Optoplasmonic detection of single particles and molecules in motion

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Introduction

Investigations on noble metal nanoparticles revealed that the electromagnetic field can be confined in a volume smaller than the wavelength of light. The confinement is due to the interaction of electromagnetic radiation and conduction electrons at metallic nanostructures, which leads to an enhancement of the optical properties such as scattering and absorption. Due to their interesting optical properties in their resonance frequency, noble metal nanoparticles have found many applications in biosensing and nanotechnology. Since the chemistry of gold is friendly and biocompatible, it enables molecular functionalization without oxidization; also the resonance of gold falls in to the visible to near-infrared range which makes the gold nanoparticles an appropriate choice for optical microscopies. In this thesis, we use gold nanoparticles, particularly nanorods to detect single nanoparticles and proteins. We use scattering signal as a high-bandwidth platform to detect small and fast changes of the scattering signal of gold nanorods caused by the diffusion of the single nanoparticles and molecules.

1.1 Optical properties of noble metal nanoparticles

Localized surface plasmon resonance (LSPR) is a phenomenon that occurs in noble-metal nanoparticles with dimensions smaller than, or comparable to the wavelength of the incident light. Due to high free-electron densities and nano-scale sizes of metal nanoparticles, a dipole is created as electrons within the metal outer layer oscillate at frequencies comparable to that of the applied light. This effect is especially noticeable for wavelengths which overlap with the localized surface plasmon resonance (LSPR) band, and yields the evanescent waves and a local electric field around the nanoparticle [1]. For gold and silver nanoparticles the resonance falls into the visible to near-infrared region of the electromagnetic spectrum. Gustav Mie in 1908 [2] provided an exact solution of Maxwell's equations for the scattering of a plane wave incident on a spherical gold nanoparticle. His solution showed an enhancement of the absorption and scattering of the nanoparticles at resonance.

The excitation of LSPR bands generates enhanced electromagnetic fields in regions of space that are controllable through the particle shape. The fields themselves give rise to light absorption and scattering depending on physical and chemical parameters of the nanoparticles [3]. A broad range of applications take advantage of the enhanced absorption and scattering of gold and silver nanoparticles [4–6]. For example, much work has been done to use gold nanoparticles as labels for biophysical studies by attaching a nanoparticle to a biomolecule of interest [7–9]; chemical inertness and photostability of gold nanoparticles make them suitable for biochemical applications.

When a nanoparticle is excited by an electric field, the nanoparticle responds by acquiring an electric dipole moment which varies linearly with the applied electric field. The tensor connecting these two vector quantities is called polarizability [10]. For particles much smaller than the wavelength of light, the polarizability α of a spherical nanoparticle with volume V is isotropic and can be written as:

$$\alpha = 3\epsilon_0 V \frac{\epsilon(\omega) - \epsilon_m}{\epsilon(\omega) + 2\epsilon_m}, \tag{1.1}$$

where ϵ_0 is the permittivity of vacuum, $\epsilon(\omega)$ is the permittivity of the metal as a function of the incoming excitation frequency ω and ϵ_m is the permittivity of the surrounding medium [11, 12]. The absorption and scattering cross section can thus be calculated as:

$$\sigma_{abs} = kIm(\alpha) \quad and \quad \sigma_{sca} = \frac{k^4}{6\pi}|\alpha|^2, \tag{1.2}$$

respectively, where $k = \omega n_m/c$ is the wavevector of the light in the surrounding medium. From equation 1.1 it is possible to see that a resonance will appear when $Re(\epsilon(\omega)) = -2\epsilon_m$. The energy of this resonance depends not only on the particle's material properties but also on the surrounding medium's optical constants. For elongated nanoparticles, some correction factors must be introduced to the polarizability [12].

Gold nanorod

The width, position and number of surface plasmon resonance (SPRs) are determined by the shape of the gold nanoparticle [13]. The spatial distribution of the polarization charges over the surface and the separation of positive and negative charges depend on the size and shape of the nanoparticle [14, 15]. Throughout this thesis, we will perform investigations based on gold nanorods (GNR)s. Wet-chemically-synthesized gold nanorods have attracted attention due to their chemical stability. Their controllable synthesis provides tunability of their diameter and length. GNRs as elongated nanoparticles support two primary plasmonic modes, the transverse mode and the longitudinal mode, corresponding to electron oscillations perpendicular or parallel to the longitudinal axis, respectively [16]. Intraband absorption of gold is at wavelengths between 200 nm and 550 nm and causes strong damping and broadening of the plasmon resonance of gold nanospheres, which falls around 520 nm. However, elongated particles such as nanorods present a longitudinal resonance significanlty red-shifted from the interband transitions of gold. Therefore, the damping effect is much weaker for GNRs and gives rise to a narrow plasmon resonance band. Also, the longitudinal resonance peak wavelength of GNRs strongly depends on their aspect ratio (length divided by diameter) and consequently provides tunability for different applications. GNRs also provide a very large field enhancement near their tips which has been widely utilized for single-molecule or -particle detection by fluorescence enhancement or Raman scattering. The longitudinal plasmon modes of GNRs are also very sensitive to the surrounding environment [17, 18].

1.2 Refractive-index-based plasmonic detection

A change in dielectric constant, corresponding to a change in the refractive index of the surrounding medium of a gold nanostructure has been used for label-free optical biosensing [19–21]. The investigation of single plasmonic nanoparticles makes it possible to perform observation at the single-molecule level [22]. So, single metal-

lic nanoparticles [23, 24] have joined dielectric resonators [25–27] as possible tools to detect single molecules [28] or particles [29] thanks to their confined electric field in a small volume. There are some advantages in the refractive-index-based plasmonic detection over fluorescence-based detection techniques which make the label-free method promising for future application. Fluorescence-based detection techniques require chemical attachment of the dye molecule to the target molecule and the detection bandwidth is limited by the comparatively slow fluorescence emission of the dye. Also, the observation time is restricted by the dye blinking and bleaching. So, detecting fast biochemical processes (in the nanosecond time scale) by fluorescence techniques is challenging and requires thousands of single molecules [30]. Therefore, label-free methods with high bandwidth able to resolve such fast processes are highly desirable. Optoplasmonic methods provide promising high-bandwidth sensing platforms, and their single-molecule sensitivity has been demonstrated repeatedly [16, 31–34].

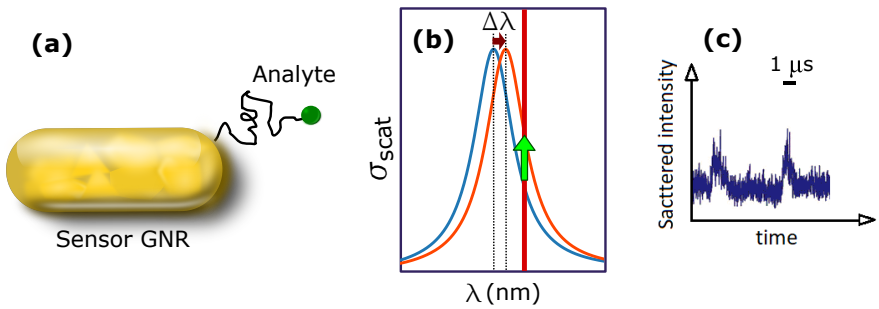

Figure 1.1: **a)** Schematic of a sensor GNR with a freely diffusing analyte through the near-field volume of the GNR. **b)** Schematic of the scattering cross section σ_{scat} changes of the sensor GNR due to the increase of the local refractive index around the sensor rod which yields a red shift in the spectrum. The wavelength of the probe laser has been shown by a vertical red line. **c)** Experimental results of the scattered intensity of a GNR as a function of time, showing clear fast fluctuations due to the diffusion of 5 nm gold nanospheres around the sensor GNR.

In this thesis we have used a single GNR as a plasmonic sensor to detect single molecules and particles. As shown in figure 1.1 the presence of an analyte in the near-field volume of the sensor rod yields a red shift in the scattering cross section σ_{scat} of the sensor rod. The amount of red shift is exaggerated in this schematic to be more clear. For the specific probe wavelength (most often in the red flank of the spectrum), we will have an increase in the scattering signal.

1.3 Single nanoparticles and molecules in motion

Biomolecules are responsible for various dynamic biological functions which involve diffusion and other dynamical process, for example protein folding and DNA extrusion. Also, translational and rotational motion carries information about such protein properties as size and shape. So, new methods enabling detection of such motions are highly desirable to understand the mechanism of many biochemical processes. The motion of single fluorescently labeled molecules has been measured using fluorescence correlation spectroscopy (FCS) [35, 36]. Single-molecule imaging together with fluorescence correlation spectroscopy have been used to measure conformational dynamics and biomolecule interactions. In particular, fluorescence resonance energy transfer (FRET) between single fluorophores has become a popular tool and found many applications [37]. Single-molecule FRET has also been used for protein folding studies and sub-populations of folded and denatured conformations of proteins freely diffusing in solution were determined by confocal microscopy [38, 39].

Many of the biological processes occur at kilohertz to gigahertz rates. For instance, the myosin motors responsible for muscle cell contraction and nutrient transport along actin filaments can have stepping frequencies up to a few kilohertz [40], enzymes such as ATPase and carbonic anhydrase have turnover rates ranging from hundreds of hertz to a megahertz [41], while some biomolecular conformational changes can take place on times as short as nanoseconds [42]. Current fluorescence-based single-molecule microscopy techniques such as STED (Stimulated Emission Depletion Microscopy), FRET (Fluorescence Resonance Energy Transfer) and PALM (Photo Activated Localization Microscopy) struggle to reach these timescales and frequencies, since the fluorescent labels they use typically emit only around ten thousand photons per second [43, 44]. Meanwhile, these frequencies and time scales may be obtainable using scattering signal. Scattering provides us with a new tool to investigate many important nanoscale processes with high frequency bandwidth. In this thesis, we propose observations of the diffusing motion of whole proteins as a starting point for the development of such methods.

Conventional optoplasmonic methods typically rely on analyte immobilization to facilitate detection, and therefore exhibit low temporal resolutions, on the order of milliseconds. The need for immobilization is a direct consequence of the minuscule dimensions of plasmonic near fields typically providing sub-attoliter-sized detection volumes. The direct detection of molecules in motion through such small volumes therefore demands sub-microsecond temporal resolution. Scattering by plasmonic nanostructures

in opto-plasmonic detectors provides enough detected photons to detect Brownian diffusion of single particles and of proteins on such short timescales. We study the freely diffusing single proteins around an immobilized GNR used as an optoplasmonic sensor in chapter 3 of this thesis.

Translational vs. rotational diffusion

Conformational changes of biomolecules [21] do not only change the distance between protein subdomains but often involve their reorientation with respect to each other which can occur at sub-microsecond time scale. Also, studying rotational diffusion of biomolecules as a whole and single nanoparticles is desirable to investigate binding effects and biochemical interactions [45]. Rotational diffusion is an important process which is crucial for the understanding of protein complex formation [46], self-assembly of proteins with multiple binding sites [47], or substrate-enzyme recognition [48].

Fluorescence methods can measure rotational diffusion by using the dependence of a fluorophore's absorption and emission of light on its dipole orientation. According to the electric-dipole approximation, the absorption is proportional to $|\vec{\mu}.\vec{E}|^2 \propto cos^2\theta$, where $\vec{\mu}$ is the transition dipole, $\vec{E}$ is the light electric field and θ is the angle between $\vec{\mu}$ and $\vec{E}$. For small molecules, the overlap of the rotational diffusion time with triplet blinking times, fluorescence lifetime and antibunching times makes it difficult to extract rotational correlation times from FCS measurements. An additional problem is the attachment of the dye molecule to the biomolecule which, being often done with a single tether, will allow the dye molecule to rotate almost freely with respect to the macromolecule [49]. Due to time consuming labeling processes and limitations of the fluorescence methods, label-free optical signals such as scattering are highly demanded [50–53]. The visualization of plasmonic nanoparticles by scattering or absorption is possible thanks to their enhanced scattering and absorption signal at their resonance frequency. Also, according to equation. 1.3 colloidal nanoparticles due to their small enough size R exhibit separated time scales for rotational τ_r and translational τ_t diffusion. Translational D_t and rotational Θ diffusion coefficients for a spherical nanoparticles are given by:

$$\begin{aligned} D_t &= \frac{k_B T}{6\pi\eta R} \qquad & \tau_t &= \frac{L^2}{4D_t} \\ \Theta &= \frac{k_B T}{8\pi\eta R^3} \qquad & \tau_r &= \frac{1}{6\Theta}, \end{aligned} \tag{1.3}$$

where k_B is the Boltzmann constant, T is the temperature, η is the viscosity of the

host medium, and L is the detection length (confocal radius in the case of confocal measurements). Since the rotational diffusion coefficient Θ is proportional to the temperature T and inversely proportional to the surrounding viscosity η and volume of the diffuser R^3, it can be used as a tool to probe the particle and its local environment, temperature, viscosity, and binding events to the diffuser.

To observe the rotational diffusion of nanoparticles, the optical properties of the nanoparticle have to be anisotropic. For gold nanorods, it has been shown that scattering, absorption and two-photon luminescence are highly polarized and correlate well with the orientation of the nanorod [54, 55]. Therefore, the rotational diffusion of individual nanorods can be measured by scattering on sub-microsecond time scales. In chapter 4 of this thesis, we have studied rotational diffusion of single GNRs in a confocal volume and investigated the effect of the laser power which cause heating of the GNRs. We also revealed an increase in the rotational diffusion time of the GNRs upon binding of single PVA chains to the nanorod. In chapter 5 of this thesis, we investigate the rotational diffusion of small GNRs around a sensor GNR by plasmonic coupling effect.

1.4 Optical microscopy

Since the absorption and scattering cross sections of nanoparticles scale respectively with their volume and squared volume, detecting very small particles in presence of background is challenging and requires a specific approach. The fluorescence signal can be easily isolated from the background caused by the excitation light using proper filters, yielding a high signal to noise ratio. For scattering measurements, either the scattered light alone (in dark field) or the interference of the scattered wave with a reference wave (interferometric scattering) can be used. In dark-field microscopy, light that is directly scattered from a nanoparticle must be detected on a dark background [8, 54]. Interferometric scattering microscopy (iSCAT) is a powerful tool for label-free sensitive detection and imaging of nanoparticles with high spatio-temporal resolution. When the particle is sufficiently small, the scattered light intensity I_s becomes much weaker than the interference term which is linearly proportional to the scattered field. As a result, interferometric detection of scattering is more favorable than the dark-field schemes for very small nanoparticles and protein molecules.

The biggest limitation inherent in optical microscopy is its lateral spatial resolution Δx, which is determined by the wavelength of the light λ used in the microscope and the numerical aperture (NA) of the objective lens ($\Delta x \approx 0.6\frac{\lambda}{NA}$). Another impor-

tant limitation is the axial resolution along the optical axis, conventionally called Δz, which is related to the depth of the field ($\Delta z \approx \frac{\lambda}{NA^2}$) [56]. In contrast to wide-field (WF) microscopy where every plane of the specimen is evenly illuminated while the image is recorded [57, 58], in a scanning confocal microscope, the illuminating beam is rapidly scanned over the specimen. In confocal microscopy the central idea is to irradiate the sample with focused light originating from a point source and direct the light transmitted, scattered or emitted by the sample onto a pinhole [56]. The principal advantage of confocal microscopy for biological imaging is that the out-of-focus light is eliminated from detection by optical arrangement yields a high contrast. The schematic of the confocal setup used in this thesis is shown in figure. 1.2a, the setup will be discussed in details in chapter 1 of this thesis.

1.4.1 iSCAT

The principal concept in interferometric microscopy is to superpose a reference light beam with the light scattered by the sample, as illustrated in figure. 1.2b. By considering $E_s = |E_s|e^{i\phi_s}$ as the field scattered by the nanoparticle and $E_{ref} = |E_{ref}|e^{i\phi_r}$ as the complex electric field of the reference beam, the signal on the detector is:

$$I_{det} \propto |E_{ref} + E_s|^2 = I_{ref} + I_s + 2E_{ref}E_s cos\phi, \tag{1.4}$$

where $\phi = \phi_r - \phi_s$ is the phase difference between the reference field and the scattered field. Neglecting the scattered intensity (I_s) which is vanishingly weak for very small particles, the iSCAT signal of interest is mainly determined by the interferometric cross-term ($I_{ref} + I_s + 2E_{ref}E_s cos\phi$).

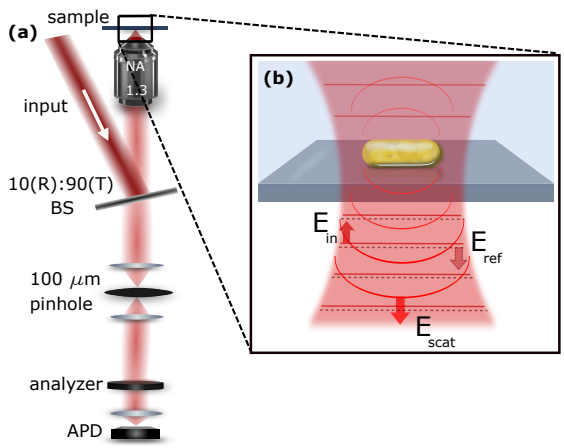

Figure 1.2: **a)** Schematic of the confocal setup used throughout this thesis. Different elements will be discussed in detail in chapters 1 and 2. **b)** iSCAT microscopy in a confocal mode, shows reference, scattered and incident wave on GNR immobilized on a glass substrate.

1.4.2 Photothermal contrast microscopy

As mentioned in section 1.1, the scattering cross section σ_{scat} depends on the surrounding refractive index. Since the refractive index is typically sensitive to the temperature, absorption by nanoparticles causes a perturbation of the surrounding material's refractive index. This perturbation can serve as an optical contrast which can be detected optically in standard microscopy setups. The technique called photothermal microscopy [59–63], normally requires two laser beams. The heating beam is intensity-modulated at high frequency, and used to heat up the object and create a time-dependent temperature and refractive index profile in the medium around the absorber [64] (see figure 1.3b). The probe beam, which is spatially overlapped with the heating beam, is scattered by the local index gradient and interferes with a reference field, such as the reflection of the incident probe beam by the interface.

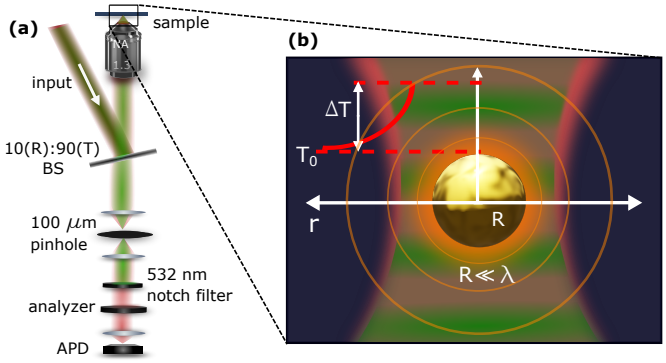

Figure 1.3: **a)** Schematic of the optical setup used for photothermal measurements. An intensity-modulated beam with 532 nm wavelength heats the absorbing particle. **b)** The probe beam (red) detects the index change. Both beams are focused on a gold nanosphere (with radius R). The thermal lens induced by the temperature gradient (orange circles) is illustrated as a cloud around the nanosphere.

In chapter 2 of this thesis we have used photothermal spectro-microscopy as a benchmarking tool for the characterization and optimization of our optoplasmonic detection technique.

1.5 Outline of this Thesis

In this thesis, we will investigate the physics and applications of immobilized and freely diffusing single gold nanorods as optoplasmonic sensors to detect single particles or molecules and local temperature changes. We explore this general question in four individual chapters: photothermal spectro-microscopy, optoplasmonic detection of single proteins, measurement of rotational diffusion in confocal volume, scattering measurements in view of creating a plasmonic goniometer.

In **chapter 2** we provide a measurement of the optoplasmonic bio-detection as an assay to probe the response of plasmonic nanostructures by changes in their dielectric environment. We introduce photothermal spectro-microscopy as a benchmarking tool for the characterization and optimization of optoplasmonic detection assays.

Chapter 3 reports the optical detection of individual proteins by reading out changes in the resonantly scattered field of an individual gold nanorod interferometrically. We also use photothermal spectroscopy to optimize the experiment's parameters.

This interferometric plasmonic scattering enables the observation of single proteins as they traverse plasmonic near fields of gold nanorods with unprecedented temporal resolution in the nanosecond-to-microsecond range.

In **chapter 4** we record dark-field scattering bursts of individual gold nanorods, 52×15 nm^2 in average size, freely diffusing in water suspension. We deduce their Brownian rotational diffusion constant from autocorrelation functions on a single-event basis. As rotational diffusion depends on particle hydrodynamic volume, viscosity and temperature, it can sense those parameters at the single-particle level. We demonstrate measurements of hot Brownian rotational diffusion of nanorods in temperature and viscosity gradients caused by plasmonic heating. Further, we monitor hydrodynamic volumes of gold nanorods upon addition of very low concentrations of the water-soluble polymer PVA, which binds to the particles, leading to measurable changes in their diffusion constant corresponding to binding of one to a few polymer coils.

Chapter 5 demonstrates a study of rotational diffusion via opto-plasmonic sensing by a large gold nanorod with 40 nm in diameter, 112 nm in length as a sensor. We detect the rotational motion of single small analyte gold nanorods with 5 nm in diameter, 15.5, 19.1 and 24.6 nm in length with less than 100 ns time resolution.