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Interaction of Colloidal Gold Nanoparticles with Model Serum Proteins: The Nanoparticle-Protein ‘Corona’ from a Physico-Chemical Viewpoint

by

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ABSTRACT

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When nanoparticles come in contact with biological fluids they become coated with a mixture of proteins present in the media, forming what is known as the nanoparticle-protein ‘corona’. This corona changes the nanoparticles’ original surface properties and plays a central role in how these get screened by cellular receptors. In the context of biomedical research, this presents a bottleneck for the transition of nanoparticles from research laboratories to clinical settings. It is therefore fundamental to probe these nanoparticle-protein interactions in order to understand the different physico-chemical mechanisms involved. This thesis is aimed to investigate the exposure of colloidal gold nanoparticles to model serum proteins, particularly serum albumin, the main transporter of molecular compounds in the bloodstream of mammals. A set of experimental tools based on optical microscopy and spectroscopy were developed in order to probe these interactions in situ. First, the intrinsic photoluminescence and elastic scattering of individual gold nanoparticles were investigated in order to understand its physical origin. These optical signals were then used to measure the size of the nanoparticles while in Brownian diffusion using fluctuation correlation spectroscopy. This spectroscopic tool was then applied to detect the binding of serum albumin onto the nanoparticle surface, increasing its hydrodynamic size. By performing a binding isotherm
as a function of protein concentration, it was determined that serum albumin follows an
anti-cooperative binding mechanism on negatively charged gold nanoparticles. This
protein monolayer substantially enhanced the stability of the colloid, preventing their
aggregation in saline solutions with ionic strength higher than biological media. Cationic
gold nanoparticles in contrast, aggregated when serum albumin was present at a low
protein-to-nanoparticle ratio, but prevented aggregation if exposed in excess. Single-
molecule fluorescence microscopy revealed that under low protein-to-nanoparticle
binding ratios, serum albumin irreversibly unfolds upon adsorption and spreads across the
available nanoparticle surface area. Unfolded proteins then interact with one another,
triggering nanoparticle aggregation. Fibrinogen and globulin also triggered aggregation
when exposed to cationic nanoparticles. In an effort to relate these physico-chemical
observations to relevant biological parameters, the uptake of protein coated gold
nanoparticles by a model cancer cell line was investigated under different incubation
conditions. Those nanoparticles pre-incubated with bovine serum albumin before fetal
bovine serum were found to be uptaken three times more than those only incubated in
serum.
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**Figure 5.6** Simultaneous photobleaching steps are unlikely. With the expected labeling density of three fluorophores per BSA molecule, the average photobleaching step as a percent of the maximum intensity should be ~33%. We observe 30 ± 10% average step size as a percent of the maximum intensity. A ~66% change or greater would be observed to indicate
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# Nomenclature

<table>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>AuNPs</td>
<td>Gold nanoparticles</td>
</tr>
<tr>
<td>AuNRs</td>
<td>Gold nanorods</td>
</tr>
<tr>
<td>AuNSs</td>
<td>Gold nanospheres</td>
</tr>
<tr>
<td>FCS</td>
<td>Fluctuation correlation spectroscopy</td>
</tr>
<tr>
<td>$R_h$</td>
<td>Hydrodynamic radius</td>
</tr>
<tr>
<td>$\tau_D$</td>
<td>Characteristic diffusion (decay) time</td>
</tr>
<tr>
<td>BIFA</td>
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1.1. Motivation: Nanoparticles from the Lab to the Real World

A significant fraction of engineered nanoparticles have now transitioned from research laboratories to real world environments. As of 2015, the worldwide market of products incorporating nanotechnology is estimated to be ~1 trillion USD.¹ Nanoparticles are now essential components in catalytic processes in industry,² computer and telecommunication devices,³ healthcare⁴ products, and cosmetics.⁵ This technological revolution is accompanied by an increase in both the production quantity of nanomaterials and the breadth of their exposure to consumers.⁶

In the context of biomedical research, nanoparticles are expected to revolutionize the way diseases are diagnosed and treated.⁷,⁸ Due to their small size,
nanoparticles can enter biological compartments at the cellular level and deliver drugs, antibodies, or molecular agents carried on their surface. Unlike administration of drugs or molecules alone, nanoparticles can potentially transport large amounts of material with very little volume as they have sufficiently large surface to volume ratios. For example, a few milliliters of a microgram per milliliter suspension of spherical nanoparticles can have an available surface area comparable to a football field. Depending on the constituent material of the nanoparticles, the core can also serve as a photothermal agent and hence can potentially be visualized as they accumulate in tumors and kill diseased cells by hyperthermia.

Synthetic protocols for engineering inorganic nanoparticles allow controlling their size, shape and surface chemistry. Typically, an organic ligand shell makes them soluble and protects them from aggregation in aqueous solutions. The combination of core materials and surface ligands constitutes the nanoparticle ‘synthetic identity’. However, when nanoparticles come in contact with biological fluids, proteins bind onto their surface changing the nanoparticle’s original surface properties. This layer of proteins known as the nanoparticle-protein ‘corona’ likely determines their interaction with cellular membranes in a biological environment, as what cells would eventually screen is not the original nanoparticle core or ligand shell, but the layer of bound proteins that constitute the nanoparticle ‘biological identity’.
1.2. Background: The Nanoparticle-Protein Corona

The interaction of nanoparticles with living organisms has received growing attention in the past decade. This interest has been partly motivated by the development of novel therapeutic and diagnostic tools, but also by growing concerns regarding the safety of nanomaterials in vivo. Understandably, research in this field has focused on the study of the association of nanoparticles and proteins, as proteins are present in biological fluids and interact with the nanoparticle surface before cellular screening.

Early studies by Dawson and co-workers showed that a protein corona is formed on nanoparticles in the presence of blood plasma, coating the original nanoparticle surface and dramatically altering the synthetic properties of the nanomaterial. Using a variety of analytical techniques such as isothermal titration calorimetry, size exclusion chromatography, and surface plasmon resonance sensing, they were able to quantify the affinities and stoichiometries of proteins for model co-polymer nanoparticles. From these seminal studies and others that followed, it was strongly suggested that the binding constants of proteins to nanoparticles depend both on nanoparticle size and surface chemistry.

The fact that the nanoparticle protein corona could be quantified using readily-available tools in standard analytical chemistry labs motivated others to expand the characterization of the protein corona to different types of nanoparticles with different surface properties. A standard ex-situ three-step protocol was followed by most studies: 1) Incubation of nanoparticles in blood serum or plasma,
followed by 2) isolation of bound proteins using centrifugation or size exclusion chromatography, and finally 3) protein identification via gel electrophoresis or mass spectrometry. Protein coronas were identified for a variety of polystyrene, silica, carbon, and gold nanoparticles of different size, surface charge, and surface chemistry.

Complementary optical spectroscopy approaches that are in principle less disruptive than the protocol mentioned above have also been developed to study the interaction between proteins and nanoparticles. Payne and co-workers conjugated proteins with fluorescent dyes and using optical microscopy co-localized bovine serum albumin (BSA) and fluorescent polysterene nanoparticles in the presence of cells. This proof-of-concept experiment demonstrated that what the cellular receptors actually screen is not the bare nanoparticle core but adsorbed proteins on their surface. Nienhaus and colleagues applied fluorescence correlation spectroscopy to measure the thickness of the protein corona. They measured monolayer adsorption of human serum albumin (HSA), transferrin, and apolipoprotein A-1 (Apo-A1) on FePt and CdSe/ZnS core/shell particles. Also, exploiting the environmental sensitivity of gold nanoparticles to refractive index changes at their surface has allowed the detection of single protein adsorption events.

Researchers have identified adsorbed proteins and quantified affinities and equilibrium binding constants for particular nanoparticle systems. However, the experimental conditions were adjusted so that proteins and nanoparticles were in a
dynamic equilibrium (or arguably ‘in a pseudo-equilibrium’), leading to controversial results for the time scales of the corona formation. In absence of direct temporal information, two hypotheses have been postulated in the literature: 1) High-abundance proteins such as serum albumin and fibrinogen bind rapidly (in seconds) without necessarily having high affinity for the nanoparticle surface. These proteins are then slowly replaced in tens to hundreds of seconds by less abundant proteins with more specific interactions such as apolipoproteins. This model is inspired by the Vroman effect that has been historically used to explain protein binding on flat surfaces. 2) Protein adsorption occurs rapidly within 30 seconds and over time significantly changes in terms of the amount of bound protein, but very little changes in composition occur.

These two distinct kinetic mechanisms for the protein corona are expected to lead to quite different biological responses to nanoparticles. In addition, it is possible that the large range of observed time scales of protein corona formation could be linked to protein unfolding and a distribution of unfolding pathways. Unfolding of adsorbed proteins is a potential adverse effect of nanoparticles because it could lead to exposure of amino acid sequences that otherwise remain buried and inactive, activation of self-defense mechanisms, or triggering of protein aggregation. While very little information is known on what happens to a protein when it is adsorbed onto a nanoparticle surface, a couple of studies partially support this possibility of protein unfolding, where it was found that poly(acrylic acid) coated gold nanoparticles can selectively bind and unfold specific amino acid chains of fibrinogen. Moreover, it was demonstrated that these unfolded
chains interacted with the integrin receptor of macrophages, activating an inflammatory response.

While this field has slowly transitioned from the mere identification of adsorbed proteins to the recognition of the physiology associated to the protein corona, it is becoming more and more relevant to investigate the underlying physico-chemical principles ruling the formation of the protein corona.

1.3. Overview: Understanding the Bio-Nano Interface from a Physico-Chemical Viewpoint

This thesis focuses on understanding the interactions between model serum proteins, mainly bovine serum albumin, and colloidal gold nanoparticles by optical microscopy and spectroscopy. My goal was to identify the impacts of these interactions on the nanoparticle state of aggregation and protein structure, as these are equally important from a physico-chemical perspective and are of strong biological significance.

Gold nanoparticles are a promising model nanoparticle system. When visible light interacts with this type of nanoparticles, the highly polarizable conduction band electrons at the surface collectively move in resonance at the frequency of the excitation light, a phenomenon known as the “surface plasmon resonance.” The plasmon resonance gives rise to strong absorption, scattering and, albeit weaker, photoluminescence of visible light, with effective extinction cross sections several orders of magnitude larger than those of conventional fluorophores and without the
drawback of permanent photobleaching.\textsuperscript{57} Due to these exceptional optical properties, there are many proposed applications of gold nanoparticles currently under development, such as their use as imaging labels in biology,\textsuperscript{58-60} carriers for drug delivery,\textsuperscript{61} sensors for molecular recognition\textsuperscript{62, 63} and as supports for catalytic reactions.

Before studying the interactions of gold nanoparticles with model serum proteins it was necessary to investigate their optical properties and measure their hydrodynamic size in aqueous environments. Chapter 2 focuses on characterizing the photoluminescence properties of gold nanospheres and nanorods using single particle spectroscopy and its application in fluctuation correlation spectroscopy (FCS). Optical spectroscopy was used to investigate the still controversial physical mechanism giving rise to photoluminescence of gold nanoparticles. FCS was used to measure their hydrodynamic size and probe their translational and rotational dynamics (for the case of nanorods). Chapter 2 contains part of my work on photoluminescence published by my colleague Alexei Tcherniak and me in \textit{The Journal of Physical Chemistry C} in 2011\textsuperscript{64}, and part of my work in FCS of nanoparticles currently under review in \textit{Annual Reviews of Physical Chemistry}.

After having established FCS as an optical method for measuring the hydrodynamic size of colloidal gold nanoparticles, I then moved on to measure the binding of a protein onto the nanoparticle surface. Chapter 3 shows a measurement of BSA protein with three sizes of colloidal gold nanospheres. FCS allowed quantifying small changes in the nanoparticle hydrodynamic radius due to protein
binding without having to remove excess protein out of solution. Using this technique, I was also able to demonstrate that BSA can form a protein monolayer on gold nanospheres when there is a large protein-to-nanoparticle ratio. Interestingly, the formation of this protein monolayer followed an anti-cooperative binding mechanism. This work was published in *Langmuir* in the spring of 2012.65

Most nanoparticle colloids aggregate and precipitate in saline solutions below the salt concentration of many bodily fluids such as blood serum. This presents a problem as nanoparticle aggregation is undesirable and potentially toxic in a biological environment. Chapter 4 shows that negatively charged gold nanoparticles aggregate and precipitate in the presence of sodium chloride, the most abundant salt in serum. A similar aggregation behavior was observed in the presence of phosphate, calcium and zinc ions. However, binding of a monolayer of BSA protein onto these gold nanoparticles completely prevented this aggregation process, even in solutions where the salt concentration was beyond the isotonic point. This work was published in *ACS Sustainable Chemistry & Engineering* in early 2013 and led to a provisional patent (US 61/837,359) in the United States Patent and Trademark Office.66

In contrast, cationic gold nanoparticles –which do not readily aggregate in saline solutions- aggregated in the presence of serum albumin at low protein-to-nanoparticle ratios. However, at high ratios serum albumin forms a monolayer and enhances the stability of the colloid. Chapter 5 presents a combination of spectroscopic techniques including single-molecule experiments aimed to
understand this intriguing interaction. Single-molecule fluorescence microscopy revealed that under low protein-to-nanoparticle ratios, serum albumin irreversibly unfolds upon adsorption onto positively charged nanoparticles and spreads across the available surface area. Unfolded proteins then interact with one another, triggering nanoparticle aggregation. Fibrinogen and globulin also triggered aggregation when exposed to cationic nanoparticles, but unlike serum albumin this was independent of the protein-to-nanoparticle ratio. In an effort to relate these physico-chemical observations to relevant biological parameters, the uptake of protein coated gold nanoparticles by a model cancer cell line was investigated under different incubation conditions. Those nanoparticles pre-incubated with bovine serum albumin before fetal bovine serum were found to be uptaken three times more than those only incubated in serum. This work is currently under review in an interdisciplinary journal and was done in collaboration with my colleagues in the Zubarev and Landes research groups at Rice University.

Finally, Chapter 6 contains the summary of the different observations of enhanced colloidal stability (Chapters 3 & 4) and nanoparticle aggregation (Chapter 5) resulting from proteins and nanoparticles interacting at different binding ratios. This chapter contains the overall concluding remarks of the thesis and will hopefully serve as a reference for future applications of nanoparticles requiring their suspension in biological media.
Chapter 2

The Intrinsic Photoluminescence of Gold Nanoparticles: Measuring the Hydrodynamic Size of Nanoparticles via Fluctuation Correlation Spectroscopy

2.1. Abstract

Fluctuation correlation spectroscopy (FCS) is a well-established analytical technique initially used to monitor molecular diffusion in dilute solutions, the dynamics of chemical reactions, and molecular processes inside living cells. This chapter presents the recent use of FCS for measuring the size of colloidal nanoparticles in solution. In order to do this, it was necessary to first investigate the photoluminescence originated from gold nanospheres and nanorods under visible laser excitation. The experimental results from these investigations suggest fast...
interconversion between surface plasmons and hot electron-hole pairs and show that the luminescence occurs via emission by a surface plasmon. Using the information obtained from these studies, we were able to successfully employ one-photon luminescence for FCS measurements and to correctly interpret autocorrelation functions, which were used to determine the hydrodynamic sizes of several gold nanoparticle samples and to extract rotational dynamics of nanorods.

The photoluminescence studies published in this chapter have been published in an article with equal contribution between Dr. Alexei Tcherniak and myself and has the following citation:


*Equal contribution

The remaining sections of this chapter on the application of FCS on nanoparticles are currently under review in Annual Reviews of Physical Chemistry and can be temporarily cited as:


2.2. Basic Principles of Fluctuation Correlation Spectroscopy

Fluctuation correlation spectroscopy is an optical technique that measures the temporal autocorrelation of photon fluctuations arising from molecules and
particles entering and leaving a well-defined microscopic observation volume.\textsuperscript{67} When these dynamic fluctuations arise from an analyte freely diffusing in solution, they are proportional to the analyte’s diffusion coefficient and therefore its hydrodynamic dimension. FCS is derived from fundamental studies of the nature of thermodynamic fluctuations in supercritical fluids using optical interference (i.e. dynamic light scattering, DLS), where the goal was to characterize kinetics for molecular transport (e.g. diffusion coefficients) and chemical reactions (e.g. rate constants).\textsuperscript{68,69} This early approach was however limited by the negligible scattering cross section of most organic molecules, requiring high illumination powers that would damage most samples and result in poor signal to background ratios.\textsuperscript{70} Seminal work by Elson and Webb showed that the intercalation of fluorescent markers with bio-molecules greatly reduced the need of damaging illumination powers,\textsuperscript{71} and the miniaturization of FCS using confocal microscopy by Koppel \textsuperscript{72} and Rigler\textsuperscript{73} ignited a variety of applications of FCS in biology and chemistry as the signal to background ratio increased by six orders of magnitude.\textsuperscript{70} Since then, FCS has been widely applied to quantify the dynamics of conjugated organic molecules,\textsuperscript{74-76} proteins,\textsuperscript{77,78} nucleic acid,\textsuperscript{79} bacteria,\textsuperscript{80} and most recently, nanoparticles.\textsuperscript{81-87}

The simplest experimental implementation of FCS is based on an inverted confocal microscope using a single CW laser line, a high numerical aperture objective and a single element detector (Figure 2.1). The laser beam focused by the objective defines the observation volume that can be further shaped with a confocal pinhole. Molecules and nanoparticles diffusing into this observation volume are
typically either electronically excited by the light and consequently emit photons or scatter the incident radiation. Depending on the type of interaction between the analyte and the focused laser beam (photoluminescence vs. scattering) different filters are inserted before the detector that counts the arrival of individual photons. In particular, for photoluminescence that is shifted to longer wavelengths compared to the excitation wavelength, dichroic and long pass filters are employed to achieve almost background-free signals from the diffusing analytes.

Figure 2.1 Fluctuation correlation spectroscopy as a tool to measure nanoparticle hydrodynamic dimensions. Simplified diagram of a fluctuation correlation spectroscopy setup with a magnified view of the observation volume, with beam waist radius $r_0$ and beam height $z_0$. Fluctuations in the observation volume due to nanoparticle diffusion are recorded with a single element detector attached to a photon counter board and a computer.

When a particle diffuses inside the observation volume, due to the interaction with the laser radiation, it produces an intensity fluctuation $\delta I(t)$, which
is the difference between the intensity $I$ at time $t$ and the average background intensity $\langle I(t) \rangle$. The mathematical expression of the normalized temporal autocorrelation $G(t)$ is defined as:\(^{67}\)

$$G(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t + \tau) \rangle}{\langle I(t) \rangle^2}$$

Equation 2.1

The autocorrelation represents the self-similarity of the recorded signal after a lag time $\tau$, typically defined by the detection system’s temporal resolution. However, in order to retrieve meaningful diffusion constants Equation 2.1 needs to be integrated for a broad range of lag times and for the entire observation volume. Given that photon collection and particle diffusion are both probabilistic processes, the physical interpretation of Equation 2.1 can be fairly complicated and depending on the system there may not be an analytical solution.\(^{70}\) However, assuming a three-dimensional Gaussian illumination profile - such as the one resulting from focusing a laser in a confocal microscope - and low analyte concentrations inside the observation volume, Equation 2.1 has been solved by Aragon and Pecora:\(^{88}\)

$$G(\tau) = \frac{1}{\langle N \rangle} \frac{1}{1 + \frac{\tau}{\tau_D}} \left( 1 + \frac{r_0}{z_0} \right) \left( 1 + \frac{\tau}{\tau_D} \right)^{1/2}$$

Equation 2.2
<N> represents the average number of particles in the observation volume, \( \tau_D \) is the characteristic diffusion time of the particles across the observation volume of waist \( r_0 \) and height \( z_0 \). Considering that the focal plane is determined with a confocal pinhole placed at the image plane of an inverted microscope, Hess noted that the recorded intensity fluctuations represent the two dimensional projection of a three dimensional motion.\(^{89}\) This realization allows one to quantify the translational diffusion coefficient \( D_{tr} \) of the analyte if the radius \(-r_0^2\) of the focal volume is known:

\[
\tau_D = \frac{1}{4D_{tr}} r_0 \tag{2.3}
\]

**Equation 2.3**

where \( \tau_D \) is obtained from the characteristic decay of the autocorrelation function (Equation 2.2). For unconstrained three dimensional Brownian diffusion, \( D_{tr} \) can be related to the hydrodynamic radius \( R_h \) of the particle using the Stokes-Einstein relationship, if the viscosity \( \kappa \) of the solution at a given temperature \( T \) is known:

\[
R_h = \frac{k_B T}{6\pi\kappa D_{tr}} \tag{2.4}
\]

**Equation 2.4**
Combining Equation 2.3 and Equation 2.4, it can be seen that the particle’s hydrodynamic radius \( R_h \) is directly proportional to the experimental diffusion time \( \tau_D \), with the proportionality constant being \( \frac{2k_BT}{3\pi \eta_0} \). The autocorrelation curve of smaller particles decays faster than that of larger or aggregated particles as it takes them less time to cross the observation volume under the same experimental conditions. (Figure 2.2)

**Figure 2.2** Sizing of nanoparticles using fluctuation correlation spectroscopy. Normalized autocorrelation curves representing small (blue) and large (red) particles. The decay time \( \tau_D \) of the autocorrelation function \( G(\tau) \) is directly proportional to the nanoparticle hydrodynamic radius \( R_h \). Smaller particles diffuse faster across the observation volume than larger particles or aggregates composed of smaller particles \( (\tau_{D,1} < \tau_{D,2}) \).

### 2.3. The Photoluminescence of Gold Nanoparticles as an Optical Signal for FCS

The photoluminescence is analogous to the fluorescence of organic molecules, and hence it is in principle a background-free technique. An advantage of
using photoluminescence over elastic scattering is that the FCS analysis is less
biased by the presence of nanoparticle aggregates as photoluminescence scales with
the nanoparticle volume $V$ compared to a $V^2$ dependence for scattering. To measure
the diffusion of nanoparticles with correlation spectroscopy using polarized
excitation and detection, the polarization of the measured signal needs to be first
understood for a stationary nanoparticle.

While plasmon scattering in gold nanorods (AuNRs) is polarized along the
long rod axis for the longitudinal mode and polarized in the perpendicular direction
for the transverse mode, only a few studies exist on one-photon luminescence of
gold nanoparticles,$^{90-98}$ and the mechanism is still a subject of debate. Two general
explanations have been proposed. One mechanism hypothesizes that the
luminescence is due to interband electron-hole pair recombination, which is
enhanced by the spectrally overlapping plasmon field.$^{91, 92, 95, 96, 98}$ The other
explanation is that the luminescence is caused by the radiative decay of a plasmon.$^{93}$

To quantify the polarization dependence of the luminescence but also to gain
a deeper insight into the mechanism we correlated the spectroscopy of individual
gold nanoparticles with their size and orientation using scanning electron
microscopy (SEM). This approach takes the effects due to size and shape
heterogeneity directly into account while eliminating the possibility that the signal
is caused by other luminescent impurities. Interestingly, we were unable to observe
luminescence in bulk measurements with lamp excitation. Because of the high
excitation rate in the single particle measurements using a laser excitation source,
enough signal could, however, be collected for recording single particle spectra. Dichroic mirrors and notch filters were placed in the detection beam path of the optical setup to ensure that the more intense scattered excitation light was blocked out.

2.4. Experimental Methods

2.4.1. Nanoparticle Characterization

Gold nanospheres (AuNSs) and AuNRs were purchased from Nanopartz and characterized with bulk UV-Vis spectroscopy (Shimadzu UV-3101PC) and TEM (JEOL 2010), as shown in Figure 2.3. This allowed quantifying the extent of size and shape heterogeneity of these AuNS and AuNR samples. The UV-Vis spectra in Figure 2.3A show single plasmon peaks for the 57 nm (dashed blue line) and 96 nm (solid black line) AuNSs at 535 and 564 nm, respectively. The AuNRs in Figure 2.3B exhibit two well-defined plasmon peaks in their extinction spectra. The resonance near 520 nm has contributions from both interband transitions and the transverse surface plasmon resonance. The spectral position of the longitudinal plasmon resonance depends mainly on the aspect ratio of the AuNRs for this particular size regime. The maximum of the longitudinal plasmon resonance is at 620 nm for the 34x60 nm AuNR sample (dashed purple line), at 700 nm for the 28x76 nm AuNR sample (solid black line), and at 735 nm for the 24x68 nm AuNR sample (dashed blue line). The samples are labeled according to their mean dimensions. Representative TEM images for these samples are given in Figure 2.3.
Figure 2.3 UV-Vis spectra and TEM images for (A) AuNS samples and (B) AuNR samples used in this study. Vertical lines denote the two excitation wavelengths that we used: 514 nm (green) and 633 nm (red). Representative TEM images for each sample are also included.

2.4.2. Correlation Spectroscopy Setup

Correlation spectroscopy experiments were performed on a home-built instrument based on an inverted Zeiss microscope described previously and in Figure 2.1. Excitation was carried out using the 514 nm line of an Ar⁺ laser (Modu-Laser) and a 633 nm He-Ne laser (JSD Uniphase). To avoid sampling NP diffusion at the interface and to minimize reflected light from the glass coverslips, the lasers were focused about 4 and 7 µm deep inside the sample chamber for luminescence and scattering correlation measurements, respectively. For luminescence correlation spectroscopy experiments, light collected from the sample was passed through a dichroic mirror and a notch filter (z532rdc, Chroma Technology, and RNF-514.5, CVI, for 514 nm excitation and z633rdc, Chroma
Technology, and RNF-632.8, CVI, for 633 nm excitation), which were used to assure that all excitation light was removed from the signal. No other filters were used and therefore spectrally integrated intensities were collected for the luminescence correlation measurements. To switch from luminescence to scattering correlation spectroscopy, the dichroic mirror was switched to a 50/50 beam splitter and the notch filter was removed. Sample preparation for correlation measurements was carried out according to procedures published previously.\textsuperscript{101}

**2.4.3. Negligible Heating and Changes in Viscosity in FCS Experiments**

It was important to ensure that the excitation power used for FCS experiments did not lead to a change in local solvent viscosity caused by NP heating. We therefore estimated the increase in temperature at the NP surface.

The dynamic viscosity $\kappa \text{[Pa}\cdot\text{s]}$ of liquid water can be described by the following functional form with respect to temperature $T \text{[Kelvin]}$: \textsuperscript{102}

$$\kappa \approx 0.001^* e^{a+b \ln T}$$

Equation 2.5

Where $a = 29.76$, $b = -5.24$

Experiments were carried out in a temperature-controlled environment at $T = 296 \pm 1 \text{ K}$. We assume that the sample is in thermal equilibrium with the
surroundings before laser irradiation. At this temperature, the dynamic viscosity of water is \( \kappa = 9 \times 10^{-4} \text{ Pa}\cdot\text{s} \).

The temperature increase at the surface of the NP due to laser induced heating is given by:

\[
\Delta T_{\text{surf}} = \frac{\sigma_{\text{abs}} I}{4 \pi \varepsilon R}
\]

**Equation 2.6**

Assuming that absorption cross sections \( \sigma_{\text{abs}} \) at 514 and 633 nm for our samples are of the order of \( \sigma_{\text{abs}} \approx 10^3 \text{ nm}^2 \), the laser irradiances \( I \) heating the samples are \( I_{\text{scattering}} \approx 15 \text{ W/cm}^2 \) and \( I_{\text{luminescence}} \approx 15 \text{ kW/cm}^2 \) for scattering and luminescence, the thermal conductivity of water is \( \varepsilon \approx 0.6 \text{ W/(m·K)} \), and the AuNSs and AuNRs radii fall in the range of \( R \approx 10^{-10} \text{ nm} \), the local temperature increase at the NP surface can be estimated to be around \( \Delta T \approx 2 \times 10^{-3} \text{ K} \) for scattering and \( \Delta T \approx 2 \text{ K} \) for luminescence. We can relate these estimated changes in temperature to changes in the dynamic viscosity. Using Equation 2.5, we calculated the changes in viscosity be \( \Delta \kappa \approx 10^{-8} \text{ Pa}\cdot\text{s} \) for scattering and \( \Delta \kappa \approx 10^{-5} \text{ Pa}\cdot\text{s} \) for luminescence. Hence, by considering both the estimated values for the temperature increase at the NP surface and the decrease in the dynamic viscosity of water, we can calculate using the Stokes-Einstein equation (Equation 2.4) that the translational diffusion constant \( D_{tr} \) increases by about 1% per degree of temperature increase. Even in the case of
photoluminescence ($\Delta T = 2 \text{ K}, \Delta \kappa = -10^{-5} \text{ Pa}\cdot\text{s}$), a variation of 2% in the translational diffusion constant $D_{tr}$ would be within our experimental error. In conclusion, local temperature changes around the NPs due to laser heating can therefore be neglected for the range of laser power used.

2.4.4. Optical Spectroscopy of Single Nanoparticles

Single particle spectra were acquired using the same instrument as described above for FCS. For scattering spectra, a halogen lamp was used for excitation in a dark-field geometry and the scattered light was redirected to a spectrometer equipped with a CCD camera (Princeton Instruments PIXIS 400BR). For luminescence spectra, the same laser lines were used for excitation as for the correlation spectroscopy experiments. The luminescence was then collected in an epi-illumination geometry and sent to the same spectrometer. As needed, specific polarizations of the incident and emitted light were controlled by half wave plates and placing a polarizer in the detection light path. Unless otherwise specified a polarizer angle of $\theta = 0$ selects the polarization direction that is parallel to the laboratory x-axis. A 100X Zeiss Epiplan objective was used to collect all spectra. Correlation between single particle spectra and the dimensions of the NPs was accomplished using a patterned substrate for identification in the optical setup and a SEM (FEI Quanta 400 ESEM FEG) as previously described.$^{56,103,104}$
2.4.5. Calibration of the Focal Volume for Fluctuation Correlation Spectroscopy

In order to measure the correct hydrodynamic dimensions of nanoparticles using FCS, one must first accurately determine the dimensions of the focal observation volume. Three calibration methods have been reported in the literature, each one with its advantages and limitations. We will discuss these methods and demonstrate them experimentally using fluorescently labeled polystyrene beads under 638 nm laser excitation (Figure 2.4).

**Amplitude Fitting.** The focal volume dimensions can be obtained by using a sample with known concentration \( C \) and measuring the amplitude of the autocorrelation function, that is \( G(\tau = 0) \). From Equation 2.2 it follows that:

\[
G(\tau = 0) = \frac{1}{\langle N \rangle}
\]

Equation 2.7

with \( \langle N \rangle \) being the average number of particles in the focal volume. Given that \( C \) is defined as number of particles per unit of volume according to:

\[
\langle C \rangle = \frac{\langle N \rangle}{V}
\]

Equation 2.8
the focal volume $V$ can be easily extracted from this simple relationship. We have verified that this linear relationship is valid for a wide range of concentrations going from 1 pM to 1 μM using fluorescently-labeled polystyrene nanoparticles ($d = 36$ nm, Figure 2.4a). From these measurements, $V$ was found to be $1.6 \pm 0.05$ fl using the slope of the linear fit in Figure 2.4a. A similarly wide linear trend was also demonstrated by Ruttinger et al. using the fluorescent molecule ATTO 665. The advantage of this method is that $G(0)$ can be obtained without having to fit the autocorrelation function (i.e. only the data point for $\tau = 0$ is important), and it furthermore allows measuring the concentration of unknown samples by comparing its amplitude to the reference. The limitation is that this method is prone to error propagation in the dilution series, and that only the volume $V$ and not the actual dimensions $r_0$ and $z_0$ of the observation volume are determined. This approach is therefore best suited to measure absolute concentration or relative changes in nanoparticle dimensions.

**Lifetime Fitting.** Because the sample concentration and/or extinction coefficient $\epsilon$ of the sample are not always known, the calibration of the focal volume can be achieved by measuring a sample of known $D_{tr}$ (and therefore known $R_h$), and solving Equation 2.3 and Equation 2.4 for $r_0$. This approach is perhaps the most popular method, as researchers have used a variety of fluorescent dyes as calibration samples, measuring $D_{tr}$ independently with alternative techniques such as pulsed-field gradient NMR, dual-focus FCS, and plug-broadening capillary flow. For example, Enderlein and coworkers calibrated their experimental setup with ATTO 665, while Rigler, Schwille and others have
used the dye Rhodamine 6G. Given that most of our FCS experiments are done with nanoparticles, our calibration samples are usually fluorescently labeled polystyrene beads previously characterized via transmission electron microscopy (TEM). This choice of standard allows us to not change any of the acquisition parameters discussed below. Using this method, the dimensions of the focal volume extracted from the fit of the autocorrelation function (Figure 2.4b) were found to be \( r_0 = 370 \pm 40 \) nm and \( z_0 = 3.3 \pm 0.3 \) µm, which yield an effective volume of \( V = \pi^{3/2} r_0^2 z_0 = 2.5 \pm 0.8 \) fl. The advantage of this method is that the sample concentration does not need to be known beforehand and that the dimensions of the focal volume can be extracted in principle from a single set of experiments. The main disadvantage is that the accuracy of the experiment is determined by the quality of the fit to the autocorrelation function and the accuracy of the independent measurement of the diffusion coefficient or size of the calibration sample.

**Image Fitting.** An alternative way to extract the dimensions of the focal volume is by visualizing the three dimensional microscope point spread function via raster scanning a particle over the diffraction-limited size of the Gaussian laser beam in both \( xy \) and \( xz \) directions. This method requires drying a sample on a surface, and recording the signal of an isolated particle using a scanning stage. Using the width at \( 1/e^2 \) of the Gaussian profiles of such a fluorescent bead image (Figure 2.4c), the dimensions of the focal volume were measured to be \( r_0 = 315 \pm 20 \) nm and \( z_0 = 3.2 \pm 0.3 \) µm, which yield a calculated volume of \( V = \pi^{3/2} r_0^2 z_0 = 1.7 \pm 0.5 \) fl. The laser excitation conditions and sample environment used should be the same as for the FCS experiments, therefore we added \( \sim 30 \) µl of water on top of the surface to
mimic the water environment of the FCS experiments. This calibration method is commonly used by commercial FCS instruments,\textsuperscript{112} and inspired the development of temporal fluorescence image correlation methods.\textsuperscript{113-115} The main advantage of this calibration method is that it does not require knowing the particle size and concentration beforehand as long as its size is below the diffraction limit. The main limitation is that this method relies on the accuracy of the scanning stage (usually a piezo-electric scanner), the alignment of the optics, and the mono-dispersity of the sample after being dried on a surface. Also, piezo-electric scanning stages and their associated controllers are usually costly, and this method does not consider possible optical aberrations of the focal volume in solution. Table 2.1 summarizes the dimensions of the focal volume obtained with each calibration method, showing fair agreement among them.
Figure 2.4 Three experimental methods to determine the confocal observation volume using 36 nm fluorescently labeled polystyrene beads under 638 nm laser excitation. A. **Amplitude Fitting.** Average number of particles in the observation volume (<N>) as a function of concentration (blue squares). The volume V is extracted from the slope of the linear regression (dashed red line). B. **Lifetime Fitting.** Experimental autocorrelation function (blue squares) fit to a 1 species diffusion model (Equation 2.2, red lines). Inset: Histogram for r₀ and z₀ obtained from fits to τ₀ of at least 28 autocorrelations using a fixed R₀. The solid red line shows a Gaussian fit. C, D. **Image Fitting.** Scanning confocal fluorescence image of an isolated bead in the x-y (C) and x-z (D) planes. Water was added on top to mimic the refractive index of the FCS experiments. The side panels show line sections (blue dots) of the images across the center maxima of the point spread functions, with their respective Gaussian fits (red lines).
### Table 2.1 Summary of focal volume parameters ($r_0$, $z_0$ and $V$) of the data shown in Figure 2.4

<table>
<thead>
<tr>
<th>Method</th>
<th>$V$ (fl)</th>
<th>$r_0$ (nm)</th>
<th>$z_0$ (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude Fitting (Figure 2.4A)</td>
<td>1.6 ± 0.05</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Lifetime Fitting (Figure 2.4B)</td>
<td>2.5 ± 0.8</td>
<td>370 ± 40</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>Image Fitting (Figure 2.4C, D)</td>
<td>1.7 ± 0.5</td>
<td>315 ± 20</td>
<td>3.2 ± 0.3</td>
</tr>
</tbody>
</table>

#### 2.4.6. Considerations for the Size of the Observation Volume

Having noted the advantages and disadvantages of each calibration method, it should be remembered that the lateral $\sigma_{xy}$ and axial $\sigma_z$ spatial resolutions of an optical microscope are determined by the laser excitation wavelength $\lambda$ and numerical aperture $NA$ of the objective used, as derived by Ernst Abbe in the late 19th century:\(^{116}\)

\[
\sigma_{x,y} = \frac{\lambda}{2 \cdot NA} \quad \sigma_z = \frac{2\lambda}{NA^2}
\]

**Equation 2.9**

Therefore shorter excitation wavelengths and higher $NA$'s improve the spatial resolution. While a higher spatial resolution usually implies higher signal to background ratios as less background fluid is inside the observation volume, it also requires higher particle concentrations in order to record a statistically meaningful number of diffusion events. The search for higher spatial resolutions has inspired clever designs for FCS experiments using zero mode waveguides\(^{117}\) and gold bowtie antennas\(^{118}\) in order to explore biological processes occurring at micromolar
concentration that could not be explored otherwise with conventional femtoliter FCS volumes more appropriate for nanomolar concentrations.

In contrast, larger observation volumes are usually attractive for studies of nanoparticles. The theory of FCS assumes that $R_h$ of the analyte is sufficiently small when compared to $r_0$ and $z_0$. This is not a concern for organic molecules as $R_h \ll r_0$, but because nanoparticles come in a wide range of sizes, $R_h$ can be closer in magnitude to $r_0$. As we will show later, an easy solution as well as a necessity is then to enlarge $r_0$ by using an objective with a smaller NA. While this focal volume expansion comes at the cost of reducing the signal to background ratio, we found that this is a feasible approach for measuring gold nanoparticles larger than 100 nm in diameter, especially because signal intensities increase with the size of the nanoparticles.

2.5. Results and Discussions

2.5.1. Photoluminescence of Gold Nanoparticles

For a single AuNS the luminescence follows the scattering spectrum and shows a weak polarization dependence. The AuNSs are not perfect spheres and interact with light differently depending on their orientation relative to the incident light polarization as was observed previously for single particle scattering and absorption.\textsuperscript{56, 119, 120} Figure 2.5A compares the scattering and the luminescence spectra, excited by a lamp (500 - 1000 nm) and with a 514 nm laser, respectively, for the same AuNS that was also imaged by correlated SEM. The size of the AuNS was 45
nm. The luminescence spectra vary with the detected polarization angle, as shown in the inset of Figure 2.5A.

**Figure 2.5 (A):** Unpolarized scattering (blue) and 514 nm excited luminescence (red) spectra of a 45 nm diameter AuNS. The inset shows polarized luminescence spectra that change in amplitude as the detection polarization was varied. (B): Unpolarized scattering (blue) and luminescence spectra excited at 514 nm (red) and 633 nm (black) of a 27x75 nm AuNR shown in the inset. (C): Unpolarized scattering (blue and cyan) and 514 nm excited luminescence (red and magenta) spectra of a 33x70 nm AuNR shown in the SEM image, which was immobilized on a glass coverslip and surrounded by air and water, respectively. (D): Polarized luminescence spectra with 514 nm excitation of a 34x68 nm AuNR shown in the SEM image. The inset illustrates the intensity as a function of detected polarization obtained by integrating the area under the spectra for the corresponding entire spectral ranges and then normalized to the maximum value, for luminescence excited at 514 nm (red) and 633 nm (black) as well as for scattering (blue). The error bars were computed from at least 3 spectra that were acquired for each polarization. The excitation light is unpolarized and circularly polarized for the scattering and luminescence spectra, respectively. All scale bars correspond to 100 nm.
The luminescence spectra of the AuNRs are independent of the excitation wavelength. For the single AuNR, shown in the SEM image in the inset of Figure 2.5B, unpolarized spectra of scattering, 514 nm, and 633 nm excited luminescence are displayed in the main part of Figure 2.5B. The three spectra are almost indistinguishable from each other for the longitudinal plasmon resonance. Thus we can conclude that these spectra excited at a single laser wavelength are indeed due to luminescence and not elastic scattering and also that luminescence can be excited both at the transverse (514 nm) or the longitudinal (633 nm) plasmon resonance giving nearly identical responses that spectrally overlap with the longitudinal plasmon band. The luminescence spectrum, when excited at 514 nm, also has a peak near 520 nm that coincides with the transverse plasmon resonance, which is visible in the bulk extinction spectrum, but too weak to be seen in the single particle scattering spectra.\textsuperscript{121} The exact location of this short wavelength peak cannot be determined because of the dichroic filter that was used.

To investigate if the luminescence is caused by the absorption of one or multiple photons, we carried out a power dependence of the luminescence intensity. Figure 2.6 shows that the luminescence intensity indeed scales linearly with laser power for AuNRs excited at 514 and 633 nm, confirming a one-photon excitation process. It is also important to note that the luminescence from immobilized AuNRs does not show any signs of intensity blinking.
Figure 2.6 Luminescence intensity vs. laser power for 514 nm (A) and 633 nm (B) excitation. Each data point represents the average for at least 6 single AuNRs that are also imaged by SEM to confirm that the signal originates from individual AuNRs. The linear trend confirms a one-photon excitation process.

The luminescence spectrum shifts with a change of the dielectric constant of the surrounding medium in the same way as the scattering spectrum. Figure 2.5C shows single particle scattering (blue and cyan) and 514 nm excited luminescence (red and magenta) spectra for a 33x70 nm AuNR supported on glass taken in air and water, respectively. The effective refractive index changes from ~1.25 as in the case of the spectra taken in air, to 1.3 for the spectra taken with water on top of the sample. The shift in the resonance maximum is about 50 nm for both scattering and luminescence. The transverse plasmon resonance did not shift measurably, but is resolved better in the luminescence spectrum with the added water because the shoulder of the longitudinal resonance no longer overlaps significantly. These results suggest that the observed luminescence is caused by emission of a plasmon. This mechanism can be probed in more detail by studying the polarization dependence of the luminescence excited at 514 and 633 nm.
The polarized luminescence spectra of a single 34x68 nm AuNR in Figure 2.5D show a strong polarization dependence for the longitudinal mode which modulates in phase with the scattering signal. In contrast, the short wavelength peak is only weakly polarization dependent. To calculate the modulation depth of the scattering and luminescence intensity as a function of the detection polarization, we recorded spectra for 10 different AuNRs as a function of polarizer angle and integrated the entire area under the spectra for each of them. An example for scattering and luminescence of the same AuNR is shown in the inset of Figure 2.5D. The resulting polarization traces were fit to $I(\theta) = N(1 + M \cos 2(\theta - \phi))$, where $N$ is a normalization factor, $M$ is the modulation depth, $\theta$ is the polarizer angle, and $\phi$ represents the angle of the longest projected dipole axis with respect to a reference frame.\textsuperscript{122} Scattering exhibits a perfect dipole behavior with an average modulation depth of $0.95 \pm 0.05$ obtained from 10 individual AuNRs. Luminescence for 633 nm excitation closely follows amplitude and phase of scattering and its modulation depth is $0.94 \pm 0.05$. The integrated luminescence for 514 nm excitation has a slightly lower modulation depth of $0.90 \pm 0.07$ because the short wavelength peak does not change in amplitude for different detection polarizations.

In addition to the emission dipole, the polarization of the absorption dipole is also important to understand the correlation spectroscopy measurements. We therefore probed it by recording spectra as a function of the excitation polarization. We found that the longitudinal plasmon luminescence is independent of the excitation polarization at 514 nm and always occurs along the long axis of the AuNR.
This was determined by independently controlling the polarization angles of both excitation and detection as shown in Figure 2.7A. When the excitation is set parallel to the short axis of the AuNR and the detected polarization is parallel to the long AuNR axis the highest intensity is obtained (90°-0°, cyan). No changes are observed when the excitation polarization is rotated by 90° (0°-0°, green). On the other hand, rotating the emission polarizer by 90° erases the longitudinal peak independent of the excitation polarization. Therefore we can conclude that luminescence of the AuNR is always aligned parallel to the long axis, regardless of the excitation polarization. The independence of the excitation polarization is further illustrated in the inset of Figure 2.7A, where the unpolarized integrated luminescence intensity is plotted as a function of excitation polarization yielding an almost negligible modulation depth.

Excitation at 633 nm on the other hand exhibits the expected pure dipole behavior and matches the phase of the luminescence. Therefore absorption and emission dipoles are collinear for 633 nm excitation. Unpolarized luminescence spectra of a 35x72 nm AuNR for different excitation polarizations are shown in Figure 2.7B. Plotting the integrated area under the spectra versus the excitation polarization yields the curve shown in the inset of Figure 2.7B and a modulation depth of 0.97 confirming a perfect dipole behavior.
Figure 2.7 (A): Luminescence spectra of a 34x68 nm AuNR excited at 514 nm taken at varying orientations of excitation and detection polarizations relative to the long axis of the AuNR. The integrated intensity as a function of excitation polarization is given in the inset. For this data the detection polarizer was removed. For this experiment only, the reported angles are defined with respect to the long axis of the AuNR. (B): Unpolarized luminescence spectra of a 35x72 nm AuNR shown in the SEM image excited at 633 nm for different excitation polarizations. The integrated intensity as a function of excitation polarization is shown in the inset. All scale bars correspond to 100 nm.

We thus assign the luminescence to the radiative decay of surface plasmons. Figure 2.8 shows a suggested mechanism for one-photon plasmon emission of AuNRs. Luminescence occurs from both the transverse and longitudinal surface plasmon resonances with the latter being the dominant decay channel. This model is supported by the nearly perfect spectral overlap of the luminescence with scattering in both air and water environments and the high polarization dependence of the longitudinal surface plasmon emission. The spectral overlap between scattering and luminescence observed here and the suggested mechanism are consistent with a previous ensemble study on the one-photon luminescence of AuNSs\(^9\) as well as two-photon luminescence recorded from individual AuNRs.\(^{12,3}\)
Figure 2.8 Schematic diagram of the mechanism for one-photon plasmon luminescence of AuNRs. The solid green lines represent excitation with 514 nm laser light, which excites both d-sp interband transitions creating electron-hole (e-h) pairs and the transverse surface plasmon resonance (TSPR). The solid red line depicts excitation of the longitudinal surface plasmon resonance (LSPR) with 633 nm laser light. The wavy green and red lines represent emission from the TSPR and LSPR, respectively. Nonradiative relaxation occurs through recombination of e-h pairs as indicated by the dashed gray line. For simplicity no distinction is made in this diagram between e-h pairs excited by interband vs. intraband transitions. Furthermore, note that the threshold for interband transitions lies above the LSPR.

The observation that the emission of the AuNSs also follows the plasmon resonance validates the assignment of the short wavelength peak in the AuNR luminescence spectrum to emission of the transverse plasmon. At 514 nm, both d-sp interband transitions and the transverse surface plasmon are excited due to their spectral overlap. Interband absorption creates electron-hole pairs that can relax very efficiently through nonradiative pathways because of the large density of states in the sp-conduction and d-valence bands. However, analyzing the excitation and emission polarizations of the short wavelength peak in the AuNR luminescence
spectra revealed a significant depolarization of the emission compared to previous polarization sensitive photothermal imaging of the absorption at 514 nm.\textsuperscript{122} This loss of polarization is assigned to a fast interconversion between electron-hole pairs and the transverse surface plasmon resonance that subsequently decays radiatively. Because of the limited spectral range for the transverse mode due to the dichroic used, other luminescence mechanisms like plasmon amplified electron-hole pair recombination\textsuperscript{91, 92, 95, 96} cannot be completely ruled out at the moment for this wavelength range. In contrast, 633 nm corresponds to energies below the interband absorption threshold and hence radiative recombination between d-valence band holes and sp-conduction band electrons should not be possible. At a wavelength of 633 nm direct excitation of the longitudinal surface plasmon resonance occurs as illustrated in Figure 2.8. Because of the large bandwidth of the plasmon resonance, excitation at the blue edge is possible and emission occurs within the spectral bandwidth of the plasmon resonance so that a distinct Stokes shift as seen in molecular fluorescence is not observed here.

The importance of hot electron-hole pairs and the fast interconversion between them and surface plasmons becomes even more evident when considering the longitudinal surface plasmon resonance. Direct excitation at 633 nm is highly polarized parallel to the long AuNR axis for both absorption and emission and leads to either direct plasmon emission or nonradiative decay through the generation of electron-hole pairs. However, excitation at 514 nm also gives rise to mainly longitudinal surface plasmon emission, which does not depend on the excitation polarization. Because the transverse and longitudinal modes are orthogonal to each
other, this observation can only be rationalized if hot electron-hole pairs can also create surface plasmons. The decay of surface plasmons into electron-hole pairs is a well accepted energy relaxation channel, but our results also show that the opposite is possible, although most likely with a much smaller yield.

Estimating a detection efficiency of 5% for our microscope setup, we determined the quantum yields of plasmon emission for different excitation wavelengths. Using calculated absorption cross sections of $1.44 \times 10^3$ nm$^2$ and $1.87 \times 10^3$ nm$^2$ at 514 and 633 nm for a 24x76 nm AuNR, and respective incident laser powers of 37.5 µW and 1.5 µW (or 13 kW/cm$^2$ and 0.5 kW/cm$^2$) at the sample, luminescence quantum yields of $8 \times 10^{-6}$ and $3 \times 10^{-4}$ are obtained for 514 and 633 nm excitation, respectively. Luminescence excited at the longitudinal surface plasmon resonance is significantly more efficient, which can be explained by the fact that the longitudinal surface plasmon energy is below the threshold for interband absorptions. Nonradiative recombination of electrons with holes in the valence band is hence no longer possible, consisted with longer plasmon dephasing times. Most of the energy is, however, dissipated via the nonradiative recombination of electron-hole pairs as shown by the gray line in Figure 2.8.

**2.5.2. Measuring the Hydrodynamic Size of Gold Nanoparticles via FCS**

We have applied FCS using the scattering and photoluminescence response of gold nanoparticles, and accurately determined their hydrodynamic dimensions in water. Figure 2.9A plots the hydrodynamic diameters of 6 gold nanospheres samples with different sizes obtained via scattering correlation spectroscopy
against the average diameter determined from TEM analysis in comparison to DLS measurements. All sizes are summarized in Table 2.2. While we found excellent agreement between these size characterization methods for sizes smaller than 100 nm, we also observe a systematic underestimation of the nanoparticle size for larger diameters using FCS. It appears that DLS is less sensitive to this underestimation artifact compared to FCS. Following this observation and our previous discussion of the focal volume size, we tested the hypothesis that when the nanoparticle’s diameter becomes comparable in size to $r_0$, the size of the focal volume sets an upper limit above which nanoparticles cannot be sized adequately with FCS (Table 2.2). Similar particle size effects have also been recently reported by Deptula et al. for large fluorescent spheres of radius between 200 nm and 600 nm.\textsuperscript{125} It is important to note that DLS is less sensitive to this artifact as it does not rely on a microscopic observation volume,\textsuperscript{126} but requires at least 10 times larger sample volumes and 10 times higher concentrations (> nM) while being negatively affected if the nanoparticles are strongly luminescent.
Figure 2.9 Sizing of colloidal gold nanoparticles using scattering and photoluminescence correlation spectroscopy. A. Hydrodynamic diameters from scattering FCS (teal blue) and DLS (gray) measurements of 6 gold nanoparticle samples with different sizes plotted against their mean diameter determined by TEM. B. Hydrodynamic diameters from photoluminescence FCS measurements of the same gold nanoparticles using a $NA = 1.4$ (blue) and a $NA = 0.7$ (red). The latter reduces the spatial and axial resolutions, enlarging the confocal observation volume $V$. For both panels, the dashed black line is shown as a guide for the eye, indicating that the sizes determined by TEM and FCS are equal, i.e. TEM = FCS.

Table 2.2 Summary of nanoparticle diameters (in nm) from the data shown in Figure 2.9. *Values in parenthesis refer to the measurements with an enlarged focal volume using $NA = 0.7$

<table>
<thead>
<tr>
<th>TEM</th>
<th>DLS</th>
<th>Photoluminescence</th>
<th>Scattering</th>
</tr>
</thead>
<tbody>
<tr>
<td>19.3 ± 2.5</td>
<td>23 ± 1</td>
<td>24 ± 15</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>40.8 ± 3.8</td>
<td>47 ± 2</td>
<td>43 ± 10</td>
<td>48 ± 11</td>
</tr>
<tr>
<td>48.8 ± 4.1</td>
<td>52 ± 3</td>
<td>50 ± 11 (51 ± 7)</td>
<td>52 ± 8 (51 ± 8)</td>
</tr>
<tr>
<td>61.7 ± 6.2</td>
<td>60 ± 3</td>
<td>62 ± 21</td>
<td>67 ± 10</td>
</tr>
<tr>
<td>87.6 ± 7.7</td>
<td>80 ± 4</td>
<td>71 ± 17 (97 ± 22)</td>
<td>91 ± 25</td>
</tr>
<tr>
<td>176.4 ± 18.5</td>
<td>158 ± 8</td>
<td>136 ± 26 (180 ± 30)</td>
<td>142 ± 27 (172 ± 29)</td>
</tr>
</tbody>
</table>
As mentioned earlier, the use of larger focal volumes allows us to measure larger nanoparticles accurately. Figure 2.9B shows the hydrodynamic diameters of the same six nanoparticles measured in Figure 2.9A using the photoluminescence signal of the gold nanoparticles. The larger focal volume was achieved by decreasing the $NA$ from 1.4 to 0.7 using an objective with an adjustable aperture. This change in $NA$ effectively increased $r_0$ from 250 ± 20 nm to 350 ± 30 nm as determined by calibration with a sample of known size. These values of $r_0$ are smaller than the ones shown in Table 2.1 because for this experiment we used a 532 nm laser closer to the resonance wavelength of the gold nanoparticles, hence producing larger scattering and photoluminescence intensities. The larger focal volume with the lower $NA$ significantly improved the accuracy for measuring the 87 nm and 176 nm gold nanoparticles using FCS (Table 2.2, parenthetical data). Moreover, this change did not affect our ability to characterize smaller nanoparticles as the size of the 48 nm nanoparticles was identical for both $NA$ settings.

2.5.3. Polarization-Sensitive FCS as a Probe of Nanorod Rotational Diffusion

The rotation of anisotropic nanoparticles such as rod-shaped particles produces polarized intensity fluctuations that can be detected with a polarization sensitive detection scheme. In the context of FCS, the characteristic decay time of nanoparticle rotations ($\tau_{rot}$) is proportional to the rotational diffusion coefficient ($D_{rot}$). Kask et al. expanded the conventional model of FCS (Equation 2.2) by adding a term describing the rotational diffusion of rod-shape particles:127
\[ G(\tau) = \frac{1}{\langle N \rangle} \left( \frac{1}{1 + \frac{\tau}{\tau_D}} \right) \left( 1 + \left( \frac{r_0}{z_0} \right)^2 \left( \frac{\tau}{\tau_D} \right) \right)^{1/2} \cdot \left( 1 + A \cdot B_i \cdot \exp(-6D_{\text{rot}} \tau) \right) \]

Equation 2.10

\( A \) is the anisotropy of the system (1 for a perfect dipole) and \( B_i \) is a coefficient describing the relative orientations between the excitation and detection polarizations. \( D_{\text{rot}} \) can be related to \( R_h \) using the Stokes-Einstein relationship for rotational diffusion:

\[ D_{\text{rot}} = \frac{k_B T}{8\pi \kappa R_h^3} \]

Equation 2.11

Moreover, Tirado et al. derived expressions for a rod-shaped macromolecule relating \( D_{\text{trans}} \) and \( D_{\text{rot}} \) to its length \( L \) and diameter \( d \):

\[ D_{\text{trans}} = \frac{k_B T}{3\pi \kappa L} \left[ \ln \left( \frac{L}{d} \right) + \nu \right] \]

Equation 2.12

\[ D_{\text{rot}} = \frac{3k_B T}{\pi \kappa L^3} \left[ \ln \left( \frac{L}{d} \right) + \sigma \right] \]
Equation 2.13

Where $v = 0.312 + 0.565 \frac{d}{L} - 0.1 \left(\frac{d}{L}\right)^2$ and $\sigma = -0.662 + 0.917 \left(\frac{d}{L}\right) - 0.05 \left(\frac{d}{L}\right)^2$.

An important conclusion of this model is that $D_{\text{rot}}$ is more sensitive to changes in nanorod length ($D_{\text{rot}} \propto \frac{1}{L^3}$) compared to $D_{\text{trans}}$ ($\propto \frac{1}{L}$). The Weiss group performed an experimental verification of this model using polarization-sensitive FCS and three peptide coated CdS/ZnS nanorods of the same diameter but different length.\(^{129}\) Similarly, Bassam and Toshiharu used polarized light scattering microscopy with FCS to measure the rotational diffusion of gold nanorods.\(^{130}\)

Clever implementations of polarization-sensitive FCS have been developed in recent years to measure the rotational dynamics of nanoparticles and molecules. For example, Enderlein and colleagues used single-photon counting electronics to measure the fast rotational diffusion of fluorescently-labeled bovine and human serum albumin proteins on the order of tens of nanoseconds. This fast time resolution was achieved by measuring the cross-correlation between two orthogonally polarized detectors following a picosecond laser pulse.\(^{131}\) Loumaigne \textit{et al.} developed an interesting time-of-flight (TOF) photon spectroscopy method that can simultaneously measured the photoluminescence spectrum and rotational diffusion of gold nanorods in water using photon correlation analysis.\(^{132}\) Orrit and coworkers on the other hand used a polarization-sensitive FCS scheme under optical trapping conditions and resolved the Brownian fluctuations of individual
gold nanorods in a glycerol/water mixtures. Orientational fluctuations in more complex and crowded environments can also be extracted in 2D and 3D with polarization resolved FCS.

We have also applied FCS to measure the hydrodynamic dimensions of colloidal gold nanorods by simultaneously detecting their rotational and translational diffusion in water (Figure 2.10). This measurement was accomplished by placing a polarization beam splitter in the detection beam path of our FCS setup, dividing the signal into two orthogonal polarization components. The inset of Figure 2.10A shows a simplified diagram of the two-channel polarization sensitive FCS setup used to measure nanorod rotational diffusion. Each channel can independently detect rotations using the autocorrelation of the signal with itself or the cross-correlation between the two. The main panel of Figure 2.10A shows the autocorrelation curve of 34 (± 8) x 60 (± 13) nm gold nanorods in water. The autocorrelation curve shows two characteristic decays due to nanorod rotation and diffusion. A fit of the data to Equation 2.10 yields a fast decay arising from rotational diffusion ($\tau_{\text{rot}} = 23 \pm 3 \, \mu\text{s}$) corresponding to a $R_h = 29 \pm 5 \, \text{nm}$ ($D_{\text{rot}} = 7300 \pm 1100 \, \text{s}^{-1}$). The slower decay associated with the nanorod’s translational diffusion is $\tau_{\text{D}} = 11 \pm 0.4 \, \text{ms}$ corresponding to a $R_h = 25 \pm 1 \, \text{nm}$ ($D_{\text{trans}} = 9.6 \pm 0.3 \, \text{μm}^2 \, \text{s}^{-1}$). These experimentally determined sizes are in excellent agreement with the expected hydrodynamic radius ($R_{h,\text{theor}} = 26 \pm 8 \, \text{nm}$) based on the size distribution of the nanorods determined by TEM. Figure 2.10B furthermore illustrates the hydrodynamic radii for three gold nanorods samples with different sizes computed from the measured rotational and translational diffusion coefficients. The values
measured via FCS all fall inside the error boundaries calculated based on a 10% size dispersion of each nanorod samples as determined by TEM.

**Figure 2.10** Polarization sensitive FCS as a tool to size gold nanorods by simultaneously measuring their rotational and translational diffusion. A. Representative autocorrelation curve of 34 x 60 nm gold nanorods in water (blue squares) along with a fit to Equation 2.10 which includes both the translational and rotational autocorrelation function (dashed red line). \( \tau_{\text{rot}} \) and \( \tau_D \) indicate the characteristic rotational and translational diffusion times extracted from the fit line. A simplified diagram of the polarization sensitive two-channel luminescence detection scheme is shown as an inset. The laser propagates along the z coordinate. B. Hydrodynamic radius \( R_h \) for 3 sizes of gold nanorods obtained via FCS measurements using the average translational (dark gray) and rotational (blue) component of at least 7 autocorrelation curves. The data is plotted against the theoretical \( R_h \) based on the dimensions of the gold nanorods measured from TEM images. The red-shaded area between the dashed red lines indicates the 10% error boundaries expected from the size distribution of the nanorods as measured by TEM.
2.6. Summary

In summary, this chapter has presented experimental implementation of fluctuation correlation spectroscopy to measure the size of colloidal nanoparticles in solution. We presented three different methods to calibrate the focal observation volume, essential to obtaining correct hydrodynamic dimensions of nanoparticles. By recording single particle photoluminescence spectra using polarization sensitive excitation and detection, we found that one-photon luminescence of AuNSs and AuNRs closely follows the plasmon-scattering spectrum. With this knowledge about the mechanism of one-photon luminescence, we demonstrated the use of FCS to measure the size of gold nanospheres and nanorods, which can be detected by their intrinsic plasmonic scattering and photoluminescence signals. Moreover, we determined that larger nanoparticles ($d > 80$ nm) required larger focal volumes to avoid under-sizing artifacts. We anticipate that FCS will continue to contribute in the study of nanoparticle behavior and transport in solution, deepening our understanding of the environmental impacts of nanoparticles.

2.7. Acknowledgments

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Vaughn Fellowship from Rice University. This work was also supported by the Nanoscale Science and Engineering Initiative of the National Science Foundation under NSF Award Numbers EEC-0647452 and CHE-0955286, the Robert A. Welch Foundation (Grant C-1664), the ACS Petroleum Research Fund (50191-DNI6), and a 3M Nontenured Faculty Grant. W.S.C. acknowledges support from the Richard E. Smalley Institute for a Peter and Ruth Nicholas fellowship. P.S. acknowledges support from the Royal Thai Government and L.S.S. thanks the NSF IGERT Nanophotonics fellowship program. C.F.L. also thanks the Norman Hackerman Welch Young Investigator Program at Rice University and the Donors of the American Chemical Society Petroleum Research Fund for partial support of this research. We thank Prof. James Tour for allowing us to use his absorption spectrometer, and Saumyakanti Khatua for assistance with acquiring the single particle spectra. We also thank Profs. Naomi Halas, Peter Nordlander, and Carsten Sönnichsen for insightful discussions.
3.1. Abstract

This chapter presents *in situ* observations of adsorption of bovine serum albumin (BSA) on citrate-stabilized gold nanospheres. We implemented scattering correlation spectroscopy as a tool to quantify changes in the nanoparticle Brownian motion resulting from BSA adsorption onto the nanoparticle surface. Protein binding was observed as an increase in the nanoparticle hydrodynamic radius. Our results indicate the formation of a protein monolayer at similar albumin concentrations as those found in human blood. Additionally, by monitoring the frequency and intensity of individual scattering events caused by single gold nanoparticles passing the observation volume, we found that BSA did not induce
colloidal aggregation, a relevant result from the toxicological viewpoint. Moreover, to elucidate the thermodynamics of the gold nanoparticle-BSA association, we measured an adsorption isotherm which was best described by an anti-cooperative binding model. The number of binding sites based on this model was consistent with a BSA monolayer in its native state. In contrast, experiments using poly-ethylene glycol capped gold nanoparticles revealed no evidence for adsorption of BSA.

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3.2. Motivation and Introduction

The safe use of nanoparticles in *in vivo* applications requires a clear understanding of the nanoparticle interface.14,16,135 How nanoparticles diffuse and interact with biomolecules in complex biological fluids is an intriguing question that receives growing attention.25,52,136 For instance, the interaction of nanoparticles with blood plasma is of special interest27, 137, 138 because many of their intended biomedical applications (e.g. drug delivery,139-141 disease diagnosis and treatment17-19,142,143) often require an intravenous approach.

Recent studies have demonstrated that once nanoparticles are introduced into plasma they can become coated by the vast collection of biomolecules present
in this medium, forming a ‘corona’ that surrounds the nanoparticles and shields their original surface properties.\textsuperscript{26,28,29} Serum proteins play a major role in these interactions because they constitute the majority of the plasma fluid and have comparable nano-scale sizes.\textsuperscript{12} It is thought that the affinity of a certain protein to bind to a nanoparticle surface is determined by the nanoparticle size,\textsuperscript{30,33} shape,\textsuperscript{144} and surface chemistry.\textsuperscript{33,144}

The role of a corona masking the designed surface chemistry of nanoparticles is a critical issue as cellular uptake and its associated physiological response are regulated by chemical interactions at the nanoparticle surface.\textsuperscript{12,21} Doorley \textit{et al.}\textsuperscript{43} demonstrated the co-localization of 200 nm polystyrene beads and serum albumin, the most abundant protein in human blood,\textsuperscript{145} at the membrane of kidney cells. This has the subtle yet critical implication that what the cell actually ‘sees’ is not the nanoparticle core or the initially synthesized organic interface but the bound proteins forming a corona on the nanoparticle surface. A recent report by Deng \textit{et al.}\textsuperscript{50} showed how 5 and 20 nm poly-acrylic acid coated gold nanoparticles bound to and induced unfolding of fibrinogen, thereby exposing a specific amino acid combination that interacts with an important receptor on human leukemia cells causing the release of cytokines and hence inflammation. Such protein-nanoparticle hybrids could show long term stability under \textit{in vivo} conditions and trigger undesired signals in cellular receptors that would otherwise remain inactive. The necessary knowledge of these unwanted side effects encourages fundamental research on the characterization of the nanoparticle interface when exposed to physiological environments.
Nanoparticle-protein associations have been characterized by several different methods that typically require the separation of the nanoparticles from the adsorbed proteins, followed by the identification of the isolated organic compounds. A common approach involves isolation via size exclusion chromatography or differential centrifugation, followed by protein identification via gel electrophoresis and mass spectrometry. Deposition of a dried sample on a substrate has been useful for structural imaging via transmission electron microscopy, while the identification of the protein corona is done by staining of the organic compounds. Most strategies therefore involve the removal of the nanoparticle-protein complexes from their original physiological environments.

A complimentary in situ approach to these previous studies measures diffusion parameters before and after protein adsorption. This allows one to address such important issues as the amount of adsorbed protein and the fate of the nanoparticle-protein complexes directly in physiological environments. The latter is especially critical from a toxicological viewpoint if adsorbed proteins induce nanoparticle aggregation, which may complicate cellular uptake and clearance, or if nanoparticles induce protein aggregation, which is often associated with a variety of diseases. Therefore, in situ observations of protein adsorption on nanoparticles under physiological conditions are required for the safe development of the emerging field of nanomedicine.

Gold nanoparticles (AuNPs) have emerged as a favorable platform for potential applications in nanomedicine because of the established non-toxicity of
the gold core.\textsuperscript{148} Furthermore AuNPs are attractive because their strong plasmon resonance makes it possible to simultaneously use them as optical probes.\textsuperscript{149-151} However, \textit{in situ} characterization of AuNPs interacting with proteins are scarce and, to some extent, contradicting as both monolayer\textsuperscript{26, 147} and multilayer\textsuperscript{28, 144} adsorption of serum albumin have been reported for AuNPs in a physiological environment. Additionally, disagreement exists in the literature on whether BSA stabilizes the NP colloid\textsuperscript{26, 31, 147} or induces NP aggregation\textsuperscript{28, 144}. The mechanism for BSA adsorption on negatively charged citrate-stabilized AuNPs is also under debate. Both an electrostatic attraction via the positively charged lysine groups of BSA\textsuperscript{26, 31} and a ligand exchange reaction with the sulfur of the cysteine residue that binds to the AuNP surface\textsuperscript{147} have been suggested. Because nanoparticle-protein interactions and the possible formation of a protein corona are thought to depend on many parameters including nanoparticle size and shape as well as surface chemistry, further quantitative \textit{in situ} measurements are necessary.

Here, we use plasmon scattering correlation spectroscopy\textsuperscript{64} to explore \textit{in situ} protein adsorption on AuNPs by monitoring the changes in the Brownian diffusion due to protein binding. Bovine serum albumin was used as a model protein and shares almost identical structure, function, and pH-dependent transitions with its human counterpart.\textsuperscript{145, 152} Our results indicate the formation of a BSA monolayer at blood plasma concentrations on citrate-stabilized AuNPs independent of AuNP size in the range of 51 - 93 nm. Citrate-stabilized AuNPs were used because they are the most common spherical AuNPs used. Burst intensity frequency analysis (BIFA)\textsuperscript{153} of scattering transients furthermore reveals that BSA adsorption does not induce
colloid aggregation for these AuNPs. Based on the results of an adsorption isotherm, we demonstrate that BSA adsorption occurs spontaneously via an anti-cooperative binding mechanism. Finally, experiments with poly-ethylene glycol (PEG) functionalized AuNPs show that the PEG coating provides protection from BSA adsorption.

3.3. Experimental Section

3.3.1. Materials

Citrate-stabilized AuNPs with three distinct diameters (51, 70, and 93 nm) and 56 nm AuNPs capped with PEG (M$_w$ = 5,000) were purchased from Nanopartz Inc. BSA (≥98% lyophilized powder, M$_w$ = 66,430) was obtained from Sigma-Aldrich Corp. The purity of the protein was comparable to previous nanoparticle studies.$^{26,28,29,31,144,147}$ Contamination by small molecules can be neglected as they do not cause a measurable effect in the correlation spectroscopy measurements. The protein powder was suspended and diluted to the desired concentration (0.75 mM unless noted otherwise) in 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer, Life Technologies$^{TM}$), 20 mM NaCl (Sigma-Aldrich) and Ultrapure$^{TM}$ molecular biology grade water (Gibco). The BSA solutions were freshly prepared for each experiment, or otherwise stored at -20 °C to reduce protein aggregation and sedimentation. The pH of this solution was in the physiological range (7.0 - 7.5). The AuNPs were also diluted in this buffer to picomolar concentrations ($\sim 10^{10}$ particles/mL). For protein binding experiments, AuNP and
BSA solutions were mixed using equal volumes and then measured immediately. Microscope cover glass slides (25 x 25 mm², Fisherbrand) were sonicated in acetone (ACS spectrophotometric grade, Sigma-Aldrich), 1% Liquinox™ (Alconox Inc.), and Milli DI™ water (>25 MΩ, Millipore) followed by drying with ultra high purity N₂ (Matheson) and O₂ plasma cleaning for 1 minute at 300 mTorr (Harrick Plasma). To avoid unwanted protein adsorption to the substrate, the cover glass was treated with Vectabond reagent (Vector Labs) and PEG-5000 (Sigma-Aldrich) following a procedure described elsewhere. Silicon chambers (50 µL, Grace Bio-Labs) were placed on top of the microscope coverslips and filled with the solution of interest.

3.3.2. Gold Nanoparticle Characterization

The AuNPs were characterized using transmission electron microscopy (TEM, JEOL 2010) and ensemble UV/Vis spectroscopy (Ocean Optics) as shown in Figure 3.1 and 3.2. The size distribution of each sample was obtained from the TEM images using an automated Matlab program. Two perpendicular axes were measured per AuNP and the results are summarized in Table 3.1. The AuNPs were mostly spherical as indicated by the black dashed lines in Figure 3.1. Zeta potentials were measured for AuNPs only and AuNPs with BSA added using a Malvern Zen 3600 (Zetasizer Nano). The samples were prepared in the same way as described above. The Smoluchowski approximation was used as an input parameter of the Henry equation, corresponding to the electrophoretic mobility of small particles in aqueous media.
Figure 3.1 Size distributions obtained via TEM images for 51 (A), 70 (B), 93 (C) and 56 nm PEG-coated (D) nm AuNPs. The dashed black lines represent an aspect ratio of 1, or perfect spherical shape, shown as a guide to the eye. Representative TEM images are given below each analysis of the AuNP size. All scale bars are 100 nm.
<table>
<thead>
<tr>
<th>Sample $R_{TEM}$</th>
<th>Mean AuNP dimensions long axis [nm] x short axis [nm]</th>
<th>Number of AuNPs analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>51 nm</td>
<td>50.4 (± 6.7) x 51.9 (± 7.0)</td>
<td>320</td>
</tr>
<tr>
<td>70 nm</td>
<td>71.0 (± 8.7) x 69.4 (± 8.6)</td>
<td>194</td>
</tr>
<tr>
<td>93 nm</td>
<td>94.4 (± 6.8) x 92.1 (± 6.5)</td>
<td>206</td>
</tr>
<tr>
<td>56 nm – PEG</td>
<td>56.3 (± 4.1) x 55.1 (± 4.1)</td>
<td>675</td>
</tr>
</tbody>
</table>

Table 3.1 AuNP dimensions measured from TEM images using an automated program in Matlab. $R_{TEM}$ is the average of the two mean dimensions. The uncertainty represents one standard deviation from the mean values.

### 3.3.3. Optical Setup for Scattering Correlation Spectroscopy

Scattering correlation spectroscopy was performed using a home-built inverted confocal microscope (Observer.D1, Zeiss) as described in Chapter 2. Briefly, light from a 532 nm laser (Verdi-6, Coherent Inc.) was collimated and expanded to overfill the back aperture of a microscope objective (Fluar, Zeiss: 100X, N.A. = 1.3). The beam was circularly polarized using a $\lambda/4$ waveplate (Newport). The laser power was attenuated to ~ 150 nW using neutral density filters (Thorlabs). This power gave a signal to background ratio of ~ 10, yet ensured negligible heating of the sample.\(^{64}\) The focal plane of the objective was set to approximately ~ 6 µm inside the solution to avoid excessive scattered light from the glass-water interface. Orange fluorescent polystyrene beads (100 nm in diameter, Molecular Probes®) were used for alignment and calibration of the focal volume, as described in Chapter 2. AuNPs diffusing across this diffraction limited focal volume scattered light due to their inherent surface plasmon resonance. Plasmon scattering was chosen for these experiments instead of one-photon plasmon luminescence of the AuNPs\(^{64}\) because of an interfering background signal from auto-fluorescence by the BSA solution. For
the scattering geometry, fluorescence was negligible at the excitation powers used. The scattered light was collected in the backwards direction and redirected to a 50 µm pinhole (Thorlabs) before focusing onto an avalanche photodetector (SPCM-AQRH, Perkin Elmer).

3.3.4. Analysis of Scattering Correlation Spectroscopy

Correlation spectroscopy yields the characteristic diffusion time $\tau_D$ of an analyte of interest as it diffuses through a diffraction limited focal volume. Due to the low concentrations used (typically $< nM$), it is assumed that only one analyte crossed the focal volume ($< 1 fL$) at a time. Fluctuations in the scattering intensity $I(t)$ were observed when AuNPs were optically excited while diffusing across this focal volume. Temporal autocorrelation analysis of the scattering signal was performed over a range of lag times $\tau$ from $\tau_{min}$ to $\tau_{max}$:

$$G(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t + \tau) \rangle}{\langle I(t) \rangle^2}$$

Equation 3.1

Here $G(\tau)$ represents the autocorrelation function and $\delta I(t)$ an intensity fluctuation, which is mathematically represented as the signal at time $t$ minus the average: $\delta I(t) = I(t) - <I(t)>$. For the case of a Gaussian excitation profile, Aragon et al.\textsuperscript{156} derived the three dimensional autocorrelation function in terms of the average...
number of species crossing the focal volume $<N>$, the beam waist $r_0$, and beam height $z_0$:

$$G(\tau) = \frac{1}{\langle N \rangle} \left( 1 + \frac{\tau}{\tau_D} \right) \left( 1 + \left( \frac{r_0}{z_0} \right)^2 \left( \frac{\tau}{\tau_D} \right) \right)^{1/2}$$

**Equation 3.2**

The experimental autocorrelation was fitted to Equation 3.2, where $\tau_D$ and $\left( \frac{r_0}{z_0} \right)$ were used as fit parameters. The amplitude was normalized for better comparison of the differences in $\tau_D$ associated with BSA binding. The characteristic diffusion time is related to the translational diffusion coefficient $D_{tr}$ by the following relationship:

$$\tau_D = \frac{r_0^2}{4D_{tr}}$$

**Equation 3.3**

The Stokes-Einstein equation then yields the hydrodynamic radius $R_h$:

$$D_{tr} = \frac{kT}{6\pi\eta R_h}$$

**Equation 3.4**
Where \( k \) is the Boltzmann constant, \( T \) is the temperature \((T = 296 \pm 0.5 \text{ K})\), and \( \eta \) is the solvent viscosity. The effect of BSA on the viscosity of the solution was considered using a linear approximation based on the intrinsic viscosity of the protein provided by the manufacturer, \([\eta] = 4.13 \text{ cm}^3 \text{ g}^{-1}\).

The minimum and maximum lag times were set to \( \tau_{\text{min}} = 10 \mu\text{s} \) and \( \tau_{\text{max}} = 40 \text{ s} \), respectively. This ensured reproducibility and accuracy in the analysis for all AuNP sizes studied here, according to the guidelines for correlation spectroscopy published previously.\(^{157}\) To avoid artifacts related to undersampling, the AuNP concentration was always kept in the picomolar regime, which guaranteed that at least 100 nanoparticle diffusion ‘events’ were sampled per 40 s transient.

### 3.4. Results and Discussions

Citrate-stabilized AuNPs interacted with BSA, the most abundant protein in the circulatory system, at concentrations similar to those found for albumin in human blood (\(~ 0.75 \text{ mM}\)). The normalized UV/Vis extinction spectrum of 51 nm citrate-stabilized AuNPs in Figure 3.2B (solid blue line) shows a strong surface plasmon resonance peak at 534 nm. The plasmon resonance maximum red-shifted by \(~ 3 \text{ nm}\) after the AuNPs were mixed with a 0.75 mM BSA solution (dashed red line). Similarly, the UV/Vis extinction spectra of citrate-stabilized 70 and 93 nm AuNPs red-shifted by \(2 ~ 3 \text{ nm}\) in the presence of BSA. This suggests that BSA interacted with citrate-stabilized AuNPs and caused a change in the refractive index at the AuNP surface and consequently a shift of the plasmon resonance energy. To
establish that BSA indeed binds to citrate-stabilized AuNPs, quantify the amount of adsorbed protein, and to check if protein adsorption leads to AuNP aggregation we performed *in situ* equilibrium binding experiments using scattering correlation spectroscopy in combination with analysis of the intensity transients by BIFA.

**Figure 3.2** (A) Size distribution of 51 nm AuNPs obtained by TEM. The dashed black line represents an aspect ratio of 1, or perfect spherical shape. (B) Normalized UV/Vis ensemble extinction spectra of 51 nm AuNPs before (solid, blue) and after (dashed, red) mixing with BSA at 0.75 mM. The lower left inset contains a representative TEM image of the sample. The upper right inset zooms into the region of the surface plasmon resonance maximum $\lambda_{\text{max}}$.

The characteristic diffusion time of AuNPs increased in the presence of BSA. Figure 3.3A shows the average of 56 autocorrelation curves ($\tau_{\text{max}} = 40$ s each) of 51
nm AuNPs before and after mixing with BSA at physiological concentrations. By combining Equation 3.3 and 3.4, the increase in \( \tau_D \) can be related to the change in \( R_h \), which is found to be \( \Delta R_h = 4.6 \pm 1.9 \) nm (Figure 3.3A inset). Because BSA in its native (N) state can be approximated as a triangular equilateral prism with sides of 8 x 8 nm\(^2\) and a height of 3.4 nm,\(^{145}\) the increase in \( R_h \) corresponds to no more than monolayer adsorption of BSA bound to the AuNP surface with the triangular base. This is consistent with previous experiments investigating the adsorption of human (and bovine) serum albumin on the surfaces of AuNPs\(^{26, 147}\) and semiconductor nanoparticles.\(^{29}\) The small discrepancy between the change in \( R_h \) and the BSA dimensions reported by x-ray crystallography\(^{145}\) could be due to a solvent hydration shell and the heterogeneous AuNP size distribution.
Figure 3.3 Autocorrelation curves of AuNPs and AuNPs + BSA [0.75 mM], for three AuNPs samples with mean diameters of 51 nm (A), 70 nm (B) and 93 nm (C). The amplitude of the autocorrelation is normalized to 1 for better comparison of the characteristic diffusion time, $\tau_D$. The average hydrodynamic radius $<R_h>$ obtained from the autocorrelation curves is shown as squares in the insets. The expected increase in $R_h$ due to BSA binding is 3.4 nm per layer (dashed lines). The error bars represent the experimental uncertainty of three to five independent measurements.
BSA adsorption was independent of AuNP size as illustrated in Figure 3.3B and 3.3C, which show the same monolayer adsorption for 70 and 93 nm AuNPs, respectively. The characteristic diffusion times and diffusion constants for all three AuNPs samples with and without BSA as measured via scattering correlation spectroscopy are summarized in Table 3.2. The hydrodynamic radii $R_h$ are also given. The values for the AuNP-BSA system were corrected for the change in viscosity when adding the BSA solution. Ignoring the contribution of the citrate capping layer, the sizes obtained by scattering correlation spectroscopy are in excellent agreement with those measured by TEM.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$R_{\text{TEM}}$ [10^{-9} m]</th>
<th>$\tau_{D, \text{AuNPs}}$ [10^{-3} s]</th>
<th>$\tau_{D, + \text{BSA}}$ [10^{-3} s]</th>
<th>$D_{\text{tr, AuNPs}}$ [10^{-6} m^2 s^{-1}]</th>
<th>$D_{\text{tr, + BSA}}$ [10^{-6} m^2 s^{-1}]</th>
<th>$R_{h, \text{AuNPs}}$ [10^{-9} m]</th>
<th>$R_{h, + \text{BSA}}$ [10^{-9} m]</th>
</tr>
</thead>
<tbody>
<tr>
<td>51 nm</td>
<td>25.6 ± 3.4</td>
<td>1.7 ± 0.3</td>
<td>2.9 ± 0.5</td>
<td>9.3 ± 0.5</td>
<td>6.1 ± 0.4</td>
<td>26.0 ± 1.3</td>
<td>30.6 ± 2.4</td>
</tr>
<tr>
<td>70 nm</td>
<td>35.1 ± 4.3</td>
<td>2.1 ± 0.1</td>
<td>3.0 ± 0.3</td>
<td>7.2 ± 0.5</td>
<td>4.7 ± 0.4</td>
<td>35.9 ± 2.3</td>
<td>39.8 ± 3.2</td>
</tr>
<tr>
<td>93 nm</td>
<td>46.6 ± 3.3</td>
<td>2.5 ± 0.3</td>
<td>3.2 ± 0.2</td>
<td>5.6 ± 0.4</td>
<td>4.3 ± 0.2</td>
<td>43.6 ± 2.8</td>
<td>47.7 ± 2.2</td>
</tr>
<tr>
<td>56 nm PEG</td>
<td>27.9 ± 3.3</td>
<td>2.1 ± 0.4</td>
<td>2.4 ± 0.5</td>
<td>8.5 ± 0.4</td>
<td>6.9 ± 0.4</td>
<td>28.6 ± 1.3</td>
<td>28.9 ± 1.4</td>
</tr>
</tbody>
</table>

Table 3.2 Characteristic diffusion time $\tau_D$, translational diffusion coefficient $D_{tr}$ and hydrodynamic radius $R_h$ obtained via autocorrelation analysis of AuNPs. The size distribution obtained via TEM ($R_{\text{TEM}}$) is shown for comparison.

It is important to establish whether the change in $R_h$ is indeed due to BSA adsorption or because of the formation of a small number of aggregates. Correlation spectroscopy is in principle an ensemble measurement technique and therefore it is difficult to exclude based on only the autocorrelation curves in Figure 3.3 that the increase in diffusion times was caused by the presence of a few larger AuNP aggregates instead of the formation of a BSA monolayer.29 The fact that the change in $R_h$ is the same for all three samples suggests that aggregation is not a dominant
factor though. To further verify this, we analyzed the individual 40 s intensity transients by autocorrelation analysis independently instead of averaging them together as was done for Figure 3.3, because the formation of rare, large aggregates would cause a broad distribution of $R_h$ values. This allowed us to access the distribution of $R_h$ and analyze any changes in size heterogeneities associated with protein binding. The resulting histograms for $R_h$ without and with BSA are shown in Figure 3.4A and 3.4B for the 51 nm AuNPs, respectively. The distribution of $R_h$ from 56 individual 40 s intensity transients for the AuNPs only (Figure 3.4A) shows a Gaussian profile consistent with the size distribution observed via TEM and lacks a trailing tail of large $R_h$ values that would be expected if aggregates were present.

**Figure 3.4** Distribution of $R_h$ obtained by autocorrelation analysis of individual 40 s intensity transients. The error quoted in the figure legend is the standard deviation of the distribution.

Further evidence for the absence of AuNP aggregation and the formation of a BSA monolayer on citrate-stabilized AuNPs under physiological conditions was obtained by BIFA of individual events in the intensity transients (Figure 3.5). The
number of events $N_{events}$ counted per 40 s intensity transient, a good estimate of the average number of AuNPs sampled, is nearly identical for the AuNP and AuNP-BSA samples. Figure 3.5A shows an intensity histogram of individual bursts for the representative time transient given in the inset. The corresponding data for 51 nm AuNPs after addition of BSA is given in Figure 3.5B. No significant reduction in the number of events was observed. In addition, because the scattering intensity scales as $\sim d^6$ for AuNPs with diameters $d < 100$ nm, the presence of aggregates with significantly larger scattering intensities would be evident in the intensity distribution. However, neither a decrease in the number of events nor a change in the intensity histograms towards higher values was observed, confirming that the stability of the colloid is preserved after protein adsorption. This is also the case for the 70 and 93 nm AuNPs samples, as shown in Figure 3.5C, where the average number $<N_{events}>$ of events per 40 seconds is presented for at least 30 time transients per sample. The small decrease in $<N_{events}>$ is within the experimental uncertainty and can be explained by the increase in the viscosity due to the presence of BSA. After aggregation has now been ruled out, we further quantified BSA-AuNP binding.
Figure 3.5 Blip intensity frequency analysis (BIFA) of 51 nm AuNPs before (A) and after (B) binding to BSA [0.75 mM]. The events are counted based on 40 s intensity transients displayed in the insets. (C) Average number of events before (blue) and after (red) binding to BSA for the three AuNP sizes measured. The error bars represent one standard deviation from the average value.

The adsorption isotherm for 51 nm AuNPs was found to follow an anti-cooperative binding model reaching saturation at physiological concentrations. This
was achieved by constructing an adsorption isotherm over a wide range of BSA concentrations. Figure 3.6 shows the experimental $R_h$ as a function of BSA concentration (red squares). Following the approach by Rocker et al.\textsuperscript{29} for adsorption of human serum albumin on smaller FePt and CdSe/ZnS nanoparticles, the experimental data is fitted to a modified Langmuir model given in Equation 3.5:

$$R_h([\text{BSA}]) = R_h(0) \left(1 + \frac{V_{\text{BSA}}}{V_{\text{NP}}} \frac{N}{1 + \left(\frac{K_D}{[\text{BSA}]}\right)^n}\right)$$

\textbf{Equation 3.5}

Here $V_{\text{BSA}}$ and $V_{\text{NP}}$ are the volumes of the BSA and the AuNP, respectively, $N$ is the number of proteins bound, $K_D$ is the dissociation constant, and $n$ is the Hill coefficient, which measures the cooperativity of the binding. A Hill coefficient of $n = 0.4 \pm 0.1$ and a dissociation constant of $K_D = 256 \pm 50$ µM returned the best fit (dashed blue line). A Hill coefficient smaller than 1 indicates anti-cooperative binding and implies strong repulsion between bound and free BSA molecules, increasing as more surface binding sites are filled. This furthermore predicts that adsorption beyond a monolayer is negligible, in agreement with the results in Figure 3.3. For comparison, the traditional Langmuir model with $n = 1$ is based on the assumption that binding occurs independent of surface coverage and results in a steeper increase of $R_h$ as a function of BSA concentration, as illustrated by the dashed black line in Figure 3.6. The dissociation constant corresponding to the Langmuir model is therefore an order of magnitude lower ($K_D = 20 \pm 10$ µM). While
a Langmuir adsorption isotherm was reported for BSA binding to citrate-capped gold films,\textsuperscript{31} more recent studies on nanoparticles also show an anti-cooperative binding model.\textsuperscript{28, 29, 144} This difference between a nanoparticle and a film surface is presumably due to the limited number of binding sites and the surface curvature of a nanoparticle.

![Figure 3.6 Adsorption isotherm relating the concentration of BSA with the nanoparticle $R_h$ (red squares). The data is fitted to the Langmuir model given by Equation 3.5. A Hill coefficient $n$ of 0.4 returns the best fit (blue line). A comparison to a non-cooperative binding model ($n = 1$) is shown by the black line.]

Knowing both $n$ and $K_D$ from the measured isotherm we can extract further information for BSA binding to AuNPs. First, the fit with $n = 0.4 \pm 0.1$ yields $N = 295 \pm 30$ proteins bound to the surface of the 51 nm AuNPs. The theoretically available number of binding sites for a monolayer coverage on a 51 nm AuNP is estimated to be $\sim 250$ by dividing the surface area of the AuNP ($S = 4 \times \pi \times 25.5 \text{ nm}^2 = 8171.3 \text{ nm}^2$) by the triangular cross section of the folded BSA ($1/2 \times 8 \times 8 \text{ nm}^2 = 32 \text{ nm}^2$). This excellent agreement gives additional confirmation for the adsorption of a monolayer of BSA in its native (N) state on citrate-capped AuNPs. Denaturation
from the N-state to a partially unfolded (F) state (N-F transition)\textsuperscript{159} after adsorption cannot be ruled out though, but all of our experiments suggest that spreading of BSA on the surface of the AuNPs is unlikely, in agreement with previous studies on AuNPs and semiconductor nanoparticles.\textsuperscript{26, 29, 144, 147} Second, using $K_D$ we can also calculate the free energy of adsorption $\Delta G_0$ according to:\textsuperscript{160}

$$\Delta G_0 = -RT \ln(K)$$

Equation 3.6

where $R$ is the gas constant and $K$ is the binding constant, which is obtained as the inverse of $K_D$. Equation 3.6 yields $\Delta G_0 = -4.9$ kcal/mol for the anti-cooperative binding model, indicating a spontaneous process for the binding of BSA to the AuNPs.

Finally, we investigated PEG-coated AuNPs as PEG has been commonly used to prevent protein adsorption to surfaces.\textsuperscript{161, 162} We indeed found no significantly adsorption of BSA on PEG-coated AuNPs. Figure 3.7A shows UV/Vis extinction spectra of 56 nm PEG-functionalized AuNPs before and after addition of BSA under the same experimental conditions as for the citrate-stabilized AuNPs. No shift in the plasmon resonance maximum was observed, in contrast to the citrate-stabilized AuNPs. The autocorrelation curves presented in Figure 3.7B also did not show an increase in the characteristic diffusion time, and therefore the relative change in the AuNP $R_h$ due to BSA adsorption is negligible. A protein monolayer therefore did not form on top of the PEG-coated AuNPs.
However, we cannot rule out sub-monolayer formation due to electrostatic interactions, hydrophobicity, or van der Waals forces of a few BSA molecules with the PEG-coated AuNPs. Future experiments will test this scenario, with fluorescence correlation spectroscopy using dye-labeled BSA.

Figure 3.7 (A) Normalized UV/VIS extinction spectra of 56 nm PEG-coated AuNPs before (solid green line) and after (dashed red line) addition of BSA. The inset zooms into the region of $\lambda_{\text{max}}$. (B) Autocorrelation curves of 56 nm PEG-coated AuNPs before (green) and after (red) addition of BSA. The inset shows the average hydrodynamic radius $<R_h>$ obtained from autocorrelation analysis. For comparison, the expected increase in $R_h$ based on one layer of BSA is shown by the dashed red line.

BSA is a globular protein and consists of 583 amino acids, 60 of which are lysine residues, 17 disulphide bridges, a single tryptophan, and a free thiol
It has been proposed that BSA adsorption on citrate-capped gold surfaces could be either due to an electrostatic attraction with the positively charged lysine residues,\textsuperscript{26,31} or by a thiol ligand displacement reaction through the unpaired cysteine.\textsuperscript{147} In particular, Brewer \textit{et al.} compared BSA adsorption on bare and citrate-coated flat gold surfaces using a quartz crystal microbalance and interpreted the higher binding constant for citrate-coated surfaces as an electrostatic attraction mechanism.\textsuperscript{31} Casals \textit{et al.} reached the same conclusion based on the observation that for small 4 nm negatively charged AuNPs formation of a protein corona was slow, which they argued was inconsistent with a ligand replacement reaction because smaller nanoparticles should have better access to the exposed thiol.\textsuperscript{26} In contrast, Tsai \textit{et al.} suggest a ligand exchange reaction on citrate-stabilized AuNPs via the thiol-terminated cysteine residue based on the binding constants obtained from adsorption isotherms.\textsuperscript{147}

Because of the small size of the citrate molecules it is not possible to distinguish between ligand replacement and binding on top of the citrate stabilizing layer by correlation spectroscopy. We also measured the zeta potential of the citrate-capped AuNPs before and after addition of BSA. Figure 3.8 shows that the surface potential of the AuNPs became less negative due to BSA adsorption, consistent with an electrostatic binding mechanism via the positively charged lysine residues. However, replacing the negatively charged citrate molecules with BSA is expected to have a similar effect because BSA is also overall negatively charged at physiological pH.\textsuperscript{152}
Furthermore, we note that many surface functionalization procedures of the same citrate-capped AuNPs involve the initial replacement of the citrate, which is used for the AuNP growth, with especially sulfur terminated ligands.\textsuperscript{163} In the case of the PEG-coated AuNPs, the zeta potential measurements showed that the AuNPs are only slightly negatively charged (Figure 3.8). Following the electrostatic binding mechanism, this reduction in surface charge could therefore explain the absence of BSA adsorption. It should be pointed out though that because the length of the PEG molecules is about 5 nm for a fully stretched conformation and therefore comparable with the height of the BSA, the correlation spectroscopy measurements presented here make it difficult to distinguish between no BSA adsorption and a thiol-thiol exchange reaction. However, as the PEG is also bound via a sulfur group to the AuNP surface the latter scenario is rather unlikely as the free energy for the replacement of one thiol with another one has been determined to be $\Delta G_0 \sim +30$ kcal/mol in the case of self-assembled monolayers on flat gold surfaces.\textsuperscript{164} Further
experiments with different surface charges and chemistries are planned to obtain a
more complete picture and to establish the role of different adsorption mechanisms
with distinct effects on the biological activity. Significant more insight into the
adsorption mechanism at the nano-bio interface can be obtained by labeling the
protein and possibly the ligands with dye molecules and performing multi-color
(excitation and detection) auto- and cross-correlation spectroscopy.

3.5. Summary

This chapter has shown in situ evidence for adsorption of BSA on citrate-
stabilized AuNPs. UV/Vis extinction spectroscopy revealed a red-shift in the surface
plasmon resonance for the citrate-stabilized AuNPs. Quantitative analysis of the
diffusion parameters before and after protein adsorption showed an increase in the
AuNP hydrodynamic radius. The change in hydrodynamic radius corresponds to the
formation of a BSA monolayer independent of AuNP size for diameters ranging
between 51 and 93 nm. Combining correlation spectroscopy with BIFA, we
furthermore demonstrated that protein adsorption does not lead to AuNP
aggregation. An adsorption isotherm showed that the association of BSA to citrate-
stabilized AuNPs can be described by an anti-cooperative binding model, which
yielded a number of bound protein molecules in agreement with the estimated
number of binding sites based on the equilateral base of N-state BSA and the AuNP
surface area. These results are in good agreement with previous in situ
measurements of smaller 10 nm citrate-stabilized AuNPs interacting with BSA,
studied by dynamic light scattering. Furthermore, we demonstrated that BSA
adsorption is negligible on PEG-coated AuNPs. Although the system studied here was specific - BSA interacting with citrate-stabilized AuNPs, the in situ approach using correlation spectroscopy and BIFA presented here can be directly applied to other nanoparticle-protein interactions.

3.6. Acknowledgments

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4.1. Abstract

We find that citrate-stabilized gold nanoparticles aggregate and precipitate in saline solutions below the NaCl concentration of many bodily fluids and blood plasma. Our experiments indicate that this is due to complexation of the citrate anions with Na\(^+\) cations in solution. A dramatically enhanced colloidal stability is achieved when bovine serum albumin is adsorbed to the gold nanoparticle surface, completely preventing nanoparticle aggregation under harsh environmental conditions where the NaCl concentration is well beyond the isotonic point. Furthermore, we explore the mechanism of the formation of this albumin ‘corona’ and find that monolayer protein adsorption is most likely ruled by hydrophobic
interactions. As for many nanotechnology-based biomedical and environmental applications, particle aggregation and sedimentation are undesirable and could substantially increase the risk of toxicological side-effects, the formation of the BSA corona presented here provides a low-cost bio-compatible strategy for nanoparticle stabilization and transport in highly ionic environments.

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### 4.2. Motivation and Introduction

Human blood plasma, the liquid part of blood, is a complex environment with thousands of proteins, antigens and antibodies diffusing in a solution with relatively high ionic strength (NaCl concentration of ~ 150 mM).65,66 Nanoparticles are currently investigated for their clinical use in disease diagnosis and treatment.141,163,167-169 Any practical application of nanoparticles in biological fluids, such as blood plasma, should ensure monodispersity of the material under these harsh conditions, as particle agglomeration and sedimentation170,171 may affect the biodistribution of the material and lead to harmful side effects.148,172

Alongside the issue of particle dispersity, exposure of nanoparticles to blood leads to adsorption of plasma proteins onto their surface,12,25,27,29,30,32 affecting
how cellular receptors screen nanomaterials. While studies characterizing this protein ‘corona’ are rising, quantitative investigations of how this corona may affect the dispersity and mobility of nanoparticles are still scarce. In addition, the role of the ionic strength of biological fluids in the affinity between proteins and nanoparticles is a fundamental aspect that needs further investigations.

Serum albumin, the most abundant protein in circulation, will likely play a key role in the transport of nanoparticles across the bloodstream, as its main biological function is the transport of non-polar compounds in the circulatory system. Bovine serum albumin (BSA) often serves a model protein for nanoparticle-protein studies, as it shares 98 % of its aminoacid sequence with its human variant. Although this protein is negatively charged at physiological pH, it also contains eleven hydrophobic binding domains, 66 positively charged lysines and an unpaired sulfhydryl group (cysteine). Given the complexity of this protein, its interaction with nanoparticles is by no means trivial.

Gold nanoparticles (AuNPs) are ideal candidates for fundamental studies of protein-nanoparticle interactions. Upon interaction with light, high absorption and scattering cross sections across the visible and near IR spectral region arise from the collective oscillation of the conduction electrons, a phenomenon known as the surface plasmon resonance. The field of plasmonics has promising applications in biology and medicine such as high contrast nanoparticle imaging, tracking, and sensing. In addition, recent
in vivo studies have found low-toxicity associated with the gold core for nanoparticle sizes around 50 nm in diameter.\textsuperscript{148, 163, 184} While many ligand-exchange strategies have been realized for AuNPs,\textsuperscript{164, 185-190} citrate stabilization via electrostatic repulsion is still the simplest low-cost route for particle synthesis that does not require covalent attachment of molecules to the surface.\textsuperscript{190-193}

The interaction of citrate-stabilized AuNPs and bovine and human serum albumin has been reported in the literature and is now extensive.\textsuperscript{26, 28, 31, 144, 147, 194-197} The proposed mechanisms to explain this interaction can be grouped into two categories: 1) Electrostatic attraction between the positively charged lysine groups of the protein and the citrate-layer covering the gold surface;\textsuperscript{26, 31, 194, 196} 2) Thiol-binding of the cysteine residues, in particular cys-34, directly to the gold surface, most likely displacing the citrate layer on the gold surface.\textsuperscript{147, 195}

Here we show that aqueous solutions with the same pH and ionic strength as human blood plasma cause aggregation and precipitation of citrate-stabilized AuNPs. Our experiments suggest that this occurs due to complexation of the citrate molecules with counter ions in solution. Adsorption of BSA onto the nanoparticle surface enhances the colloidal stability at salt concentrations beyond the isotonic point (> 100 mM), a positive side effect of the BSA protein-corona. Additionally, we address the ongoing debate of the binding mechanism of BSA on citrate-stabilized AuNPs by testing the adsorption of the protein under different saline conditions and with a sulfhydryl-blocked form of BSA. Finally, we demonstrate that the ionic strength of the solution drives the adsorption of BSA to AuNPs, as the binding
affinity increases in the presence of salts due to an overall increase of the nanoparticle hydrophobicity.

4.3. Experimental Section

4.3.1. Materials

Citrate-stabilized AuNPs with a nominal diameter of 50 nm and an approximate concentration of $10^{10}$ AuNPs/mL were purchased from BBInternational. The sample was further characterized by transmission electron microscopy (TEM, JEOL 2010), yielding an average diameter of 51 ± 7 nm. A representative TEM image is shown in Figure 4.1, along with the size distribution obtained using a home-built image analysis program written in Matlab.

NaCl, CaCl$_2$, and ZnCl$_2$ were purchased from Sigma-Aldrich. Solutions of these salts were prepared in Milli DI water (> 25 MΩ, Millipore). The behavior of AuNPs in saline solutions was studied by mixing equal volumes of citrate-stabilized AuNPs and salt solutions. The salt concentration reported throughout the manuscript is the final concentration after mixing with AuNPs. Phosphate buffer saline (PBS) and Tris saline buffer (TBS, NaCl = 150 mM) were purchased from Sigma-Aldrich. The pH of all saline solutions was measured and found to be in the physiological range (7.0 – 7.5).
Figure 4.1 Top: Size distribution obtained from TEM images containing 351 individual AuNPs yields an average diameter of 51 ± 7 nm. The dashed line represents the aspect ratio of 1 (perfect spherical shape) and is shown as a guide to the eye only. Bottom: Representative TEM image.

NaCl, CaCl₂, and ZnCl₂ were purchased from Sigma-Aldrich. Solutions of these salts were prepared in Milli DI water (> 25 MΩ, Millipore). The behavior of AuNPs in saline solutions was studied by mixing equal volumes of citrate-stabilized AuNPs and salt solutions. The salt concentration reported throughout the manuscript is the final concentration after mixing with AuNPs. Phosphate buffer saline (PBS) and Tris saline buffer (TBS, NaCl = 150 mM) were purchased from Sigma-Aldrich. The pH of all saline solutions was measured and found to be in the physiological range (7.0 – 7.5).
Albumin from Bovine Serum was purchased from Sigma-Aldrich (≥ 98% lyophilized powder, product number A7906, \( M_W = 66430 \)). Sulfhydryl-blocked Bovine Serum Albumin (SB-BSA) was purchased from Lee Biosolutions (≥ 98% lyophilized powder, product number 100-10SB, \( M_W = 66317 \)). The purity of these BSA samples is comparable to other studies of nanoparticle-protein interactions.\(^{26,28,31,147}\) The proteins were diluted to the desired concentration in molecular biology grade water at the required salt concentration. To prevent protein aggregation, protein solutions were freshly prepared for each experiment. The BSA lyophilized powder was stored at 2 °C. For protein binding experiments, AuNPs and BSA were mixed using equal volumes of each solution. The protein concentration reported throughout the manuscript is the final concentration after mixing.

Ellman’s reagent (5,5’-dithiobis-(2-nitrobenzoic acid), Sigma Aldrich) was used to quantify the thiol activity of BSA and SB-BSA using UV/Vis spectroscopy.\(^{198}\) Figure 4.2A shows the UV/Vis extinction of Ellman’s reagent after reacting with BSA and SB-BSA, as well as with positive (thiol-PEG, Sigma Aldrich) and negative controls (\( \text{H}_2\text{O}, \text{Millipore} \)). The lack of thiol activity of SB-BSA is confirmed by its weak absorbance at 412 nm (Figure 4.2B) when compared to the sample with just water.
Figure 4.2 (A) UV/Vis extinction of Ellman’s reagent upon reacting with \( \text{H}_2\text{O} \), sulfhydryl-blocked BSA (SB-BSA), BSA and thiol-PEG (SH-PEG). (B) Extinction at 412 nm extracted from the spectra shown in (A). Note that 412 nm is the wavelength used in Ellman’s original paper\textsuperscript{198} to quantify the thiol-activity of tissues and biomolecules.

Microscope cover glass slides (22 x 22 mm\(^2\), Fisherbrand) were sonicated in acetone (ACS spectrophotometric grade, Sigma-Aldrich) and Milli Di water for 20 min. The slides were then dried with ultra-high purity \( \text{N}_2 \) (Matheson) and stored in SampleStorr\textsuperscript{TM} vacuum containers (Ted Pella, Inc) to avoid contamination. Before use, the microscope slides were cleaned with \( \text{O}_2 \) plasma for 90 s at 200 mTorr (Harrick Plasma). Silicon chambers (50 µL, Grace Bio-Laboratories) were placed on top of the microscope cover glass and filled with the solution of interest using a micropipette (Thermo Scientific).

4.3.2. UV/Vis Spectroscopy and Zeta-Potential Measurements

Ensemble UV/vis spectroscopy of the AuNPs solutions was measured using a home-built optical setup with a fiber spectrometer from Ocean Optics (SD1024DW).
A cuvette containing approximately 1 mL of solution was illuminated using a white LED light source (MWWHL3, Thorlabs). The incident power was adjusted with a 15 V power supply unit and a LED driver (Thorlabs) to avoid saturation of the detector. The experiment was controlled using the SpectraSuite spectroscopy software supplied by Ocean Optics. Zeta-potential values were acquired using a Malvern Zen 3600 (Zetasizer Nano). The Smoluchowski approximation was used as an input parameter of the Henry equation, corresponding to the electrophoretic mobility of small particles in aqueous media.

4.3.3. Scattering Correlation Spectroscopy

Scattering correlation spectroscopy was performed using a home-built inverted microscope (Observer.D1, Zeiss) described in Chapters 2 and 3. Light from a 532 nm laser (Verdi-6, Coherent Inc.) was circularly polarized using a λ/4 wave plate, then collimated and expanded to overfill the objective (Fluar, Zeiss: 100 X, NA = 1.3). The laser power was attenuated using neutral density filters (Thorlabs) to approximately ~ 400 nW, measured before the microscope. Heating of the sample in this power range is negligible. The focal plane of the objective was set to approximately ~ 6 µm inside the sample, to avoid excessive scattered light from the glass-water interface. The scattered light was collected in the backwards direction, and redirected to a 50 µm pinhole before focusing into an avalanche photodetector (SPCM-AQRH, Perkin Elmer). To ensure that only elastic scattering is collected, a 532 ± 2 nm band pass filter was placed in the detection beam path.
While the effect of the salts (NaCl, ZnCl₂, and CaCl₂) on the viscosity of the aqueous solutions is negligible for the range of concentrations tested, the effect of BSA was considered using a linear approximation based on the intrinsic viscosity of the protein according to the provider, [η] = 4.13 cm³ g⁻¹. This same approximation was used for SB-BSA.

Following previously published criteria for consistent correlation spectroscopy analysis, the minimum and maximum lag times were set to τ_min = 10 µs and τ_max = 40 s. The dimensions of the focal volume were obtained using 110 nm latex beads purchased from Invitrogen (C37485). Figure 4.3 shows the autocorrelation function of 110 nm latex beads which were used as a standard to extract the hydrodynamic dimensions of the AuNPs (Table 4.1).

**Figure 4.3** Autocorrelation traces of 110 nm latex beads used for calibration of the focal volume (red squares) and 51 nm AuNPs (black squares). The solid lines represent the fit to Equation 3.2. The size of the AuNPs extracted from the scattering correlation analysis agrees with the value obtained from TEM.
Table 4.1 Characteristic diffusion time and hydrodynamic radii extracted from Figure 4.3.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\tau_D$ (ms)</th>
<th>$R_h$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>51 nm AuNPs</td>
<td>0.75 ± 0.05</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>110 nm latex beads</td>
<td>1.61 ± 0.2</td>
<td>110 (fixed)</td>
</tr>
</tbody>
</table>

4.4. Results and Discussions

Citrate-stabilized AuNPs precipitate in NaCl solutions at physiological pH. Figure 4.4A shows the extinction spectra of AuNP solutions for different NaCl concentrations. The spectra were acquired after 24 hours of equilibration. The extinction of the surface plasmon resonance at its maximum $\lambda_{\text{max}} = 535$ nm decreases with increasing NaCl concentration. To better understand the underlying mechanism of AuNP precipitation, spectra were also acquired as a function of time after initial mixing as shown in the inset of Figure 4.4A.

While the spectra at equilibrium show that AuNPs precipitate out of solution in the presence of NaCl, the spectral acquisition as a function of time after initial mixing strongly indicates particle aggregation in the early stages of precipitation, observed as a red-shift and broadening of the plasmon band. The time dependent spectra were acquired at a higher NaCl concentration of 100 mM for a faster visualization of this process. It should be noted however that the lower concentrations of NaCl shown in the main panel induce similar aggregation and precipitation but on longer time scales (data not shown). The red-shift of the plasmon peak occurs as a result of plasmon coupling within AuNP aggregates,178
as time increases this red-shifted band vanishes as the aggregates fall out of solution.

**Figure 4.4** (A) UV/Vis extinction spectra of citrate-stabilized AuNPs for increasing NaCl concentrations after 24 hours of storage at room temperature. The inset shows extinction spectra as a function of time for the first 600 s of the precipitation process. (B) Extinction at $\lambda_{\text{max}}$ as a function of NaCl concentration extracted from equilibrated spectra in (A), along with zeta-potential measurements of the same NP solutions. Pictures of two representative solutions are shown as insets. The error bars are calculated from the peak value of the zeta-potential acquired from three independent measurements.

The decrease in the extinction of the plasmon resonance occurs together with an increase in the AuNPs zeta-potential. Figure 4.4B shows the extinction at $\lambda_{\text{max}}$ extracted from Figure 4.4A, alongside with zeta-potential measurements of the
AuNP colloids also acquired at equilibrium. The zeta-potential increases with increasing NaCl concentration. Note that at NaCl concentrations higher than 15 mM the error bars substantially increase as the solution concentration decreases. Pictures of the AuNPs solutions are also shown in Figure 4.4B. Whereas at 1 mM NaCl the solution is bright red due to the plasmon resonance of the colloidal AuNPs, the solution turns clear at 30 mM because the AuNPs have formed aggregates and precipitated.

Two hypotheses can be formulated to explain the increase in zeta-potential shown Figure 4.4B: 1) The formation of an electric double layer of Na\(^+\) cations binding to the citrate, or 2) complexation of the citrate anions with counter ions in solution, as it is well known that citrate can form complexes with metallic salts in aqueous environments.\(^{199}\) To gain more insight into the salt-triggered aggregation mechanism, extinction spectra of the same citrate-stabilized AuNPs were acquired in CaCl\(_2\) and ZnCl\(_2\) solutions (Figure 4.5), thereby changing the cations but keeping the same amount of net charge for these two conditions(Ca\(^{2+}\), Zn\(^{2+}\)). As seen in Figure 4.5A, as little as 80 µM of CaCl\(_2\) can precipitate the colloid almost entirely. ZnCl\(_2\), on the other hand, requires a concentration of at least an order of magnitude higher (~800 µM) in order to achieve complete precipitation.
Figure 4.5 UV/Vis extinction spectroscopy of citrate-stabilized AuNPs in the presence of increasing concentrations of CaCl$_2$ (A) and ZnCl$_2$ (B).

Because Ca$^{2+}$ and Zn$^{2+}$ have the same net charge, the dramatically different concentrations required for nanoparticle precipitation does not support the formation of an electric double layer. The experiments with NaCl shown in Figure 4.4 do not support this hypothesis either because 30 mM NaCl was required to induce colloid precipitation, an order of magnitude higher concentration than for Ca$^{2+}$, despite having only half the charge. If an electric double layer were formed, the amount of salts required to crash the nanoparticles would not differ by an order of magnitude between the salts tested. The order of the complexation (Ca$^{2+}$ > Zn$^{2+}$ > Na$^+$) also agrees well with the reported complexation affinity of citrate with these metal ions. We therefore conclude that nanoparticle aggregation and subsequent precipitation likely occur because the citrate anions form complexes with counter cations in solution. This process removes the citrate layer, thereby exposing the bare, unprotected nanoparticle surface.
Further support of the formation of nanoparticle aggregates in the presence of NaCl is observed via scattering correlation spectroscopy. Because of the diffraction limited detection volume, and the scaling of the scattering intensity with $V^2$, scattering correlation spectroscopy is a powerful technique for the detection of small amounts of aggregates in heterogeneous samples. In our previous work we observed that as small as a 2% contamination of a colloid with larger nanoparticles dramatically bias the autocorrelation curves to longer diffusion times. Figure 4.6A shows the autocorrelation traces of AuNPs before and after addition of 40 mM NaCl approximately 10 min after preparation. Note that from the data in Figure 4.4A, this NaCl concentration can precipitate the colloid entirely in about a day. The increase of one order of magnitude in the characteristic diffusion time $\tau_D$ from $\sim 1$ ms to $\sim 10$ ms implies a similar increase in the hydrodynamic radius $R_h$ from 26 nm to $\sim 250$ nm, resulting from particle aggregation. Therefore, Figure 4.6A demonstrates that large and slow aggregates are diffusing through the observation volume in the presence of NaCl.

The raw intensity traces used in the autocorrelation analysis also contain useful information for the detection of particle aggregates. Figure 4.6B shows a blip intensity frequency analysis (BIFA) of the same autocorrelation traces shown in Figure 4.6A. BIFA plots the frequency and intensity (photon counts) of scattering events for a fixed amount of time for an intensity vs. time trace (40 s in this case as shown in the inset of Figure 4.6B). The formation of aggregates not only shifts the intensity histograms toward larger values, but also decreases the number of events detected per time interval. Fewer but brighter events are a result of larger particle
aggregates formed in solution. Having discussed the salt-induced aggregation and precipitation of citrate-stabilized AuNPs, we now explore the effect of BSA adsorption on the stability and diffusion of AuNPs in saline solutions.

**Figure 4.6** (A) Autocorrelation traces of citrate-stabilized AuNPs in water (black) and after addition of 40 mM of NaCl (red). (B) Blip intensity frequency analysis (BIFA) extracted from the same raw intensity traces (inset) used in the autocorrelation analysis presented in (A).

Addition of BSA to the AuNPs in the range of physiological concentrations ([BSA] = 375 µM), before mixing with NaCl stabilizes the colloid even at concentrations beyond the isotonic point ([NaCl] > 100 mM). Figure 4.7A shows the extinction spectra of AuNP solutions with increasing NaCl concentrations, measured
after 24 hours of preparation. The AuNPs were stable even at similar NaCl concentrations to that found in seawater (~ 1000 mM), and in commonly used buffer solutions such as phosphate buffer saline (PBS) and tris buffer saline (TBS) (Figure 4.7C). In contrast to the salt-dependent precipitation observed in Figure 4.4, the AuNPs remain stable in the presence of large amounts of NaCl.

This enhanced colloidal stability in environments with high ionic strength is observed even if BSA and NaCl are simultaneously added to the citrate-stabilized AuNPs. The inset of Figure 4.7A shows extinction spectra acquired immediately after simultaneous addition of 375 µM of BSA and 100 mM of NaCl (t = 0 s, solid cyan line), and ten min later (t = 600 s, dashed red line). The extinction spectrum remains completely unchanged, strongly suggesting that particle stabilization via protein adsorption occurs at a faster time scale than particle aggregation and precipitation.

Adsorption of BSA onto citrate-stabilized AuNPs increases the nanoparticle zeta-potential. Figure 4.7B shows the extinction at λ_{max} of the same spectra shown in Figure 4.7A, alongside with zeta-potential measurements of the same AuNP solutions. The offset in the zeta-potential observed at 0 mM NaCl (~ 30 mV) in the presence of BSA, in comparison to the zeta potential in the absence of BSA (~ 50 mV, Figure 4.7B) is strong evidence of protein adsorption. Although the intensity and shape of the extinction spectra remain unaffected in a wide range of NaCl concentrations, the nanoparticle zeta-potential increases as the NaCl concentration increases. The fact that the zeta-potential increases toward neutral values as the
NaCl concentration increases could imply that more BSA is bound to the nanoparticle surface, as this protein is just slightly negatively charged at physiological pH. As the protein concentration is kept constant at 375 µM, this conclusion is not obvious and would imply that the salt concentration drives the affinity between BSA and AuNPs, as will be discussed in more below.
Figure 4.7 (A) UV/Vis extinction spectra of citrate-stabilized AuNPs mixed with BSA (375 µM) before addition of increasing NaCl concentrations, measured after 24 hours of storage at room temperature. The inset shows the extinction spectra of AuNPs after simultaneous addition of BSA (375 µM) and NaCl (100 mM) at t = 0 s (solid-cyan line) and at t = 600 s (dashed-red line). (B) Extinction at $\lambda_{\text{max}}$ as a function of NaCl concentration extracted from the spectra in (A), along with zeta-potential measurements of the same solutions. (C) UV/Vis extinction spectra of citrate-stabilized AuNPs mixed with BSA (375 µM) before addition of PBS and TBS, measured after 24 hours of storage at room temperature.
Further support of the enhanced colloidal stability provided by BSA in saline solutions is shown via scattering correlation spectroscopy. Figure 4.8A shows the autocorrelation curve of the original citrate-stabilized AuNP solutions (0 mM NaCl + 0 mM BSA, blue squares). Addition of BSA at concentrations in the physiological range (375 µM) increases the characteristic diffusion time $\tau_D$, corresponding to an increase in the nanoparticle hydrodynamic radius from $R_h = 26 \pm 2$ nm (blue squares) to $R_h = 31 \pm 2$ nm (black squares), in agreement with our previously published results of BSA monolayer adsorption onto citrate-stabilized AuNPs shown in Chapter 3. The observed change in hydrodynamic radius after addition of BSA to the AuNPs corresponds to the formation of a BSA monolayer on the citrate-stabilized AuNPs, based on the dimensions of the native form of the protein. Addition of 40 mM NaCl to this BSA-AuNP system yields identical autocorrelation traces (red squares).

In agreement with Figure 4.8, BIFA (Figure 4.8B) shows almost identical intensity histograms for BSA-stabilized (375 µM) AuNPs in 0 mM (black) and 40 mM (red) NaCl solutions. In addition, the number of events counted for a time interval of 40 s remains similar for both solutions. Scattering correlation spectroscopy and BIFA therefore demonstrate that AuNP diffusion is unaffected by the NaCl concentration of the solution and that the nanoparticles do not aggregate once BSA is adsorbed onto the AuNP surface.
Figure 4.8 (A) Autocorrelation curves of citrate-stabilized AuNPs (blue squares), and BSA-bound AuNPs in 0 mM (black squares) and 40 mM NaCl (red squares) solutions. (B) Blip intensity frequency analysis (BIFA) extracted from the same intensity traces (inset) used in the autocorrelation analysis presented in (A).

Addition of BSA after adding NaCl immediately stops the salt-induced aggregation. Figure 4.9A shows UV/Vis extinction spectra of citrate-stabilized AuNPs for the first 600 s after mixing with 100 mM NaCl. This NaCl concentration was chosen to speed-up the salt-induced precipitation before BSA addition. Following a similar trend as observed in the inset of Figure 4.4A, the AuNP colloid starts to aggregate and precipitate quickly. Addition of 50 µM BSA after 10 s immediately stops the precipitation process, as the extinction spectra of the AuNPs
are all identical after BSA has been added to the solution. This particular BSA concentration was selected to reduce the effect of the protein's viscosity in the mixing with AuNPs, but larger BSA concentrations are expected to produce the same enhanced stability. BSA adsorption must therefore occur on a faster time-scale than the NaCl induced aggregation and citrate-complexation, however further experiments would be necessary to quantitatively measure the competitive kinetics between these two processes.

Figure 4.9 (A) UV/Vis extinction spectra of citrate-stabilized AuNPs as a function of time after addition 100 mM NaCl. 50 µM BSA is added at t = 10 s. (B) Same experiment as presented in (A), except that a sulfhydryl-blocked form of BSA (SB-BSA) is added at t = 10 s. The blue dots highlight the maximum extinction of each spectrum. The drop in extinction after BSA addition is due to dilution of the AuNP solution.

Having discussed the enhanced stability provided by BSA to AuNPs in environments that would otherwise induce aggregation, we now explore the question of the physico-chemical mechanism for BSA adsorption on AuNPs. As stated in the introduction, some studies have proposed that BSA binds to AuNPs
using the unpaired sulfhydryl group of cysteine-34, forming a thiol bond with the gold surface. If this is the case, a sulfhydryl-blocked form of BSA (SB-BSA) should not adsorb onto the AuNP surface and hence not provide the enhanced colloidal stability in environments with high ionic strength as native BSA does.

The sulfhydryl-blocked form of BSA provides the same enhanced colloidal stability as native BSA in salt solutions. In a similar manner as Figure 4.9A, Figure 4.9B shows UV/Vis extinction spectra of AuNPs in 100 mM NaCl as a function of time for the first 600 s after adding salt. The salt-induced precipitation is stopped after 10 s by addition of 50 µM SB-BSA, as evidenced by the identical extinction spectra observed for the remaining 590 s of spectra acquisition. To further verify that the enhanced colloidal stability provided by SB-BSA comes from adsorption onto the AuNPs surface, scattering correlation spectroscopy experiments were performed.

BSA and SB-BSA are both adsorbed on citrate-stabilized AuNPs. Scattering correlation spectroscopy experiments demonstrate that in the presence of physiological concentrations of BSA (Figure 4.10A) and SB-BSA (Figure 4.10B), the autocorrelation curves shifts to slower characteristic diffusion times as a result of protein adsorption onto the nanoparticle surface. The calculated hydrodynamic radius of citrate-stabilized AuNPs grows from $R_h = 26 \pm 2$ nm to $R_h = 31 \pm 2$ nm in BSA and $R_h = 30 \pm 2$ nm in SB-BSA. These changes in hydrodynamic radii correspond to no more than monolayer adsorption of both forms of BSA onto citrate-stabilized
AuNPs. These results strongly suggest that the dominant mechanism for adsorption is not via the unpaired-thiol group of BSA.

Figure 4.10 Autocorrelation traces of citrate-stabilized AuNPs before and after addition of BSA (A) and SB-BSA (B) at physiological concentrations (375 µM). The insets contain the calculated in hydrodynamic radius of the AuNPs based on the measured characteristic diffusion times.

So far we have established that salts in solution assist the complexation of citrate anions with salt-cations (such as Na⁺, Ca²⁺ or Zn²⁺), leaving the AuNP surface with little electrostatic protection for effective particle-particle repulsion. We also found that BSA binding onto the AuNP surface occurs faster than the citrate-complexation induced precipitation of the colloid. Because the enhanced colloidal stability comes from BSA-adsorption, we therefore further hypothesize that the presence of salts must have an effect on the binding affinity between BSA and citrate-stabilized AuNPs. To explore this in detail, BSA adsorption isotherms at two distinct NaCl concentrations were obtained using scattering correlation spectroscopy.
The binding affinity of BSA to AuNPs increases in the presence of NaCl. Figure 4.11 shows the AuNP hydrodynamic radius, extracted from scattering correlation spectroscopy analysis (blue squares), as a function of BSA concentration for 0 mM (Figure 4.11) and 20 mM NaCl solutions (Figure 4.11B). Note that this NaCl concentration was chosen because it allowed us to obtain the binding isotherms in a wide range of BSA concentrations, as 20 mM NaCl does not precipitate the AuNPs entirely at low BSA concentrations. The data is fitted to the Hill equation for ligand-receptor interactions (solid red line)\(^29\) to extract the dissociation constant \(K_D\) and the Hill coefficient \(n\), which measure the affinity and cooperativity of the interaction, respectively. \(K_D\) decreases about an order of magnitude when the NaCl concentration is increased from 0 mM to 20 mM. BSA adsorption reaches monolayer saturation at a BSA concentration < 100 µM in the presence of 20 mM NaCl, while in the absence of NaCl saturation is reached only after 1000 µM. Also, the Hill coefficient slightly increases from 0.6 to 0.9 in the presence of NaCl, indicating that the interaction becomes less anticooperative. Although the hydrodynamic radius at the highest BSA concentration tested (1.5 mM) in the absence of NaCl could indicate the formation of a protein double layer, we cannot discard protein unfolding upon adsorption.\(^53\) Further experiments beyond the scope of this study are required to investigate the possibility of protein conformational changes at low salt concentrations.

In addition to the adsorption isotherms obtained via scattering correlation spectroscopy, we measured the zeta-potential of the AuNPs solutions in the same range of BSA concentrations. The zeta-potential also suggests an increase in affinity
for BSA to citrate-stabilized AuNPs in the presence of NaCl. Figure 4.11C shows zeta-potential measurements as a function of BSA concentration for citrate-stabilized AuNPs at 0 mM (light blue) and 20 mM (dark blue) NaCl. As discussed earlier, the increase in zeta-potential is indicative of BSA adsorption.\textsuperscript{26, 194} In agreement with the adsorption isotherms presented in Figure 4.11A and 4.11B, in the presence of 20 mM NaCl the zeta-potential reaches saturation at a BSA concentration < 100 µM, whereas in the absence of salt, saturation in the zeta-potential is not observed until at least 1000 µM BSA.
Figure 4.11 Adsorption isotherms of BSA onto citrate-stabilized AuNPs in 0 mM (A) and 20 mM NaCl (B). The experimental data points obtained via scattering correlation spectroscopy (blue squares) are fitted to the Hill equation (red line). The NP core ($R_h = 26$ nm) and theoretical protein layers ($\Delta R_h \approx 4$ nm) are shown as eye guides. (C) Zeta-potential experiments of citrate-stabilized AuNPs as a function of BSA concentration in the presence of 0 mM and 20 mM NaCl. Error bars show the standard deviation calculated out of at least three independent measurements.
The increase in the affinity between BSA and AuNPs in the presence of NaCl suggests that hydrophobic interactions may rule the mechanism of adsorption of BSA onto citrate-stabilized AuNPs. If BSA was binding via electrostatic interactions to the citrate anions bound to the AuNP surface, the binding affinity would have to decrease in the presence of salts because fewer citrate anions are available at the nanoparticle surface, as they are forming complexes with the metal cations as shown above.26, 31, 194, 196 Also, if BSA was forming a thiol bond with the AuNP surface,147, 195 the sulfhydryl-blocked form of BSA should not provide the enhanced colloidal stability in saline solutions and should not form a similar monolayer adsorption as found for native BSA. Instead it is most likely that the complexation between the citrate anions and the metal cations increases the hydrophobicity of the AuNPs, as the ‘naked’ AuNP surface becomes increasingly exposed with increasing salt concentration. While it is possible that some citrate molecules are still left bound to the AuNP surface and BSA forms a monolayer on top of citrate molecules, the overall increase in hydrophobicity shifts the equilibrium between BSA and AuNPs toward higher binding affinities. This finding is in agreement with earlier data that suggested that hydrophobic interactions tend to dominate the energy balance between plasma proteins and nanoparticles.32 In addition, our results complement recent findings of an enhanced stability of BSA-coated AuNPs in simulated intestinal fluid,200 and negligible hemolytic activity and cytotoxic responses of BSA-stabilized AuNPs in the presence of red blood cells.201
4.5. Summary

In summary, this chapter showed that citrate-stabilized AuNPs aggregate and precipitate in aqueous solutions with the same NaCl concentration as human blood plasma and commonly used saline buffers. This salt-induced aggregation likely occurs due to the ability of citrate to form complexes with counter ions in solution, leaving the AuNP surface unprotected from Van der Waals interparticle attraction. Adsorption of BSA onto the nanoparticle surface enhances the stability of the colloidal solution to a degree, where monodisperse nanoparticle solutions are stable at NaCl concentrations above the isotonic point (> 100 mM) and similar to commonly used saline buffers and seawater. In addition, we demonstrated that the ionic strength of the solution plays a key role in the formation of the BSA-corona with citrate-stabilized AuNPs. The binding affinity between BSA and AuNPs increases in the presence of salts, likely due to the complexation of the citrate anions with salt cations in solution, thereby increasing the hydrophobicity of the nanoparticle surface. Our study confirms the role of BSA as the multifunctional transporter of non-polar compounds in environments with high ionic strength such as blood plasma, highlights a positive effect of the BSA protein-corona in the transport of nanoparticles across the bloodstream, and provides a bio-compatible and low-cost strategy for nanoparticle protection in highly ionic environments.
4.6. Acknowledgements

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Chapter 5

Adsorption and Unfolding of a Single Protein Triggers Nanoparticle Aggregation

5.1. Abstract

The response of living systems to nanoparticles depends on the protein corona that forms shortly after exposure of nanoparticles to physiological fluids. This protein corona is linked to a wide array of pathophysologies ranging from increased bio-compatibility to macrophage activation and protein fibrillation. Here we observe increased cancer cell uptake of gold nanomaterials by adding an incubation step in relatively low concentrations of bovine serum albumin before exposing to serum. To understand how the chemistry of the protein corona changes with this pre-incubation at low concentrations, we follow the adsorption of single proteins onto single nanorods.
using fluorescence microscopy. We observe a strong, irreversible association that heavily disrupts the α-helical structure of the protein, inducing cooperative interactions between unfolded proteins and ultimately, nanoparticle aggregation. Our approach demonstrates how to quantify non-equilibrium binding dynamics at the single protein limit, and may help elucidate strategies to control the associated pathophysiology of nanoparticle exposure in vivo.

This chapter was prepared as a letter with equal contributions from my colleague Lydia Kisley and myself, and is currently under review in an interdisciplinary journal. It can temporarily be cited as:


*Equal Contribution

5.2. Results and Discussions

When nanoparticles are pre-incubated in bovine serum albumin (BSA) at lower than physiological concentrations, properties such as uptake by MCF-7 cancer cells, BSA adsorption, and nanoparticle aggregation are strongly influenced (Figure 5.1). Cationic mercaptoundecyltrimethylammonium bromide coated gold nanorods (MUTAB-AuNRs) incubated in 10 % fetal bovine serum (FBS) are uptaken by MCF-7 cancer cells (Figures 5.1a, b). However, by pre-incubating the MUTAB-AuNRs in 1 % w/v BSA the uptake is increased three-fold (Figure 5.1b). Because BSA is the most highly abundant serum protein and is thus present in higher concentrations in FBS,
the pre-incubation with low concentrations of BSA seems key for increased uptake. This result also suggests that a BSA-based protein corona might be protein-concentration dependent.

Luminescence correlation spectroscopy provides strong support that the BSA protein corona that forms on MUTAB-AuNRs is concentration dependent (Figure 5.1c). At high BSA concentrations, a protein monolayer forms, consistent with earlier reports of BSA monolayer formation on gold and other types of nanoparticles (Table 5.1). In contrast, at lower BSA concentration the luminescence correlation decay shows two important trends. First, a strong shift to longer average decay times indicates that the nanoparticle-protein complex has become much larger than can be explained by monolayer adsorption. Most importantly, the average decay curve could not be fit to the equilibrium adsorption model, suggesting non-equilibrium behavior.
Figure 5.1 The uptake of MUTAB-AuNRs by MCF-7 cells is increased by incubating the nanorods in BSA prior to exposure to serum and culturing. a, Cartoon representation of (left, blue) pre-incubation of MUTAB-AuNRs in low concentration of BSA where three times higher cellular uptake of AuNRs was observed compared to (right, orange) MUTAB-AuNRs exposed to high concentration of proteins in serum without pre-incubation. b, Number of MUTAB-AuNRs uptaken per cell for MUTAB-AuNRs pre-incubated with 1% BSA before suspending in 10% fetal bovine serum (FBS) and Eagle's minimum essential media (EMEM), compared to MUTAB-AuNRs only suspended in 10% FBS and EMEM. Cells suspended in 10% FBS and EMEM without addition of nanorods are shown as a control. c, d, Aggregation of MUTAB-AuNRs is observed at (blue) low concentrations of BSA that would be present in pre-incubation conditions vs. stable MUTAB-AuNRs in (orange) serum-level protein concentrations. MUTAB-AuNRs alone (gray) shown as a control. c, Autocorrelation curves with respective decay fits shown as solid lines. Under low concentration BSA the fit did not follow a predictable model; dashed line is instead shown as a guide for the eye. d, UV/vis spectra under similar conditions as c suggest MUTAB-NR aggregation occurs when incubated at low concentrations of BSA.
Table 5.1 Hydrodynamic radius ($R_h$) of MUTAB-AuNRs before and after addition of BSA at different concentrations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$R_h$, trans (nm)</th>
<th>$R_h$, rot (nm)</th>
<th>$R_h$, theor (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUTAB-AuNRs alone</td>
<td>24 ± 2</td>
<td>25 ± 2</td>
<td>24</td>
</tr>
<tr>
<td>MUTAB-AuNRs + 2 nM BSA</td>
<td>N/A**</td>
<td>N/A**</td>
<td>-</td>
</tr>
<tr>
<td>MUTAB-AuNRs + 10 µM BSA</td>
<td>32 ± 3</td>
<td>33 ± 3</td>
<td>-</td>
</tr>
<tr>
<td>MUTAB-AuNRs + 100 µM BSA</td>
<td>30 ± 3</td>
<td>33 ± 3</td>
<td>-</td>
</tr>
<tr>
<td>MUTAB-AuNRs + 500 µM BSA</td>
<td>31 ± 3</td>
<td>34 ± 3</td>
<td>-</td>
</tr>
</tbody>
</table>

*Average values ± standard deviations out of three independent measurements.  
**Poor fitting, as shown in Figure 5.1c

Further support for the strong differences of BSA corona formation at low and high concentrations is found in the UV-VIS adsorption spectra (Figure 5.1d). As expected, at high BSA concentration the extinction spectrum of the MUTAB-AuNRs is red-shifted, consistent with equilibrium formation of a BSA monolayer corona. However, at low BSA concentrations the resultant spectrum is both decreased in intensity and shifted in resonance. The loss of a strong plasmon resonance suggests that the MUTAB-AuNRs aggregate in the presence of low concentrations of BSA whereas they are stabilized with a protein monolayer at high BSA concentrations.

The combination of results on cancer cell uptake, protein adsorption, and nanoparticle colloidal stability strongly suggest that local protein concentrations must be considered when assessing corona chemistry and its influence on nanoparticles’ ultimate physiological fate. For example, low protein-to-nanoparticle ratios can occur under controlled pre-incubation conditions after injection into a
living organism and upon accumulation in cellular compartments. Thus, we examine more closely the structure of protein and nanoparticles when they interact at low BSA concentrations, suggest how these processes lead to the ultimate fate of the BSA-nanoparticle colloid, and discuss whether our observations are generalizable for other systems. We use BSA and MUTAB-NRs as example nanoparticle supports and serum proteins, respectively, however fibronogen and globulin, the second and third most abundant proteins in serum, have also been tested.

When a low concentration of Alexa647-labeled BSA is flowed over individual MUTAB-AuNRs, single molecule analysis reveals that only one BSA protein binds irreversibly to each AuNR (Figure 5.2). BSA adsorption onto single MUTAB-AuNRs is identified by co-localization of dye fluorescence and intrinsic AuNR luminescence (Figure 5.2a). SEM image analysis confirms that 99% of the analyzed BSA adsorption events occur at single MUTAB-AuNRs with little non-specific BSA interaction with the substrate because of surface passivation. Similar results are also observed for MUTAB-functionalized gold spheres and nanowires and cationic NH$_2$-PEG gold nanowires as well as in the presence of fibrinogen and globulin (Figure 5.3, 5.4).
Figure 5.2 Single BSA proteins adsorb to single MUTAB-AuNRs. a, Co-localization of single MUTAB-AuNRs, confirmed by SEM (inset; scale bar, 50 nm), with fluorescently labeled BSA. Fluorescence images are 74 frames binned with the same intensity; scale bar 500 nm. AuNRs are visible through their weak intrinsic photoluminescence using long exposure times before introducing BSA. b, Intensity transient (black) of BSA adsorbed to a MUTAB-AuNR with state and step identification (teal dashed line) of photobleaching steps. Simultaneous photobleaching was discounted by probability analysis. c, Distribution of the number of photobleaching steps for 86 transients.
Figure 5.3 Single molecule imaging of BSA interactions with nanomaterials of varying geometries (sphere, wire, rod) and surface chemistries (cationic MUTAB, cationic NH$_2$-PEG-SH, anionic citrate). Images show nanomaterial (left) before and (right) after 2 nM Alexa-BSA is introduced. Images are binned, ranging from 10-100 frames. These results show that BSA binding occurs independent of the nanoparticle geometry. Furthermore, a different surface chemistry also facilitates binding as long as the surface charge remains positive (weaker adsorption for negatively charged citrate capped nanoparticles).
Figure 5.4 Adsorption of serum proteins to MUTAB-AuNRs inducing aggregation occurs with BSA in protein mixtures and with fibrinogen. a-c, Irreversible adsorption of BSA occurs in the presence of other serum proteins, including fibrinogen, gamma globulin fragments, and heparin sulfate. a, MUTAB-AuNRs were preincubated with 10 nM of the unlabeled protein mixture and were observed by single particle photoluminescence. b, Then, 2 nM Alexa 647-BSA was introduced and the increased intensity indicates adsorption of BSA. c, The adsorbed Alexa 647-BSA was attempted to be removed by a rinse of buffer for 15 min, but similar amounts of adsorbed BSA were observed. d-f, Similar irreversible adsorption of fibrinogen, another serum protein occurs to MUTAB-AuNRs as observed by single molecule imaging. Fibrinogen makes up 7% of serum. d, MUTAB-AuNRs observed by single particle photoluminescence. e, Adsorbed 2 nM Alexa 647-fibrinogen to MUTAB-AuNRs. f, Alexa 647-fibrinogen was not removed from the MUTAB-AuNRs by a rinse of buffer for 15 min. Images in a-f taken from different representative areas of the sample to avoid dye photobleaching. MUTAB-AuNRs aggregation in the presence of 2 nM fibrinogen (g) and 2 nM gamma globulin (h).
The possible presence of multiple proteins on each MUTAB-AuNR is tested with fluorescence time transient analysis (Figure 5.2 b,c). Although a single BSA molecule in its native conformation is smaller than the surface of a AuNR, there is no evidence for multiple BSA adsorption. Protein adsorption is observed as an increase in fluorescence intensity, followed by a step-wise decrease as each of the dye molecules on the protein photobleach (Figure 5.2b). The number of photobleaching steps identified by an established step-finding algorithm\textsuperscript{206} is thus a direct measure of the BSA molecules per AuNR (Figure 5.2b, line). Statistical analysis for 86 AuNRs yields an average of 3.2 ± 1.2 photobleaching steps (Figure 5.2c). This number matches the labeling density provided by the supplier and strongly implies that only one BSA molecule binds to a single AuNR (Molecular Probes, A34785). Further controls involve discarding the possibility of reversible protein desorption/re-adsorption and simultaneous photobleaching of multiple dyes (Figure 5.5 and 5.6).
Evidence for the duration of the BSA dissociation time being limited by photobleaching. Single molecule fluorescence images were collected by varying the collection frame rate where unnecessary excitation/emission cycles of the fluorophores—when data is not being collected—was prevented using an acoustooptic modulator and frequency generators within the total internal reflection fluorescence microscope setup. Using a 30 ms integration time, the frame collection rate was varied between 0.25–16 Hz. The BSA dissociation times (on-times) on single MUTAB-AuNRs were extracted by first identifying BSA adsorption events and then measuring the time until fluorescence was no longer observed in consecutive frames from the same adsorption site. These on-times of all identified events are plotted as cumulative distributions $P$ as a function of frame rate. It is expected that when decreasing frequencies produce the same distribution of dissociation times, the data collection frequency is optimized to observe desorption and not photobleaching of adsorbed molecules. However, the dissociation time distributions for BSA on MUTAB-AuNRs did not converge (i.e., 0.25 Hz shows events much longer than any greater frequency), indicating that photobleaching and not BSA desorption dominates the measured residence times. Beyond 0.25 Hz stage stability and focal drift did not allow for accurate quantification of the dissociation times. Therefore, photophysics of the fluorophore labels on the protein are observed, and based on these experiments we can only estimate that the dissociation time is on the order of 10’s of seconds or greater.
Figure 5.6 Simultaneous photobleaching steps are unlikely. With the expected labeling density of three fluorophores per BSA molecule, the average photobleaching step as a percent of the maximum intensity should be ~33%. We observe 30 ± 10% average step size as a percent of the maximum intensity. A ~66% change or greater would be observed to indicate simultaneous photobleaching. Out of the 213 photobleaching steps we analyzed, we observed < 4% of steps greater than 60%.

The adsorption of single BSA proteins to single MUTAB-AuNRs is irreversible on a time scale of many hours. The percentage of MUTAB-AuNRs occupied by single BSA molecules before and after rinsing remains constant over a rinsing period of 8 hours (Figure 5.7). It is not possible to test if our observation of only one protein adsorbing to each MUTAB-AuNR extends to the solution case, as the free NR surface available in solution is larger than that presented on the glass surface. However, we can say that these results strongly suggest that in the colloidal solution at the low BSA limit, many fewer proteins adsorb than are predicted by monolayer formation. The only hypothesis that explains how the strong, irreversible adsorption of single (or few) BSA molecules can lead to the results shown in Figures 5.2, 5.7 is if BSA is undergoing structural changes upon adsorption.
Figure 5.7 Irreversible adsorption of BSA to MUTAB-AuNRs. a, Representative images of BSA/MUTAB-AuNRs identified based on the stronger BSA signal (circles) before (No BSA), after (t = 0 min) adding a 2 nM BSA solution, and after 500 min of rinsing with buffer. False identification (No BSA) is likely due to <1% of AuNR aggregates with higher photoluminescence intensities. Scale bar, 5 µm. b, Percent of MUTAB-AuNRs with adsorbed BSA molecules identified before and after rinsing. Values calculated from five different, uncorrelated 400 µm² areas for each data point to avoid photobleaching.

Using circular dichroism (CD) spectroscopy we find that BSA undergoes a large loss in α-helical structure when adsorbed to MUTAB-AuNRs (Figure 5.8a). BSA is primarily α-helical in secondary structure and therefore increases in the negative peaks at 209 and 222 nm and decreases in the positive peak at 192 nm in the UV CD spectrum indicate a loss of BSA α-helical structure in the presence of MUTAB-AuNRs (Figure 5.8a). Similar behavior is observed for MUTAB-coated Au nanospheres (Figure 5.8b). From the UV CD signal, we calculate the loss of protein order using the
DichroWeb K2d neural network secondary structure analysis\textsuperscript{208,209} to be 20 ± 9%, similar to results obtained on cationic-functionalized silica particles.\textsuperscript{210} In contrast, no decrease in conformational order occurs for BSA adsorbed to anionic citrate-capped Au nanospheres or in the presence of only MUTAB without any nanostructures (Figure 5.9). This analysis, however, provides only a low estimate of structural loss because of obscurance from excess native BSA in solution. This assertion is supported by centrifugation and removal of non-adsorbed BSA from the supernatant solution, as without free BSA in solution the CD signal in the UV is much weaker.

**Figure 5.8** a, Unfolding of BSA upon adsorption to MUTAB-AuNRs. CD spectra of 1 mg/mL of BSA alone (black), BSA with MUTAB-AuNRs (red), centrifuged BSA with MUTAB-AuNRs (green), and MUTAB-AuNRs alone (blue). b, Unfolding of BSA upon adsorption to MUTAB-AuNPs.
Figure 5.9 Circular dichroism (CD) spectra of controls experiments. Control far UV CD spectra of BSA (0.1 mg/ml, ~ 1.5 µM) in the presence of free MUTAB ligand in solution (0.1 mg/ml) and BSA (same concentration) with spherical citrate-capped Au nanoparticles (citrate-AuNPs, concentration = 75 pM, diameter $d = 48 \pm 4$ nm). No change in the CD spectrum of BSA is observed under these conditions when compared to BSA in buffer at the same concentration. Note that BSA binds to citrate-AuNPs and starts to form a monolayer at this protein-to-nanoparticle ratio.

To ensure that BSA is still adsorbed to the MUTAB-AuNRs despite the harsh centrifugation process we turn to surface plasmon coupled CD spectroscopy in the visible spectral region (Figure 5.10b). The MUTAB-AuNRs show a strong CD signal in the visible region at the plasmon resonance due to the chiral BSA protein adsorbed onto the non-chiral MUTAB-AuNRs. Recent literature has proposed that plasmonic nanomaterials can act as strong visible light antennas for the chirality of surface adsorbed molecules, manifested by a chiral response of the plasmon resonance itself.\textsuperscript{211-213} The otherwise weak UV CD of the chiral aminoacids is amplified at visible wavelengths by coupling to the plasmon resonance.\textsuperscript{214} After centrifugation, the surface plasmon coupled CD signal is still observed confirming the presence of BSA (Figure 5.10b, green line). The decrease in CD intensity after centrifugation matches the decrease in optical density in the UV/vis...
extinction spectrum (Figure 5.10d), telling that some AuNRs are lost in the separation step.

**Figure 5.10** CD and extinction spectra of BSA and MUTAB-AuNRs before and after a centrifugation step to remove unbound BSA. a, UV and b, surface plasmon coupled CD spectra of 1 mg/mL of BSA alone (black), BSA/MUTAB-AuNRs (blue), centrifuged BSA/MUTAB-AuNRs (green), and MUTAB-AuNRs alone (red). c, d. Extinction spectra of the same solutions as in a, b acquired simultaneously. The redshift, decrease in maximum intensity, and appearance of a longer wavelength shoulder for the surface plasmon resonance of the MUTAB-AuNPs with BSA added (red) compared to only MUTAB-AuNPs are clear signatures of nanoparticle aggregation.
Sub-diffraction localization mapping of protein binding to nanowires supports the hypothesis of protein unfolding (Figure 5.11a). Larger Au nanowires functionalized with MUTAB (width: ~70 nm; length: 1 - 10 µm) are exposed to 1 nM BSA and multiple protein binding events are imaged. The smallest distance between nearest-neighbor BSA proteins is determined by cross-correlating the intensity transients of neighboring pixels (Figure 5.11b). Figure 5.11a shows the most likely locations of individual BSA proteins adsorbed onto a large MUTAB-Au nanowire. We determined that the average distance between neighboring BSA proteins was larger than 200 nm. This average separation distance on wires helps explain why only a single BSA was ever observed to bind to the smaller 20 x 58 nm MUTAB-AuNRs discussed earlier. Unfolded BSA restricts adsorption of additional BSA to the MUTAB-AuNR as the bound protein covers most of the available surface area. With this new knowledge of the adsorbed protein structure it is possible to explain the nanoparticle aggregation in the presence of low BSA concentrations presented in Figure 5.1d and 5.1d.
Figure 5.11 Unfolding and ‘spreading’ of BSA on MUTAB-gold nanowires. a. Super-resolution map of identified individual BSA molecules, represented by different colored outlines, overlaid on an average fluorescence image. Representative distances between neighboring BSA molecules are shown. b. Cross-correlation analysis of the same nanowire shown in a. High correlation values signifies higher confidence on the locations of individual proteins.

An unfolded BSA corona on MUTAB-AuNRs causes AuNR aggregation (Figure 5.12). MUTAB-AuNRs pre-incubated with BSA form aggregates with immobilized MUTAB-AuNRs that were similarly pre-incubated with BSA. Figure 5.12a shows a cartoon of the experiment conditions, in which large aggregates are formed on the substrate as observed by SEM imaging (Figure 5.12b, insets). Particle size analysis quantifies this conclusion as the sample with substrate and solution BSA/MUTAB-AuNR shows a large increase in both the number of structures (single AuNRs and aggregates) and distribution of surface areas in comparison to control samples consisting of only one of the components (Table 5.2). Anionic citrate-capped Au nanospheres, which adsorb BSA but do not induce unfolding, do not aggregate.
Figure 5.12 Nanoparticle aggregation is induced by interactions between unfolded proteins. Cartoon showing a MUTAB-AuNR immobilized on a substrate (i) and aggregation of such supported AuNR with BSA coated AuNRs in solution when 2 nM BSA/MUTAB-AuNRs is introduced into the flow cell (ii). Samples were imaged by SEM (insets, scale bar, 500 nm). The log-log scale distribution quantifies the AuNR particle surface areas observed within 235 µm² of the SEM images. Both single AuNRs and isolated aggregates were counted as individual particles. Case (ii) shows a large increase in the number of particles n and the distribution of surface areas A (blue, n = 333; A = (17,000 ± 50,000) nm²), while for case (i) exclusively single particles are found (purple, n = 152; A = (1,300 ± 200) nm²). (iii) Control nanoparticle aggregation measurement of 2 nM BSA/MUTAB-AuNRs flowed over a blank coverslip. (Scale bar 1 µm).
Thus, we propose a non-equilibrium mechanism in which irreversible adsorption occurs at low protein-to-nanoparticle ratios and is followed by BSA unfolding (Figure 5.12c). Unfolded BSA-BSA interactions$^{215}$ drive the nanoparticle aggregation process. Although protein-induced nanoparticle aggregation$^{50,144}$ has been reported in the literature, we believe we have provided a mechanistic explanation of how this process can happen under specific conditions and how it leads to the formation of a ‘hard’ protein corona. It is worth noting that fibrinogen and globulin also triggered nanoparticle aggregation, but independent of the protein-to-nanoparticle ratio, further supporting the specificity of the BSA-MUTAB-AuNR interaction. These results show how the protein-to-nanoparticle ratio influences the physical chemistry of this corona, and that achieving a single protein level of mechanistic insight will deepen our understanding of the connections between protein corona composition, structure and potential physiological pathways. More generally, we anticipate that protein aggregation$^{216,217}$ could be
studied with nanostructure scaffolds that have appropriately adjusted surface chemistries to induce different degrees of protein unfolding.

### 5.3. Materials

#### 5.3.1. Nanoparticle Uptake by MCF-7 Cells

Bovine serum albumin (7.5% in DBPS), Penicillin-streptomycin (10,000 U-10 mg/mL), HCl (Trace Metals Grade) and HNO3 (Trace Metals Grade) were purchased from Sigma-Aldrich. MCF-7 cells were acquired from ATCC (HTB-22.) EMEM (with EBSS and L-Glutamine) was purchased from Lonza. Fetal Bovine Serum (Heat-Inactivated) was purchased from Seradigm. Lactate Dehydrogenase (LDH) activity assay kit was purchased from Thermo Scientific. All chemicals were used without further purification.

#### 5.3.2. Gold Nanorods

Commercially available gold nanorods (AuNRs; A12-25-750, Nanopartz, Loveland, CO, USA) suspended in water and coated with cetyl-trimethylammounium bromide (CTAB) were functionalized with mercaptoundecyltrimethylammonium bromide (MUTAB), a cationic ligand that covalently attaches to the gold surface via a sulfur bond. Excess CTAB was removed by centrifugation of the stock solution for 10 minutes at 7,500 rpm. The AuNRs were then resuspended in 1 mg/ml MUTAB in Millipore H2O (>1 MΩ) and placed overnight in a water bath at 35° C. Excess MUTAB was removed by centrifugation at 7,500 rpm for 10 minutes and replaced with
Millipore H₂O for storage. We estimate that the final concentration of free MUTAB in solution after this centrifugation step is less than 0.01 mg/ml. The MUTAB-AuNR solutions were positively charged (\(\zeta = 35 \pm 5\) mV) at pH 7.2 according to zeta-potential measurements (Malvern Zen 3600). MUTAB-AuNRs were characterized in the buffer conditions of the experiments (20 mM HEPES, 20 mM NaCl in Molecular Biology Grade H₂O) by UV/vis spectroscopy (Figure 5.13) and by transmission electron microscopy (Figure 5.14).

![Image](image_url)

**Figure 5.13** Resistance against aggregation in buffer for MUTAB-AuNRs. Almost identical spectra of MUTAB-AuNRs in water and in buffer show that no colloidal aggregation occurred when changing to the buffer conditions of the experiments (20 mM HEPES, 20 mM NaCl in Molecular Biology Grade H₂O, Thermo Scientific).
Figure 5.14 Distribution of sizes of AuNRs and representative transmission electron microscopy (TEM) image. Distributions of length (top right), width (bottom left), and aspect ratio (bottom right) of the AuNRs used for this study were obtained from analyzing more than 600 nanoparticles in TEM images such as the one shown in the top left. TEM micrographs were obtained with a JEOL 2010.

5.3.3. Gold Nanospheres

Commercially available citrate-coated gold nanospheres (AuNPs) with a nominal diameter of 50 nm were purchased from BBI solutions (Cardiff, UK). Sizing performed by the manufacturer using TEM shows that their actual size is 48 ± 4 nm (Batch # 16659, ~ 75 pM concentration based on the mass of gold per milliliter used for the synthesis). Unless otherwise noted, citrate-AuNPs were functionalized with MUTAB using the same procedure as described above for the gold nanorods. The
zeta-potential of the AuNPs went from negatively charged ($\zeta = -35 \pm 5 \text{ mV}$) in the presence of citrate, to positively charged once functionalized with MUTAB ($\zeta = 40 \pm 5 \text{ mV}$), confirming the ligand exchange reaction occurred.

### 5.3.4. Gold Nanowires

Gold nanowires (Au nanowires, diameter $\sim 70 \text{ nm}$, length $1 \sim 10 \mu\text{m}$) were synthesized using a modified three-step seeding synthesis originally developed for gold nanorods.\textsuperscript{218,219} Seed particles were prepared by a rapid reduction of HAuCl$_4$ (gold precursor) using NaBH$_4$ (reducing agent). These particles were then grown in a solution containing HAuCl$_4$, ascorbic acid and hexadecyl-trimethylammounium bromide (CTAB). The presence of ascorbic acid reduces the gold salt from its Au(III) to a stable Au(I) state. Further addition of seeds to this solution induces an autocatalytic reaction of Au(I) that enlarges the seed particles. While this method would typically yield nanowires with an aspect ratio smaller than 10, aspect ratios larger than 100 can be achieved when the reaction takes place in an acidic environment (pH $\sim 2$) and less seeds are added to the growth solution.

CTAB on the as-synthesized Au nanowires was replaced with MUTAB. The CTAB-Au nanowires sedimanted to the bottom of the vial after 1-2 hours at room temperature without mixing or shaking. Carefully, CTAB was removed out of solution without removing the nanowires. An equal volume of MUTAB (1 mg/ml in Millipore H$_2$O) is added to fill the volume left by removed CTAB. The solution was mixed using a micro-pipette and placed overnight in a water bath at 35$^\circ$ C. The MUTAB-Au nanowires were then re-suspended in solution by gently shaking the
vial, and then left untouched for 1-2 hours at room temperature to allow for sedimentation. Finally, excess MUTAB was removed out of solution (carefully without removing the nanowires), and an equal volume of Millipore H₂O was added to fill the volume left by excess MUTAB.

5.3.5. Microscope Glass Coverslips

Glass coverslips (22 x 22 mm², VWR) were cleaned by sonication for 10 minutes in acetone, 10 minutes in water/detergent mixture, and 10 minutes in deionized water. The coverslips were then immersed in a bath of base (6:1:1 water, 30% H₂O₂, NH₄OH) for 90 seconds at 80 °C followed by rinsing with copious amounts of deionized water. The glass was then cleaned for 2 minutes in O₂ plasma (Harrick Plasma). Cleaned coverslips were placed under vacuum for long term storage. Details of the passivation of the surface for correlation spectroscopy experiments and single protein binding experiments can be found in Figure 5.15.
Figure 5.15 Passivation of substrates for luminescence correlation spectroscopy (a) and single molecule imaging experiments (b). The passivation of the cleaned coverslips depended on the type of experiment and the analyte of interest. For correlation spectroscopy (a), the success of the experiment required minimal interaction of the positively charged MUTAB-AuNRs with the substrate. The coverslips were immersed in a 2% solution of Vectabond reagent (Vector Labs) for 5 minutes followed by rinsing with DI water. Vectabond ensured minimal interaction with the MUTAB-AuNRs in solution. Negligible adsorption of MUTAB-AuNRs was observed by fluorescence imaging on these Vectabond passivated coverslips (a).

Single protein binding experiments (b) on the other hand, required free diffusion of Alexa647-labeled BSA molecules and negligible non-specific adsorption to the coverslip surface without affecting their binding interaction with immobilized MUTAB-AuNRs. Using a custom silicon isolator with a small elliptical opening (43018C, Grace Biolabs), the cleaned glass coverslips were passivated with 30 μl of 25 mg/ml unlabeled BSA in buffer and dried at room temperature for three hours. Excess BSA was washed with 15 soft rinses of 1 ml Molecular Biology H₂O. This BSA layer acted as an excellent passivating agent against Alexa647-labeled BSA as negligible non-specific binding of Alexa647-labeled BSA was observed by fluorescence imaging (b). Both of these fluorescence images demonstrate the effectiveness of these passivation protocols and were collected under the same conditions of the single-protein binding experiments (incident excitation intensity of 5 mW/cm², an integration time of 100 ms, frame rate of 7.5 Hz, and electron multiplying gain of 300).
5.4. Experimental Methods

5.4.1. Luminescence Correlation Spectroscopy

Luminescence of MUTAB-AuNRs was recorded with a home-built confocal microscope described previously. The instrument is based on an inverted epifluorescence microscope (Observer.D1, Zeiss) equipped with a 638 nm diode laser (CUBE, Coherent). The laser light was collimated and delivered to the back aperture of an oil immersion objective (Apochromat 64X, NA = 1.4, Zeiss). The objective focused the light onto a small liquid cell placed on top of a glass coverslip containing 45 μl of sample solution. The emitted light was collected by the same objective and passed through a dichroic mirror (ZT 532/638 rpc, Chroma Technology), a long pass filter (XLP-647, CVI Melles-Griot), and a 50-μm-diameter pinhole (Thorlabs) placed at the focal plane of the microscope. The luminescence signal was detected by a single element diode detector (PDM 50ct, Micro-Photon-Devices) and processed by a home-built photon counting module (LabView). All measurements were recorded at a controlled laboratory temperature of 20 ± 1 °C, and using an excitation power of $1.5 \times 10^3$ mW/cm$^2$ at the sample. This excitation power ensured minimal heating and negligible auto-fluorescence background of unlabeled BSA. For protein binding isotherms, ~75 pM MUTAB-AuNR solutions with varying concentrations of BSA were prepared by mixing equal volumes of both solutions in the buffer conditions of the experiment described above (Catalog #A7906, Sigma-Aldrich; BSA concentrations: 0.1 nM, 0.5 nM, 1 nM, 1.5 nM, 2 nM, 3
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5.4.2. Super-localization Single-Molecule Microscopy

Samples containing single immobilized MUTAB-AuNRs and MUTAB-AuNPs were prepared by dropcasting ~40 µl of ~12.5 pM of nanoparticle solutions (1:6 dilution of the concentration used for luminescence correlation spectroscopy experiments) on microscope coverslips passivated with unlabeled BSA (Figure 5.15). The droplet was then dried for 10 minutes at room temperature, and the coverslips were rinsed with 1 ml of Millipore H₂O and gently dried with nitrogen gas. A home-built total internal reflection wide field microscope with 637 nm excitation (Coherent OBIS-FP 637 LX) as described elsewhere was used with a custom flow chamber (1 mm height, elliptical opening of 12×5 mm; 43018C, Grace BioLabs) placed over the sample containing immobilized MUTAB-nanoparticles. A solution of 2 nM Alexa647-labeled BSA (A34785, Molecular Probes) was flowed over the sample at 5 µl/min using a Genie Plus flow system (Kent Scientific). Data was collected at an incident excitation intensity of 5 mW/cm², an integration time of 100 ms, frame rate of 7.5 Hz, and electron multiplying gain of 300. Analysis was possible at the super-localization level, as the fluorescent BSA can be selectively observed only when adsorbed to the interface, and is unobservable by motion blur when freely diffusing (D ~ 60 µm²/s). Increases in signal to noise ratio of each frame, identification of adsorbed BSA, and super-localization analysis of the location by radial symmetry fitting was performed using MATLAB 2011b as described
Samples containing single immobilized MUTAB-Au nanowires were prepared using the same drying procedure, but because the concentration was unknown, the solution was repeatedly diluted until single isolated nanowires were observed on the microscope coverslip under the optical microscope.

5.4.3. Circular Dichroism Spectroscopy (CD)

Ensemble CD measurements were taken on a J-815 circular dichroism spectrometer (JASCO) with a 1 mm pathlength quartz cuvette at 20 ± 0.1 °C using a Peltier temperature controller. Equal volumes of ~1 nM nanoparticle solutions (MUTAB-AuNRs or AuNPs) and 0.1 mg/ml BSA solutions were mixed ~30 minutes before each measurement. Wavelength ranges of 190-260 nm and 500-750 nm were collected every 1 nm under standard sensitivity, bandwidth of 1.00 nm, and rate of 50 nm/min. Four scans were collected for each trial and averaged. The spectra were baseline-corrected and presented as mean residue ellipticity. The solvent for CD measurements was 1 mM phosphate buffer (pH 7.2) due to its low absorbance in the far UV. This solvent did not affect the stability of MUTAB-AuNRs, MUTAB-AuNPs or BSA.

5.4.4. Scanning Electron Microscopy (SEM) Imaging

SEM images were taken using a FEI Quanta 400 ESEM FEG, operated at 10 kV under low vacuum conditions. Analysis of aggregate size was performed in ImageJ.
5.4.5. Pre-incubation of MUTAB-AuNRs under Different Media Conditions Before Incubation with MCF-7 Cells

For the experiments shown in Figure 5.1a, 5.1b, MUTAB-AuNRs were incubated in two different media conditions:

1) 1 mL of MUTAB-AuNRs were mixed with 133 uL of 7.5% (w/v) BSA (final concentration of BSA ~1 % w/v) and incubated overnight. This formed a BSA-corona onto the MUTAB-AuNRs before exposure to serum. Next, the BSA-MUTAB-AuNRs were diluted (1:10) in EMEM with 10% FBS (with 1% PCN) and incubated overnight.

2) 1 mL of MUTAB-AuNRs were directly diluted (1:10) in EMEM with 10% FBS (with 1% PCN) and incubated overnight.

Both MUTAB-AuNR solutions were found to be stable in EMEM in within the time scale of the experiments, as observed via UV/Vis spectroscopy.

5.4.6. Nanoparticle Incubation with MCF-7 Cells

MCF-7 cells were seeded into two 6-well plates with each well containing 2 mL of 100,000 cells/mL solution and cultured for three days in EMEM with 10% FBS and 1% PCN (same as incubation conditions for MUTAB-AuNRs). On the third day, the media was removed and each well was carefully washed three times with PBS to remove adherent proteins.
Two wells were filled with 2 mL media containing either 1% BSA, 10% FBS in EMEM with 1% PCN or 10% FBS in EMEM with 1% PCN. The remainder of the wells contained 2 mL of the corresponding BSA-MUTAB-AuNR solutions, each in triplicate.

The plates were incubated at 37°C in 4.5% CO₂ for 6 hours. After incubation for 6 hours, 50 µL of media from each well was removed in triplicate using a pipette and kept for the LDH assay. The wells were washed with PBS three times to remove adherent proteins and nanorods.

The cells were then detached via incubation in 0.05% trypsin with EDTA and counted using a standard hemacytometer (Figure 5.16). After counting, the cells were transferred to a solution of 0.1% Triton X-100 and placed in a -20°C freezer overnight.
Figure 5.16 Number of cells/mL under the two different incubation conditions used for the nanoparticle uptake experiments (BSA-FBS: 1% BSA followed by 10% FBS; FBS: 10 % FBS). Cells were also counted without MUTAB-AuNRs as a control.

5.4.7. LDH Cytotoxicity Assay of MCF-7 Cells Incubated with BSA-MUTAB-AuNRs

A LDH assay was performed by adding 50 μL of formazan dye (Thermo Scientific) to each well containing media from the nanoparticle-cell incubation experiments (Figure 5.17). The plates were placed in an incubator at 37° C with 4.5% CO₂ for 30 minutes and read on a BioTek Uniread 800 plate reader. The purpose of this assay was to measure the cytotoxicity of the MUTAB-AuNRs under the different incubation conditions with BSA and FBS, compared to MCF-7 cells alone.
Figure 5.17 Normalized LDH release under the two different incubation conditions used for the nanoparticle uptake experiments (BSA-FBS: 1% BSA followed by 10% FBS; FBS: 10% FBS only). LDH were also monitored without MUTAB-AuNRs as a control. These results demonstrate that the MUTAB-AuNRs are not significantly more cytotoxic to MCF-7 cells (independent of the incubation conditions).

5.4.8. ICP-MS Measurement of BSA-MUTAB-AuNRs with MCF-7 Cells

The lysed cells were thawed and centrifuged at 8000 g for 10 minutes. The supernatant was removed and the pellets were digested using aqua regia (3:1 solution of HNO3 and HCl). Following digestion, the pellets were diluted to 10 mL using 5% HCl in milli-Q water. The samples were measured for Au concentration using a Perkin-Elmer Nexion 300 ICP-MS. The concentration of Au was determined for each cell using the cell count collected after detaching the cells (Figure 5.18).
Figure 5.18 Concentration of Au (ppb) in MCF-7 cells under the two different incubation conditions used for the nanoparticle uptake experiments (BSA-FBS: 1% BSA followed by 10% FBS; FBS: 10% FBS) determined via ICP-MS. MCF-7 cells alone were also measured without MUTAB-AuNRs as a control.

5.4.9. Calculation of the Number of Nanorods Uptaken per Cell

The average number of nanorods uptaken per cell was calculated by first determining the number of nanorods in the different solutions with cells from the concentration of Au determined via ICP-MS. The number of nanorods was then divided by the average number of cells. The addition of BSA to the MUTAB-AuNRs increases the uptake by MCF-7 cells by 320% compared to the conventional incubation conditions in EMEM with 10% FBS.
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Chapter 6

Concluding Remarks: The Importance of the Protein-to-Nanoparticle Ratio at the Nano-Bio Interface

6.1. Concluding Remarks

This thesis has presented a comprehensive study of the nanoparticle-protein corona from a physico-chemical perspective. The interaction between colloidal gold nanoparticles and bovine serum albumin—a model serum protein and the most abundant in mammals—has been investigated in situ using microscopy and spectroscopy techniques. To achieve such studies, the optical properties of gold nanoparticles were characterized in aqueous solutions. Using single particle spectroscopy, the photoluminescence of gold nanospheres and nanorods was probed with different excitation and detection polarization geometries and its physical origin attributed to the radiative decay of surface plasmons, despite scaling
as a one-photon excitation process. This by itself is an interesting result as it relates the semi-classical electromagnetic process of the surface-plasmon resonance that involves the collective oscillation of thousands of electrons, with a quantum-mechanical process as the excitation of single photons resulting from electron-hole pairs. While the underlying physical mechanism of the photoluminescence of plasmonic nanoparticles is still investigated, the work presented in Chapter 2 led to future investigations of the photoluminescence quantum yield of gold nanorods and gold nanoparticle dimers that further supported the ‘plasmonic’ origin of the photoluminescence.

Using the intrinsic optical signals of gold nanoparticles (e.g. photoluminescence and scattering), fluorescence correlation spectroscopy was applied to measure the size of colloidal gold nanoparticles and nanorods while freely diffusing in aqueous environments. Three calibration methods were implemented to determine the dimensions of the femtoliter observation volume. Accurate nanoparticle sizing was achieved with just a few microliters of picomolar nanoparticle suspensions and less than 5 minutes of data acquisition. Nanoparticles larger than 80 nm in diameter required larger focal observation volumes to avoid under-sizing artifacts, achieved by using an objective with a lower numerical aperture.

Fluctuation correlation spectroscopy was then applied to measure protein binding on nanoparticles using colloidal gold nanoparticles and bovine serum albumin protein. Protein binding was detected as an increase in the nanoparticle’s
hydrodynamic radius without having to remove excess protein out of solution, an advancement to hitherto used analytical techniques that required separation of proteins and nanoparticles. This technique therefore allowed a direct measurement of the thickness of the protein corona and to perform a protein binding isotherm as a function of the nanoparticle’s hydrodynamic radius. This physico-chemical analysis determined that BSA formed a monolayer on colloidal gold nanospheres at micromolar concentrations, and the binding isotherm was best described by an anticooperative model derived from the Hill equation.

A protein monolayer of serum albumin on colloidal gold nanoparticles provided remarkable protection against aggregation and precipitation in saline solutions. This is particularly relevant in a physiological environment, as nanoparticle aggregation is unwanted and potentially toxic. Citrate-stabilized gold nanoparticles aggregated in saline solutions below the NaCl concentration of many bodily fluids including blood plasma. Aggregation was also observed in the presence of phosphate, calcium and zinc ions. Binding of a monolayer of serum albumin onto the nanoparticle’s surface completely prevented this aggregation phenomenon, even when the salt concentration was almost an order of magnitude higher than that of physiological fluids. It was found that higher salts concentrations increase the complexation of the nanoparticle’s citrate anions with salt cations in solution, increasing the hydrophobicity of the nanoparticle surface and the affinity of serum albumin for the bare nanoparticle surface.
A protein monolayer occurs when there is a sufficient excess of proteins to nanoparticles, granted that there is enough binding affinity between the two. In contrast, when the ratio of concentrations of proteins to nanoparticles is close to $\sim 1$, the binding cannot be described as an equilibrium process, an interaction known as the 'hard' protein corona. Using fluorescence microscopy, individual bovine serum albumin proteins were observed as they adsorbed onto single, positively charged gold nanoparticles. Binding occurred within seconds after exposure and was found to be irreversible for many hours, even in the presence of massive serum proteins such as fibrinogen and globulin. Interestingly, only one serum albumin protein adsorbed per nanoparticle. Circular dichroism spectroscopy revealed that unfolding of the protein occurs shortly after binding, and further super-resolution fluorescence imaging showed that a single protein may spread across the entire available surface area of a single nanoparticle. In contrast to the enhanced colloidal stability observed at large protein-to-nanoparticle ratios, low binding ratios induced protein unfolding which then triggered nanoparticle aggregation.

Nanoparticles pre-exposed to serum albumin before the usual incubation step in fetal bovine serum were found to be uptaken by a model cancer cell line three times more than those only incubated in serum before exposure to cells. Whether this enhanced uptake is beneficial for anticancer therapeutics or not will still require a great amount of research efforts. This thesis thus makes the case for awareness of the relative concentration ratio of proteins and nanoparticles, as it determines the physical chemistry of the protein corona and plays a central role in how nanoparticles interact with living organisms.
References


