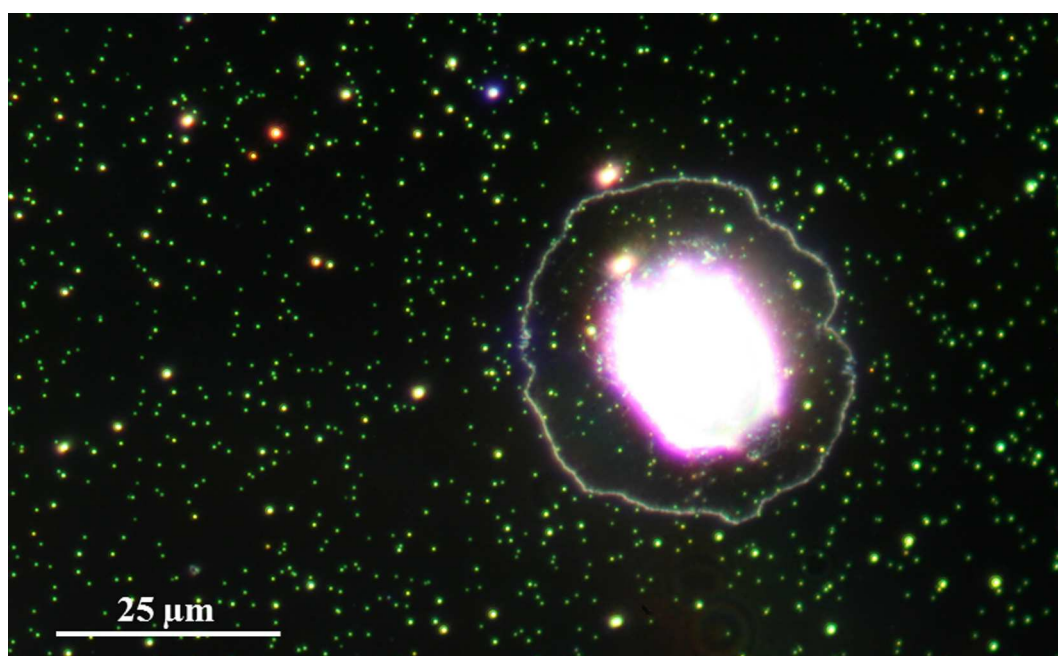


# Biological Applications of Plasmonic Metal Nanoparticles

Dissertation zur Erlangung des Grades  
"Doktor der Naturwissenschaften" im Promotionsfach Chemie  
am Fachbereich Chemie, Pharmazie und Geowissenschaften  
der Johannes Gutenberg-Universität in Mainz



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**Frontpage:** True color optical dark-field microscopy image of an adherent epithelial cell (MDCKII) growing on a glass substrate patterned with single gold nanospheres. Herein, each green dot corresponds to light scattered by an individual nanoparticle due to its plasmon resonance. The structure resembling a fried egg originates from the cell. The area around the nucleus appears white due to efficient scattering of the white light illumination by the dense material in the cytoplasm. In contrast, the thin spread out membrane appears translucent around the bright area.

This thesis presents results of work carried out from October 2008 till October 2013 in the Nanobiotechnology group of Prof. Dr. Carsten Sönnichsen at the Johannes Gutenberg-University in Mainz (D77).

Dekan:

Erster Berichterstatter:

Zweiter Berichterstatter:

Tag der mündlichen Prüfung: 10/12/2013

For Katharina and Werner

You'll never shine if you don't glow!  
- Smash Mouth -





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## Abstract

Plasmons in metal nanoparticles respond to changes in their local environment by a spectral shift in resonance. This discovery extended the field of application from immunolabeling for electron microscopy to sensing purposes. Here, the potential of plasmonic metal particles for label-free detection and observation of biological systems is presented.

The extent of the response to changes in the surrounding defines the plasmonic sensitivity of nanoparticles. Comparing the material silver and gold, silver particles exhibit a higher sensitivity voting for a favored application. However, silver particles are chemically unstable under light exposure limiting their general usage.

To sense processes of living organisms, an influence of the particles on the native behavior has to be excluded. For localization and quantification of particles within a cell, I developed a new approach combining results gained from optical dark-field microscopy and transmission electron microscopy. However, the metabolic activity of cells with a significant number of particles internalized is reduced. Hence the investigated nanoparticles are not suited for sensing applications within a cell. In contrast, cells growing on top of particles exposing a negatively charged biocompatible polymer show the same behavior in viability and proliferation as cells growing on a bare substrate making them promising candidates to sense membrane fluctuations.

Many small nanoparticles being specific sensing elements can build up an overall sensor for parallel analyte detection without any need of labeling. This multiplexed sensor is easy to fabricate, re-usable, and has a sensitivity down to nanomolar concentrations providing a convincing route to an inexpensive mass-produced product. Furthermore, the binding kinetics of various partner proteins interacting with one protein of interest are accessible in parallel. This application has currently the status of proof-of-principle and will be improved concerning sensor fabrication and measurement speed.

The small particles are able to sense local oscillations in the surface density of proteins on a lipid bilayer, which are not accessible by surface plasmon resonance spectroscopy due to averaging over the whole chip area. Studies on the fluorescently labeled system and the native system identify an influence of the label on the kinetics of a diffusion controlled process. Most remarkably, the results give evidence for a constant protein layer on top of the lipid bilayer, which was not considered from other studies so far.



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# Scientific Publications

## Parts of this thesis have been published in scientific journals

### **"Highly Sensitive Plasmonic Silver Nanorods"**

A. Jakab, C. Rosman, Y. Khalavka, J. Becker, A. Trügler, U. Hohenester, and C. Sönnichsen

*ACS Nano* **2011**, 5, 6880-6885.

### **"A New Approach to Assess Gold Nanoparticle Uptake by Mammalian Cells: Combining Optical Dark-Field and Transmission Electron Microscopy"**

C. Rosman, S. Pierrat, A. Henkel, M. Tarantola, D. Schneider, E. Sunnick, A. Janshoff, and C. Sönnichsen

*Small* **2012**, 8, 3683-3690.

### **"Multiplexed Plasmon Sensor for Rapid Label-Free Analyte Detection"**

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*Nano Letters* **2013**, 13, 3243-3247.

## Parts of this thesis are in preparation for scientific publication

### **"Mammalian Cell Growth on Gold Nanoparticle Patterned Substrates is Influenced by the Particle Coating"**

C. Rosman, S. Pierrat, and C. Sönnichsen

### **"Plasmonic Nanoparticles as Sensors for Simultaneous Quantification of Protein-Protein Interactions"**

R. Ahijado-Guzmán, C. Rosman, J. Prasad, A. Henkel, and C. Sönnichsen

### **"Single Particle Plasmon Sensors Reveal Constant Fraction of MinD Bound to the Membrane during Min Wave Propagation"**

C. Rosman, A. Martos, A. Henkel, A. Neiser, P. Schwille and C. Sönnichsen

### Additional scientific publications (not presented here)

**"Cytotoxicity of Metal and Semiconductor Nanoparticles Indicated by Cellular Micromotility"**

M. Tarantola, D. Schneider, E. Sunnick, H. Adam, S. Pierrat, C. Rosman, V. Breus, C. Sönnichsen, T. Basché, and A. Janshoff  
*ACS Nano* **2009**, 3, 213-222.

**"Toxicity of Gold-Nanoparticles: Synergistic Effects of Shape and Surface Functionalization on Micromotility of Epithelial Cells"**

M. tarantola, A. Pietuch, D. Schneider, J. Rother, E. Sunnick, C. Rosman, S. Pierrat, C. Sönnichsen, J. Wegener, and A. Janshoff  
*Nanotoxicology* **2011**, 5, 254-268.

**"Angular Trapping of Anisometric Nano-Objects in a Fluid"**

M. celebrano, C. Rosman, C. Sönnichsen, and M. Krishnan  
*Nano Letters* **2012**, 12, 5791-6796.

### Additional scientific publications in preparation

**"Size Variation Accounts for One Third of Interparticle Variation in Plasmonic Sensitivity of Gold Nanorods"**

A. Henkel, Y. Khalavka, A. Neiser, C. Rosman, S. Schmachtel, and C. Sönnichsen

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# 1 Chapter 1.

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## Introduction

Plasmonic metal nanoparticles find more and more their way into biological applications due to their tiny size and their strong interaction with light. First usage in biological samples was reported in 1971<sup>1</sup> and benefited from the material itself and the size being on the nanometer scale: gold nanoparticles treated with antibodies were used as contrast agents for electron microscopy to specifically label bacteria according to their antigen. Already a decade later, the so-called immunogold labeling was established as a routine method giving rise to a commercial market for antibody functionalized gold nanoparticles and expanding the use as contrast agent to bright-field light microscopy.<sup>2</sup> However, in bright-field light microscopy, the resolution is not powerful enough to image single nanoparticles as in electron microscopy, but the amount of material associated with the specimen of interest was high enough to create contrast.

The strong interaction of metal nanoparticles with light is attributed to their plasmon, which is a collective oscillation of the electrons in the conduction band. Light scattering of gold particles was first observed with an optical microscope operating in dark-field geometry in 1902.<sup>3</sup> Herein, each gold particle appears as bright colored spot on a black background. Optical dark-field microscopy was used in 1977 to visualize the tracks of cell migration as voids in an otherwise dense nanoparticle 'carpet'.<sup>4</sup> The first single particle scattering spectrum was acquired in 1998<sup>5</sup> giving rise to sensing applications of changes in the local environment, which are reported by a

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<sup>1</sup> Faulk, Taylor, "Immunocolloid Method for the Electron Microscope."

<sup>2</sup> Van Laere et al., "Immuno Gold Staining (IGS) and Immuno Gold-Silver Staining (IGSS) for the Identification of the Plant Pathogenic Bacterium *Erwinia-Amylovora* (Burril) Winslow *et al.*"

<sup>3</sup> Siedentopf, Zsigmondy, "Über Sichtbarmachung und Größenbestimmung ultramikroskopischer Teilchen mit besonderer Anwendung auf Goldrubingläser."

<sup>4</sup> Albrecht-Bühler, "Phagokinetic Tracks of 3T3-Cells."

<sup>5</sup> Klar et al., "Surface-Plasmon Resonances in Single Metallic Nanoparticles."

response of the plasmon.<sup>6</sup> The first application involved measurement of the specific molecular recognition between streptavidin and biotinylated BSA on a gold nanosphere.<sup>7</sup> Later, this study was extended by investigation of streptavidin binding to biotinylated lipids using gold nanorods as single sensor elements.<sup>8</sup> Within this time, setups for optical dark-field spectroscopy were improved from recording the response of one particle to gaining statistics over 20 particles.<sup>9,10</sup> Further setup improvements by Dr. Andreas Henkel increased statistics to a few hundreds of particles<sup>11</sup> giving access to the parallel detection of multiple molecular recognition motifs within one sample, which was achieved during the time of my PhD.<sup>12</sup> The principle of parallel detection pushes optical dark-field spectroscopy to a high-throughput method.

Following the plasmon response over time, adsorption or binding processes are reported in real time without need of any label or marker like in surface plasmon resonance spectroscopy, but on a much smaller scale. Recently, the ultimate sensitivity was demonstrated by the detection of single proteins adsorbing to a gold nanorod.<sup>13</sup> Using the same improved setup described before, I was able to gain statistics of the binding process by observing the plasmon resonance of few tens of particles within one experiment. Combined with the concept of parallel binding detection for multiple motifs, binding constants of several protein mutants responding to the same target can be extracted from one experiment. Therefore, an immobilization of the protein mutants on the particles without denaturation is necessary to investigate the native system, which I established. Finally, I investigated a dynamic protein system using single gold nanorods. The results demonstrate that the technique is non-invasive and superior compared to fluorescence based techniques and surface plasmon resonance.

Since gold nanoparticles have proven as useful tools to study proteins, the next step in system complexity are living organisms like cells. Therefore, I performed initial studies to gain a basic understanding of the interaction of gold nanoparticles with mammalian cells. Besides the results demonstrating the successful extension in the field of applications, I worked as well on the synthesis of plasmonic metal nanoparticles and investigated their

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<sup>6</sup> Kreibig, Vollmer, *Optical Properties of Metal Clusters*.

<sup>7</sup> Raschke et al., "Biomolecular recognition based on single gold nanoparticle light scattering."

<sup>8</sup> Bacia et al., "Protein-membrane interaction probed by single plasmonic nanoparticles."

<sup>9</sup> Raschke et al., "Biomolecular recognition based on single gold nanoparticle light scattering."

<sup>10</sup> Bacia et al., "Protein-membrane interaction probed by single plasmonic nanoparticles."

<sup>11</sup> Henkel, *Rod-Shaped Plasmonic Sensors: Synthesis and Single Particle Spectroscopy*.

<sup>12</sup> Rosman et al., "Multiplexed Plasmon Sensor for Rapid Label-Free Analyte Detection."

<sup>13</sup> Ament et al., "Single Unlabeled Protein Detection on Individual Plasmonic Nanoparticles."



basic properties. This thesis is structured as follows: In the next chapter (Chapter 2), I will explain the physical properties, which made plasmonic metal nanoparticles finding their way to biological applications. Herein, the plasmon response to changes in the local environment of the particle is the key feature for sensing applications. The technique to follow the plasmon response of single nanoparticles is optical dark-field spectroscopy, which is introduced as well.

In Chapter 3, I present studies on the plasmonic sensitivity of silver nanorods, which I synthesized and characterized. It turned out that these silver particles show a more pronounced plasmon response to changes in the local environment than gold particles of a comparable size. This higher sensitivity favors in principle the application of silver particles compared to gold particles in sensing experiments. However, silver particles are chemically unstable under light exposure limiting their sensing capabilities and the material itself is not as inert as gold, which are barriers for routine use.

The following two chapters cover the interaction of gold nanoparticles and mammalian cells. In Chapter 4, I investigate the uptake of gold particles by a model cell line depending on shape and functionalization. Therefore, I developed a new approach to quantify the uptake by combining the results of transmission electron microscopy and optical dark-field microscopy. The extent of uptake varies significantly for the investigated stabilizing agents. Herein, particles stabilized by a weakly bound surfactant enter the cells in a high degree impairing their metabolic activity. The results of this study suggest the need of further experiments till intended delivery of a nanoparticle into a cell works out without influencing its native behavior. For cells growing on top of differently functionalized gold nanorods, I found already a system showing a comparable behavior to unaffected cells (Chapter 5). Here, particles with a biocompatible polymer exposing a negatively charged head-group to the cell do not seem to interact with the membrane. Therefore, these particles are promising candidates for the sensing of membrane fluctuations from which mechanical properties are deduced.

In Chapter 6, I present a sensor having single gold nanorods as sensing elements for the parallel, label-free detection of multiple analytes from one solution of interest. Besides this multiplexed target detection, I demonstrate the easy fabrication, re-usability, and sensitivity down to nanomolar concentrations, which provides a convincing route to an inexpensive mass-produced product. Fields of application are abundant, for example, identifying diseases, monitoring food quality and composition, screening for

drugs or hazards.

Extending the multiplexed investigation of different species by time resolved measurements, parallel kinetic investigations are possible. In order to study binding of a protein to various mutants of the partner protein, the observation of proteins in their native form keeping their functionality is essential. Therefore, I transferred the functionalization strategy used to immobilize native proteins on gold chips developed for surface plasmon resonance spectroscopy to gold nanoparticles. With these functionalized particles, not only reported binding constants could be reproduced, but also the binding of FtsZ to three binding partners could be monitored in parallel. The results are presented in Chapter 7.

Chapter 8 presents the application of single gold nanorods to sense the dynamic oscillation of the Min protein system label-free and in real-time. This oscillation takes place on such a small length scale (about 100  $\mu\text{m}$ ) that surface plasmon resonance spectroscopy misses the dynamics due to averaging over the area of the chip. Here, the nanoparticles being small in size and therefore having a local sensing capability are better suited to the length scales under investigation than surface plasmon resonance spectroscopy. In addition, the nanoparticle sensors perform superior to fluorescence based techniques, since there is no bleaching of the signal and the results even demonstrate a change in the dynamics when labeled proteins are investigated underlining the need of native protein observation. Furthermore, the nanoparticles identified a constant fraction of protein not taking part in the oscillation, which was not considered so far.

The summary in Chapter 9 is followed by an Appendix with material for supporting information to the main chapters.

## 1.1. References

1. Faulk, W. P.; Taylor, G. M. *Immunochemistry* **1971**, *8*, 1081–1083.
2. Van Laere, O.; De Wael, L.; De Mey, J. *Histochemistry* **1985**, *83*, 397–399.
3. Siedentopf, H.; Zsigmondy, R. *Annalen der Physik* **1902**, *315*, 1–39.
4. Albrecht-Bühler, G. *Cell* **1977**, *11*, 395–404.
5. Klar, T.; Perner, M.; Grosse, S.; Von Plessen, G.; Spirk, W.; Feldmann, J. *Physical Review Letters* **1998**, *80*, 4249–4252.
6. Kreibig, U.; Vollmer, M., *Optical Properties of Metal Clusters*; Springer Series in Materials Science, Vol. 25; Springer: 1995.
7. Raschke, G.; Kowarik, S.; Franzl, T.; Sönnichsen, C.; Klar, T. A.; Feldmann, J.; Nichtl, A.; Kürzinger, K. *Nano Letters* **2003**, *3*, 935–938.
8. Baci, C. L.; Becker, J.; Janshoff, A.; Sönnichsen, C. *Nano Letters* **2008**, *8*, 1724–1728.
9. Henkel, A., *Rod-Shaped Plasmonic Sensors: Synthesis and Single Particle Spectroscopy*; PhD Thesis: 2012.
10. Rosman, C.; Prasad, J.; Neiser, A.; Henkel, A.; Edgar, J.; Sönnichsen, C. *Nano Letters* **2013**, *13*, 3243–3247.
11. Ament, I.; Prasad, J.; Henkel, A.; Schmachtel, S.; Sönnichsen, C. *Nano Letters* **2012**, *12*, 1092–1095.



## Chapter 2.

# 2 Theory and Background

Plasmonic metal nanoparticles gave vivid colors to glass already in ancient times (e.g. Lycurgus Cup).<sup>1</sup> Admittedly, this staining was the result of 'lucky coincidences' and its origin was unknown. Systematic studies describing physical properties of colored solutions containing colloidal gold particles evolved in the 19th century.<sup>2,3</sup> However, it took nearly one century more until the first biological application of gold nanoparticles was reported.<sup>4</sup> This application did not take advantage of the outstanding optical properties, but rather used the the high electron density of the particles as label. The single particle plasmon was first utilized as sensor only 15 years ago,<sup>5</sup> but since then a huge number of studies has been reported (ISI Web of Knowlegde: more than 4000 publications tagged with 'nanoparticle sensor' on 30/10/2013). In this chapter, I present the development in biological applications of plasmonic metal nanoparticles and I relate these applications to the physical properties of the particles.

### 2.1. Label in Electron Microscopy

In electron microscopy, contrast is generated by interaction of the electron beam with the specimen. Herein, contrast is stronger for elements with higher atomic numbers (i.e. more electrons interfere with the electron beam). For biological samples, contrast is poor due to the light atoms (mainly H, C, O, N, S) composing the tissue. Therefore, techniques to enhance contrast by staining or labeling with electron rich materials were developed since

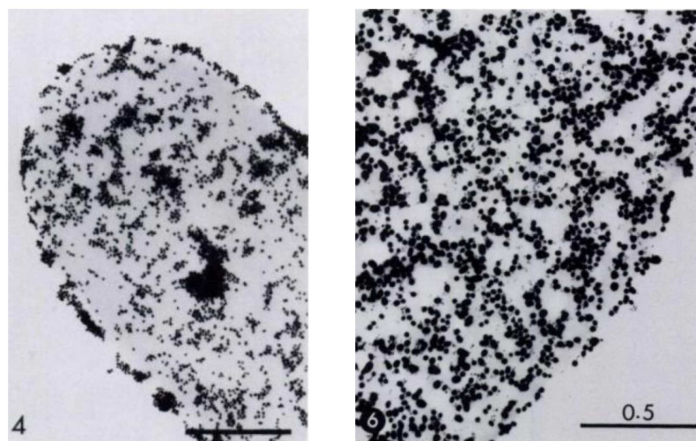
<sup>1</sup> Mulvaney, "Surface plasmon spectroscopy of nanosized metal particles."

<sup>2</sup> Faraday, "The bakerian lecture: Experimental relations of gold (and other metals) to light."

<sup>3</sup> Rayleigh, "On the transmission of light through an atmosphere containing small particles in suspension, and on the origin of the blue of the sky."

<sup>4</sup> Faulk, Taylor, "Immunocolloid Method for the Electron Microscope."

<sup>5</sup> Sönnichsen et al., "Spectroscopy of single metallic nanoparticles using total internal reflection microscopy."



**Figure 2.1.:** Transmission electron microscopy image of bacterium labeled with gold nanoparticles **Left)** Overview, scale bar represents 1  $\mu\text{m}$ . **Right)** Magnified view, scale bar represents 0.5  $\mu\text{m}$ . Images are taken from Horisberger and Rosset.<sup>8</sup>

1959.<sup>6,7</sup> Herein, specific target labeling was achieved by linking an antibody to the contrast creating metal. Inspired by this approach, Faulk and Taylor demonstrated in 1971 that gold colloids with adsorbed antibodies specifically label the corresponding antigens of a bacterial membrane. In order to localize the labels, ultra-thin sections of a conserved specimen being between 50 nm and 100 nm thick have to be prepared before imaging. Figure 2.1 shows labeling of bacteria with gold nanoparticles done by Horisberger and Rosset.<sup>8</sup>

If compartments within the biological tissue, which the particles do not assess from outside, need to be visualized, labeling can be performed after fixation and sectioning of the sample as well. Using distinguishable particle sizes functionalized specifically for different targets, discrimination of multiple compartments within one sample is possible. Besides visualization or localization of target sites, there are techniques available to create a 3D-reconstitution from consecutive specimen sections, which quantify the amount of internalized particles.<sup>9</sup> During the past 40 years, immunolabeling evolved to a routine technique and a commercial market established with various particle sizes and different conjugates available. Nevertheless, the technique takes just advantage of plasmonic metal nanoparticles as a electron

<sup>6</sup> Singer, "Preparation of an Electron-Dense Antibody Conjugate."

<sup>7</sup> Sternberger, "Electron Microscopic Immunochemistry - A Review."

<sup>8</sup> Horisberger, Rosset, "Colloidal Gold, A Useful Marker for Transmission and Scanning Electron Microscopy"

<sup>9</sup> Mayhew et al., "A review of recent methods for efficiently quantifying immunogold and other nanoparticles using TEM sections through cells, tissues and organs."

dense material, which can be reliably synthesized in a small size compared to biological tissue and which is easy to functionalize with antibodies. The plasmonic optical properties are completely neglected.

## 2.2. Label in Light Microscopy

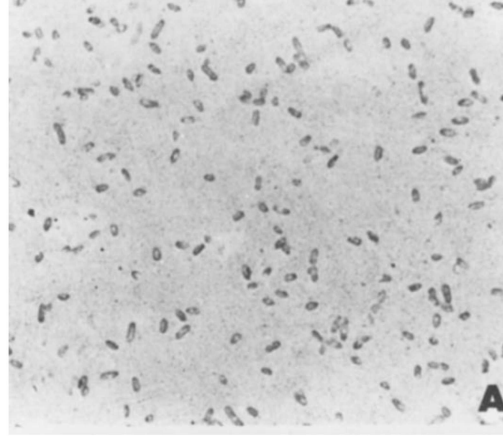
If there is no high resolution of immunolabeled samples required, the complete specimen can be imaged using light microscopy without ultra-thin sectioning, which reduces the effort in sample preparation remarkably. In 1985, plant pathogenic bacteria were identified by showing dark granules in bright-field microscopy after labeling with 20 nm gold colloids (Figure 2.2).<sup>10</sup> Contrast was in this case not generated by single particles, but simply the number of particles associated with the bacterial membrane was so high resulting in a dense coverage, which was able to interact with light to a detectable degree. However, light microscopy visualizes single particles if the illuminating light path is set up in dark-field geometry. Herein, the resolution is not increased compared to bright-field illumination and optical dark-field microscopy is not powerful enough to image the actual shape of gold nanoparticles as well, but the particles are so efficient scattering centers due to their plasmon that they appear as colorful diffraction limited spots on a dark background. Here, I will first present the physics causing the strong scattering and then I will give examples taking advantage of the strong single particle scattering for visualization.

**Plasmon.** The plasmon is a quasi-particle and it is excited by an electromagnetic wave. In a metal nanoparticle, the electromagnetic wave moves the free electron gas collectively away from the stationary lattice ions, which then act as an restoring force attracting the free electron gas back (Figure 2.3).<sup>11</sup> Therefore, a driven harmonic oscillation takes place forming a dipole of alternating charges, which exhibits a resonance frequency. For the bulk metal, the plasma frequency  $\omega_P$  is described in the free electron model including the density  $n$  and the effective mass  $m^*$  of the conduction electrons, the electron

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<sup>10</sup> Van Laere et al., “Immuno Gold Staining (IGS) and Immuno Gold-Silver Staining (IGSS) for the Identification of the Plant Pathogenic Bacterium *Erwinia-Amylovora* (Burril) Winslow *et al.*”

<sup>11</sup> Willets, Van Duyne, “Localized Surface Plasmon Resonance Spectroscopy and Sensing”



**Figure 2.2.:** There are so many gold nanoparticles attached to bacterial cells that they appear as dark granules on a pale background in bright-field light microscopy. Image taken from Van Laere *et al.*<sup>10</sup>

charge  $e$ , and the permittivity of free space  $\varepsilon_0$  by:<sup>12</sup>

$$\omega_P = \sqrt{\frac{ne^2}{m^*\varepsilon_0}} \quad (2.1)$$

In the case of plasmonic metal nanoparticles, the particle resonance frequency  $\omega_{\text{res}}$  is influenced by the surrounding and the bulk plasma frequency changes with the dielectric constant of the embedding medium  $\varepsilon_m$ :<sup>12</sup>

$$\omega_{\text{res}} = \frac{\omega_P}{\sqrt{2\varepsilon_m + 1}} \quad (2.2)$$

At the resonance frequency, the plasmonic nanoparticles scatter and absorb light strongly. Since only scattered light is detected in optical dark-field microscopy, I will focus on this property. The intensity in light scattering by the particle  $I_{\text{sca}}(\omega)$  follows from the interaction of illuminating light intensity per area  $I_0(\omega)/A$  with the frequency dependent scattering cross-section of the particle  $C_{\text{sca}}(\omega)$ :

$$I_{\text{sca}}(\omega) = \frac{I_0(\omega)}{A} C_{\text{sca}}(\omega) \quad (2.3)$$

The scattering cross-section is related to the polarizability of the particle  $\alpha$ :<sup>13</sup>

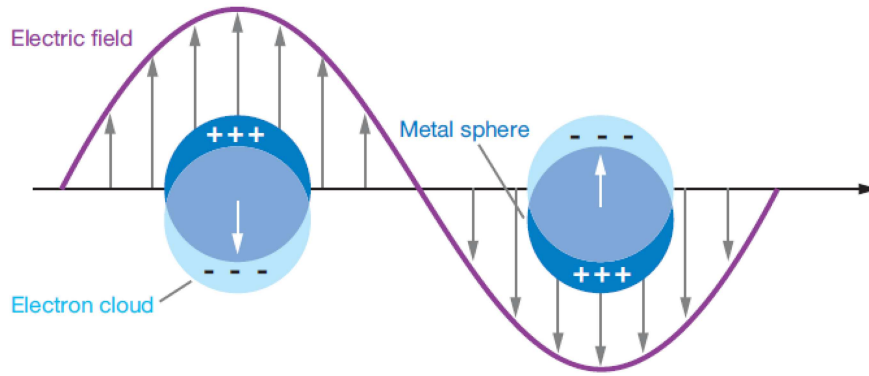
$$C_{\text{sca}} = \frac{k^4}{6\pi} \left| \frac{\alpha}{\varepsilon_0} \right|^2 \quad (2.4)$$

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<sup>12</sup> Mayer, Hafner, “Localized Surface Plasmon Resonance Sensors”

<sup>13</sup> Bohren, Huffman, *Absorption and Scattering of Light by Small Particles*





**Figure 2.3.:** The term 'plasmon' describes the collective oscillation of the free electron gas with respect to the position fixed lattice ions in a metal nanoparticle due to excitation with an electromagnetic wave. Image taken from Willets and Van Duyne.<sup>10</sup>

with  $k = 2\pi/\lambda$  with  $\lambda$  for the wavelength.

Expressions for the polarizability can be derived within the quasi-static approximation (QSA). Herein, the applied field around the particle is assumed to be constant due to the small size of the particle compared to the incident electromagnetic wave. This approximation neglects effects due to self-induction of electromagnetic fields, therefore holding true well for particle diameters up to 40 nm. For spherical particles, electrostatic calculations result in the polarizability expressed by the Clausius-Mossotti relation:<sup>13</sup>

$$\alpha = 3V\epsilon_0 \frac{\epsilon_r - 1}{\epsilon_r + 2} \quad (2.5)$$

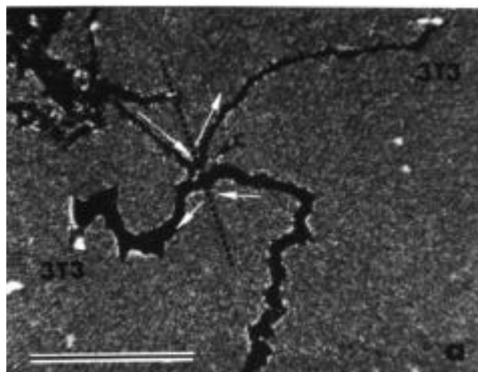
with the dielectric constant relativ to the medium  $\epsilon_r = \epsilon_{\text{metal}}/\epsilon_{\text{medium}}$  and the particle volume  $V$ . The polarizability can be extended to ellipsoidal particles. Herein, introduction of an geometry factor  $L_i$  yields for an ellipsoid in a field parallel to the axis  $i$ :<sup>13</sup>

$$\alpha_i = V\epsilon_0 \frac{\epsilon_r - 1}{1 + L_i(\epsilon_r - 1)} \quad (2.6)$$

Since nanorods are not completely described by ellipsoids, appropriate geometrical factors matching the shape and accounting for different end cap structures were developed.<sup>14</sup>

Using optical dark-field microscopy benefiting from the strong scattering due to the plasmon, tracks of cells on top of substrate covered with gold

<sup>14</sup> Prescott, Mulvaney, "Gold nanorod extinction spectra."



**Figure 2.4.:** Cell migration creates voids in the dense nanoparticle coverage of a patterned substrate. The track of the cell can be clearly seen by optical dark-field microscopy as black line in a field of dots being the scattered light of the plasmonic nanoparticles. Scale bar represents 0.5 mm. Image taken from Albrecht-Bühler.<sup>15</sup>

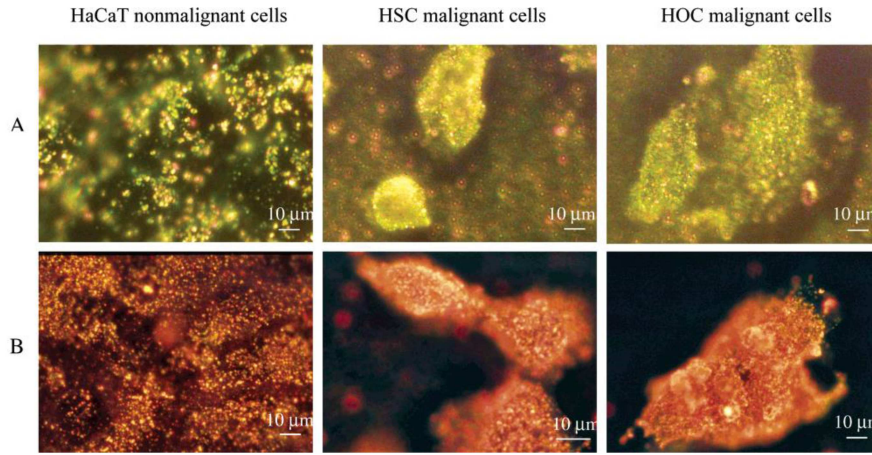
nanoparticles were visualized in 1977 (Figure 2.4).<sup>15</sup> Herein, so many particles were deposited on a substrate that a dense coverage of colorful dots was observed. Then, cells were grown on the substrate for at least one day and the sample was fixed using formaldehyde. During the incubation time, the migration of the cells left voids in the dense nanoparticle carpet indicating clearly the track.

Huang *et al.* discriminated between nonmalignant and malignant cells by labeling with gold nanoparticles (Figure 2.5).<sup>16</sup> For specific targeting of malignant cells, the authors functionalized particles with the antigen for the epidermal growth factor receptor, which is overexpressed in case of cancerous mutation. Afterwards, different cell lines were incubated with the particles carrying the antigen. Using optical dark-field microscopy, the sample of nonmalignant cells showed a random particle distribution all over, whereas malignant cells attracted the particles to such a high extent that they appeared as bright islands. Hence, visualization is straight forward in optical dark-field microscopy, but it is not the main application of plasmonic metal nanoparticles. The outstanding feature of those particles is their sensitivity to changes in the immediate local environment, which is presented in the following section.

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<sup>15</sup> Albrecht-Bühler, "Phagokinetic Tracks of 3T3-Cells"

<sup>16</sup> Huang et al., "Cancer cell imaging and photothermal therapy in the near-infrared region by using gold nanorods"



**Figure 2.5.:** Gold nanospheres and gold nanorods were functionalized to specifically target cancer cells. In optical dark-field microscopy, non-malignant cells show an even distribution of particles, whereas malignant cells appear as bright islands due to the high amount of attached nanoparticles. The upper row of images shows targeting with gold nanospheres and the lower shows targeting with gold nanorods, respectively. The different colors originate from the plasmon resonance depending on the nanoparticle shape. Images taken from Huang *et al.*<sup>16</sup>

### 2.3. Sensors for Changes in Local Environment

Plasmonic metal nanoparticles are efficient sensors for changes in their immediate environment. Herein, the plasmon resonance reports these changes by shifting the resonance wavelength. Converting Formula (2.2) from frequency to wavelength via  $\lambda = 2\pi c/\omega$  and from dielectric constant to refractive index via  $\epsilon_m = n^2$  yields:<sup>12</sup>

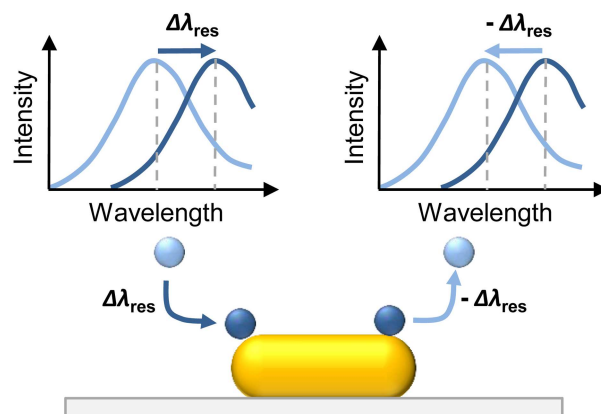
$$\lambda_{\text{res}} = \lambda_P \sqrt{2n_m^2 + 1} \quad (2.7)$$

Hence, the resonance shifts to longer wavelength if the refractive index in the surrounding is raised or the resonance shifts to shorter wavelength if the surrounding refractive index is lowered (Figure 2.6).

**Bulk refractive index change.** If the complete environment around a nanoparticle is exchanged, the magnitude of the plasmon shift  $\Delta\lambda$  with respect to the magnitude of the refractive index change  $\Delta n$  defines the bulk sensitivity  $S$ :

$$S = \frac{\Delta\lambda}{\Delta n} \quad (2.8)$$

In order to have an efficient sensor being able to resolve a refractive in-



**Figure 2.6.:** Protein attachment raises the local refractive index of the surrounding of the nanoparticle, which leads to a shift of the plasmon resonance to longer wavelength  $\Delta\lambda_{\text{res}}$ . Detachment of this protein lowers the refractive index of the surrounding back to the starting situation resulting in an inversion of the plasmon shift  $-\Delta\lambda_{\text{res}}$ .

dex change as small as possible with a detectable shift in resonance wavelength, the sensitivity value should be as large as possible. For election of the nanoparticle with most pronounced sensitivity, different materials and shapes were exposed to bulk refractive index changes. Among the materials gold, spheres,<sup>17</sup> nanoshells,<sup>18</sup> bipyramids,<sup>19</sup> rods,<sup>20</sup> and rod-shaped rattles<sup>21</sup> were tested and showed sensitivities from 40 nm/RIU for spheres to 280 nm/RIU for rattles. In addition to exposure of particles to various environments, there are also theoretical studies presented. For example, Lee and El-Sayed calculated sensitivity values for gold nanorods with an increasing length ranging from 150 nm/RIU to 650 nm/RIU.<sup>22</sup> In Chapter 3, I present experimental results comparing the sensitivity of silver nanorods and gold nanorods. Herein, silver nanorods exhibit a higher sensitivity than gold nanorods, which originates from the dielectric properties of the materials.

**Refractive index change due to molecule adsorption.** The linear relation between sensitivity and shift in resonance wavelength depicted in Formula

<sup>17</sup> Raschke et al., "Biomolecular recognition based on single gold nanoparticle light scattering."

<sup>18</sup> Mock et al., "Local refractive index dependence of plasmon resonance spectra from individual nanoparticles."

<sup>19</sup> Chen et al., "Shape- and size-dependent refractive index sensitivity of gold nanoparticles."

<sup>20</sup> Baciú et al., "Protein-membrane interaction probed by single plasmonic nanoparticles"

<sup>21</sup> Khalavka et al., "Synthesis of Rod-Shaped Gold Nanorattles with Improved Plasmon Sensitivity and Catalytic Activity."

<sup>22</sup> Lee, El-Sayed, "Gold and Silver Nanoparticles in Sensing and Imaging: Sensitivity of Plasmon Response to Size, Shape, and Metal Composition."

(2.8) holds true, if the complete environment around the particle is exchanged. In case there is a refractive index change just on the surface of the particle but within the plasmon field - like for adsorption of small molecules - the exponential decay of the shift in resonance wavelength with distance  $r$  from the surface has to be considered:<sup>23</sup>

$$\Delta\lambda(r) = S\Delta n e^{-r/l_d} \quad (2.9)$$

with  $l_d$  as the effective electromagnetic decay length defining the sensing volume  $V_S$  of the particle, which contains 95% of its sensitivity. The attachment of one molecule having a volume of  $V_M$  results in a shift per molecule  $\Delta\lambda_M$  of:

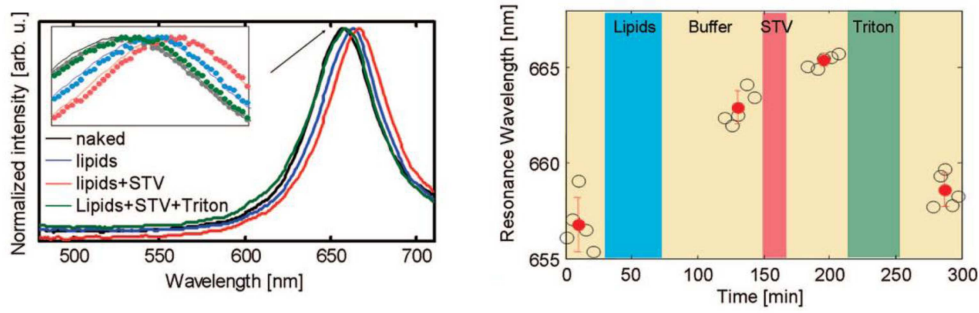
$$\Delta\lambda_M = S\Delta n \frac{V_M}{V_S} \quad (2.10)$$

Hence, in order to obtain an optimal plasmon response, the sensing volume of the particle needs to be suitable for the volume of the molecule. Ament *et al.* investigated the shift in resonance length induced by adsorption of the protein fibronectin to gold nanorods with different sensing volumes.<sup>24</sup> Herein, they found the largest plasmon response for rods with the smallest volume. However, using the particle with the smallest sensing volume is just valid for direct adsorption to the particle. In case of a functional layer for target specific detection present on the particle, the optimal sensor has to exhibit a sensing volume considerably larger than the volume already occupied by the anchoring layer. Baciú *et al.* measured the plasmon response for different states in particle coverage (Figure 2.7).<sup>20</sup> First, they covered gold nanorods with biotinylated lipids. Then, specific binding of streptavidin to the biotinylated lipids was detected. Finally, everything was removed from the particle with a surfactant recreating the starting situation. If the dimensions of the molecules involved (i.e. molecules for specificity and target molecules) are known, the optimal sensor dimensions can be estimated. Such an estimation was carried out for the Multiplexing presented in Chapter 6 and the measurements of the Min system presented in Chapter 8.

**Time-resolved measurements of the plasmon response.** If the concentration of an analyte is low and the time-resolution of the setup used to extract the plasmon resonance is fast enough, adsorption events of single

<sup>23</sup> Nusz et al., "Rational Selection of Gold Nanorod Geometry for Label-Free Plasmonic Biosensors."

<sup>24</sup> Ament et al., "Single Unlabeled Protein Detection on Individual Plasmonic Nanoparticles"



**Figure 2.7.:** Plasmon resonance wavelength for different states in nanoparticle coverage. Gold nanorods were covered with biotinylated lipids, which led to an increase of the resonance wavelength. Afterwards, binding of streptavidin to the biotin anchors further increased the resonance wavelength. Washing with the surfactant Triton removed the lipids from the particles, which reverted the resonance wavelength back to the starting value. **Left)** Single particle scattering spectra of one particle for the different states in coverage. **Right)** Statistics of several particles for the different states in coverage. Image taken from Baciú *et al.*<sup>20</sup>

molecules can be resolved.<sup>24,25</sup> Figure 2.8 shows the attachment of single fibronectin molecules as stepwise increase in the resonance wavelength. This responsivity results from the sensing volume of the nanoparticle matching better molecular dimensions than the sensing volume being active in surface plasmon resonance spectroscopy.<sup>24</sup> If an overall adsorption instead of single events is detected, kinetics are accessible like in surface plasmon resonance spectroscopy. The plasmon shift over time  $\Delta\lambda(t)$  with the maximum value  $\Delta\lambda_{\max}$  can be described by Langmuir adsorption:

$$\Delta\lambda(t) = \Delta\lambda_{\max}(1 - e^{-k/t}) \quad (2.11)$$

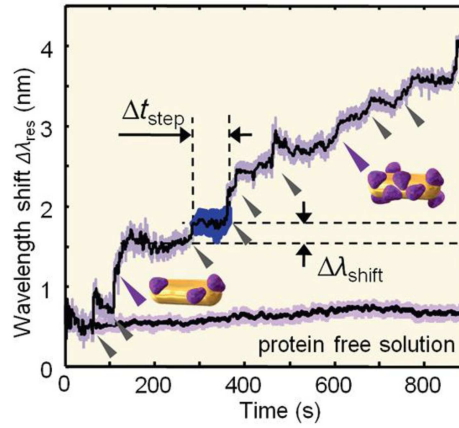
Herein,  $k$  shows a linear dependence on the analyte concentration  $c$  revealing the association rate  $k_{\text{on}}$  and the dissociation rate  $k_{\text{off}}$

$$k = k_{\text{on}}c + k_{\text{off}} \quad (2.12)$$

However, there are only a few time-resolved studies using single nanoparticle sensors demonstrating the proof-of-principle, but no kinetics were assessed on the single particle level so far.<sup>24,25,26</sup> In Chapter 6, I present the association

<sup>25</sup> Zijlstra *et al.*, “Optical detection of single non-absorbing molecules using the surface plasmon resonance of a gold nanorod”

<sup>26</sup> Mayer *et al.*, “A single molecule immunoassay by localized surface plasmon resonance.”



**Figure 2.8.:** The adsorption of fibronectin was monitored in a time-resolved measurement of the plasmon resonance. Each adsorption event led to a stepwise increase of the resonance wavelength due to the sudden change in the local refractive index of the particle surrounding. Image taken from Ament *et al.*<sup>20</sup>

and dissociation rate for the interaction of streptavidin with its target specific aptamer being extracted from measurements on single particles.

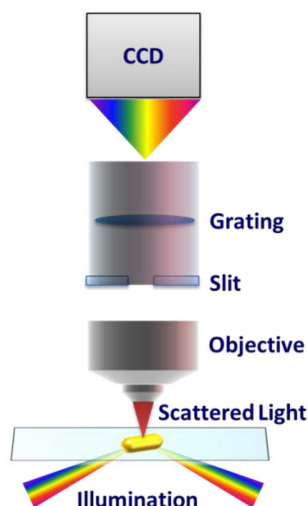
Instead of determining the actual rates, the overall dissociation constant  $K_D$  (ratio of dissociation rate to association rate) can be deduced. Herein, the surface coverage  $\Theta$  on top of the particle is indicated by the equilibrium plasmon shift  $\Delta\lambda_{eq}$ , which depends on the concentration  $c$  according to the Langmuir isotherm:

$$\Theta \sim \Delta\lambda_{eq} = \Delta\lambda_{max} \frac{c}{K_D + c} \quad (2.13)$$

The dissociation constant is a quite general information on the system, since different combinations of association and dissociation rates result in the same constant.<sup>27</sup> However, the constant is easier to access experimentally than the rates. For the rates, each concentration demands a separate, time-resolved experiment following the adsorption curve to extract  $k$  and several  $k$ -values are needed. In contrast, for the dissociation constant, the concentration can be raised stepwise within one experiment with the restriction that equilibrium has to be reached before increase of the concentration in order to determine the equilibrium plasmon shift. Hence, this kind of experiment does not necessarily require a time-resolved measurement, but following

<sup>27</sup> Önell, Andersson, “Kinetic determinations of molecular interactions using Biacore - minimum data requirements for efficient experimental design.”





**Figure 2.9.:** In optical dark-field spectroscopy, the sample is illuminated with white light in dark-field geometry so that the objective collects only scattered light. The scattered light passes the entrance slit of a spectrograph and gets dispersed. Finally, a CCD-camera captures the distribution in light intensity. (Sketch by Dr. Andreas Henkel)

the plasmon resonance over time indicates the equilibrium situation. In Chapter 7, I present dissociation constants of the protein FtsZ and several binding partners, which were determined in parallel from one sample in a multiplexed way.

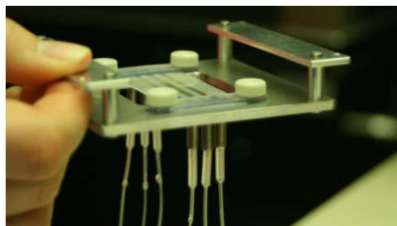
## 2.4. Optical Dark-field Spectroscopy

In optical dark-field spectroscopy, the setup is built as follows: a conventional light microscope is operated with dark-field illumination and the scattered light of the sample is passed through a spectrograph with an entrance slit to disperse the intensity according to the wavelength, which is afterwards captured by a CCD-camera (Figure 2.9). In order to acquire a single particle spectrum, an immobilized particle has to be placed below the entrance slit of the spectrograph. Dr. Andreas Henkel developed a setup design and software for fast and accurate spectra acquisition.<sup>28</sup> I built a copy of this setup, which was used for the studies presented in Chapters 6-8. Details and benefits of the method are shown in the Appendix Chapter D.

For sensing applications, the environment around the particles needs to be

<sup>28</sup> Henkel, *Rod-Shaped Plasmonic Sensors: Synthesis and Single Particle Spectroscopy*.





**Figure 2.10.:** Improved flow cell design exhibiting three separate channels for sensing experiments. The flow cell consists of two cover glasses held together by a piece of Nescofilm with voids creating the channels. In order to realize a flow, one cover glass exhibits holes for the direct attachment of tubings with the help of screws.

exchanged in order to determine the plasmon response. At the beginning of my PhD, we used flat glass capillaries with glued tubings as flow cells described in Chapter 3. Since the flow cell has to be replaced before starting a new sensing experiment, there was a loss of time between the experiments. Over time, I minimized the effort in sample replacement by increasing the number of flow channels within one flow cell to three. The improved flow cells are a composite of two cover glasses held together with a piece of Nescofilm having three separate flow channels. Herein, one piece of glass exhibits holes to attach tubings for solution exchange. The attachment of tubings changed from gluing to direct contact using screws. A picture of such an improved chamber in the microscope holder is shown in Figure 2.10.

## 2.5. References

1. Mulvaney, P. *Langmuir* **1996**, *12*, 788–800.
2. Faraday, M. *Philosophical Transactions of the Royal Society of London* **1857**, *147*, 145–181.
3. Rayleigh, L. *Philosophical Magazine (Series 5)* **1899**, *47*, 375–384.
4. Faulk, W. P.; Taylor, G. M. *Immunochemistry* **1971**, *8*, 1081–1083.
5. Sönnichsen, C.; Geier, S.; Hecker, N. E.; Plessen, G. von; Feldmann, J.; Ditzbacher, H.; Lamprecht, B.; Krenn, J. R.; Aussenegg, F. R.; Chan, V. Z. H.; Spatz, J. P.; Möller, M. *Applied Physics Letters* **2000**, *77*, 2949–2951.
6. Singer, S. J. *Nature* **1959**, *183*, 1523–1524.
7. Sternberger, L. A. *Journal of Histochemistry & Cytochemistry* **1967**, *15*, 139–159.
8. Horisberger, M.; Rosset, J. *Journal of Histochemistry & Cytochemistry* **1977**, *25*, 295–305.
9. Mayhew, T. M.; Muehlfeld, C.; Vanhecke, D.; Ochs, M. *Annals of Anatomy-Anatomischer Anzeiger* **2009**, *191*, 153–170.
10. Van Laere, O.; De Wael, L.; De Mey, J. *Histochemistry* **1985**, *83*, 397–399.
11. Willets, K. A.; Van Duyne, R. P. *Annual Review of Physical Chemistry* **2007**, *58*, 267–297.
12. Mayer, K. M.; Hafner, J. H. *Chemical Reviews* **2011**, *111*, 3828–3857.
13. Bohren, C.; Huffman, D., *Absorption and Scattering of Light by Small Particles*; Wiley & Sons: 1982.
14. Prescott, S. W.; Mulvaney, P. *Journal of Applied Physics* **2006**, *99*, 123504; Corrigendum: **2008**, *103*, 119901.
15. Albrecht-Bühler, G. *Cell* **1977**, *11*, 395–404.
16. Huang, X. H.; El-Sayed, I. H.; Qian, W.; El-Sayed, M. A. *Journal of the American Chemical Society* **2006**, *128*, 2115–2120.
17. Raschke, G.; Kowarik, S.; Franzl, T.; Sönnichsen, C.; Klar, T. A.; Feldmann, J.; Nichtl, A.; Kürzinger, K. *Nano Letters* **2003**, *3*, 935–938.
18. Mock, J. J.; Smith, D. R.; Schultz, S. *Nano Letters* **2003**, *3*, 485–491.
19. Chen, H.; Kou, X.; Yang, Z.; Ni, W.; Wang, J. *Langmuir* **2008**, *24*, 5233–5237.

20. Baciú, C. L.; Becker, J.; Janshoff, A.; Sönnichsen, C. *Nano Letters* **2008**, *8*, 1724–1728.
21. Khalavka, Y.; Becker, J.; Soennichsen, C. *Journal of the American Chemical Society* **2009**, *131*, 1871–1875.
22. Lee, K.-S.; El-Sayed, M. A. *Journal of Physical Chemistry B* **2006**, *110*, 19220–19225.
23. Nusz, G. J.; Curry, A. C.; Marinakos, S. M.; Wax, A.; Chilkoti, A. *Acs Nano* **2009**, *3*, 795–806.
24. Ament, I.; Prasad, J.; Henkel, A.; Schmachtel, S.; Sönnichsen, C. *Nano Letters* **2012**, *12*, 1092–1095.
25. Zijlstra, P.; Paulo, P. M. R.; Orrit, M. *Nature Nanotechnology* **2012**, *7*, 379–382.
26. Mayer, K. M.; Hao, F.; Lee, S.; Nordlander, P.; Hafner, J. H. *Nanotechnology* **2010**, *21*.
27. Önell, A.; Andersson, K. *Journal of Biomolecular Recognition* **2005**, *18*, 307–317.
28. Henkel, A., *Rod-Shaped Plasmonic Sensors: Synthesis and Single Particle Spectroscopy*; PhD Thesis: 2012.



## Chapter 3.

# 3 Highly Sensitive Plasmonic Silver Nanorods

The quality of a sensor depends on the magnitude of its response expressed in the sensitivity. In order to compare the material properties of silver and gold, the single particle plasmonic sensitivity of nanorods with similar resonance wavelength is determined. Herein, silver nanoparticles show a 1.2 to 2 times higher sensitivity than gold, which is in good agreement with simulations based on the Boundary-Elements-Method (BEM). According to the Drude-model of optical properties of metals together with the quasi-static approximation (QSA) for localized surface plasmons, the dominant contribution to higher sensitivity of silver originates from the lower background polarizability of the d-band electrons shown in a simple formula for the sensitivity. In this project, I synthesized and characterized the particles together with Yuriy Khalavka. Dr. Arpad Jakab, Dr. Jan Becker, and myself performed the sensitivity measurements. Dr. Arpad Jakab and Dr. Andreas Trügler worked on the theory and the simulations. This work is published in *ACS Nano* **2011**, 5, 6880-6885.

### 3.1. Introduction

Sensor devices based on the refractive index sensitivity of surface plasmons have emerged on the market in the last decade. Attempts to increase the sensitivity and reduce the sensor size focus on single particle based sensors supporting localized surface plasmons polaritons (LSPP) or particle plasmons.<sup>1,2,3</sup> As recently reported, single particle sensors can have a higher

<sup>1</sup> Baciú et al., "Protein-Membrane Interaction Probed by Single Plasmonic Nanoparticles."

<sup>2</sup> Mayer et al., "A Single Molecule Immunoassay by Localized Surface Plasmon Resonance."

<sup>3</sup> Wu, Xu, "Stable and Functionable Mesoporous Silica-Coated Gold Nanorods as Sensitive Localized Surface Plasmon Resonance (LSPR) Nanosensors."

sensitivity than propagating surface plasmon based sensors.<sup>4</sup> The spectral position of the localized surface plasmon is highly dependent on the dielectric properties of the surrounding medium.<sup>5</sup> For example, a change of the surrounding refractive index of 0.02 from 1.333 to 1.353 results in a red shift of the resonance wavelength by 5.1 nm for a gold NRs (nanorods) with an aspect ratio of 2.5. For optimal sensing of smallest dielectric changes (i.e. binding of small molecules), the spectral shift of the resonance wavelength upon changing the surrounding medium has to be maximized. Increasing the spectral shift can be achieved by various methods like: optimizing the shape and size,<sup>6,7,8,9</sup> inter-particle distance<sup>10,11</sup> or the material of the particle.<sup>9,12</sup> In recent times, efforts have been intensified to investigate silver nanoparticle sensitivity.<sup>13,14</sup> Silver and gold nanotriangle sensitivities of self assembled monolayers have been reported earlier,<sup>15</sup> but due to different monolayer thicknesses the differing sensitivities could not be attributed to optical properties of the metals. In this work, we present experimental results of single silver nanorod and gold nanorod sensitivities revealing that silver nanorods have a higher sensitivity than gold nanorods at the same resonance wavelength and that, contrary to earlier predictions,<sup>16</sup> the background polarizability of the d-band electrons strongly influences the sensitivity. We provide a theoretical discussion of the differing sensitivities

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<sup>4</sup> Otte et al., "Identification of the Optimal Spectral Region for Plasmonic and Nanoplasmonic Sensing."

<sup>5</sup> Kelly et al., "The Optical Properties of Metal Nanoparticles: the Influence of Size, Shape, and Dielectric Environment"

<sup>6</sup> Becker et al., "The Optimal Aspect Ratio of Gold Nanorods for Plasmonic Bio-sensing"

<sup>7</sup> Khalavka et al., "Synthesis of Rod-Shaped Gold Nanorattles with Improved Plasmon Sensitivity and Catalytic Activity."

<sup>8</sup> Mahmoud, El-Sayed, "Gold Nanoframes: Very High Surface Plasmon Fields and Excellent Near-Infrared Sensors"

<sup>9</sup> Liz-Marzan, "Tailoring Surface Plasmons Through the Morphology and Assembly of Metal Nanoparticles"

<sup>10</sup> Wang, Reinhard, "Monitoring Simultaneous Distance and Orientation Changes in Discrete Dimers of DNA Linked Gold Nanoparticles."

<sup>11</sup> Jain, El-Sayed, "Noble Metal Nanoparticle Pairs: Effect of Medium for Enhanced Nanosensing."

<sup>12</sup> Ringe et al., "Unraveling the Effects of Size, Composition, and Substrate on the Localized Surface Plasmon Resonance Frequencies of Gold and Silver Nanocubes: A Systematic Single-Particle Approach."

<sup>13</sup> Wei et al., "Multipolar Plasmon Resonances in Individual Ag Nanorice."

<sup>14</sup> Malinsky et al., "Nanosphere Lithography: Effect of Substrate on the Localized Surface Plasmon Resonance Spectrum of Silver Nanoparticles."

<sup>15</sup> Haes et al., "Nanoscale Optical Biosensor: Short Range Distance Dependence of the Localized Surface Plasmon Resonance of Noble Metal Nanoparticles."

<sup>16</sup> Lee, El-Sayed, "Gold and Silver Nanoparticles in Sensing and Imaging: Sensitivity of Plasmon Response to Size, Shape, and Metal Composition"

based on medium long wavelength approximation (MLWA)<sup>5,17</sup> to account for size dependent effects. Simplification with the quasi-static approximation (QSA) leads to an elegant formulation of sensitivity as function of resonance wavelength position, plasmonic material parameters and the refractive index of the embedding medium.

### 3.2. Results and Discussion

**Synthesis.** For our study, we form silver nanorods with various aspect ratios in solution adapting a two-step 'seeded growth' approach developed by Kitaev *et al.*<sup>18</sup> with small modifications in illumination and heating. In the first step, faceted silver "seeds" are produced under light illumination. We use a blue LED lamp (141 mW, peak maximum @ 459.5 nm, full-width-at-half-maximum (fwhm) 20.53 nm) instead of the high power metal halide lamp used by Kitaev and co-workers. In the second step, the seeds are added to a 'growth solution' where they develop into silver nanorods. We preheat the growth solution in a consumer microwave at 90 W for 1 min. Then AgNO<sub>3</sub> is added and the solution is placed into the microwave again for heating between 2 and 6 min resulting in rods with increasing aspect ratio (AR). Heating for 2 min results in rods with an aspect ratio of  $2.4 \pm 0.8$ , heating for 4 min yields an aspect ratio of  $2.9 \pm 1.3$ , and heating for 6 min yields an aspect ratio of  $4.0 \pm 1.6$ . (Figure 3.1, top). We compare the plasmonic sensitivity of those silver rods with two types of gold nanorods with different widths. The nanorods with small widths were synthesized by the method of Nikoobakht *et al.*,<sup>19</sup> the ones with the large widths by the method of Ni *et al.*<sup>20</sup> The large nanorods have the advantage that their widths are comparable with the silver nanorods.

**Plasmonic nanorod sensitivity.** We observe the light scattering spectra of the single nanoparticles with a scanning dark-field setup<sup>21</sup> that allows for a rapid measurement of a large number of immobilized nanoparticles

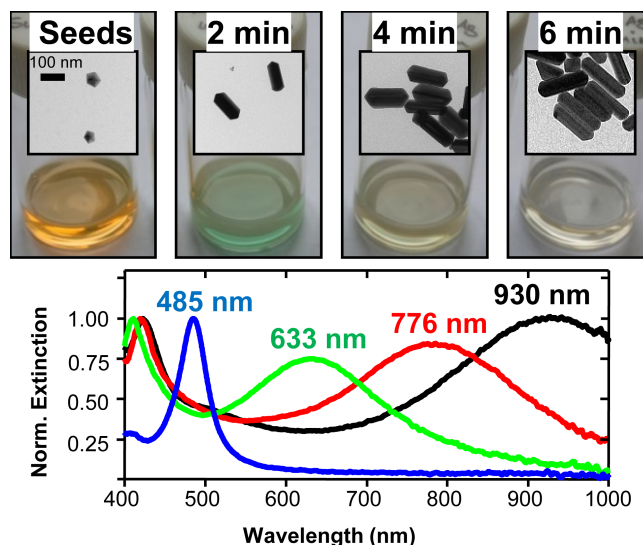
<sup>17</sup> Meier, Wokaun, "Enhanced Fields on Large Metal Particles - Dynamic Depolarization."

<sup>18</sup> Pietrobon et al., "Synthesis of Size-Controlled Faceted Pentagonal Silver Nanorods with Tunable Plasmonic Properties and Self-Assembly of These Nanorods"

<sup>19</sup> Nikoobakht, El-Sayed, "Preparation and Growth Mechanism of Gold Nanorods (Nrs) Using Seed-mediated Growth Method."

<sup>20</sup> Ni et al., "Tailoring Longitudinal Surface Plasmon Wavelengths, Scattering and Absorption Cross Sections of Gold Nanorods."

<sup>21</sup> Carbone et al., "Light-Controlled One-Sided Growth of Large Plasmonic Gold Domains on Quantum Rods Observed on the Single Particle Level."



**Figure 3.1.:** Representative silver nanorod samples are shown as photographs and TEM images (**top**) together with the corresponding ensemble extinction spectra (**bottom**). The samples correspond to Ag-seeds and Ag-nanorods grown with 2, 4, and 6 min of heating time (from left to right). Their plasmon peak increases from 485 to 633, 776, and 930 nm.

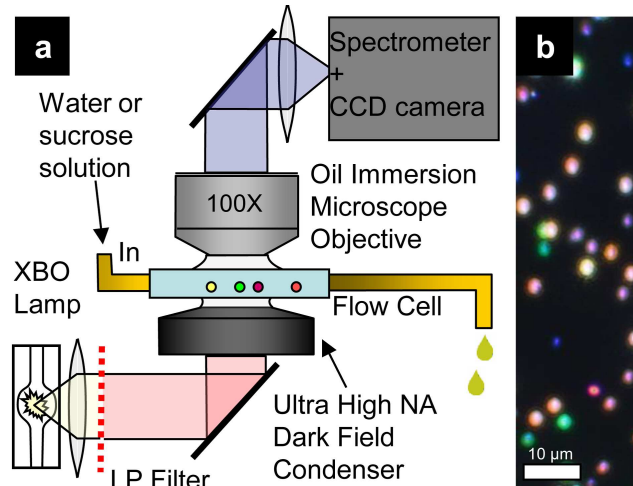
(Figure 3.2a). To measure the plasmon resonance shift upon changes in the refractive index of the environment (single particle sensitivity), the nanoparticles are immobilized in a flat glass capillary or flow cell.

To immobilize nanoparticles in the flow cell, we add a suspension of nanoparticles in water followed by a salt solution. The concentration of nanoparticles is adjusted by dilution to create a nanoparticle density in the field-of-view that allows the distinction of single particles (Figure 3.2b); i.e. an average inter-particle distance of about 10  $\mu\text{m}$ . The addition of salt screens the surface charges that stabilize the nanoparticle suspension, which leads to nonspecific adsorption of nanoparticles to the glass surface of the flow cell. For gold nanorods, we used 0.1 M NaCl, for silver 0.1 M  $\text{KNO}_3$  because, as discussed later, chloride ions can cause structural changes to silver nanoparticles.<sup>22</sup> To avoid aggregation, both liquids are separated in the source tube by a tiny air bubble that gets trapped in the entrance chamber of the flow cell thus the mixing takes only place in the flow cell.

Table 3.1 summarizes the size characteristics of the nanorods used for single particle spectroscopic measurements. We used only silver nanorods

<sup>22</sup> Tsuji et al., “Laser-induced Morphology Changes of Silver Colloids Prepared by Laser Ablation in Water - Enhancement of Anisotropic Shape Conversions by Chloride Ions”





**Figure 3.2.:** a) Experimental wet environment dark-field setup designed for single nanoparticle spectroscopy in various surrounding media. b) Typical dark-field image of the nanoparticles; the scale bar is 10  $\mu\text{m}$ .

**Table 3.1.:** Properties of the silver and gold nanorods used in our study (sizes from TEM images)

| nanorod material | ensemble maximum (nm) | width (nm)  | aspect ratio (length/width) |
|------------------|-----------------------|-------------|-----------------------------|
| Ag               | 633                   | $55 \pm 10$ | $2,4 \pm 0,7$               |
| Ag               | 776                   | $59 \pm 14$ | $2,9 \pm 1,0$               |
| Au               | 634                   | $28 \pm 4$  | $2,0 \pm 0,5$               |
| Au               | 702                   | $18 \pm 2$  | $2,7 \pm 5,2$               |
| Au               | 816                   | $11 \pm 2$  | $4,1 \pm 1,4$               |
| Au               | 680                   | $50 \pm 6$  | $2,1 \pm 0,5$               |

from samples with mean longitudinal plasmon resonances of 633 nm and 776 nm (green and red curves in Figure 3.1). Those silver nanorods had a mean width of about 55-60 nm and aspect ratios of 2.4 and 2.9, respectively. In comparison, the small gold nanorods have widths of 11-28 nm but with comparable aspect ratios, and the large gold nanorods have widths of 50 nm. More details about the nanoparticle size distribution are presented in Appendix Figures A.1-A.3 and Appendix Tables A.1 and A.2.

**Sensitivity measurements.** The plasmon sensitivity quantifies the dependence of the plasmon resonance wavelength or frequency on changes in the dielectric properties of the surrounding medium. Sensitivity  $S$  (in wavelength units) is therefore defined by  $S = d\lambda_{\text{res}}/dn$ , where  $\lambda_{\text{res}}$  is the plasmon resonance wavelength and  $n$  is the refractive index of the surrounding medium. There are more elaborate measures to quantify the sensor quality of

nanoparticles taking into account more realistic sensor concepts, for example  $FOM$ ,<sup>23,24</sup>  $FOM^*$ .<sup>6</sup> All of these quantities depend on the plasmon sensitivity. Here we focus on the sensitivity in wavelength units and refer to this term simply as "sensitivity".

We determined the plasmonic sensitivity experimentally by recording spectra of single immobilized nanoparticles in the initial environment (pure water,  $n_1 = 1.3330$ ), then immersed in 12.5% sucrose solution ( $n_2 = 1.3454$ ), and at the end again in water. The resulting experimental results are shown in Figure 3.3 (top panel). The experimentally obtained trend-lines (continuous lines) qualitatively follow the trend simulated with the boundary-element-method (BEM)<sup>25</sup> (dashed lines) but are lower than the simulation. However, the simulation was performed with a homogenous medium around the particles, whereas in the experiment, the particles are attached to a glass substrate reducing the influence of the change in medium. The fact that the experimental silver trend shows a stronger deviation from simulations than the gold nanorods might be caused by additional damping of the plasmon resonance due to their penta-twinned crystal structure.<sup>26</sup>

We find that silver sensitivity is generally higher than gold sensitivity, thick gold nanorods have a higher sensitivity than the thinner gold nanorods, and in all cases, sensitivity depends on wavelength. The sensitivity of silver nanorods reaches values above 400 nm/RIU, which is, up to now, the largest value for rod-like objects in this wavelength range.<sup>8</sup> Especially in the wavelength range of 600-700 nm, where thin gold nanorods have only a sensitivity of ca. 170 nm/RIU and thick gold nanorods of 250 nm/RIU, silver outperforms all gold NRs with sensitivities around 340 nm/RIU.

To directly compare the sensitivity of the thin gold and silver nanorods over the entire measured spectral range, we show the ratio of their sensitivity (the sensitivity enhancement of silver compared to gold  $S_{Ag}/S_{Au}$ ) in the bottom panel of Figure 3.3, both for the experimental data and the simulation. In simulations, we use 20 nm diameter for the thin gold NRs which is the average of the thicknesses of the thin gold NRs (11-28 nm) used in our experiments. Experiment and simulation agree well up to 850 nm in this

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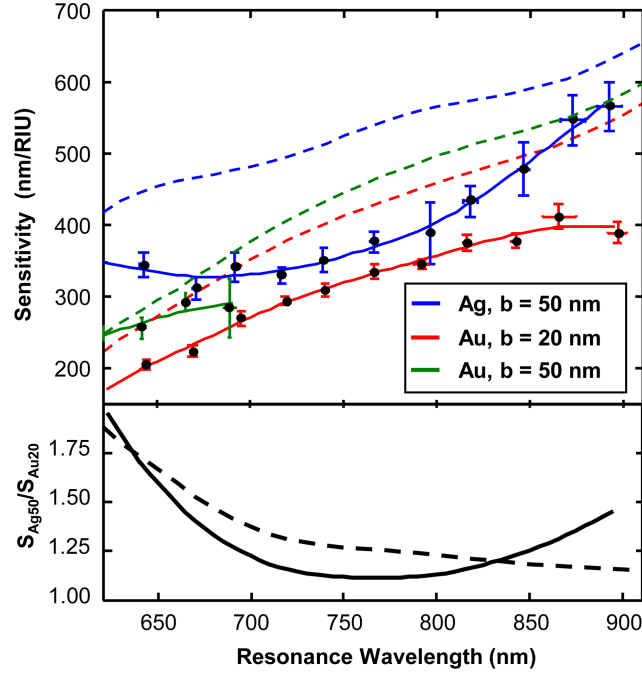
<sup>23</sup> Sherry et al., "Localized Surface Plasmon Resonance Spectroscopy of Single Silver Nanocubes."

<sup>24</sup> Unger, Kreiter, "Analyzing the Performance of Plasmonic Resonators for Dielectric Sensing."

<sup>25</sup> Hohenester, Krenn, "Surface Plasmon Resonances of Single and Coupled Metallic Nanoparticles: a Boundary Integral Method Approach."

<sup>26</sup> Tang, Ouyang, "Tailoring Properties and Functionalities of Metal Nanoparticles Through Crystallinity Engineering."

diagram; the substrate effect effectively cancels out. At shorter wavelengths, around 620 nm, the enhancement reaches a factor of 2, at longer wavelengths the enhancement drops to a value between 1.1 and 1.2 with a slow increase above 850 nm that is not followed by the simulated results.



**Figure 3.3.: Top)** Experimental results (continuous lines) and BEM-simulation without substrate (dashed lines) of single particle sensitivity of silver 50 nm thick (blue line), gold 20 nm thick (red line), and gold 50 nm thick (green line) nanorods. The error bars indicate the uncertainty of the mean in a range of 25 nm. **Bottom)** The experimentally obtained (continuous line) and BEM-simulated (dashed line) sensitivity enhancement ( $S_{Ag50}/S_{Au20}$ ) of silver nanorods with 50 nm thickness in relation to gold nanorods with 20 nm thickness.

**Model.** To understand the origin of the higher plasmonic sensitivity of silver compared to gold nanorods, we calculated sensitivities using simplified approaches, the medium long wavelength approximation MLWA and the quasi-static approximation (QSA).<sup>5</sup> MLWA includes size dependent effects in plasmon resonance calculations, allowing us to investigate the impacts of particle size. In both cases, we use a simple Drude-type model for the dielectric function for gold and silver that separates the contribution of free and d-band electrons.

Whereas the boundary elements method (BEM) mentioned above provides exact solutions of the Maxwell equations (if the number of elements is

sufficiently high), it provides little insight into the underlying physics. To understand the origin of the higher plasmonic sensitivity of silver compared to gold nanorods, it is instructive to use the quasi-static approximation to calculate the scattering cross section together with a simple Drude-type model for the dielectric function for gold and silver. Within those two approximations, the sensitivity  $S$  at a given resonance wavelength  $\lambda$  is given by the following equation (details in Appendix Section A.3):

$$S(\lambda) = \frac{\lambda}{n} \left( 1 - \frac{\lambda_p^2}{\lambda^2} n_\infty^2 \right) \quad (3.1)$$

where  $\lambda_p = 2\pi c_0/\omega_p$  is the bulk plasmon wavelength ( $\lambda_p = 136$  nm for gold and silver),  $n_\infty = (\varepsilon_\infty)^{1/2}$  is the background refractive index (square root of the background dielectric constant) from the polarizability of the d-band electrons ( $\varepsilon_{\infty,\text{Au}} = 9.84$  for gold and  $\varepsilon_{\infty,\text{Ag}} = 3.7$  for silver correspond to  $n_{\infty,\text{Au}} = 3.1$  and  $n_{\infty,\text{Ag}} = 1.9$ , respectively), and  $n$  is the refractive index of the surrounding medium. The equation above reveals that, contrary to earlier reports,<sup>16</sup> the sensitivity depends on the material through the strong difference in the background polarizability  $\varepsilon_\infty$  of gold and silver (the sensitivity decreases with increasing  $\varepsilon_\infty$  or  $n_\infty$ ). Despite the simplifications of the quasi-static approach and the Drude model for the dielectric function, the equation above predicts the sensitivity of nanorods remarkably well. Additional calculations using the medium long wavelength approximation (MLWA) that includes size effects, show that the above result is not drastically changed if radiation damping and retardation are taken into account. At a given wavelength, particle size influences plasmon sensitivity much less than the material (Appendix Figure A.10).

Interestingly, all geometry related factors cancel out in the simple equation for the sensitivity shown above. Consequently, for small thickness/wavelength ratios (QSA) the sensitivity of a plasmonic nanorod at a given resonance wavelength is independent from its size and shape and the formula could be valid more generally for metal structures of other shapes. Our results are in accordance with the findings of Miller *et al.* that within the QSA the sensitivity of gold nanodiscs, nanocylinders and nanoshells depends only on the location of the plasmon resonance peak and the dielectric properties of the involved materials.<sup>27</sup>

**Sensing reversibility.** Whereas sensitivity alone seems to favor silver

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<sup>27</sup> Miller, Lazarides, “Sensitivity of Metal Nanoparticle Surface Plasmon Resonance to the Dielectric Environment.”

over gold, another important aspect for sensors is the sensing reversibility upon repeated changes of the environment. Given the fact that silver is less noble than gold, we carefully checked the reversibility. For this purpose, our measurements always went back to the initial environment at the end and we recorded the difference in resonance wavelength between initial and final resonance wavelength, a quantity we call the sensing cycle shift. For a reliable sensor, the mean sensing cycle shift should remain close to the experimental uncertainty ( $\pm 0.5$  nm) and we disregarded all particles that did not show a full reversibility in the datasets for estimating particle sensitivity (reversibility within one standard deviation). Halide ions ( $\text{Cl}^-$  and especially  $\text{Br}^-$ ) induce a reshaping of silver nanoparticles above a threshold concentration of  $\sim 10^{-8}$  M, so we took care to avoid any use of those ions.<sup>22,28,29</sup> Generally, we found that even without halide ions present, silver nanoparticles showed much worse reversibility performance than gold. Light is known to modify silver nanoparticles.<sup>22,30</sup> We therefore suspected a light induced process, so we measured the reversibility with different filters in the light path.

In Figure 3.4, we show the sensing cycle shift of single nanorods plotted as a cumulative probability distribution (an integrated histogram that allows an easy visualization of the median value) measured under different illumination conditions where each dot corresponds to the sensitivity cycle shift of a single particle. The sensing cycle shift is decreasing with increasing cut-off wavelength of the long pass filter. The mechanism of this light-induced irreversible shift remains unclear and is probably a combination of heating<sup>31,32</sup> due to the absorbed light, (we believe that heat probably changes the arrangement of the crystalline domains in the pentatwinned silver nanorods) and photoetching.<sup>22</sup> The shift has nothing to do with changing the environment as we checked in experiments that show a red shift of nanoparticles monitored continuously in water (Appendix Figure A.9). Our experiments indicate that a sufficiently adjusted long pass filter allows the use of silver nanoparticles for sensing in environments without halide ions. However, these silver NRs are less suitable for other measures of plasmonic sensor performance like the "figure of merit" (*FOM*) due to the relatively large linewidth of our silver NRs (Appendix Figure A.8). We believe that the reduced radiation damping

<sup>28</sup> Jiang, Yu, "Silver Nanoplates: a Highly Sensitive Material Toward Inorganic Anions."

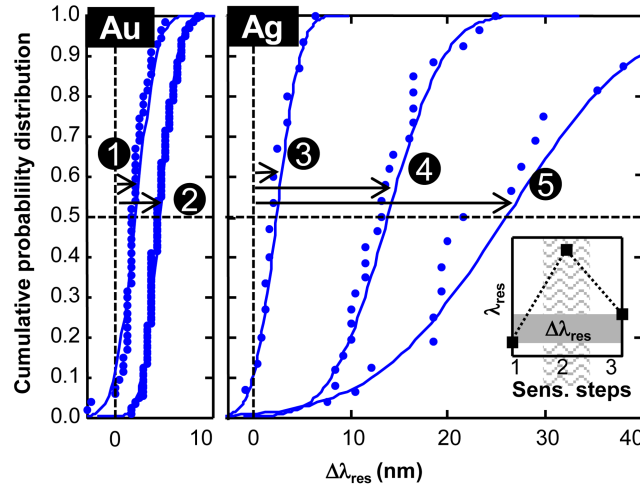
<sup>29</sup> Cathcart et al., "Silver Nanoparticles with Planar Twinned Defects: Effect of Halides for Precise Tuning of Plasmon Resonance Maxima from 400 to  $> 900$  nm."

<sup>30</sup> Jin et al., "Photoinduced Conversion of Silver Nanospheres to Nanoprisms."

<sup>31</sup> Mock et al., "Shape Effects in Plasmon Resonance of Individual Colloidal Silver Nanoparticles."

<sup>32</sup> Plech et al., "Time-resolved X-ray Diffraction on Laser-excited Metal Nanoparticles."

of silver NRs with a smaller volume could provide a substantially better performance for *FOM* sensitivity.



**Figure 3.4.:** Cumulative distribution of single nanoparticle resonance wavelength shifts after one sensing measurement cycle (the sensing cycle shift). The continuous lines are Gaussian fits, the arrow indicates the mean cycle shift. **Left graph:** 1) small Au rods with 610 nm long pass (LP) filter, 2) small Au rods without LP filter. **Right graph:** 3) Ag rods with 610 nm LP filter, 4) Ag rods with 530 nm LP filter, 5) Ag rods without LP filter. **Inset at bottom right:** definition of sensing cycle shift  $\Delta\lambda_{\text{Res}}$  in the cycle  $n_1 \rightarrow n_2 \rightarrow n_1$ .

### 3.3. Conclusion

We have presented experimental results of silver nanorod sensitivity and compared them to results found for gold nanorods. Silver nanorods have up to two times higher sensitivity than gold nanorods in the spectral range of 600-700 nm. Increasing nanorod width leads to higher sensitivity but the dominant factor that influences the sensitivity is the material of the nanorods. In the region of 700-900 nm, the sensitivity is still about 10 to 20% higher. Calculations using MLWA (medium long wavelength approximation) and simulations with BEM (boundary element methods) confirm both material and size dependency. From simple theoretical arguments (Drude's model of optical properties of metals and the quasi-static-approximation QSA), we developed a simple expression for the sensitivity, and we could identify the lower background (d-band electron) polarizability of silver compared to gold as the main reason for the higher sensitivity. The reversibility of silver

nanorod based sensors is, generally, worse than gold nanorod based sensors, but adding a 610 nm cut-off long-pass filter in the light path, resulted in a reasonably reversible sensor for both cases.

### 3.4. Methods

Deionized water from a Millipore system ( $>18\text{ M}\Omega$ , Milli Q) was used in all experiments. Suppliers of chemicals are given in the Appendix Chapter A.

**Synthesis of silver nanorods.** Silver nanorods were synthesized following mainly the procedure developed by Kitaev *et al.*<sup>refPietrobonag</sup> Decahedral Ag nanoparticles were used as seeds. For their synthesis, a precursor solution of silver nanoparticles was first prepared using a mixture of 1.000 mL of 0.05 M sodium citrate, 0.060 mL of 0.05 M PVP, 0.100 mL of 0.005 M L-arginine, 0.400 mL of 0.005 M  $\text{AgNO}_3$  and 14.0 mL of deionized water in a 20 mL vial and then reduced by adding 0.160 mL of 0.10 M  $\text{NaBH}_4$ . The resulting yellow solution was stirred for several minutes. To produce decahedra, a blue diode array lamp with  $\lambda_{\text{max}} \approx 460\text{ nm}$  with an exposure time of 10 hours was used (LED spectrum Appendix Figure A.4). In the next step, 2 mL of the freshly prepared decahedral Ag seed solution were centrifuged to replace the supernatant with 1.0 mL of pure deionized water. Having prepared the decahedra seed solution, 1 mL of the purified decahedra solution, 2.0 mL of water, 0.400 mL of 0.05 M sodium citrate, and 0.066 mL of 0.05 M PVP were heated to  $95^\circ\text{C}$  in a 20 mL vial in microwave oven at 90 W power. After temperature equilibration, 0.400 mL of silver nitrate (0.005 M) were added at once to produce rods with an aspect ratio up to 4-5 in high yield in 2-6 min of the reaction under 90 W microwave power.

**Characterization.** Transmission electron microscopy was performed using FEI Tecnai F30 and Phillips EM-420 microscopes. Nanoparticle solutions were deposited on a carbon-coated Formvar grid (Plano). Operating voltage was varied from 120-300 kV. The average size and standard deviation were determined from TEM images by averaging diameters of about 100 particles. Ensemble optical spectra were acquired with Ocean Optics USB 2000 spectrometer.

**Single particle microscopy.** The single particle spectroscopy was performed with a self-built microscopy setup employing a 2D piezo scanner. The setup shown in Figure 3.2 consists of a positioning stage (PI P-542.2CD Nanopositioning and Scanning Stage), microscope optics (Zeiss Achroplan

Oil-Iris, 100X, NA = 0.7-1.25), an imaging spectrometer (Princeton Instruments / ACTON, SpectraPro 2300), and a CCD camera (Princeton Instruments, Pixis 400B). During a spectral scan, the sample is moved in steps of 1  $\mu\text{m}$  along the x-axis. At each step, the spectrum of the particles in the center column is taken. The scan window width is adjusted to the step size to ensure gapless coverage of the scanned area.

**Flow cell.** The glass capillary (Vitrotube W5010-100 by Vitrocom) is initially UV-irradiated for 15 minutes and left for another 15 minutes in the reactive atmosphere, then we attach thin polyethylene tubing using epoxy glue to construct the flow cell system. The flow cell is purged with 1% Helmanex solution for 24 hours, and finally cleaned with hot deionized water for 1 hour.



### 3.5. References

1. Baciu, C. L.; Becker, J.; Janshoff, A.; Sönnichsen, C. *Nano Letters* **2008**, *8*, 1724–1728.
2. Mayer, K. M.; Hao, F.; Lee, S.; Nordlander, P.; Hafner, J. H. *Nanotechnology* **2010**, *21*, 255503.
3. Wu, C. L.; Xu, Q. H. *Langmuir* **2009**, *25*, 9441–9446.
4. Otte, M. A.; Sepulveda, B.; Ni, W. H.; Juste, J. P.; Liz-Marzan, L. M.; Lechuga, L. M. *ACS Nano* **2010**, *4*, 349–357.
5. Kelly, K. L.; Coronado, E.; Zhao, L. L.; Schatz, G. C. *Journal of Physical Chemistry B* **2003**, *107*, 668–677.
6. Becker, J.; Trügler, A.; Jakab, A.; Hohenester, U.; Sönnichsen, C. *Plasmonics* **2010**, *5*, 161–167.
7. Khalavka, Y.; Becker, J.; Sönnichsen, C. *Journal of the American Chemical Society* **2009**, *131*, 1871–1875.
8. Mahmoud, M. A.; El-Sayed, M. A. *Journal of the American Chemical Society* **2010**, *132*, 12704–12710.
9. Liz-Marzan, L. M. *Langmuir* **2006**, *22*, 32–41.
10. Wang, H. Y.; Reinhard, B. M. *Journal of Physical Chemistry C* **2009**, *113*, 11215–11222.
11. Jain, P. K.; El-Sayed, M. A. *Nano Letters* **2008**, *8*, 4347–4352.
12. Ringe, E.; McMahon, J. M.; Sohn, K.; Cobley, C.; Xia, Y. N.; Huang, J. X.; Schatz, G. C.; Marks, L. D.; Van Duyne, R. P. *Journal of Physical Chemistry C* **2010**, *114*, 12511–12516.
13. Wei, H.; Reyes-Coronado, A.; Nordlander, P.; Aizpurua, J.; Xu, H. X. *ACS Nano* **2010**, *4*, 2649–2654.
14. Malinsky, M. D.; Kelly, K. L.; Schatz, G. C.; Van Duyne, R. P. *Journal of Physical Chemistry B* **2001**, *105*, 2343–2350.
15. Haes, A. J.; Zou, S. L.; Schatz, G. C.; Van Duyne, R. P. *Journal of Physical Chemistry B* **2004**, *108*, 6961–6968.
16. Lee, K. S.; El-Sayed, M. A. *Journal of Physical Chemistry B* **2006**, *110*, 19220–19225.
17. Meier, M.; Wokaun, A. *Optics Letters* **1983**, *8*, 581–583.
18. Pietrobon, B.; McEachran, M.; Kitaev, V. *ACS Nano* **2009**, *3*, 21–26.

19. Nikoobakht, B.; El-Sayed, M. A. *Chemistry of Materials* **2003**, *15*, 1957–1962.
20. Ni, W.; Kou, X.; Yang, Z.; Wang, J. F. *ACS Nano* **2008**, *2*, 677–686.
21. Carbone, L.; Jakab, A.; Khalavka, Y.; Sönnichsen, C. *Nano Letters* **2009**, *9*, 3710–3714.
22. Tsuji, T.; Okazaki, Y.; Higuchi, T.; Tsuji, M. *Journal of Photochemistry and Photobiology a-Chemistry* **2006**, *183*, 297–303.
23. Sherry, L. J.; Chang, S. H.; Schatz, G. C.; Van Duyne, R. P.; Wiley, B. J.; Xia, Y. N. *Nano Letters* **2005**, *5*, 2034–2038.
24. Unger, A.; Kreiter, M. *Journal of Physical Chemistry C* **2009**, *113*, 12243–12251.
25. Hohenester, U.; Krenn, J. *Physical Review B* **2005**, *72*, 195429.
26. Tang, Y.; Ouyang, M. *Nature Materials* **2007**, *6*, 754–759.
27. Miller, M. M.; Lazarides, A. A. *Journal of Physical Chemistry B* **2005**, *109*, 21556–21565.
28. Jiang, X. C.; Yu, A. B. *Langmuir* **2008**, *24*, 4300–4309.
29. Cathcart, N.; Frank, A. J.; Kitaev, V. *Chemical Communications* **2009**, 7170–7172.
30. Jin, R. C.; Cao, Y. W.; Mirkin, C. A.; Kelly, K. L.; Schatz, G. C.; Zheng, J. G. *Science* **2001**, *294*, 1901–1903.
31. Mock, J. J.; Barbic, M.; Smith, D. R.; Schultz, D. A.; Schultz, S. *The Journal of Chemical Physics* **2002**, *116*, 6755–6759.
32. Plech, A.; Kurbitz, S.; Berg, K. J.; Graener, H.; Berg, G.; Gresillon, S.; Kaempfe, M.; Feldmann, J.; Wulff, M.; Plessen, G. von *Europhysics Letters* **2003**, *61*, 762–768.

## Chapter 4.

# 4

### A New Approach to Assess Gold Nanoparticle Uptake by Mammalian Cells: Combining Optical Dark-Field and Transmission Electron Microscopy

Toxicological effects of nanoparticles are associated with their internalization into cells. Hence, there is a strong need for techniques revealing the interaction between particles and cells as well as quantifying the uptake at the same time. For that reason, herein optical dark-field microscopy is used in conjunction with transmission electron microscopy to investigate the uptake of gold nanoparticles into epithelial cells with respect to shape, stabilizing agent, and surface charge. The number of internalized particles is strongly dependent on the stabilizing agent, but not on the particle shape. A test of metabolic activity shows no direct correlation with the number of internalized particles. Therefore, particle properties besides coating and shape are suspected to contribute to the observed toxicity. In this project, I synthesized, functionalized, and characterized the particles. I performed cell culture together with Dr. Marco Tarantola, Dr. David Schneider, and Dr. Eva Sunnick, who performed the cytotoxicity tests. I prepared the samples for microscopy and imaged them. Together with Dr. Sebastien Pierrat and Dr. Andreas Henkel, I developed the data analysis. This work is published in *Small* **2012**, 8, 3683-3690.

## 4.1. Introduction

Nanoparticle use for biomedical and cosmetic purposes has considerably expanded in the last decades.<sup>1</sup> Herein, metal and especially gold nanoparticles have received attention because of their strong light scattering and absorption at their plasmon resonance which can be tuned to the infrared part of the electromagnetic spectrum (including the 'optical window' of biological tissue in the wavelength region of 650-900 nm).<sup>2</sup> These optical properties make them suitable for in vivo and medical applications, for example, as contrast agents in imaging,<sup>3,4,5</sup> as biosensors,<sup>6</sup> as delivery systems,<sup>7,8</sup> and also as tools for photothermal therapy.<sup>9</sup> Moreover, consumer products like face cream containing gold nanoparticles are on the market<sup>10</sup> exposing a large population to a possible threat. Consequently, gold nanoparticle cellular uptake, toxicity, effects on living organisms, and fate in mammals after exposure has to be addressed.

In 1538, Paracelsus postulated that the dose makes the poison.<sup>11</sup> Therefore, the amount of internalized nanomaterial serves as a basis to interpret biological responses and thus plays a key role in toxicity assessment.<sup>12,13,14</sup> But besides quantification, a definite particle localization as well as determination of their distribution and aggregation state inside cells is the next step for a detailed understanding of potential risks. Thus, in order to gain this set of information efficiently in terms of time and effort, it is desirable to have an appropriate technique showing the amount of particles, their position, and their degree of aggregation inside cells from one experimental sample. To date, several methods are available to quantify nanoparticle

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<sup>1</sup> De et al., "Applications of Nanoparticles in Biology."

<sup>2</sup> Weissleder, "A clearer vision for in vivo imaging."

<sup>3</sup> Wax, Sokolov, "Molecular imaging and darkfield microspectroscopy of live cells using gold plasmonic nanoparticles"

<sup>4</sup> Xu, Wang, "Photoacoustic imaging in biomedicine."

<sup>5</sup> Murphy et al., "Gold Nanoparticles in Biology: Beyond Toxicity to Cellular Imaging."

<sup>6</sup> Parak et al., "Biological applications of colloidal nanocrystals."

<sup>7</sup> Sperling et al., "Biological applications of gold nanoparticles."

<sup>8</sup> Ghosh et al., "Gold nanoparticles in delivery applications."

<sup>9</sup> Lal et al., "Nanoshell-Enabled Photothermal Cancer Therapy: Impending Clinical Impact."

<sup>10</sup> Kington, "Gold Face Cream: A Costly Leap of Faith."

<sup>11</sup> Paracelsus, *Septem Defensiones. Die Selbstverteidigung eines Aussenseiters.*

<sup>12</sup> Lewinski et al., "Cytotoxicity of nanoparticles"

<sup>13</sup> Khlebtsov, Dykman, "Biodistribution and toxicity of engineered gold nanoparticles: a review of in vitro and in vivo studies."

<sup>14</sup> Boisselier, Astruc, "Gold nanoparticles in nanomedicine: preparations, imaging, diagnostics, therapies and toxicity."

uptake. Fluorescence-activated cell sorting (FACS) allows quantification of the uptake of fluorescent or fluorescently labeled nanoparticles<sup>15</sup> and simple optical extinction spectra show the uptake of strongly colored particles.<sup>16</sup> Using inductively coupled plasma mass spectrometry (ICP-MS)<sup>17</sup> or atomic emission spectrometry (ICP-AES)<sup>18</sup> the metal ion concentration after dissolving cells and nanoparticles is determined and used to calculate the number of metal nanoparticles incorporated into cells. Unfortunately, none of those techniques allows for resolving the location of particles within the cell and great care has to be taken to avoid counting particles attached to the outside of the cell.<sup>19,20</sup> In order to gain information about the particle location within cells in addition to quantification, microscopic methods displaying the cell interior are essential. There are different approaches available with the three most prominent being confocal microscopy,<sup>21</sup> two-photon luminescence microscopy,<sup>22</sup> and transmission electron microscopy (TEM).<sup>23</sup> Among those, optical techniques with a resolution lower than the particle size (confocal, two-photon luminescence microscopy) serve to screen whole cells quickly for particles, but the particle number is estimated from signal intensities, which need to be calibrated. Besides calibration, the differentiation of various particle shapes and sizes is challenging, which restricts the techniques to an investigation of nanoparticle samples with low polydispersity. TEM, however, has the advantage of showing the prominent particle shapes and sizes in addition to their location owing to its high resolution. Unfortunately, TEM needs extensive sample preparation (fixation, drying, staining, and slicing) and allows the investigation of only a tiny fraction of a single cell in an individual experiment. This spatial restriction demands a complex

<sup>15</sup> Luhmann et al., "Cellular uptake and intracellular pathways of PLL-g-PEG-DNA nanoparticles."

<sup>16</sup> Yen et al., "Cytotoxicity and Immunological Response of Gold and Silver Nanoparticles of Different Sizes."

<sup>17</sup> Alkilany et al., "Cellular Uptake and Cytotoxicity of Gold Nanorods: Molecular Origin of Cytotoxicity and Surface Effects."

<sup>18</sup> Chithrani et al., "Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells"

<sup>19</sup> Cho et al., "Understanding the Role of Surface Charges in Cellular Adsorption versus Internalization by Selectively Removing Gold Nanoparticles on the Cell Surface with a I-2/KI Etchant"

<sup>20</sup> Liu et al., "Synthesis, stability, and cellular internalization of gold nanoparticles containing mixed peptide-poly(ethylene glycol) monolayers."

<sup>21</sup> Clift et al., "The impact of different nanoparticle surface chemistry and size on uptake and toxicity in a murine macrophage cell line."

<sup>22</sup> Huff et al., "Controlling the cellular uptake of gold nanorods"

<sup>23</sup> Mayhew et al., "A review of recent methods for efficiently quantifying immunogold and other nanoparticles using TEM sections through cells, tissues and organs"

unbiased sample analysis for 3D reconstruction of particle aggregates for a correct calculation of the total particle number.<sup>23,24</sup>

Here, we report a technique to quantify nanoparticle uptake while localizing and resolving the particles. For this purpose, we combine the imaging of gold nanoparticles in optical dark-field light microscopy (DF)<sup>3</sup> with the high resolution power of TEM. We demonstrate the feasibility of the method by analyzing the uptake of gold nanoparticles of different shapes as well as surface coatings into epithelial cells (MDCK II). In order to validate the quantitative results gained by the current approach, we relate the uptake numbers to reported values and we conduct an extensive error estimation of the method. Furthermore, we compare the number of internalized particles with the metabolic activity of the cells. Herein, we find that the resulting toxicity may not exclusively originate from the extent of gold nanoparticle uptake. A more detailed study of the cellular response to the investigated particles regarding their extent of uptake is presented elsewhere.<sup>25</sup>

## 4.2. Results

**Technique.** In this study, we take advantage of the plasmonic properties of gold nanoparticles to visualize them in DF.<sup>3</sup> Since whole cells themselves scatter light strongly under dark-field illumination, particles can only be seen in areas with low tissue density or of small tissue thickness.<sup>26</sup> Hence, we fixed and cut the cells into 1  $\mu\text{m}$  slices to investigate cell interiors for particles. Due to the length scale under observation, these slices allow the fast assessment of large areas, i.e. many cell slices. However, the optical resolution limit prevents identifying and counting single particles in an aggregate, a restriction originating from the properties of a plasmon. These include that signal intensities do not simply scale with the particle volume but depend on the precise particle alignment as well as surrounding. We overcome this drawback by TEM, which resolves single particles with atomic resolution. Unfortunately, effective electron penetration requires very thin cell slices (in the order of 50 nm) rendering the investigation of enough cell

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<sup>24</sup> Brandenberger et al., “Quantitative Evaluation of Cellular Uptake and Trafficking of Plain and Polyethylene Glycol-Coated Gold Nanoparticles”

<sup>25</sup> Tarantola et al., “Toxicity of gold-nanoparticles: Synergistic effects of shape and surface functionalization on micromotility of epithelial cells”

<sup>26</sup> Wang et al., “Size-dependent endocytosis of gold nanoparticles studied by three-dimensional mapping of plasmonic scattering images.”

slices for meaningful statistics cumbersome. Therefore, optical and electron microscopy are combined to determine the average number of nanoparticles in a cell. Herein, we use in one step DF operating on the cell length scale to obtain the number of slices per cell as well as the number of aggregates per slice. In another step, the number of particles per aggregate is gained by TEM suitable for the particle length scale. Finally, the combination of these three factors (number of slices per cell, number of aggregates per slice, number of particles per aggregate) results in the total number of particles per cell.

**System.** Using the approach based on DF and TEM, we investigate the uptake of gold nanorods (38 nm × 17 nm) and gold nanospheres (43 nm), each with three different surface coatings since the particle bound molecules (nanoparticle 'functionalization') are expected to be a major factor influencing uptake by cells.<sup>27</sup> Herein, one kind of surface functionalization is built by molecules of cetyltrimethylammonium bromide (CTAB), which is a relatively toxic cationic surfactant<sup>12</sup> used for particle preparation. We compare these CTAB-particles with particles functionalized by the inert polymer polyethylene glycol (PEG) known for its biocompatibility.<sup>28</sup> To investigate the influence of molecule charge, we use PEG chains terminated with either amine (NH<sub>2</sub>-PEG) or carboxy groups (COOH-PEG). Hence, we test the uptake of positively charged (CTAB,  $\zeta$ -potential  $\approx$  50 mV),<sup>29,30</sup> neutral (NH<sub>2</sub>-PEG,  $\zeta$ -potential  $\approx$  0 mV), and negatively charged (COOH-PEG,  $\zeta$ -potential  $\approx$  -20 mV) nanoparticles, respectively ( $\zeta$ -potentials of PEG-particles from gel electrophoresis, Appendix Figure B.3). The cells were incubated at culture conditions for 20 hours after exposure to a dose of 1.5 pg gold per cell (i.e. about 10000 nanorods or about 2000 nanospheres, respectively), prepared for microscopy, and then imaged by optical dark-field microscopy and TEM (for details see method section).

**DF and TEM images.** Figure 4.1 shows representative DF and TEM images of cells exposed to the same dose of nanoparticles (rods or spheres, each with three different surface coatings, namely CTAB, NH<sub>2</sub>-PEG, and COOH-PEG). Among these pictures, 1  $\mu$ m cell slices imaged by DF show bright spots corresponding to gold nanoparticles (see Figure 4.1a1-f1). Herein, the spot color displays the plasmon frequency depending on the particle

<sup>27</sup> Verma, Stellacci, "Effect of Surface Properties on Nanoparticle-Cell Interactions."

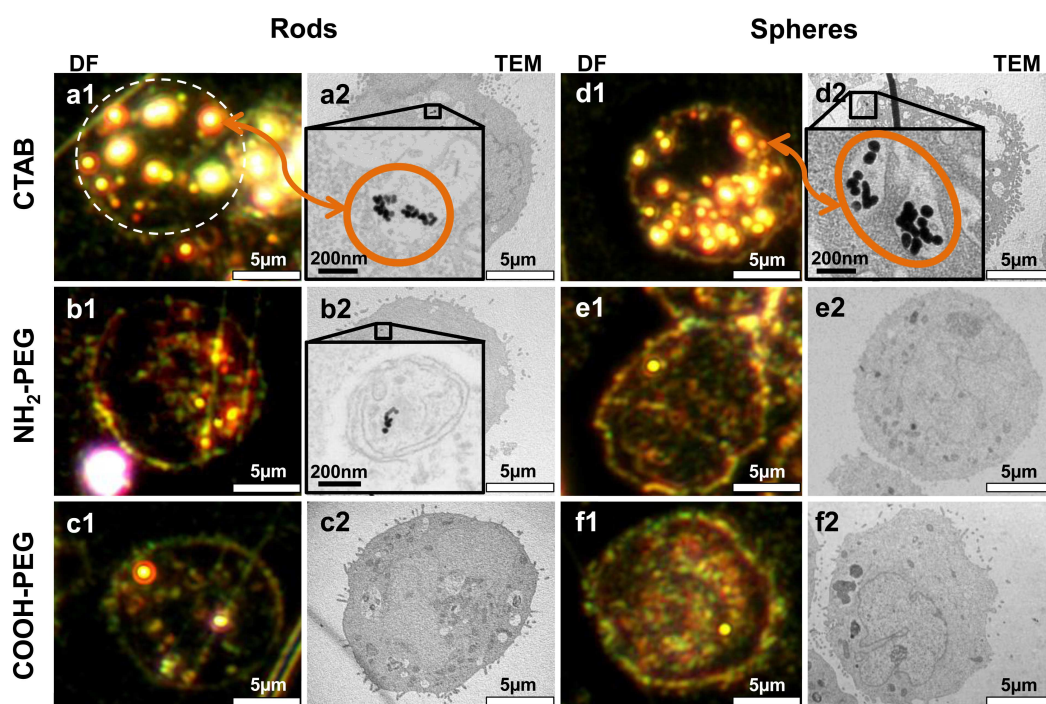
<sup>28</sup> Ryan et al., "Advances in PEGylation of important biotech molecules: delivery aspects"

<sup>29</sup> Hauck et al., "Assessing the effect of surface chemistry on gold nanorod uptake, toxicity, and gene expression in mammalian cells"

<sup>30</sup> Rayavarapu et al., "In vitro toxicity studies of polymer-coated gold nanorods."



shape: Single gold nanospheres appear as green spots, while individual gold nanorods are represented by red spots. However, when two or more particles aggregate, they show an orange or white spot, respectively. Examples of TEM images used to investigate the number of particles in an aggregate within the ultra-thin cell slices (50 nm thickness) are shown in Figure 4.1a2-f2 and in Appendix Figures B.4-B.6.



**Figure 4.1.:** 1) DF images of 1  $\mu\text{m}$  slices and 2) TEM images of 50 nm thick slices of cells incubated for 20 hours with a) CTAB-rods, b)  $\text{NH}_2$ -PEG-rods, c) COOH-PEG-rods, d) CTAB-spheres, e)  $\text{NH}_2$ -PEG-spheres, and f) COOH-PEG-spheres. DF images show particle aggregates as bright orange spots within the cells (the area of one cell is marked with a dashed circle in a1). In the case of CTAB-particles, there are many large aggregates visible, whereas for PEG-particles there are only a few, much smaller aggregates showing up. Therefore, while CTAB-particles are easily identified in TEM images, there are so few PEG-particles internalized that we find hardly any  $\text{NH}_2$ -PEG-rods and neither  $\text{NH}_2$ -PEG-spheres nor COOH-PEG-particles. Particles marked with an orange circle in a2 and d2 provide examples of how aggregates seen by DF might look in TEM (these are not exactly the same aggregates).

**Uptake.** Qualitatively, one observes immediately a strong cellular uptake and aggregation of CTAB coated particles. Fewer particles are visible inside cells if PEG coated nanoparticles were applied. None of the investigated surface coatings showed a significant difference in uptake between rods



and spheres. Generally, the images show particles distributed over the cell interior and not accumulated in some place. We find no particles outside the cells confirming the effective removal of particles by our washing steps before cell fixation. However, on some TEM images, we see particles in close proximity to the cell membrane and microvilli, which we believe are in the process of uptake (Appendix Figure B.7).

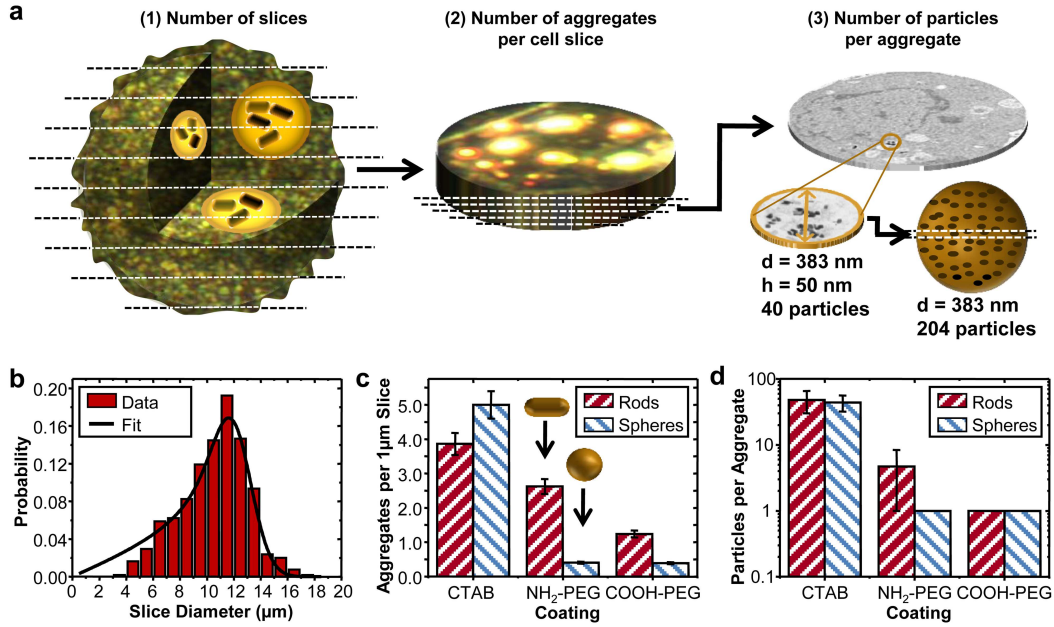
Beyond the qualitative description of the extent of uptake described above, the investigation of many cell slices (>50 per particle type for TEM and >150 cell slices for light microscopy) allows for quantifying the amount of internalized nanoparticles. Figure 4.2a schematically shows how the mean number of particles per cell is calculated. We determine the mean number of internalized particles  $N$  as the product of the mean number of slices per cell  $N_{\text{slices}}$ , the mean number of aggregates per cell slice  $N_{\text{aggregates}}$ , and the mean number of particles per aggregate  $N_{\text{particles}}$  yielding the formula  $N = N_{\text{slices}} N_{\text{aggregates}} N_{\text{particles}}$ . The three factors needed can be derived individually from the corresponding images.

$N_{\text{slices}}$  depends on the diameter of the cells and the slice thickness by their ratio  $N_{\text{slices}} = \text{diameter} / \text{thickness}$ . The slice thickness is set to 1  $\mu\text{m}$  in the microtome and the cell size can be deduced from the cell diameters measured on the DF images. We measure cell diameters on 550 cell slices and fit the resulting distribution numerically by Gaussian size distributed cells cut at random slice positions in 1  $\mu\text{m}$  slices. This procedure yields a mean cell diameter of  $(12.5 \pm 0.6) \mu\text{m}$ . Our value is slightly larger than a diameter of  $(11.6 \pm 0.2) \mu\text{m}$  calculated from quartz crystal microbalance analysis by Wegener et al. for the same cell line,<sup>31</sup> an effect possibly resulting from cell treatment during preparation for microscopy. Finally, we get  $N_{\text{slices}} = 12.5 \pm 0.6$  as the mean number of slices per cell (see Figure 4.2b).

$N_{\text{aggregates}}$  from counting the aggregates within about 150 slices imaged by DF. Since the particles are restricted to a definite volume, namely the cell, we assume the number of aggregates to be Poissonian distributed inside the complete cell. Consequently, a cutting of the cell yields slices exhibiting an exponentially distributed number of aggregates.<sup>32</sup> Therefore, we obtain the mean number of aggregates by fitting the measured probability for the aggregate number with an exponential distribution (for fits see Appendix Figure B.8). Results are shown in Figure 4.2c and are summarized in Table 4.1.

<sup>31</sup> Wegener et al., "Cell adhesion monitoring using a quartz crystal microbalance: comparative analysis of different mammalian cell lines"

<sup>32</sup> Weiers, *Introduction to Business Statistics*.



**Figure 4.2.:** **a)** Scheme to illustrate the determination of the total number of particles per cell  $N$  as the product of (1) the number of slices per cell  $N_{\text{slices}}$ , (2) the number of aggregates per cell slice  $N_{\text{aggregates}}$ , and (3) the number of particles per aggregate  $N_{\text{particles}}$  yielding the formula  $N = N_{\text{slices}}N_{\text{aggregates}}N_{\text{particles}}$ . (1)  $N_{\text{slices}}$  results from the cell diameter divided by the slice thickness  $N_{\text{slices}} = \text{diameter}/\text{thickness}$ . The mean cell diameter is determined from DF images. (2)  $N_{\text{aggregates}}$  is derived from the number of bright spots within the cell area observed by DF. (3)  $N_{\text{particles}}$  is computed from the particles in one aggregate as counted on TEM images and corrected for the three dimensional volume of the aggregate. **b)** Histogram of the diameters of  $1 \mu\text{m}$  cell slices measured in optical microscopy and fitted numerically assuming spherical cells with a Gaussian size distribution cut at random positions in  $1 \mu\text{m}$  slices. The fit yields a mean cell diameter of  $(12.5 \pm 0.6) \mu\text{m}$ , i.e.  $N_{\text{slices}} = (12.5 \pm 0.6)$  slices per cell of  $1 \mu\text{m}$  thickness. **c)**  $N_{\text{aggregates}}$  and its uncertainty determined from DF images. **d)**  $N_{\text{particles}}$  and its uncertainty determined from TEM images. If no particle was found, the number of particles per aggregate was set to one in order to assign the lowest meaningful value.

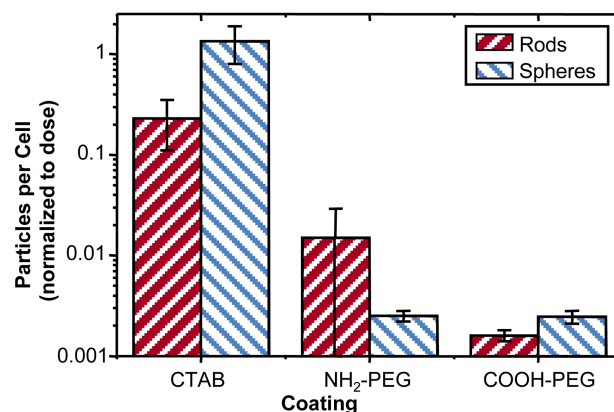
**Table 4.1.:** The measured number of aggregates per 1  $\mu\text{m}$  cell slice  $N_{\text{Aggregates}}$ , the number of particles per aggregate  $N_{\text{Particles}}$ , and the resulting number of particles per cell  $N$ . Cells were incubated for 20 hours with 2000 and 10000 particles per cell for spheres and rods, respectively.

| Particles        | $N_{\text{Aggregates}}$ | $N_{\text{Particles}}$ | $N$             |
|------------------|-------------------------|------------------------|-----------------|
| CTAB-rods        | $3.86 \pm 0.32$         | $48 \pm 18$            | $2300 \pm 1200$ |
| CTAB-spheres     | $5.00 \pm 0.40$         | $44 \pm 12$            | $2700 \pm 1100$ |
| NH2-PEG-rods     | $2.62 \pm 0.22$         | $4.7 \pm 3.7$          | $150 \pm 140$   |
| NH2-PEG-spheres  | $0.40 \pm 0.03$         | 1                      | $5.0 \pm 0.6$   |
| COOH-PEG-rods    | $1.24 \pm 0.10$         | 1                      | $16 \pm 2$      |
| COOH-PEG-spheres | $0.39 \pm 0.03$         | 1                      | $4.9 \pm 0.7$   |

Lastly, we determine by TEM how many particles form the aggregates found in the dark-field light microscope images. For the calculation of  $N_{\text{particles}}$ , the TEM slice thickness has to be considered because it is so thin (50 nm) that it shows only a cut-out of the larger aggregates. In practice, we assume to see in the TEM image the largest diameter of a spherical aggregate with the same particle density as the cut-out and extrapolate the number of particles in the aggregate (scheme see Figure 4.2a(3)). The mean values of  $N_{\text{particles}}$  and their errors are shown in Figure 4.2d and Table 4.1.

The procedure comprising three steps, namely  $N_{\text{slices}}$ ,  $N_{\text{aggregates}}$ , and  $N_{\text{particles}}$ , permits to calculate the total number of particles per cell as their product (results shown in Table 4.1). We find about 2500 CTAB-particles per cell regardless of shape. The number of internalized PEG-particles is about 200 times smaller with no obvious influence of the PEG end-groups or charge, respectively. Since we now know the number of internalized particles, we can assess the extent of gold nanoparticle uptake if we normalize the number of internalized particles to the applied dose. Normalization shows an uptake of all applied CTAB-spheres within the error, whereas one-fifth of the CTAB-rods and only a fraction of a percent for the PEG-particles are internalized (see Figure 4.3).

**Test of metabolic activity.** Furthermore, the knowledge about the particle number within the cells allows us to relate biological activity to nanoparticle uptake. For this purpose, we determine the metabolic activity of cells exposed for 24 hours to gold nanoparticles by a conventional cytotoxicity assay, namely the MTS test using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (Figure 4.4). The test shows that the metabolic activity of cells is strongly reduced by CTAB-spheres, moderately reduced by CTAB-rods, slightly reduced by



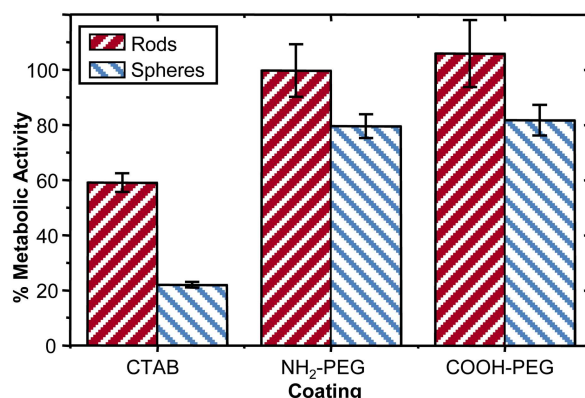
**Figure 4.3.:** Cells are exposed to suspensions of gold nanoparticles with the same gold concentration (in  $\mu\text{g}$  per mL). Normalization of the number of particles per cell calculated from the data in Figure 4.2 to the applied particle dose (in nanoparticles per mL) shows the extent of uptake. The data indicate that all CTAB-spheres and one-fifth of the CTAB-rods end up inside the cells, whereas the uptake of the PEG-particles is orders of magnitude lower regardless of their end-group. This difference in uptake between CTAB-particles and PEG-particles is so huge that a logarithmic scale for the internalized dose is needed to present the data.

PEG-spheres, and unaffected by PEG-rods with no significant influence of the chemical nature of PEG endgroups.

We have about the same amount of CTAB-spheres and CTAB-rods present inside the cell, but the metabolic activity of cells exposed to spheres is much more diminished than in the case of rods (20% compared to 60%). Similar amounts of PEG-spheres and PEG-rods were internalized into cells but only PEG-spheres show an appreciable response in metabolic activity. This slight reduction in metabolic activity by the presence of so few PEG-spheres (less than 10 per cell) is quite remarkable.

### 4.3. Discussion

**Uptake mechanism.** We developed an approach based on microscopy in order to visualize gold nanoparticles internalized into cells with regard to particle amount, location, distribution as well as shape and size. From TEM images, we can infer how the particles interact with the cell. The internalized particles or aggregates are located in multivesicular bodies or late endosomes, which is especially true for CTAB coated nanoparticles (Appendix Figures B.4-B.6). The large protrusion of the cell membrane around



**Figure 4.4.:** Metabolic activity of cells after 24 hour incubation with nanoparticles determined by the MTS test. Metabolic activity is strongly reduced by CTAB-spheres, moderately reduced by CTAB-rods, slightly reduced by PEG-spheres, and unaffected by PEG-rods. Error bars represent the standard deviation of three parallel and independent tests.

the particles close to the membrane (Appendix Figure B.7) points to a non-specific (non-clathrin/caveolae dependent) endocytosis or macropinocytosis as reported previously for the internalization of polymer nanoparticles into MDCK cells.<sup>33</sup>

We find a severe uptake of highly positively charged CTAB-particles, whereas neutral NH<sub>2</sub>-PEG-particles and slightly negative COOH-PEG-particles are hardly internalized by the cells. A possible route of internalization consistent with a strongly enhanced uptake of positively charged CTAB-particles is the retrograde pathway described by Orr *et al.*<sup>34</sup> This mechanism includes an interaction between positively charged particles and negatively charged membrane proteins being directly or indirectly in contact with the actin filament within filopodia and microvilli. By addition of actin monomers to the distal end of the structure, a retrograde motion takes place where particles caught by membrane proteins get transported and finally internalized. In another study, Cho *et al.* observed a favored adsorption of positively charged particles compared to neutral and negatively charged ones on the negatively charged cell membrane.<sup>19</sup> Finally, the favored adsorption results in a higher internalization level of positively charged particles compared to neutral and negatively charged ones, whereas there is not much difference in uptake between the neutral and negatively charged particles.

<sup>33</sup> Harush-Frenkel *et al.*, “Surface charge of nanoparticles determines their endocytic and transcytotic pathway in polarized MDCK cells.”

<sup>34</sup> Orr *et al.*, “Submicrometer and nanoscale inorganic particles exploit the actin machinery to be propelled along microvilli-like structures into alveolar cells.”

**Particle aggregation.** Looking at the internalized CTAB-particles, we observe a high degree of aggregation. It is an open question whether the particles cluster together in the culture medium, settle down, and then reach the cell surface for internalization, or get attached to the cell membrane as single particles and are grouped together during the process of uptake. An aggregation on the cell membrane is reported by Bartczak *et al.* who attribute the clustering of particles specific for the NRP-1 receptor to the clustering of the addressed receptor.<sup>35</sup> However, we do not find particle aggregates attached directly to the cell membrane but rather in its close proximity which is a consequence of the different internalization routes (Bartczak *et al.*: receptor-mediated uptake; our case: non-specific uptake, Appendix Figure B.7). Hence, the observed aggregation does not result from receptor clustering but seems to occur already in solution. In order to test for this hypothesis, we followed the behavior of CTAB-particles in cell medium as well as water over time in a previous study.<sup>25</sup> There, we detect sedimentation and cluster formation of CTAB-particles in medium within about 15 hours, whereas they remain well dispersed in water. It is therefore very likely that the applied CTAB-particles settle down on top of the cell monolayer in a high number and are already available in a clustered state. In contrast, PEG particles remain well dispersed in solutions even at high ionic strength.<sup>36</sup> Hence, a smaller number of single PEG-particles compared to aggregated CTAB-particles is present in close proximity of the cells ready for internalization. This sedimentation behavior might account for a higher number of CTAB-particles compared to PEG-particles found in the cells.<sup>37,38</sup>

**Uptake numbers.** Quantitatively, we find about 2500 CTAB-particles per cell whereas the uptake of PEG-particles is greatly reduced (a few tens per cell). It is, however, difficult to compare the absolute particle numbers per cell to literature values derived in various studies since there are so many parameters influencing the uptake (for example cell line, dose, incubation time and conditions, shape and surface coating of the particles).<sup>39</sup> Hence, we focus on the magnitude and trend in particle uptake. A number of several

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<sup>35</sup> Bartczak et al., "Receptor-Mediated Interactions between Colloidal Gold Nanoparticles and Human Umbilical Vein Endothelial Cells."

<sup>36</sup> Pierrat et al., "Self-assembly of small gold colloids with functionalized gold nanorods"

<sup>37</sup> Teeguarden et al., "Particokinetics in vitro: Dosimetry considerations for in vitro nanoparticle toxicity assessments."

<sup>38</sup> Cho et al., "The effect of sedimentation and diffusion on cellular uptake of gold nanoparticles."

<sup>39</sup> Bartczak et al., "Interactions of Human Endothelial Cells with Gold Nanoparticles of Different Morphologies."



thousand particles per cell without surface functionalization after synthesis is in agreement with literature:<sup>18,24,29</sup> Chithrani *et al.* incubated HeLa cells for 6 hours with CTAB-rods (40 nm × 14 nm in size) as well as citric acid spheres (50 nm in diameter) and find about 2000 and 6000 particles per cell using ICP-AES. Unfortunately, it is unclear, which particle dose was used.<sup>18</sup> In another study, the number of 15 nm spherical particles inside epithelial alveolar cells determined by TEM amounts to about 4000 particles per cell after incubation for 4 hours with a density of  $16.1 \times 10^{11}$  particles per cm<sup>2</sup>.<sup>24</sup> Incubation of HeLa cells with a 150 µm solution of CTAB-rods (40 nm × 18 nm in size) for 6 hours results in about 15000 particles per cell, which was quantified using ICP-AES by Hauck *et al.*<sup>29</sup> A reduction in particle uptake due to the replacement of CTAB by methoxy-functionalized PEG was also found in other *in vitro* experiments,<sup>22,24</sup> as well as in *in vivo* experiments.<sup>40</sup> Herein, Huff *et al.* identify a nearly 20 times lower uptake of PEG-rods into KB cells compared to CTAB-rods using two-photon luminescence microscopy.<sup>22</sup> TEM counts about a four times lower quantity of 15 nm PEG-spheres compared to not functionalized ones after incubation of epithelial alveolar cells with  $14.1 \times 10^{11}$  PEG-coated particles per cm<sup>2</sup>.<sup>24</sup> ICP-MS used to determine the distribution of PEG-rods and CTAB-rods (65 nm × 11 nm in size) in mice half an hour after intravenous injection detects only few percent of the PEG-particle dose inside the organs, whereas it is about 40% in case of CTAB-particles.<sup>40</sup> Hence, the good agreement of literature and our results shows the applicability of the presented approach to quantify gold nanoparticle uptake.

**Quality of method.** The relative errors of the particle numbers per cell in our study are about 50%, but the differences in particle uptake between CTAB and PEG coated particles are remarkably significant (see Table 4.1). These deviations in the uptake numbers are caused by the combination of several factors: First of all, we investigate single cells with a statistically distributed number of internalized particles. Secondly, also the number of particles in an aggregate varies statistically. Lastly, the ultra-thin cell slices investigated by TEM resemble only a fraction of a percent of the volume of the whole cell. This part is so small that we need to extrapolate the total number of particles per aggregate from a small fraction (see Figure 4.2a(3)). However, the method permits to assign standard deviations to the mean value from a single experiment, which is often missing in reported values. Most reports give standard deviations from three individual test runs showing only the

<sup>40</sup> Niidome *et al.*, “PEG-modified gold nanorods with a stealth character for *in vivo* applications”

reproducibility. If a consecutive stack of slices of several complete cells were investigated, our microscopic technique could show the variation in particle uptake among the cells within the investigated ensemble. Another potential advantage of our technique is that a size or shape selective uptake could be found on the TEM images.

Combining the high resolution power of TEM with the capability of DF to quickly investigate large cell volumes, we present an efficient method to study the uptake of gold nanoparticles. Herein, the method reveals not only the mean number of internalized particles, but also their location within the cell, their degree of aggregation, as well as the nanoparticle shape and size. The localization within the cell and the discrimination of nanoparticles bound to the outer surface of the cell is a major advantage compared to other spectroscopic techniques that measure the total amount of gold inside a large cell ensemble (e.g. ICP-MS and ICP-AES). On the other hand, TEM alone resolves single particles and their position within cells, but it is rarely used for uptake quantification due to high effort in stereological means and the high number of cell slices required for meaningful statistics.<sup>23,24</sup> These drawbacks in data collection and statistics get reduced by the combination with DF in our technique.

As further development of the technique, one could envision that a calibration of the spot intensity observed in DF may lead to quantification without TEM use. However, such a calibration is not straight forward since the scattering intensity is not proportional to particle volume but to the volume squared and depends on the precise geometry of the aggregate and the particle surrounding.

**Cytotoxicity.** From our test for the metabolic activity of cells exposed to nanoparticles, we detect no direct correlation to the number of internalized particles per cell. Apparently, factors beside the particle number influence the cell activity. One possibility for the strong metabolic effect of CTAB-particles could probably result from the release of surface bound CTAB, which is a relatively toxic cationic surfactant.<sup>12</sup> In a previous study, we compared the cellular response to pure CTAB in a concentration matching the situation if all surface bound molecules were released into solution with the cellular response to particles stabilized by CTAB. In that study, we found a more pronounced metabolic effect for CTAB-particles compared to pure CTAB solution.<sup>41</sup> This result points to particles serving as carriers for CTAB re-

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<sup>41</sup> Tarantola et al., "Cytotoxicity of Metal and Semiconductor Nanoparticles Indicated by Cellular Micromotility."



leasing their CTAB coating after internalization, which could cause more cell damage than CTAB acting from outside the cell.<sup>25</sup> A potential point for damage inside the cell could arise from the ability of CTAB to cause complexation of nucleic acids as well as protein/polysaccharide aggregation. Indeed, this carrier model would explain the stronger metabolic effect of spheres compared to rods because of their larger surface area per particle (nearly three times) making the spheres able to channel three times more CTAB molecules into the cell. However, the carrier model alone does not explain the reduced metabolic activity of cells incubated with PEG-spheres because PEG is known for its biocompatibility and inertness.<sup>28</sup> Further investigations are required to clarify the impact of particle shape on nanocytotoxicity.

## 4.4. Conclusion

Here, a new approach to address nanoparticle uptake based on microscopic methods is presented. We combined the high resolution power of TEM with the ability of DF to quickly access large cell volumes. We use the technique to quantify the number of nanoparticles present in the cells and their degree of aggregation. An advantage of this microscopic method compared to ensemble based methods such as ICP-MS is additional access to information on the nanoparticle location within the cell, their shape and size, as well as some hints to the type of interaction with the cell. In addition, the variation in particle uptake among a cell ensemble is accessible – a parameter often neglected but valuable for application considerations. Hence, the procedure gains versatile insight into the interaction between cells and gold nanoparticles and allows to analyze cytotoxicity data regarding extent of nanoparticle uptake.

## 4.5. Methods

Deionized water from a Millipore system ( $>18\text{ M}\Omega$ , Milli Q) was used in all experiments. Suppliers of chemicals are given in the Appendix Chapter B.

**Particle synthesis.** Gold nanorods and gold nanospheres were prepared as follows: The synthesis of rod-shaped particles were carried out according to the seeded growth method published by Nikoobakht.<sup>42</sup> In a first step, seeds

<sup>42</sup> Nikoobakht, El-Sayed, "Preparation and growth mechanism of gold nanorods (NRs) using

were prepared by adding 0.6 mL of ice-cold 0.010 M sodium borohydride ( $\text{NaBH}_4$ ) to a 10 mL solution of 0.1 M cetyltrimethylammonium bromide (CTAB) containing 50  $\mu\text{L}$  of 0.1 M tetrachloroauric acid ( $\text{HAuCl}_4$ ) under vigorous shaking. In a second step, rods were prepared by adding 12  $\mu\text{L}$  of seed solution to a growth solution consisting of 75  $\mu\text{L}$  of 0.1 M  $\text{HAuCl}_4$ , 10 mL of 0.1 M CTAB, 7  $\mu\text{L}$  of 0.04 M silver nitrate ( $\text{AgNO}_3$ ), and 105  $\mu\text{L}$  of 0.0788 M ascorbic acid (AA). Spherical particles were purchased from British Biocell International. In order to have the same surface chemistry as the gold rods, 1 mL aliquots of particles were precipitated by centrifugation (10 min, 10000 g) and resuspended in 1 mL of 0.1 M CTAB solution. Both spheres and rods were stored in 0.1 M CTAB solution. Shortly before the experiment, particles were washed in two centrifugation steps with water to remove unbound CTAB. Particle characterization. We determined the nanoparticle size using transmission electron microscopy (TEM). In order to prepare the samples, a 10  $\mu\text{L}$  drop of a solution with an approximated concentration of  $6 \times 10^{13}$  particles. $\text{mL}^{-1}$  was dropped on a carbon-coated copper grid (Plano) placed on a filter paper. After drying, images were taken on a Philips EM420 using an operating voltage of 120 kV. The mean nanoparticle size and its standard deviation resulted from the size measurement of at least 270 particles. Representative TEM images and histograms of size distributions are given in Appendix Figure B.1. We determined a length of  $(37.8 \pm 6.5)$  nm and a width of  $(17.2 \pm 2.9)$  nm for the nanorods and a diameter of  $(42.9 \pm 4.0)$  nm for the nanospheres. These values correspond to a particle volume of  $8000 \text{ nm}^3$  and  $41000 \text{ nm}^3$  for rods and spheres, respectively.

**Particle concentration.** Concentrations of nanoparticle solutions were calculated from their optical extinction value at 400 nm (examples are shown in Appendix Figure B.2). The molar extinction coefficient was calculated within the Mie-Gans theory in the quasi-static approximation<sup>43</sup> yielding  $1.1 \times 10^9 \text{ L.mol}^{-1}.\text{cm}^{-1}$  and  $6.2 \times 10^9 \text{ L.mol}^{-1}.\text{cm}^{-1}$  for rods and spheres with the sizes found by TEM, respectively. With these values, the concentrations of our stock solutions were  $6.2 \times 10^{11}$  particles. $\text{mL}^{-1}$  for the nanorods and  $1.1 \times 10^{11}$  particles. $\text{mL}^{-1}$  for the nanospheres, both corresponding to  $88.9 \mu\text{g gold.mL}^{-1}$ .

**Particle functionalization.** We can control the charge of the particles by introducing a stabilizing agent other than CTAB. In order to replace CTAB, various functionalized polyethylene glycol thiols ( $\text{X-PEG-SH}$ ;  $\text{X}=\text{COOH}$ ,  $\text{NH}_2$ ,

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seed-mediated growth method.”

<sup>43</sup> Prescott, Mulvaney, “Gold nanorod extinction spectra.”

CH<sub>3</sub>O; M<sub>w</sub>5000) were grafted on the gold surface.<sup>36</sup> For this purpose, we incubated the nanoparticle pellet overnight with 100 µL of an aqueous 2 mM mixture of 75% NH<sub>2</sub>-PEG-SH and 25% CH<sub>3</sub>O-PEG-SH (NH<sub>2</sub>-PEG-particles) or 75% COOH-PEG-SH and 25% CH<sub>3</sub>O-PEG-SH (COOH-PEG-particles), respectively. The next day, excess PEG was removed by centrifugation. Success of PEGylation was tested by gel electrophoresis, which also reveals the surface charge of the particles (Appendix Figure B.3).

**Cell culture.** In our studies, we used epithelial MDCK (type II) cells. They were cultured in Earle's minimum essential medium supplemented with 4 mM glutamine, 100 µg.mL<sup>-1</sup> of both penicillin and streptomycin, 10% (v/v) fetal calf serum and stored in an incubator with 5% CO<sub>2</sub> atmosphere at 37°C (HERA cell 150, Heraeus). Subculture was performed weekly after cells reached confluence. After medium was removed, the cell monolayer was washed twice with 4 mL of phosphate-buffered saline (PBS) without magnesium and calcium ions and incubated with 2 mL of the chelating agent ethylenediaminetetraacetic acid (EDTA) for 10 minutes in the incubator. Then, EDTA was removed and cells were detached from the substrate by incubation with 1 mL of trypsin/EDTA for 10 minutes in the incubator. Trypsination was stopped by adding 10 mL of medium, which was removed afterward by centrifugation at 110 g for 10 minutes. Cells were resuspended in 10 mL of fresh medium and seeded into a new culture flask at a ratio of 1:10.<sup>44</sup>

**Cell treatment and fixation.** For the experiment, roughly 10<sup>6</sup> cells were seeded in a Petri dish and cultured until confluence within 4 days. The confluent monolayer consisted of about 11×10<sup>6</sup> cells.<sup>31</sup> Then, medium was exchanged with 1.1 mL of fresh medium containing 16.3 µg of gold. This concentration corresponded to a dose of 1.5 pg gold per cell, which were about 10000 nanorods and 2000 nanospheres, respectively. After 20 h of incubation in 5% CO<sub>2</sub> atmosphere at 37°C, cells were detached from the substrate as described before. The detached cells were fixated and embedded in an epoxy resin for preparation of cell slices for DF and TEM. For fixation, cells were incubated for 1 h with 3 mL of 2.5% glutaraldehyde in cacodylate buffer (0.1 M sodium cacodylate and 0.1 M sucrose at pH 7.4), washed three times with cacodylate buffer, incubated for 1 h with 1 mL of 2% osmium tetroxide (OsO<sub>4</sub>) in cacodylate buffer, and washed two times with water. For embedding, cells were put in 2 wt% agar, which was dehydrated using an increasing ethanol row (30%, 50%, 70%, 80%, 96%, absolute), and transferred

<sup>44</sup> Freshney, *Culture of Animal Cells*.

into araldite epoxy resin with the help of propylene oxide.<sup>45</sup> The resin was polymerized by heating to 65°C for three days.

**Cell sectioning and staining.** Cells in resin were cut into 1 µm or 50 nm slices using a Reichert Ultracut S ultramicrotome (Leica). Thin slices of 1 µm thickness were placed on dust-free microscope slides for DF and ultra-thin slices of 50 nm thickness on copper TEM grids. Contrast enhancement of biological tissue for TEM was achieved by incubation in a solution of 2 % uranyl acetate in 50 % ethanol for 2 min<sup>46</sup> and a lead salt solution prepared according to Sato's method for 2 min.<sup>47</sup> Before investigation in TEM, samples were rinsed thoroughly with water and dried in air.

**Cytotoxicity test.** We determined effects of particles on the metabolic activity of cells. The metabolic activity was tested using the MTS assay according to the manufacturer protocol. For the assay, cells were grown to a predetermined optimal number of 12000/well in a 96-well plate. Subsequently, medium was exchanged with 0.2 mL of fresh medium containing 3.0 µg of gold. Successful control experiments were carried out using cell-free wells and vital cells not exposed to particles. After 24 h of incubation, samples were rinsed three times with PBS and full medium to remove any nanoparticles that were not internalized. Afterward, cells were incubated with the tetrazolium educt for 45 minutes and the extinction at 490 nm was recorded using a 96-well plate photometer (Thermomax, Molecular Devices). Cytotoxicity experiments were performed in three parallel and independent tests.

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<sup>45</sup> Mersseman et al., "Exogenous introduction of an immunodominant peptide from the non-structural IE1 protein of human cytomegalovirus into the MHC class I presentation pathway by recombinant dense bodies."

<sup>46</sup> Hayat, *Principles and Techniques of Electron Microscopy*.

<sup>47</sup> Hanaichi et al., "A Stable Lead by Modification of Sato Method."

## 4.6. References

1. De, M.; Ghosh, P. S.; Rotello, V. M. *Advanced Materials* **2008**, *20*, 4225–4241.
2. Weissleder, R. *Nature Biotechnology* **2001**, *19*, 316–317.
3. Wax, A.; Sokolov, K. *Laser & Photonics Reviews* **2009**, *3*, 146–158.
4. Xu, M. H.; Wang, L. H. V. *Review of Scientific Instruments* **2006**, *77*, 041101.
5. Murphy, C. J.; Gole, A. M.; Stone, J. W.; Sisco, P. N.; Alkilany, A. M.; Goldsmith, E. C.; Baxter, S. C. *Accounts of Chemical Research* **2008**, *41*, 1721–1730.
6. Parak, W. J.; Gerion, D.; Pellegrino, T.; Zanchet, D.; Micheel, C.; Williams, S. C.; Boudreau, R.; Le Gros, M. A.; Larabell, C. A.; Alivisatos, A. P. *Nanotechnology* **2003**, *14*, R15–R27.
7. Sperling, R. A.; Riveragil, P.; Zhang, F.; Zanella, M.; Parak, W. J. *Chemical Society Reviews* **2008**, *37*, 1896–1908.
8. Ghosh, P.; Han, G.; De, M.; Kim, C. K.; Rotello, V. M. *Advanced Drug Delivery Reviews* **2008**, *60*, 1307–1315.
9. Lal, S.; Clare, S. E.; Halas, N. J. *Accounts of Chemical Research* **2008**, *41*, 1842–1851.
10. Kingson, J. A. *The New York Times* **2010**, May 27.
11. Paracelsus, T., *Septem Defensiones. Die Selbstverteidigung eines Aussen-seiters*; Schwabe: 2003 (1538), p 63.
12. Lewinski, N.; Colvin, V.; Drezek, R. *Small* **2008**, *4*, 26–49.
13. Khlebtsov, N.; Dykman, L. *Chemical Society Reviews* **2011**, *40*, 1647–1671.
14. Boisselier, E.; Astruc, D. *Chemical Society Reviews* **2009**, *38*, 1759–1782.
15. Luhmann, T.; Rimann, M.; Bitterman, A. G.; Hall, H. *Bioconjugate Chemistry* **2008**, *19*, 1907–1916.
16. Yen, H.-J.; Hsu, S.-h.; Tsai, C.-L. *Small* **2009**, *5*, 1553–1561.
17. Alkilany, A. M.; Nagaria, P. K.; Hexel, C. R.; Shaw, T. J.; Murphy, C. J.; Wyatt, M. D. *Small* **2009**, *5*, 701–708.
18. Chithrani, B. D.; Ghazani, A. A.; Chan, W. C. W. *Nano Letters* **2006**, *6*, 662–668.

19. Cho, E. C.; Xie, J. W.; Wurm, P. A.; Xia, Y. N. *Nano Letters* **2009**, *9*, 1080–1084.
20. Liu, Y. L.; Shipton, M. K.; Ryan, J.; Kaufman, E. D.; Franzen, S.; Feldheim, D. L. *Analytical Chemistry* **2007**, *79*, 2221–2229.
21. Clift, M. J. D.; Rothen-Rutishauser, B.; Brown, D. M.; Duffin, R.; Donaldson, K.; Proudfoot, L.; Guy, K.; Stone, V. *Toxicology and Applied Pharmacology* **2008**, *232*, 418–427.
22. Huff, T. B.; Hansen, M. N.; Zhao, Y.; Cheng, J. X.; Wei, A. *Langmuir* **2007**, *23*, 1596–1599.
23. Mayhew, T. M.; Mühlfeld, C.; Vanhecke, D.; Ochs, M. *Annals of Anatomy-Anatomischer Anzeiger* **2009**, *191*, 153–170.
24. Brandenberger, C.; Mühlfeld, C.; Ali, Z.; Lenz, A. G.; Schmid, O.; Parak, W. J.; Gehr, P.; Rothen-Rutishauser, B. *Small* **2010**, *6*, 1669–1678.
25. Tarantola, M.; Pietuch, A.; Schneider, D.; Rother, J.; Sunnick, E.; Rosman, C.; Pierrat, S.; Sönnichsen, C.; Wegener, J.; Janshoff, A. *Nanotoxicology* **2011**, *5*, 254–268.
26. Wang, S. H.; Lee, C. W.; Chiou, A.; Wei, P. K. *Journal of Nanobiotechnology* **2010**, *8*.
27. Verma, A.; Stellacci, F. *Small* **2009**, *6*, 12–21.
28. Ryan, S. M.; Mantovani, G.; Wang, X. X.; Haddleton, D. M.; Brayden, D. J. *Expert Opinion on Drug Delivery* **2008**, *5*, 371–383.
29. Hauck, T. S.; Ghazani, A. A.; Chan, W. C. W. *Small* **2008**, *4*, 153–159.
30. Rayavarapu, R. G.; Petersen, W.; Hartsuiker, L.; Chin, P.; Janssen, H.; Leeuwen, F. W. B. van; Otto, C.; Manohar, S.; Leeuwen, T. G. van *Nanotechnology* **2010**, *21*.
31. Wegener, J.; Janshoff, A.; Galla, H. J. *European Biophysics Journal with Biophysics Letters* **1998**, *28*, 26–37.
32. Weiers, R. M., *Introduction to Business Statistics*; South-Western Cengage Learning: 2004, p 228.
33. Harush-Frenkel, O.; Rozentur, E.; Benita, S.; Altschuler, Y. *Biomacromolecules* **2008**, *9*, 435–443.
34. Orr, G.; Panther, D. J.; Phillips, J. L.; Tarasevich, B. J.; Dohnalkova, A.; Hu, D. H.; Teeguarden, J. G.; Poundsl, J. G. *Acs Nano* **2007**, *1*, 463–475.

35. Bartczak, D.; Sanchez-Elsner, T.; Louafi, F.; Millar, T. M.; Kanaras, A. G. *Small* **2011**, *7*, 388–394.
36. Pierrat, S.; Zins, I.; Breivogel, A.; Sönnichsen, C. *Nano Letters* **2007**, *7*, 259–263.
37. Teeguarden, J. G.; Hinderliter, P. M.; Orr, G.; Thrall, B. D.; Pounds, J. G. *Toxicological Sciences* **2007**, *95*, 300–312.
38. Cho, E. C.; Zhang, Q.; Xia, Y. N. *Nature Nanotechnology* **2011**, *6*, 385–391.
39. Bartczak, D.; Muskens, O. L.; Nitti, S.; Sanchez-Elsner, T.; Millar, T. M.; Kanaras, A. G. *Small* **2012**, *8*, 122–130.
40. Niidome, T.; Yamagata, M.; Okamoto, Y.; Akiyama, Y.; Takahashi, H.; Kawano, T.; Katayama, Y.; Niidome, Y. *Journal of Controlled Release* **2006**, *114*, 343–347.
41. Tarantola, M.; Schneider, D.; Sunnick, E.; Adam, H.; Pierrat, S.; Rosman, C.; Breus, V.; Sönnichsen, C.; Basché, T.; Wegener, J.; Janshoff, A. *ACS Nano* **2009**, *3*, 213–222.
42. Nikoobakht, B.; El-Sayed, M. A. *Chemistry of Materials* **2003**, *15*, 1957–1962.
43. Prescott, S. W.; Mulvaney, P. *Journal of Applied Physics* **2006**, *99*, 123504; Corrigendum: **2008**, *103*, 119901.
44. Freshney, R. I., *Culture of Animal Cells*, 4th ed.; Wiley-Liss: 2000.
45. Mersseman, V.; Besold, K.; Reddehase, M. J.; Wolfrum, U.; Strand, D.; Plachter, B.; Reyda, S. *Journal of General Virology* **2008**, *89*, 369–379.
46. Hayat, M. A., *Principles and Techniques of Electron Microscopy*, 4th ed.; Cambridge University Press: 2000.
47. Hanaichi, T.; Sato, T.; Iwamoto, T.; Malavasiyamashiro, J.; Hoshino, M.; Mizuno, N. *Journal of Electron Microscopy* **1986**, *35*, 304–306.





## Chapter 5.

# 5

# Mammalian Cell Growth on Gold Nanoparticle Patterned Substrates is Influenced by the Particle Coating

Gold nanorods should be suitable sensors for cell membrane fluctuations characterizing their mechanical properties. Before application, an influence of the particles on the native behavior has to be excluded. This chapter presents a study on cells growing on top of gold nanorods with various surface coatings. Herein, live cell imaging for a period of three days depicted the viability and proliferation of the cells. Particles functionalized with a biocompatible polymer exposing a negatively charged end group towards the cell membrane turned out to be promising candidates to sense membrane fluctuations. In this project, I performed cell culture, synthesized, characterized, and functionalized the particles and evaluated the data. With the help of Dr. Sebastien Pierrat, I improved the microscope setup in a way enabling live cell imaging over a prolonged period of time and we shared the experiments.

## 5.1. Introduction

Cellular processes like mobility, proliferation, and endo/exocytosis depend on mechanical properties of a bilayer membrane, such as its viscosity, bending rigidity, and tension.<sup>1,2</sup> Since the processes are spatially restricted and change over time, quantitative mapping of the membrane properties in

<sup>1</sup> Helfrich, "Elastic Properties of Lipid Bilayers - Theory and Possible Experiments."

<sup>2</sup> Morris, Homann, "Cell surface area regulation and membrane tension."

a noninvasive manner is desirable to understand underlying mechanisms.<sup>3,4</sup> So far, visualization of membrane fluctuations is used to extract mechanical properties, but this approach suffers from various drawbacks: there exist light microscopy based methods neglecting however spatial distributions<sup>5,6</sup> or techniques focussing on complete height changes instead of pure membrane displacement like atomic force microscopy<sup>7</sup>, digital holographic microscopy<sup>3</sup> or phase microscopy.<sup>8,9</sup> In contrast, plasmonic metal nanoparticles exhibit a high sensitivity to changes in their local environment, which they report by a shift in resonance wavelength<sup>10</sup> detectable by optical dark-field spectroscopy.<sup>11</sup> Compared to conventional SPR, the sensing volume for plasmons in metal nanoparticles are better matched to molecular dimensions and fluctuations do not cancel out over area.<sup>12</sup> However, before using nanoparticles as fluctuation sensors for membranes, an influence of the particles on the native membrane behavior must be excluded. Therefore, we investigate the growth behavior of epithelial cells (MDCK II) on glass substrates patterned with various functionalized gold nanorods. We find an impact of the particle coating on the viability and proliferation of the cells correlating with known toxic effects of the stabilizing agent. Furthermore, there seems to be an interaction between the particles and the cell membrane depending on the functional end-group of the stabilizer. This interaction has an impact on viability and proliferation as well. Hence, the nanoparticle coating used for fluctuation recording is the key parameter for reliable measurements.

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<sup>3</sup> Rappaz et al., "Spatial analysis of erythrocyte membrane fluctuations by digital holographic microscopy"

<sup>4</sup> Girard et al., "Cellular chemomechanics at interfaces: sensing, integration and response"

<sup>5</sup> Evans et al., "Fluctuations of the red blood cell membrane: Relation to mechanical properties and lack of ATP dependence."

<sup>6</sup> Levin, Korenstein, "Membrane Fluctuations in Erythrocytes are Linked to MgATP-Dependent Dynamic Assembly of the Membrane Skeleton."

<sup>7</sup> Nowakowski et al., "Imaging erythrocytes under physiological conditions by atomic force microscopy."

<sup>8</sup> Popescu et al., "Fourier phase microscopy for investigation of biological structures and dynamics."

<sup>9</sup> Popescu et al., "Erythrocyte structure and dynamics quantified by Hilbert phase microscopy."

<sup>10</sup> Sönnichsen et al., "Spectroscopy of single metallic nanoparticles using total internal reflection microscopy."

<sup>11</sup> Sönnichsen et al., "Drastic reduction of plasmon damping in gold nanorods."

<sup>12</sup> Ament et al., "Single Unlabeled Protein Detection on Individual Plasmonic Nanoparticles."

## 5.2. Results

**System.** We follow the growth of epithelial cells (MDCK II) on glass substrates patterned with gold nanorods ( $38\text{ nm} \times 17\text{ nm}$ ), each with three different surface coatings since the particle bound molecules (stabilizing agents) are expected to promote interaction with the cell membrane.<sup>13,14</sup> Herein, one kind of surface functionalization is built by molecules of cetyltrimethylammonium bromide (CTAB), which is a relatively toxic cationic surfactant<sup>15</sup> used for particle preparation. We compare these CTAB-particles with particles functionalized by the inert polymer polyethylene glycol (PEG) known for its biocompatibility.<sup>16</sup> To investigate the influence of present reactive groups, we use PEG chains terminated with either amine ( $\text{NH}_2$ -PEG) or carboxy groups ( $\text{COOH}$ -PEG). A comparison for unaffected cell growth is performed using a bare glass substrate.

**Live cell imaging.** We immobilize single gold nanorods with a density of about 1 particle per  $\mu\text{m}^2$  on the glass bottom of a special petri dish with a diameter of  $3.5\text{ cm}^2$  and add about 6000 cells to ensure seeding of single cells. We image about 50 cells per sample by optical dark-field microscopy using a home-built setup for live cell imaging (Appendix Figure C.1), during which culture conditions of a 5%  $\text{CO}_2$  atmosphere at  $37^\circ\text{C}$  are maintained. In optical dark-field microscopy, we collect only scattered light by the sample, hence the cell tissue appears bright on top of small colored spots generated by the gold nanorods. By using a mapping system of crosses scratched to the glass, we are able to track the same 50 cells chosen in the beginning daily over a period of three days (Figure 5.1a). Afterwards, we judge on the growth behavior of the cells by their viability and proliferation. Figure 5.1b shows an example for a cell, which is alive at day one indicated by its presence and which has disappeared after its death at day two. From the portion of viable cells, we rate their proliferation behavior shown by an active cell division. Figure 5.1c shows a cell, which is alive at day one and which has considerably expanded its spreading area at day two.

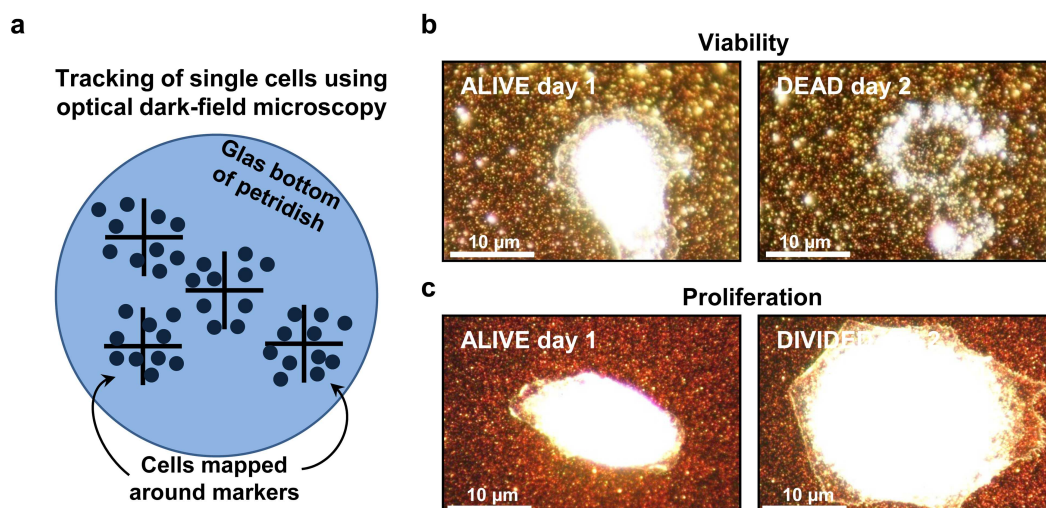
**Viability.** We determine the viability of cells as amount of living cells

<sup>13</sup> Cho et al., "Understanding the Role of Surface Charges in Cellular Adsorption versus Internalization by Selectively Removing Gold Nanoparticles on the Cell Surface with a I-2/KI Etchant."

<sup>14</sup> Orr et al., "Submicrometer and nanoscale inorganic particles exploit the actin machinery to be propelled along microvilli-like structures into alveolar cells."

<sup>15</sup> Lewinski et al., "Cytotoxicity of Nanoparticles"

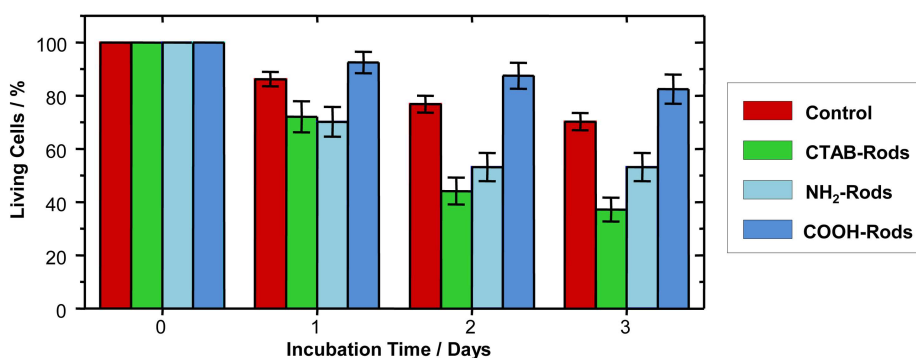
<sup>16</sup> Ryan et al., "Advances in PEGylation of important biotech molecules: delivery aspects"



**Figure 5.1.:** a) We scratch crosses in the glass bottom of a special petri dish and map the position of single cells located close to these crosses. Therefore, we are able to find the same cells for several days. b) We determine the viability of the cells. The left picture shows a optical dark-field image of an adherent, living cell. On the right image, the cell detached from the substrate and is therefore rated to be dead. c) From the portion of living cells, we determine their proliferation by occurrence of cell division. The left image shows a living cell, whose spreading area has considerably expanded after one more day of incubation.

normalized to the starting cell number (Figure 5.2). In order to identify the number of living cells, we image each of the cells being chosen in the beginning of the experiment. In these images, presence of the cell indicates viability, whereas an absent cell is rated to be dead. The control sample shows the viability of cells grown on a bare glas substrate. There is a small percentage of cells dying attributed to the fact that not every cell starts proper development after seeding. A considerably lower amount of viable cells is present in the sample with a CTAB-rods patterned substrate. A slight reduction in viability compared to the control results from the cell growth on  $\text{NH}_2$ -PEG-rods patterned substrate, whereas patterning of  $\text{COOH}$ -PEG-rods does not have an influence.

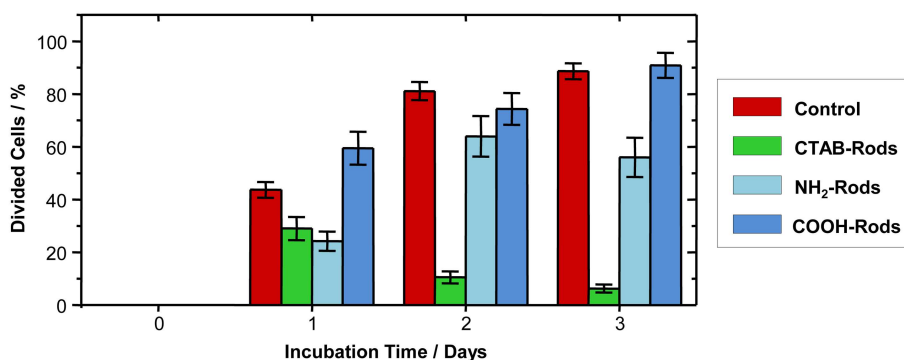
**Proliferation.** The portion of divided cells among the living cells depicts the proliferation behavior (Figure 5.3). In order to quantify the divided cells, a living cell with an expanded spreading area is rated as poliferting, whereas a living cell, which has not further spread out, is considered to be not proliferating. Unaffected cells growing on a bare glass substrate as control sample show over time an increasing number of cells enlarging



**Figure 5.2.:** The cell viability is the ratio of cells being still alive among the tracked cells compared to the starting cell number. In the control sample consisting of cells growing on bare glass, there are a few cells dying due to seeding of a low cell density. Patterning the substrate with CTAB-rods has the strongest impact on cell viability. NH<sub>2</sub>-PEG-rods reduce slightly the portion of living cells, whereas COOH-PEG-rods do not affect viability. (Error bar indicates s.e.m. for binomial distribution of 43 - 55 investigated cells.)

their spreading area. In contrast, the number of dividing cells growing on a CTAB-rods patterned substrate decreases more and more until proliferation nearly stops after three days of incubation. In the case of a NH<sub>2</sub>-PEG-rods patterned substrate, more and more cells start division between day one and two, but then the ratio of dividing cells among the living cells stagnates. For the COOH-PEG-rods, we observe similar proliferation behavior as for cells growing on bare glass, which is an increasing number of divided cells.

**Nanoparticle - cell interaction.** In the sample, where we grow the cells on top of NH<sub>2</sub>-PEG-rods, we observe movement of particles below the cell membrane. Figure 5.4a shows a cell area, which is enlarged on the four images aside. This set of images displays a time series with a wait time about two seconds. The white arrow indicates a particle, which clearly changes its position. After three days of incubation, there are a few particles moving below almost every living cell. On the other hand, the cell does not remove the particles from the glass substrate to a recognizable extent. On areas, where the cell retracted its membrane, an even pattern of particles is present without any irregular holes or clefts (Figure 5.4b). In a control experiment, we incubated cells on particles for seven days and removed the cell layer by trypsin. The nanoparticle pattern was not affected by cell growth (Appendix Figure C.2). The filamentous residues of the membrane observable in Figure 5.4b occur in the control sample as well (Appendix Figure C.3) and therefore they are no characteristic features for nanoparticle-cell interaction.

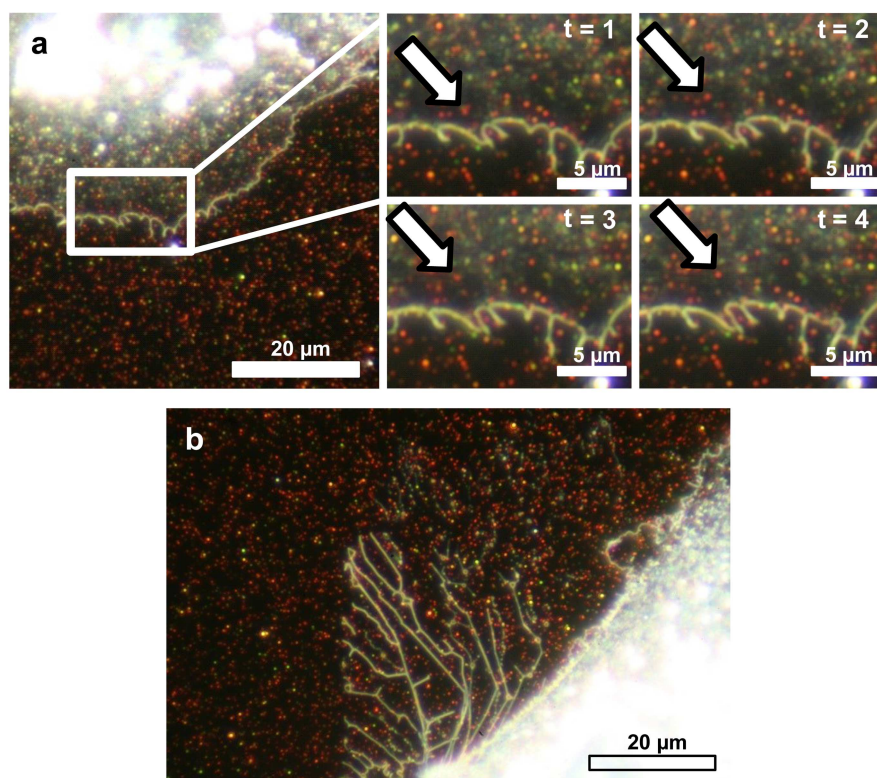


**Figure 5.3.:** Proliferation results from the number of divided cells among the remaining tracked living cells. In the control sample, more and more cells divide, whereas proliferation nearly stops in the case of CTAB-rods. For the NH<sub>2</sub>-PEG-rods, there is an initially increasing number of dividing cells, which rests after two days of incubation. We observe the same proliferation behavior for the COOH-PEG-rods as for the control sample. (Error bar indicates s.e.m. for binomial distribution of 16 - 45 living cells.)

### 5.3. Discussion

We tracked the growth behavior of living cells on glass substrates patterned with gold nanorods using optical dark-field microscopy. Herein, three different stabilizing agents present on the particle surface were investigated regarding their impact on the cells compared to growth on bare glass substrates. We observe for the cell viability as well for the proliferation a strong influence by CTAB-rods, a minor effect by NH<sub>2</sub>-PEG-rods, and no difference compared to the control sample for COOH-PEG-rods. Since there is at least one sample showing no impact, COOH-PEG-rods, we exclude negative effects on cell growth due to patterning of the substrate. Therefore, the stabilizing agent, which comes into direct contact with the cell membrane, causes diminished viability and proliferation. Among the investigated stabilizers, CTAB is known to be toxic<sup>15</sup> causing complexation of nucleic acids as well as protein/polysaccharide aggregation. Toxic effects of CTAB emerge clearly in the proliferation, since after three days of incubation nearly all cells stopped division while about 40% of the starting cells are judged to be alive. Here, we deduce viability simply from the cell presence, which might be incorrect: the outer cell structure could be fixed by CTAB preserving the shape while any proliferation is prohibited. In order to have a reliable measure on viability, a staining test for live/dead has to be performed, which is, however, not the goal of this study. With respect to live cell sensing applications, CTAB-rods





**Figure 5.4.:** a) In the indicated and enlarged cell area, we observe movement of  $\text{NH}_2$ -PEG-rods. The four images show a time series with a wait time of about two seconds, the moving particle is marked with a white arrow. b) Areas, which are uncovered by a retracking cell membrane, show an even particle pattern. Hence, the cell does not remove the particles to a remarkable extent.

are not suitable due to their impact on the native cell behavior.

The other investigated stabilizer, PEG, is considered to be biocompatible by itself.<sup>16</sup> This biocompatibility explains the observed similarity in cell growth of the control sample and the sample patterned with  $\text{COOH}$ -PEG-rods. In contrast, the presence of  $\text{NH}_2$ -PEG-rods slightly lowers viability and proliferation, which is inconsistent with the PEG biocompatibility. Therefore, the impaired cell metabolism is assumed originating from the interaction of cells and particles,<sup>4</sup> which we observe in the movement of  $\text{NH}_2$ -PEG-rods in regions covered by the cell membrane. However, the cells do not internalize particles as there are no obvious irregularities in the nanoparticle patterned substrate after retraction of the cell membrane. In regions, where the cell membrane retracted, there remain filamentous residues, but these filaments are no indicator for an enhanced nanoparticle membrane interaction, since they are present in the control sample as well. Although the interaction

of NH<sub>2</sub>-PEG-rods and the cell membrane seems to be weak, its occurrence makes NH<sub>2</sub>-PEG-rods unsuitable for membrane sensing applications. In contrast, COOH-PEG-rods show no interaction with the cell membrane and cells grown in their presence exhibit similar viability and proliferation behavior as control samples fulfilling therefore basic requirements on a sensor for membrane fluctuations.

## 5.4. Conclusion

Gold nanoparticles exhibit optical properties to sense membrane fluctuations, but the sensor presence has to keep these fluctuations unaffected. In order to test for influences of particle presence on cell growth, we investigated cell viability and proliferation on top of gold nanorod patterned substrates. Herein, a strongly impaired cell growth correlates with the toxicological properties of the stabilizing agent. In case of a biocompatible stabilizer, an interaction between nanoparticles and cells results in a slightly altered growth behavior compared to control samples. Our study included gold nanorods stabilized with COOH-PEG, which result in the same viability and proliferation rates as control samples. Therefore, these COOH-PEG-rods are a promising sensor scheme to perform membrane fluctuation studies on living cells.

## 5.5. Methods

Deionized water from a Millipore system (>18 M $\Omega$ , Milli Q) was used in all experiments. Suppliers of chemicals are given in the Appendix Chapters B and C.

**Particle synthesis, functionalization, and cell culture.** See Section 4.5.

**Cell growth on patterned substrates.** Markers for cell tracking shown in Figure 5.1a were scratched with a diamond-tip pencil into the glass bottom of a petri dish. Afterwards, the petri dish was treated with oxygen plasma for 45 s to make the surface hydrophilic. For sample preparation, 100  $\mu$ L of 1 M sodium chloride and 100  $\mu$ L of the particle solution or 100  $\mu$ L of water in case of the control sample were put into the petri dish. After incubation for 30 minutes, samples were rinsed three times with 3 mL of water to remove particles, which were not immobilized. Water was replaced by 500  $\mu$ L of



culture medium filtered through a sterile syringe filter with 200 nm pore size to minimize the risk of big scattering centers, which could add background in optical dark-field microscopy. Finally 10  $\mu$ L of cell suspension (~6000 cells) were seeded and samples were kept under cell culture conditions.

**Live cell imaging.** Optical dark-field microscopy was performed after 4, 24, 48, and 72 hours while maintaining 37°C and a 5% CO<sub>2</sub> environment. In order to improve contrast for imaging, cell culture medium was replaced by a version without phenol-red pH-indicator and any nutrients. As soon as imaging was finished, medium was changed back to full culture medium and samples were kept in a cell culture incubator.

## 5.6. References

1. Helfrich, W. *Zeitschrift Für Naturforschung C - a Journal of Biosciences* **1973**, *C 28*, 693–703.
2. Morris, C. E.; Homann, U. *Journal of Membrane Biology* **2001**, *179*, 79–102.
3. Rappaz, B.; Barbul, A.; Hoffmann, A.; Boss, D.; Korenstein, R.; Depeursinge, C.; Magistretti, P. J.; Marquet, P. *Blood Cells Molecules and Diseases* **2009**, *42*, 228–232.
4. Girard, P. P.; Cavalcanti-Adam, E. A.; Kemkemer, R.; Spatz, J. P. *Soft Matter* **2007**, *3*, 307–326.
5. Evans, J.; Gratzer, W.; Mohandas, N.; Parker, K.; Sleep, J. *Biophysical Journal* **2008**, *94*, 4134–4144.
6. Levin, S.; Korenstein, R. *Biophysical Journal* **1991**, *60*, 733–737.
7. Nowakowski, R.; Luckham, P.; Winlove, P. *Biochimica Et Biophysica Acta-Biomembranes* **2001**, *1514*, 170–176.
8. Popescu, G.; Deflores, L. P.; Vaughan, J. C.; Badizadegan, K.; Iwai, H.; Dasari, R. R.; Feld, M. S. *Optics Letters* **2004**, *29*, 2503–2505.
9. Popescu, G.; Ikeda, T.; Best, C. A.; Badizadegan, K.; Dasari, R. R.; Feld, M. S. *Journal of Biomedical Optics* **2005**, *10*.
10. Sönnichsen, C.; Geier, S.; Hecker, N. E.; Plessen, G. von; Feldmann, J.; Ditlbacher, H.; Lamprecht, B.; Krenn, J. R.; Aussenegg, F. R.; Chan, V. Z. H.; Spatz, J. P.; Möller, M. *Applied Physics Letters* **2000**, *77*, 2949–2951.
11. Sönnichsen, C.; Franzl, T.; Wilk, T.; Plessen, G. von; Feldmann, J.; Wilson, O.; Mulvaney, P. *Physical Review Letters* **2002**, *88*.
12. Ament, I.; Prasad, J.; Henkel, A.; Schmachtel, S.; Sönnichsen, C. *Nano Letters* **2012**, *12*, 1092–1095.
13. Cho, E. C.; Xie, J.; Wurm, P. A.; Xia, Y. *Nano Letters* **2009**, *9*, 1080–1084.
14. Orr, G.; Panther, D. J.; Phillips, J. L.; Tarasevich, B. J.; Dohnalkova, A.; Hu, D.; Teeguarden, J. G.; Poundsl, J. G. *Acs Nano* **2007**, *1*, 463–475.
15. Lewinski, N.; Colvin, V.; Dreze, R. *Small* **2008**, *4*, Times Cited: 766, 26–49.
16. Ryan, S. M.; Mantovani, G.; Wang, X.; Haddleton, D. M.; Brayden, D. J. *Expert Opinion on Drug Delivery* **2008**, *5*, 371–383.

## Chapter 6.

# 6 Multiplexed Plasmon Sensor for Rapid Label-free Analyte Detection

Efficient and cost-effective multiplexed detection schemes for proteins in small liquid samples would bring drastic advances to fields like disease detection or water quality monitoring. In this chapter, I present a novel multiplexed sensor with randomly deposited aptamer functionalized gold nanorods. Among the individual nanorods, each particle exhibits a plasmon resonance responding specifically to the presence of a different protein by a shift in the spectral position, which is monitored by optical dark-field spectroscopy. As striking properties of the sensor, I demonstrate nanomolar sensitivity, sensor recycling and the potential to upscale to hundreds or thousands of targets. In this project, Dr. Jonathan Edgar synthesized and characterized the gold nanorods. Janak Prasad established the aptamer functionalization of the particles. Dr. Andreas Henkel developed the setup and conducted calculations. I performed the measurements with the help of Andreas Neiser and Janak Prasad and I coordinated the project. This work is published in *Nano Letters* **2013**, *13*, 3243-3247.

## 6.1. Introduction

Inexpensive sensors verifying quickly the presence of multiple analytes within a small drop would bring drastic improvements for medical diagnosis, e.g. in discriminating SARS from normal flu patients.<sup>1,2</sup> Common approaches for parallel analyte detection in small liquid samples couple specific receptor

<sup>1</sup> Peiris et al., “Current concepts: The severe acute respiratory syndrome.”

<sup>2</sup> Webby, Webster, “Are we ready for pandemic influenza?”

molecules to spectrally encoded markers (e.g. SERS barcode,<sup>3</sup> luminex)<sup>4</sup> limiting the number of independent targets in a parallel assay to a few dozen. A (potentially) larger number of targets are possible on micro-spot arrays (e.g. DNA microarrays,<sup>5</sup> multiplexed ELISA)<sup>6</sup> where the type of target is encoded in the position of the corresponding spot. However, current multiplexed detection schemes are too complex, slow and/or expensive for routine use 'in the field'.<sup>7</sup> We show a new approach to detect multiple analytes simultaneously in a microfluidic flow cell using randomly deposited gold nanorods. Random deposition allows an inexpensive, simple and high-throughput sensor fabrication. Each nanorod responds with a spectral shift of its plasmon resonance<sup>8</sup> specifically to one target, acting effectively as a 'nano-SPR' device. Using four distinct proteins as targets, we demonstrate the feasibility of the concept, sensitivity down to nanomolar concentrations, the reactivation and reuse of the sensor over several cycles, and estimate the potential for up-scaling the concept to hundreds or thousands of targets. Our technique has the potential to simplify multiplexed detection and reduce the costs of each sensor to negligible dimensions, especially if combined with advanced nanofabrication methods like nano-stamping<sup>9</sup> or optical trapping.<sup>10</sup> Such inexpensive sensor platform could be used, for example, to discriminate influenza subtypes in a doctor's office. Even a simplified home version is imaginable provided the readout machine is produced with costs compatible to consumer budgets. Besides medical uses, applications for inexpensive multiplexed sensors are abundant, for example identifying plant diseases, monitoring food composition, quality and safety, screening for explosives, drugs or environmental hazards.

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<sup>3</sup> Cao et al., "Nanoparticles with Raman Spectroscopic Fingerprints for DNA and RNA Detection."

<sup>4</sup> Vignali, "Multiplexed particle-based flow cytometric assays."

<sup>5</sup> Schena et al., "Quantitative monitoring of gene-expression patterns with a complementary-DNA microarray."

<sup>6</sup> Mendoza et al., "High-throughput microarray-based enzyme-linked immunosorbent assay (ELISA)"

<sup>7</sup> Giljohann, Mirkin, "Drivers of biodiagnostic development."

<sup>8</sup> Famulok, Mayer, "Aptamer Modules as Sensors and Detectors"

<sup>9</sup> Nedev et al., "Optical Force Stamping Lithography"

<sup>10</sup> Celebrano et al., "Angular Trapping of Anisometric Nano-Objects in a Fluid"

## 6.2. Results and Discussion

**Sensor scheme.** To measure many analytes in small sample volumes (e.g. a drop of blood), the actual sensing elements need to be as small as possible, ultimately in the form of nanoparticles.<sup>11</sup> Designing multiplexed nanoparticle sensors poses two challenges: a cost-effective sensor fabrication strategy and the reliable translation from target recognition to a measurable signal, the target detection method. For many existing multiplexed sensors, the sensor fabrication strategy requires to deposit prefabricated nanoparticles by micro-spotting, a slow serial process resulting in expensive sensors with integration densities far below the theoretical limit. Our approach is to simply deposit nanoparticles functionalized with receptors for specific targets  $T_i$  randomly from solution disregarding the order and control of spotted arrays. By flowing in one nanoparticle batch at a time while recording the position of each deposited nanoparticle, we know the specific target for each particle. This serial process results in a 'mapped sensor' with a position encoding similar to a spotted array but without the geometric order (see Figure 6.1a). An even simpler sensor fabrication strategy is to mix all batches of nanoparticles before deposition. This dramatically simplifies sensor production for the cost of losing the position encoding. Such an 'unmapped sensor' lacks then the *a priori* ability to discriminate the targets  $T_i$  but rather reports on the presence of any of the targets (see Figure 6.1b). We believe that in many (pre-)screening applications, the precise identification of the target is not required. For example, if a patient reports flu-like symptoms, the presence of any type of influenza virus (in contrast to virus causing common colds) justifies more in-depth treatment. Such approaches are often referred to as 'chemical nose' or fingerprint sensors.<sup>12</sup>

Plasmons in noble metal nanoparticles react to protein binding by a shift of the resonance wavelength (see Figure 6.1c).<sup>13</sup> We use this simple and direct principle as the target detection method for both the mapped and unmapped sensors by monitoring individual nanoparticles by optical dark-field spectroscopy.<sup>14</sup> An advantage over fluorescence based techniques<sup>12</sup> (e.g. ELISA)<sup>6</sup> is the direct sensor response to target binding - without labeling or multi-step procedures - which enables continuous monitoring. Compared

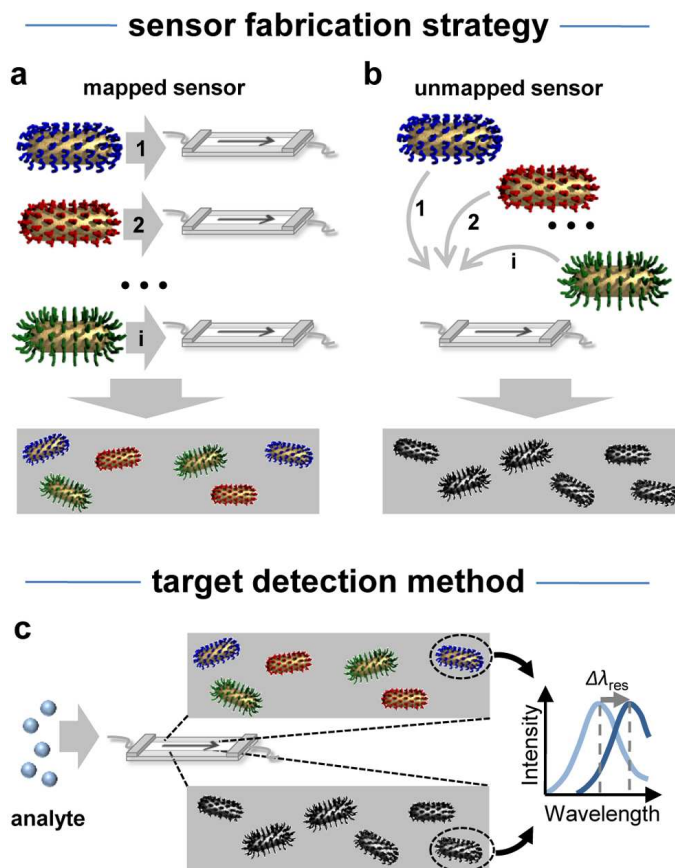
<sup>11</sup> Rosi, Mirkin, "Nanostructures in biondiagnostics."

<sup>12</sup> Saha et al., "Gold Nanoparticles in Chemical and Biological Sensing"

<sup>13</sup> Sönnichsen et al., "Spectroscopy of single metallic nanoparticles using total internal reflection microscopy."

<sup>14</sup> Raschke et al., "Biomolecular recognition based on single gold nanoparticle light scattering"

to conventional SPR or SPR imaging,<sup>15</sup> the sensing volume for plasmons in metal nanoparticles are better matched to molecular dimensions<sup>16</sup> and the maximal integration density is at least an order of magnitude higher.<sup>17</sup>



**Figure 6.1.:** **a)** To fabricate a mapped or position encoded sensor, aptamer coated nanoparticles 1...i are deposited consecutively (recording their positions after each step) in a microfluidic flow cell. **b)** An unmapped sensor is produced by mixing all particles 1...i before deposition. Hence, position encoding is not available. **c)** To detect an analyte in a solution, it is injected into the flow cell (in both cases), the targets bind specifically to their corresponding aptamer coated nanoparticles and induce shifts  $\Delta\lambda_{res}$  in their plasmon resonances.

**Single particle plasmon sensing.** The magnitude of plasmon shifts depends on particle dimensions and falls off quickly with distance.<sup>18</sup> Therefore,

<sup>15</sup> Li et al., "Fabrication and characterization of RNA aptamer microarrays for the study of protein-aptamer interactions with SPR imaging"

<sup>16</sup> Ament et al., "Single Unlabeled Protein Detection on Individual Plasmonic Nanoparticles"

<sup>17</sup> Rueemle et al., "A Localized Surface Plasmon Resonance Imaging Instrument for Multiplexed Biosensing."

<sup>18</sup> Anker et al., "Biosensing with plasmonic nanosensors."

we use small DNA aptamers as receptors (instead of commonly employed but much larger antibodies)<sup>19</sup> and couple them to gold nanorods with dimensions optimized for maximal plasmon shift (Appendix Figures D.1-D.4 for finding optimal dimensions, the surface functionalization and the estimation of aptamer load for our gold nanorods).

To record plasmon shifts for a multiplexed sensor, we improved the dark-field spectroscopy method by building a fully automated, temperature stable, user friendly and fast optical microscopy system optimized for spectral precision (Appendix Figure D.5). This system allows recording routinely the response of hundreds of particles with a precision of about 0.3 nm (corresponding to only a fraction of a percent of the plasmon linewidth or binding of about 30 proteins per particle). With a similar system, we already demonstrated the ultimate sensitivity of single protein adsorption detection.<sup>16</sup>

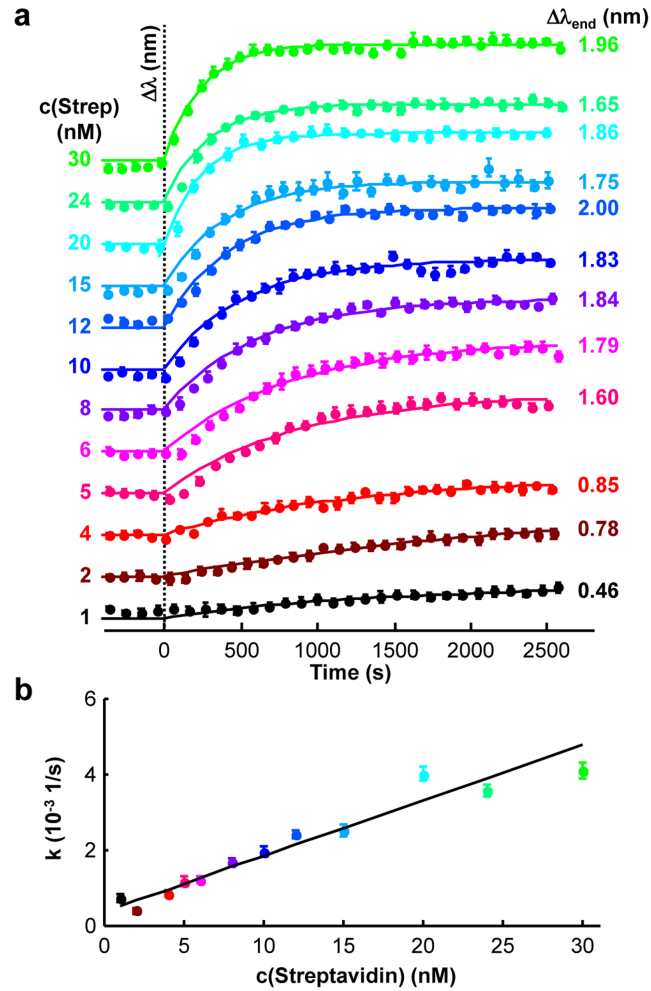
**Limit of detection.** To explore the limit of detection (LoD) of our sensor/setup, we continuously record the concentration-dependent response of particles targeting Streptavidin for 45 minutes, which is our arbitrarily chosen maximum allowable incubation time for a useful sensor (Figure 6.2a). This experiment closely resembles the well-established SPR technique. A 1 nM analyte concentration results at the end in a shift of about  $\Delta\lambda_{\text{end}} \approx 0.5$  nm, which we later set as our threshold value for successful detection. A LoD of about 1 nM is comparable to published data on multiplexed single-step sensors<sup>15,20,21</sup> and therefore most likely limited by the receptor-target affinity rather than measurement accuracy. The response  $\Delta\lambda(t)$  follows a trend according to  $\Delta\lambda(t) = \Delta\lambda_{\text{max}} (1 - e^{-kt})$ , where  $k$  shows a linear dependence on the concentration  $c$  with  $k = c k_{\text{on}} + k_{\text{off}}$ . We extract values of  $k_{\text{on}} = (0.146 \pm 0.012) \times 10^{-3} \text{ s}^{-1} \text{ nM}^{-1}$  and  $k_{\text{off}} = (0.36 \pm 0.14) \times 10^{-3} \text{ s}^{-1}$  that can be used to determine unknown concentrations (Figure 6.2b).

**Sensor regeneration.** Sensor regeneration is an important aspect of sensor design. The ability for repeated measurements is not only commercially interesting but allows much better controls, verification protocols and even to ‘train’ sensors (recording its response to known samples). Most current sensors based on antibody recognition allow only one identification step. An advantage of our DNA-aptamer and nanoparticle based detection method is

<sup>19</sup> Jayasena, “Aptamers: An emerging class of molecules that rival antibodies in diagnostics.”

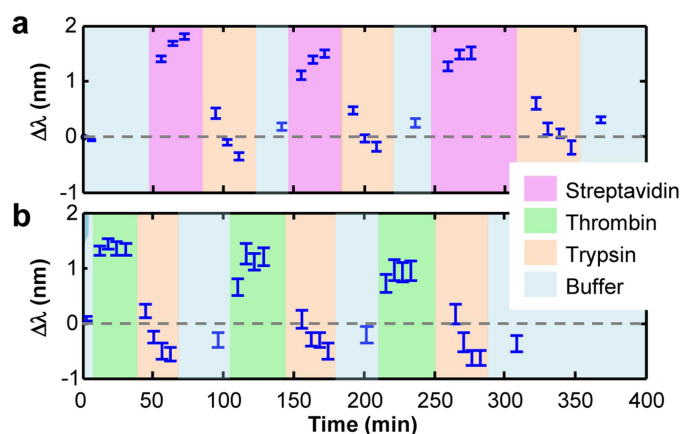
<sup>20</sup> De et al., “Sensing of proteins in human serum using conjugates of nanoparticles and green fluorescent protein.”

<sup>21</sup> Zheng et al., “Multiplexed electrical detection of cancer markers with nanowire sensor arrays.”



**Figure 6.2.:** **a)** The sensor response to different concentrations  $c$  of Streptavidin (as shown on the left) is displayed as function of time. The points show the median plasmon shift and its error (s.e.m.) for about 20 particles, the solid lines are fits to the exponential  $\Delta\lambda(t) = \Delta\lambda_{\text{max}} (1 - e^{-kt})$  with the value at 2700 s ( $\Delta\lambda_{\text{end}}$ ) indicated at the right. **b)** As function of concentration  $c$ , the parameters  $k$  follow a linear trend (black solid line).





**Figure 6.3.:** **a)** Sensor response (median and s.e.m. of 23 particles) after repeated incubation with Streptavidin (purple shaded areas), protease trypsin (orange shaded areas), and buffer (blue shaded areas). **b)** Sensor response (33 particles) after Thrombin incubation. In both cases, the target binding results in a strong plasmon shift  $\Delta\lambda$ , which is completely reversed to its original value after incubation with trypsin and rinsing with buffer.

that there are no proteins involved in the recognition, which allows us to remove protein targets by selective degradation (proteolysis). We demonstrate this regeneration concept by successfully removing the target protein with the protease Trypsin within 35 minutes (Figure 6.3 and Section 6.4: Sensor regeneration).

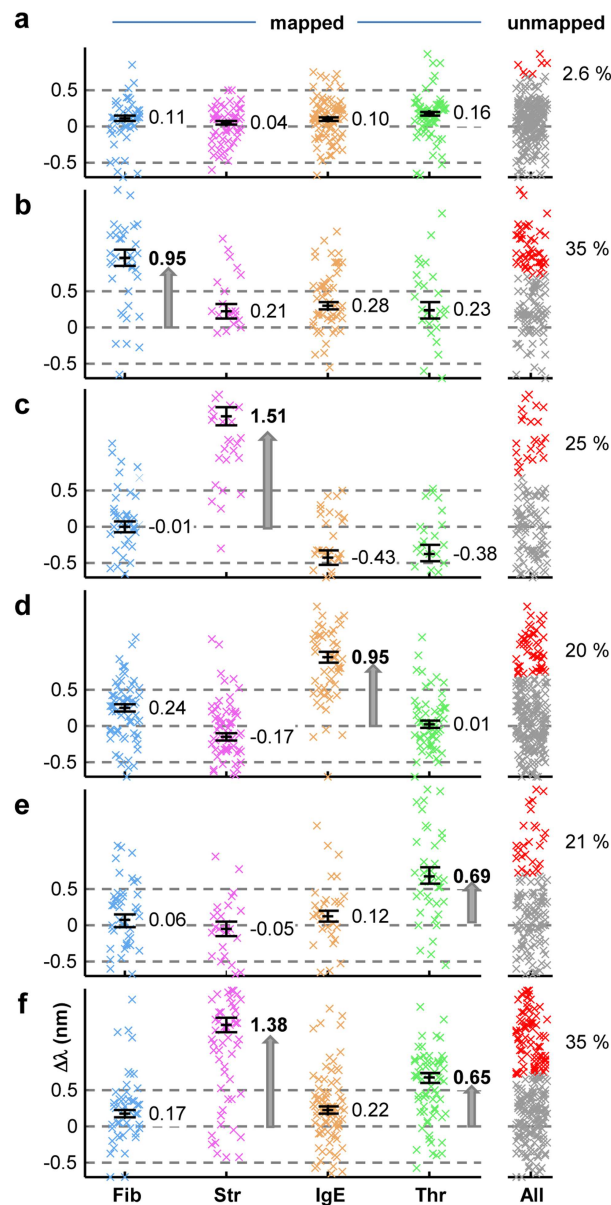
**Multiplexed detection.** To show the feasibility of multiplexed detection, we use gold nanorods with aptamers against Thrombin ( $M_W$  37 kDa), Immunoglobulin E (IgE) ( $M_W$  184 kDa), Streptavidin ( $M_W$  66 kDa), and Fibronectin ( $M_W$  440 kDa). We prepare the sensors for our experiments with the ‘mapping’ method with sequential deposition steps (Figure 6.1a) and evaluate the response both with and without the mapping information. Figure 6.4 shows the resulting response after flushing in either no target (a), one of the four target proteins (b-e) or two target proteins at the same time (f) (further data in Appendix Table D.1, Appendix Figure D.6). In all cases, an additional protein (BSA,  $M_W$  67 kDa) is present in large excess providing an unspecific ‘background’. The first thing to note is a different median shift for the four target groups originating from the different sizes of the aptamer sequences and target proteins. Furthermore, we observe a scattering of the single plasmon shifts around their median value of about  $\pm 0.5$  nm, even for the control experiment. Within this scattering, we do not see a dependency of the shift on the resonance wavelength of the particle (Appendix Figure D.7).

At our analyte concentrations, the nano-sensor surface is completely covered by proteins (Appendix Figure D.8) excluding effects arising from binding to different locations on the gold nanorods (i.e. tip and side). Hence, the variation in shift values is probably caused by a combination of measurement uncertainty, poorly functionalized particles, conformational changes, and unexpected binding events. The scattering implies the need to collect adequate statistics (i.e. recording many particle spectra) for precise and reliable sensor response evaluation.

Using the mapping information, we determine the median plasmon shift  $\Delta\lambda_i$  for each particle batch  $T_i$ . The median shift  $\Delta\lambda_i$  is clearly larger for the correct group (0.65 nm to 1.51 nm) compared to the other groups (-0.43 nm to 0.28 nm, c.f. Figure 6.4). A simple way to define a positive sensor response to the target  $T_i$  is to require the corresponding median shift  $\Delta\lambda_i$  (with its error) to be larger than an empirically chosen limit  $L$ . We find that a value of  $L = 0.5$  nm is well above the non-specific response (Appendix Figure D.9) and allows us to correctly identify any combination of the four target molecules in 'blind experiments' where both operator and data evaluator are not aware of the targets present. A mapped sensor therefore allows to identify different target molecules with one sensor chip, even if more than one type of target molecules is present, but requires the sequential preparation procedure.

Evaluating the experiments without the mapping information (effectively emulating a sensor prepared in a one-step procedure as in Figure 6.1b), we find again a significant response for each target (Figure 6.4, right column). We define a significant response simply by requiring the fraction of shifts  $p$  above an empirically set limit  $L$  ('positives') to be significantly larger than the corresponding fraction  $p_0$  without target molecules present. For our unmapped sensor, we find the optimal value for  $L = 0.7$  nm (Appendix Tables D.2-D.5 for considerations to choose  $L$ ).

**Upscaling.** Using the mapping information, we know exactly which particles should show a significant shift and which not, i.e. we are able to separate  $p$  into 'true positives' and 'false positives'  $p = p_{\text{true}} + p_{\text{false}}$ . The parameters  $p_{\text{true}}$  and  $p_{\text{false}}$  depend on the quality of the receptor (mainly the specificity of the receptor molecule) and the nanoparticle functionalization efficiency and effectiveness. We evaluated both parameters for  $L = 0.7$  nm for each batch  $T_i$  separately and find typical values for  $p_{\text{true}}$  around 60% and  $p_{\text{false}}$  around 10%, depending on the kind of target. These parameters let us calculate roughly the number of particles  $N$  needed for up-scaling our unmapped sensor to  $n$  analytes, provided there is no cross-reactivity among the receptors



**Figure 6.4:** a-f) show the plasmon shifts of 147-272 particles in a multiplexed sensor after exposure to a) no analyte (control), b) Fibronectin (Fib), c) Streptavidin (Str), d) IgE, e) Thrombin (Thr), f) Streptavidin and Thrombin. Each cross represents the response of an individual nanoparticle. The four columns on the left show the response of particles grouped according to their functionalization (23-90 particles per group) with the median and its error (s.e.m.) indicated by black symbols and numbers. Median responses above the detection limit  $L = 0.5$  nm, indicated with gray arrows, correctly identify the analytes in all cases. The column on the right shows the combined response, resembling the information from an unmapped sensor, with shifts above the significance limit  $L = 0.7$  nm shown in red. The corresponding fractions  $p$  are displayed as percentage.

and assuming Poissonian statistics. With these assumptions, the following formula must hold for significant detection of the target (Appendix Section D.11.):

$$\frac{n}{\sqrt{N}} \leq \frac{p_{\text{true}}}{\sqrt{p_{\text{false}}}} \equiv \alpha \quad (6.1)$$

Our particles show  $\alpha$  values around  $\alpha \approx 2$  (Appendix Table D.5), thus requiring roughly  $N = 10^6$  particles for  $n = 1,000$  analytes. Even with a mean particle spacing of several  $\mu\text{m}$ ,  $10^6$  particles easily fit on a sensor area of a few  $\text{mm}^2$ . However, this theoretical performance is limited by the number of suitable aptamers at present.<sup>8</sup>

The statistics behind up-scaling the mapped sensor is much simpler as each sensor group is evaluated separately. For the four investigated targets, between 3 and 58 nanoparticles are enough to ensure a significant response to the target (Appendix Table D.6). For detecting  $n = 1,000$  analytes, one needs therefore about  $N = 50,000$  particles, a factor of 20 less than for the unmapped sensor, fitting easily on an area of less than  $1 \text{ mm}^2$ . Obviously, the challenge for up-scaling a mapped sensor to 1,000 analytes lies more in the fabrication process than in the subsequent use. However, nanoparticle patterning techniques such as DNA-tiling<sup>22</sup> or optical stamping<sup>9,10</sup> could drastically simplify position encoding.

For sub-picomolar target detection, the sensitivity could be further increased with secondary antibodies or nanotags<sup>23</sup> or replacing the gold nanorods with more sensitive plasmonic geometries such as EIT structures<sup>24</sup> or fano resonances.<sup>25</sup> Analyte detection with plasmonic sensors is not restricted to a specific class of biochemical receptors, i.e. replacing aptamers with conventional antibodies<sup>14</sup> or Fab fragments could result in more specific and reliable target recognition.

## 6.3. Conclusion

The simple fabrication process of our randomly deposited nanoparticle sensors and their re-usability provide a convincing route to an inexpensive

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<sup>22</sup> Seeman, “DNA in a material world.”

<sup>23</sup> Osterfeld et al., “Multiplex protein assays based on real-time magnetic nanotag sensing.”

<sup>24</sup> Liu et al., “Plasmonic analogue of electromagnetically induced transparency at the Drude damping limit.”

<sup>25</sup> Wu et al., “Fano-resonant asymmetric metamaterials for ultrasensitive spectroscopy and identification of molecular monolayers.”

mass-produced product. Together with the extremely small required sample volume, the fast and direct response, as well as nanomolar sensitivity, such sensors could revolutionize point of care screening at doctor's offices or even at home.

## 6.4. Methods

Deionized water from a Millipore system ( $>18\text{ M}\Omega$ , Milli Q) was used in all experiments. Suppliers of chemicals are given in the Appendix Chapter D.

**Synthesis of gold nanorods.** A stock of gold nanorods (AuNRs) was synthesized according to a modified literature procedure.<sup>26,27</sup> Seeds were prepared by rapid addition of ice cold 0.01 M sodium borohydride ( $\text{NaBH}_4$ ) to a solution of 0.1 mM tetrachloroauric acid ( $\text{HAuCl}_4$ ) and 25 mM cetyltrimethylammonium bromide (CTAB) under vigorous stirring. 1 mL of this seed solution was stirred into 1000 mL of growth solution containing 0.1 M CTAB, 0.5 mM  $\text{HAuCl}_4$ , 0.6 mM ascorbic acid and 70  $\mu\text{M}$  silver nitrate ( $\text{AgNO}_3$ ). The reaction was allowed to proceed for one hour at 30°C. The nanorods were separated from the excess surfactant by centrifugation at 2,790 g for 20 min. Centrifugation was repeated twice, the supernatant was discarded each time and replaced with 0.05 M CTAB.

**Buffers.** The following 'binding buffer' was used for particle functionalization and target detection: 20 mM Tris acetate, 140 mM sodium chloride, 5 mM potassium chloride, 2.5 mM magnesium chloride, 1 mM calcium chloride. The pH was adjusted with NaOH or HCl to 7.4. For the target detection, the binding buffer was supplemented with 450  $\mu\text{g/mL}$  (6.82  $\mu\text{M}$ ) bovine serum albumin ('BSA binding buffer') to provide an unspecific protein background. For target removal, we used the following regeneration buffer: 20 mM Tris acetate, 140 mM sodium chloride, 5 mM potassium chloride, adjusted to pH = 8.0.

**Aptamer functionalization.** We extracted aptamer sequences from literature, checked their cross-reactivity by SPR (Appendix Table D.7), and found four aptamers exhibiting high specificity for Fibronectin,<sup>28</sup> Streptavidin,<sup>29</sup>

<sup>26</sup> Jana et al., "Wet chemical synthesis of high aspect ratio cylindrical gold nanorods."

<sup>27</sup> Nikoobakht, El-Sayed, "Preparation and growth mechanism of gold nanorods (NRs) using seed-mediated growth method."

<sup>28</sup> Ogawa et al., "Aptamer selection for the inhibition of cell adhesion with fibronectin as target."

<sup>29</sup> Bing et al., "Bifunctional combined aptamer for simultaneous separation and detection of

Immunoglobulin E (IgE),<sup>30</sup> and Thrombin<sup>31</sup> (Appendix Figures D.10 and D.11). In a typical procedure, 7  $\mu\text{L}$  of 500  $\mu\text{M}$  thiolated DNA aptamer solution was diluted in 43  $\mu\text{L}$  of binding buffer supplemented with 0.4% sodium dodecyl sulfate (SDS) and 5 mM tris(2-carboxyethyl)phosphine (TCEP), and incubated for 30 minutes to cleave disulfide bonds. After incubation, the solution was heated at 95°C for 10 min and allowed to cool for another 60 min. Subsequently, a 50  $\mu\text{L}$  solution of CTAB stabilized AuNRs (O.D. 400 = 0.9, [AuNR]  $\approx$  0.14 nM) was centrifuged once at 6,290 g for 5 min. The supernatant was discarded and nanoparticles were then gently dispersed in the aptamer solution and incubated at room temperature overnight. The successful functionalization of nanorods was verified by UV-Vis spectroscopy and gel electrophoresis (Appendix Figures D.2 and D.3). The aptamer functionalized AuNRs prepared by the above protocol were diluted 1:800 in binding buffer for further experiments.

**Preparation of the sensor.** We use a flow cell consisting of two cover slips held together by a piece of Nescofilm with a void area. One cover slip has two small holes for the connection of thin tubings as in- and outlet. The flow cell was cleaned by rinsing with 100  $\mu\text{L}$  of Hellmanex II special cleaning concentrate and 1 mL of water. Now, aptamer functionalized AuNRs were injected and incubated until an adequate surface density of attached particles was reached. Subsequently, unbound nanoparticles were removed by washing with binding buffer. At the end, binding buffer containing 4 mM thiolated methoxy-polyethylene glycol (mPEG, MW 3000 Da) was added for 40 min. The mPEG is supposed to fill any voids in the aptamer layer on the AuNRs. The flow cell surface is then blocked with BSA binding buffer for 60 min to avoid analyte depletion by unspecific adsorption to the glass surfaces.

**Mapping procedure.** We recorded the position of each deposited nanoparticle after rinsing with binding buffer by taking a photograph of the entire field of view under dark-field illumination and software identification of bright spots. By flowing in one nanoparticle batch at a time, we know the specific target for each particle and group their responses accordingly.

**Limit of detection.** The plasmon resonances of about 20 particles targeting Streptavidin were recorded repeatedly while exposing them to a given

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thrombin.”

<sup>30</sup> Wang et al., “Surface plasmon resonance imaging for affinity analysis of aptamer-protein interactions with PDMS microfluidic chips.”

<sup>31</sup> Tasset et al., “Oligonucleotide inhibitors of human thrombin that bind distinct epitopes.”

concentration of Streptavidin in BSA binding buffer under continuous flow. After monitoring the protein attachment, we rinsed with BSA binding buffer to remove loosely bound proteins. This showed a decrease of up to 0.2 nm in the signal, influencing the parameter  $\Delta\lambda_{\text{end}}$  but not  $k$ .

**Sensor regeneration.** The sensor was regenerated after target binding by a treatment with 20  $\mu\text{M}$  trypsin in regeneration buffer for 25 min. This treatment degraded all aptamer-bound target proteins without deactivating the aptamer. A 10 min rinse with BSA binding buffer removed the trypsin.

**Target detection measurements.** The measurements began by recording the reference plasmon resonance wavelengths of all immobilized AuNRs in BSA binding buffer. Then, a solution containing one or several target proteins was added (typical target molecule concentration was 20  $\mu\text{g/mL}$ ) in BSA binding buffer for 30 min. After washing with BSA binding buffer for 15 min, the plasmon resonance wavelengths were recorded again to calculate the shifts  $\Delta\lambda$ .



## 6.5. References

1. Peiris, J. S. M.; Yuen, K. Y.; Osterhaus, A.; Stohr, K. *New England Journal of Medicine* **2003**, *349*, 2431–2441.
2. Webby, R. J.; Webster, R. G. *Science* **2003**, *302*, 1519–1522.
3. Cao, Y. C.; Jin, R.; Mirkin, C. A. *Science* **2002**, *297*, 1536–1540.
4. Vignali, D. A. A. *Journal of Immunological Methods* **2000**, *243*, 243–255.
5. Schena, M.; Shalon, D.; Davis, R. W.; Brown, P. O. *Science* **1995**, *270*, 467–470.
6. Mendoza, L. G.; McQuary, P.; Mongan, A.; Gangadharan, R.; Brignac, S.; Eggers, M. *Biotechniques* **1999**, *27*, 778–780.
7. Giljohann, D. A.; Mirkin, C. A. *Nature* **2009**, *462*, 461–464.
8. Famulok, M.; Mayer, G. *Accounts of Chemical Research* **2011**, *44*, 1349–1358.
9. Nedev, S.; Urban, A. S.; Lutich, A. A.; Feldmann, J. *Nano Letters* **2011**, *11*, 5066–5070.
10. Celebrano, M.; Rosman, C.; Sönnichsen, C.; Krishnan, M. *Nano Letters* **2012**, *12*, 5791–5796.
11. Rosi, N. L.; Mirkin, C. A. *Chemical Reviews* **2005**, *105*, 1547–1562.
12. Saha, K.; Agasti, S. S.; Kim, C.; Li, X.; Rotello, V. M. *Chemical Reviews* **2012**, *112*, 2739–2779.
13. Sönnichsen, C.; Geier, S.; Hecker, N. E.; Plessen, G. von; Feldmann, J.; Ditlbacher, H.; Lamprecht, B.; Krenn, J. R.; Aussenegg, F. R.; Chan, V. Z. H.; Spatz, J. P.; Möller, M. *Applied Physics Letters* **2000**, *77*, 2949–2951.
14. Raschke, G.; Kowarik, S.; Franzl, T.; Sönnichsen, C.; Klar, T. A.; Feldmann, J.; Nichtl, A.; Kürzinger, K. *Nano Letters* **2003**, *3*, 935–938.
15. Li, Y.; Lee, H. J.; Corn, R. M. *Nucleic Acids Research* **2006**, *34*, 6416–6424.
16. Ament, I.; Prasad, J.; Henkel, A.; Schmachtel, S.; Sönnichsen, C. *Nano Letters* **2012**, *12*, 1092–1095.
17. Ruemmele, J. A.; Hall, W. P.; Ruvuna, L. K.; Van Duyne, R. P. *Analytical Chemistry* **2013**, *85*, 4560–4566.
18. Anker, J. N.; Hall, W. P.; Lyandres, O.; Shah, N. C.; Zhao, J.; Van Duyne, R. P. *Nature Materials* **2008**, *7*, 442–453.



19. Jayasena, S. D. *Clinical Chemistry* **1999**, *45*, 1628–1650.
20. De, M.; Rana, S.; Akpınar, H.; Miranda, O. R.; Arvizo, R. R.; Bunz, U. H. F.; Rotello, V. M. *Nature Chemistry* **2009**, *1*, 461–465.
21. Zheng, G.; Patolsky, F.; Cui, Y.; Wang, W. U.; Lieber, C. M. *Nat Biotech* **2005**, *23*, 1294–1301.
22. Seeman, N. C. *Nature* **2003**, *421*, 427–431.
23. Osterfeld, S. J.; Yu, H.; Gaster, R. S.; Caramuta, S.; Xu, L.; Han, S.-J.; Hall, D. A.; Wilson, R. J.; Sun, S.; White, R. L.; Davis, R. W.; Pourmand, N.; Wang, S. X. *Proceedings of the National Academy of Sciences* **2008**, *105*, 20637–20640.
24. Liu, N.; Langguth, L.; Weiss, T.; Kästel, J.; Fleischhauer, M.; Pfau, T.; Giessen, H. *Nature Materials* **2009**, *8*, 758–762.
25. Wu, C.; Khanikaev, A. B.; Adato, R.; Arju, N.; Yanik, A. A.; Altug, H.; Shvets, G. *Nature Materials* **2012**, *11*, 69–75.
26. Jana, N. R.; Gearheart, L.; Murphy, C. J. *Journal of Physical Chemistry B* **2001**, *105*, 4065–4067.
27. Nikoobakht, B.; El-Sayed, M. A. *Chemistry of Materials* **2003**, *15*, 1957–1962.
28. Ogawa, A.; Tomita, N.; Kikuchi, N.; Sando, S.; Aoyama, Y. *Bioorganic & Medicinal Chemistry Letters* **2004**, *14*, 4001–4004.
29. Bing, T.; Liu, X.; Cheng, X.; Cao, Z.; Shangguan, D. *Biosensors and Bioelectronics* **2010**, *25*, 1487–1492.
30. Wang, Z.; Wilkop, T.; Xu, D.; Dong, Y.; Ma, G.; Cheng, Q. *Analytical and Bioanalytical Chemistry* **2007**, *389*, 819–825.
31. Tasset, D. M.; Kubik, M. F.; Steiner, W. *Journal of Molecular Biology* **1997**, *272*, 688–698.



## 7 Chapter 7.

# Plasmonic Nanoparticles as Sensors for Simultaneous Quantification of Protein-Protein Interactions

Understanding complex biological processes results from studies on the interaction of the involved species. In this chapter, I present an advancement of the multiplexed plasmon sensor presented in Chapter 6 to assess in parallel the binding kinetics of a protein interacting with various binding partners. Therefore, nanoparticles immobilized on a surface are functionalized with the partner proteins keeping their functionality to interact with another protein. Addition of the protein of interest induces a response in the plasmon resonance of the particles, which is monitored by optical dark-field spectroscopy. As a proof-of-principle, I present the immobilization of the partner proteins on the nanoparticles while maintaining bioactivity and I present the dissociation constants of three protein systems determined in parallel from one experiment. Furthermore, I give ideas to improve the technique for high-throughput in multiplexed measurements. In this project, I established basic functionalization of the nanoparticles for attachment of His-tagged proteins and prove proper interaction with another protein. Janak Prasad refined the functionalization strategy enabling multiplexed experiments. Dr. Rubén Ahijado-Guzmán performed measurements on the repeated immobilization of His-tagged proteins on the nanoparticles and he assessed the dissociation constants in a multiplexed manner.

## 7.1. Introduction

The study of protein-protein interactions builds up a main field in understanding biological processes.<sup>1</sup> Common approaches for the determination of binding affinities investigate either the interaction of molecules free in solution being limited to the study of one interacting system at a time (e.g. Microscale Thermophoresis,<sup>2</sup> Isothermal Titration Calorimetry)<sup>3</sup> or having one binding partner immobilized on a substrate giving access to the parallel study of multiple systems due to position encoding (e.g. Quartz Crystal Microbalance,<sup>4</sup> Surface Plasmon Resonance Spectroscopy).<sup>5</sup> Recently, randomly deposited individual gold nanorods have been proven as multiplexed plasmon sensor for rapid label-free analyte detection with each nanorod acting effectively as a 'nano-SPR'.<sup>6</sup> Here, we demonstrate that the same sensor scheme of randomly deposited gold nanoparticles is capable of simultaneous quantification of protein-protein interactions. In order to sense binding, the first protein is specifically immobilized on a nanorod and binding of the second protein freely diffusing in solution is detected by a shift in plasmon resonance.<sup>7</sup> We prove immobilization of the protein keeping its functionality and using three distinct first proteins, we demonstrate the feasibility of the method for parallel assessment of binding kinetics. In order to improve the technique from proof-of-principle to a high-throughput method, several developments have to be established: preparation of the sensing elements by immobilization of the first protein in solution,<sup>8</sup> sensor production by nanofabrication methods like nano-stamping<sup>9</sup> or optical trapping,<sup>10</sup> as well as parallel acquisition of multiple plasmon spectra.<sup>11</sup> The presented technique is an alternative to SPR with the sensing volume better matched to molecular

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<sup>1</sup> Schwille, Diez, "Synthetic biology of minimal systems."

<sup>2</sup> Wienken et al., "Protein-binding assays in biological liquids using microscale thermophoresis."

<sup>3</sup> Leavitt, Freire, "Direct measurement of protein binding energetics by isothermal titration calorimetry."

<sup>4</sup> Tuantranont et al., "A review of monolithic multichannel quartz crystal microbalance: A review."

<sup>5</sup> Rich, Myszka, "Higher-throughput, label-free, real-time molecular interaction analysis."

<sup>6</sup> Rosman et al., "Multiplexed Plasmon Sensor for Rapid Label-Free Analyte Detection"

<sup>7</sup> Raschke et al., "Biomolecular recognition based on single gold nanoparticle light scattering."

<sup>8</sup> De et al., "Nickel-Ion-Mediated Control of the Stoichiometry of His-Tagged Protein/Nanoparticle Interactions"

<sup>9</sup> Nedev et al., "Optical Force Stamping Lithography"

<sup>10</sup> Celebrano et al., "Angular Trapping of Anisometric Nano-Objects in a Fluid"

<sup>11</sup> Bingham et al., "Localized Surface Plasmon Resonance Imaging: Simultaneous Single Nanoparticle Spectroscopy and Diffusional Dynamics"

dimensions than in the case of SPR.<sup>12</sup> If finally optimized, variations from experiment to experiment could potentially be minimized compared to SPR, since the overall sensor could be built up from sensing elements processed once in batch instead of chip by chip functionalization for SPR.

## 7.2. Results and Discussion

**Sensor scheme.** To measure the protein-protein interaction of multiple systems in parallel, the actual sensing elements need to be as small as possible. A small sensor unit is given by nanoparticles<sup>13</sup> and a high number of these entities was used for the multiplexed detection of proteins from an analyte solution.<sup>6</sup> In order to probe the binding of a protein to various partner proteins, these partner proteins need to be immobilized on the nanoparticles. Therefore, we randomly deposit nanoparticles with anchor points for protein immobilization from solution and add the protein. By sequentially flowing in the nanoparticles while recording the position of each deposited particle followed by one kind of protein, we can relate each first protein to a position resulting in a mapped sensor (Figure 7.1).

Protein binding induces a shift of the plasmon resonance wavelength of a noble metal nanoparticle.<sup>14</sup> Herein, the shift magnitude correlates with the extent of surface coverage and can be monitored continuously due to the direct sensor response to target binding without the need of labeling or multi-step procedures.<sup>15</sup> Compared to SPR techniques, the sensing volume for plasmons in metal nanoparticles are better matched to molecular dimensions<sup>12</sup> and the maximal integration density is at least an order of magnitude higher.<sup>16</sup>

To follow plasmon shifts due to protein binding over time, we use the optical dark-field spectroscopy setup described in Appendix Chapter D. This setup is fully automated, temperature stable, and optimized for spectral sprecision. The speed in acquisition of optical spectra allows measurement of 12 particles per minute.

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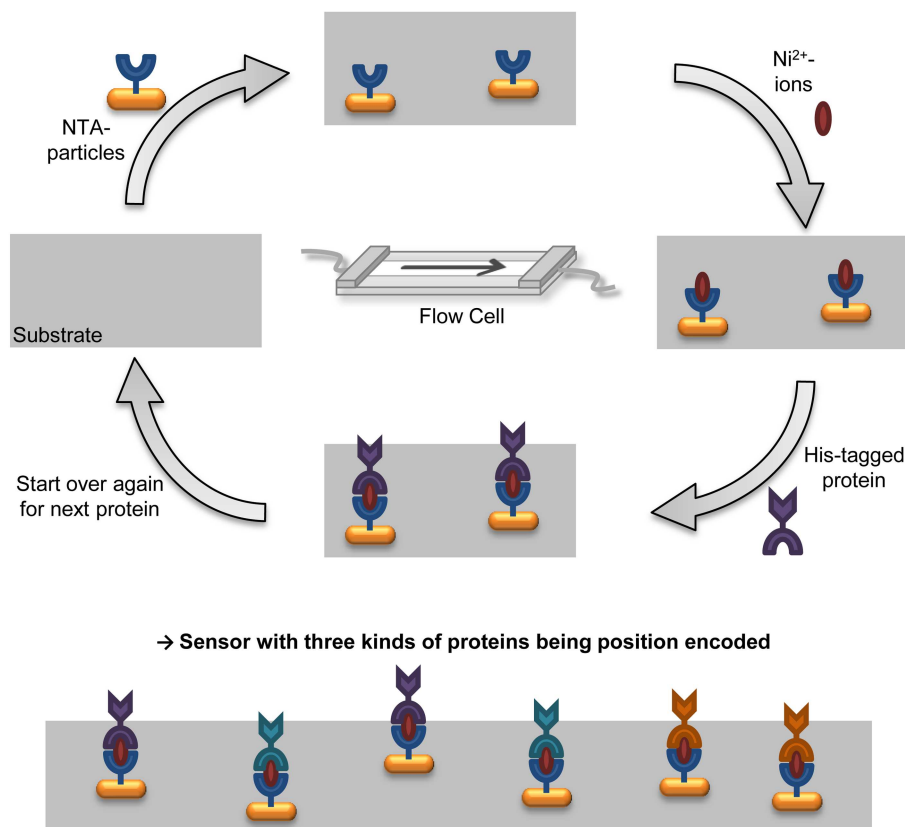
<sup>12</sup> Ament et al., "Single Unlabeled Protein Detection on Individual Plasmonic Nanoparticles"

<sup>13</sup> Rosi, Mirkin, "Nanostructures in biondiagnostics."

<sup>14</sup> Sönnichsen et al., "Spectroscopy of single metallic nanoparticles using total internal reflection microscopy."

<sup>15</sup> Saha et al., "Gold Nanoparticles in Chemical and Biological Sensing."

<sup>16</sup> Ruemmele et al., "A Localized Surface Plasmon Resonance Imaging Instrument for Multiplexed Biosensing."



**Figure 7.1.:** Fabrication of a position encoded sensor for the parallel assessment of the dissociation constants of one protein interacting with three partner proteins. NTA functionalized gold nanorods are deposited in a microfluidic flow cell while recording their positions. Afterwards, NTA is complexed with nickel ions for immobilization of a His-tagged protein, which is subsequently flushed into the flow cell. The steps of particle deposition, complexation with nickel ions, and immobilization of His-tagged protein are repeated for every partner protein.

In this study, we determine the dissociation constant  $K_D$  of FtsZ binding to two different mutants of ZipA as well as FtsZ binding to MinC. These proteins are essential components for bacterial cell division.<sup>17</sup> FtsZ is a 40 kDa soluble GTPase homolog of eukaryotic tubulin<sup>18</sup> and self-associates in the presence of GDP forming oligomers.<sup>19</sup> ZipA is a 36 kDa transmembrane protein and consists of an amino-terminal helix integrating into the bacterial

<sup>17</sup> Martos et al., "Towards a bottom-up reconstitution of bacterial cell division."

<sup>18</sup> Erickson et al., "FtsZ in Bacterial Cytokinesis: Cytoskeleton and Force Generator All in One."

<sup>19</sup> Martos et al., "Characterization of Self-Association and Heteroassociation of Bacterial Cell Division Proteins FtsZ and ZipA in Solution by Composition Gradient-Static Light Scattering"

membrane, a flexible linker, and a cytoplasmic domain, which specifically interacts with FtsZ.<sup>20</sup> The two mutants of ZipA, which we investigate, differ from the wild type protein by removing the part ranging of the N-terminal domain (amino acids 1-25 for s1ZipA, amino acids 1-188 for s2ZipA), but bioactivity is preserved (FtsZ binding by amino acids 189-328).<sup>19</sup> MinC is a 13 kDa protein and influences self-association of FtsZ,<sup>21</sup> therefore inhibiting the *in vivo* formation of the Z-ring, which is needed for cell division.

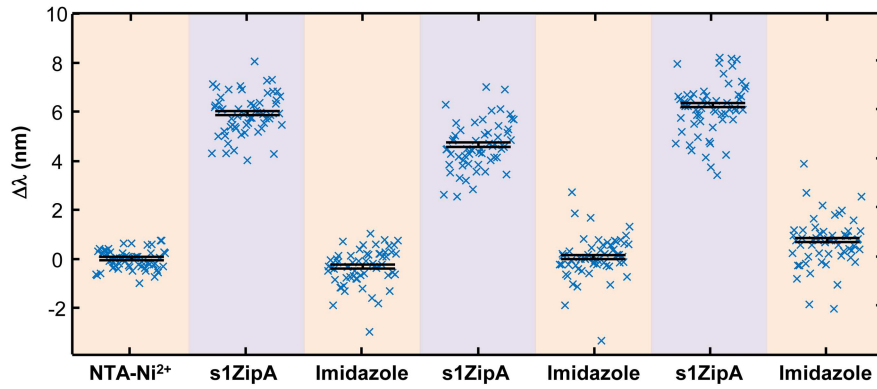
**Sensor functionality.** To immobilize a mutant of ZipA or MinC on gold nanoparticles without affecting bioactivity, we functionalize the particles with nitrilotriacetic acid (NTA) moieties and complex those with nickel ions, which act as specific anchor points for a His-tag attached to the proteins<sup>8</sup> s1ZipA, s2ZipA, and MinC. In a first approach, we performed the functionalization with NTA on particles already immobilized in the flow cell (Appendix Figure E.1), but this strategy was not suitable for a multiplexed experimental design, since a sequential attachment of binding partner proteins was not possible. For sequential deposition, we improved the functionalization to a method, where the particles are pre-functionalized in solution with NTA before immobilization in the flow cell. In order to proof proper interaction of NTA, nickel ions, and His-tagged proteins, we repeatedly bound His-tagged s1ZipA and removed it using imidazole. The results are shown in Figure 7.2 and demonstrate functionality due to complete reversibility.

Furthermore, we performed control experiments using particles of different states in the functionalization with NTA and exposed them to His-tagged s2ZipA (Appendix Figure E.2). In those experiments, we observe significant binding of His-tagged s2ZipA only in the case of NTA-particles complexed with nickel ions. This His-tagged s2ZipA bound to the particles is able to bind FtsZ underlining the intact bioactivity of the nanoparticle bound protein. Hence, we are able to immobilize a protein on the nanoparticle surface while maintaining its functionality to interact with another protein.

**Multiplexed determination of  $K_D$ .** To show the feasibility of multiplexed detection, we prepare a substrate by sequential preparation of three kinds of gold nanorods, namely one kind with s1ZipA, one kind with s2ZipA, and one kind with MinC. Then, we flow a low concentration of FtsZ (30 nM) for 30 minutes and we determine the plasmon shift of the three individual

<sup>20</sup> Ohashi et al., “Structural evidence that the P/Q domain of ZipA is an unstructured, flexible tether between the membrane and the C-terminal FtsZ-binding domain.”

<sup>21</sup> Dajkovic et al., “MinC spatially controls bacterial cytokinesis by antagonizing the scaffolding function of FtsZ.”



**Figure 7.2.:** Sensor response (single particle response displayed as blue crosses, median and s.e.m. of the 56 particles marked in black) after repeated incubation with His-tagged s1ZipA and imidazole. Reversibility of the immobilization process proves successful binding of the protein via the His-tag.

groups. Afterwards, we repeat the procedure with increasing FtsZ concentrations (100 nM, 300 nM, 1  $\mu$ M, 2  $\mu$ M, 3  $\mu$ M, 6  $\mu$ M, 10  $\mu$ M, 30  $\mu$ M, 100  $\mu$ M). From the concentration  $c$  dependent equilibrium plasmon shifts  $\Delta\lambda_{eq}$  with the maximum value  $\Delta\lambda_{max}$ , the overall dissociation constant  $K_D$  (ratio of dissociation rate to association rate) can be deduced according to the Langmuir isotherm:

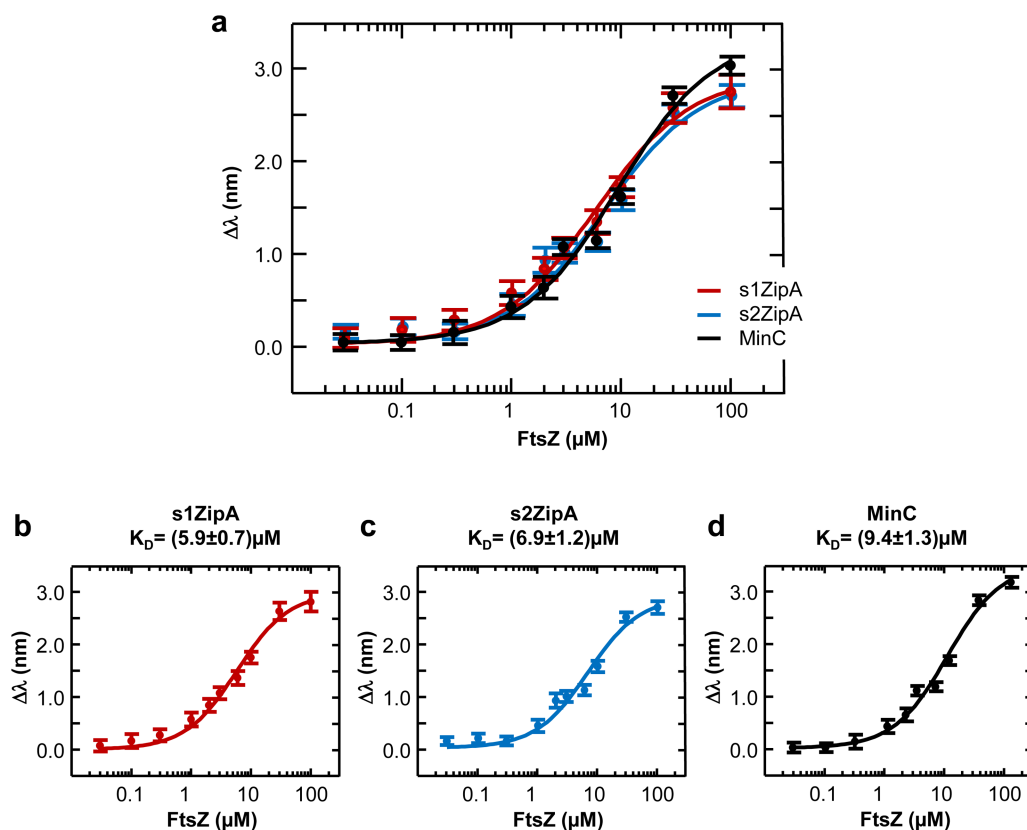
$$\Delta\lambda_{eq} = \Delta\lambda_{max} \frac{c}{K_D + c} \quad (7.1)$$

Figure 7.3 shows the equilibrium shifts depending on the various FtsZ concentrations for the three investigated binding partners and fits according to the Langmuir equation. We obtain the following  $K_D$  values: FtsZ-s1ZipA  $K_D = (5.9 \pm 0.7) \mu$ M, FtsZ-s2ZipA  $K_D = (6.9 \pm 1.2) \mu$ M and FtsZ-MinC  $K_D = (9.4 \pm 1.3) \mu$ M. These values are in agreement with previous reports.<sup>19,22</sup> Therefore, our sensor consisting of single gold nanorods with various proteins on top has successfully proven to detect the dissociation constant with a protein of interest in a multiplexed manner.

**Room for improvements.** To assess the binding of a protein of interest to a high number of partner proteins in parallel, there are three main fields, which need to be improved: the particle preparation, the sensor fabrication, and the detection of the plasmon response. In the particle preparation, binding of the partner proteins to the particles in solution would simplify the method, since it avoids sequential preparation steps by complexation with

<sup>22</sup> Hernandez-Rocamora et al., “Dynamic Interaction of the Escherichia coli Cell Division ZipA and FtsZ Proteins Evidenced in Nanodiscs.”





**Figure 7.3.:** Equilibrium plasmon shifts (median and s.e.m. of 24-36 particles) of a multiplexed sensor consisting of s1ZipA, s2ZipA, and MinC exposed to increasing concentrations of FtsZ. Fitting a Langmuir isotherm (straight line) in the case of each partner protein yields the corresponding dissociation constants. **a)** Results of the three systems displayed all together. **b), c), d)** The results of each system displayed separately for clarity.

nickel ions and immobilization of the proteins. Therefore, we are adapting a protocol recently presented by De *et al.*<sup>8</sup> In the sensor fabrication, we currently need to deposit one protein on the particles after the other, so that we can map the particle positions for encoding of the different studied systems. If we could reliably prepare the sensor without monitoring the particle positions during deposition, we would speed up the experiment. Hence, techniques for automated particle placement like nano-stamping<sup>9</sup> or optical trapping<sup>10</sup> would be desirable. Finally, acquisition of single particle scattering spectra to determine the plasmon response of each particle is time consuming, since every particle has to be measured individually at the moment (details in Appendix Chapter D: Setup Improvements). A method to assess scattering spectra of all particles within the field of view at the

same time was presented by Bingham *et al.* in 2009,<sup>11</sup> which we call spectral imaging. Herein, the sample is illuminated with white light through a dark-field condenser. Scattered light collected by the objective is sent through a liquid crystal tunable filter with a continuously adjustable transmission having a bandwidth of 7 nm. Tuning the bandwidth across the visible range of white light and detecting the scattering intensity for every bandwidth yield the information to extract the plasmon spectrum of each particle. Hence, hundreds of single particle scattering spectra are assessable within seconds of tuning the filter, whereas our current particle positioning technique allows measurement of 12 particles per minute. Furthermore, due to the higher time resolution between measurements of spectral imaging compared to particle positioning, the rate constants of association and dissociation ( $k_{\text{on}}$  and  $k_{\text{off}}$ ) could be determined from the adsorption curves resulting from binding of the protein of interest to the various partner proteins (Chapter 2). These rates characterize the system in more detail than the overall dissociation constant, since different combinations of association and dissociation rates result in the same constant.<sup>23</sup> Currently, Dr. Arpad Jakab is building up a setup using a liquid crystal tunable filter for high-throughput assessment of single particle scattering spectra with spectral imaging.

### 7.3. Conclusion

The small size of metal nanoparticles together with the direct plasmon response as read-out signal provide a sensor platform for parallel investigation of the binding kinetics of one protein to a high number of partner proteins within one experiment. At the moment, the status of the technique is a proof-of-principle with the parallel assessment of the dissociation constants of three systems. To push the technique forward to high throughput with a high number of systems under parallel investigation, the particle preparation and sensor fabrication needs to be simplified. Furthermore, improvements in the detection of the plasmon response would raise the time resolution to follow the binding process, which is needed for determination of the association rates and the dissociation rates of the systems. Finally, the presented technique could be an alternative to SPR with the sensing volume better matched to molecular dimensions.

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<sup>23</sup> Önell, Andersson, “Kinetic determinations of molecular interactions using Biacore - minimum data requirements for efficient experimental design.”

## 7.4. Methods

Deionized water from a Millipore system (>18 M $\Omega$ , Milli Q) was used in all experiments. Suppliers of chemicals are given in the Appendix Chapter E.

**Working buffer.** The working buffer consists of 50 mM trizma hydrochloride, 100 mM potassium chloride, and 5 mM magnesium chloride with an adjusted pH of 7.5. Before usage, the buffer was filtered through a syringe filter with a pore size of 20 nm.

**Protein purification.** *Escherichia coli* wild-type FtsZ was expressed and purified by the calcium-induced precipitation method.<sup>24</sup> The mutagenesis of the ZipA gene as well as overexpression and purification of the His-tagged ZipA mutants were carried out by removing the part ranging of the N-terminal domain of wild type ZipA (amino acids 1-25 for s1ZipA, and amino acids 1-188 in the case of s2ZipA).<sup>19</sup> His-MinC was expressed and purified as previously described by Loose *et al.*<sup>25</sup> All proteins were dialyzed in working buffer and stored at -80°C in 20  $\mu$ L aliquots.

**Synthesis of gold nanorods.** See Section 6.4.

**Functionalization of gold nanorods with NTA.** 300  $\mu$ L of gold nanorods were centrifuged at 6,220 g for 5 minutes. The supernatant was discarded and the particles were resuspended in 300  $\mu$ L of 0.3 % sodium dodecyl sulfate (SDS). The suspension was sonicated for 3.5 hours in order to replace the stabilizer cetyltrimethylammonium bromide (CTAB) on the nanoparticles by SDS. Afterwards, particles were centrifuged at 6,220 g for 5 minutes and the supernatant was discarded. Particles were resuspended in 300  $\mu$ L of an aqueous solution containing 6 mM 3,3'-dithiobis[N-(5-amino-5-carboxypentyl)propionamide-N,N'-diacetic acid] dihydrochloride (C2-NTA) and 1 mM tris(2-carboxyethyl)phosphine (TCEP) with pH adjusted to 7. In this solution, particles keeping colloidal stability can be stored for several months. Absorbance spectra and gel electrophoresis of particles in different states in the functionalization are shown in Appendix Figures E.3 and E.4.

**Preparation of the sensor.** We use a flow cell consisting of two cover slips held together by a piece of Nescofilm with a void area. One cover slip has two small holes for the connection of thin tubings as in- and outlet. The flow cell was cleaned by rinsing with 100  $\mu$ L of Hellmanex II special cleaning

<sup>24</sup> Rivas *et al.*, "Magnesium-induced linear self-association of the FtsZ bacterial cell division protein monomer - The primary steps for FtsZ assembly."

<sup>25</sup> Loose *et al.*, "Min protein patterns emerge from rapid rebinding and membrane interaction of MinE."

concentrate and 1 mL of water. Now, NTA functionalized nanorods were injected and incubated until an adequate surface density of attached particles was reached. Unbound nanoparticles were removed using working buffer. Flushing an aqueous solution of 5 mM nickel chloride for 5 minutes and afterwards a 1  $\mu$ M solution of a His-tagged protein in working buffer resulted in immobilization of the protein on the nanoparticles. This concentration is enough to saturate the particle surface with the His-tagged protein. Deposition of particles, complexation with nickel ions and immobilization of the protein was repeated for each protein species.

**Removal of His-tagged protein.** For release of His-tagged s1ZipA from the particles, we flushed 600 mM imidazole in working buffer for 5 minutes. Excess of imidazole was removed by flushing with working buffer for 15 minutes.

**Measurements for dissociation constant.** We prepared a multiplexed sensor with particles having s1ZipA, s2ZipA, and MinC. Particles were exposed to increasing concentrations of FtsZ (100 nM, 300 nM, 1  $\mu$ M, 2  $\mu$ M, 3  $\mu$ M, 6  $\mu$ M, 10  $\mu$ M, 30  $\mu$ M, 100  $\mu$ M) with a flow rate of 30  $\mu$ L per minute for 30 minutes each and the plasmon response for each concentration was measured. From the equilibrium plasmon shifts depending on the FtsZ concentrations, we determined the dissociation constants for FtsZ and the three partner proteins according to Langmuir adsorption.

## 7.5. References

1. Schwille, P.; Diez, S. *Critical Reviews in Biochemistry and Molecular Biology* **2009**, *44*, 223–242.
2. Wienken, C. J.; Baaske, P.; Rothbauer, U.; Braun, D.; Duhr, S. *Nature Communications* **2010**, *1*.
3. Leavitt, S.; Freire, E. *Current Opinion in Structural Biology* **2001**, *11*, 560–566.
4. Tuantranont, A.; Wisitsora-at, A.; Sritongkham, P.; Jaruwongrungrunsee, K. *Analytica Chimica Acta* **2011**, *687*, 114–128.
5. Rich, R. L.; Myszk, D. G. *Analytical Biochemistry* **2007**, *361*, 1–6.
6. Rosman, C.; Prasad, J.; Neiser, A.; Henkel, A.; Edgar, J.; Sönnichsen, C. *Nano Letters* **2013**, *13*, 3243–3247.
7. Raschke, G.; Kowarik, S.; Franzl, T.; Sönnichsen, C.; Klar, T. A.; Feldmann, J.; Nichtl, A.; Kürzinger, K. *Nano Letters* **2003**, *3*, 935–938.
8. De, M.; Rana, S.; Rotello, V. M. *Macromolecular Bioscience* **2009**, *9*, 174–178.
9. Nedev, S.; Urban, A. S.; Lutich, A. A.; Feldmann, J. *Nano Letters* **2011**, *11*, 5066–5070.
10. Celebrano, M.; Rosman, C.; Sönnichsen, C.; Krishnan, M. *Nano Letters* **2012**, *12*, 5791–5796.
11. Bingham, J. M.; Willets, K. A.; Shah, N. C.; Andrews, D. Q.; Van Duyne, R. P. *Journal of Physical Chemistry C* **2009**, *113*, 16839–16842.
12. Ament, I.; Prasad, J.; Henkel, A.; Schmachtel, S.; Soennichsen, C. *Nano Letters* **2012**, *12*, 1092–1095.
13. Rosi, N. L.; Mirkin, C. A. *Chemical Reviews* **2005**, *105*, 1547–1562.
14. Sönnichsen, C.; Geier, S.; Hecker, N. E.; Plessen, G. von; Feldmann, J.; Ditlbacher, H.; Lamprecht, B.; Krenn, J. R.; Aussenegg, F. R.; Chan, V. Z. H.; Spatz, J. P.; Möller, M. *Applied Physics Letters* **2000**, *77*, 2949–2951.
15. Saha, K.; Agasti, S. S.; Kim, C.; Li, X.; Rotello, V. M. *Chemical Reviews* **2012**, *112*, 2739–2779.
16. Ruemmele, J. A.; Hall, W. P.; Ruvuna, L. K.; Van Duyne, R. P. *Analytical Chemistry* **2013**, *85*, 4560–4566.
17. Martos, A.; Jimenez, M.; Rivas, G.; Schwille, P. *Trends in Cell Biology* **2012**, *22*, 634–643.

18. Erickson, H. P.; Anderson, D. E.; Osawa, M. *Microbiology and Molecular Biology Reviews* **2010**, *74*, 504–528.
19. Martos, A.; Alfonso, C.; Lopez-Navajas, P.; Ahijado-Guzman, R.; Mingorance, J.; Minton, A. P.; Rivas, G. *Biochemistry* **2010**, *49*, 10780–10787.
20. Ohashi, T.; Hale, C. A.; De Boer, P. A. J.; Erickson, H. P. *Journal of Bacteriology* **2002**, *184*, 4313–4315.
21. Dajkovic, A.; Lan, G.; Sun, S. X.; Wirtz, D.; Lutkenhaus, J. *Current Biology* **2008**, *18*, 235–244.
22. Hernandez-Rocamora, V. M.; Reija, B.; Garcia, C.; Natale, P.; Alfonso, C.; Minton, A. P.; Zorrilla, S.; Rivas, G.; Vicente, M. *Journal of Biological Chemistry* **2012**, *287*, 30097–30104.
23. Önell, A.; Andersson, K. *Journal of Molecular Recognition* **2005**, *18*, 307–317.
24. Rivas, G.; Lopez, A.; Mingorance, J.; Ferrandiz, M. J.; Zorrilla, S.; Minton, A. P.; Vicente, M.; Andreu, J. M. *Journal of Biological Chemistry* **2000**, *275*, 11740–11749.
25. Loose, M.; Fischer-Friedrich, E.; Herold, C.; Kruse, K.; Schwille, P. *Nature Structural & Molecular Biology* **2011**, *18*, 577–U195.

# 8

## Single Particle Plasmon Sensors Reveal Constant Fraction of MinD Bound to the Membrane during Min Wave Propagation

The observation of label-free constituents in real-time gives access to the unbiased characterization of protein systems. In this chapter, I present single gold nanoparticles being efficient sensors for the dynamics of native proteins. Herein, the read-out signal is the response of the sensor to changes in the local environment by a spectral shift in the plasmon resonance. Due to the small size of the sensors, local oscillations in the attachment of the reversible, dynamic Min system to a lipid bilayer can be detected. Measurements on the native system and on the system having a fraction of the proteins fluorescently labeled show an impact of the fluorescence label on the dynamics of the system. Since the particles sense the total amount of protein associated with the lipid bilayer, a constant fraction of protein associated with the dynamic system was detected. This constantly bound material is identified as one protein species of the Min system. For this project, I had the idea and I initiated the cooperation. Furthermore, I performed the single particle measurements. Dr. Andreas Henkel developed the setup and conducted calculations. Dr. Ariadna Martos expressed, purified, and characterized the proteins and performed the fluorescence measurements.

## 8.1. Introduction

The Min system is a spatial regulator of the cytokinesis in *Escherichia coli* (E.coli) that cooperates with the nucleoid occlusion to block the division assembly elsewhere than at mid-cell.<sup>1,2</sup> The so called Min system, comprises three proteins - MinC, MinD, and MinE. Upon ATP hydrolysis, self-organization processes and binding/unbinding events on the membrane, the system oscillates from one pole of the cell to the other.<sup>3,4</sup> As a result of this dynamic behavior, the concentration of the inhibitor of FtsZ assembly - MinC -, exhibits a time-average local minimum at mid-cell thus targeting the formation of the Z-ring there.<sup>5</sup> Following a minimal bottom-up approach, the dynamic behaviour of Min proteins has been successfully reconstructed in vitro on artificial bilayers (SLBs) mimicking the inner membrane of E.coli.<sup>6</sup> Upon addition of ATP, the Min proteins assemble as spatiotemporal patterns able to propagate across the membrane surface. Approached by single molecule techniques, these studies have rendered relevant mechanistic information; however, the detailed molecular mechanism of the protein assembly has not been fully understood yet<sup>7</sup> and current knowledge originates from biochemical assays, which cannot follow the dynamics,<sup>8,9,10,11</sup> as well as fluorescence microscopy visualizing dye labeled proteins during oscillation.<sup>6,12,13</sup> Here, we sense the assembly and disassembly of native proteins in vitro

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<sup>1</sup> De Boer et al., "A Division Inhibitor and a Topological Specificity Factor Coded for by the Minicell Locus Determine Proper Placement of the Division Septum in Escherichia-Coli."

<sup>2</sup> Rothfield et al., "Spatial control of bacterial division-site placement."

<sup>3</sup> Raskin, deBoer, "Rapid pole-to-pole oscillation of a protein required for directing division to the middle of Escherichia coli"

<sup>4</sup> Hu, Lutkenhaus, "Topological regulation of cell division in Escherichia coli involves rapid pole to pole oscillation of the division inhibitor MinC under the control of MinD and MinE"

<sup>5</sup> Lutkenhaus, "Assembly dynamics of the bacterial MinCDE system and spatial regulation of the Z ring."

<sup>6</sup> Loose et al., "Spatial regulators for bacterial cell division self-organize into surface waves in vitro"

<sup>7</sup> Loose et al., "Protein Self-Organization: Lessons from the Min System"

<sup>8</sup> De Boer et al., "The MinD Protein is a Membrane ATPase Required for the Correct Placement of the Escherichia-Coli Division Site."

<sup>9</sup> Hu, Lutkenhaus, "Topological regulation of cell division in E-coli: Spatiotemporal oscillation of MinD requires stimulation of its ATPase by MinE and phospholipid."

<sup>10</sup> Lackner et al., "ATP-dependent interactions between Escherichia coli Min proteins and the phospholipid membrane in vitro."

<sup>11</sup> Szeto et al., "Membrane localization of MinD is mediated by a C-terminal motif that is conserved across eubacteria, archaea, and chloroplasts."

<sup>12</sup> Loose et al., "Min protein patterns emerge from rapid rebinding and membrane interaction of MinE"

<sup>13</sup> Schweizer et al., "Geometry sensing by self-organized protein patterns"



by single plasmonic nanoparticles. Comparing the oscillation behavior of systems having labeled and having only native proteins, we observe the same process but with a different speed pointing to an influence on the dynamics by fluorescence labeling. Furthermore, our study identifies that the dynamics of the Min system do not include complete protein removal from the bilayer. We found a high fraction of MinD present on the bilayer not taking part in one oscillation cycle, which has been shown for the complete bacterium<sup>14</sup> but was not considered from *in vitro* studies so far. Furthermore, this majority of MinD is completely constant bound to the bilayer, since it cannot be actively removed, suggesting an oscillation of MinD and MinE on top of a MinD covered lipid bilayer. Thus, a restructuring of the protein to leave the bilayer after attachment seems to be unlikely.

## 8.2. Results and Discussion

The basic interaction behavior of the Min proteins among each other and with lipids was identified by biochemical assays: in the presence of ATP, MinD probably dimerizes and exposes a membrane targeting sequence as an anchor to bind to a lipid bilayer.<sup>15</sup> MinD by itself has a low ATPase activity and does not leave the membrane. Binding of MinE to MinD on a lipid bilayer raises the ATPase activity<sup>16</sup> and the proteins are thought to get released into solution.<sup>12</sup> The third protein of the Min system, MinC, is passed by just as a passenger and it is not essential for the creation of an oscillation pattern.<sup>3,4</sup> The protein dynamics can be visualized *in vitro* by fluorescence microscopy. These experiments gave recently information on protein ratios and the protein diffusion behavior at different positions within the wave.<sup>12</sup> However, the studies focused on changes in the surface coverage and not the overall protein density on top of the lipid bilayer. Total surface coverages can be easily determined by surface plasmon resonant spectroscopy (SPR), which shows a change in the plasmon resonance of a thin gold film by increasing the refractive index on top due to analyte binding. The gold films have an active area of several mm<sup>2</sup>, where changes in the

<sup>14</sup> Meacci, Kruse, "Min-oscillations in *Escherichia coli* induced by interactions of membrane-bound proteins"

<sup>15</sup> Wu et al., "Determination of the structure of the MinD-ATP complex reveals the orientation of MinD on the membrane and the relative location of the binding sites for MinE and MinC"

<sup>16</sup> Hu et al., "Dynamic assembly of MinD on phospholipid vesicles regulated by ATP and MinE."

surface coverage due to the Min system showing local oscillations on a  $\mu\text{m}$  scale cancel out. In contrast, single gold nanoparticles are plasmonic sensors for the local environment. Herein, each nanoparticle acts effectively as a 'nano-SPR' with a sensing volume better matching molecular dimensions than SPR.<sup>17</sup> In this study, we form a supported bilayer on top of the gold nanorods previously immobilized in a microfluidic flow cell consisting of two coverglasses. Upon the incubation of MinD and MinE in the presence of ATP, we used the plasmon resonance as reporter for changes in the layer thickness.

**Optical Dark-field spectroscopy on Min system.** In order to monitor the propagation of the Min system on supported membranes, we perform optical dark-field spectroscopy using the setup described in Appendix Chapter D. Herein, the resonance wavelength  $\lambda_{\text{res}}$  of a position fixed gold nanorod is determined from its single particle scattering spectrum. This resonance wavelength shifts to longer wavelength by attachment of a protein ( $\Delta\lambda_{\text{res}}$ , Figure 2.6) due to the higher refractive index of the protein compared to the buffer environment.<sup>18</sup> This shift reverses by detachment of the protein ( $-\Delta\lambda_{\text{res}}$ , Figure 2.6). Hence, the particle reports directly the layer thickness at its specific position by the plasmon response. Since the sensing distance of a particle is limited correlating with its size,<sup>19</sup> we determined the optimal particle dimensions for the Min system, which adds on top of the particle 5 nm by the lipid bilayer, 4.4 nm by MinD,<sup>15</sup> and 2.6 nm by MinE<sup>20</sup> (protein sizes from crystal structure). Therefore, we use rods having an average size of 31 nm by 68 nm (Appendix Figure F.1).

These gold nanorods were first immobilized on the surface of a flow cell (Figure 2.10), and the whole surface was covered afterwards with an artificial lipid bilayer mimicking the inner membrane from *E.coli* (Appendix Figure F.2). In the presence of ATP and upon incubation of the proteins at physiological ratios (see Methods section), the Min proteins self-organize on the membrane leading to periodic travelling patterns. When these samples are observed by confocal microscopy - using MinE-Alexa488 as the tracer -, the dynamic pattern is visible as a travelling wave, where the fluorescence intensity correlates with the protein-density distribution within the wave. No

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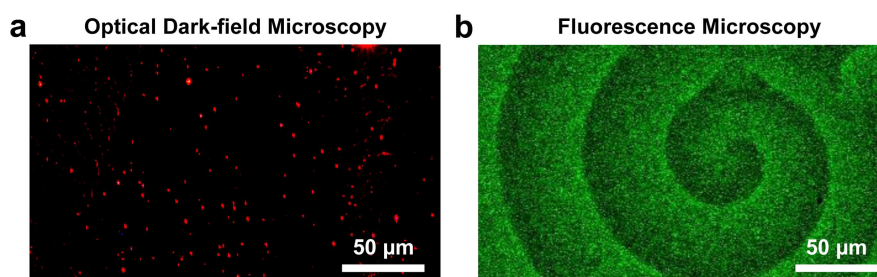
<sup>17</sup> Ament et al., "Single Unlabeled Protein Detection on Individual Plasmonic Nanoparticles."

<sup>18</sup> Sönnichsen et al., "Spectroscopy of single metallic nanoparticles using total internal reflection microscopy."

<sup>19</sup> Anker et al., "Biosensing with plasmonic nanosensors."

<sup>20</sup> Kang et al., "Crystal structure of *Helicobacter pylori* MinE, a cell division topological specificity factor."

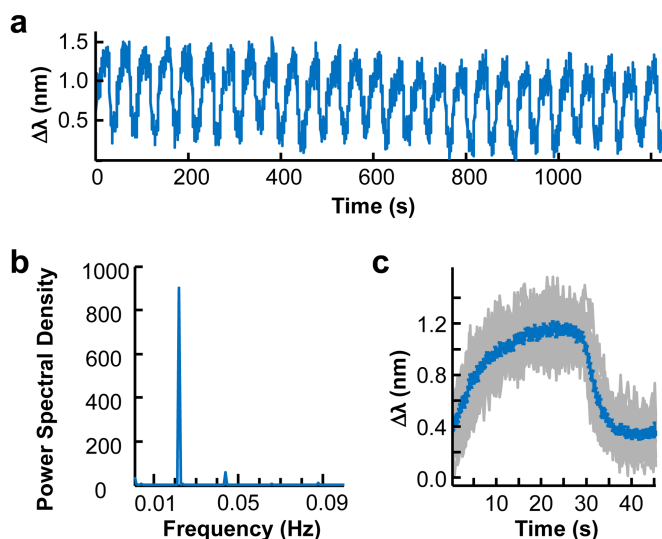
differences were observed in the behavior of these dynamic protein patterns regarding those previously reconstructed on simple supported bilayers.<sup>6</sup> Figure 8.1a shows a color image of the sample in dark-field illumination with single gold nanorods appearing as red dots and Figure 8.1b displays a confocal fluorescence microscopy image of the protein oscillation with 40% of MinE labeled with Alexa-488.



**Figure 8.1.:** a) Optical dark-field microscopy image with single gold nanorods appearing as red dots. b) Fluorescence microscopy image of the protein wave having 40% of MinE labeled with Alexa-488. Areas of high intensity show a maximum of proteins present on the bilayer, areas of low intensity a minimum, respectively.

**Label-free and non-invasive sensing.** Figure 8.2a shows the development of the plasmon shift of one single particle over time in the presence of native MinD and MinE without fluorescence labeling. Consistently, the shift increases and decreases periodically depicting the varying protein amount associated with the lipid bilayer at one fixed position as the wave passes by. The overall signal and amplitude (difference of peak and trough in oscillation) is regular and stable without drift (the timetrace shows raw data without any corrections). The power spectral density exhibits a sharp peak at a frequency of 22 mHz (i.e. period between to similar states in layer coverage of 45.5 s) underlining the high periodicity of the system (Figure 8.2b). With this periodicity, we divide the timetrace in sections starting from the first minimum, so that we end up with 26 single oscillation cycles characterizing the average oscillation behavior (Figure 8.2c). The process starts with a steep increase in resonance wavelength, which levels off and finally decreases abruptly. Within the process, we follow the overall change in protein coverage on top of the particle without the ability to discriminate the species and their particular extent inducing the signal. Therefore, we compare this profile with the concentration distributions obtained by fluorescence measurements. To obtain the relative and overall contributions of the two different proteins to the overall profile, we performed the self-organization assays using MinD-

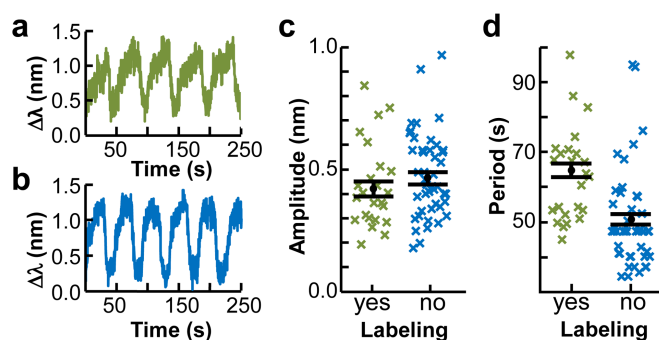
Cy3 and/or MinE-Cy3, both exhibiting the same degree of labeling (mol fluorophore/mol protein). The resulting overall fluorescence intensity profile - obtained when MinD-Cy3 and MinE-Cy3 are incubated together - is in good agreement with the one observed in the single particle measurements (Appendix Figure F.3).



**Figure 8.2.:** a) The change in  $\Delta\lambda$  of a position fixed rod over time reports the extent of protein coverage on top of the rod as the protein wave passes by. b) The power spectral density shows a pronounced characteristic frequency underlining the periodicity of the oscillation. The wave frequency is 0.022 Hz (period 45.5 s). c) With the periodicity determined in b), we divide the timetrace in sections starting from the first minimum to get the average oscillation behavior. The 26 single oscillation cycles are presented in grey. Median and s.e.m. of the 26 cycles are presented in blue.

**Impact of labeling on period.** In order to investigate, whether protein labeling has an influence on the oscillation behavior, we compared the amplitude and period of the plasmon shift gained from experiments having 40% of MinE labeled with Alexa-488 and having no labeled protein at all. Figure 8.3 shows characteristic timetraces and the results. In case of the amplitude, we obtain the same results using labeled or non-labeled protein. Since the particle size has an impact on the sensing ability, which therefor could influence the amplitude, we evaluated the amplitude with respect to the particle diameter. However, the investigated particles show no trend, which we attribute to the limited range of sizes investigated (Appendix Figure F.4). From the similar amplitude found for the labeled and non-labeled system, we conclude that addition of a fluorescence label does not influence

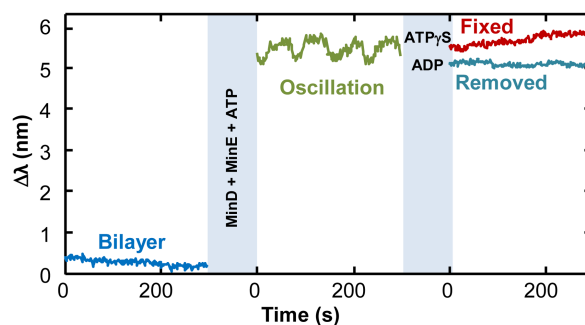
the protein coverage on top of the lipid bilayer. In contrast, we observe a significant difference for the period: the Min system with 40% of MinE labeled shows a period, which is about 20% longer than for the Min system with native proteins. Hence, labeling slows down the dynamics of the system. However, Loose *et al.* determined a similar ATPase activity for the labeled and non-labeled protein,<sup>6</sup> so that a change in the ATPase activity can be excluded. Therefore, parameters building up the dynamic equations in a reaction-diffusion model<sup>13</sup> (concentrations, attachment/detachment rates, and diffusion constants) need to be different for the labeled and non-labeled protein. Most obvious among the parameters, diffusion constants involving MinE get lowered by the label due to an increase of the hydrodynamic radius of the protein. The slowed dynamics of the Min system having labeled proteins, which is found by single particle dark-field spectroscopy, underlines the need of label-free techniques to identify properly the behavior of native proteins.



**Figure 8.3.:** Impact on oscillation behavior using fluorescently labeled MinE. **a)** Timetrace of Min oscillation using 40% of MinE labeled with Alexa-488. **b)** Timetrace of Min oscillation using solely native MinE. **c)** The amplitude of the oscillation (average  $\Delta\lambda$ ) using labeled and native proteins. We observe a similar median amplitude. Hence, there is no effect on the overall oscillation process/behavior adding a fluorescence dye to the protein. **d)** The wave period of the oscillation extracted from the maximum in the power spectral density using labeled and native proteins. Adding a fluorescence dye slows down the dynamics of the system, which is characteristic for diffusion controlled processes. In this case, having 40% of MinE labeled increases the period by about 20%. (Median and s.e.m from 24 particles using labeled MinE and from 45 particles using native MinE)

**Constant layer MinD.** We performed experiments following the resonance shift of one particle for different protein coverage situations on top of the lipid bilayer (Figure 8.4). Addition of the Min system to the bilayer shifts the

resonance wavelength by  $\sim 5$  nm (statistics shown in Appendix Figure F.5). The oscillation shows an amplitude of  $\sim 1$  nm, in which the state of a completely empty bilayer coverage is not reached and the majority of material does not participate within one oscillation cycle. Fixing the protein coverage on top of the bilayer by addition of the non-hydrolyzable ATP analogon  $\text{ATP}\gamma\text{S}$  equals the maximum in the oscillation (we made an offset correction for  $\text{ATP}\gamma\text{S}$  intercalating into the bilayer, Appendix Figure F.6). Therefore, the maximum in the oscillation resembles full coverage of the lipid bilayer. In an experiment contrary to fixation of the proteins, we tried to remove the proteins by flushing buffer and ADP solution. Herein, the signal did only drop down to the minimum in one oscillation cycle instead of reverting back to the empty bilayer (statistics shown in Appendix Figure F.6b). Hence, material not taking part in the oscillation is tightly attached to the bilayer and is considered to remain constantly bound.



**Figure 8.4.:** Adding the Min system to the lipid bilayer increases  $\lambda_{\text{res}}$ . In one oscillation cycle, the signal does not go down to the state of a pure lipid bilayer and the amplitude covers only a fraction of the maximum shift value. Hence, there is material on the lipid bilayer, which is constant during one oscillation cycle.  $\text{ATP}\gamma\text{S}$  (non-hydrolyzable analogon of ATP) fixes the proteins bound to the lipid bilayer indicating the maximum protein coverage on the lipid bilayer. On the other hand, ADP is supposed to remove the proteins from the lipid bilayer. The minimum lipid coverage corresponds to the minimum in the oscillation. Therefore, a lot of material stays on the lipid bilayer once bound.

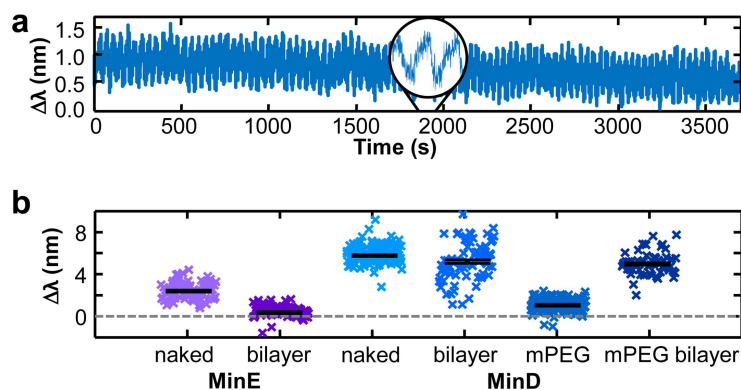
To investigate the constantly bound material concerning accumulation, we followed the Min system oscillation for one hour. We find a stable signal concerning amplitude and period (Figure 8.5a) indicating that no material accumulates over time on the lipid bilayer after reaching saturation. To consider impurities, which could build up the constant material, we determined the amount of byproducts of the proteins by SDS-PAGE (Appendix Figure F.7). Assuming impurities of 1%, this material could cover only less than 10%

of the whole surface, which can not explain a shift of  $\sim 5$  nm by addition of the Min system. Since impurities are excluded, MinD and/or MinE builds up the constantly bound material. In order to identify the constant protein, we incubated particles and particles covered with a lipid bilayer with each of the Min species alone in the presence of ATP (Figure 8.5b). Adding MinE to particles results in a non-specific attachment of the protein. This attachment is completely suppressed when adding MinE to particles covered with a lipid bilayer, since MinE exhibits no membrane affinity.<sup>3</sup> Furthermore, the absence of non-specific interactions underlines the defectless spreading of the lipid bilayer on top of the particles, so there is no protein-particle interaction if the particle is covered by a bilayer. In the case of MinD, the protein gets non-specifically attracted by particles, such as MinE, but the plasmon shift is larger due to the bigger size of MinD compared to MinE. Since MinD dimerizes in the presence of ATP and exposes a membrane targeting sequence,<sup>15</sup> we sense MinD with particles covered by a lipid bilayer. In this case, the shift is slightly smaller compared to naked particles due to blocking of some sensing volume by the bilayer. In order to investigate, whether the membrane targeting sequence of MinD acts an anchor, which sticks through the bilayer getting trapped by the particle, we used particles coated with a short thiolated methoxy polyethylene glycol (mPEG-SH,  $M_w$  735 Da) to avoid non-specific protein attachment (Figure 8.5b).<sup>21</sup> Adding MinD to mPEG-particles, the shift gets reduced to about one fifth of the value obtained with uncoated particles indicating an efficient suppression of non-specific attachment. However, we find a comparable shift for particles and mPEG-particles in the presence of the lipid bilayer. Furthermore, the oscillation behavior of the Min system sensed with mPEG-particles does not differ from the oscillation behavior described before (Appendix Figure F.8). The results demonstrate a specific interaction of MinD and the bilayer without an unintended contribution of the particles.

Our experiments give evidence for a MinD layer, which remains bound on the lipid bilayer once it attached. On top of this constant layer, the oscillation of the Min system takes place (Figure 8.6). This picture refines the process proposed by Loose *et al.*<sup>12</sup> The authors describe that, before detachment of all proteins, MinE could bind transiently to the bilayer or it could rebind to a neighboring MinD present on the bilayer. Here, we exclude an interaction of MinE with the bilayer and rather propose a transient handover from

<sup>21</sup> Walkey *et al.*, "Nanoparticle Size and Surface Chemistry Determine Serum Protein Adsorption and Macrophage Uptake."





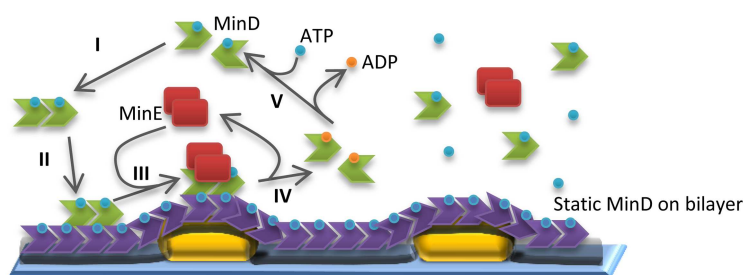
**Figure 8.5.:** a) Observation of the Min oscillation for 1h. There is no increase or reduction in the amount of proteins, which do not leave the membrane, excluding an accumulation of material by the nanoparticles. b) Adding MinD or MinE to pure gold nanorods results in non-specific binding. MinE does not interact with the lipid bilayer on top of the particles. MinD binds to a similar degree to the lipid bilayer as to the naked particles. Covering the rods with mPEG reduces significantly non-specific binding in the absence of a lipid bilayer, but in the presence of a bilayer, the degree of binding compares to the degree of binding to bilayer covered pure rods. Hence, the MinD gets not trapped by the particle, but interacts solely with the membrane. (Each colored cross shows the response of one single particle. Median and s.e.m. of 56 - 137 particles are marked in black.)

MinD being active in the oscillation process to constant MinD on the bilayer. A constant portion of MinD was also detected by Meacci and Kruse in experiments observing fluorescently labeled MinD in living *E.coli*.<sup>14</sup> They found an oscillating MinD fraction of 50% and they predicted 30% of MinD being mobile by numerical calculations, which agrees with our findings of a constant MinD layer. Hence, single particle dark-field spectroscopy is not only capable of detecting the surface coverage of the Min system on the lipid bilayer, but it can also give insight into the underlying mechanism.

### 8.3. Conclusion

Optical dark-field spectroscopy using single gold nanorods as individual sensing elements is a label-free real-time technique to study protein dynamics. So far, these dynamics are assessed by SPR, but the sensor size is not suitable to assess locally restricted density changes in surface coverage. Taking advantage of the small size of a nanoparticle, whose sensing volume





**Figure 8.6.:** Our results show the presence of a constant MinD layer covering the lipid bilayer. On top of this static layer, the oscillation of the Min system takes place. In the presence of ATP, MinD dimerizes and interacts with the constant MinD layer. Afterwards, binding of MinE to the active MinD removes the mobile fraction of proteins and the cycle can start over again.

matches molecular dimensions, we demonstrate the need of native protein observation to correctly analyze the system. Furthermore, we refine the current view of the process by detection of a constant MinD layer. This insight into the Min system could initiate further experimental designs towards a bottom-up reconstitution of bacterial cell division *in vitro*.

## 8.4. Methods

Deionized water from a Millipore system ( $>18\text{ M}\Omega$ , Milli Q) was used in all experiments. Suppliers of chemicals are given in the Appendix Chapter F.

**Synthesis of gold nanorods.** See Section 6.4.

**Buffers and nucleotides** Buffer for the formation of supported lipid bilayers (SLB-buffer): 50 mM Tris-HCl, 150 mM KCl, pH 7.5. Buffer for the Min system (Min-buffer): 50 mM Tris-HCl, 200mM KCl, 5 mM  $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ , pH 7.5. ATP, ADP, and  $\text{ATP}\gamma\text{S}$  were present at final concentrations of 2.5 mM, 2.5 mM, and 1 mM, respectively.

**Purification of the proteins and labeling of MinE.** His-MinD and His-MinE, here referred as MinD and MinE, were purified with an N-terminal His-tag as previously described by Loose *et al.*<sup>6</sup> MinE was labeled with AlexaFluor-488  $\text{C}_5$  maleimide according to the manufacturer's instructions.

**Home-made flow chambers.** We use a flow cell consisting of two cover slips held together by a piece of Nescofilm with a void area. One cover slip has two small holes for the connection of thin tubings as in- and outlet. First,

the flow cell was cleaned by rinsing with 100  $\mu$ L of Hellmanex II special cleaning concentrate and 1 mL of water. After cleaning, gold nanorods were injected and incubated until an adequate surface density of attached particles was reached.

**Formation of supported lipid bilayers (SLBs) by vesicle fusion.** The *E.coli* polar extract in chloroform was dried by nitrogen flow for 30 minutes and kept under vacuum for 2 hours to remove organic traces. Upon hydration of the dried film with SLB-buffer, the multilamellar vesicles (MLVs) obtained were sonicated for 10 minutes to form small unilamellar vesicles (SUVs). We used the method of vesicle fusion on glass<sup>22</sup> in order to form a supported lipid bilayer over the glass surface of the flow chamber and the gold particles there immobilized. Therefore, a suspension of these SUVs (1 mg/mL) was flushed into the flow cell in the presence of 3.3 mM  $\text{CaCl}_2$  and incubated for 30 minutes. After that, the non-fused vesicles were washed out by flowing 1 mL of SLB-buffer. Exchange of the SLB-buffer for the Min-buffer was carried out in a last washing step.

**Self-organization assays.** Self-organization assays of Min proteins on the SLBs formed inside the flow chambers were conducted essentially as previously described by Loose *et al.*<sup>6</sup> Experimental concentrations in the assays described in this paper were: 0.75  $\mu$ M MinD and 0.75  $\mu$ M MinE in the presence of 2.5 mM ATP. Alternatively, when MinE-Alexa488 was used as a tracer, the same sample contained 0.45  $\mu$ M MinE and 0.30  $\mu$ M MinE-Alexa488 instead. The flow cell was flushed with 180  $\mu$ L of the described protein mixture and incubated for 1 h to allow the Min proteins to self-assemble in travelling waves on the membrane surface. The experiments were carried out at 23°C.

**Incubation of particles with one Min species.** Pure gold nanorods or particles covered with a lipid bilayer were incubated with a Min-buffer solution containing 2.5 mM ATP and 0.75  $\mu$ M MinE or MinD, respectively. For functionalization of the gold nanorods with mPEG-SH, we incubated particles immobilized in the flow cell with a 2 mM aqueous solution of mPEG-SH over night. Afterwards, we determined the response of the functionalized particles and the functionalized particles covered with a lipid bilayer to a Min-buffer solution containing 2.5 mM ATP and 0.75  $\mu$ M MinD.

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<sup>22</sup> Brian, McConnell, "Allogeneic stimulation of cytotoxic T cells by supported planar membranes."

## 8.5. References

1. De Boer, P. A. J.; Crossley, R. E.; Rothfield, L. I. *Cell* **1989**, *56*, 641–649.
2. Rothfield, L.; Taghbalout, A.; Shih, Y. L. *Nature Reviews Microbiology* **2005**, *3*, 959–968.
3. Raskin, D. M.; deBoer, P. A. J. *Proceedings of the National Academy of Sciences of the United States of America* **1999**, *96*, 4971–4976.
4. Hu, Z. L.; Lutkenhaus, J. *Molecular Microbiology* **1999**, *34*, 82–90.
5. Lutkenhaus, J. *Annual Review of Biochemistry* **2007**, *76*, 539–562.
6. Loose, M.; Fischer-Friedrich, E.; Ries, J.; Kruse, K.; Schwille, P. *Science* **2008**, *320*, 789–792.
7. Loose, M.; Kruse, K.; Schwille, P. *Annual Review of Biophysics* **2011**, *40*, 315–336.
8. De Boer, P. A. J.; Crossley, R. E.; Hand, A. R.; Rothfield, L. I. *Embo Journal* **1991**, *10*, 4371–4380.
9. Hu, Z. L.; Lutkenhaus, J. *Molecular Cell* **2001**, *7*, 1337–1343.
10. Lackner, L. L.; Raskin, D. M.; deBoer, P. A. J. *Journal of Bacteriology* **2003**, *185*, 735–749.
11. Szeto, T. H.; Rowland, S. L.; Rothfield, L. I.; King, G. F. *Proceedings of the National Academy of Sciences of the United States of America* **2002**, *99*, 15693–15698.
12. Loose, M.; Fischer-Friedrich, E.; Herold, C.; Kruse, K.; Schwille, P. *Nature Structural & Molecular Biology* **2011**, *18*, 577–U195.
13. Schweizer, J.; Loose, M.; Bonny, M.; Kruse, K.; Moench, I.; Schwille, P. *Proceedings of the National Academy of Sciences of the United States of America* **2012**, *109*, 15283–15288.
14. Meacci, G.; Kruse, K. *Physical Biology* **2005**, *2*, 89–97.
15. Wu, W.; Park, K.-T.; Holyoak, T.; Lutkenhaus, J. *Molecular Microbiology* **2011**, *79*, 1515–1528.
16. Hu, Z. L.; Gogol, E. P.; Lutkenhaus, J. *Proceedings of the National Academy of Sciences of the United States of America* **2002**, *99*, 6761–6766.
17. Ament, I.; Prasad, J.; Henkel, A.; Schmachtel, S.; Sönnichsen, C. *Nano Letters* **2012**, *12*, 1092–1095.

18. Sönnichsen, C.; Geier, S.; Hecker, N. E.; Plessen, G. von; Feldmann, J.; Ditlbacher, H.; Lamprecht, B.; Krenn, J. R.; Aussenegg, F. R.; Chan, V. Z. H.; Spatz, J. P.; Möller, M. *Applied Physics Letters* **2000**, *77*, 2949–2951.
19. Anker, J. N.; Hall, W. P.; Lyandres, O.; Shah, N. C.; Zhao, J.; Van Duyne, R. P. *Nature Materials* **2008**, *7*, 442–453.
20. Kang, G. B.; Song, H. E.; Youn, H. S.; Lee, J. G.; An, J. Y.; Chun, J. S.; Jeon, H.; Eom, S. H. *Molecular Microbiology* **2010**, *76*, 1222–1231.
21. Walkey, C. D.; Olsen, J. B.; Guo, H.; Emili, A.; Chan, W. C. W. *Journal of the American Chemical Society* **2012**, *134*, 2139–2147.
22. Brian, A. A.; McConnell, H. M. *Proceedings of the National Academy of Sciences of the United States of America* **1984**, *81*, 6159–6163.

## Chapter 9.

# 9 Summary

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The results presented in this thesis demonstrate the potential of plasmonic metal nanoparticles for real-time label-free sensing of changes in the local environment due to their direct plasmon response. Here, I will briefly summarize the main achievements during my PhD, which range from basic research to applications of nanoparticles as tool to study the dynamics of biological systems.

Concerning basic research, I compared the plasmonic sensitivity of gold and silver nanorods (Chapter 3). Herein, the silver particles exhibited experimentally a more pronounced plasmon response to changes in the local environment than gold particles. Theoretical considerations according to Drude's model of optical properties of metals and the quasi static approximation identified the lower background polarizability of silver compared to gold as the main reason for the higher sensitivity. However, the silver particles are chemically unstable under light exposure, which limits their sensing capabilities for routine use.

To employ nanoparticles as sensors for living organisms, any unintended interaction needs to be excluded. For localization and quantification of gold nanoparticles inside mammalian cells at the same time, I developed a new approach by combining the results of transmission electron microscopy and optical dark-field microscopy (Chapter 4). Investigating the uptake of gold nanospheres and nanorods with different stabilizing agents, I found a significant variation in the extent of uptake depending on the particle coating. Herein, particles stabilized by a weakly bound surfactant entered the cells in a high degree impairing their metabolic activity, whereas there was internalized only a tiny fraction of particles coated with a biocompatible polymer. Hence, intended delivery without affecting the native behavior of a cell needs further development. In contrast, growing cells on gold nanorods with the same stabilizing agents, particles coated with a biocompatible polymer exposing a negatively charged headgroup to the cell seemed neither to im-

pair the growth behavior nor to interact with the cell membrane (Chapter 5). Thus, these particles are promising candidates for the sensing of membrane fluctuations, which give access to mechanical properties of the cell.

Taking advantage of the small nanoparticle size in combination with the direct response to changes in the local environment, I demonstrated a set of gold nanorods - each specifically functionalized to interact with a certain protein - being efficient sensing elements for the parallel, label-free detection of multiple analytes from a solution of interest (Chapter 6). Besides multiplexed detection, the sensor is easy to fabricate, re-usable, and sensitive down to nanomolar concentrations. Hence, this promising concept for an inexpensive mass-produced product could find application in abundant fields like disease identification, food/water quality and composition monitoring, and drug or hazard screening.

As an extension of multiplexed target detection, I assessed the binding of one protein of interest to partner proteins attached individually to gold nanorods (Chapter 7). Herein, observation of proteins in their native form keeping their functionality was essential for conclusive results. Therefore, proper attachment of the partner proteins was established using a His-Tag as well as particles functionalized with nitrilotriacetic acid and complexed with nickel ions. Proof-of-principle was given by the reproduction of three reported binding constants from one experiment and efforts for upscaling the technique are currently in progress.

Finally, I used single gold nanorods as real-time reporter for the reversible, dynamic oscillation of the Min protein system without need of labeling. Since the periodicity of the oscillation is in the range of 100  $\mu\text{m}$ , the sensing capability of the particles for the local environment can resolve the variation in protein density. In contrast, surface plasmon resonance spectroscopy misses the dynamics due to averaging over the chip area. Compared to fluorescence techniques, the particles show no bleaching and observation of the system over a prolonged period (at least 1 hour) was possible. Furthermore, the results identified fluorescence labeling having an impact on the dynamics of the system, which demands an investigation of native proteins for proper characterization. Most remarkable, the gold nanorods uncovered a constant fraction of protein not taking actively part in the oscillation, which was not considered so far from any other study on the minimal system for protein oscillation.

## Appendix





# A Appendix A.

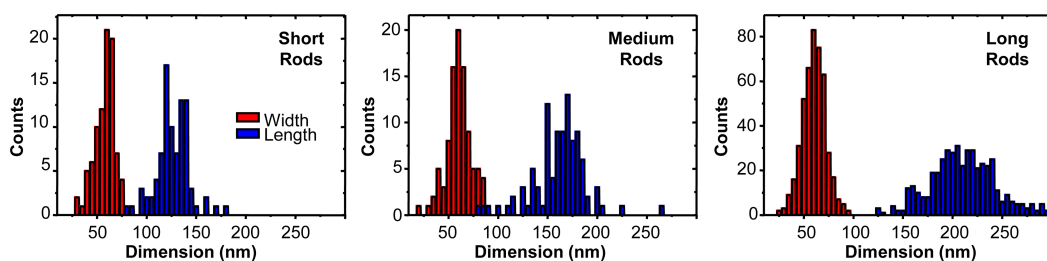
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## Highly Sensitive Plasmonic Silver Nanorods

### A.1. Materials

Silver nitrate (99%), sodium citrate tribasic dihydrate (99%), polyvinylpyrrolidone (PVP,  $M_w$  40K), sodium borohydride (99%), L-arginine (98%) were purchased from Aldrich and used as received. Millipore deionized water was used as solvent.

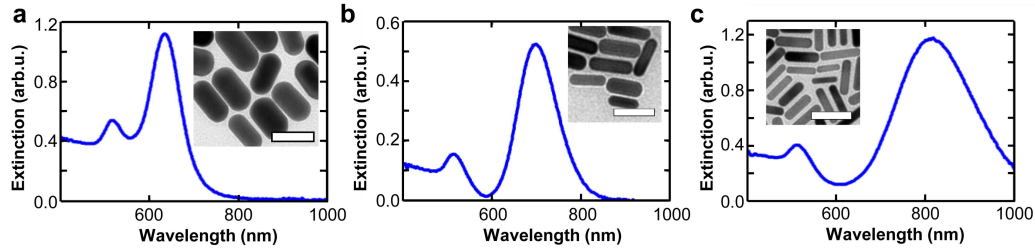
### A.2. Figures



**Figure A.1.:** Histogram of width (red) and length (blue) for Ag rod samples with different average lengths (from TEM images).

**Table A.1.:** Dimensional characterization of the silver nanoparticles that were used for single particle sensitivity measurements. The relative standard deviation of the widths is slightly larger (20%) than of the lengths (15%).

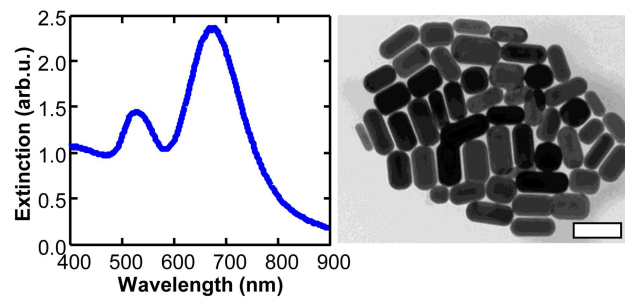
| Sample Rods | Ens.Peak (nm) | No. of Particles | Width (nm)  | Length (nm)  |
|-------------|---------------|------------------|-------------|--------------|
| Short       | 633           | 88               | $55 \pm 10$ | $124 \pm 16$ |
| Medium      | 776           | 98               | $59 \pm 13$ | $159 \pm 27$ |
| Large       | 930           | 459              | $58 \pm 12$ | $212 \pm 29$ |



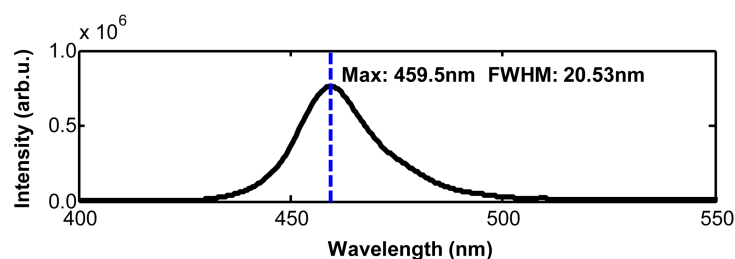
**Figure A.2.:** TEM images and ensemble extinction spectra of the small gold NRs used to measure the sensitivity. The longitudinal ensemble peak is at (a) 630nm, (b) 702 nm and (c) 816 nm. The gold NRs have spherical or slightly flattened endcaps. The scale bars are 50 nm.

**Table A.2.:** Dimensional characterization of the gold nanoparticles that were used for single particle sensitivity measurements.

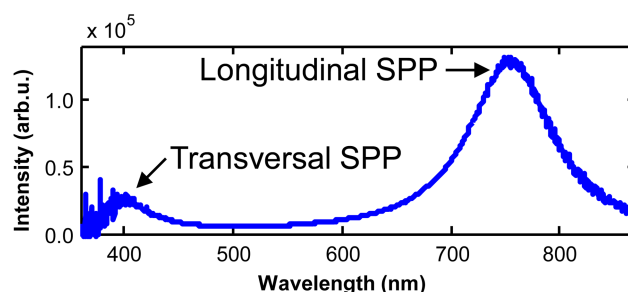
| Sample ( $\lambda_{\text{Res}}$ ) | Width (nm) | Length (nm) |
|-----------------------------------|------------|-------------|
| 634 nm                            | $28 \pm 4$ | $57 \pm 6$  |
| 702 nm                            | $18 \pm 2$ | $49 \pm 4$  |
| 816 nm                            | $11 \pm 2$ | $43 \pm 8$  |



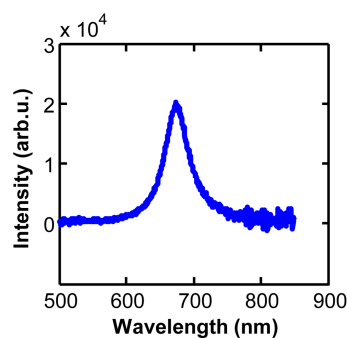
**Figure A.3.:** Ensemble extinction spectrum and TEM image of the large gold NRs used to measure the sensitivity. These gold NRs have lengths of  $107 \text{ nm} \pm 12 \text{ nm}$ , and widths of  $50 \text{ nm} \pm 6 \text{ nm}$ , these dimensions are similar to the silver NR dimensions. The scale bar is 100 nm.



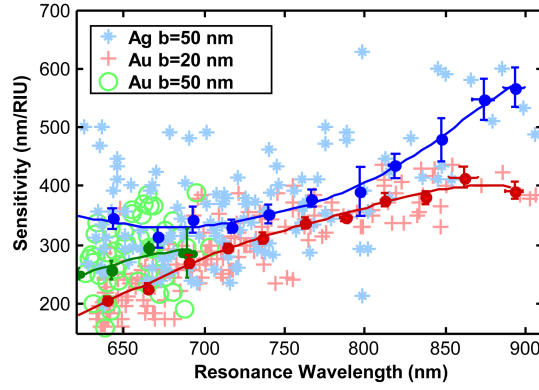
**Figure A.4.:** The spectrum of the blue LED lamp used to grow silver seeds.



**Figure A.5.:** Example of a white light scattering spectrum of a single silver nanorod. For unpolarized excitation, both the long axis (or longitudinal) and the short axis (or transversal) plasmon resonances are visible.



**Figure A.6.:** Example of the scattering spectrum of a single gold nanorod from the batch with 702nm ensemble peak. For unpolarized excitation only the long axis (or longitudinal) plasmon resonances is visible, the short axis (or transversal) plasmon resonance is very weak at widths of 18 nm (see gold nanoparticle size data in Table A.2).



**Figure A.7.:** Single particle sensitivity vs. resonance wavelength for each measured NR ( $b$  = avg. thickness = short axis). The straight lines are splines through the binned averages of the experimental data (solid circles) with horizontal bin size of 25 nm (same like in Figure 3.3 of the main article).

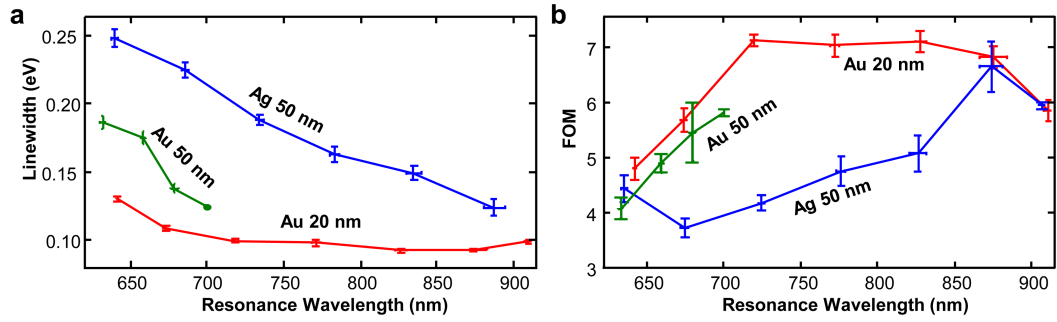
### A.3. Theoretical Description

The scattering cross-section  $C_{\text{sca}}$  of a metal nanoparticle is proportional to the square of the absolute value of the polarizability:  $C_{\text{sca}} \propto |\alpha|^2$ . The polarizability  $\alpha$  is obtained from the material dependent complex electric permittivity (or dielectric constant)  $\varepsilon$  of both the metal and the embedding medium by solving Maxwell's Equations. The embedding medium has a constant and real-valued dielectric constant ( $\varepsilon_m$ ), the metal's dielectric constant is a frequency dependent complex number  $\varepsilon(\omega)$ . We use the numerical boundary element method (BEM), the medium long wavelength approximation (MLWA), and the quasi-static approximation (QSA) to calculate the nanoparticle response  $\alpha$  for two different embedding media to get the plasmonic sensitivity  $S$ .

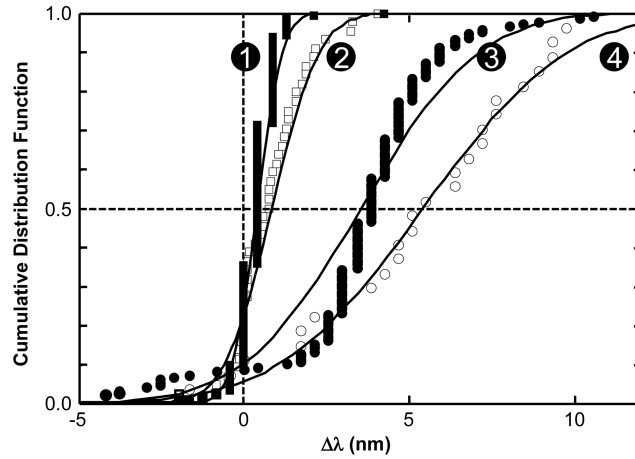
**Drude.** Drude's model describes the electric permittivity  $\varepsilon$  of gold and silver in the visible and near infrared very well (for light energies below the minimum required to excite d-band electrons into the conduction band):

$$\varepsilon(\omega) = \varepsilon_\infty - \frac{\omega_p^2}{\omega^2 + \gamma^2} + i \frac{\omega_p^2 \gamma}{\omega^3 + \omega \gamma^2} \quad (\text{A.1})$$

where  $\omega_p$  is the plasma frequency ( $\hbar\omega_p = 9.1$  eV for both gold and silver), and  $\varepsilon_\infty$  the background permittivity arising from the bound d-band electrons ( $\varepsilon_{\infty,\text{Au}} = 9.84$  for gold and  $\varepsilon_{\infty,\text{Ag}} = 3.7$  for silver). The lower value for background permittivity of silver reflects the deeper lying d-band with an offset



**Figure A.8.:** a) Linewidth vs. resonance wavelength of silver (blue line), 20 nm thick gold (red line) and 50 nm thick gold (green line) NRs. Silver has larger linewidth due to the large radiation damping and multi-crystalline structure. 50 nm width gold NRs have larger linewidth than 20 nm gold NRs due to increased radiation damping. The characteristic onset of linewidth increase due to interband damping is observed at the gold NRs @ 650 nm. b) Figure of merit (FOM) vs resonance wavelength of silver, 50 nm thick gold and 20 nm thick gold NRs (color code like in the top panel). Due to the broad spectral peak of the silver NRs, FOM of gold is larger than that of silver.



**Figure A.9.:** Spectral stability of nanoparticles measured under identical conditions as during sensitivity measurements, except that here the surrounding medium was always water. The graph shows the influence of the long pass filter on the spectral stability of Au and Ag nanoparticles by plotting the cumulative probability (integrated histogram) of the spectral shift  $\Delta\lambda_{\text{Res}}$  1) Au with 610 nm long pass filter, 2) Ag with 610 nm long pass filter, 3) Au without long pass filter, 4) Ag without long pass filter. The spectral stability of gold is better (smaller average shift) than of silver. We assume that some activation energy related photochemical process is inducing an irreversible reshaping of the nanoparticle.

to the Fermi-energy of 4 eV compared to 2 eV for gold. The damping term  $\gamma$  ( $\hbar\gamma_{\text{Au}} = 67 \text{ meV}$  and  $\hbar\gamma_{\text{Ag}} = 19 \text{ meV}$ )<sup>1</sup> has a small value compared to optical frequencies and is therefore often omitted (however, it contributes to the size dependent resonance red shift in the MLWA theory). Neglecting  $\gamma$ , we get for the real part of the dielectric function:

$$\Re[\varepsilon(\omega)] \approx \varepsilon_{\infty} - \frac{\omega_p^2}{\omega^2} \quad (\text{A.2})$$

**Quasi-static approximation (QSA).** Within the quasi-static approximation (i.e. for particle dimensions much smaller than the light wavelength in the metal), the polarizability  $\alpha$  for an ellipsoidal nanoparticle with volume  $V$  is given by:

$$\alpha = V\epsilon_0 \frac{\varepsilon_r - 1}{1 + L(\varepsilon_r - 1)} \quad (\text{A.3})$$

where  $\varepsilon_r = \varepsilon(\omega)/\varepsilon_m$  is the relative dielectric function of the particle  $\varepsilon(\omega)$  to the embedding medium  $\varepsilon_m$ ,  $L$  is a geometric depolarization form factor that is well approximated by  $L = (1 + AR)^{-1.6}$  for rods with aspect ratio (AR) between 1 to 8.

If the imaginary part of the dielectric function depends only weakly on frequency, the plasmon resonance occurs at the frequency  $\omega_{\text{Res}}$  where the real part of the denominator becomes zero:

$$1 + L(\varepsilon_r - 1) = 0 \quad (\text{A.4})$$

With the simplified Drude dielectric function (Equation (A.2)), we can express  $\omega_{\text{Res}}$  or the resonance wavelength  $\lambda_{\text{Res}} = 2\pi c_0/\omega_{\text{Res}}$  with  $c_0$ , the speed of light in vacuum. Introducing the refractive index  $n = \sqrt{\varepsilon_m}$  of the surrounding medium, Equation (A.4) can be cast into the form:

$$\lambda_{\text{res}} = \lambda_p \sqrt{\varepsilon_{\infty} + n^2 \left( \frac{1}{L} - 1 \right)} \quad (\text{A.5})$$

where  $\lambda_p = 2\pi c_0/\omega_p = 136 \text{ nm}$  is the bulk plasmon wavelength with  $c_0$ , the speed of light in vacuum. Since the sensitivity is defined by the change of the resonance wavelength  $\lambda_{\text{Res}}$  with the surrounding refractive index  $n$ , we arrive at the equation for the sensitivity:

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<sup>1</sup> Sönnichsen, *Plasmons in metal nanostructures*.

$$S = \frac{d}{dn} \lambda_{\text{Res}} = \frac{\lambda_p n}{\sqrt{n_\infty^2 \left(\frac{L}{1-L}\right)^2 + n^2 \left(\frac{L}{1-L}\right)}} \quad (\text{A.6})$$

where  $n_\infty = \sqrt{\varepsilon_\infty}$  is the background refractive index. To be able to directly compare the experimental data to the results of the analytic expression in Equation (A.6), we need to express the sensitivity as function of the resonance wavelength. Thus we use Equation (A.5) and rearrange it to express  $L/(1-L)$  as function of  $n_\infty$ ,  $n$ ,  $\lambda_p$  and  $\lambda_{\text{Res}}$ :

$$\left(\frac{L}{1-L}\right) = n^2 / \left(\frac{\lambda_{\text{Res}}^2}{\lambda_p^2} - n_\infty^2\right) \quad (\text{A.7})$$

Inserting the right side of Equation (A.7) into Equation (A.6) and writing  $\lambda$  for  $\lambda_{\text{Res}}$ , we arrive at the simple equation:

$$S(\lambda) = \frac{\lambda}{n} \left(1 - \frac{\lambda^2}{\lambda_p^2} n_\infty^2\right) \quad (\text{A.8})$$

where all geometry related factors have canceled out.

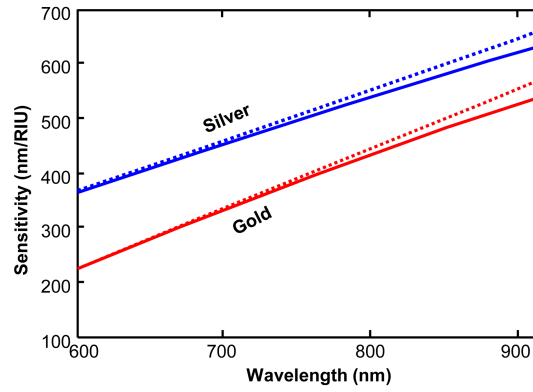
**Effect of particle size on plasmon sensitivity.** To study the effects of particle size on the plasmonic sensitivity of noble metal nanorods, we use the numeric boundary element method (BEM) and the medium long wavelength approximation (MLWA) approach of Kelly<sup>2</sup> and Meier<sup>3</sup>. The latter contains the Mie coefficients (thus the exact solution of the Maxwell Equations) to the third order of the wave vector  $k$ . BEM gives an exact solution of the Maxwell equation but offers little insight into the physics behind the observed trends. The MLWA shows nicely the origin of the deviations from the QSA for larger particle sizes.

**Medium long wavelength approximation (MLWA).** Using the wavelength inside the metal:  $\lambda' = \lambda/n'$  with  $n' = \Re[\tilde{n}]$  where  $\tilde{n}$  is the complex refractive index of the metal, the polarization of an ellipsoidal NR with volume  $V = 1/6\pi ab^2$  can be expressed within MLWA<sup>2</sup> as:

$$\alpha \propto \frac{(\varepsilon_r - 1)}{1 + (\varepsilon_r - 1) \left[ L - \frac{\pi}{3} \left( 2\pi \frac{n'b}{\lambda} \right)^2 - i \frac{\pi}{9} AR \left( 2\pi \frac{n'b}{\lambda} \right)^3 \right]} \quad (\text{A.9})$$

<sup>2</sup> Kelly et al., "The Optical Properties of Metal Nanoparticles: the Influence of Size, Shape, and Dielectric Environment"

<sup>3</sup> Meier, Wokaun, "Enhanced Fields on Large Metal Particles - Dynamic Depolarization."



**Figure A.10.:** Sensitivity of gold and silver vs the resonance wavelength  $\lambda_{\text{Res}}$  calculated with MLWA (Au: red lines, Ag: blue lines). The continuous lines correspond to a nanoparticle width of 20 nm, dotted lines correspond to a width of 50 nm. In case of MLWA, the sensitivity of nanorods with 20 nm width overlap with the sensitivity calculated using the QSA approach of Equation (A.8).

Where  $a$  is the length,  $b$  is the width of the NR (Attention: in Kelly's article  $b$  denotes the long axis of the ellipsoid which is half the length of the NR), and  $AR = a/b$  is the aspect ratio. The corrections compared to Equation (A.3) reduce the geometric depolarization factor  $L$  with two terms. The term  $\propto (n'b/\lambda)^2$  is called dynamic depolarization factor and is responsible for the red shift with increasing width, whereas the imaginary term  $\propto (n'b/\lambda)^3$  is causing an additional linewidth broadening with increasing volume (radiation damping). The resonance wavelength can be obtained by finding the position of the maximum of the squared norm of  $\alpha$ .

**Discussion of the size effect.** Calculations of the sensitivity for nanorods with different volumes show an increased sensitivity for larger nanorods compared to smaller nanorods with the same aspect ratio. Both BEM and MLWA show this effect. MLWA allows to identify the dynamic depolarization factor as the dominant contribution. We also find that including the imaginary part in the Drude dielectric function is necessary to observe an effect of size on the plasmon sensitivity.

Figure A.10 shows the sensitivity calculated with MLWA for two different particle thicknesses (20 nm and 50 nm). The difference between the different thicknesses is much smaller than the difference between the two materials (gold and silver). This finding matches the experimental results shown in Figure 3.3.



## A.4. References

1. Sönnichsen, C., *Plasmons in metal nanostructures*; Cuvillier Verlag Göttingen: 2001.
2. Kelly, K. L.; Coronado, E.; Zhao, L. L.; Schatz, G. C. *Journal of Physical Chemistry B* **2003**, *107*, 668–677.
3. Meier, M.; Wokaun, A. *Optics Letters* **1983**, *8*, 581–583.



## **B** Appendix B.

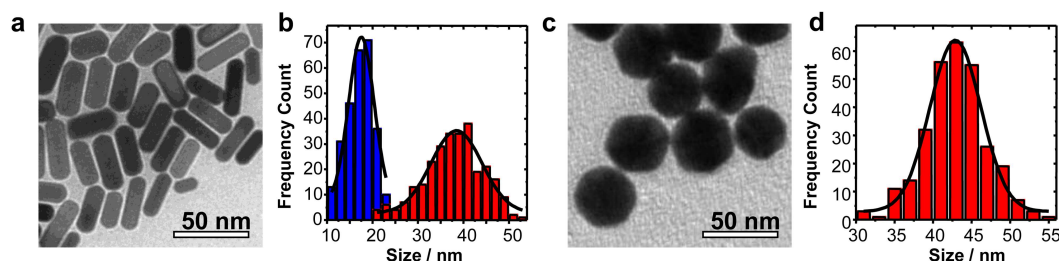
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# **A New Approach to Assess Gold Nanoparticle Uptake by Mammalian Cells: Combining Optical Dark-Field and Transmission Electron Microscopy**

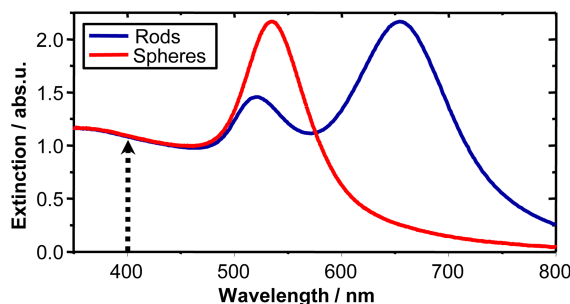
### **B.1. Materials**

Hydrogen tetrachloroaurate trihydrate, sodium borohydride, ascorbic acid, silver nitrate, cetyltrimethylammonium bromide, glutaraldehyde, and uranyl acetate were purchased from Sigma. Thiolated methoxy-polyethylene glycol, thiolated amine-polyethylene glycol, and thiolated carboxy-polyethylene glycol ( $M_w$  5000 Da) were purchased from Iris Biotech. Earle's minimum essential medium, L-glutamine, penicillin, streptomycin, phosphate-buffered saline without magnesium and calcium ions, ethylenediaminetetraacetic acid, and trypsin 0.05% were purchased from Biochrom. Fetal calf serum was purchased from PAA. Sucrose was purchased from Fluka. Agar was purchased from Roth. Ethanol abs. was purchased from Riedel-deHaën. Sodium cacodylate, osmium tetroxide, propylene oxide, epoxy resin (26 g of Araldite CY212, 24 g of Araldite hardener HY964, 1 g of 2,4,6-Tris(dimethylamonimethyl) phenol), lead salt solution (0.2 g of anhydrous lead citrate, 0.15 g of lead nitrate, 0.15 g of lead acetate trihydrate, 1.0 g of sodium citrate dehydrate) were purchased from Serva. Sodium hydroxide was purchased from Merck. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) was purchased from Promega.

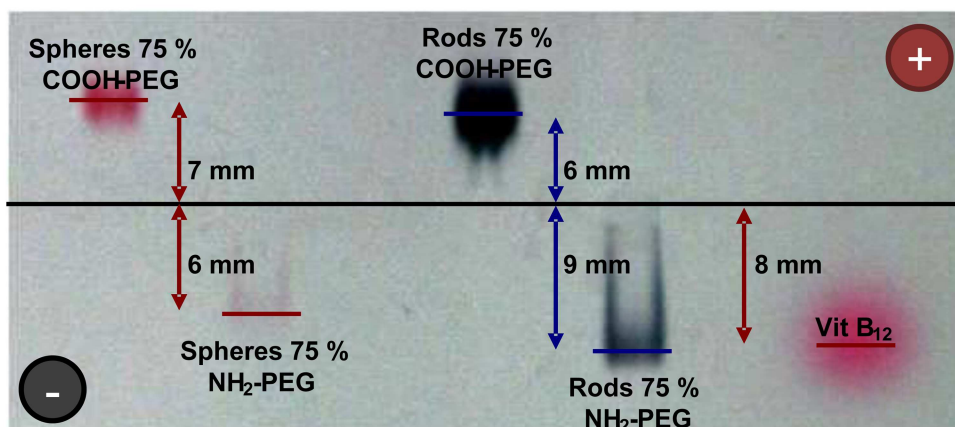
## B.2. Figures



**Figure B.1.:** Gold nanoparticle characterization. **a)** Representative TEM image of gold nanorods. **b)** Measurement of 274 particles gives a width of  $17.2 \text{ nm} \pm 2.9 \text{ nm}$  (left side of the histogram, blue bars) and a length of  $37.8 \text{ nm} \pm 6.5 \text{ nm}$  (right side of the histogram, red bars). Black lines are Gaussian fits for mean and standard deviation. **c)** Representative TEM image of gold nanospheres. **d)** Measurement of 291 particles gives a diameter of  $42.9 \text{ nm} \pm 4.0 \text{ nm}$ . The black line is a Gaussian fit for mean and standard deviation.



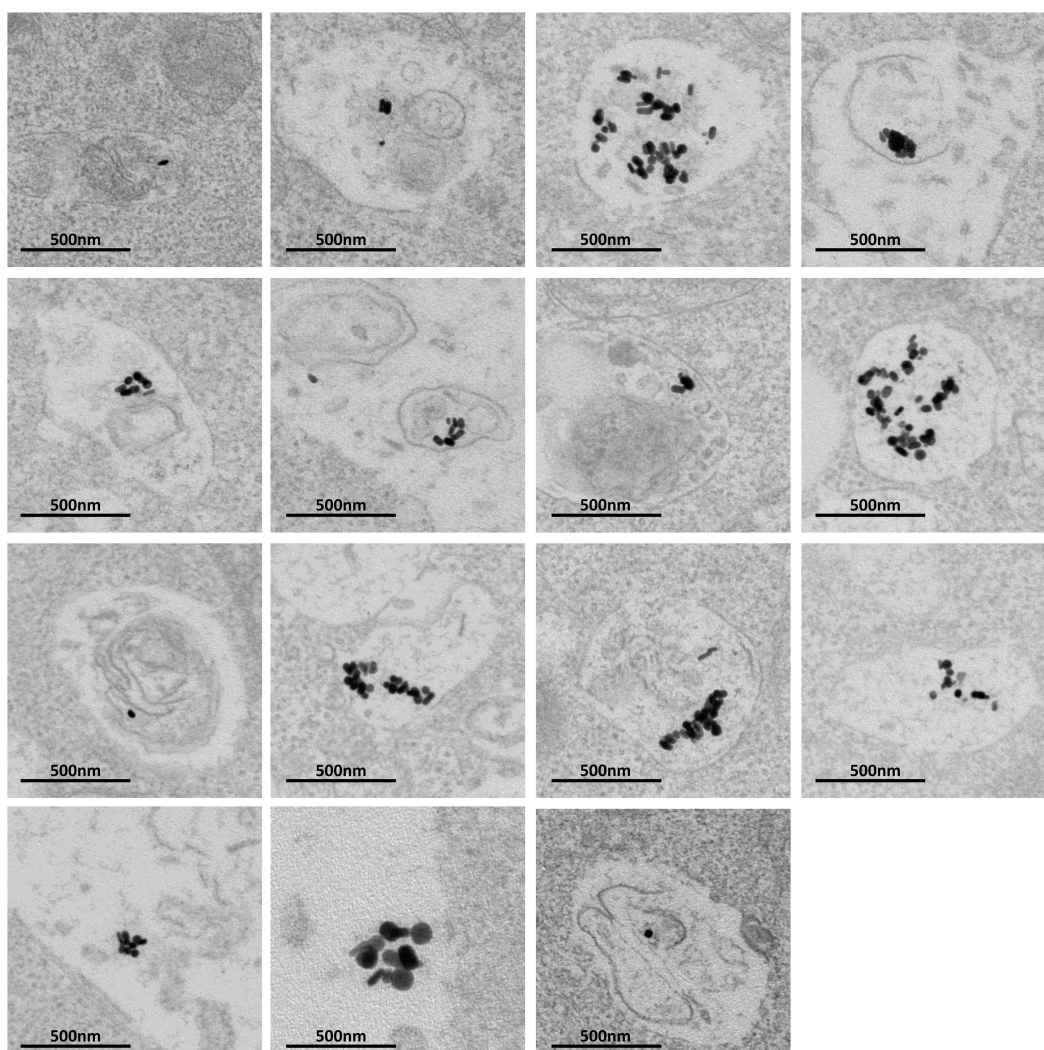
**Figure B.2.:** Extinction spectra of our gold nanoparticle stock solutions. The rod solution as well as the sphere solution has an extinction value of 1.1 at 400 nm. Using the extinction coefficients at 400 nm of  $1.1 \times 10^9 \text{ L.mol}^{-1}.\text{cm}^{-1}$  for the rods and  $6.2 \times 10^9 \text{ L.mol}^{-1}.\text{cm}^{-1}$  for the spheres, we get a particle concentration of  $6.2 \times 10^{11} \text{ rods.mL}^{-1}$  and  $1.1 \times 10^{11} \text{ spheres.mL}^{-1}$  which means  $88.9 \text{ } \mu\text{g gold.mL}^{-1}$  for both particle shapes.



**Figure B.3.:** Gel electrophoresis shows successful particle functionalization. The image shows a true color photograph of a 0.2 weight % Agarose (Agarose, 'For Routine Use', Sigma) gel in 0.5×TBE (Tris-borate EDTA) buffer (prepared by diluting a 10×stock solution, Sigma) ran for 30 minutes at 150 V in a horizontal electrophoresis system with an electrode spacing of 15 cm (Mini-Sub Cell GT, Biorad). Before starting the electrophoresis, concentrated samples were mixed with 2.5  $\mu\text{L}$  of loading buffer (30 % D(+)-Sucrose in 0.5×TBE buffer) and 10  $\mu\text{L}$  of these solutions were placed into small pockets located at the black line. One pocket was filled with the uncharged molecule Vitamin B<sub>12</sub> in loading buffer to observe the electroosmotic effect. Colored lines indicate the travel distance of the different particles. Particles functionalized with 75 % COOH-PEG-SH and 25 % CH<sub>3</sub>O-PEG-SH ran clearly to the positive electrode and particles functionalized with 75 % NH<sub>2</sub>-PEG-SH and 25 % CH<sub>3</sub>-PEG-SH ran to the negative pole. According to our experience, particles with a low surface coverage of the stabilizing polymer precipitate at their starting point due to the high ionic strength of TBE buffer. Therefore, the observed particle movement in the gel proved a successful functionalization of the particles. Using the travel distances, we calculated the electrophoretic mobility of the particles and then their zeta potential according to Hanauer et al.<sup>1</sup> We get for the NH<sub>2</sub>-PEG-particles  $\zeta$ -potential  $\approx 0$  mV and for the COOH-PEG-particles  $\zeta$ -potential  $\approx -20$  mV.

<sup>1</sup> Hanauer et al., "Separation of nanoparticles by gel electrophoresis according to size-and shape."

**CTAB-rods**



**Figure B.4.:** TEM images of CTAB-rods within the cell.

**CTAB-spheres**

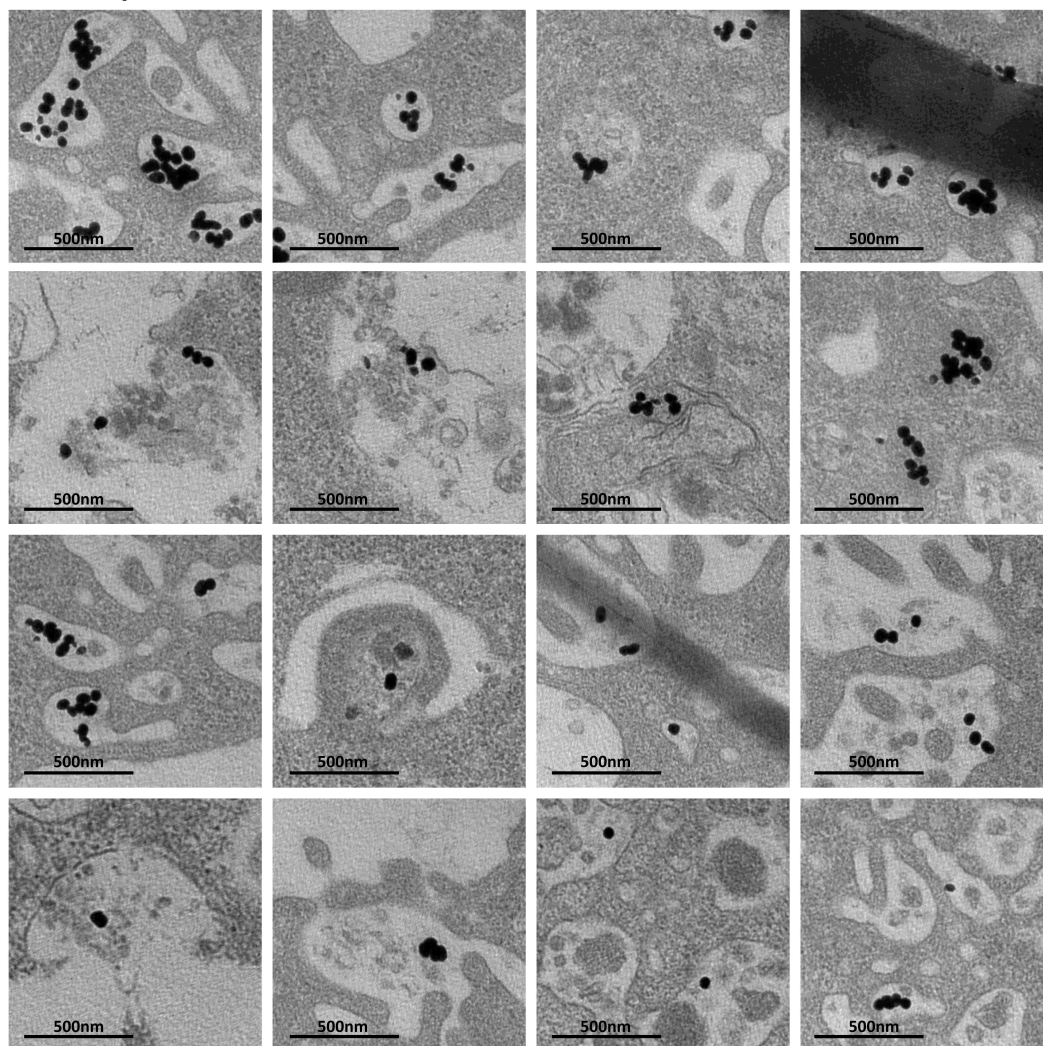


Figure B.5.: TEM images of CTAB-spheres within the cell.

**NH<sub>2</sub>-PEG-rods**

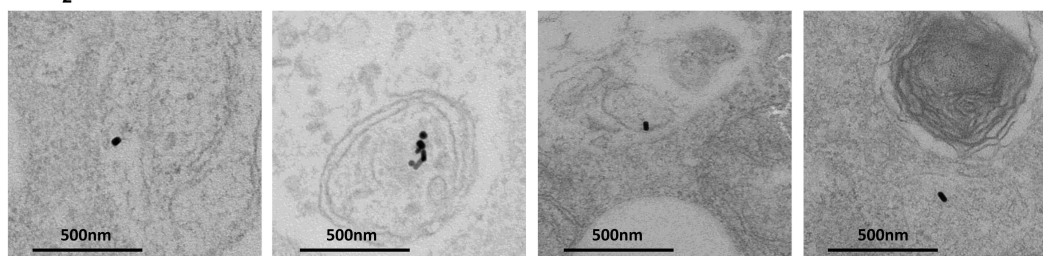
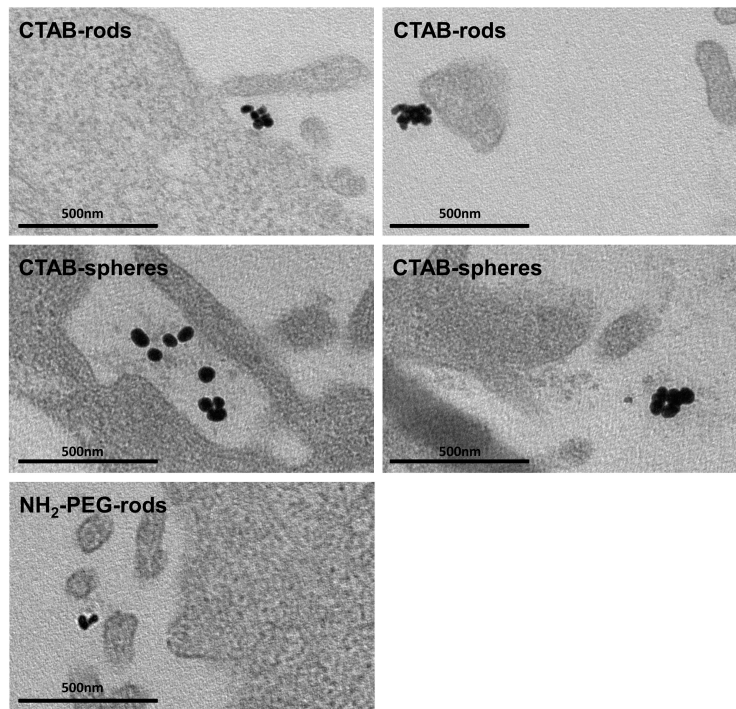
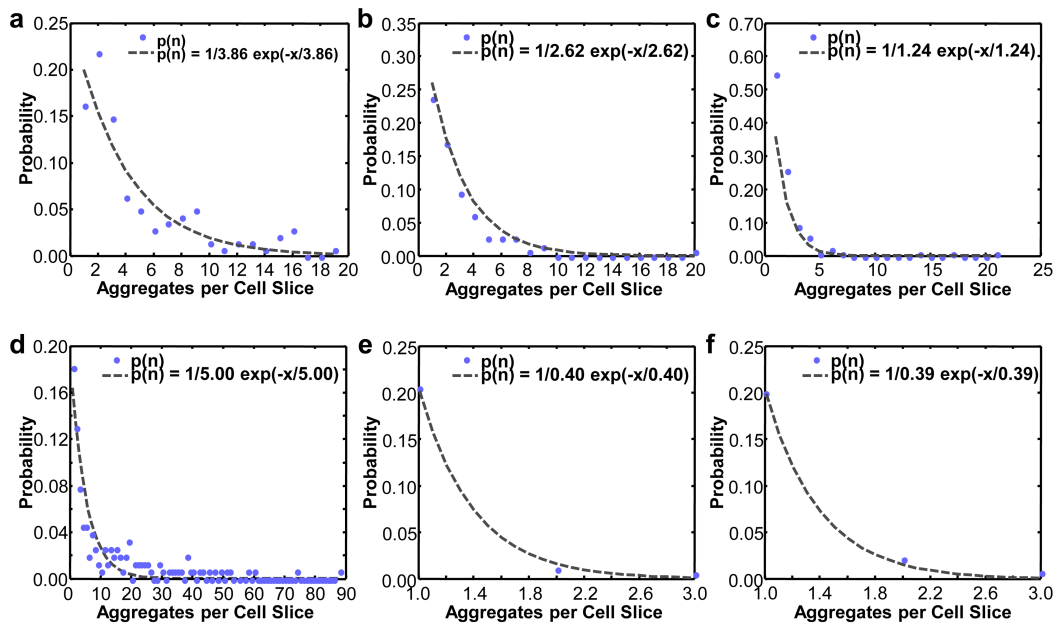


Figure B.6.: TEM images of NH<sub>2</sub>-PEG-rods within the cell.





**Figure B.7.:** TEM images of CTAB-particles and  $\text{NH}_2$ -PEG-rods in close proximity to the cell membrane and the microvilli.



**Figure B.8.:** Experimental data of the number of aggregates per cell slice (blue dots) are fitted with exponential distributions (grey dashed lines) to estimate the mean number of aggregates per cell slice. **a)** CTAB-rods, **b)**  $\text{NH}_2$ -PEG-rods, **c)** COOH-PEG-rods, **d)** CTAB-spheres, **e)**  $\text{NH}_2$ -PEG-spheres, and **f)** COOH-PEG-spheres.



### B.3. References

1. Hanauer, M.; Pierrat, S.; Zins, I.; Lotz, A.; Sönnichsen, C. *Nano Letters* **2007**, *7*, 2881–2885.

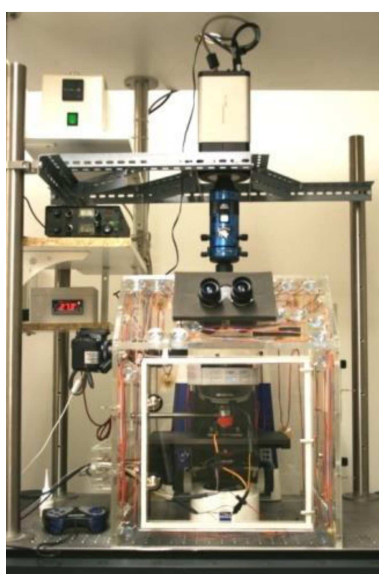


# C Mammalian Cell Growth on Gold Nanoparticle Patterned Substrates is Influenced by the Particle Coating

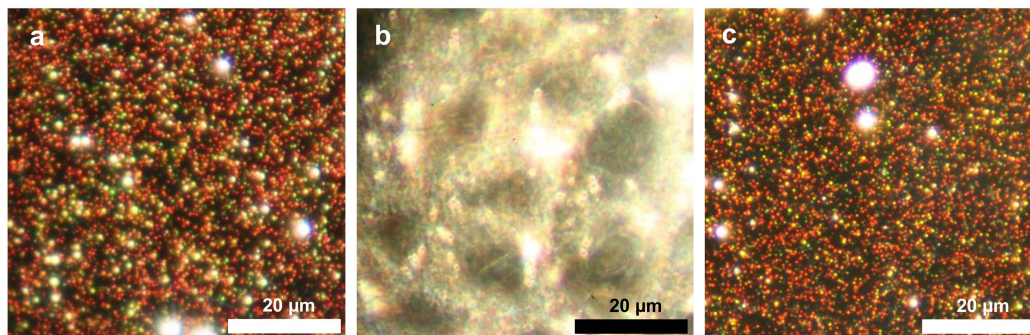
## C.1. Materials

Sterile, untreated petri dishes with glass bottoms ( $\mu$ -dish, 35 mm) were purchased from ibidi. Sodium chloride was purchased from Sigma. Sterile Anotop 25 disposable syringe filters with a pore size of 200 nm were purchased from VWR.

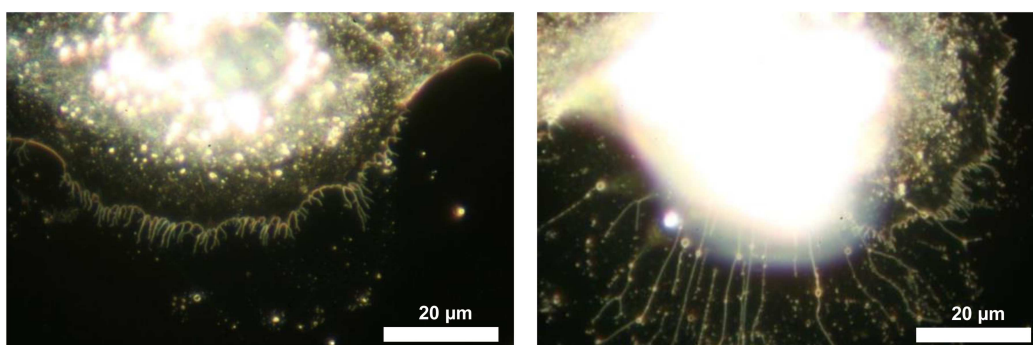
## C.2. Figures



**Figure C.1.:** We equipped a Zeiss Axio Imager A.1 with a home-built cage consisting of acrylic glass. Within this cage, we can maintain cell culture conditions, i.e. a temperature of 37°C and 5% CO<sub>2</sub> environment. Temperature control is carried out by a heating cable spanning along the cage connected to a regulation system. The CO<sub>2</sub> content is controlled by a gas sensor (Vaisala) giving feedback to an automated valve, which regulates gas support.



**Figure C.2.:** a) Substrate patterned with CTAB-nanorods. b) After seven days of incubation, a monolayer of cells covers the substrate. c) Cells were removed by trypsin. The substrate pattern before and after cell growth shows a comparable density. Hence, MDCK II cells do not internalize the particles in a remarkable extent.



**Figure C.3.:** These images show examples of filamentous residues on a bare glass substrate remaining after retraction of the cell membrane.



# D Multiplexed Plasmon Sensor for Rapid Label-free Analyte Detection

## D.1. Materials

Hydrogen tetrachloroaurate trihydrate ( $\text{HAuCl}_4$ ), sodium borohydride, ascorbic acid, silver nitrate, sodium chloride ( $\text{NaCl}$ ), potassium chloride ( $\text{KCl}$ ), magnesium chloride hexahydrate ( $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ ), calcium chloride dihydrate ( $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ ), sodium dodecyl sulfate (SDS), hydrochloric acid ( $\text{HCl}$ ) and tris(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma. Cetyltrimethylammonium bromide (CTAB) was purchased from Fluka. DNA oligonucleotides, purified by Reverse Phase HPLC (RP-HPLC), were purchased from Biomers (Germany) and supplied in dry state. Thiolated methoxy-polyethylene glycol (mPEG  $M_w$  3000 Da, mPEG  $M_w$  5000 Da) were purchased from Iris Biotech. Bovine serum albumin (BSA), Thrombin (Human), Streptavidin and Fibronectin (Bovine), Trypsin (Porcine) were purchased from Sigma. Immunoglobulin E (Human myeloma IgE) was purchased from Merck.

## D.2. Gold Nanorod Dimensions Optimized for Maximal Plasmon Shift

Using the Boundary Element Method (BEM), we calculated the resonance wavelength for particles covered with only an aptamer layer, and the resonance wavelength for the same particles covered with the aptamer layer and an additional protein layer. We use these results to determine the shift in

resonance wavelength due to target binding. For Thrombin, the size of the aptamer is about 5.5 nm and the protein size is 4.6 nm. The biggest shift can be obtained by rods having a diameter of 32 nm and an aspect ratio (ratio of length to diameter) of 2.5. Our synthesis resulted in rods being  $31.1 \text{ nm} \pm 5.6 \text{ nm}$  (mean and s.d.) in diameter having an aspect ratio of  $2.22 \pm 0.30$  (mean and s.d.). In addition, we performed an experiment to determine the plasmonic sensitivity of the particles (resonance wavelength shift per refractive index unit change). For this, we measured the non-functionalized particles in water (refractive index 1.332) and in a glycerol solution (refractive index 1.368) making sure that the shift is reversible upon shifting back to water. There is no change in sensitivity with wavelength (aspect ratio), since the variation of the particle dimensions within our nanorods sample is rather small.

### D.3. Surface Functionalization of Gold Nanorods

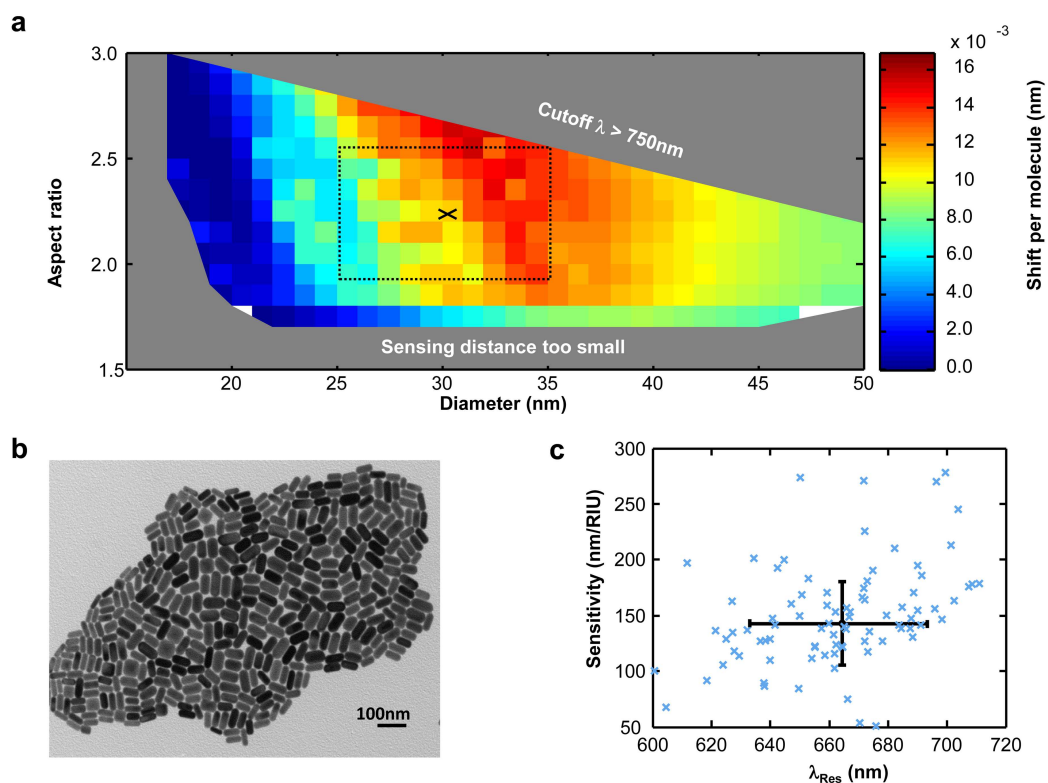
The proper conformation of nucleic acid aptamers requires a buffered environment of high ionic strength. However, surface functionalization of CTAB stabilized gold nanorods often leads to particle aggregation when the process is done in buffer. Simply adding 5'-thiol terminated aptamer molecules to CTAB stabilized gold nanorods is therefore not possible. Our solution to this problem consists of adding aptamer molecules along with SDS, a negatively charged surfactant that is known to prevent nanoparticle aggregation. The addition of SDS makes the nanoparticles more tolerant to ions present in the buffer, thus allowing the CTAB layer to be displaced slowly by the thiolated aptamers.

The spectra of gold nanorods collected post incubation with 0.4% SDS in water, thiolated aptamer (in 0.4% SDS in binding buffer), and (as a control) thiolated mPEG (in 0.4% SDS in binding buffer) show the robustness of this functionalization strategy (Figure D.2). All spectra exhibit a similar full width at half maximum of the longitudinal plasmon peak which is a good indicator for a lack of aggregation. The plasmon peak shifts to lower wavelength or higher energies after replacing CTAB to SDS, indicating a negative charging of the particles. The addition of the DNA aptamer (also highly negatively charged) further shifts the plasmon peak to the blue.<sup>1</sup>

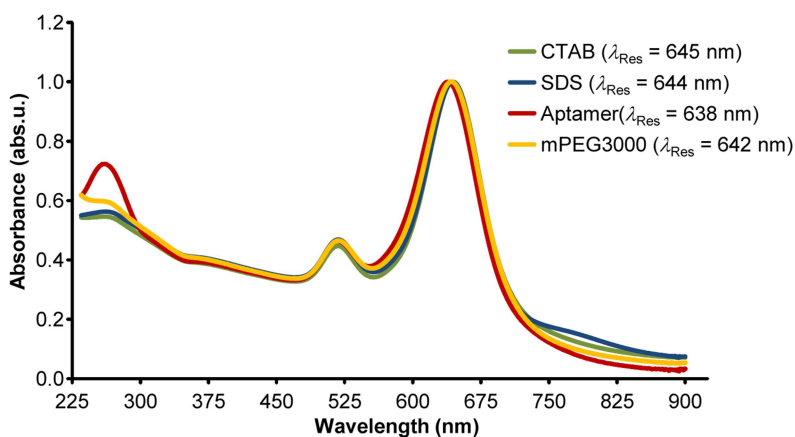
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<sup>1</sup> Novo et al., "Direct observation of chemical reactions on single gold nanocrystals using surface plasmon spectroscopy."





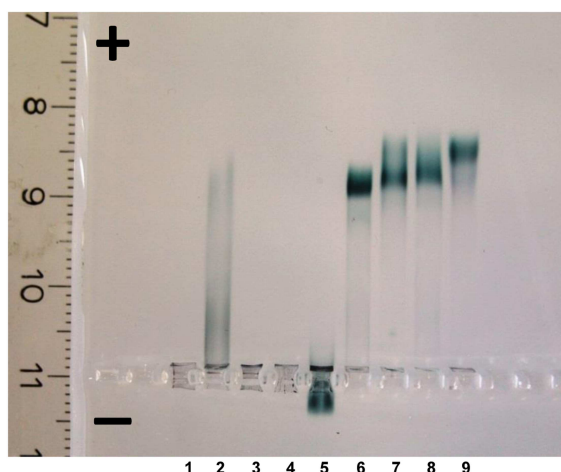
**Figure D.1:** **a)** Calculation of the shift in resonance wavelength induced by adding a layer of Thrombin on top of aptamer coated particles. The color code corresponds to the magnitude of the shift in resonance wavelength induced per bound target molecule. On the lower and left part of the diagram (gray area), the particle have sensing distances too small to sense target binding, hence we have not calculated their expected response. We can only measure resonance wavelength up to 750 nm, thus restricting the accessible aspect ratios on the top area of the graph. This inaccessible area is shaded in transparent grey. The mean diameter and the mean aspect ratio of the particles used for the sensor are indicated by a black cross. The dotted square around the cross marks the area of one standard deviation, showing which region is covered by the sample we used. **b)** Transmission electron microscopy image of the gold nanorods used in our sensor. These rods have a width of  $31.1 \text{ nm} \pm 5.6 \text{ nm}$  and a length of  $67.8 \text{ nm} \pm 9.2 \text{ nm}$  (values are mean and standard deviation determined from 486 particles). **c)** Plasmonic sensitivity ( $S = \Delta\lambda/\Delta n$ ) of the particles used in this study determined from a measurement in water (refractive index 1.332) and glycerol (refractive index 1.368), each blue cross corresponds to a single particle. Median and s.d. from 93 particles are marked in black (resonance wavelength  $664.2 \text{ nm} \pm 31.8 \text{ nm}$ ; sensitivity  $143.1 \text{ nm/RIU} \pm 39.5 \text{ nm/RIU}$ ).



**Figure D.2.:** UV visible spectra of gold nanorods after incubation with CTAB (green), SDS (blue), thiolated aptamer (red) and thiolated methoxy-PEG (yellow). The wavelength of the plasmon peak is indicated in the legend. In the UV region, the presence of the DNA aptamer is clearly visible by the characteristic DNA peak at 260 nm.

In order to further check successful nanoparticle functionalization, we performed gel-electrophoresis, where an applied external electric field is used to migrate particles of the same size according to their surface charge in a 0.5% Agarose gel.<sup>2</sup> Figure D.3 shows the resulting unstained gel for the differently functionalized nanoparticles. Lane 6-9 contains functionalized particles prepared by the method above, lane 1-5 various controls. As clearly evident from this gel, only correctly treated particles are stable under the conditions of gel electrophoresis. More specifically: Lane 1: CTAB stabilized particles aggregate in the well Lane 2: SDS stabilized particles (in water) show a smeared out lane indicating a loss of stabilization over time. Lane 3 and 4: SDS-AuNRs (in buffer) and AuNRs with non-thiolated DNA (in SDS buffer) aggregate in the well Lane 5: Thiol-mPEG-AuNRs (in SDS buffer) maintain their integrity and migrate slightly towards the negative end Lane 6-9: Thiol-aptamer functionalized-AuNRs (in SDS buffer) remain stable after functionalization and move towards the positive end in sharp bands attesting of the effective binding of thiol aptamers to nanorods in buffer solution. The migration of thiolated m-PEG-AuNR conjugates to the negative end proves that SDS did not compete or interfere during the PEG-binding process.

<sup>2</sup> Hanauer et al., "Separation of nanoparticles by gel electrophoresis according to size-and shape."

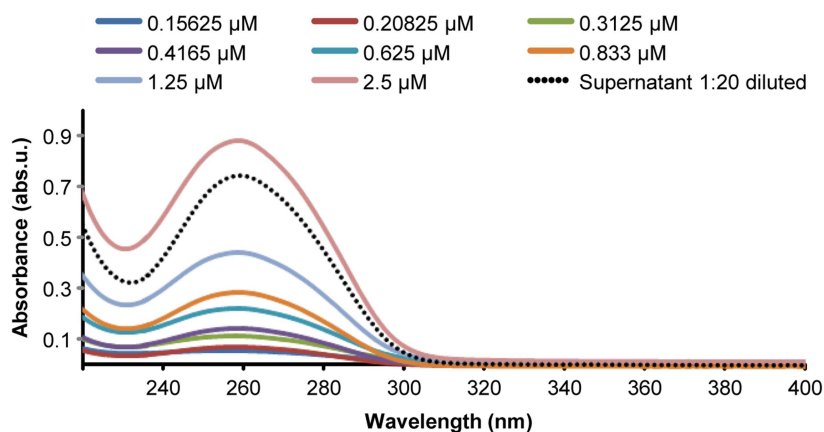


**Figure D.3.:** Electrophoretic mobility of nanorods incubated with:  
 Lane 1: Cetyltrimethylammonium bromide-AuNR  
 Lane 2: Sodium dodecylsulphate- AuNR (in water)  
 Lane 3: Sodium dodecylsulphate- AuNR (in buffer)  
 Lane 4: 50  $\mu$ M non-thiol DNA-AuNR (in SDS + buffer)  
 Lane 5: 50  $\mu$ M thiol-methoxy-PEG-AuNR (in SDS + buffer)  
 Lane 6: 50  $\mu$ M thiol-antifibronectin aptamer-AuNR (in SDS + buffer)  
 Lane 7: 50  $\mu$ M thiol-antistreptavidin aptamer-AuNR (in SDS + buffer)  
 Lane 8: 50  $\mu$ M thiol-antiimmunoglobulin E aptamer-AuNR (in SDS + buffer)  
 Lane 9: 50  $\mu$ M thiol-antithrombin aptamer-AuNR (in SDS + buffer).

## D.4. Estimation of Aptamer Load on the Gold Nanorods

Thrombin aptamer (5'-SH-(T)<sub>10</sub>AGTCCGTGGTAGGGCAGGTTGGGGTGACT-3') was used as model for determining the number of aptamers bound to the gold nanorod surface. Using UV absorbance at 260 nm, we determined the aptamer concentration in the supernatant after conjugation to gold nanorods. Three independent experiments resulted in concentrations of aptamer bound to nanorods of 0.403  $\mu$ M, 0.3929  $\mu$ M and 0.562  $\mu$ M, respectively. In order to quantify the number of rods, we calculate the molar extinction coefficient with the Mie-Gans theory<sup>3</sup> for the mean rod dimensions determined by transmission electron microscopy (67.8 nm  $\times$  31.1 nm). The corresponding values for aptamer load on particles were 2878, 2806, and 4015, respectively, yielding an average aptamer load of  $3233 \pm 553$  aptamers per particle (mean and s.d.).

<sup>3</sup> Prescott, Mulvaney, "Gold nanorod extinction spectra."



**Figure D.4.:** Calibration curve obtained with anti-thrombin aptamer in binding buffer. Dotted curve represents the aptamer in supernatant (dilution 1:20) after nanoparticle functionalization.

In order to verify these numbers, we performed dark-field spectroscopy on single particles. Herein, we measured the shift in the plasmon resonance wavelength of 29 particles after overnight incubation with 33  $\mu\text{M}$  Thrombin aptamer and 10 mM TCEP in buffer. We obtained an average shift of 9.3 nm with a standard deviation of 1.6 nm. From the single shift values of the individual particles, we calculated the number of bound aptamers according to Nusz et al.<sup>4</sup> using an aptamer size of 1.6 nm in length, 0.7 nm in width, and 7.5 nm in height.<sup>5</sup> We end up with a number of  $2700 \pm 600$  aptamers per rod (mean and s.d.). Hence, the results derived by UV spectroscopy and dark-field spectroscopy are in reasonable agreement. The number of aptamers per gold nanorods is roughly  $3000 \pm 500$  (mean and s.d.).

## D.5. Setup Improvements

We equipped a Zeiss Axio Observer Z1 inverted microscope with a PI542 XY-piezo stage ( $200 \mu\text{m} \times 200 \mu\text{m}$ , closed-loop) and a PI721 Z-piezo ( $100 \mu\text{m}$ , closed-loop). Furthermore, for VIS-NIR-spectroscopy, we added an Inspector V10E transmissive imaging spectrograph with an Andor Luca R EM-CCD. The true colour images are taken with a Canon EOS 5D Mark II (IR-filter removed). For the automated acquisition and analysis of many single particle

<sup>4</sup> Nusz et al., “Rational Selection of Gold Nanorod Geometry for Label-Free Plasmonic Biosensors.”

<sup>5</sup> Lin et al., “Studies of the binding mechanism between aptamers and thrombin by circular dichroism, surface plasmon resonance and isothermal titration calorimetry.”

spectra, we used MATLAB based control software. The particle positions are saved with the spectra allowing to identify changes of plasmon peaks for each particle individually after the surrounding of the particles is altered. Particles that have a corrupt spectrum (no clear resonance peak) or are simply lost due to the exchange of solutions are sorted out in subsequent measurements.

A measurement consists of 3 steps:

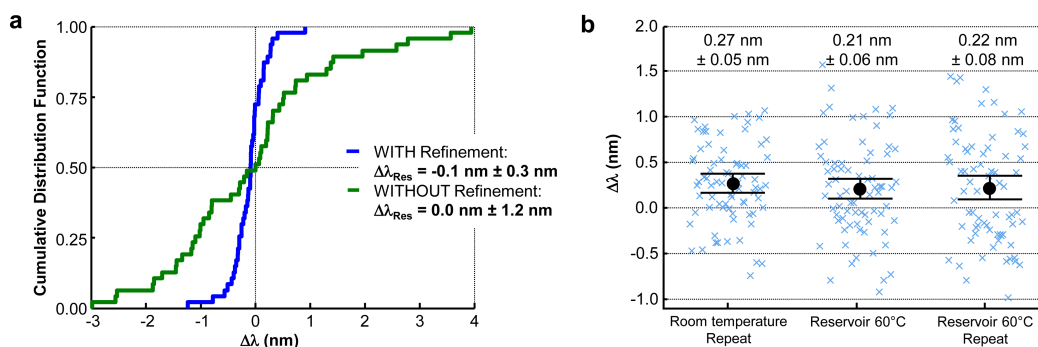
1. Acquisition of a true color image.
2. Recognition of the bright spots in the image.
3. Acquisition of spectra of each bright spot including automated position refinement in X-position and Z-position (autofocus).

For acquisition of the spectrum, each identified bright spot needs to be centered individually below the entrance slit of the spectrograph, which is done by a piezo stage. The movement of the stage is limited to an area of  $200\text{ }\mu\text{m} \times 200\text{ }\mu\text{m}$ , so only particles located in this area can be measured. For optimal positioning of the bright spot below the slit, we use an automated refinement in X-position. Within this X-refinement, the bright spot is moved in steps of  $0.25\text{ }\mu\text{m}$  over a distance of  $1.5\text{ }\mu\text{m}$  below the slit and spectra with an exposure time of 50 ms are acquired. The position with maximum intensity is identified as optimum. Afterwards, the focus is optimized by a refinement in Z-position. The Z-focus moves in steps of  $0.2\text{ }\mu\text{m}$  over a distance of  $1.2\text{ }\mu\text{m}$  and again spectra with an exposure time of 50 ms are acquired. As for the X-refinement, the Z-position with maximum intensity is identified as optimum. Finally, the spectrum of the particle is collected at the optimal X- and Z-position as an average of 3 individual spectra having each an exposure time of 250 ms. Using the refinement method with the described settings, we are able to investigate about 12 particles per minute.

The position refinement results in measurement repeatability with a standard deviation of 0.3 nm, compared to a standard deviation of about 1.2 nm without any refinement (Figure D.5a). This improved accuracy was a key factor for the successful detection of protein targets as it greatly reduces the number of particles needed for meaningful statistics and hence reduced the overall measurement time to manageable dimensions.

In order to test for temperature effects, we measure gold nanorods in water of different temperatures (room temperature and  $60^{\circ}\text{C}$ ). Figure D.5b shows the resonance wavelength shift of 74 particles compared to a measurement

at room temperature. Repeated measurements at the same temperature give comparable shifts (due to the measurement accuracy) as for changing the temperature. Thus, we can rule out a significant effect of temperature within normal operating conditions on the plasmon wavelength making a controlled temperature obsolete.



**Figure D.5.:** a) Measurement repeatability with (blue) and without (green) position refinement. Measuring the same 47 particles under identical conditions twice (repeated) results in a median plasmon shift of  $-0.1 \text{ nm} \pm 0.3 \text{ nm}$  (median and s.d.) with position refinement, compared to  $0.0 \text{ nm} \pm 1.2 \text{ nm}$  (median and s.d.) with no refinement. b) Temperature influence on the shift magnitude. Measuring in a flow of water, which comes from a reservoir having either room temperature or  $60^\circ\text{C}$ , results in the same average plasmon shift. Hence, there is no observable temperature influence in the investigated temperature range.

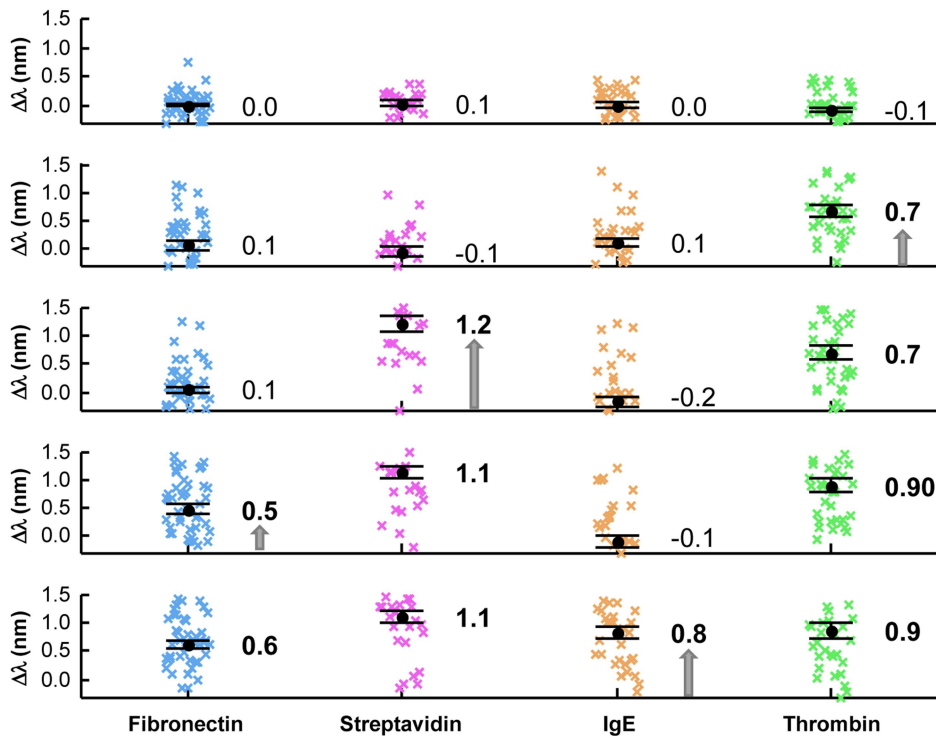
## D.6. Additional Data for Multiplexed Sensing

Table D.1 shows the statistical data displayed in Figure 6.4.

In an additional experiment, we added all proteins consecutively using the protocol described for Figure 6.4. The order of target addition is: Thrombin, Streptavidin, Fibronectin, IgE. Each matching particle group showed a significant response in each step, demonstrating the orthogonality of all particle groups.

**Table D.1.:** Statistics of Figure 6.4

|                               | <b>Fib</b> | <b>Str</b> | <b>IgE</b> | <b>Thr</b> | <b>#</b> | <b><math>\Delta\lambda &gt; 0.7 \text{ nm}</math></b> |
|-------------------------------|------------|------------|------------|------------|----------|-------------------------------------------------------|
| <b>No target</b>              |            |            |            |            |          |                                                       |
| # particles                   | 52         | 64         | 90         | 66         | 272      | 7                                                     |
| Median                        | 0.11       | 0.04       | 0.10       | 0.16       |          |                                                       |
| s.e.m.                        | 0.03       | 0.03       | 0.03       | 0.02       |          |                                                       |
| <b>Fibronectin (Fib)</b>      |            |            |            |            |          |                                                       |
| # particles                   | 40         | 23         | 56         | 28         | 147      | 52                                                    |
| Median                        | 0.95       | 0.21       | 0.28       | 0.23       |          |                                                       |
| s.e.m.                        | 0.10       | 0.11       | 0.06       | 0.11       |          |                                                       |
| <b>Streptavidin (Str)</b>     |            |            |            |            |          |                                                       |
| # particles                   | 51         | 43         | 40         | 33         | 167      | 41                                                    |
| Median                        | -0.01      | 1.51       | -0.43      | -0.38      |          |                                                       |
| s.e.m.                        | 0.08       | 0.13       | 0.10       | 0.11       |          |                                                       |
| <b>Immunoglobulin E (IgE)</b> |            |            |            |            |          |                                                       |
| # particles                   | 68         | 69         | 57         | 62         | 256      | 52                                                    |
| Median                        | 0.24       | -0.17      | 0.95       | 0.01       |          |                                                       |
| s.e.m.                        | 0.06       | 0.05       | 0.07       | 0.05       |          |                                                       |
| <b>Thrombin (Thr)</b>         |            |            |            |            |          |                                                       |
| # particles                   | 48         | 27         | 33         | 43         | 151      | 31                                                    |
| Median                        | 0.06       | -0.05      | 0.12       | 0.69       |          |                                                       |
| s.e.m.                        | 0.08       | 0.09       | 0.07       | 0.11       |          |                                                       |
| <b>Str and Thr</b>            |            |            |            |            |          |                                                       |
| # particles                   | 52         | 64         | 90         | 66         | 272      | 95                                                    |
| Median                        | 0.17       | 1.38       | 0.22       | 0.65       |          |                                                       |
| s.e.m.                        | 0.05       | 0.11       | 0.04       | 0.06       |          |                                                       |



**Figure D.6.:** Consecutive detection of Thrombin, Streptavidin, Fibronectin, and IgE.

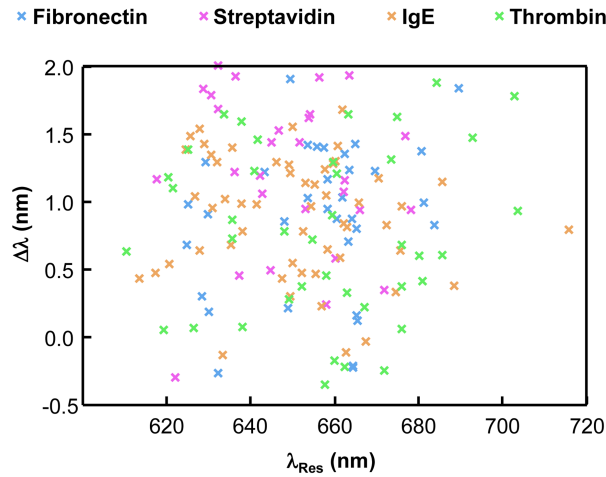
## D.7. Dependence of Shift Magnitude on Resonance Wavelength

It is well known that the sensitivity of gold nanorods (shift in resonance wavelength per refractive index unit change in the surrounding) increases with aspect ratio. Accordingly, particles with a higher resonance wavelength are supposed to show larger shifts. We check for this effect in Figure D.7. However, within the small variation of resonance wavelengths (from 620 nm to 680 nm), this effect is hidden in the overall scattering caused by measurement uncertainty. The scattering is therefore caused by other effects.

## D.8. Extent of Target Binding

We analyze in more detail the protein coverage on the particles using the data from Figure 6.2 (performance of the sensor). We define a complete surface coverage  $\Theta$  as the maximal plasmon shift  $\Delta\lambda_{\text{end}}$  (extrapolated to infinite incubation time and maximum protein concentration). The plas-





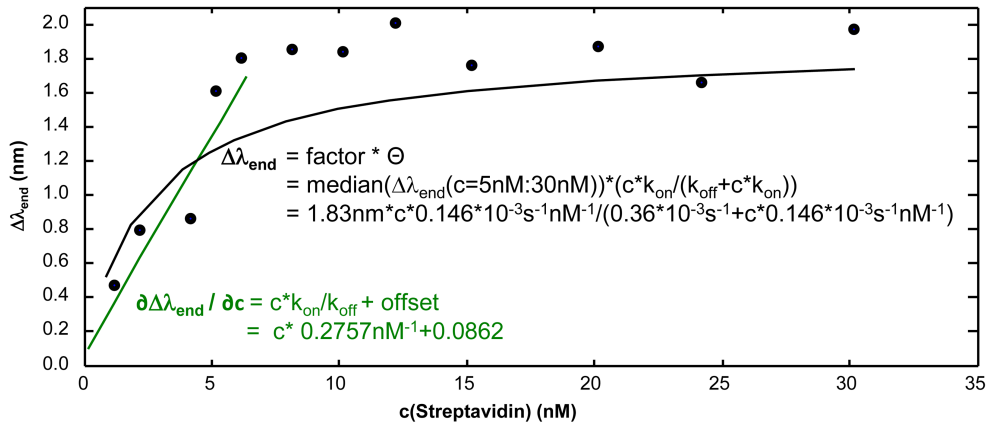
**Figure D.7.:** Dependence of plasmon shift on resonance wavelength.

mon shift  $\Delta\lambda_{\text{end}}$  reaches a constant value already for concentrations above 5 nM, which lets us determine the proportionality factor to convert plasmon shift to surface coverage from the mean value between 5 nM and 30 nM as  $factor = 1.83 \text{ nm}$ . Simple adsorption theory predicts an equilibrium surface coverage of  $\Theta = ck_{\text{on}}/(k_{\text{off}} + ck_{\text{on}})$  which is shown as black line in Figure D.8 using the values for  $k_{\text{on}}$  and  $k_{\text{off}}$  we determined before. The agreement is reasonable well demonstrating the self-consistency of the parameters.

For lower Streptavidin concentrations (between 1 nM and 5 nM), there is an approximately linear relation between  $\Delta\lambda_{\text{end}}$  and the protein concentration (displayed as green line) with a slope of  $0.2757 \text{ nM}^{-1}$ . This value gives a measure of the sensitivity of our sensors to changes in concentration in the low concentration regime (below 5 nM), which is mostly a measure for the receptor target binding affinity.

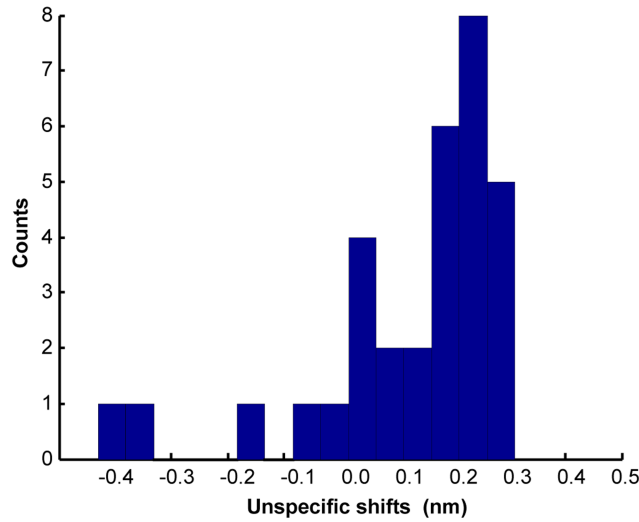
## D.9. Determination of the Significant Target Detection Limit $L$ for a Mapped Sensor

We conducted a total of 15 measurements where a group of at least 20 particles should not respond to the analyte due to non-matching functionalization. The resulting histogram (Figure D.9) shows that none of these measurements showed a median shift of more than 0.3 nm. To be on the safe side, we chose  $L = 0.5 \text{ nm}$  as a threshold value for the median shift of functionalized particles to identify positive target binding, i.e. we define a



**Figure D.8.:** Dependence of plasmon shift on resonance wavelength.

positive sensor response to target  $i$  if the corresponding group of particles shows a median plasmon shift significantly above 0.5 nm.



**Figure D.9.:** Histogram for 15 experiments showing non-specific sensor response.

## D.10. Determination of the Significant Target Detection Limit $L$ for an Unmapped Sensor

To evaluate the response of an unmapped sensor, we simply set a limit  $L$  and determine the fraction of particles with shifts  $p$  above this limit ('positives'). We define a significant response simply by requiring this fraction  $p$

to be significantly larger than the corresponding fraction  $p_0$  without target molecules present.  $L$  needs to be chosen in such a way that it minimizes the probability of finding a particle that randomly shifts above this limit ('false positive') and maximizes the probability that a particle that should show a positive response actually shifts above this limit ('true positive').

In an ideal situation, after adding target  $i$ ,  $p$  should correspond to the fraction of particles of type  $i$ . The choice of the 'significance limit'  $L$  determines how many false positives  $p_{\text{false}}$  (i.e. particles showing significant shift although not targeting the analyte) and true positives  $p_{\text{true}}$  (i.e. particles showing significant shift due to intended target detection) are counted. Without target molecules, the number of false positive shifts should fluctuate around the sensor ground state  $p_0 = p_{\text{false}}N$  according to Poisson statistics by  $\pm\sqrt{p_{\text{false}}N}$ . With the target molecules, one expects to find  $p_{\text{true}}N/n$  shifted particles, yielding  $p_{\text{true}}N/n \geq \sqrt{p_{\text{false}}N}$  for significant target detection. To be able to identify a large number of different analytes  $n$  with a given sensor containing  $N$  particles, one needs to optimize  $p_{\text{true}}/\sqrt{p_{\text{false}}} \equiv \alpha$ .

In order to determine an optimal value of  $L$ , we varied  $L$  between 0.1 nm and 0.9 nm and determined fraction of true positives (particles showing a shift above  $L$  due to presence of the matching analyte, Table D.2) and false positives (particles showing a shift above the limit although no matching analyte was present, Table D.3). The optimal  $L$  is given by the biggest value for  $\alpha$  (Table D.4). For our aptamers, the choice of  $L = 0.7$  nm yields the best results. Table D.5 summarizes the data for this choice of  $L$  in simplified form.

**Table D.2.:** True positive values  $p_{\text{true}}$  (median from 3-5 experiments)

| $L/\text{nm}$ | 0.10 | 0.20 | 0.30 | 0.40 | 0.50 | 0.60 | 0.70 | 0.80 | 0.90 |
|---------------|------|------|------|------|------|------|------|------|------|
| <b>Fib</b>    | 0.88 | 0.80 | 0.77 | 0.77 | 0.74 | 0.74 | 0.69 | 0.67 | 0.54 |
| <b>Str</b>    | 0.91 | 0.90 | 0.89 | 0.89 | 0.89 | 0.79 | 0.79 | 0.79 | 0.79 |
| <b>IgE</b>    | 0.83 | 0.81 | 0.78 | 0.74 | 0.72 | 0.68 | 0.59 | 0.54 | 0.47 |
| <b>Thr</b>    | 0.83 | 0.79 | 0.73 | 0.69 | 0.64 | 0.58 | 0.47 | 0.40 | 0.32 |

**Table D.3.:** False positive values  $p_{\text{false}}$  (median from 4-7 experiments)

| $L/\text{nm}$ | 0.10 | 0.20 | 0.30 | 0.40 | 0.50 | 0.60 | 0.70 | 0.80 | 0.90 |
|---------------|------|------|------|------|------|------|------|------|------|
| <b>Fib</b>    | 0.52 | 0.46 | 0.35 | 0.25 | 0.17 | 0.15 | 0.12 | 0.08 | 0.08 |
| <b>Str</b>    | 0.50 | 0.39 | 0.25 | 0.23 | 0.20 | 0.16 | 0.16 | 0.13 | 0.09 |
| <b>IgE</b>    | 0.50 | 0.39 | 0.30 | 0.21 | 0.18 | 0.15 | 0.12 | 0.09 | 0.09 |
| <b>Thr</b>    | 0.51 | 0.41 | 0.31 | 0.29 | 0.25 | 0.18 | 0.13 | 0.10 | 0.09 |

**Table D.4.:**  $\alpha \equiv p_{\text{true}}/\sqrt{p_{\text{false}}}$ 

| $L/\text{nm}$ | 0.10 | 0.20 | 0.30 | 0.40 | 0.50 | 0.60 | 0.70 | 0.80 | 0.90 |
|---------------|------|------|------|------|------|------|------|------|------|
| <b>Fib</b>    | 1.22 | 1.18 | 1.30 | 1.54 | 1.79 | 1.91 | 1.99 | 2.37 | 1.91 |
| <b>Str</b>    | 1.29 | 1.44 | 1.78 | 1.86 | 1.99 | 1.98 | 1.98 | 2.19 | 2.63 |
| <b>IgE</b>    | 1.17 | 1.30 | 1.42 | 1.61 | 1.70 | 1.76 | 1.70 | 1.80 | 1.57 |
| <b>Thr</b>    | 1.16 | 1.23 | 1.31 | 1.28 | 1.28 | 1.37 | 1.30 | 1.26 | 1.07 |

**Table D.5.:** Statistical values evaluated for a limit of  $L = 0.7$  nm

|                     | $p_{\text{true}}$ | $p_{\text{false}}$ | $\alpha$ |
|---------------------|-------------------|--------------------|----------|
| <b>Fibronectin</b>  | 69%               | 12%                | 1.99     |
| <b>Streptavidin</b> | 79%               | 16%                | 1.9      |
| <b>IgE</b>          | 59%               | 12%                | 1.70     |
| <b>Thrombin</b>     | 47%               | 13%                | 1.30     |

## D.11. Statistics for Up-Scaling a Mapped Sensor

We want to determine the minimal number of particles per group  $n_{\text{min}}$  needed to ensure a significant response of the group. For a confidence level of 95%, the median has to exceed the limit  $L$  by two standard errors ( $2s.e.m. = 2s.d./\sqrt{n_{\text{min}}}$ ):

$$\frac{2s.d.}{\sqrt{n_{\text{min}}}} + \text{median} = L \Leftrightarrow n_{\text{min}} = \left(\frac{2s.d.}{L - \text{median}}\right)^2 \quad (\text{D.1})$$

Table D.6 shows those numbers for  $n_{\text{min}}$ . Between 3 and 58 nanoparticles are enough for reliable detection.

**Table D.6.:** Statistics of sensor experiments and calculated number of particles needed for 95% confidence in target detection

|                     | <b>N</b> | <b>median</b> | <b>s.e.m.</b> | <b>s.d.</b> | $n_{\text{min}}$ |
|---------------------|----------|---------------|---------------|-------------|------------------|
| <b>Fibronectin</b>  | 40       | 0.95          | 0.10          | 0.63        | 8                |
| <b>Streptavidin</b> | 43       | 1.51          | 0.13          | 0.85        | 3                |
| <b>IgE</b>          | 57       | 0.95          | 0.07          | 0.53        | 6                |
| <b>Thrombin</b>     | 43       | 0.69          | 0.11          | 0.72        | 58               |

## D.12. Verification of Aptamer-Target Interaction by Surface Plasmon Resonance (SPR)

SPR measurements were conducted on Reichert SPR Instrument to check the response of eleven aptamers against selected proteins. One channel of the dual channel instrument was blocked with the thiolated mPEG ( $M_w$  5000 Da) as reference whereas the other channel was covered with thiolated aptamers. The aptamer containing channel was further passivated with thiolated mPEG as well to prevent unspecific protein binding to the gold surface. The procedure was performed in binding buffer throughout.

The channels were successively rinsed with BSA, non-target proteins and the target protein for 20 min. Only those aptamers, which show negligible affinity to BSA and non-specific targets, were finally selected for multiplexed sensing. Table D.7 shows the results with aptamers used in the study.

As shown in Table D.7, only 6 out of 11 aptamer sequences reported in literature responded to their respective targets in our tests. Lysozyme was found to bind transiently to the most other aptamers at a concentration of 2.85  $\mu$ M. This can be attributed to the positive charge of Lysozyme under the specified set of conditions and therefore electrostatic interaction with DNA. The other protein causing non-specificity is human neutrophil elastase (HNE). These non-specific interactions can be removed by prolong rinse with binding buffer (Figure D.10) and are therefore weak. Substituting the aptamers by a modified versions (such as Photoaptamers<sup>6</sup> and SOMAmers<sup>7</sup>) could be possible way to overcome the issue of non-specificity.

The SPR sensograms demonstrating specific detection of Fibronectin, Streptavidin, Thrombin and IgE are shown in Figure D.11. Interferents like Lysozyme and HNE were excluded from this study as their meticulous detection demands an appropriate modification in existing aptamers.

<sup>6</sup> Bock et al., "Photoaptamer arrays applied to multiplexed proteomic analysis."

<sup>7</sup> Gold et al., "Aptamer-Based Multiplexed Proteomic Technology for Biomarker Discovery."

Table D.7.: Cross-talk studies on 11 different aptamers

| Target              | Sequence of DNA-aptamer                                                               | Cross reactivity      |
|---------------------|---------------------------------------------------------------------------------------|-----------------------|
| <b>Thrombin</b>     | 5'-SH-(T <sub>10</sub> )-GGTTGGTGTGGTTGG-3' <sup>8</sup>                              | Lysozyme              |
| <b>Fibronectin</b>  | 5'-SH-(T <sub>10</sub> )-AGTCCGTGGTAGGGCAGGTTGGGG-TGACT-3' <sup>9</sup>               | Lysozyme              |
| <b>IgE</b>          | 5'-SH-(T <sub>10</sub> )-CATGGCGCTTCCCCCTGTGGTTGGT-GTCAGTCGGCCTGTG-3' <sup>10</sup>   | HNE, Lysozyme         |
| <b>Streptavidin</b> | 5'-SH-(T <sub>10</sub> )-GGGGCAGTTTATCCGTCCCTCCTA-GTGGCGTGCCCC-3' <sup>11</sup>       | HNE, Lysozyme         |
| <b>HNE</b>          | 5'-SH-(T <sub>10</sub> )-CACGGTAGTGCTACCAGATGGTTAT-GTTAC-3' <sup>13</sup>             | Lysozyme              |
| <b>Lysozyme</b>     | 5'-SH-(T <sub>10</sub> )-ATCTACGAATTCATCAGGGCTAAAG-AGTGCAGAGTTACTTAG-3' <sup>14</sup> | -                     |
| <b>Insulin</b>      | 5'-SH-(T <sub>10</sub> )-GGTGGTGGGGGGGTTGGTAGGGT-GTCTTC-3' <sup>15</sup>              | Target binding failed |
| <b>PDGF</b>         | 5'-SH-(T <sub>10</sub> )-CAGGCTACGGCAGCTAGAGCATCAC-CATGATCCTG-3' <sup>16</sup>        | Target binding failed |
| <b>VEGF</b>         | 5'-SH-(T <sub>10</sub> )-TGTGGGGGTGGACGGGCCGGGTAGA-3' <sup>17</sup>                   | Target binding failed |
| <b>L-Selectin</b>   | 5'-SH-(T <sub>10</sub> )-CAAGGTAACCAGTACAAGGTGCTAA-ACGTAATGGCTTCG-3' <sup>18</sup>    | Target binding failed |
| <b>Nucleolin</b>    | 5'-SH-(T <sub>10</sub> )-GGTGGTGGTGGTTGTGGTGGTGGT-GG-3' <sup>19</sup>                 | Target binding failed |

<sup>8</sup> Bock et al., "Selection of single-stranded DNA molecules that bind and inhibit human thrombin."

<sup>9</sup> Tasset et al., "Oligonucleotide inhibitors of human thrombin that bind distinct epitopes."

<sup>10</sup> Ogawa et al., "Aptamer selection for the inhibition of cell adhesion with fibronectin as target."

<sup>11</sup> Wang et al., "Surface plasmon resonance imaging for affinity analysis of aptamer-protein interactions with PDMS microfluidic chips."

<sup>12</sup> Bing et al., "Bifunctional combined aptamer for simultaneous separation and detection of thrombin."

<sup>13</sup> Charlton et al., "In vivo imaging of inflammation using an aptamer inhibitor of human neutrophil elastase."

<sup>14</sup> Cheng et al., "Aptamer-Based Biosensors for Label-Free Voltammetric Detection of Lysozyme."

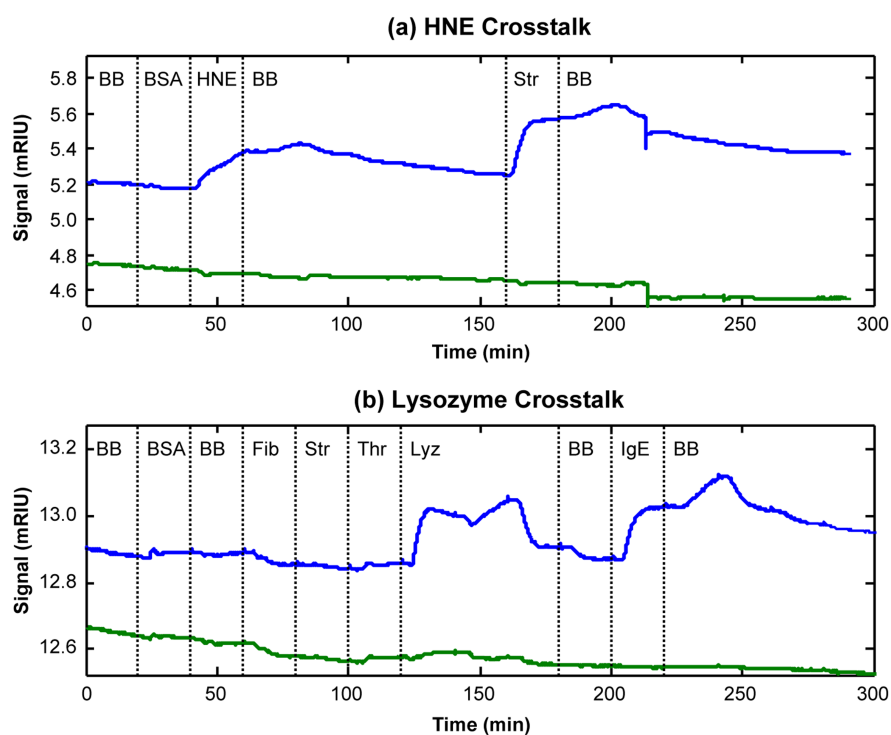
<sup>15</sup> Cha et al., "Optical Nanosensor Architecture for Cell-Signaling Molecules Using DNA Aptamer-Coated Carbon Nanotubes."

<sup>16</sup> Fang et al., "Synthetic DNA Aptamers to Detect Protein Molecular Variants in a High-Throughput Fluorescence Quenching Assay."

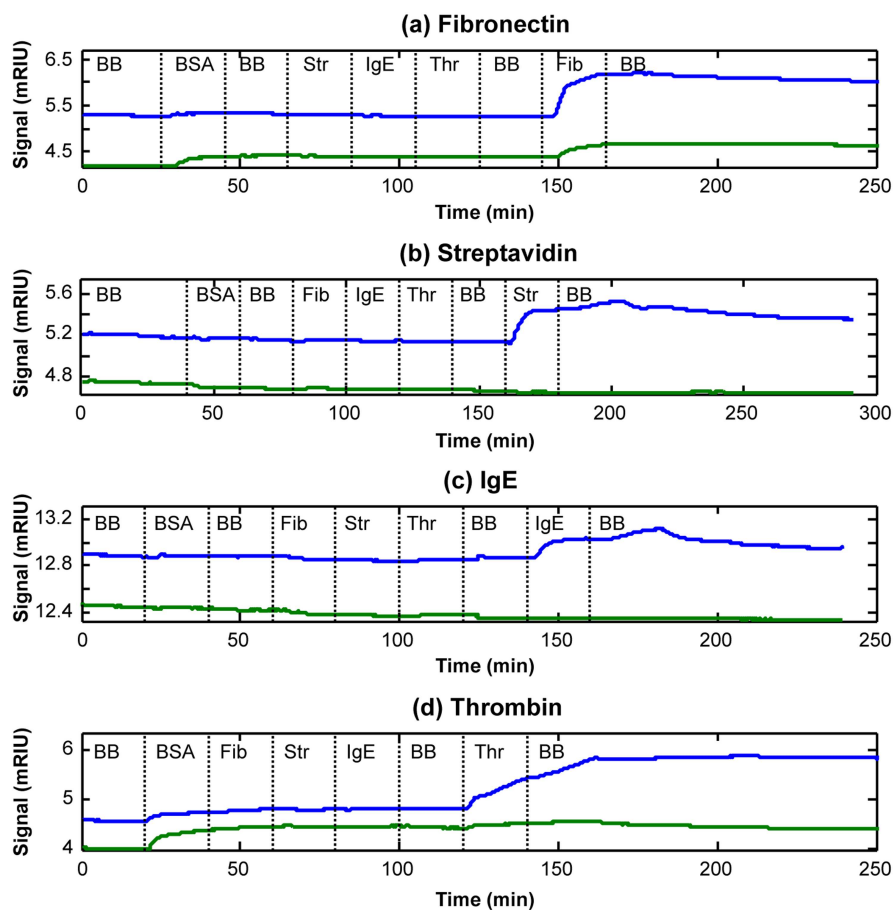
<sup>17</sup> Nonaka et al., "Screening and Improvement of an Anti-VEGF DNA Aptamer."

<sup>18</sup> Hicke et al., "DNA aptamers block L-selectin function in vivo. Inhibition of human lymphocyte trafficking in SCID mice."

<sup>19</sup> Soundararajan et al., "The Nucleolin Targeting Aptamer AS1411 Destabilizes Bcl-2 Messenger RNA in Human Breast Cancer Cells."



**Figure D.10.:** SPR response showing **a)** HNE cross talk in anti-streptavidin aptamer, **b)** Lysozyme cross talk in anti-IgE aptamer. Green - reference, blue - sample. BB: binding buffer, BSA: Bovine Serum Albumin, Fib: Fibronectin, Str: Streptavidin, Thr: Thrombin, Lyz: Lysozyme, HNE: human neutrophil elastase.



**Figure D.11.:** SPR response showing aptamer-target specific interaction for **a)** anti-fibronectin aptamer, **b)** anti-streptavidin aptamer, **c)** anti-IgE aptamer, **d)** anti-thrombin aptamer. Green - reference, blue - sample. BB: binding buffer, BSA: Bovine Serum Albumin, Fib: Fibronectin, Str: Streptavidin, Thr: Thrombin, Lyz: Lysozyme, HNE: human neutrophil elastase.



## D.13. References

1. Novo, C.; Funston, A. M.; Mulvaney, P. *Nature Nanotechnology* **2008**, *3*, 598–602.
2. Hanauer, M.; Pierrat, S.; Zins, I.; Lotz, A.; Sönnichsen, C. *Nano Letters* **2007**, *7*, 2881–2885.
3. Prescott, S. W.; Mulvaney, P. *Journal of Applied Physics* **2006**, *99*, 123504; Corrigendum: **2008**, *103*, 119901.
4. Nusz, G. J.; Curry, A. C.; Marinakos, S. M.; Wax, A.; Chilkoti, A. *ACS Nano* **2009**, *3*, 795–806.
5. Lin, P.-H.; Chen, R.-H.; Lee, C.-H.; Chang, Y.; Chen, C.-S.; Chen, W.-Y. *Colloids and Surfaces B: Biointerfaces* **2011**, *88*, 552–558.
6. Bock, C. et al. *Proteomics* **2004**, *4*, 609–618.
7. Gold, L. et al. *PLoS ONE* **2010**, *5*, e15004.
8. Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J. *Nature* **1992**, *355*, 564–566.
9. Tasset, D. M.; Kubik, M. F.; Steiner, W. *Journal of Molecular Biology* **1997**, *272*, 688–698.
10. Ogawa, A.; Tomita, N.; Kikuchi, N.; Sando, S.; Aoyama, Y. *Bioorganic & Medicinal Chemistry Letters* **2004**, *14*, 4001–4004.
11. Wang, Z.; Wilkop, T.; Xu, D.; Dong, Y.; Ma, G.; Cheng, Q. *Analytical and Bioanalytical Chemistry* **2007**, *389*, 819–825.
12. Bing, T.; Liu, X.; Cheng, X.; Cao, Z.; Shangguan, D. *Biosensors and Bioelectronics* **2010**, *25*, 1487–1492.
13. Charlton, J.; Sennello, J.; Smith, D. *Chemistry & Biology* **1997**, *4*, 809–816.
14. Cheng, A. K. H.; Ge, B.; Yu, H.-Z. *Analytical Chemistry* **2007**, *79*, 5158–5164.
15. Cha, T.-G.; Baker, B. A.; Sauffer, M. D.; Salgado, J.; Jaroch, D.; Rickus, J. L.; Porterfield, D. M.; Choi, J. H. *ACS Nano* **2011**, *5*, 4236–4244.
16. Fang, X.; Sen, A.; Vicens, M.; Tan, W. *ChemBioChem* **2003**, *4*, 829–834.
17. Nonaka, Y.; Sode, K.; Ikebukuro, K. *Molecules* **2010**, *15*, 215–225.
18. Hicke, B. J. et al. *The Journal of Clinical Investigation* **1996**, *98*, 2688–2692.

19. Soundararajan, S.; Chen, W.; Spicer, E. K.; Courtenay-Luck, N.; Fernandes, D. J. *Cancer Research* **2008**, *68*, 2358–2365.

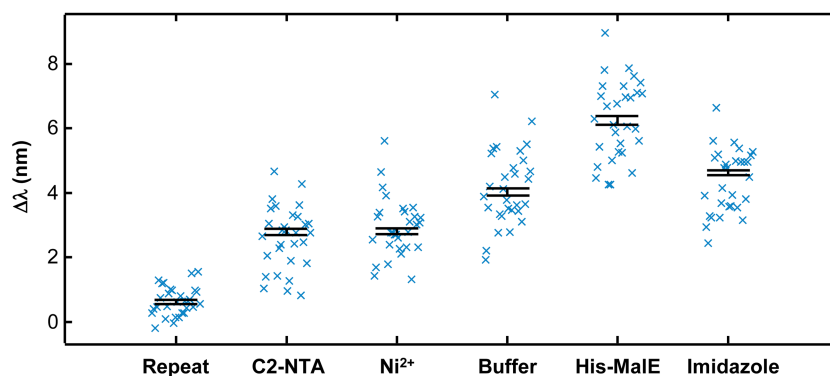
# Plasmonic Nanoparticles as Sensors for Simultaneous Quantification of Protein-Protein Interactions

## E.1. Materials

Trizma hydrochloride (Tris-HCl), potassium chloride (KCl), magnesium chloride hexahydrate ( $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ ), sodium dodecyl sulfate (SDS), tris(2-carboxyethyl)phosphine (TCEP), nickel chloride hexahydrate ( $\text{NiCl}_2 \times 6\text{H}_2\text{O}$ ), and imidazole were purchased from Sigma. Sterile Anotop 25 disposable syringe filters with a pore size of 200 nm were purchased from VWR. 3,3'-dithiobis[N-(5-amino-5-carboxypentyl)propionamide-N,N'-diacetic acid] dihydrochloride (C2-NTA) was purchased from Dojindo.

## E.2. Particle Functionalization in Flow Cell

To avoid aggregation during nanoparticle functionalization, we deposited gold nanorods in a flow cell. After washing with water, we incubated with an aqueous solution of 130  $\mu\text{M}$  C2-NTA over night. For complexation with nickel ions, particles were exposed to an aqueous solution of 200 mM  $\text{NiCl}_2$  for 30 minutes. Afterwards, we changed the environment from water to a buffer solution (50 mM Tris-HCl, 50 mM NaCl, pH 8) being suitable for the His-tagged test protein (maltose-binding protein from *Escherichia coli*, His-MalE). The protein was incubated for 45 minutes at a concentration of 104  $\mu\text{g}/\text{mL}$  in buffer for attachment to the NTA- $\text{Ni}^{2+}$  complex. Removal of the protein was achieved by incubation with 500 mM imidazole in buffer for 20 minutes.

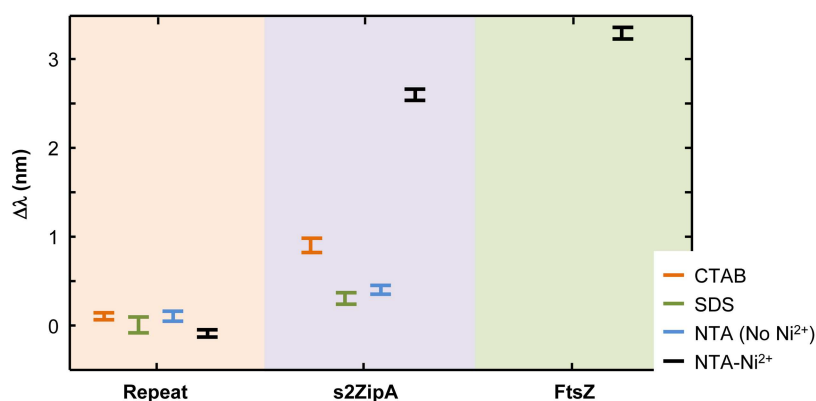


**Figure E.1.:** Successful nanoparticle functionalization is indicated by a positive shift in resonance wavelength due to the increase in refractive index of the particle surrounding compared to water. The nickel ions do not induce a plasmon response. A change from water to buffer induces a small shift due to increased ionic strength compared to water. Attachment of the His-MalE induces a plasmon response, again due to an increase in the refractive index of the particle surrounding, which reverts by exposure to imidazole indicating removal of the protein.

## E.3. Particles of Different Functionalization States Exposed to His-tagged s2ZipA

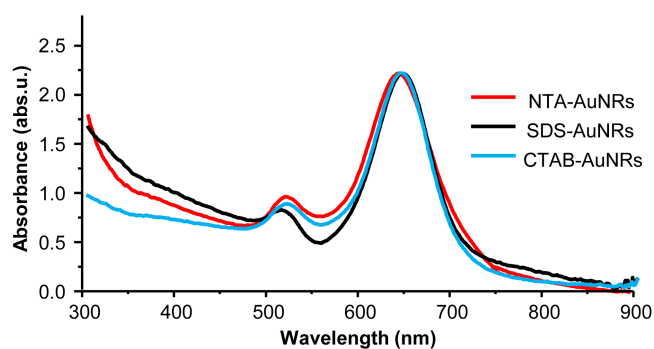
To investigate the specificity in attachment of His-tagged proteins to NTA-particles complexed with nickel ions, we exposed particles of different states in functionalization to 500 nM His-tagged s2ZipA for 30 minutes. The inves-

tigated states are: CTAB-particles as synthesized, SDS-particles after removal of SDS, NTA-particles without complexation with nickel ions, and NTA-particles complexed with nickel ions. Among these particle groups, only particles functionalized with NTA and complexed with nickel ions show a significant plasmon response reporting efficient attachment of His-tagged s2ZipA. The other groups show a small shift in plasmon response, which is attributed to unspecific adsorption of the protein. To proof functionality for interaction with a second protein, we exposed particles with attached s2ZipA to 1  $\mu$ M FtsZ. Successful protein-protein interaction is indicated by an increase of the plasmon shift.

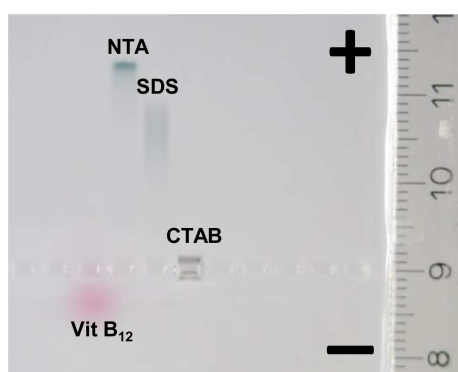


**Figure E.2.:** Particles with different coatings during functionalization are exposed to His-tagged s2ZipA. Only particles being properly functionalized with NTA and complexed with nickel ions for attachment of a His-tagged protein show a significant plasmon response. Hence, we conclude proper attachment of the protein via the His-tag. Furthermore, these particles with properly immobilized s2ZipA are able to interact with FtsZ, which is reported by a positive plasmon response after exposure to FtsZ.

## E.4. Particle Functionalization in Solution



**Figure E.3.:** UV visible spectra of gold nanorods in different states of the functionalization with NTA moieties. All spectra exhibit a similar full width at half maximum of the longitudinal plasmon peak which is a good indicator for a lack of aggregation.



**Figure E.4.:** In order to check successful nanoparticle functionalization, we performed gel-electrophoresis, where an applied external electric field is used to migrate particles of the same size according to their surface charge in a 0.5% Agarose gel. The image shows the unstained gel for the differently functionalized nanoparticles after application of an electric field of 150 V for 30 minutes. Particles stabilized with CTAB do not withstand the ionic strength of the buffer and aggregate in the well. Particles stabilized with SDS and particles functionalized with NTA moieties clearly migrate towards the positive pole indicating a negative surface charge, which is in agreement with the properties of the molecules attached to the particles.





## Appendix F.

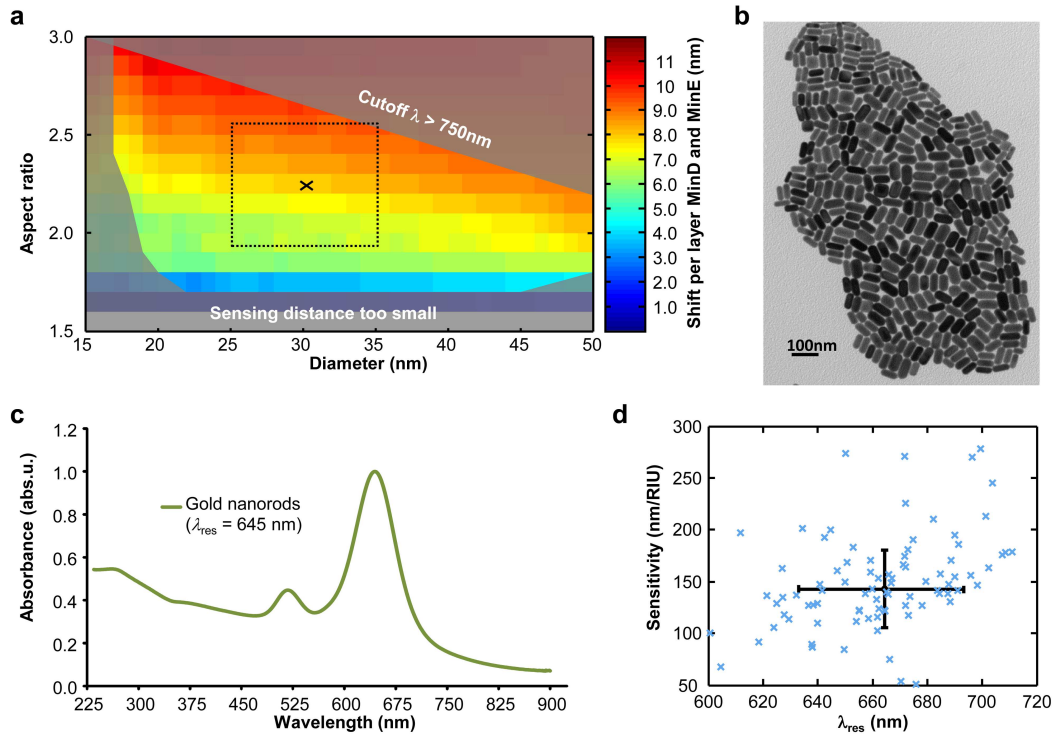
# F Single Particle Plasmon Sensors Reveal Constant Fraction of MinD Bound to the Membrane during Min Wave Propagation

### F.1. Materials

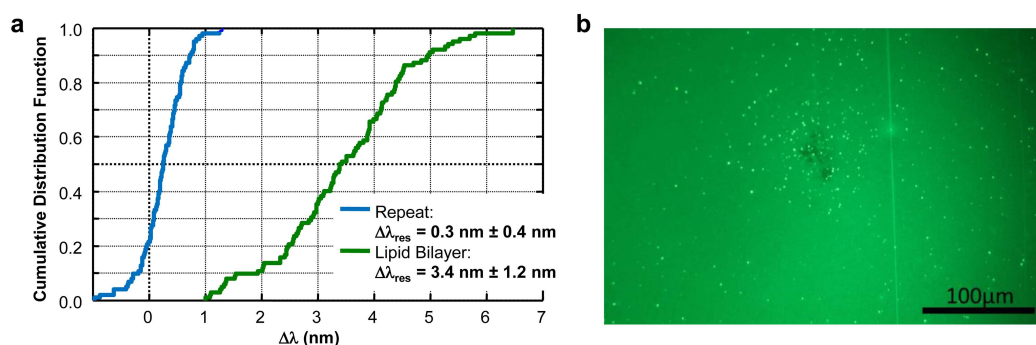
Trizma hydrochloride (Tris-HCl), potassium chloride (KCl), magnesium chloride hexahydrate ( $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ ), calcium chloride dihydrate ( $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ ), ADP, and ATP $\gamma$ S were purchased from Sigma. ATP was purchased from Roche Applied Science. *E.coli* polar extract was purchased from Avanti. Thiolated methoxy-polyethylene glycol (mPEG-SH,  $M_w$  735 Da) was purchased from Iris Biotech.

### F.2. Figures

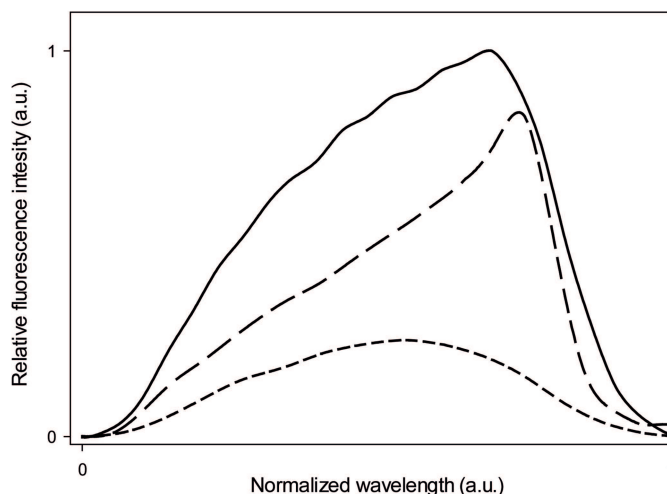
Using the Boundary Element Method (BEM), we calculated the resonance wavelength for particles covered with a lipid bilayer and for the same particles with the bilayer having an additional layer of MinD and MinE. From this, we determined the corresponding resonance shifts. Furthermore, we assessed the plasmonic sensitivity of the particles (resonance shift per refractive index unit change) by measuring the non-functionalized particles in water (refractive index  $n = 1.332$ ) and in a glycerol solution ( $n = 1.368$ ) making sure that the shift is reversible. There is no change in sensitivity with wavelength (aspect ratio), since the variation in dimensions is small.



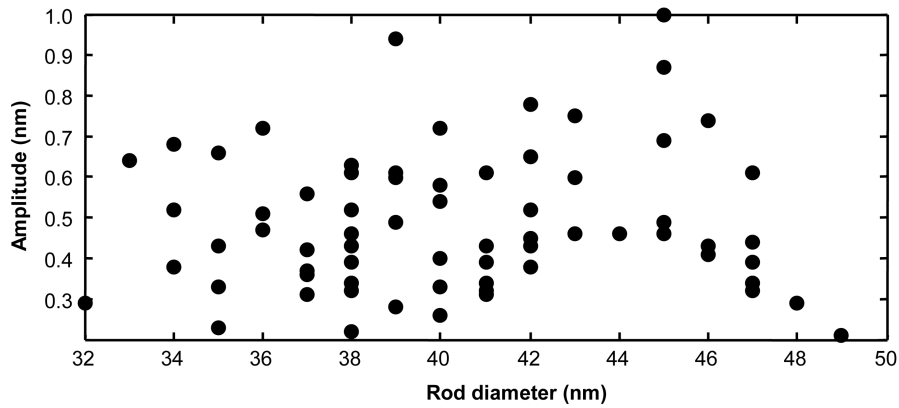
**Figure F.1:** **a)** Calculation of the shift in resonance wavelength induced by adding the Min system to particles covered with a lipid bilayer. The color code corresponds to the magnitude of the shift in resonance wavelength induced per layer MinD and MinE. On the lower and left part of the diagram (gray area), the particles have sensing distances too small to sense the presence of a MinD and MinE layer. We can only measure resonance wavelength up to 750 nm, thus restricting the accessible aspect ratios on the top area of the graph. This inaccessible areas are shaded grey. The mean diameter and the mean aspect ratio of the particles used for the sensor are indicated by a black cross. The dotted square around marks one standard deviation, showing which region is covered by the actual sample. **b)** Transmission electron microscopy image of the gold nanorods used to sense Min oscillations. The rods have a width of  $31.1\text{ nm} \pm 5.6\text{ nm}$  and a length of  $67.8\text{ nm} \pm 9.2\text{ nm}$  (mean and s.d. from 486 particles). **c)** Extinction spectrum of the gold nanorod sample. **d)** Plasmonic sensitivity ( $S = \Delta\lambda/\Delta n$ ) of the particles determined from a measurement in water (refractive index  $n = 1.332$ ) and glycerol ( $n = 1.368$ ), each blue cross corresponds to a single particle. Median and s.d. from 93 particles are marked in black (resonance wavelength  $664.2\text{ nm} \pm 31.8\text{ nm}$ ; sensitivity  $143.1\text{ nm/RIU} \pm 39.5\text{ nm/RIU}$ ). There is no change in sensitivity with wavelength (aspect ratio), since the variation of the dimensions is rather small.



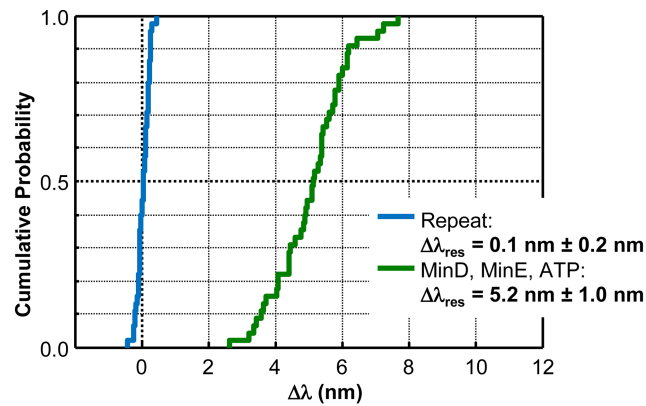
**Figure F.2.:** **a)** Covering the gold nanorods with a lipid bilayer as described in Section 8.4 shifts the plasmon resonance to longer wavelength ( $3.4 \text{ nm} \pm 1.2 \text{ nm}$ , median and s.d. from 102 particles). **b)** Fluorescence image of the same lipid bilayer measured in a), which was afterwards stained with Vybrant DiO (Molecular Probes). Staining was carried out by incubation with  $2 \mu\text{M}$  DiO for 30 minutes. Excess stain was removed by rinsing with SLB-buffer. The center of the image shows a defect in the bilayer, which were rarely present. The bright dots correspond to unfused vesicles, which were not removed by rinsing.



**Figure F.3.:** Relative fluorescence intensity for three different samples having a 1:3 ratio of protein:dye (in this case Cy3). One sample shows the change in relative fluorescence intensity for MinD (short dashed line), the other for MinE (long dashed line), and the third one for MinD and MinE (line). The displayed intensities are the average profile of three distinct waves, which are normalized to the maximum fluorescence intensity of the sample having labeled MinD and MinE while keeping the intensity ratio among the samples. The width of the profile is normalized to the average wavelength of the oscillations. The profile of the sample having MinD and MinE labeled with the same fluorescence dye reflects the change in surface coverage on the bilayer sensed by a single nanoparticle presented in Figure 8.2c.

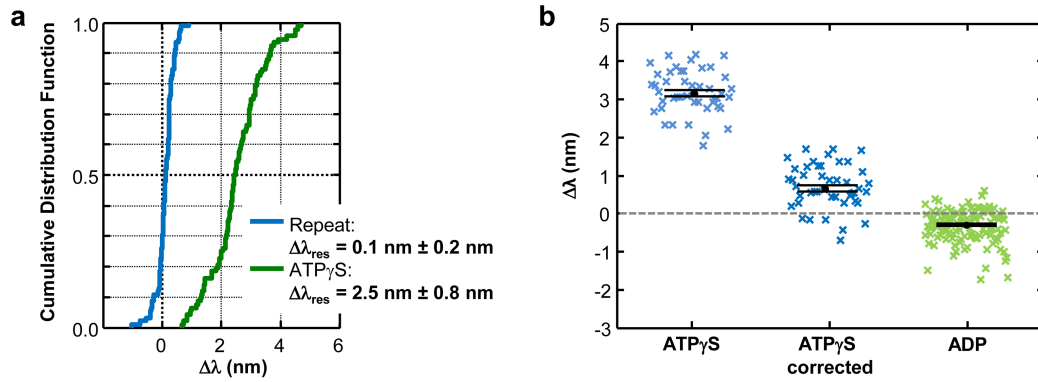


**Figure F.4.:** The gold nanorod diameter determines the sensing volume of the particle. In order to check for an optimal particle diameter showing the largest amplitude in the wavelength oscillation due to complete fill of the sensing volume by the Min system, we plot the amplitude depending on the nanorod diameter. Herein, we determined the nanorod dimensions from the single particle dark-field spectrum according to Henkel.<sup>1</sup> Among the particle diameters investigated, we do not observe a value showing predominantly large amplitudes.

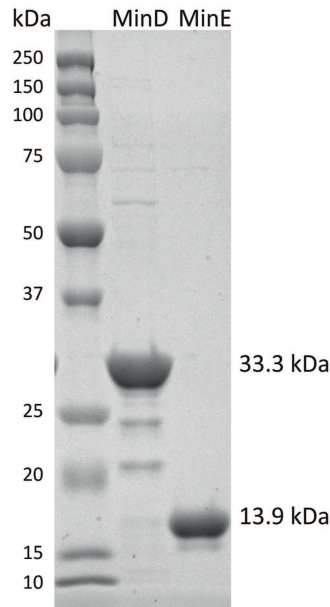


**Figure F.5.:** We added the Min system consisting of MinD, MinE, and ATP to gold nanorods covered with a lipid bilayer and waited for proper oscillation of the waves. Afterwards, we determined the median shift and its s.d. induced by the Min system, which amount to  $3.4 \text{ nm} \pm 1.2 \text{ nm}$  (statistics from 45 particles).

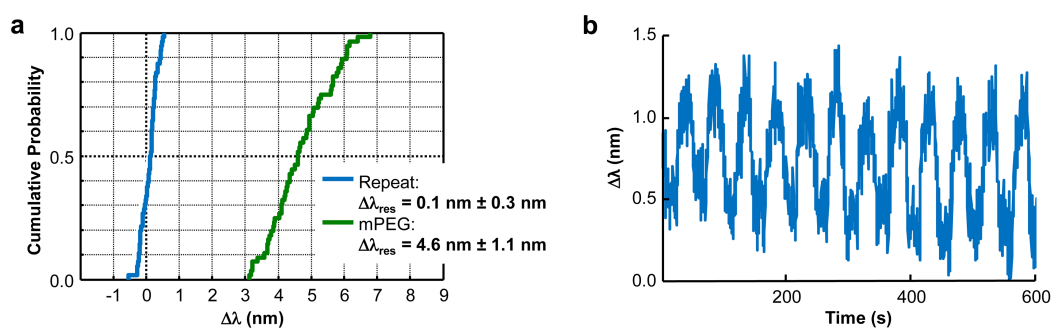
<sup>1</sup> Henkel, *Rod-Shaped Plasmonic Sensors - Synthesis and Single Particle Spectroscopy*.



**Figure F.6.:** a) Incubation of a lipid bilayer with ATP $\gamma$ S (1.25 mM in Min-buffer, 1 hour) results in a median shift and its s.d. of  $2.5 \text{ nm} \pm 0.8 \text{ nm}$  (statistics from 45 particles). Hence, ATP $\gamma$ S intercalates into the lipid bilayer. b) We fixed the Min system after showing waves using ATP $\gamma$ S. We observe a shift compared to the state of the Min system showing waves of  $3.2 \text{ nm} \pm 0.1 \text{ nm}$  (median and s.e.m. from 46 particles). Since ATP $\gamma$ S alone induces a shift of 2.5 nm, we correct for this offset and get a shift of  $0.7 \text{ nm} \pm 0.1 \text{ nm}$  (median and s.e.m. from 46 particles). Removal of the Min system after showing waves using ADP shows a shift of  $-0.3 \text{ nm} \pm 0.0 \text{ nm}$  (median and s.e.m. from 117 particles).



**Figure F.7.:** a) Coomassie stained 12% PAGE-SDS gel of the purified proteins MinD-Histag and MinE-Histag. 16  $\mu\text{g}$  of MinD and 8  $\mu\text{g}$  of MinE were loaded to each lane. From the intensities of the bands, we assume impurities amounting to 1% of the protein concentration.



**Figure F.8.:** **a)** Functionalization of the gold nanorods with mPEG-SH shifts the resonance wavelength by  $4.6 \text{ nm} \pm 1.1 \text{ nm}$  (median and s.d. from 56 particles). **b)** Using functionalized particles, we observe the same oscillation pattern in the resonance wavelength as using uncoated gold nanorods as sensors with a period of 50 s and an amplitude of 1 nm.

### F.3. References

1. Henkel, A., *Rod-Shaped Plasmonic Sensors - Synthesis and Single Particle Spectroscopy*; PhD Thesis: 2012.





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# Acknowledgement



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# Curriculum Vitae