rate constant $k_a$ changed very little (Tab. 5.1). As expected, the cleavage of the dimerized peptide by disulfide reduction restored the behavior of the monomeric peptide (cf. Tab. 5.1), and the affinity was found to decrease again to a $K_d$ value
of ~34 nM (compared to $K_d \approx 38$ nM with monomeric Orai1 CMBD, as shown in Fig. 5.16a). In the case of smMLCK, dimerization had little effect on the affinity for immobilized CaM and resulted even in a slightly higher $K_d$ value (Fig. 5.20b); accordingly, splitting of the smMLCK dimer also induced minor changes only (cf. Tab. 5.4).
5.1.3 AFM Single Molecule Recognition Force Spectroscopy

The binding of one CaM to two N-terminal helices in the Orai oligomer proposed recently\cite{131} is so unique and new that extensive characterization is required to support this hypothesis. One indication comes from the two-step binding seen with CaM and monomeric Orai peptides (Fig. 5.6). Another evidence is the expected coalescence of the two binding steps into one high-affinity step when dimerizing Orai via disulfide bonds (Figs. 5.8a and 5.20a). Bivalent binding was also indicated from the SPR data fit when soluble CaM interacted with two peptides on the chip surface (Figs. 5.13 and 5.14a) but this evidence for bivalency is indirect because the two binding steps were not explicitly resolved by SPR.

This problem was overcome by studying the interaction of CaM with the CMBD peptides of Orai1, Orai3, and smMLCK on the single-molecule level with a time resolution of $10^{-2}$ s where monovalent and bivalent binding was well resolved. By using AFM single molecule recognition force spectroscopy (SMRFS), we were able to determine the forces that govern bond formation between CaM and the CMBD segments of Orai and explore the energy landscape of this interaction. For this purpose, CaM was flexibly tethered onto the apex of an AFM cantilever tip through PEG-linkage and, in parallel, the Orai peptides were similarly linked to an ultra-flat support (or vice versa, cf. Fig. 4.3 on page 57 and Secs. 3.3.3.1 on page 43 and 4.2.3 on page 56). Single-molecular force measurements\cite{89, 71, 121} (cf. Sec. 4.2.3.6 on page 58) were performed by lowering the functionalized tip towards the surface-bound peptides to allow for binding of CaM.

In case of interaction, the subsequent retraction of the tip resulted in a downward deflection of the cantilever which jumped back to the resting position when the CaM-Orai bond was ruptured (vertical jump in Fig. 5.21a).

Figure 5.21: Detection and extraction of unbinding forces using single molecule force spectroscopy. a) Typical force-distance cycle with CaM-functionalized tip and surface-immobilized Orai in 2 mM Ca$^{2+}$. The blue and red trace reflect monovalent and bivalent binding (tip retraction rate 400 nm s$^{-1}$). (b) Histogram and pdf of the unbinding forces after 20 ms contact time, together with two fitted Gaussian distributions which represent the most probable monovalent and bivalent unbinding forces. Figure adapted from \cite{210}.
5.1 Interaction Between Calmodulin and Orai

Figure 5.22: Binding probability for the interaction between tip-tethered CaM and surface-immobilized Orai1 or Orai3 CMBD.[210] In 2 mM Ca^{2+}, the binding probability (i.e. the fraction of force-distance cycles showing an unbinding event) was 19% for Orai1 CMBD and 26% for Orai3 CMBD. Without Ca^{2+} (in 2 mM EGTA), the binding probability dropped significantly to 2 or 3%, respectively (statistical significance p < 0.0001).

Force distance curves where no interaction occurred showed very similar trace and retrace curves with no characteristic nonlinear stretching of the PEG-linker (inset in Fig 5.21a). The unbinding force was determined from the vertical jump seen during tip retraction, as explained in Fig. 4.5 on page 60. Two distinctly different unbinding forces were observed with Orai1 or Orai3 peptides on the surface and CaM on the tip, as exemplified by two force-distance curves in Fig. 5.21a (blue and red trace, only the retrace curves are shown). The force distribution was calculated as empirical probability density function (according to Eq. 3.59 on page 41) of the unbinding events in >1000 force-distance cycles and is shown as black solid line in Fig. 5.21b. As explained in more detail in Sec. 4.2.3.8 on page 59, pdfs represent the original data and can be seen as the equivalent of continuous histograms with the advantage of taking into account the variances of each unbinding event.

That the measured unbinding events indeed arose from specific interactions was verified by performing the same experiments in Ca^{2+}-free buffer (2 mM EGTA). Both for Orai1 and Orai3 CMBD, the amount of force-distance curves that showed unbinding events (i.e., binding probability) decreased significantly (p < 0.0001, Fig. 5.22), proving the Ca^{2+}-dependency of the interaction and the specificity of our single-molecule measurement approach.

For tip-tethered CaM and immobilized Orai1, the calculated force distributions consisted of two Gaussian components (Fig. 5.23a), with the mean unbinding forces of ~45 pN (blue area) and ~75 pN (red area); for Orai3, similar values were found (~50 pN and ~95 pN, respectively; Fig. 5.23b).

Using force spectroscopy allowed us not only to actively approach tip-tethered CaM to surface-immobilized peptides, but also to accurately control the tip-surface contact time to follow the kinetics of multimeric bond forma-
tion with millisecond resolution. For Orai CMBD, at a low contact time of 20 ms most unbinding events were occurring at lower forces (blue marked Gaussian distribution in Fig. 5.23, also shown in Fig. 5.24, top row) and only about 35% showed significant higher forces (red marked distribution, Fig. 5.23). Gradually increasing the tip-surface contact time to 90 ms and 120 ms resulted in distinct changes in force distribution and shifted the most probable unbinding force (i.e., the highest peak in the pdf) progressively to higher forces, as expected for a time-dependent transition from monovalent to bivalent binding of CaM (Fig. 5.24a, blue and red pictograms in Fig. 5.21). At 120 ms, almost 90% of unbinding events were attributed to the higher-force Gaussian distribution, indicating an almost complete transition from monovalent to bivalent binding within 100 ms. The same contact time dependence was also seen with Orai3 (Fig. 5.24b). In the AFM experiments, the transition from mono- to bivalent binding of CaM was certainly influenced by the long polymer linker (9 nm, cf. Fig. 4.3 on page 57) which gave the second Orai CMBD much motional freedom and thereby slowed its binding to the second lobe of CaM. In the native Orai hexamer, the close alignment of the CMBD helices is expected to provide for a much faster transition from mono- to bivalent binding of CaM.

As the force required to disrupt a molecular bond is critically depending on how fast an external force is applied,[66] we varied the velocity \( v \) of tip retraction. The maxima of both Gaussian distributions in Fig. 5.23, that were attributed to monovalent and bivalent binding, were separately plotted against the logarithm of the loading rate \( r \) (\( r = v \cdot \text{effective spring constant} k_{\text{eff}} \)); Fig. 5.25, blue and red data points). Both for Orai1 and Orai3, the monovalent interactions (blue) were well fitted by the Bell-Evans model (straight blue line), indicating the crossing of a single energy barrier in the thermally activated regime (cf. Sec. 3.3.3 on page 38).[13, 66] Extrapolation to zero force...
Figure 5.24: Distribution of unbinding forces for Orai1 and Orai3 as a function of tip-surface contact time.[210] By varying the time the CaM-functionalized tip is in contact with the surface, different unbinding force distributions were observed, both for Orai1 CMBD (a) and Orai3 CMBD (b). For all plots, two Gaussian distributions (dashed blue and red lines) were fitted to the experimental pdf (black line), yielding the sum of fitted Gaussians (gray line) that reproduces the pdf. The maxima of the populations that are representing either monovalent or bivalent interactions of CaM with Orai CMBD, are at \(-45\) pN and \(-75\) pN for Orai1 CMBD and \(-50\) pN and \(-95\) pN for Orai3 CMBD, respectively. Extension of contact time from 20 ms to 120 ms progressively increased the fraction of the higher force unbinding events (red dashed lines). The tip retraction velocity was set to \(400\) nm s\(^{-1}\).
Figure 5.25: Loading rate dependence plot of the interaction between tip-tethered CaM and (a) surface-immobilized Orai1 or (b) Orai3 CMBD. The most probable unbinding forces for mono- and bivalent binding per loading rate (the respective peak of the Gaussian fits as shown in Fig. 5.23) are plotted over the logarithm of the loading rate. The monovalent unbinding forces (blue squares) were fitted by the Bell-Evans model\cite{13, 66} (blue line), the bivalent ones (red spheres) were compared to the prediction for \( n = 2 \) bonds according to the framework of Williams\cite{223} (red line). Error bars for the standard deviation of loading rates are not shown but were considered for the fitting procedure. Figure adapted from \cite{210}.

yielded the thermal dissociation rate constant (\( k_d = 0.42 \text{ s}^{-1} \) for Orai1) which was similar to the one determined by SPR (\( k_d = 0.25 \text{ s}^{-1} \); for Orai3 the values were also similar, as depicted in Tab. 5.1), hereby showing that the probed energy barrier is also dominant during spontaneous thermodynamic dissociation. In our experiments, the rupture of bivalently bound CaM occurred quasi-simultaneous due to the finite time resolution of the AFM. As proposed by Williams\cite{223}, the rupture forces of multiple uncorrelated parallel bonds can be predicted from the parameters of the monovalent bond ruptures (cf. Sec. 4.2.3.9 on page 61). Assuming \( n = 2 \) bonds for our experimental system (with one CaM on the tip and a pair of Orai peptides on the surface, pictograms in Fig. 5.21a), the experimental unbinding forces of the bivalent interaction between one CaM and two Orai1 or Orai3 CMBDs (red spheres in Fig. 5.25) were well explained by this prediction (red solid line in Fig. 5.25).

To further prove that the higher-force rupture events derived from bivalent binding, we reversed the functionalization of tip and substrate, whereby only one Orai peptide was tethered to the apex of the AFM tip (pictograms in Fig. 5.26b). Now only one Gaussian-like force distribution at lower unbinding force was seen (Fig. 5.26b, gray area), with the same most probable unbinding force as for the monovalent population in Fig. 5.26a (blue area).
5.1 Interaction between Calmodulin and Orai

Figure 5.26: Force distribution of the interaction between CaM and Orai3 CMBD. (a) Distribution of unbinding forces for tip-tethered CaM and surface-functionalized Orai3 CMBD as shown in Fig. 5.23b. Most unbinding events were found to be of monovalent nature (blue area), but a significant fraction showed higher unbinding forces (bivalent interactions, red area; see pictograms). (b) When the measurement configuration was reversed (CaM on the surface and Orai3 CMBD tethered to the AFM tip, see pictogram), only one force population at lower unbinding forces was found. For both configurations, the tip retraction velocity was set to 400 nm s\(^{-1}\). Very similar results were obtained for Orai1 CMBD. Image section from [210].

Accordingly, when we varied the loading rate and plotted the most probable unbinding force against the logarithm of the loading rate, the fit with the Bell-Evans model (Fig. 5.27, solid black lines) was almost identical with the fit for the monovalent interactions of the previous configuration (solid blue lines in Fig. 5.25, also shown in Fig. 5.27 for comparison).

Figure 5.27: Loading rate dependence of the reversed functionalization scheme with CaM on the substrate and (a) Orai1 or (b) Orai3 on the tip. As expected (see pictograms), only monovalent interactions were seen (black diamonds), the resulting Bell-Evans fit (black line) was very similar to that in Fig. 5.25 (blue line, together with the error boundaries on the basis of a 95% confidence interval (dotted lines)). Error bars were omitted for better readability. Figure adapted from [210].

In the above described MST and SPR experiments, the CMBD of smMLCK had always shown only monovalent interactions (Figs. 5.5, 5.8b, 5.9b and Figs. 5.15, 5.16b, 5.20b). By analogy, in the AFM only one force peak was
90 results and discussion

Figure 5.28: Unbinding forces and Bell-Evans fit for support-bound smMLCK peptide and tip-tethered CaM. Only one single force population was observed per loading rate as expected for a strictly 1:1 interaction. The most probable unbinding forces per loading rate were fitted by the model of Bell and Evans as described for Orai CMBD. (b) The distributions of unbinding forces were determined for different contact times. Elongation of contact time did not result in significant changes in unbinding forces; the most probable unbinding force (determined by fitting a Gaussian fit to the respective pdf) yielded a mean unbinding force of 56 pN ± 1 pN. For all measurements, the tip retraction velocity was set to 400 nm s$^{-1}$. Figure adapted from [210].

observed for surface-tethered smMLCK at any given loading rate (Fig. 5.28a), also at longer tip-surface contact times (Fig. 5.28b).
Table 5.1: Overview of the determined kinetic rates and equilibrium constants of the interaction between CaM and different CMBDs. The parameters are grouped according to the respective methods (SPR, MST, and AFM).

<table>
<thead>
<tr>
<th>CMBD</th>
<th>( k_{a1} ) (M(^{-1})s(^{-1}))</th>
<th>( k_{a1} ) (s(^{-1}))</th>
<th>( k_{d2} ) (s(^{-1}))</th>
<th>( K_{d2} ) (nM)</th>
<th>( K_{d2} ) (RU)</th>
<th>( K_d ) (nM)</th>
<th>( K_d ) (nM)</th>
<th>( x_0 ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orai1 (^{a, b})</td>
<td>5.29 (\times) 10(^{5})</td>
<td>0.25</td>
<td>0.07</td>
<td>0.24</td>
<td>4.67</td>
<td>3.31</td>
<td>0.41</td>
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<tr>
<td>Orai1, reversed (^{a, b})</td>
<td>8.02 (\times) 10(^{4})</td>
<td>0.03</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>37.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orai3 (^a)</td>
<td>7.44 (\times) 10(^{5})</td>
<td>0.04</td>
<td>0.12</td>
<td>0.55</td>
<td>5.01</td>
<td>4.58</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>Orai3, reversed (^{a, b})</td>
<td>1.27 (\times) 10(^{4})</td>
<td>0.01</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>7.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>smMLCK (^a)</td>
<td>7.92 (\times) 10(^{5})</td>
<td>0.02</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2.45</td>
<td></td>
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<tr>
<td>smMLCK, reversed (^{a, b})</td>
<td>1.80 (\times) 10(^{5})</td>
<td>7.11 (\times) 10(^{-4})</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.40</td>
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</tr>
<tr>
<td>Orai1 dimer, reversed (^{a, b})</td>
<td>4.51 (\times) 10(^{5})</td>
<td>2.63 (\times) 10(^{4})</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.58</td>
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<tr>
<td>Orai1 dimer, reduced, reversed (^{a, c, d})</td>
<td>7.94 (\times) 10(^{4})</td>
<td>2.69 (\times) 10(^{-3})</td>
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<td>--</td>
<td>--</td>
<td>33.80</td>
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<td></td>
</tr>
<tr>
<td>smMLCK dimer, reversed (^{a, b})</td>
<td>2.70 (\times) 10(^{3})</td>
<td>2.55 (\times) 10(^{-3})</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>smMLCK dimer, reduced, reversed (^{a, b, c})</td>
<td>6.13 (\times) 10(^{-3})</td>
<td>1.67 (\times) 10(^{-3})</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orai1b3a4a (^{e})</td>
<td>5.33 (\times) 10(^{4})</td>
<td>0.05</td>
<td>3.69 (\times) 10(^{-5})</td>
<td>0.02</td>
<td>9.21</td>
<td>5.47</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Orai1b3a4a, reversed (^{a})</td>
<td>3.02 (\times) 10(^{-3})</td>
<td>0.10</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>32.90</td>
<td></td>
<td></td>
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<tr>
<td>Orai32a4a (^{e})</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>dOrai, reversed (^{a, b})</td>
<td>4.65 (\times) 10(^{-5})</td>
<td>0.02</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>39.10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Peptide immobilized on the SPR chip or on the AFM support, CaM in solution or on the AFM tip. \(^b\) CaM immobilized on the SPR chip or on the AFM support, peptide in solution or on the AFM tip. 
\(^c\) SPR data not shown in any figure. \(^d\) The kinetic and equilibrium constants obtained from SPR are stated without standard deviations because they were almost negligible. \(^e\) This \(K_d\) was determined from a 1:1 Langmuir fit, in the case of two binding steps it represents an approximate estimate for the effective overall affinity of both steps.
5.2 Homomeric Interaction Between the Orai1 N-Terminus and Loop2

The C-terminal domain of Orai is supposed to represent the main coupling site for STIM1 (cf. Sec. 2.3.1 on page 12). The Orai N-terminus, however, is indispensable for Orai channel gating.[239, 141, 51] Although the extended transmembrane Orai N-terminal (ETON) region (for Orai1 it comprises aa73-91, for Orai3 aa48-65; it also contains the CMBD) is fully conserved between Orai1 and Orai3, Orai3 tolerates larger N-terminal truncations than Orai1. Orai3 requires approximately 1.5 helix turns less of the ETON region than Orai1 for store-operated activation, which indicates that different molecular requirements are needed for the activation of Orai1 and Orai3 channels.[51, 15] Analysis of different Orai mutants and chimeras as well as molecular dynamics (MD) simulations suggest that the communication between the Orai N-terminus and the cytosolic loop that connects transmembrane region TM2 and TM3 (loop2 or L2) plays a crucial role for channel activation (Fahrner et al. [68], currently under review).

Figure 5.29: Binding probability between surface-immobilized Orai1_{70-91} or Orai1_{79-91} and tip-tethered Orai1-L2 or Orai3-L2, respectively. Control experiments were performed with unfunctionalized tips carrying only the crosslinker (striped bars). The shown binding probability was calculated as the average of the values of three tips.

In order to examine whether a longer or shorter Orai N-terminus might form altered interactions with either Orai1-L2 or Orai3-L2, possibly accounting for the inhibitory effects on Orai1 channel activation, we performed in vitro force measurements using atomic force microscopy.

This technique allows for specifically bringing the two strands in close contact to each other and to determine the resulting interaction forces. For this purpose, the fragment of Orai1-L2 was covalently conjugated onto the apex of the AFM cantilever tip via a flexible polyethylene glycol (PEG)-linker
as described in Sec. 4.2.3 on page 56. Single-molecular force measurements were performed by repeatedly approaching and withdrawing the functionalized tip towards surface-bound Orai170–91 peptides which comprise the full ETON-region, or towards Orai179–91 peptides which were lacking the first 6 aa (aa73–78) of the ETON region. In the case of an interaction, a pulling force was developed during retraction, causing a downward bending of the cantilever. At a certain critical force, the bond between the two fragments was ruptured, allowing for quantitative determination of the single-molecular unbinding force (cf. Sec. 3.3.2.1 on page 35 and Sec. 4.2.3.6 on page 58).

To investigate if isoform-specific structural elements in loop2 of Orai1 and Orai3 influence the interaction with the truncated N-terminal ETON domain, we performed the same experiments with Orai3-L2 instead of Orai1-L2 on the tip. As control experiment to examine if the determined interaction forces were indeed arising from specific binding of Orai-L2 to Orai-NT, we used tips which carried only the PEG-linker without Orai-L2. With these tips, both for surface immobilized Orai170–91 and Orai179–91, the number of force-distance curves that showed unbinding events (i.e., binding probability) decreased significantly (striped bars in Fig. 5.29).

Our measurements indicate that the truncation of the ETON region of Orai1 (Orai179–91) leads to an increased probability for the interaction with Orai L2 (black and blue bars in Fig. 5.29), showing statistically stronger evidence for the interaction with Orai1-L2 (black bar, p = 0.008) than with Orai3-L2 (blue bar, p = 0.02) (Fig. 5.29). However, as the binding probability between truncated Orai179–91 and Orai3-L2 (blue bar) is very similar to the one of non-truncated Orai NT and Orai1-L2 (red bar, i.e., ~13%), we assume that this value might represent a certain threshold of interaction probability, above which inhibitory effects are likely to occur.

![Figure 5.30: Box plot of the prevailing interaction forces at a loading rate of ~5000 pN s⁻¹, based on three data sets (three tips). The boxes are determined by the 25th and 75th percentiles, the whiskers are determined by the 5th and 95th percentiles, respectively.](image-url)
In addition to the higher interaction frequency, truncated Orai\textsubscript{79-91} also showed significantly higher unbinding forces during dissociation from Orai\textsubscript{1-L2} (p < 0.001), while no significant increase was observed for the interaction with Orai\textsubscript{3-L2} (Fig. 5.30). The force pdfs (Fig. 5.31) revealed a rather distinct peak (i.e., most probable unbinding force) with only few unbinding events at higher forces. Fig. 5.31 shows the respective pdfs normalized to the highest binding probability as seen for Orai\textsubscript{179-91}. Measurements without Orai-L2 on the tip (dashed lines), resulted only in few unbinding events with lower forces, most probably due to non-specific adhesion.

Figure 5.31: Overlay of the force distributions (shown as pdfs) of the interactions between tip-tethered Orai\textsubscript{1-L2} and surface immobilized Orai\textsubscript{179-91} (black solid line) or Orai\textsubscript{170-91} (red solid line). The pdfs that are shown as dashed lines represent the respective control experiment without Orai\textsubscript{1-L2} on the tip. All pdfs were normalized to the highest binding probability (i.e. Orai\textsubscript{179-91}, black solid line); the area under each pdf therefore represents the respective binding probability in relation to the one of Orai\textsubscript{179-91}. Each pdf represents one exemplary data set measured with one tip.

By varying the pulling velocity and plotting the most probable unbinding force as a function of the loading rate, we further obtained information about the molecular transition during dissociation. In accordance with the model of Bell and Evans[13, 66] (cf. Sec. 3.3.3 on page 38), we observed a linear increase in unbinding force with logarithmically increasing loading rate (Fig. 5.32). Taking into account the standard deviation of the respective model fit, the determined dissociation rate $k_d$ is not significantly changed upon truncation of Orai\textsubscript{NT} and interaction with Orai\textsubscript{3-L2} ($k_d = 0.09\pm0.10$ s\textsuperscript{-1} for Orai\textsubscript{179-91} versus $k_d = 0.23\pm0.21$ s\textsuperscript{-1} for Orai\textsubscript{170-91}, Fig. 5.32b), but shows an about ten times lower dissociation rate for the interaction with Orai\textsubscript{1-L2} ($k_d = 0.13\pm0.08$ s\textsuperscript{-1} for Orai\textsubscript{179-91} versus $k_d = 1.90\pm0.47$ s\textsuperscript{-1} for Orai\textsubscript{170-91}, Fig. 5.32a).

The combination of more frequent interactions (Fig. 5.29), higher interaction forces (Fig. 5.30) and slower dissociation rate thus indicates a stronger
interplay between truncated Orai\textsubscript{1\textsubscript{70--91}} and Orai\textsubscript{L2}, which might account for the inhibited, non-permissive channel conformations observed with the N-terminal truncation of Orai wild-type and constitutively active Orai forms.

\cite{68}

Figure 5.32: Loading rate dependence plots of the interaction between tip-tethered (a) Orai\textsubscript{L2} and surface-immobilized Orai\textsubscript{1\textsubscript{79--91}} (black data points and lines) or Orai\textsubscript{1\textsubscript{70--91}} (red), or (b) Orai\textsubscript{3\textsubscript{L2}} and surface-immobilized Orai\textsubscript{1\textsubscript{79--91}} (blue) or Orai\textsubscript{1\textsubscript{70--91}} (magenta). For the sake of clarity, error bars for the standard deviation of loading rates are not shown but were considered for the fitting procedure. Together with the fit (solid lines), confidence bands (CI = 95\%) of the respective fits are illustrated by dashed lines.

Additional experiments with further mutants and chimeras as well as MD-simulations strongly support this hypothesis, and together suggest that maintenance of Orai channel function requires a permissive communication between its N-terminus and loop\textsubscript{2}, which is likely governed in an isoform-specific manner.\cite{68}
The major objective of this PhD thesis was to investigate the interaction between different N-terminal Orai segments and calmodulin. Based on different biochemical and biophysical in vitro methods, this interaction was characterized using three different approaches: MST detects a relative change in the thermophoretic movements of a fluorescence-labeled molecule and allows for determining binding affinities, SPR measures the change in refractive index at the surface-solution interface and yields kinetic and equilibrium constants, and AFM-based force spectroscopy makes it possible to determine the single-molecular interaction forces.

Altogether, the results of these three complementary methods presented within this thesis provide a detailed picture of the mechanism by which CaM interacts with the CMBD of Orai proteins (Fig. 6.1): Step-wise binding of two CMBD peptides was seen with two distinct affinities, kinetic constants, and single-molecular interaction forces.

MST experiments in solution with labeled CaM and unlabeled Orai CMBD segments revealed a two-step binding of CaM to two Orai peptides with one high affinity step ($K_{d,1} \approx 10^{-9}$ M) as typically reported for CaM targets, and a second binding step with distinctively lower affinity ($K_{d,2} \approx 10^{-6}$ M). In the corresponding SPR assay where the native situation could be reconstituted more closely, a similar bivalent binding of soluble CaM to two immobilized Orai CMBD segments was observed. Analysis of the kinetic parameters and deconvolution of the overall binding response showed that the bivalent
complex greatly stabilized the interaction and slowed down the dissociation process.

In addition, the time-dependent transition from monovalent to bivalent binding was quantitatively followed in SPR and AFM measurements. By a step-wise increase of the time during which the tip with immobilized CaM was in contact with the Orai peptides at the surface, distinct changes in the force distribution and a shift of the most probable unbinding force to higher values was observed, the latter dominating for tip-surface contact times of over 100 ms. In control experiments with one Orai peptide on the tip and CaM on the surface, only one force population was seen, which was fully unaffected from extension of the contact time.

The fact that the dissociation rate constants derived from AFM experiments were similar to the ones determined by SPR indicates that the same energy barrier was responsible for the dissociation kinetics measured by these two different techniques.

Quasi-simultaneous binding of CaM with an extreme affinity \( K_d \approx 10^{-11} \) M was seen when two Orai CMBD peptides were interlinked in a parallel fashion, thereby mimicking the parallel alignment of the conserved N-terminal helical segments in the native Orai\(_1\) hexamer. Interestingly, this more than 100-fold increase in affinity seemed to be mainly caused by a dramatic decrease of the kinetic dissociation rate constant, while the association rate constant only changed little.

As a control, all assay methods were also applied to the CMBD of smMLCK and here only the well-known canonical binding mode\([142]\) was observed, proving the validity of our approach.

Taken together, the findings presented in this thesis support, extend, and specify the bivalent interaction model proposed recently by Liu et al.\([131]\); moreover, we showed that the same bivalent interaction mode also applies to the CMBD of Orai\(_3\), as well as of dOrai in *drosophila melanogaster*.

The fact that Liu et al.\([131]\) did not see co-crystallization of one CaM with two Orai\(_1\) CMBDs was probably due to the low affinity of the second binding step. This problem should readily be overcome by co-crystallization of CaM with dimerized CMBD peptides. The same strategy appears promising to resolve the geometry of the complex in solution by nuclear magnetic resonance (NMR) spectroscopy. The exceptionally high affinity of bivalently bound CaM for the dimerized CMBD indicates that CaM should also tightly interact with two out of the six helices that are grouped into a parallel bundle in the native Orai hexamer. The occurrence of such an interaction in live cells was recently questioned.\([149]\) However, it seems unlikely that the extraordinarily high affinity which we found to be conserved from *drosophila* to human Orai proteins should be irrelevant in the native situation, although the physiological role of the CaM-Orai interaction is admittedly still unclear.

Besides the novel insights into the binding mechanism between CaM and Orai segments, the concerted approach of the three complementary meth-
ods used in this study might serve as a novel analysis strategy for an ex-
act biophysical characterization of other biological interactions. A combined
determination of equilibrium constants, kinetic rates, and single-molecular
interaction forces might not only increase the experimental reliability and ro-
bustness, but also provides access to transient phenomena and facilitates an
in-depth characterization of interactions at the solution-surface interface as
well as in solution.
In this part, two self-contained studies and a comprehensive book chapter about the reversible immobilization of biotinylated molecules on regenerative sensor chips as used within this thesis are enclosed.
The article


is enclosed is the following.*

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* My contributions include the following: I performed SPR experiments (in particular for Fig. 1 and Fig. 2), analyzed and discussed data, and assisted in reviewing and editing of the manuscript.
Regenerative biosensor chips based on switchable mutants of avidin—A systematic study

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ABSTRACT

Biotinylated bait molecules can be immobilized on biotinylated sensor chips by formation of biocytin–avidin–biocytin bridges which are very stable when using wild-type (strept)avidin. Stable immobilization of biotinylated baits is important for monitoring reversible binding and dissociation of prey molecules. For measurements with another bait molecule, however, it is desirable to replace all immobilized proteins by fresh (strept)avidin and new biotinylated bait. In this study, five avidin mutants have been characterized with respect to their ability to form switchable biotin–avidin–biotin bridges on biotinylated chip surfaces, as needed for complete chip regeneration. All five mutants formed stable biotin–avidin–biotin bridges at pH 7, were more or less stable at pH 2–3, and required the combination of pH 2 with SDS for quantitative removal from the chip surface. Mutant #3 ("switchavidin") showed the best combination of properties, i.e., low nonspecific adsorption of protein and nucleic acids, high binding capacity, and good stability at pH 2–3, as typically used for quantitative removal of prey molecules in repeated measurement cycles.

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1. Introduction

Biosensors monitor binding of soluble prey molecules to immobilized bait molecules. Subsequently, all bound prey molecules must be removed before the next sample can be measured [1]. Usually the bait molecules are immobilized by covalent bonds or by avidin–biocytin interaction [2–5]. Since neither is reversible in a reasonable time window, a new measurement series can only be started after exchanging the sensor chip and immobilizing another bait molecule. A desirable alternative is replacement of the old bait molecules for new ones. Exchange of the bait on the chip confers significant advantages: (i) it saves the cost and time of chip exchange. (ii) No human intervention is required, allowing for switching of baits in programmed overnight runs. (iii) It eliminates chip-to-chip variation, which is a problem with some product lines. (iv) The most urgent need for in situ exchange of bait molecules is encountered if the harsh conditions (e.g., 100 mM HCl or NaOH) typically used for removal of bound prey molecules [1] cause denaturation of the bait.

In order to be useful in practical application, any method of bait exchange must obey strict criteria: (a) the bait must remain stably bound for hours or days, until all planned measurements have been completed with one kind of bait molecule. (b) It must be possible to quantitatively remove all bound bait molecules within minutes. (c) The binding capacity for new bait molecules must be fully retained for a large number of regeneration cycles. (d) The reagents used for chip regeneration must be compatible with the flow cells of common biosensors. (e) The reagents and the chip surface must be stable under ambient conditions (i.e., not sensitive to oxidation or hydrolysis). (f) The sensor surface used for switchable bait immobilization must not bind any prey or side components of the sample. (g) Preferably, the method should be easy to implement in many types of biosensors.

Two published methods fulfill these criteria to a high degree. In both methods, avidin or (strept)avidin is reversibly immobilized, providing for immobilization of biotinylated bait molecules before a measurement series, and for rapid removal of (strept)avidin plus bait at the end. In the first method, (strept)avidin is

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Fig. 1. Reversible functionalization of biotinylated biotinylated probe molecules. (a) Illustration of chip recycling with switchable biotin–avidin–biotin bridges. (b) Testing of different avidin mutants for reversible immobilization of biotinylated antibodies. (c) SPR traces showing binding of mutant #1 in both flow cells and of biotin–IgG in FC2 (blue dashed line), as well as removal by SDS/citric acid. The numbers 1–4 in (c) correspond to the stages 1–4 in (b). (d) Comparison with respect to the mutant layers in both cells, biotin–IgG bound in FC2, and baseline drift from stage 1 to 4. (e) Calculated pI of the avidin mutants (Table S1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reversibly immobilized on carboxymethyl dextran via DNA double strand formation (BIACore application note “Biotin CAPture Kit”, GE data file 28-9577-47 AA, https://www.gelifesciences.com/gecis_images/GELS/Related%20Content/Files/1514787424814/1litdoc28957747AA1_20110831132219.pdf). This method fulfills criteria (a)–(e) but it is not applicable to DNA-binding proteins and only available for one brand of biosensors. In the second method, the avidin mutant M96H is used as a switchable link between biotinylated sensor surfaces and biotinylated bait molecules [6,7]. Mutation M96H is located at the subunit interface (Supplementary Fig. S1) and it confers sensitivity to low pH [6]. In the biotin-bound state, however, this mutant retains full function down to pH 2.7, unless avidin M96H is intentionally dissociated into four non-functional subunits by combination of citric acid with sodium dodecyl sulfate (SDS) as outlined in Fig. 1a [6].

An obvious drawback of avidin M96H is its positive charge at neutral pH (pI ~ 9.5, see Fig. 1e). Most proteins and all nucleic acids are negatively charged at neutral pH, resulting in nonspecific adsorption to immobilized avidin M96H, especially in case of DNA [6,7]. Nonspecific protein adsorption was largely suppressed by blocking with biotin–BSA [6], whereas suppression of DNA adsorption required additional mutations, which lowered the pI towards 7 (mutant #3 in Fig. 1e and Table S1) [7]. In the present study, five avidin mutants (Table S1 and Fig. S1) were characterized along criteria (a)–(g) and the limits of the method were identified. Mutant #3 (“switchavidin” [7]) was found to be the optimal choice, combining high stability of the biotin–avidin–biotin bridge with low nonspecific adsorption of protein and DNA.

2. Materials and methods

2.1. Materials

The avidin mutants were constructed for bacterial expression in Escherichia coli by introducing mutations to cDNA encoding chicken avidin containing ompA signal peptide in pET101/D [9,10] by QuikChange mutagenesis according to manufacturer's
instructions (Stratagene, La Jolla, CA, USA) or by using standard PCR techniques, where multiple mutations were introduced by overlapping mutated DNA fragments amplified using DNA oligonucleotides containing the desired mutations, followed by subcloning to pET101/D with the help of TOPO cloning (for details see Ref. [7]). All DNA constructs were confirmed by DNA sequencing. The proteins were produced and purified as described [7]. The components of the mixed self-assembled monolayer (SAM) were synthesized and mixed as described in Ref. [6]. Biotin-cap-NHS, biotinylated protein G (biotin–protein G), immunoglobulin G (IgG) from goat, human IgG2x, and lysozyme were obtained from Sigma–Aldrich. Bovine serum albumin (BSA, fatty acid-free) was purchased from Roche Applied Science. Biotin–IgG (with 6–7 biotins/IgG) was prepared as published [11] and biotin–BSA was prepared using the same mass concentrations of protein and biotin-cap-NHS. All single-stranded DNA molecules were custom-synthesized by VBC Genomics (Vienna) with 99 ± 0.5% coupling efficiency and the uncapped final product was purified by HPLC (positive selection for the 5'-terminal dimethoxytrityl group by reversed phase chromatography). The biotin-probe had the structure 5′-biotin-GCAGTCTCAGCTGCAAAGTCTGGGCT-3′ [5], the “unlabeled probe” was the same sequence but lacked biotin. The digoxigenin-labeled analyte was complementary to the probe (digoxigenin-5′-ACGCCAGACTTCTCCACAGGAGTCAGGTGC-3′) and the “unlabeled analyte” had the same sequence but lacked digoxigenin [5]. Biotin-N₂-N₂-T contained 1:1:1:1 mixtures of A:C:G:T in positions 1–19 and thymine in position 20. Phosphate-buffered saline (PBS 7.3) contained 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, yielding pH 7.3. It was degassed by sterile filtration (0.2 μm) with strong aspirator suction every day. Biotin–BSA, Biotin–IgG, BSA, IgG, and lysozyme were purified by gel filtration in PBS 7.3 on Superdex 200 (1 × 30 cm, GE Healthcare) at 0.5 ml/min to remove aggregates. The protein concentrations were adjusted to 1 mg/ml and small aliquots were frozen in liquid nitrogen and stored at −25 °C. Biotin–protein G, IgG2x and the oligonucleotides were dissolved in PBS (20 μM, 7 μM, and 10 μM, respectively) and stored at −25 °C. The aliquots were thawed by short immersion in water (−20 °C), diluted to the desired concentration by addition of PBS 7.3, stored at 4 °C, and used within four days.

2.2. Surface plasmon resonance experiments

Cleaning of bare glass chips, evaporation of chromium (3 nm) and gold (41 nm), as well as cleaning of the gold surface and coating with a mixed biotin SAM (Fig. S2) was performed as described [6]. The chips were mounted on the chip supports with double-sided adhesive tape (non-permanent) and inserted in a BiAcore X device for measurement of binding by surface plasmon resonance (SPR). Degassed buffer (PBS 7.3) was run over the chip surface at 10–20 μl/min, as stated in the figure legends. The resonance angle was recorded at 1 s intervals in both flow cells and expressed in resonance units (1 RU = 0.0001 ◦).

3. Results and discussion

Fig. 1a shows the two kinds of chip regeneration used in this study. “Normal regeneration” means quantitative removal of prey molecules only, as conventionally performed on all kinds of biosensors [1]. “Rigorous regeneration” means quantitative dissociation of the biotin–avidin–biotin bridges on the chip surface, thereby removing the bare biotinylated chip on which fresh avidin and a new kind biotinylated bait is immobilized for a new series of experiments. In this study, we examined the reproducibility of chip regeneration (Section 3.1), nonspecific binding of protein (Section 3.2) and nucleic acids (Section 3.6), multivalent bait–prey interactions (Section 3.3), as well as perfect differentiation between sample cell and reference cell (Section 3.5). Of particular interest was the suitability of methods for quantitative removal of prey (“normal regeneration”) without losing biotinylated bait molecules from the chip surface (Section 3.4).

3.1. Binding of biotinylated protein to biotinylated surfaces by using different avidin mutants

In preceding studies [6,7], three different avidin mutants were shown to provide for reversible formation of biotin–avidin–biotin bridges, as outlined in Fig. 1a. Reversibility is made possible by mutation M06H which is located at the subunit interface (Fig. S1), causing subunit dissociation when treated with SDS/citric acid [6]. Mutant #2 contains the additional mutation R114L which lowers the pl value, reduces nonspecific binding, and enhances the affinity for biotinylated molecules [7], due to its location next to the biotin-binding site (Fig. S1). The three additional mutations in mutant #3 are located on the outer surface of avidin (Fig. S1, Table S1) and shifted the pl value towards 7 (Fig. 1e), resulting in very low nonspecific adsorption of proteins and nucleic acids [7].

In the present study, the newly prepared avidin mutant #4 was analogous to mutant #3, except that mutation R114L was replaced by mutation R26N which had little effect on the pl value (Fig. 1e). The intention was to demonstrate the beneficial effect of mutation R114L for the stability of the biotin-bound state, and this was confirmed by the data (see below). Finally, mutant #5 contained all mutations at the same time, causing further lowering of the pl value (Fig. 1e, Table S1).

Mutants #1–5 were systematically tested for their performance in reversible biosensor functionalization. The first test concerned binding of the avidin mutants to the biotinylated chip and of biotin–IgG on top of avidin (stages 2 and 3 in Fig. 1b), as well as removal of the bound proteins with a mixture of SDS and citric acid (stage 4 in Fig. 1b). As exemplified in Fig. 1c, the avidin mutant was injected in both flow cells and biotin–IgG in FC2 only. The experiments were performed in triplicates with all five avidin mutants and the signal amplitudes were highly reproducible (Fig. 1d).

The extent of mutant binding (stage 2 minus stage 1) was similar for mutants #1–4 (−2000 RU, circles in Fig. 1d), only mutant #5 was less effective. Binding of biotin–IgG (stage 3 minus stage 2) showed a significant decrease with increased mutant number (triangles in Fig. 1d). All mutants allowed for good reversibility of binding (stage 4 minus stage 1, squares in Fig. 1d). Mutant #3 performed best in this respect, with a drift of 13 ± 18 RU in FC1 and 13 ± 14 RU in FC2.

It is important to note that all tested chips could be regenerated for an unlimited number of cycles, except that the binding capacity of the chips started to decrease after three weeks of continued use.

3.2. Nonspecific adsorption of protein on monolayers of different avidin mutants

Sensitive and selective biosensing implies that no binding/adsorption of any component of the sample occurs on the chip surface, except for specific capture of prey to bait. Fig. 2a exemplifies the standard test [6] which consists of consecutive injections of lysozyme, BSA, and goat IgG.

The results for BSA and IgG are summarized in Fig. 2b. Mutant #3 showed the lowest adsorption of BSA (circles) and IgG (trian-
Fig. 2. Non-specific adsorption of proteins on monolayers of the avidin mutants #1–5. (a) Test for protein adsorption to mutant #2. (b) The experiment in (a) was performed with mutants #1–5. The amounts of protein which remained bound at the end of the injections of BSA and goat IgG are shown. “BSA injection 2” and “IgG after BSA” for mutants #1–3 were reported before [7].

Fig. 3. Kinetics of human IgG2a binding to biotin–protein G on top of mutant #3. (a) Schematic binding to immobilized protein G in FC1 (red trace in (c)), (b) Schematic of the control injection in FC2 (blue trace in (c)). (c) Repeated association and dissociation of IgG2a, using 100 mM glycine (pH 2.7) for removal of IgG2a. The different IgG2a samples were prepared by serial dilution of 7 μM IgG2a (in PBS 7.3) with sample buffer (1 μM BSA in PBS 7.3). Double referencing [6,14] was used to obtain the experimental binding curves (solid traces in (d) and (e)). (d) The dotted lines show the best global fit of the Langmuir model to the experimental binding curves (solid lines). (e) Analogous fit as in (d), using the “bivalent analyte model”. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3 Biological interaction analysis on avidin mutant-functionalized chip surfaces

In a preceding study [6] we showed that biotinylated protein G and human IgG2a provide for a critical functional test of chip performance, for two reasons: (i) the interaction is multivalent in the

sense that one soluble IgG molecule is captured by two biotinylated protein G molecules on the chip surface (Fig. 3a). (ii) In this case, the repeated removal of IgG after each IgG injection (Fig. 3c) is typically performed with 100 mM glycine buffer (pH 2.7). GE Healthcare Data File 18-1012-91 AC, entitled “Affinity Chromatography: Protein G Sepharose 4 Fast Flow”, available at the internet from https://www.gehealthsciences.com/gehcs_images/GELS/Related%20Content/Files/1314774443672/litdoc18101291AC_20110831095008.pdf) which seems dangerously close to “rigorous regeneration” (Fig. 1a) with SDS/citric acid.

In Ref. [6] it was shown that the biotin–avidin–biotin bridges formed by mutant #1 were only affected by SDS/citric acid (pH 2.0) but not by glycine (pH 2.7). We now show that the same is also true for mutants #2 (Fig. 3c) and #3 (Fig. 3c). The avidin mutant under inspection was immobilized in both flow cells, FC2
was blocked with biotin–BSA (Fig. 3b) and FC1 was functionalized with biotin–protein G (Fig. 3a). After further passivation of both flow cells with biotin–BSA and BSA, different concentrations of IgG2x were injected and repeatedly removed with glycine (pH 2.7), as shown in Fig. 3c. FC2 was subtracted from FC1 and the resulting trace for sample buffer injection (Fig. 3c) was subtracted from all other injection traces (“double referencing method” [14]), resulting in the experimental binding curves (solid traces in panels (d) and (e), Figs. 3 and S4). The kinetic data could well be fitted by the “bivalent analyte model” (Figs. 3 e and S4e, dotted lines), which assumes binding of each IgG molecule by two adjacent protein G molecules on the chip surface (Fig. 3a), in contrast to the simple Langmuir model (Figs. 3 d and S4d, dotted lines) which assumes 1:1 binding.

The usefulness of all three avidin mutants (#1–3) for biological interaction analysis is proven by the good agreement of the calculated kinetic constants (Table 1), yielding averages with small standard deviations (last column). In spite of the less perfect fit, the Langmuir model has the advantage that it yields an effective $K_d$ value with the usual dimension “nM” (not “RU”), as in the bivalent analyte model, see Table 1, allowing for comparison with literature data. $K_d$ = 710 nM was reported from affinity adsorption of mixed human IgG [15]. The Fc fragment of human IgG1 gave $K_d$ values of 47 nM in BiAcore experiments on a CM5 chip [16] and 310 nM in a homogeneous fluorescence assay [17]. The discrepancy with $K_d$ = 2 nM in Table 1 is in part explained by the fact that bivalent interaction was only possible on our dense avidin monolayers which allow for close proximity of immobilized biotin–protein G (Fig. 3a). Obviously our chip is able to mimic the natural function of protein G, which is also present at high lateral density on the surface of Streptococcus sp. [18].

### Table 1

<table>
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<th>#3</th>
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#### 3.4. Sensitivity of avidin mutant-functionalized chips to acid or SDS

As mentioned above, the selective removal of IgG from protein G by 100 mM glycine (pH 2.7) in Fig. 3c seems rather close to the SDS/citric acid mixture (pH 2.0, Fig. S5) used for removal of all proteins at the end of Fig. 3c. A close look at the first IgG2x injection indeed shows that the subsequent injection of glycine (pH 2.7) leads to a lower baseline (~188 RU) than before injection of 200 mM IgG2x. Probably a small fraction of mutant #3 is more sensitive to pH 2.7 than the rest, being bound to only one biotin residue on
the biotin–SAM [19]. However, such baseline drift was not seen in the subsequent cycles with lower concentrations of IgG2k. Thus, such a baseline shift during the bait–prey interaction study can easily be avoided when applying a dummy injection of glycine (pH 2.7) before the first injection of IgG2k. The same observations were made with mutants #1 [6] and #2 (Fig. S4c).

Unfortunately, pH 2.7 is insufficient for removal of antigens from immobilized antibodies (or vice versa). Reported regeneration conditions range from pH 2.5 to pH 1.75 [1]. We, therefore, examined the resistance of the chip-bound avidin mutants #1–5 to low pH, using 2.5% citric acid without salt (pH 2.0) or with 150 mM NaCl (pH 1.9, Fig. S5). The results are shown in Figs. S6–S9. At pH 2, only mutant #1 showed stable binding to the biotinylated chip (red squares in Fig. S7b). Fortunately, the sensitivity to pH 2 was only seen if the avidin mutants were the only protein on the chip surface (state 2 in Fig. 1b). If biotinylated antibody was bound on top of avidin (state 3 in Fig. 1b) then all five avidin mutants were resistant to pH 2 (blue circles in Fig. S7b).

In spite of its lower pH (1.9, Fig. S5), the combination of citric acid with 150 mM NaCl caused much less removal of the avidin mutants from the biotinylated chip (Figs. S6–S9). A similar beneficial effect of elevated ionic strength is also expected for the 100 mM glycine buffers typically used for antibody–antigen separation.

Detergents are also used for removal of prey from bait [1]. Therefore, we tested the resistance of mutants #1–5 to 0.5% SDS at neutral pH (Figs. S10 and S11). Mutant #1 was very stable, while mutant #3 showed losses of ~5% (irrespective of whether biotin–lgG was bound on top of the avidin layer or not). Inclusion of NaCl afforded increased resistance to SDS.

The reported experiments with citric acid, NaCl, and SDS resulted in the following rules for “normal regeneration” (Fig. 1a) of chips functionalized with the tested avidin mutants:

i. The regeneration buffer (pH 1.9 or SDS) should have physiological ionic strength (e.g., 150 mM NaCl) because then much less avidin mutant (and biotinylated bait) is dissociated from the chip.

ii. Mutant #3 is most attractive because of low nonspecific adsorption, but this mutant will resist pH 1.9 only if it is crosslinked by a multiply biotinylated protein (sketch (d) in Fig. S7). Pronounced stabilization of avidin by such crosslinking has been demonstrated before [6]. This stabilization is high in case of IgG carrying 6–7 biotin residues on average (blue circles in Fig. S7b).

iii. The extent of crosslinking, and of stabilization at pH 1.9, will be much weaker in case of small proteins with few biotin residues and no stabilization is expected for baits with only one biotin residue. The latter situation is equivalent to a simple avidin monolayer (sketch (c) in Fig. S7). Here, only mutant #1 is sufficiently resistant to pH 1.9 (red squares in Fig. S7b). Fortunately, it is possible to eliminate nonspecific adsorption of protein on mutant #1 [6]. In case of DNA, however, it is necessary to use mutant #3 and other methods than pH 1.9 for “normal regeneration” (see Section 3.6).

iv. A small loss of avidin plus biotinylated bait will frequently be expected during the first regeneration round, even with milder regeneration at pH 2.7 (Fig. 3). Fortunately, no such loss is seen in subsequent injections. Therefore, a dummy injection with regeneration buffer should be performed before repeated binding and dissociation of prey molecules. Initial removal of the weekly bound avidin molecules has no adverse effects on subsequent measurement cycles of bait–prey interaction.

In conclusion, the choice of regeneration conditions and the choice of mutant #1 versus #3 must be made with care, in order to exploit the full potential of these mutants for regenerative biosensing.

3.5. How to prevent bait molecule immobilization in the reference cell

Label-free biosensing requires injection of the sample in the active cell with immobilized bait molecule and in a reference cell lacking the bait molecules (see Fig. 3a,b). Fig. 3c (and Fig. S12c) show that the bait (biotin–protein G) was only present in the sample cell if the reference cell was functionalized with bait–BSA prior to injection of biotin–protein G to the active cell. In contrast, pronounced contamination of the reference cell with biotin–protein G was observed if protein G was injected before bait–BSA (Fig. S12b). The reason lies in the design of microfluidic flow cells which allows for diffusion between the cells even if the flow is blocked in one cell (see Fig. S12a).

Fig. 4c shows another successful strategy by which bait immobilization is restricted to the active cell. Mutant #5 was first injected into the active cell only (FC2, blue trace in Fig. 4c), followed by injection of biotinylated bait (biotin-probe). In this situation, mutant #5 is still absent in the reference cell (FC1, red trace) and no bait–DNA can be immobilized in FC1, even if a trace of it diffuses into FC1. Subsequently, mutant #5 was injected into the reference cell (FC1, red trace), generating an inert surface where no DNA was bound in the next steps.

The protocol in Fig. 4c always ensures restriction of bait to the active flow cell. The simpler protocol in Fig. 3c is only applicable to large baits (such as proteins) which cannot bind on avidin after injection of bait–BSA.

3.6. Specific and nonspecific binding of DNA on avidin-mutant-functionalized chip surfaces

In a preceding study [7], mutants #1 and #2 exhibited high nonspecific adsorption of DNA, whereas only specific binding (hybridization, Fig. 4a) was seen on mutant #3. The same experiment was now performed on mutants #4 (Fig. S15) and #5 (Fig. 4c).

The sample cell was functionalized with avidin and biotinylated probe DNA, followed by avidin binding in the reference cell. Subsequently, absence of nonspecific protein adsorption was seen with BSA and absence of nonspecific DNA adsorption when injecting unlabeled probe DNA which had same nucleotide sequence as the biotinylated DNA on the chip surface (Fig. 4c). Subsequent injection of unlabeled analyte DNA gave pronounced hybridization in the sample cell (blue traces in Fig. 4c and d) and no nonspecific response in the reference cell (red traces in Fig. 4c and d). However, when the experiment was repeated using digoxigenin-labeled DNA (Fig. 4b) in place of unlabeled DNA (Fig. 4a), pronounced binding was observed in the reference cell (red trace in Fig. 4e). No such effect was seen with mutant #4 (Fig. S15) and mutants #1–3 (see below) even when using digoxigenin-labeled DNA.

Fig. 5 compares mutants #1–4 with respect to specific binding (hybridization) and nonspecific adsorption. The top row (a)–(c) concerns experiments with mutant #1. Panel (a) shows pronounced binding of analyte DNA, both in the active cell which contained the complementary biotinylated probe DNA (blue trace), and in the reference cell with the bare monolayer of mutant #1 (red trace). We suspected that the contribution of nonspecific DNA adsorption in the active cell (blue trace) was much lower than the purely nonspecific signal in the reference cell (red trace), because the positively charged mutant #1 was covered with negatively charged biotin-DNA in the active cell but not in the reference cell. This hypothesis was verified in panel (b) where the reference cell contained a degenerated oligonucleotide (biotin-N3P).
Fig. 5. DNA hybridization experiments performed on monolayers of mutant #1 (panels a–c) and of mutants #2, #3, and #4 (panels d–f), using the protocol of Fig. 4c. Red traces show adsorption of analyte DNA to the avidin mutants in FC1, blue traces the sum of specific and nonspecific binding in FC2 to avidin carrying biotin-labeled probe DNA. The green traces in (g), (h), and (i) were calculated by subtraction of the red traces from the blue traces in (d), (e), and (f), respectively. The analyte DNA always carried a digoxigenin label, except in (c). In (b) and (c) both flow cells were treated with biotin-N19T before injection of analyte DNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

while the active cell contained biotinylated probe DNA plus subsequently injected biotin-N19T. This resulted in low nonspecific binding not only in the reference cell (red trace) but also in the active cell (blue trace). Panel (c) confirms the beneficial effect of biotin-N19T and a very minor contribution of the digoxigenin label to nonspecific adsorption of digoxigenin-labeled DNA to mutant #1.

The center row (d)–(f) shows the performance of mutants #2–4 under the same conditions as used for mutant #1 in panel (a) (i.e., with digoxigenin-labeled DNA, in absence of biotin-N19T). Nonspecific binding of DNA on mutant #2 (red trace in (d)) was only slightly lower than on mutant #1. Nonspecific DNA adsorption was absent on mutants #3 and #4 (red traces in (e) and (f)). The small transient displacement of the red trace in panel (f) is due to a bulk effect (different refractory index of sample and running buffer). The findings are well explained by the high pI values of mutants #1 and #2 (Fig. 1e), which implies a positive net charge, and by the neutral pI of mutants #3 and 4, which eliminates electrostatic attraction of DNA.

The bottom row (g)–(i) shows the extent of specific binding (hybridization), as calculated by subtraction of nonspecific binding in the reference cell (red traces in (d)–(f)) from total DNA binding in the active cell (blue traces in (d)–(f)). The biphasic curve for mutant #2 (panel (g)) is caused by much higher nonspecific adsorption in the reference cell than in the active cell (as explained above for mutant #1). In contrast, panels (h) and (i) reflect the true hybridization signals on mutants #3 and #4, due to absence of nonspecific adsorption on both mutants (red traces in (e) and (f)). Mutant #3 exhibits a higher binding capacity than mutant #4, therefore it appears best suited for measurement of DNA-containing samples.

In this study we provide no method for the "normal regeneration" (Fig. 1a) of DNA-functionalized chips, i.e., for complete dissociation of analyte DNA without loss of biotinylated DNA. Mutant #3 is not sufficiently stable to use 100 mM HCl (as used in the "Biotin CAPTURE Kit") and the more acid-resistant mutant #1 strongly adsorbs DNA. The most promising reagent seems concentrated urea; it ensures complete dissociation of DNA duplexes [20], while avidin is known to resist 9 M urea without losing its biotin-binding capacity [21]. However, establishing the exact conditions will require a substantial amount of experiments in a future study.

4. Conclusions

All five tested avidin mutants are suitable for reversible immobilization of biotinylated baits on biotinylated sensor chips. The stably formed biotin–avidin–biotin bridges can be quantitatively dissolved when desired.

Mutant #1 showed the highest stability at pH 2 and the highest binding capacity for biotinylated bait molecules. Nonspecific binding of proteins was moderate, especially when biotin–BSA was used to passivate the reference cell and the unoccupied biotin-binding sites in the active cell. The major weakness of mutant #1 was high
nonspecific binding of nucleic acids. Passivation with degenerate biotin-NHS-T caused a strong reduction of DNA adsorption but did not eliminate it completely. In conclusion, mutant #1 may be ideal for biological interaction analysis between purified proteins, but it appears unsuitable for biosensing where nucleic acids are likely to be present. Mutant #2 resembles mutant #1 in all respects, except that all positive and negative aspects are somewhat moderated.

Mutant #3 showed slightly lower binding capacity and stability at pH 2, nevertheless it can well be used for interaction studies between antibodies and antigens where pH 2.3 is typically used for repeated removal of the analyte. The reason is that statistically biotinylated antibodies or antigens cause crosslinking of mutant #3, which confers high stability down to pH 2. This fact is important because mutant #3 appeared ideal in all other aspects: it showed the lowest nonspecific binding of protein and DNA and the highest performance in chip recycling with SDS/citric acid.

Mutants #4 and #5 exhibited also low nonspecific binding of protein and DNA. However, they were not stable at pH 2 and their binding capacity for biotinylated bait molecules was considerably lower than that of mutant #3. In conclusion, mutant #3 showed the best overall performance in chip recycling and application of recycled chips in biosensing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2016.02.039

References


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Hermann J. Gruber received his Ph.D. in chemistry from the Institute of Organic Chemistry at Karl–Franzens–University in Graz, Austria in 1983. He was postdoctoral fellow in the group of Prof. P. S. Low in the Biochemistry Division of the Chemistry Department at Purdue University, West Lafayette (1983–1985) and in the group of Prof. H. Schnidler at the Institute of Biophysics of Johannes Kepler University Linz, Linz, Austria (1985–1995), where he became assistant professor (1995–2001) and associate professor (since 2001) at the same institute. He has published >130 scientific articles. In particular, he established a toolbox of linkers and procedures for flexible tethering of single biomolecules to the measuring tip of force micro-
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The article


is enclosed is the following.*

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* My contributions include the following: I maintained the SPR measurement system and the chemical reagent library, co-supervised master students, labeled and purified proteins and peptides, functionalized SPR sensor chips, planned and performed SPR experiments (in particular for Fig. 4 and Fig. 5), analyzed and discussed data, and I assisted in reviewing and editing of the manuscript.
Label-free biosensors are ideally suited for the quantitative analysis of specific interactions among biomolecules or of biomolecules with drugs, as well as for quantification of diagnostic markers in biosamples. In contrast to the label-dependent methods, a new assay for a particular prey molecule can be set up within few minutes by immobilizing the corresponding bait molecule on the sensor surface, using one of the common immobilization procedures. Unfortunately, the widespread use of label-free biosensors is still hampered by the fact that the immobilization of the bait molecule is usually irreversible; for that reason, a new chip (which is expensive) is required for every successful or futile attempt. Here, we present a general method for the switchable immobilization of biotinylated bait molecules on a new desthiobiotin surface, using wild-type streptavidin as a robust bridge between the chip and the biotinylated bait. The immobilization of the bait is very stable, so that many cycles of prey injection and subsequent prey removal can be carried out. For the latter, common reagents like HCl, Na2CO₃, glycine buffer, or SDS are employed. When desired, however, streptavidin plus the biotinylated bait can be completely removed by 3 min injections of biotin, guanidinium thiocyanate, pepsin, and SDS, which makes it possible to immobilize new biotinylated bait. The number of in situ regeneration cycles is unlimited during the lifetime of the chip (2–3 weeks). One chip can easily be shared by many users with unrelated tasks (as is typical in academics), or used for the fully automated screening of many different interactions (for example in pharmaceutical research). In comparison to other regenerative chips, the new chip surface has much wider applicability and all of its structural and functional parameters have been disclosed.
avidin-biotin bridge was no longer perfectly stable under conditions typically used for prey removal (Zauner et al., 2016). Unfortunately, this kind of avidin mutant had serious disadvantages; the high stability of avidin M96H was associated with high non-specific adsorption, whereas multiply mutated avidin, which had a low non-specific adsorption, was not sufficiently stable, and therefore could not be used for routine application in biosensing.

The goal of the present study was to find a solution which simultaneously provides for (i) low non-specific adsorption, (ii) high stability of the biotin-avidin-biotin bridge under typical working conditions, and (iii) complete rupture of the biotin-avidin-biotin bridge when this was desired. This goal was achieved by using wild-type streptavidin in place of the avidin mutants (see Scheme 1A). The advantages of streptavidin are its low $p_I$, its low non-specific adsorption of DNA and protein, and its robustness under a wide range of buffer conditions (Green, 1990). The major difficulty was in finding a method for switching off the robust bond between streptavidin and the biotin chip. Initial attempts on the biotin-SAM of Pollheimer et al. (2013) were unsuccessful, even when extreme denaturants were used (see section S8 in the Supplementary Information). The replacement of biotin by iminobiotin was not an option because the affinity of that latter substance for streptavidin is too weak (Raphael et al., 2011). Although desthiobiotin initially appeared more promising, in all published examples (Knoll et al., 2000; Yoon et al., 2001; Hirsch et al., 2002) the dissociation of streptavidin from desthiobiotin surfaces was rather incomplete, even after hours of washing with biotin-containing buffer.

The aforementioned desthiobiotin surfaces are unfavorable for biosensing because they are not protein-resistant. PAMAM particles as used by Yoon et al. (2001) are known to cause non-specific adsorption of proteins (Matsumoto et al., 2011). The same applies to the desthiobiotin-SAM used by Knoll et al. (2000) which is depicted in Fig. 1A. Ninety percent of this SAM consisted of mercaptoundecanol (1), which strongly adsorbs proteins (Prime et al., 1991).

In a recent study, we replaced the desthiobiotin-SAM of Fig. 1A with the new desthiobiotin-SAM shown in Fig. 1B. It exhibits the same high protein resistance as the analogous biotin-SAM and allows for complete regeneration according to Scheme 1A (Gruber, 2017). As shown below in much greater detail, this strategy enabled us to accomplish all of our desired goals: (i) very low non-specific adsorption of protein and nucleic acids, (ii) stable binding of streptavidin and biotinylated baits for as long as was desired, and (iii) complete removal of streptavidin and biotinylated baits in a matter of minutes when a novel washing protocol was employed.

2. Materials and methods

2.1. Materials

The matrix alkanethiol derivative (MAT, 3) was synthesized as described (Pollheimer et al., 2013). The desthiobiotin alkanethiol derivative (DBAT, 4) was synthesized and mixed with MAT under reducing conditions, as described in the Supplementary information (Charts S1 and S2, respectively). Most of the buffers, proteins, and nucleic acids were the same as the ones used in the previous studies with switchable avidin mutants (Zauner et al., 2016). A detailed description of their preparation is given in the Supplementary information (section S3). The same also applies to the additional components used in Section 3.7.

Biotin (200 mM, pH 8–8.5) was prepared by dissolving 1 mmol biotin and 1.3 mmol Tris base in water in a final volume of 5 ml and then, if needed, correcting the pH (according to pH paper) by further addition of Tris base. It was divided into single-use aliquots and stored at $-25^\circ C$. Guanidinium thiocyanate (GTC, 6 M) was prepared by dissolving 7.09 g GTC in 4.28 ml water and stored at room temperature, whereby exposure to light was avoided (CAUTION: GTC must not be mixed with acid because this will result in the release of toxic HSCN gas!). Pepsin (2 mg/ml) was dissolved in 1 M glycine (pH 2.5 adjusted with HCl) and stored for 2 weeks at 4 °C. SDS (0.5%) was dissolved in water and kept at room temperature.

2.2. Surface plasmon resonance experiments

Cleaning of the bare glass chips, evaporation of the chromium (3 nm) and the gold (41 nm), as well as the cleaning of the gold surface and the coating with the mixed SAM was performed as described (Pollheimer et al., 2013), except that the biotin alkanethiol derivative (BAT, Chart S4) was replaced by DBAT (Fig. 1B). Details are found in the Supplementary information (Section S2). The chips were mounted on the chip supports with double-sided adhesive tape (non-permanent) and inserted into a BIACore X® device for measurement of binding by surface plasmon resonance (SPR). Filtered (0.45 μm) and degassed buffer was run over the chip surface, typically at 20 μl/min, as stated in the figure legends. The resonance angle was recorded at 1 s intervals and expressed in resonance units (1 RU = 0.0001°).
In most experiments the desthiobiotin chip was first covered with a monolayer of streptavidin (injected at 2 µM concentration for 3 min) in both flow cells. Then, a biotinylated bait or biotinylated bovine serum albumin (biotin-BSA) was separately applied to flow cell 1 (FC1) or flow cell 2 (FC2), as specified in the figures. The prey molecules were injected in both flow cells, whereby the sample buffer and the running buffer usually contained a low concentration of BSA, as is stated. All experiments were terminated by complete chip regeneration, typically by sequential injections of free biotin, guanidinium thiocyanate (GTC), pepsin, and SDS, as is specified.

3. Results and discussion

3.1. Reversible immobilization of streptavidin on the mixed desthiobiotin-SAM

The new desthiobiotin component (DBAT, 4) was synthesized, mixed with a 4-fold molar amount of matrix component (MAT, 3) under reducing conditions (Chart S2), and used to prepare the mixed SAM depicted in Fig. 1B. The binding capacity for streptavidin was high (2340 ± 70 RU, Fig. 1C). Initially, we tried to remove bound streptavidin with guanidinium chloride (6 M), or HCl (100 mM), or SDS, but most of the streptavidin remained bound (data not shown). Guanidinium thiocyanate, however, caused complete removal within a few seconds, as can be seen from the initial spike of each GTC injection (Fig. 1C). Binding and desorption of streptavidin could be repeated with perfect reproducibility, as demonstrated in Fig. 1C.

3.2. Reverse immobilization of biotinylated bait molecules

GTC was able to remove a streptavidin monolayer (Fig. 1C and red trace in Fig. 1D) but not the double layer formed between biotinylated IgG and streptavidin (blue trace in Fig. 1D). Fortunately, complete removal of all the protein molecules was achieved during the subsequent 3 min injection of pepsin (blue trace in Fig. 1D). As a consequence, the chip could repeatedly be functionalized with streptavidin and biotinylated IgG, whereby the extent of protein immobilization was fully reproducible (Fig. 1C).

The need for pepsin is explained by the illustration in Scheme 1B.

At the given desthiobiotin density (20 mol-%) on the chip, streptavidin binds two desthiobiotin groups, while the two remaining binding sites are available for biotinylated baits (Jung et al., 2000). At the same time, the biotinylated IgG carries several biotin labels per protein. Consequently, a network is formed by the two proteins on the chip surface; it can only be dissolved by digestion with a protease. This behavior was not only observed with biotin-IgG (Figs. S1-S2, see Supplementary information) but also with biotin-BSA (Fig. S3) and biotin-protein G (Fig. S5). In contrast, no pepsin was required for the removal of mono-biotinylated BSA (Fig. S4), because no crosslinking is possible if the bait carries a single biotin residue (see Scheme 1C). Together, these findings indicate (i) that GTC is only able to dissociate desthiobiotin but not biotin groups from the streptavidin on the chip surface; it can only be dissolved by digestion with a protease. (ii) The GTC, the base and the acidic TCEP are available for biotinylated baits (Jung et al., 2000). At the same time, it makes sense to first denature the protein by GTC, which makes it more vulnerable to the protease. (iii) In the optimized protocol (Fig. 2), the initial biotin injection must be followed by GTC (see chapter S7), so that pepsin and SDS serve as backups for the removal of the last remnants.

Admittedly, GTC was able to remove a fraction of biotin-IgG when it was mixed with triis(2-carboxyethyl)phosphine (TCEP, see blue trace in Fig. 1D). The combination of GTC and TCEP was much more effective than either reagent alone (Fig. S1), yet even repeated injections never achieved complete removal of the biotin-IgG and the streptavidin (Fig. S1). In the end, TCEP was eliminated from the list of regeneration reagents for the following reasons: (i) TCEP can only enhance the decomposition of proteins like IgG, where multiple subunits are connected via disulfide bridges. (ii) The TCEP, the base and the acidic TCEP hydrochloride must be mixed immediately before the injection, which is inconvenient and cannot be done in standard autosamplers. (iii) The addition of acidic TCEP to GTC before the base will result in the decomposition of proteins like IgG, where multiple subunits are connected via disulfide bridges.
3.3. The roles of free biotin, GTC, pepsin, and SDS in rapid chip regeneration

As mentioned in the introduction, several studies described the use of free biotin for removal of (strept)avidin from desthiobiotin surfaces (Knoll et al., 2000; Yoon et al., 2001; Hirsch et al., 2002) but the extent of removal was always well below 100%, even after extensive washing with free biotin at concentrations ranging from 0.1 to 50 mM. Chip regeneration protocols are only useful if the complete regeneration occurs within a few minutes. For this reason, we tested whether much higher concentrations of free biotin can remove streptavidin and biotinylated proteins from the desthiobiotin chip on this short time scale. Indeed, almost complete removal of streptavidin and biotin-BSA occurred during a 3-min injection of 200 mM biotin at pH 8 (Fig. 2), and the minor remnants were removed by a 3-min injection of GTC. In Fig. 2, the subsequent injections of pepsin and SDS appeared unnecessary but later it turned out that this is not generally true (see Fig. 3B). The only protocol which never failed was the sequence "biotin, GTC, pepsin, and SDS", and therefore it was adopted as the standard regeneration protocol.

Biotin concentrations above 200 mM (or longer washing with 200 mM biotin than for 3 min) turned out to be counterproductive, because free biotin progressively binds to the SAM with progressive tightness (Figs. S8-S10). The adsorption of free biotin was fully reversed by 6 M GTC, but only if ≤200 mM biotin was injected for ≤3 min (as in Fig. 2), and not after more extensive application of free biotin (Figs. S8-S10).

The dramatic effect of 200 mM biotin in Fig. 2 indicates that this high biotin concentration is able to dissociate not only the desthiobiotin-streptavidin bonds but also the biotin-streptavidin bonds which form the network depicted in Scheme 1B. It came as a great surprise that 200 mM biotin can do this, while 6 M GTC cannot (see Fig. 1D). We therefore examined whether 200 mM biotin (pH 8) acts by a double mechanism: (i) specific competition for biotin-binding sites and (ii) general denaturation of protein folding.

The latter aspect was tested by measuring the melting transition (ΔT_m) of myoglobin in 100–500 mM biotin (pH 8) and parallel comparison with the effect of different urea concentrations (Figs. S6–S7). In these experiments, 200 mM biotin had the same effect as 1.5 M urea (ΔT_m = −5 °C), and 500 mM biotin was equi-effective with 3 M urea (ΔT_m = −12 °C). The data indicate that free biotin is a strong denaturant when used at high concentration. These findings can be explained by the structural features of biotin: (i) At pH 8, biotin has a detergent-like structure, with a long hydrophobic chain and a carboxylate anion. (ii) Thioether structures are known to undergo enhanced interaction with proteins, as exploited in thiophilic adsorption chromatography (Porath et al., 1985). (iii) The ureido structure of biotin resembles urea, which is known for its denaturation potency. At 200 mM biotin, the synergy between the three structural aspects appears to cause a general destabilization of streptavidin, leading to the accelerated replacement of desthiobiotin and the biotinylated bait by free biotin.

The efficient dissociation of biotinylated baits from streptavidin by 200 mM biotin nourished hopes that high concentrations of free biotin might be able to remove streptavidin from the analogous biotin-SAM (Chart S4). Unfortunately, this idea turned out to be wrong. Although free biotin was much less effective than GTC and pepsin on the regular biotin-SAM, the latter reagents were also unable to remove all of the proteins from the biotin-SAM (Figs. S11-S12).

In conclusion, only the desthiobiotin-SAM (and not the analogous biotin-SAM) turned out to be fully regenerative, and the regeneration protocol in Fig. 2 was found to be superior to all of the alternatives concerning reagent concentrations and injection times.

3.4. Low non-specific adsorption of protein and nucleic acids

Fig. 3A reveals that the desthiobiotin-SAM depicted in Fig. 1B meets an essential criterion for practical application in biosensing: low non-specific adsorption of protein, both before and after the binding of streptavidin. The first injection with SDS caused no positive or negative change of the baseline, which means that SDS did not bind and that nothing had been adsorbed on the chip before this injection. Injection of a high BSA concentration showed only a transient bulk effect but also did not result in binding. The same was true for the injection of biotin-blocked streptavidin in flow cell 1 (FC1, see red text in Fig. 3A). Unblocked streptavidin, however, was bound with high efficiency in both flow cells (2430 and 2390 RU in FC1 and FC2, respectively). Subsequently, lysozyme and BSA (2×) were injected at high concentrations (1 mg/ml), resulting in almost undetectable levels of non-specific adsorption (see numbers in Fig. 3A). The final injection of goat IgG (1 mg/ml) gave slightly higher adsorption levels, but the extent of ≤25 RU corresponds to less than 1% of a dense IgG monolayer (compare Fig. 1D) and still falls into the category of exceptionally low non-specific adsorption (Ostuni et al., 2001).

Non-specific adsorption of DNA was also tested and found to be insignificant (Fig. S13). At the same time, a biotinylated oligonucleotide (a 30-mer) was seen to bind on top of streptavidin with high efficiency (420 RU) and to hybridize with the complementary single-stranded DNA (see Fig. S13).

3.5. Stability of the biotin-streptavidin-biotin bridge during prey removal

Conventionally, bait molecules are immobilized on the chip surface by covalent coupling (Cooper, 2009). In this context, the term "chip...
regeneration” means that all bound prey molecules are removed from the chip, making it ready for the next injection of a prey-containing sample (see Scheme 1A). Typical methods for prey removal make use of short pulses of HCl (1–100 mM), glycine buffer (pH 1.7–2.5), NaOH (6–100 mM), SDS, or denaturants, as reviewed recently (Goode et al., 2015).

Although our new chips allow for “full regeneration” of the protein-free desthiobiotin surface (Scheme 1A), it is very important that we also have the option of performing many cycles of prey binding and prey removal (see Scheme 1A). The characterization of a biospecific interaction requires many injections of the same prey at different concentrations during all of which the biotinylated bait should remain firmly immobilized on the streptavidin surface (see Figs. 4 and 5). On a conventional chip with covalently bound baits, pulses of acid or base or SDS can only cause denaturation of the bait but the bait cannot get lost. On our desthiobiotin chips, however, streptavidin and the biotinylated bait are bound via non-covalent bonds, therefore it was essential to find out whether these persist while using common methods of prey removal.

Here we tested the stability of streptavidin-bound biotin-IgG during applications of 15 s pulses of HCl (Fig. 3B), sodium carbonate (Fig. S14), NaOH (Fig. S15), and glycine buffer (pH 2.5, Figs. 4 and 5 and S16-S17). The different HCl (or NaOH) concentrations were prepared by mixing 100 mM HCl (or 100 mM NaOH) with 100 mM NaCl, so that the ionic strength remained uniform in spite of varying HCl (or NaOH) concentrations. The different sodium carbonate buffers were prepared by adjusting a 100 mM Na₂CO₃ solution to pH 11.0, 10.5, or 10.0 with concentrated HCl.

The double layer of streptavidin and biotin-IgG was resistant to 10–100 mM HCl (Fig. 3B, blue solid trace), while the single layer of...
streptavidin was only fully stable at 10 mM HCl (red dashed trace). These findings leave us with two options for prey removal by HCl: (i) the use of 10 mM HCl, or (ii) the stabilization of the streptavidin layer in the control cell with an inert biotinylated protein (e.g., biotin-BSA), which enables us to use up to 100 mM HCl for bait removal.

Sodium carbonate buffers (pH 10–11) also proved to be well applicable for prey removal (Fig. S14). A minor unexpected response was the small increase in the resonance angle of the streptavidin monolayer, which was regularly seen after the first injection of basic buffer (red trace in Fig. S14). Nevertheless, sodium carbonate can readily be used for bait removal if a dummy injection with carbonate is applied before the first injection of the bait; if this is done, the baseline reaches a new stable value.

NaOH (10–100 mM) appears unsuitable for bait removal, since it causes a significant loss of the chip-bound streptavidin (see Fig. S15). The unfavorable effect of 10 mM NaOH (pH 11) is obviously due to its low ionic strength, because 100 mM sodium carbonate (pH 11) caused no undesired bait removal (Fig. S14).

Perfect stability of the chip-bound bait was found when 1 M glycine (pH 2.5, for 3 min) was used for bait removal (Fig. S16). On the other hand, small losses were seen when 100 mM glycine (pH 2.5, for 3 min, Fig. S17) was employed. These observations confirm the hypothesis that high ionic strength protects chip-bound baits from being dissociated by the reagent that is used for prey removal. The same effect had consistently been found when avidin mutants were used for the immobilization of biotinylated baits on biotin-SAMs (Zauner et al., 2016).

It should be mentioned that 0.5% SDS can also be applied for the removal of the prey from streptavidin-bound bait so long as that prey is not denatured by SDS. We found this method to be applicable for the calmodulin-binding peptide; the removal of calmodulin by glycine (pH 2.5, Figs. 5A and S17) could also be performed with 0.5% SDS (data not shown).

In conclusion, the desthiobiotin-streptavidin-biotin bridge between the chip surface and the bait molecule is resistant to the reagents like HCl, Na2CO3, or SDS which are commonly used for in the sense of “prey removal” (see Scheme 1A). Hereby, an ionic strength of ≥ 200 mM was found to be advantageous. As a consequence, a chip with an immobilized biotinylated bait can be used in the same way as a chip with a covalently bound bait. Only when desired, the chip-bound streptavidin plus bait is removed by the “full regeneration” method that is illustrated in Scheme 1A and exemplified in Fig. 2.

3.6. Measurement of the interaction between protein G and human IgG2ε

The reversible binding of human IgG2ε to chip-bound biotin-protein G had previously been measured to test the performance of regenerative chips which relied on switchable avidin mutants (Pollheimer et al., 2013; Zauner et al., 2016). In our study, the same test was applied to the new desthiobiotin chip where wild-type streptavidin was used for immobilization of biotinylated protein G.

The entire experiment is presented in Fig. S16. Both cells were functionalized with streptavidin, whereby biotin-BSA was immobilized in the control cell (see Fig. 4B) and biotin-protein G was bound in the sample cell (see Fig. 4A). After a dummy injection of 1 M glycine (pH 2.5), many different concentrations of IgG2ε were injected; 1 M glycine (pH 2.5) was used for prey removal after each injection. The data were processed by the double referencing method (Richard and Myszka, 2000; Pollheimer et al., 2013), yielding the experimental bindings curves which are shown in both Fig. 5C and D as solid colored curves.

A perfect fit of the binding curves was obtained with the “bivalent analyte model” (dashed traces in Fig. 4C), which assumes two binding steps: pre-binding of one IgG2ε to one protein G molecule, followed by bivalent binding of IgG2ε to two adjacent protein G molecules on the streptavidin surface (see Fig. 4A). The kinetic and equilibrium constants obtained from the fit were quite similar to those previously obtained on chips covered with avidin mutants instead of streptavidin (Zauner et al., 2016). As previously found on avidin surfaces, it was impossible to fit the binding curves with the simple Langmuir model, which assumes only 1:1 binding between soluble IgG2ε and streptavidin-bound protein G (dashed traces in Fig. 4D).

In conclusion, the test with immobilized protein G and soluble IgG2ε demonstrates that the streptavidin monolayer on the regenerative chip is well suited for the reconstitution of the interaction mechanism between native protein G of streptococcus sp. and IgG molecules. Since Protein G is also found at high density on the surface of the microorganism (Fahnstock et al., 1986), the binding mechanism described in Fig. 4 is likely to also occur in vivo.

3.7. Measurement of calmodulin-peptide interactions

Fig. 5 presents two alternative strategies for studying the specific interaction between calmodulin (CaM) and the CaM-binding segment of smooth muscle myosin light chain kinase (smMLCK). These two proteins are known to interact in a simple 1:1 fashion, with a Kd of ~1 nM (O’Neil and DeGrado, 1995).

In the first approach, the CaM-binding peptide was anchored to the streptavidin monolayer by a C-terminal bait group (Fig. 5A), and soluble CaM was injected at different concentrations in presence of 2 mM Ca2+ (Fig. 5C). Interestingly, the immobilization of the peptide was much more efficient if maleimide-PEG21-biotin was first bound to streptavidin and the peptide was then covalently coupled to the maleimide group (see Chart S5 and Fig. S17). This is very fortunate, because in situ-coupling of a thiol-peptide (or of thiol-DNA) is much faster and easier than biotinylation in solution and purification of the biotinylated peptide by reversed phase chromatography. The complete SPR experiment (with the coupling step) is shown in Fig. S17. The resulting binding curves (solid traces in Fig. 5C) could readily be fitted to a simple 1:1 binding model (dashed traces in Fig. 5C) with a Kd value of 0.4 nM.

In the complementary approach (Fig. 5B), CaM was first labeled with biotin, purified by gel filtration, immobilized on the streptavidin surface, and then probed with different concentrations of nearly the same smMLCK peptide (it lacked only the C-terminal cysteine). The corresponding binding curves (solid traces in Fig. 5D) could also be fitted to the simple 1:1 binding model (dashed traces in Fig. 5D), yielding the same Kd value (0.4 nM) as with the immobilized peptide in Fig. 5C.

3.8. Quantification of antigens in crude biofluids

Above, the new regenerative chip was shown to be very useful for biological interaction analysis with purified components. In parallel it was interesting to find out whether the new chip can be used for quantification of analyte molecules in crude biofluids, preferably by the standard addition technique.

For comparison we chose a study where a monoclonal anti-His6 tag antibody had been covalently coupled to a CMS chip at an immobilization level of >13600 RU and a linear dose-response (from 45 to 1850 RU) of GFP-His6 binding had been reported between 3 and 170 nM antigen concentration, as needed for standard addition (Ekström, 2012).

In our experiments (section S13 in the Supplementary Information) we used His6-tagged enhanced GFP (His6-eGFP) as the analyte and a biotinylated monoclonal anti-GFP antibody as the bait molecule. As expected for our two-dimensional surface, the immobilization level of the biotinylated antibody was much lower (2508 RU, Fig. S20) than that on the CMS chip. Accordingly, the linear regime of the dose-response to the antigen was less pronounced (from 3 to ~25 nM) and the responses for these concentrations of antigen were much smaller (from 6 to 48 RU, Fig. S22). When potential matrix effects on the dose
response were ignored, then the comparison of the calibration curve (Fig. S22) with the dose-response of crude lysate (Fig. S23) allowed for estimation of antigen concentration with an accuracy of ~20% (Fig. S24). Explicit determination of the matrix effect by the standard addition method was, however, precluded by the narrow range of the linear dose-response on our chip.

Overall the results with serum and bacterial lysate were much better than what we had dared to expect. For the following reasons, we find them very encouraging: (i) On antibody-functionalized surfaces, the non-specific adsorption from crude biofluids was surprisingly low. (ii) The problem of low antibody immobilization levels and low dose-responses to antigen will become irrelevant when label-enhanced SPR is employed, since the signal-to-noise ratio is about 100-fold better there than in label-free SPR (Eng et al., 2016; Granqvist et al., 2013). In addition, label-enhanced SPR amplifies only the specific response but not the non-specific responses. For these reasons, we think that it could be very well possible to exploit the potential of our regenerative chip surfaces not only for biological interaction analysis but also for quantification of analytes in crude biofluids.

4. Conclusions

The new desthiobiotin-SAM shows the same high protein resistance and binding capacity for streptavidin as previously found for the analogous biotin-SAM (Pollheimer et al., 2013). In contrast to the latter, however, the new desthiobiotin-SAM can be re-used with different bait molecules as often as desired within a period of 2–3 weeks. Even tenaciously bound double layers consisting of streptavidin plus biotinylated proteins are completely removed by 3 min injections of biotin, GTC, pepsin, and SDS, without any negative impact on the chip performance.

Fortunately, streptavidin and the biotinylated bait can only be removed by the reagents named above, while no loss of biotinylated bait is observed with most of the reagents (HCl, Na2CO3, and SDS) that are commonly used for removal of prey molecules. This means that numerous cycles of prey binding and prey removal can be performed with one type of biotinylated bait.

Due to the fact that it makes it possible to strictly distinguish between mere prey removal on the one hand, and rigorous removal of streptavidin along with the bait on the other hand, the new desthiobiotin chip appears to be ideal for the rapid testing of numerous bait-prey interactions. Its costs are very low, and it can be employed in a fully automated manner.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2017.07.033.

References


The book chapter

Reversible immobilization of biotinylated baits on regenerative sensor chips: comparison of switchable avidin mutants with wild-type streptavidin.

is enclosed is the following.*

My contributions include the following: I maintained the SPR measurement system and the chemical reagent library, co-supervised master students, labeled and purified proteins and peptides, functionalized SPR sensor chips, planned and performed SPR experiments, analyzed and discussed data, and I assisted in reviewing and editing of the manuscript.
Chapter 1

Reversible immobilization of biotinylated baits on regenerative sensor chips: comparison of switchable avidin mutants with wild-type streptavidin

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1. Introduction

1.1 Label-free biosensing

Label-free biosensors are convenient tools for the quantitative analysis of specific interactions between biomolecules [1] as well as for the quantification of specific analytes in biofluids such as serum or lysates of cultured cells [2]. First, one of the interacting molecules (the "bait") is immobilized on the sensing surface by a standard protocol and the cognate molecule (the "prey") is injected at different concentrations. Thereby, the binding of the bait is quantitatively monitored in real time by a label-free readout such as surface plasmon resonance (SPR), a surface acoustic wave (SAW) device, or the quartz crystal microbalance (QCM) [3-5]. Label-free detection methods cannot discriminate between (i) the specific binding of the prey to the immobilized bait and (ii) the non-specific adsorption of a sample component to the sensor surface; therefore, it is essential that non-specific adsorption be suppressed by immobilizing the bait on a protein-resistant sensing surface.

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Conventionally, the bait molecules are covalently coupled to carboxymethyl- dextran-coated chips or other protein-resistant surfaces [6]. A convenient alternative is the immobilization of biotinylated bait molecules on streptavidin-coated chips; less bait is required for that procedure and the protocol is much simpler. Both immobilization methods are essentially irreversible. This is an advantage if a chip functionalyzed with one particular bait is to be used for the screening of a large number of samples with the same prey molecule.

**Fig. 1.** Comparison of the mixed self-assembled monolayers (SAMs) used for switchable immobilization of avidin mutants or of streptavidin, respectively. (A) A SAM with 20% biotin group density was employed for immobilization of acid-sensitive M96H-mutants of avidin in which methionine-96 had been replaced by histidine [7-9]. (B) A SAM with 20% desthiobiotin group density was required for switchable immobilization of wild-type streptavidin [10, 11].

### 1.2 Methods for reversible immobilization of biotinylated bait molecules on sensing surfaces

For the quick measurement of many different bait-prey interactions, it would be desirable to replace the immobilized bait with another bait molecule with the help of simple washing steps inside the biosensor device. Three methods have been developed in which biotinylated bait molecules can be replaced by new baits by means of such washing steps.

In the Biotin CAPture Kit™ (GE Healthcare Product No. 28920233), a single-stranded DNA is covalently coupled to the carboxymethyl-dextran chip and, in parallel, the complementary DNA strand is coupled to streptavidin. Streptavidin is then immobilized on the chip by DNA double strand formation, and after that the biotinylated bait molecule is bound to streptavidin. When the desired number of bait-prey interactions has been measured, the chip is washed with a pulse of 100 mM HCl, whereupon the DNA double strand is denatured and streptavidin is removed along with the immobilized bait. The chip shows excellent reproducibility, but it is not applicable to DNA-binding proteins, its binding capacity is not higher than that of flat chip surfaces, it is only available for one brand of biosensors, and its molecular parameters have not been disclosed. Moreover, the 100 mM HCl pulse that is used for the complete regeneration
of the chip is not very different from typical methods employed for the selective removal of prey molecules between the successive injections of prey-containing samples [12]; therefore, there is a risk of undesired loss of immobilized baits in an ongoing measurement series.

Fig. 2. Illustration of the reversible immobilization of biotinylated bait molecules on biotin-SAMs or desthiobiotin-SAMs. (A) Distinction between conventional regeneration (termed "prey removal") and "full regeneration" of the sensor chip (removal of streptavidin plus biotinylated bait). Avidin mutants (M96H ± further mutations) can be dissociated into non-functional monomers (state 5a [9]) whereas wild-type streptavidin remains tetrameric (state 5b [11]). (B) The removal of the network formed by streptavidin and a statistically biotinylated protein requires guanidinium thiocyanate (GTC) plus pepsin [11]. (C) A mono-biotinylated protein can be removed with GTC alone [11]. Reproduced with slight modification from ref. [11] (creative commons license CC BY).

In the second method, a self-assembled monolayer with a 20% biotin group density (Fig. 1A) is used as a sensing surface and the biotinylated bait molecules are immobilized by the formation of a biotin-avidin-biotin bridge, whereby a switchable mutant of avidin is employed (see Fig. 2A) [7-9]. Fortunately, the biotin-avidin-biotin bridge is resistant to most of the methods that are used for the selective removal of prey from immobilized bait (states 3 and 4 in Fig. 2A), which allows for many successive tests for the prey of interest. Only when desired is the biotin-avidin-biotin bridge dissociated by a mixture of sodium dodecyl sulfate (SDS) and citric acid, (state 5a in Fig. 2A). Thereby the bare biotin-SAM is recovered (state 1) for a new round of functionalization with the avidin mutant and another kind of biotinylated bait molecule (states 2 and 3).
The third method is similar to the second one, with two distinct differences: the biotin-SAM (Fig. 1A) is replaced by the analogous desthiobiotin-SAM (Fig. 1B), and wild-type streptavidin is used in place of a switchable avidin mutant (see Fig. 2A, state 5b) [10, 11]. In this chapter, methods No. 2 and 3 are compared with respect to their performance and the critical parameters are discussed in sufficient detail to give potential users full insight into the relevant practical aspects.

2. Mechanism and parameters of reversible immobilization of biotinylated baits via switchable mutants of avidin

The binding of wild-type avidin or streptavidin to biotinylated surfaces is essentially irreversible. Even very strong denaturants such as 6 M guanidinium thiocyanate were seen to remove only a fraction of streptavidin that was bound on the biotin-SAM shown in Fig. 1A [11]. The only method that has been reported to bring about the quantitative removal of streptavidin from immobilized biotin residues is treatment with distilled water at temperatures above 70°C [13]. Such heating cannot, however, be used in typical biosensor flow cells. Therefore, no aqueous reagent has yet been found which can remove wild-type avidin or streptavidin from biotinylated sensor chips while they are mounted in the biosensor device.

In this situation, we were faced with the choice of modifying either the biotin groups on the sensor surface or the (strept)avidin molecules which are used for immobilization of biotinylated baits on biotinylated surfaces (see Fig. 2A). The modification of the biotin groups on the sensor chip is described in section 3 (below), and the modification of avidin is presented in this section.

A well-known method for lowering the affinity of avidin for biotin is the nitration of tyrosine residues [14, 15]. We therefore tested this so-called "captavidin" on the biotin-SAM shown in Fig. 1A, but the affinity turned out to be too low for stable binding on the chip, and the binding capacity for biotinylated antibodies was also rather low [7]. We then considered using avidin mutants in which hydrophobic amino acids positioned at the subunit-subunit interfaces had been replaced by histidine residues [16]. The imidazole residue of histidine can be protonated by acid, resulting in a positive charge which leads to the dissociation of the avidin tetramer into four separate subunits at low pH [16]. Among all of the histidine mutants, avidin M96H (with a histidine instead of methionine at position 96) required the lowest pH for dissociation of the tetramer [16]. For this reason, we chose avidin M96H as the most promising candidate for the stable immobilization of biotinylated baits on a biotinylated chip [7], as illustrated in Fig. 2A.

In our first tests, avidin M96H was bound to the biotin-SAM shown in Fig. 1A. Then, 1% citric acid (pH 2.2) was injected, whereupon only a small fraction of the avidin mutant was removed [7]. Even less removal of avidin M96H was seen when phosphate-buffered saline (PBS) containing 0.5% SDS was injected [7]. In contrast, a rapid and complete dissociation of chip-bound avidin M96H was achieved when a mixture of SDS and unbuffered citric acid (pH 2.0) was injected [7]. This discovery enabled the
repeatable functionalization of a biotin-SAM with biotinylated baits, as illustrated in Fig. 2A. The corresponding SPR data are shown in Fig. 3A.

**Fig. 3.** Repeated immobilization of (strept)avidin with and without biotinylated antibody on a (desthio)biotin chip. (A) A chip with 20% biotin density (Fig. 1A) was treated with avidin mutant #3 (termed "switchavidin" [8]) in both flow cells. Biotin-IgG was immobilized in flow cell 2 (FC2, blue trace) and both proteins were repeatedly removed with a mixture of SDS and citric acid. Three consecutive experiments performed on the identical chip are shown on the coherent time scale, with pauses of 2708 s and 52 s between the runs [17]. (B) A chip with 20% desthiobiotin density (Fig. 1B) was used for the repeated immobilization of streptavidin (± biotin-IgG) and was regenerated by consecutive injections of guanidinium thiocyanate (GTC, 6 M, with 4 mM TCEP), pepsin (2 mg/ml in 1 M glycine, pH 2.5), and SDS (0.5%). Panel B was reproduced with slight modifications from ref. [11] (creative commons license CC BY).
Initially, PBS was run over the biotin-SAM, and SDS (0.5%) was injected to remove potentially adsorbed protein or impurities from the chip surface. Avidin M96H (with additional mutations that lower the pI to 7.0 (see the third row in Table 1) was injected in both flow cells. That resulted in the formation of a dense monolayer, as was indicated by the high SPR response (2416 ± 8 RU and 2467 ± 11 RU in flow cell 1 and 2, respectively) [18]. Subsequently, biotin-IgG was only injected into flow cell 2 (FC2, blue trace), resulting in a binding response of 2109 ± 66 RU. Finally, the mixture of SDS and citric acid was injected into both flow cells, followed by a pulse of SDS. The last two injections resulted in the complete removal of avidin and biotin-IgG from the biotin-SAM, as was shown by the return to the original baseline (see green dotted line). In Fig. 3A, the experiment was repeated three times and proved to be fully reproducible. In practice, the performance of the chip was found to remain constant for at least two weeks of continued operation (including rinsing at 5 µl/min with 5 mM NaN₃ and 100 mM NaCHO₃, pH 8.5, overnight and over the weekend). The same good performance that was shown for the avidin mutant #3 in Fig. 3A was also found with mutants #1 and #2 (see Table 1), except that their binding capacity for biotin-IgG was higher than that of mutant #3 (by 11 ± 1.3% and 3.3 ± 1.2%, respectively) [9].

An important clue to the molecular action of SDS and citric acid came from the following observation: only by combining these two reagents was it possible to remove avidin mutants containing the mutation M96H. We hypothesized that citric acid (2.5%, pH = 2.0) protonates the four non-native histidine residues in position 96 of each subunit (shown in the center of the avidin tetramer in Fig. 4A). These four positive charges are likely to attract the negative head groups of four SDS molecules, which are additionally attracted by the hydrophobic environment at the subunit-subunit interfaces (Fig. 4B). Consequently, these electrostatically-bound SDS molecules are expected to facilitate the dissociation of the avidin tetramer into separate subunits (Fig. 4B), which are known to possess a very low affinity for biotin [19].

Two observations suggested that SDS/citric acid acts by dissociating avidin into its four subunits (Fig. 2A, state 5a), and not by pulling intact tetramers off the biotinylated chip surface (Fig. 2A, state 5b): (i) Nordlund et al. [16] treated avidin M96H in solution with acidic buffer and observed its dissociation into separate subunits by gel filtration. (ii) The removal of the double layer of avidin M96H and biotin-IgG (blue trace in Fig. 3A) occurs with the same rapid kinetics as the removal of the avidin M96H monolayer alone (red trace in Fig. 3A). This is surprising, in view of the fact that biotin-IgG carried at least 7 biotin residues [11], which resulted in the extreme crosslinking of the streptavidin tetramers. The latter could only be removed from the chip when biotin-IgG was digested with a protease (as illustrated in Fig. 3B and described in section 3).

The unexpected stability of avidin M96H mutants at pH ~ 2 in the absence of SDS was very fortunate. It allowed for the stable immobilization of the biotinylated bait during repeated cycles of prey binding and prey dissociation, which involved pH 2.5 injections (states 3 and 4 in Fig. 2A) [7, 9]. Nevertheless, the avidin mutants with the mutation M96H displayed disadvantages (see sections 4 and 5), which prompted us to search for
an alternative method where wild-type streptavidin could be used in place of the avidin mutants. Fortunately, this search was successful, as is described in the next section.

**Fig. 4.** Hypothesis for the synergy between H⁺ and SDS with respect to the denaturation of M96H mutants of avidin [7]. (A) Position 96 (methionine) of each monomer is located at a subunit-subunit interface [16]. In M96H-mutants, methionine-96 was replaced by the basic amino acid histidine. The additional positions indicated in the above structure image were optionally mutated in order to lower the pI value of avidin (see Table 1). (B) The avidin tetramer has four mutated sites M96H. (C) Addition of 2.5% citric acid (pH 2.0) protonates the four new histidine residues which, in turn, are thought to bind the negatively charged head groups of SDS molecules. (D) The tightly bound SDS molecules appear to cause dissociation and denaturation of the subunits. Panel A was reproduced from ref. [9] (creative commons license CC BY) and Panel B from ref. [7] (with permission).
Table 1. Comparison of avidin mutants [9] and streptavidin [11] with respect to their pI values, non-specific adsorption of protein and DNA, as well as their stability at pH 2.

<table>
<thead>
<tr>
<th>mutant No.</th>
<th>pI a)</th>
<th>mutations</th>
<th>IgG adsorption (RU) b)</th>
<th>DNA adsorption (RU) d)</th>
<th>(strept)avidin remaining after pH-2 pulse d)</th>
<th>(strept)avidin + biotin-IgG after pH-2 pulse d)</th>
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<tr>
<td>#1</td>
<td>9.69</td>
<td>M96H</td>
<td>64 ± 7</td>
<td>270</td>
<td>102 %</td>
<td>97 %</td>
</tr>
<tr>
<td>#2</td>
<td>9.51</td>
<td>M96H, R114L</td>
<td>66 ± 8</td>
<td>200</td>
<td>81 %</td>
<td>99 %</td>
</tr>
<tr>
<td>#3 &quot;switch-avidin&quot;</td>
<td>7.06</td>
<td>M96H, R114L, K9E, R124H, K127E</td>
<td>27 ± 6</td>
<td>0</td>
<td>63 %</td>
<td>99 %</td>
</tr>
<tr>
<td>streptavidin</td>
<td>5-7</td>
<td>none (wild-type)</td>
<td>25 ± 4</td>
<td>-4</td>
<td>99 %</td>
<td>99 %</td>
</tr>
<tr>
<td>desired</td>
<td>5-7</td>
<td>none (wild-type)</td>
<td>0</td>
<td>0</td>
<td>100 %</td>
<td>100 %</td>
</tr>
</tbody>
</table>

a) The pI values for the mutants were calculated from the amino acid sequence [9]. The pI values reported for wild-type streptavidin range from 5-6 [20]. Recombinant streptavidin has a pI near 7 according to the manufacturers’ data sheets. b) Binding of goat IgG during a 5-min injection at 1 mg/ml on top of (strept)avidin (see FC1 in Fig. 6, red trace). c) Binding of unlabeled bait DNA (1 µM, 3 min injection) on a monolayer of (strept)avidin (see FC1 in Fig. 7c, red trace). For comparison, the hybridization response to injection of 1 µM analyte DNA was 280 RU (see section 6) d) 2.5% Citric acid (with 150 mM NaCl, pH = 1.9) was injected when the avidin mutants were employed [9], whereas 10 mM HCl (with 90 mM NaCl, pH ~ 2.0) was injected when streptavidin was used [11].

3. Mechanism and parameters of reversible immobilization of biotinylated baits via wild-type streptavidin

As mentioned at the beginning of section 2, the biotin-SAM shown in Fig. 1A binds wild-type streptavidin with such high affinity that no known aqueous reagent could remove it inside the biosensor [11]. One way to solve this problem was to mutate streptavidin in a manner analogous to that described above for avidin. Unfortunately, the geometry of the subunit-subunit interfaces in streptavidin is very different from that in avidin. Consequently, streptavidin does not contain a hydrophobic amino acid which is structurally equivalent to the methionine 96 in avidin (compare Fig. 4A). The search for some other mutation in streptavidin with a similar functional effect would have been laborious and the perspective for success was uncertain. We, therefore, tried to find ways to modify the biotin-SAM in a manner which would make it easier to remove the bound streptavidin.

Three derivatives of biotin with reduced affinity for (strept)avidin are well characterized in the literature: iminobiotin [21-23], N-ethylbiotin [24], and desthiobiotin [25-27]. Iminobiotin cannot be used on sensor chips for two reasons: (i) At pH 11 it shows a high
affinity for avidin [21], but at neutral pH the binding of avidin is unstable [28]. (ii) Streptavidin has a very low affinity for iminobiotin ($K_d \sim 10^{-2}$ M) at all pH values between 7 and 11 [29]. Unstable binding of (strept)avidin was also seen when $N$-ethylbiotin was employed [24]. The opposite problem was encountered with desthiobiotin: The affinity is so high that only partial removal of (strept)avidin from desthiobiotin-surfaces was achieved, even after prolonged washing with 0.1-50 mM free biotin or with 50 mM HCl [25-27].

For our intentions, such difficult removal of streptavidin from the desthiobiotin-surface was quite advantageous – as long as we could find a method for achieving the complete dissociation of streptavidin from the chip within a time scale of minutes. The advantage of tight binding is that it ensures stable binding of the biotinylated bait molecules (Fig. 2A, state 3) under the conditions which are commonly used to remove all of the bound prey molecules (Fig. 2A, transition from state 3 to state 2).

In order to test a desthiobiotin surface, we synthesized a desthiobiotin-SAM (Fig. 1B) [11] which was strictly analogous to the previously published biotin-SAM (Fig. 1A) [7]. In our first tests, a monolayer of streptavidin was bound and then treated with up to 500 mM free biotin [11], or 6 M guanidinium chloride (± 100 mM HCl), or 0.5% SDS (± 100 mM HCl) [30]. In all cases, only a partial removal of streptavidin was observed (data not shown here).

These futile attempts showed that an even stronger denaturant was required to dissociate streptavidin from the desthiobiotin chip. The only candidate was guanidinium thiocyanate (GTC). In this reagent, not only the guanidinium cation but also the thiocyanate anion is strongly chaotropic. As can be seen from Fig. 3B, complete removal of a streptavidin monolayer was indeed achieved by a 3-min injection of 6 M GTC (red trace, FC1). At the same time, Fig. 3B shows that 6 M GTC was unable to remove the double layer formed by streptavidin and biotinylated IgG (blue trace, FC2). We hypothesized that this failure might be due to crosslinking of adjacent streptavidin molecules by the IgG molecules (which carry at least 7 biotin residues per protein [11]), as illustrated in Fig. 2B. For this reason, we injected a concentrated solution of pepsin (at pH 2.5, where the activity of pepsin is high), and indeed that enzyme brought about the complete removal of all of the proteins from the chip surface (Fig. 3B, blue trace, FC2). The final injection of SDS was only done to ensure that all traces of adsorbed protein (fragments) were removed from the desthiobiotin-SAM.

Fig. 3B also shows that the binding of streptavidin and the biotinylated antibody could be repeated in a reproducible way, allowing for many cycles of chip functionalization and full regeneration. In practice, an unlimited number of regeneration cycles could be performed, but after two weeks of continued use the capacity for streptavidin and biotinylated bait molecules started to decrease. This lifetime was somewhat extended if the chip was stored in a screw-capped 50 ml polypropylene tube under argon gas at 4°C over the weekends.

The hypothesis that streptavidin is crosslinked by proteins with multiple biotin groups (Fig. 2B) was further tested by comparing statistically biotinylated bovine serum
albumin (BSA, ~3 biotin groups per protein) with site-specifically biotinylated BSA carrying only one biotin label per protein. The latter was prepared by labeling the single free cysteine residue of BSA with maleimide-PEG₂-biotin [11]. The results were in accord with the expectations: BSA with multiple biotin residues obviously caused crosslinking of adjacent streptavidin molecules (Fig. 2B), because both GTC and pepsin were required for their complete removal from the desthiobiotin-SAM [11]. In contrast, the double layer formed by streptavidin and mono-biotin-BSA (Fig. 2C) was easily removed with GTC alone, whereby no pepsin was needed [11].

The experiments described above revealed the following aspects of GTC action: (i) GTC does not dissociate the streptavidin tetramer; if it did, no pepsin would have been required. (ii) GTC is unable to dissociate the interaction between streptavidin and the normal biotin residues of the biotinylated proteins. In conclusion, it seems that GTC only acts by dissociating streptavidin from the desthiobiotin groups on the chip surface.

The action of pepsin is probably restricted to the biotinylated protein because the functional core structure of streptavidin is known to be resistant to virtually all proteases [31]. Pepsin is convenient to use because the 2 mg/ml stock solution (pH 2.5) can be stored at 4°C for several weeks without loss of activity.

In Fig. 3B, the 6 M GTC which was injected also contained 4 mM tris(2-carboxyethyl)phosphine (TCEP), which is a strong reducing agent. The idea was to cut the disulfide bonds between the subunits of the biotin-IgG that was bound on top of streptavidin in FC2 (blue trace in Fig. 3B), and thereby to break the long-range crosslinking illustrated in Fig. 2B. However, the extent of protein removal by GTC + TCEP was far from complete (blue trace in Fig. 3B), and the subsequent injection of pepsin resulted in a full regeneration of the chip surface, independent of whether TCEP had been included in the GTC injection or not. For this reason, we dismissed the idea of including TCEP in the chip regeneration protocol. The elimination of TCEP was fortunate because TCEP is sensitive to air and would have had to be manually mixed with GTC immediately before each GTC injection.

The chip regeneration protocol involving successive injections of GTC, pepsin, and SDS (Fig. 3B) often but not always sufficed to remove all of the bound components. When biotin-protein G was bound on top of streptavidin, for instance, two cycles with GTC/pepsin/SDS had to be applied for complete chip regeneration (see section 8.1). Our goal was to develop a standardized protocol which always resulted in the complete removal of streptavidin and the biotinylated bait from the desthiobiotin surface. With this goal in mind, we re-examined the possibility of using free biotin, albeit at much higher concentrations than the 0.1-50 mM reported in the literature [25-27]. Our hypothesis was that higher concentrations of deprotonated biotin (pH ≥ 8.0) might act by a double mechanism: (i) competition with the desthiobiotin groups on the chip surface for binding to streptavidin, and (ii) the interaction of many additional biotin molecules with streptavidin. This additional interaction could have a denaturation activity, and might destabilize streptavidin folding. That would weaken the interaction of streptavidin with the desthiobiotin groups of the chip surface, and possibly also with the biotin groups of the biotinylated bait molecules.
Fig. 5. Optimized protocol for the removal of streptavidin plus biotinylated bait from a mixed desthiobiotin-SAM [11]. Biotin (200 mM) was dissolved in water, using Tris base for the adjustment of pH 8-8.5. Guanidinium thiocyanate (GTC) was dissolved in water at a concentration of 6 M (62.4%, w/w). Pepsin was dissolved in 1 M glycine (pH 2.5). The figure was reproduced from ref. [11] (creative commons license CC BY).

The putative denaturation activity of free biotin was verified by independent experiments in which metmyoglobin was treated with increasing concentrations of biotin (50-500 mM) or urea (1-6 M). At each concentration of biotin or urea, the denaturation temperature of metmyoglobin ($T_m$) was measured by a temperature scan in a UV-vis spectrophotometer. The results confirmed our suspicion that deprotonated biotin is a much stronger denaturant than urea: 200 mM biotin had the same effect as 1.5 M urea ($\Delta T_m = -5^\circ C$), and 500 mM biotin was just as effective as 3 M urea ($\Delta T_m = -11^\circ C$) [11].

Fig. 5 shows that 200 mM biotin (pH 8-8.5) is very effective in removing a large fraction of the chip-bound streptavidin, along with the biotin-BSA (FC2, blue trace). Only a small amount appeared to remain bound on the desthiobiotin surface, and it was easily desorbed by the subsequent injection of GTC. In Fig. 5, the subsequent two steps involving pepsin and SDS were not actually necessary. Under other circumstances, however, the complete regeneration of the bare desthiobiotin surface was only achieved by carrying out all of the four steps [11]. For this reason, the sequence "biotin/GTC/pepsin/SDS" (Fig. 5) was adopted as the standard regeneration procedure; it is even effective in coping with worst case scenarios.

Biotin concentrations higher than 200 mM and/or biotin injection times longer than 3 min proved very disadvantageous because biotin appeared to progressively adsorb to the desthiobiotin-SAM (Fig. 1B) with increasing tightness. The only reagent which could
fully reverse this adsorption of biotin to the SAM was GTC, but only if ≤200 mM free biotin was applied to the SAM for ≤3 min [11].

The results of all of the variations of the standard regeneration protocol shown in Fig. 5 was that the chosen reagent concentrations and the 3 min injection times are optimal and should not be altered. The only change that can be made is that the pauses between the four injections may be significantly shortened, if it is possible to do so with the biosensor device that is being used. In case of imperfect results, the best advice seems to be that one should simply repeat the sequence "biotin/GTC/pepsin/SDS" shown in Fig. 5.

The almost complete removal of streptavidin and biotin-BSA by free biotin (blue trace in Fig. 5) indicated that free biotin must have ruptured (i) the bonds between streptavidin and the desthiobiotin-SAM (Fig. 1B) as well as (ii) the bonds between streptavidin and biotinylated BSA. We, therefore, suspected that free biotin might also be able to break the bonds between streptavidin and a normal biotin-SAM (Fig. 1A). This supposition turned out to be erroneous, however [11].

The overall conclusion was that if wild-type streptavidin is to be used for reversible chip functionalization by the scheme depicted in Fig. 2A, it is only possible to do so on the desthiobiotin-SAM (Fig. 1B), and not on the biotin-SAM shown in Fig. 1A. Besides, the optimal regeneration of the desthiobiotin-SAM occurs as is shown in Fig. 5.

4. Comparison of avidin mutants and wild-type streptavidin with respect to non-specific adsorption of proteins

In label-free biosensors, non-specific adsorption results in a change of the resonance angle. This cannot be distinguished from the response caused by the specific binding of prey molecules to immobilized bait molecules. Fortunately, the extent of non-specific adsorption of protein on a desthiobiotin-SAM (Fig. 1B) was very small, both before and after the binding of a monolayer of streptavidin (Fig. 6A). The bare desthiobiotin-SAM was tested with a high concentration of BSA (1 mg/ml) and with 2 µM streptavidin that had been pre-blocked with 200 µM free biotin; in neither case was a significant amount of protein adsorbed. After the formation of streptavidin monolayers in both flow cells, the streptavidin layer in FC2 (blue trace) was challenged with successive injections of lysozyme, BSA (2×) and goat IgG. The high protein concentrations of 1 mg/ml did cause transient rises in the resonance angle, but this was only due to changes in the bulk refractory index, and did not result from adsorption. The actual extent of non-specific adsorption was calculated by comparing the resonance angles before and after the protein injection.

The corresponding numbers are shown in Fig. 6A. The adsorption of IgG (21 ± 2 RU) was higher than that of lysozyme and BSA: A slightly higher response to IgG (25 ± 4 RU) was seen in FC1, where the streptavidin layer had not been pre-treated with BSA. Overall, these adsorption signals correspond to only ~1% of a monolayer of IgG that is
adsorbed side-on (~2000 RU) or to ~0.5% of a monolayer of vertically oriented IgG (~5000 RU) [18, 32]; they still fall into the category of very low non-specific adsorption [33].

**Fig. 6**: Critical properties of regenerative sensor chips, exemplified with wild-type streptavidin on a desthiobiotin SAM (Fig. 1B). (A) The extent of non-specific protein adsorption was tested by injections of lysozyme (in FC2), BSA (2× in FC2), and IgG (in both flow cells). (B) The resistance of streptavidin (± biotin-IgG) to acid was tested by 15 s injections of the specified HCl concentrations. The sum of [HCl] + [NaCl] was 100 mM in each injection. Pepsin was dissolved in 100 M glycine (pH 2.5). The figure was reproduced from ref. [11] (creative commons license CC BY).

The same test as in Fig. 6A was also performed with the analogous biotin-SAM, both before and after binding of the avidin mutants listed in Table 1. Each of these avidin
mutants contained the mutation M96H (Fig. 3A), which rendered it sensitive to the combination of citric acid and SDS (Fig. 3B) [7-9]. Mutant #2 contained the additional mutation R114L, which had two effects: it improved the affinity for biotin conjugates and it eliminated one positive charge in each subunit (Table 1) [8]. Unfortunately, a stabilization of biotin binding by mutation R114L was not seen on the biotin-SAM [9]. Mutant #3 contained three further mutations (beside M96H and R114L): R124H eliminated one positive charge because histidine is a much weaker base than arginine; in the other two mutations, positively charged arginine residues were replaced by negatively charged glutamates [8]. Except for the mutation M96H, all of the other mutations served to lower the pI value, so that it tended towards the neutral region (see column 2 in Table 1). A high pI value implies a positive net charge of the avidin mutant, which is expected to cause a high non-specific adsorption of proteins (because many proteins have a negative net charge at neutral pH) and a very high non-specific adsorption of nucleic acids (which are highly anionic at neutral pH).

The protein adsorption tests like those in Fig. 6A for the mutants #1, #2, and #3 were performed in previous studies [7-9]; the most interesting aspect of the results, namely the adsorption of IgG, is shown in column 4 of Table 1. Mutant #3 ("switchavidin") showed the same low non-specific IgG adsorption as wild-type streptavidin, whereas mutants #1 and #2 were stickier (~65 RU), in accordance with their higher pI values (see columns 2 and 4 in Table 1). Such an extent of nonspecific adsorption on mutants #1 and #2 is not optimal, but it is still compatible with biosensing (see Figure 10 in ref. [7]). Much worse was the extent of non-specific DNA adsorption on top of mutants #1 and #2 (column 5 in Table 1), as is discussed in detail in section 6 (see below).

It should be noted that in practical applications the non-specific adsorption of protein is further minimized by supplementing the running buffer with a low concentration of BSA (e.g. 1 µM = 0.066 mg/ml, see section 8) or of Tween-20 (e.g., 0.005%, see section 9).

5. Stability of biotinylated bait immobilization under the conditions used for prey removal

Conventionally, the bait molecules are irreversibly coupled to biosensor chips by covalent bonds. Consequently, only the prey molecules can bind in a reversible manner, and therefore the term "chip regeneration" is used for the short injection of a reagent which quickly dissociates all of the bound prey molecules without denaturing the immobilized baits.

In respect to our regenerative surfaces, the term "chip regeneration" can adopt two distinct meanings, as illustrated in Fig. 2A: Regeneration in the sense of "prey removal" is appropriate for what occurs after each injection of a particular sample; the chip has to be regenerated for the injection of the next test sample (alternation between states 3 and 4). In contrast, "full regeneration" means that all of the proteins are dissociated from the chip surface (state 5a or 5b) and the bare (desthio)biotin-SAM is recovered for a new round of chip functionalization. Our major concern was to ensure that the biotinylated baits remain firmly bound to the chip surface while multiple cycles of prey binding and
prey removal are being carried out in the course of a typical measurement series (1-2 h). In practical terms, this means that the biotin-avidin-biotin bridge should be stable when reagents (such as dilute HCl, NaOH, SDS, etc.) which are commonly used for the rapid dissociation of the bound prey molecules are being injected [12].

An appropriate test experiment is depicted in Fig. 6B. The desthiobiotin-SAM was covered with streptavidin in both flow cells, and biotin-IgG was additionally bound in FC2 (blue trace). Subsequently, pulses of 10, 20, 50, and 100 mM HCl were injected, whereby the ionic strength was kept constant with the help of 90, 80, 50, and 0 mM NaCl, respectively. At all of the HCl concentrations, only minute losses of protein (≤1%) were seen in FC2 (blue trace), where biotin-IgG was bound on top of streptavidin (see last two columns in Table 1). The same was also true for the streptavidin monolayer in FC1 (red trace) when 10 mM HCl (with 90 mM NaCl) was injected. HCl concentrations higher than 10 mM, however, caused significant losses from the streptavidin monolayer in FC1 (red trace); therefore, they cannot be recommended for the removal of the bait from mono-biotinylated bait molecules which cannot form crosslinks between adjacent streptavidin molecules (compare Figs. 2B and 2C).

When avidin M96H mutants on biotin-SAMs were used for the immobilization of biotinylated baits, the stability of the biotin-avidin-biotin bridge very much depended on the choice of mutant and the type of application [7, 9]. Representative findings are summarized in the last two columns of Table 1. If biotin-IgG (or another statistically biotinylated protein) was bound on top of the avidin mutant, the resulting protein double layer was very resistant to injection pulses at pH 2.0 (last column in Table 1). Hereby it was important to include ≥150 mM NaCl in the injection buffer in order to improve the stability of the avidin layer [9]. In the absence of biotin-IgG, however, only mutant #1 remained stably bound on the chip at pH 2.0, whereas 19% and 37% were lost from the chip in case of mutant #2 and #3, respectively (column before the last one in Table 1).

The divergent behavior in the absence and in the presence of biotin-IgG (last two columns in Table 1) can be readily explained if we assume that extensive crosslinking occurs in the double layer of avidin and biotin-IgG (as illustrated in Fig. 2B). The low stability of mutants #2 and #3 in the absence of biotin-IgG is attributed to the absence of crosslinking. The same reduced stability is also expected to occur when mono-biotinylated bait molecules are used, due to the fact that they are not able to crosslink adjacent (strept)avidin molecules (compare Figs. 2B and 2C).

Together, the last two columns in Table 1 indicate that both the avidin mutant #1 and wild-type streptavidin are useful in combination with all kinds of biotinylated bait molecules, whereas mutants #2 and #3 should only be used in combination with statistically biotinylated proteins which bring about stabilization by means of crosslinking. In addition, mutants #2 and #3 can also be employed in situations where conditions other than pH 2 are used for bait removal. When working with multiply biotinylated baits in the sample cell, it is important to also stabilize the (strept)avidin layer in the control cell, e.g., with biotin-BSA. That substance also causes profound crosslinking and therefore stabilizes (strept)avidin [9, 11].
The stability of chip-bound streptavidin (± biotin-IgG on top) was also tested by applying NaOH and Na₂CO₃ injections with a protocol which was analogous to that shown in Fig. 6. Unsatisfactory results were obtained with 10, 20, 50, and 100 mM NaOH (containing 90, 80, 50, and 0 mM NaCl for the attainment of a uniform ionic strength): several percent of streptavidin (± biotin-IgG) were lost in each injection [11]. In contrast, no losses were observed when 150 mM Na₂CO₃ was injected (with pH 10.0 or 10.5 or 11.0) in place of NaOH [11]. This was surprising, because 10 mM NaOH (with 90 mM NaCl) also has a pH value of 11.0. The apparent contradiction between these findings can readily be explained by taking into account the fact that the molar ionic strength (I) of 150 mM Na₂CO₃ (I = 450 mM) is much higher than that of the mixture of 10 mM NaOH with 90 mM NaCl (I = 100 mM).

High ionic strength also had a profound stabilizing effect in the above described stability tests at pH ~ 2.0, both for wild-type streptavidin [11] and for the three avidin mutants [9]. For example, losses of mutants #1 - #3 higher than those recorded in the next to the last column of Table 1 were measured when the 150 mM NaCl was omitted from the citric acid solution. For this reason, our preferred method for bait removal was by using 1 M glycine buffer, in which case the immobilized streptavidin (± biotinylated bait) remained completely stable (see section 8.1).

Besides low or high pH buffers, SDS is another popular reagent for the quantitative dissociation of prey molecules from the immobilized baits [12]. Of course, the use of SDS is only possible if the immobilized bait is not denatured by short pulses of that substance. On our regenerative chips, chip-bound streptavidin (± biotinylated bait) showed perfect stability during injections of 0.5% SDS (Traxler et al., manuscript in preparation), and the avidin mutant #1 also proved to be rather resistant to 0.5% SDS at physiological salt concentration [9]. Mutant #3 and especially mutant #2 were more sensitive to SDS [9].

A very important aspect has not yet been mentioned: In most of the cases where the (strept)avidin layer was not completely stable during the injection of acids, bases, or SDS, only the first injection of this reagent caused an undesired loss of some of the (strept)avidin (± bait) from the chip surface. Obviously, only a small fraction of (strept)avidin is weakly bound and therefore readily removed during the "prey removal" step, while the largest proportion of that substance is more stably bound. This fact can be readily exploited: it is possible to carry out a dummy injection of an acid (or a base, or SDS) before the first injection of a prey-containing sample (as exemplified in section 8). No, or only small losses of (strept)avidin were seen during the subsequent cycles of "prey removal" and sample injection (see section 8) [9, 11].

The following hypothesis offers an explanation of why a small fraction of the (strept)avidin molecules binds to the (desthio)biotin-SAM with low affinity: On our mixed SAMs, 20% of the PEG chains carried a (desthio)biotin group (Fig. 1AB). At this high lateral density, streptavidin binds with much higher stability than at a biotin density of one percent [34, 35]. The enhanced stability at a (desthio)biotin density of 20% was attributed to the bivalent binding of streptavidin, whereas at 1% biotin density the
binding was monovalent [34, 35]. However, at 20% (desthio)biotin a small fraction of streptavidin was always seen to bind with low affinity, and at 1% (desthio)biotin a small fraction of streptavidin was always seen to bind with high affinity [35]. The most reasonable explanation for that observation is that even at 20% (desthio)biotin density a small fraction of the (strept)avidin molecules can find only one (desthio)biotin group to which they can bind. When this small unstable fraction of (strept)avidin is removed by a pre-pulse of acid (or base, or SDS), the rest of the (strept)avidin layer displays the high stability which is needed to perform many cycles of prey binding and prey removal.

6. Specific and non-specific binding of DNA on monolayers of avidin mutants as compared to on streptavidin

The avidin mutants #1 - #3 (Table 1) and wild-type streptavidin were tested for (i) specific binding of biotinylated single-stranded DNA (ssDNA), (ii) specific binding of the complementary ssDNA to the biotinylated DNA, and (iii) non-specific binding of ssDNA. Thereby, the model experiment that is shown in Fig. 7 was always employed; the biotin-SAM (Fig. 1A) was used for the avidin mutants, and the desthiobiotin-SAM (Fig. 1B) was employed for streptavidin. The first two injections in Fig. 7C only took place in flow cell 2 (FC2, blue trace): streptavidin was bound to the desthiobiotin-SAM and single-stranded biotin-DNA (a 30-mer) was immobilized on top of it. The next step was the immobilization of streptavidin in the control cell (red trace in Fig. 7C). After that, all of the injections passed through both FC1 and FC2 (as illustrated in Fig. 7A); a zoom of those injections is shown in Fig. 7D.

First, BSA was applied, whereupon no non-specific adsorption was seen in any flow cell. The small transient rise was a bulk effect that resulted from the high protein concentration (1 mg/ml). The next injection contained 1 μM of a ssDNA which had the same sequence as the biotin-DNA; therefore, no hybridization was expected. No binding was seen in any of the two flow cells, indicating an absence of non-specific DNA adsorption to the streptavidin layer on the chip. Finally, a single-stranded prey DNA which was complementary to the biotin-DNA that had been immobilized in FC2 was injected. As a result, pronounced hybridization was observed in FC2 (blue trace). In FC1 (red trace in Fig. 7D) a small negative bulk effect was seen during the injection of 1 μM prey DNA, due to the fact that the 100 μM stock solution of prey DNA had been prepared in water and not in the running buffer. After the injection of prey DNA, the baseline in FC1 showed the same level as before, which indicates an absence of non-specific DNA adsorption on the chip-bound streptavidin.

The analogous SPR experiments for the avidin mutants #1 - #3 [8, 9] are not shown here, but the most important findings are presented in Table 1. Only mutant #3 exhibited the same low degree of non-specific DNA adsorption as streptavidin (column 5 in Table 1); in contrast, dramatic non-specific DNA adsorption was observed on top of mutants #1 and #2. The vigorous adsorption of ssDNA to mutants #1 and #2 is clearly explained by their high pI values (column 2 in Table 1), which indicate that they have a pronounced positive net charge. Therefore, they exert a strong electrostatic attraction on the negatively charged DNA. The obvious consequence of these findings is that mutants
#1 and #2 cannot be used in any type of experiment where nucleic acids are present; neither as the analyte-of-interest nor as side components of the matrix.

**Fig. 7.** Test for non-specific binding and for hybridization of single-stranded DNA, exemplified with streptavidin on a desthiobiotin-SAM [11]. (A) and (B) The schematic representation of a typical SPR flow cell shows the absence of a valve between FC1 and FC2; therefore, small solutes can diffuse from FC1 into FC2, or vice versa. (C) The sample cell (FC2, blue trace) was functionalized with streptavidin plus biotin-labeled single-stranded DNA (with the sequence GCACCTGACTCCTGAGAGTCTGCCGT, termed the "bait"). Subsequently, only streptavidin without any biotin-DNA was bound in the control cell. After injection of BSA, the identical 30-mer DNA, which, however, lacked a biotin residue, was injected (see black bar,
to test for non-specific adsorption. Finally, the complementary DNA strand ("prey") was injected to measure hybridization in the sample cell (FC2, blue trace). (D) Expanded view of the traces in panel (C), showing the injections of BSA, unlabeled bait DNA, and unlabeled prey DNA. The figure was reproduced from ref. [11] (creative commons license CC BY).

The undesired non-specific adsorption of DNA to mutants #1 and #2 could largely (but not completely) be suppressed by binding biotin-N19T on top of the avidin mutants [9]. This injection was performed in both flow cells after the biotinylated bait DNA had been immobilized in FC2, but not in FC1. The randomized N19 sequence ensured that (almost) no undesired hybridization with the analyte DNA occurred, and the strong negative charge of biotin-N19T formed a negatively charged "protective layer" on top of the positively charged avidin mutants.

It may be asked why the inertization of mutants #1 and #2 with biotin-N19T (or other biotin-DNA) should be of interest, since no such trick is required when mutant #3 is employed. The answer is that mutant #3 binds less biotin-DNA than mutant #1 and its stability of immobilization is lower than that of mutant #1.

The overall conclusion from Table 1 is that the functional properties of wild-type streptavidin are closest to the optimal values listed in the last row of Table 1. It exhibits the same low level of non-specific adsorption of protein and of DNA as mutant #3 and its stability at pH 2.0 is as good as that of mutant #1. Furthermore, streptavidin is more resistant to low pH, high pH, and SDS, and shows a higher binding capacity for biotinylated baits than the avidin mutants. The avidin mutants, however, have the advantage that the procedure for their complete dissociation with SDS/citric acid (Fig. 3A) is faster than the complete removal of wild-type streptavidin with biotin/GTC/pepsin/SDS (Fig. 5).

7. Strategies for selective functionalization of the sample cell as compared to the reference cell

Fig. 7A&B depicts a typical flow cell arrangement of an SPR biosensor. In the case of our instrument (BIAcore X), each flow cell has a volume of 0.06 µl and the loop between the two flow cells has a volume of 0.8 µl. The dead volume between the two flow cells can only be so small if there is no valve between them. This lack of a separating valve is compensated for by the additional outlet between the two flow cells; the outlet is closed if the flow has to pass through both flow cells (Fig. 7A) and opened if it is only supposed to pass through FC1 (Fig. 7B). In the latter case, the normal exit of FC2 is closed, and therefore no flow can occur in FC2. However, this does not eliminate the diffusion of molecules (especially of small molecules) into FC2, as indicated by the cyan color gradient in Fig. 7B.

The valve setting shown in Fig. 7B is typically used when the bait molecules are selectively immobilized in one flow cell. In this situation, it is crucial that no bait molecules diffuse into the reference cell, where they might become immobilized. Such undesired functionalization of the control cell can readily occur when biotinylated bait
molecules are utilized; they bind to streptavidin with a rate constant of $\sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$ [36], corresponding to a time constant of 1 s at a concentration of 0.1 µM.

The functionalization protocol in Fig. 7C was chosen to completely suppress undesired control cell functionalization. Initially, streptavidin was only present in FC2; therefore, the subsequently injected biotin-DNA could only bind in that flow cell. Even if a small fraction of biotin-DNA diffused into FC1, it could not bind there because there was no streptavidin for it to bind to.

A less laborious method for the avoidance of undesired immobilization of biotinylated bait in the control cell is shown in Fig. 8C. In this simplified protocol, both flow cells were simultaneously functionalized with streptavidin, but then the control cell (FC2, blue trace) was blocked by injection of biotin-BSA. Only after this blocking step could the biotinylated bait (biotin-protein G) safely be injected into the sample cell (FC1, red trace). If this precaution was omitted and biotin-protein G was injected before biotin-BSA, the control cell also contained immobilized biotin-protein G, as was concluded from the pronounced binding of antibody (human IgG2κ) [9].

The functionalization scheme in Fig. 7 shows a unique feature of our regenerative sensor chips (and the Biotin CAPture Kit™) which is not shared by conventional streptavidin chips: We can "activate" each flow cell separately and at different times, thus enabling the employment of this rigorous method of selective flow cell functionalization. On commercial streptavidin chips, only the improvised method can be used where the control cell is first blocked with an inert biotin derivative, and then the sample cell is activated with a biotinylated bait.

8. Examples for biospecific interaction analysis between biotinylated baits and soluble prey molecules

8.1 Biotinylation of bait molecules and subsequent immobilization of the (strept)avidin surface

A practical example of "Biospecific Interaction Analysis" (BIA) on a regenerative chip is presented in Fig. 8. Streptavidin was bound to the desthiobiotin chip in both flow cells, and the control cell (FC2, blue trace in panel C) was then blocked with biotin-BSA before the biotinylated bait (biotin-protein G) was immobilized in the sample cell (FC1, red trace in panel C). As explained in section 7, this sequence of injections was essential for the prevention of the undesired functionalization of the reference cell with biotin-protein G [9]. In all of the subsequent injections, the flow passed both flow cells in series (as illustrated in Fig. 7A).

The subsequent injection of biotin-BSA in both flow cells was performed to obtain complete saturation of the residual biotin-binding sites on chip-bound streptavidin. No response was seen in any flow cell, however; it is therefore clear that all of the biotin-binding sites had already been occupied. Next, a high concentration of BSA was injected to test for non-specific adsorption, and again no net response was observed. Then, 1 M
glycine (pH 2.5) was injected to test the functionalized chip for full stability under the conditions used for selective removal of prey. Fortunately, this injection caused no change in the baselines, which shows that streptavidin and the biotinylated proteins were perfectly retained at pH 2.5.

Fig. 8. Binding and dissociation kinetics of human IgG2κ on biotin-protein G which had been immobilized on a monolayer of streptavidin [11]. (A) Schematic representation of the bivalent binding of IgG2κ to adjacent biotin-protein G molecules on the streptavidin surface of the sample cell. (B) Representation of the inert surface in the control cell which had been blocked with biotin-BSA. (C) After activation of both flow cells with streptavidin, inertization of the control cell with biotin-BSA (FC2, blue trace), functionalization of the sample cell with biotin-protein G (FC1, red trace), and blocking of both flow cells with biotin-BSA and BSA, the interaction of immobilized protein G with soluble IgG2κ was tested by repeated injection at different concentrations. Glycine (1 M, pH 2.5) was used for the repeated removal of IgG2κ. The control trace (blue) was subtracted from the sample trace (red) and the buffer injection of the resulting trace was subtracted from all of the other injections ("double referencing method" [1]). This yielded the experimental binding curves which are shown as solid traces in (D) and (E). (D) The dotted lines show the best global fit of the Langmuir model to the experimental binding curves (solid lines). (E) The dotted lines represent the best global fit with the "bivalent analyte model".
Finally, a series of injections of human IgG2κ was applied at different concentrations, whereby 1 M glycine (pH 2.5) was injected after each IgG2κ concentration to remove all of the bound IgG2κ from the chip surface. The last injection was performed only with sample buffer, as is required for the double referencing method [1]. The control trace (FC2, blue color) was subtracted from the sample trace (FC1, red trace). Then the resulting difference curve from the sample buffer injection was subtracted from all of the sample injections (double referencing method [1]), resulting in the experimental binding curves which are shown as solid colored lines in Figs. 8D and 8E. The dashed black lines in Fig. 8D show the best global fit that can be obtained with the simple Langmuir model, which assumes only 1:1 binding of soluble IgG2κ to immobilized protein G. The term "global fit" means that all of the binding curves were fitted with the same association and dissociation rate constants. The large discrepancy between the experimental and the calculated binding curves indicated that the Langmuir model was clearly inappropriate. In contrast, the experimental binding curves could be perfectly fitted with the "bivalent analyte model", which assumes that the IgG2κ molecules can bind to the chip in two steps: First, one of the two heavy chains of IgG2κ binds to one immobilized protein G, and in the second step the second heavy chain of IgG2κ binds to an adjacent protein G on the chip surface. Only this final state is illustrated in Fig. 8A. Such bivalent binding is also likely to occur in vivo because the outer surface of Streptococcus sp. is densely covered with protein G [37]. It therefore appears that the laterally dense arrangement of biotin-protein G on our streptavidin surface is a valid imitation of the in vivo situation, and that the kinetic characterization in Fig. 8 is physiologically relevant.

In order to compare wild-type streptavidin with the avidin mutants listed in Table 1, biotin-protein G was immobilized on top of the avidin mutants which had been bound on a biotin SAM, and the same interaction study with human IgG2κ was performed as shown in Fig. 8 [7, 9]. The corresponding data are summarized in Table 2. Generally speaking, the results were similar for the three avidin mutants as well as for wild-type streptavidin (last column in Table 1). An interesting trend was seen for the binding signal of biotin-protein G on the different surfaces (first row in Table 2). A progressive decrease was noted for the three avidin mutants, and the highest level of protein G immobilization was observed on top of streptavidin. These findings help to explain the minor differences between the data set obtained with the avidin mutants as compared to streptavidin (Table 2). The kinetic and equilibrium constants were very similar for the three avidin mutants, but the values for streptavidin always indicated a slightly higher affinity of soluble IgG2κ for immobilized protein G. The higher lateral density of biotin-protein G on the streptavidin surface probably implied that a higher fraction of the immobilized protein G had the proper lateral distance for binding IgG2κ in a bivalent fashion, and thus with higher affinity.

The fit parameter $R_{\text{max}}$ reflects the extrapolation of IgG2κ binding to infinitely high IgG2κ concentrations. These values were also higher on streptavidin than on the avidin
mutants (in both models, see Table 2), which is in line with the higher lateral density of binding sites (i.e., with the higher level of protein G immobilization, first row in Table 2).

**Table 2.** Kinetic and equilibrium constants determined for the interaction of soluble IgG2κ with biotin-protein G that had been immobilized on one of the avidin mutants #1, #2, #3 [9] or on wild-type streptavidin [11].

<table>
<thead>
<tr>
<th></th>
<th>mutant #1</th>
<th>mutant #2</th>
<th>mutant #3</th>
<th>wild type streptavidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>biotin-protein G (RU)</td>
<td>396</td>
<td>379</td>
<td>366</td>
<td>566</td>
</tr>
<tr>
<td></td>
<td>(70%)</td>
<td>(67%)</td>
<td>(65%)</td>
<td>(=100%)</td>
</tr>
<tr>
<td><strong>Langmuir model</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_a$ ($10^5$ M$^{-1}$ s$^{-1}$)</td>
<td>4.1</td>
<td>4.1</td>
<td>4.2</td>
<td>2.8</td>
</tr>
<tr>
<td>$k_d$ ($10^4$ s$^{-1}$)</td>
<td>11</td>
<td>7.4</td>
<td>8.4</td>
<td>4.6</td>
</tr>
<tr>
<td>$K_D$ (nM)</td>
<td>2.7</td>
<td>1.8</td>
<td>2.0</td>
<td>1.7</td>
</tr>
<tr>
<td>$R_{max}$ (RU)</td>
<td>1370</td>
<td>1360</td>
<td>1290</td>
<td>2140</td>
</tr>
<tr>
<td><strong>Bival. analyte model</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{a1}$ ($10^5$ M$^{-1}$ s$^{-1}$)</td>
<td>1.8</td>
<td>1.6</td>
<td>1.8</td>
<td>0.92</td>
</tr>
<tr>
<td>$k_{d1}$ ($10^4$ s$^{-1}$)</td>
<td>17</td>
<td>14</td>
<td>14</td>
<td>6.6</td>
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<tr>
<td>$K_{D1}$ (nM)</td>
<td>9.4</td>
<td>8.8</td>
<td>7.5</td>
<td>7.3</td>
</tr>
<tr>
<td>$k_{a2}$ ($10^4$ RU$^{-1}$ s$^{-1}$)</td>
<td>1.7</td>
<td>2.7</td>
<td>3.1</td>
<td>0.98</td>
</tr>
<tr>
<td>$k_{d2}$ (s$^{-1}$)</td>
<td>0.013</td>
<td>0.013</td>
<td>0.017</td>
<td>0.0035</td>
</tr>
<tr>
<td>$K_{D2}$ (RU)</td>
<td>77</td>
<td>47</td>
<td>56</td>
<td>36</td>
</tr>
<tr>
<td>$R_{max}$ (RU)</td>
<td>1800</td>
<td>1930</td>
<td>1740</td>
<td>3430</td>
</tr>
</tbody>
</table>

Another characteristic advantage of streptavidin over the avidin mutants #1 - #3 was the higher stability of biotinylated bait immobilization. In Fig. 8, the pre-pulse of 1 M glycine (pH 2.5) had absolutely no net effect in FC1 or FC2, whereas a loss of 60 RU was seen on mutant #1 [7] and 180-185 RU on mutants #2 and #3 [9].

In conclusion, the avidin mutants #1 - #3 are well-suited for use in the biospecific interaction analysis of proteins on the biotin-SAM (Fig. 1A). Wild-type streptavidin on the desthiobiotin-SAM (Fig. 1B) provides for higher immobilization levels and a higher stability of the biotinylated bait layer, however.
8.2 Immobilization of biotin-maleimide and covalent coupling of thiolated bait molecules on the chip surface

Figs. 9 and 10 describe an interaction study which in several respects differs from the example in Fig. 8: (i) Only monovalent binding of the soluble prey to the immobilized biotinylated bait was seen. (ii) The bait molecule was a synthetic peptide consisting of 21 amino acids. In particular, the synthetic peptide had the same amino acid sequence as the calmodulin-binding segment of smooth muscle myosin light-chain kinase (smMLCK); it is known to bind calmodulin in a 1:1 fashion, with a $K_d$ of ~ 1 nM, both in vivo and as an isolated peptide [38]. (iii) The "immobilization chemistry" of the peptide was also different: First, maleimide-PEG$_{11}$-biotin was bound to the streptavidin monolayer, and only then was the peptide injected under conditions which ensured covalent coupling of the C-terminal cysteine residue to the maleimide group on the streptavidin surface (Figs. 9 and 10A).

![Diagram of peptide immobilization](image_url)

**Fig. 9.** Covalent coupling of a peptide with a free cysteine (or a nucleic acid with a thiol group) to maleimide-PEG$_{11}$-biotin which had been immobilized on chip-bound streptavidin [11]. The coupling was performed in HEPES buffer containing 1 mM EDTA, 5 mM TCEP (for reduction of oxidized disulfides), pH ~ 7.5, at a peptide concentration of 0.5 mM. Unused maleimide groups were deactivated with cysteine (20 mM). The experiment is exemplified in Fig. 10B. The figure was reproduced from ref. [11] (creative commons license CC BY).

The coupling of the peptide was monitored in the SPR biosensor (Fig. 10B). The extent of peptide coupling was found to be 263 RU. This method of peptide immobilization was preferred for two reasons: (i) Less peptide was immobilized when it had first been biotinylated in solution and then bound to the streptavidin surface. (ii) In situ coupling of the peptide as depicted in Fig. 9 was much simpler and more versatile than biotinylation in solution; the latter required purification by HPLC and characterization by mass spectrometry.
Fig. 10: Immobilization of the calmodulin-binding peptide segment from smooth muscle myosin light chain kinase (smMLCK, acetyl-RKWQTKGHAVRAGRLSSGGC-NH₂) and test for the specific binding of soluble calmodulin. (A) Illustration of peptide coupling to streptavidin-bound maleimide-PEG₁₁-biotin, and of calmodulin binding to the immobilized peptide. (B) The data show the binding of streptavidin in both flow cells, the blocking of the control cell with biotin-BSA (blue trace), the binding of maleimide-PEG₁₁-biotin in the sample cell (red trace), the coupling of the smMLCK peptide to maleimide (red trace), and the deactivation of maleimide with cysteine. (C) Repeated injections of glycine (100 mM, pH 2.5) and of calmodulin at different concentrations. The running buffer and the sample buffer contained 2 mM Ca²⁺ for the activation of calmodulin and 0.5 µM BSA for the minimization of non-specific adsorption. The figure was reproduced with slight modifications from ref. [11] (creative commons license CC BY).

After the peptide immobilization, free cysteine was injected in order to deactivate potentially unused maleimide groups on the chip surface (Fig. 10B). Although no net
change of the resonance angle was seen, this does not mean that no cysteine had coupled because cysteine is too small to be detected. Only after the deactivation step with cysteine could we switch to the sample buffer, which contained 0.5 µM BSA. In commercial BSA preparations, the number of reactive SH groups per protein is usually ~0.5 [39]. Therefore, BSA would have been coupled to unused maleimide groups if they had not been deactivated with cysteine.

The actual interaction study between the immobilized smMLCK peptide and soluble calmodulin is shown in Fig. 8C. It started with a pre-pulse of 100 mM glycine (pH 2.5), which caused some loss of the peptide. In this particular situation, the observed loss possibly reflected the desorption of a small peptide fraction which had simply adsorbed to streptavidin. Such adsorption was not unexpected because the peptide has a net charge of +5 at neutral pH (in contrast to the negatively charged streptavidin), and it contains many hydrophobic amino acids.

After the pre-pulse, calmodulin was injected at different concentrations and always removed with glycine buffer. The gradual decrease of the baseline was not caused by the glycine injections but was clearly due to a slight baseline drift in the running buffer. The control trace (FC2, blue) was subtracted from the sample trace (FC1, red), and then the buffer injection ("0 nM calmodulin") was subtracted from all of the other injections (double referencing method [1]), yielding the experimental binding curves which are shown as solid colored solid lines in Fig. 11B. In contrast to Fig. 8, these data could readily be fitted to the simple 1:1 binding model (dashed curves in Fig. 11B); this was not possible for IgG binding on protein G (see above).

In order to obtain an independent control of the kinetic data in Fig. 11B, the immobilization scheme was reversed as is illustrated in Fig. 11C. For this purpose, calmodulin was labeled with ≤1 biotin/calmodulin, purified by gel filtration and then immobilized on the streptavidin surface [11]. Subsequently, different concentrations of the smMLCK peptide (without a C-terminal cysteine!) were injected, and after each injection the bound peptide was removed with 100 mM glycine (pH 2.5). The data were processed with the "double referencing method" (as described above), yielding the experimental binding curves which are shown as solid colored lines in Fig. 11D. The noise was higher in Fig. 11D than in Fig. 11B, due to the fact that the molar mass of the peptide was one order of magnitude smaller than that of calmodulin. These data could also be fitted with the simple 1:1 binding model. The kinetic constants in Fig. 11D were ~2× smaller than in Fig. 11B but the K_d values were essentially equal.

Taken together, the results in Figs. 8 – 11 show that our regenerative biosensor surface can be used for large proteins as well as for small peptides, and the data reveal whether a given interaction is bivalent or monovalent. A convenient and efficient option is the in situ coupling of small thiol-containing bait molecules to pre-immobilized maleimide-PEG-biotin; it obviates the need for the laborious biotinylation procedure and for the HPLC purification of small biotinylated molecules which cannot be isolated by gel filtration.
Fig. 11. Measurement of the Ca\textsuperscript{2+}-dependent interaction between calmodulin and a peptide which corresponds to the calmodulin-binding segment from smMLCK [11]. (A) Configuration with immobilized smMLCK peptide (red color) and soluble calmodulin (blue color). (B) SPR data showing the association and dissociation of soluble calmodulin on immobilized peptide at different calmodulin concentrations (solid colored lines). These binding curves were calculated from the original SPR data in Fig. 10C by the "double referencing method" [1] (compare legend to Fig. 8). (C) Configuration with immobilized biotinylated calmodulin and soluble smMLCK peptide. (D) SPR data for the configuration in panel C, showing the association and dissociation at different peptide concentrations (solid colored lines). The dashed lines in panels B and D are global fits with a simple 1:1 binding model (Langmuir model), which take mass transport limitations into account. The latter was justified because the binding kinetics was dependent on the flow. The figure was reproduced with slight modifications from ref. [11] (creative commons license CC BY).
9. Quantification of soluble analytes in crude biofluids

Biospecific interaction analysis studies are usually performed with purified components in defined buffer solutions, as exemplified in sections 8.1 and 8.2. However, SPR biosensors are also used for the quantification of particular analytes in crude biofluids such as serum or the lysates of cultured cells. Such matrices contain high background concentrations of side components, some of which have a pronounced tendency for non-specific adsorption. Usually, polymer coatings such as dextran are employed to suppress the non-specific adsorption [6].

On our regenerative sensor chips, the coating is much thinner than a dextran layer. It consists of a mixed SAM shown in Fig. 1A or 1B, a monolayer of (strept)avidin, and a layer of the biotinylated bait. For this reason, we tested whether the non-specific adsorption from serum or bacterial lysate was sufficiently low to enable the quantification of the specific binding of an antigen to a biotinylated antibody. In agreement with common practice [40], fetal calf serum (FCS) or bacterial lysate was diluted by a factor of 20 with running buffer that contained 0.05% Tween 20 and then for 3 min applied to a streptavidin chip that had been covered with biotinylated goat IgG. The pleasant surprise was that the extent of non-specific binding from FCS and bacterial lysate was as low as 4 RU and 6 RU, respectively [11].

Encouraged by these findings, we attempted the quantification of hexahistidine-tagged, enhanced green fluorescent protein (His<sub>6</sub>-GFP) in bacterial lysate. First, the sensor had to be properly functionalized and calibrated. The functionalization is shown in Fig. 12A: Streptavidin was immobilized in both flow cells, followed by the binding of inert biotin-IgG (from goat) in FC2 (blue trace) and the binding of biotinylated anti-GFP-antibody in the sample cell (red trace). Subsequently, three injections were performed with purified His<sub>6</sub>-GFP at a 200 nM concentration in the running buffer, whereby 100 mM glycine (pH 2.5) was applied after each of them to dissociate all of the His<sub>6</sub>-GFP which had been captured by the anti-GFP antibody. The purpose of these three cycles was to determine (i) whether all of the bound His<sub>6</sub>-GFP had been removed and (ii) whether the anti-GFP antibody had retained its function during the 1 min injections of 100 mM glycine (pH 2.5). Fortunately, both aspects were found to be true, and therefore this protocol was used in the subsequent injection series where His<sub>6</sub>-GFP was applied at different concentrations (data not shown). The control trace (FC2) was subtracted from the sample trace (FC1); the overlay of the resulting binding responses is shown in Fig. 12B. In each binding curve, the "report point" was 30 s after the end of the sample injection (red vertical line in Fig. 12B), and the resonance angles obtained at this time point were plotted versus the known His<sub>6</sub>-GFP concentration of the injected samples (solid black line in Fig. 12C). For the quantification of the unknown His<sub>6</sub>-GFP concentration, which had been over-expressed in bacterial lysate, the bacterial lysate was diluted to different degrees with running buffer and injected on the identical chip that had been used in panels A and B shortly before. The binding curves from the different dilutions are shown in Fig. 12D. We then assumed a hypothetical concentration of 7 µM for His<sub>6</sub>-GFP in undiluted lysate and divided this value by the dilution factor of each binding curve in Fig. 12D. In this way, we were able to assign an explicit concentration
value to each of the binding curves in Fig. 10D. The response values at the report point (red vertical line in Fig. 12D) were then plotted against the hypothetical concentrations in Fig. 12C. The resulting dose-response curve (●) showed good agreement with the calibration curve (solid black line) which confirmed our assumption of 7 µM His6-GFP in the undiluted lysate. The result was quite clear because the assumption of 5 µM His6-GFP (+) was obviously too low and 10 µM was too high (×).

Fig. 12. Quantification of His6-GFP in a bacterial lysate, using a biotinylated monoclonal anti-GFP antibody on streptavidin as the immobile bait molecule. (A) Selective functionalization of the control cell with biotin-IgG (from goat) and of the sample cell with biotinylated anti-GFP antibody, followed by repeated binding of purified His6-GFP protein (100 nM) and dissociation by glycine (100 mM, pH 2.5). (B) The experiment shown in (A) was immediately repeated with different known concentrations of His6-GFP (not shown) and the binding curves (solid colored lines in panel B) were calculated by subtraction of the control trace. (C) Comparison of the dose-responses to known His6-GFP injections (vertical red line in panel B) with the dose-responses to different dilutions of a bacterial lysate (vertical red line in panel D). The responses measured at the report points were plotted versus the known His6-GFP concentration (solid black line in this panel C) or versus the assumed concentrations of His6-GFP in the bacterial lysate (based on three different assumptions for the His6-GFP concentration in the undiluted bacterial lysate, as specified in this panel C). (D) The experiment shown in (B) was immediately repeated with different dilutions of the bacterial lysate, which contained over-expressed His6-GFP. The dilution factors ranged from 40 to 2500, as specified in this panel. The figure was created by plotting data from different graphs from ref. [11] (creative commons license CC BY).
The results presented in Fig. 12 should be regarded as a feasibility test and not as a recommendation to use this method for the quantification of analytes in crude biofluids. The established method for this purpose is the "standard addition technique", which requires a strictly linear relationship between the analyte concentration and the resulting binding response over a wide concentration range.

A wide linear dose-response was reported for dextran chips that had been functionalized with a high density of anti-His<sub>6</sub> antibody (e.g., ~13600 RU [40]), provided that the injected concentration of the antigen (GFP-His<sub>6</sub>) was sufficiently low to occupy less than 50% (~1600 RU) of the maximal binding capacity for antigen (~3200 RU [40]). On our flat chip surface the level of antibody immobilization was much lower (2508 RU), and in addition the steric accessibility of the antigen-binding sites was obviously also lower, resulting in a maximal binding response of only ~120 RU (see Fig. 12B). In our dose-response curve (solid line in Fig. 7C) the only linear region was at ≤27 RU, which corresponded to a His<sub>6</sub>-GFP concentration of ≤12.5 nM. Such low dose-responses are, however, not easy to distinguish from non-specific adsorption and other imperfections of label-free biosensing.

In spite of the low dose-responses and the narrow linear region seen in Fig. 12C, the aforementioned results that were obtained with our regenerative chips are, for the following reason, encouraging: Label-enhanced SPR is able to selectively amplify the specific binding response by two orders of magnitude, without enhancing the non-specific responses [41, 42]. For this purpose, a biotinylated capture antibody is immobilized on the sensor chip, antigen is bound, and a sandwich is formed with a detector antibody that has been labeled with a dye that absorbs in the infrared region. The label-enhanced SPR response is evaluated with suitable software in common SPR biosensors [41, 42]. In combination with label-enhanced SPR, it should also be possible to perform the accurate quantification of analytes in crude samples on our regenerative surfaces. This would extend their use beyond biospecific interaction analysis, where they have been shown to work very well and where their regenerative aspect has proven to be very useful.

10. Conclusions

In this chapter, we have presented two methods for the switchable immobilization of biotinylated bait molecules on protein-resistant sensor chips. In the first one, a biotin-SAM is covered with a monolayer of a switchable avidin mutant and the biotinylated baits are bound on top of it: When desired, the biotin-avidin-biotin bridge can quickly be dissociated, resulting in the perfect regeneration of the bare biotin-SAM. In the second method, the biotin-SAM is replaced with a desthiobiotin-SAM and wild-type streptavidin is used for the immobilization of biotinylated bait molecules. The desthiobiotin-streptavidin-biotin bridge is more stable than the one employed in the first method; nevertheless, streptavidin and the biotinylated baits can be completely removed by 3-min injections of biotin, GTC, pepsin, and SDS. In both methods, the chip can be regenerated and re-functionalized with a new biotinylated bait molecule as often as
desired, except that the binding capacity of the chip starts to decrease after 2 weeks of continued use.

Fortunately, (strept)avidin and biotinylated bait are only removed under the extreme conditions that were described above; they remain in place when injecting reagents, such acidic buffer or SDS, which are commonly used for the quantitative dissociation of prey molecules which are bound on the immobile bait molecules. This means that a chip with one particular biotinylated bait can be used to measure many samples with the same prey molecule. Only when desired is the biotinylated bait removed and the bare (desthio)biotin-SAM regenerated.

The second method had a number of advantages over the first one: The immobilization of the biotinylated bait was more stable and allowed for a wider range of conditions (pH 2, pH 11.5, or SDS) in which only the prey molecules are removed, but the biotinylated bait remains attached. Moreover, streptavidin (which is used in the second method) exhibited a very low non-specific adsorption of proteins and nucleic acids. In case of the avidin mutants (which are used in the first method), the high stability is only seen with mutant #1, which however shows higher non-specific adsorption; the low non-specific adsorption of mutant #3 is associated with reduced stability and less binding of biotinylated bait molecules. The unique advantage of the avidin mutants is the faster procedure for the complete regeneration of the bare biotin-SAM.

The regenerative chips are intended for "Biospecific Interaction Analysis" (BIA), and for this application they perform very well, as described in this chapter. In comparison to the "three-dimensional" carboxymethylidextran chips, the level of bait immobilization on our flat surface is 3-4 times lower; therefore, small molecules with a molar mass of <1000 g/mol will be difficult to quantify and the standard addition technique cannot be used for quantification of analytes in crude biofluids. The latter problems should easily be solved by using label-enhanced SPR, however.

The essential advantage of our regenerative chips is that one chip can be sequentially employed by different users for unrelated measurement problems with almost any type of bait molecule. The immobilization and regeneration procedure is very simple; it therefore takes little instruction to get new users started.

In conclusion, the new regenerative chips eliminate the financial and practical barriers involved in using BIA on a daily basis. Developing simple and cheap BIA for all potential users was our intention when we initiated this work some years ago, and fortunately we have reached this goal with the above described methods.

**Acknowledgements**

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LUKAS TRAXLER
CURRICULUM VITAE

PERSONAL INFORMATION

Date of birth       June 28, 1989
Nationality        Austrian
Address            Thoman-Strasse 1, 4201 Gramastetten, Austria
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EDUCATION

11/2014–12/2017    Johannes Kepler University, Linz, Austria
PhD Studies in    Institute of Biophysics
Engineering
Thesis: A New Strategy for the Analysis of Biospecific Interactions, Exemplified for
Calmodulin and Two of its Targets
Supervisors: Prof. Hermann J. Gruber & Prof. Peter Hinterdorfer

10/2011–07/2014    Johannes Kepler University, Linz, Austria
Master Studies in  Institute of Environmental Law
Legal and Business
Thesis: Rechtliche Rahmenbedingungen bei der Zulassung von Medizinprodukten (Legal
Requirements for the Admission of Medical Devices)
Supervisor: Prof. Erika Wagner

10/2011–11/2013    University of Applied Sciences, Linz, Austria
Master Studies in  Graduated with honors
Medical
Engineering
Department for Medical Engineering
Thesis: Kinetic Investigation of the pH-dependent Biotin-Binding Behavior of Avidin and
Avidin Mutants by Use of Quartz Crystal Microbalance (QCM)
Supervisors: Prof. Andreas Ebner & Prof. Kurt Schilcher

10/2008–07/2011    University of Applied Sciences, Linz, Austria
Bachelor Studies   Graduated with honors
in Medical
Engineering
Department for Medical Engineering
Thesis: Atemfunktionsdiagnostik mit künstlichen Strömungswiderständen (Breath Diagnosis
with Artificial Flow Resistances)
Supervisor: Prof. Andreas Lindbaum

WORK EXPERIENCE

2014–date          Research Fellow
Johannes Kepler    Research fellow with teaching activities at the Institute of Biophysics
University, Linz

11/2012–10/2013    Intern
Johannes Kepler    Intern and master student at the Insitute of Biophysics
University, Linz

2007–date          Paramedic
Austrian Red       Paramedic for the Austrian Red Cross on a voluntary basis
Cross

2006–2014          Retail Employee
Merkur Waren-
handels AG, Linz
Retail employee (part-time employed)
07/2013–08/2013 Research Fellow

Research fellow for the microscopic analysis and characterization of different pharmaceuticals

Center for Advanced Bioanalysis, Linz

04/2011–07/2011 Intern

Intern for the development of an in-vitro diagnostic medical product for blood sugar analysis in intensive-care medicine

B. Braun Melsungen AG, Graz

11/2007–07/2008 Administrative Assistant

Administrative assistant in the Department for advanced training (civilian service)

Austrian Red Cross, Linz

PUBLICATIONS


Regenerative biosensor for use with biotinylated bait molecules.

Biosensors and Bioelectronics


2017 in press

Detailed Evidence for an Unparalleled Interaction Mode between Calmodulin and Orai Proteins.

Angewandte Chemie Int. Edition

Traxler L., Rathner P., Fahrner M., Stadlbauer M., Faschinger F., Charnavets T., Müller N., Romanin C., Hinterdorfer P., Gruber H. J.

2017 in press

Reversible immobilization of biotinylated baits on regenerative sensor chips: comparison of switchable avidin mutants with wild-type streptavidin.

Advances in Sensors: Reviews


2017 Biophys. J., 112(3):493a

Convenient Biological Interaction Analysis with a Regenerative Streptavidin Chip.

Biophysical Journal


2017 Biophys. J., 112(3):183-184a

Characterization of the Orai-Calmodulin Interaction as Potential Mediator of Calcium-Dependent Orai-Channel Inactivation.

Biophysical Journal

Traxler L., Faschinger F., Rathner P., Stadlbauer M., Charnavets T., Müller N., Hinterdorfer P., Gruber H. J.

2017 Biophys. J., 112(3):588a

Curli Mediate Bacterial Adhesion to Fibronectin via a Tensile Collective Binding Network.

Biophysical Journal

Oh Y. J., Hubauer-Brenner M., Gruber H. J., Cui Y., Traxler L., Siligan C., Park S., Hinterdorfer P.


Advanced portrayal of SMIL coating by alloying CZE performance with in-capillary topographic and charge-related surface characterization.

Analytica Chimica Acta


Curli mediate bacterial adhesion to fibronectin via tensile multiple bonds.

Scientific Reports

Oh Y. J., Hubauer-Brenner M., Gruber H. J., Cui Y., Traxler L., Siligan C., Park S., Hinterdorfer P.

**Mapping molecular adhesion sites inside SMIL coated capillaries using atomic force microscopy recognition imaging.**


**Regenerative biosensor chips based on switchable mutants of avidin - A systematic study.**
Zauner D., Taskinen B., Eichinger D., Flattinger C., Ruttmann B., Knoglinger C., Traxler L., Ebner A., Gruber H. J., Hytönen, V. P.

2016  Biosensors, 6:23

**Broadband 120 MHz Impedance Quartz Crystal Microbalance (QCM) with Calibrated Resistance and Quantitative Dissipation for Biosensing Measurements at Higher Harmonic Frequencies.**
Kasper M., Traxler L., Salopek J., Grabmayr H., Ebner A., Kienberger F.

**CONFERENCE CONTRIBUTIONS**

**Oral Presentations**
05/2017 · International Conference on Bio-Sensing Technology, Riva, Italy
01/2017 · Biophysical Society Meeting, New Orleans, USA
07/2016 · Doctoral College Summerschool, Strobl, Austria
06/2016 · NANOnBIO, Le Gosier/Guadeloupe, France

**Poster Presentations**
02/2017 · Biophysical Society Meeting, New Orleans, USA
07/2016 · Doctoral College Summerschool, Strobl, Austria
06/2016 · NANOnBIO, Le Gosier/Guadeloupe, France

01/2016 · Linz Winterworkshop, Linz, Austria
05/2015 · International Conference on Bio-Sensing Technology, Lisbon, Portugal
02/2015 · Linz Winterworkshop, Linz, Austria
05/2014 · International Regional Biophysics Conference, Smolenice, Slovakia
02/2014 · Linz Winterworkshop, Linz, Austria

**TEACHING ACTIVITIES**

**Academic Teaching and Tutoring**
WS 2014/15, 2015/16, 2016/17 · Methods in biochemistry I practical course
SS 2014, 2015, 2016, 2017 · Methods in biochemistry II practical course
SS 2015, 2016, 2017 · Bioanalytics II practical course
SS 2017 · Biophysics II practical course (partially)
SS 2016 · Biological interaction analysis practical course

**Students’ Supervision**
2014–2017 · (Co-)supervision of >8 bachelor and master students

**Other Tutoring Activities**
2014–2016 · “Long Night of Research”, demo sessions, Linz, Austria
2014–2017 · Linz Winter School, demo sessions, Linz, Austria

**OTHER INFORMATION**

**Grants**
2008, 2009, 2012 · Merit Scholarship awarded by the Austrian Ministry of Science

**Languages**
German · Mother tongue
English · Fluent
French · Basic knowledge

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