On the Interplay of Gold Nanoparticles and Small Molecules

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the Adaptive & Responsive Nanomaterials Group Department of Chemical Engineering University College London

August, 2019
Declaration of authorship

I, Ye Yang, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed: 

Date: 

Abstract

University College London
Faculty of Engineering Sciences
Department of Chemical Engineering

Doctor of Philosophy

**On the Interplay of Gold Nanoparticles and Small Molecules**

by Ye Yang

Gold nanoparticles (AuNPs) have been widely used in optical, catalytic, chemical and biomedical applications due to their distinct physicochemical properties. Molecular recognition and the binding of chemical and biological entities have attracted particular research interest for the relevance in drug delivery and diagnostics. By addressing material design as well as advanced characterisation methods, this thesis aims to shed light on fundamental aspects of AuNPs, including the synthesis, functionalisation and implications for relevant applications. A versatile AuNP synthesis enabled the decoupling of core size refinement and ligand shell variation in sub-10 nm thiol-capped AuNPs, thus providing a novel solution of comparative fine-tuning of the ligand shell composition. The analysis of AuNP size distribution was addressed with a comparative study employing single particle analysis (electron microscopy imaging) and ensemble methods (X-ray scattering and analytical ultracentrifugation). Subsequently, nanoscale colloidal stability and molecular interaction with small molecules were investigated both in solution by small-angle X-ray scattering and on a surface by quartz crystal microbalance with dissipation monitoring. This work establishes a versatile platform for AuNP engineering with target size and surface functionality and provides a powerful toolbox for systematic studies on colloidal behaviour as well as ligand-shell mediated nanoparticle-stimuli interactions for biomedical applications.

Keywords: gold nanoparticle, molecular interaction, colloidal stability, SAXS, QCM
Impact statement

The 21st century has seen a rise of nanoscience and nanotechnology thanks to the groundbreaking advances in nanoscale fabrication and characterisation techniques. Increased understanding of the structure-property-function interplays in nanomaterials has fostered their relevance, especially in chemical and life sciences. Unlike top-down fabrication methods typically used in inorganic nanomaterials, the self-assembly of both natural and synthetic materials enables bottom-up morphology control on the 1–10 nm length scale as well as biochemical functionality and biocompatibility. This may be harvested for nanoparticles that interact selectively with chemical and biological entities, the intriguing potential of which has been demonstrated especially for biomedical research.

To this end, hybrid gold nanoparticles are an interesting model system, as they offer, via the rational assembly of their organic ligand shell, pathways to achieve the structural fidelity required for specific molecular interaction. By addressing material design as well as advanced characterisation methods, this doctoral research aims to shed light on fundamental aspects of gold nanoparticles, i.e. the synthesis, functionalisation and implications for relevant applications.

A versatile platform for gold nanoparticle engineering is established in this thesis, which provides a powerful toolbox for systematic studies on colloidal behaviour as well as ligand-shell mediated nanoparticle-stimuli interactions. Importantly, polar and hydrophobic interactions dominate most biophysical processes with ionic functional groups and non-polar domains commonly displayed by biological entities. However, the intriguing behaviour of nanoscale macromolecules, such as the regulation of intracellular protein interactions, extends beyond the current understanding of colloidal systems. From a molecular chemistry perspective, proteins and functionalised nanoparticles share a plethora of structural similarities. They are amphiphilic colloidal systems with distinct interfacial properties at similar nanoscopic length scale, governed by the diversity and complexity of their surface morphologies. Building on the systematic evaluation of the colloidal behaviour of amphiphilic gold nanoparticles and their molecular interaction with small molecules, this research offers a rich resource to explore structure-property relationships of protein-mimetic gold nanoparticles, thus promoting a better understanding of many unresolved questions around protein behaviour, such as protein folding and protein aggregation. By doing so, the work in this thesis shall help to inform material design for a variety of applications, including diagnostics, drug delivery and a combination thereof.
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<td>1D</td>
<td>One-dimensional</td>
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<tr>
<td>2D</td>
<td>Two-dimensional</td>
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<tr>
<td>3D</td>
<td>Three-dimensional</td>
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<tr>
<td>5CB</td>
<td>4-Cyano-4'-pentylbiphenyl</td>
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<tr>
<td>AcA</td>
<td>Acetic acid</td>
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<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
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<tr>
<td>AM</td>
<td>Areal mass</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>Analytical ultracentrifugation</td>
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<tr>
<td>AuNP</td>
<td>Gold nanoparticle</td>
</tr>
<tr>
<td>AUT</td>
<td>11-Amino-1-undecanethiol hydrochloride</td>
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<tr>
<td>BA</td>
<td>Boronic acid</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>BuA</td>
<td>Butyric acid</td>
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<tr>
<td>Cryo-TEM</td>
<td>Cryogenic transmission electron microscopy</td>
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<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
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<td>DCM</td>
<td>Dichloromethane</td>
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<td>diOT</td>
<td>1,8-Octanedithiol</td>
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<td>DLS</td>
<td>Dynamic light scattering</td>
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<td>DLVO</td>
<td>Derjaguin-Landau-Verwey-Overbeek</td>
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<td>DMAP</td>
<td>4-((N,N\text{-Dimethylamino})\text{pyridine})</td>
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<td>DNA</td>
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<td>DOA</td>
<td>Dioctylamine</td>
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<td>dPBA</td>
<td>Phenylboronic acid disulfide</td>
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<tr>
<td>EDL</td>
<td>Electric double layer</td>
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<td>ESA</td>
<td>Extreme small angle</td>
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<td>LC</td>
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<td>LOD</td>
<td>Limit of detection</td>
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<td>LSA</td>
<td>Linear-superposition approximation</td>
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<td>Localised surface plasmon resonance</td>
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<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionisation</td>
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<td>MDDCBO</td>
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<tr>
<td>MUS</td>
<td>11-Mercapto-1-undecanesulfonate</td>
</tr>
<tr>
<td>MUTAB</td>
<td>(11-Mercaptoundecyl)trimethylammonium bromide</td>
</tr>
<tr>
<td>NMM</td>
<td>N-Methylmorpholine</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>OAm</td>
<td>Oleylamine</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>ODA</td>
<td>Octadecylamine</td>
</tr>
<tr>
<td>OHP</td>
<td>Outer Helmholtz Plane</td>
</tr>
<tr>
<td>OT</td>
<td>1-Octanethiol</td>
</tr>
<tr>
<td>PAA</td>
<td>Poly(acrylic acid)</td>
</tr>
<tr>
<td>PBA</td>
<td>Phenylboronic acid</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PET</td>
<td>2-Phenylethanethiol</td>
</tr>
<tr>
<td>PPh₃</td>
<td>Triphenylphosphine</td>
</tr>
<tr>
<td>PS</td>
<td>Polystyrene</td>
</tr>
<tr>
<td>QCM-D</td>
<td>Quartz crystal microbalance with dissipation monitoring</td>
</tr>
<tr>
<td>RET</td>
<td>Resonance energy transfer</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Restricted primitive model</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-assembled monolayer</td>
</tr>
<tr>
<td>SANS</td>
<td>Small-angle neutron scattering</td>
</tr>
<tr>
<td>SAXS</td>
<td>Small-angle X-ray scattering</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SERS</td>
<td>Surface-enhanced Raman scattering</td>
</tr>
<tr>
<td>SLD</td>
<td>Scattering length density</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SV</td>
<td>Sedimentation velocity</td>
</tr>
<tr>
<td>STM</td>
<td>Scanning tunnelling microscopy</td>
</tr>
<tr>
<td>tBAB</td>
<td>t-Butylamine-borane complex</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>ThyT</td>
<td>Thyminethiol</td>
</tr>
<tr>
<td>TMA</td>
<td>Trimethylammonium</td>
</tr>
<tr>
<td>TOAB</td>
<td>Tetraoctylammonium bromide</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-visible</td>
</tr>
<tr>
<td>xDLVO</td>
<td>Extended Derjaguin-Landau-Verwey-Overbeek</td>
</tr>
</tbody>
</table>
Thesis overview

The aim of this doctoral study lies in the use of sub-10 nm AuNPs with tailored surface functionality as a model system to study nanoscale colloidal behaviour and molecular interaction with small molecules. In light of the underlying principles and accomplished results, the structure of this thesis is outlined as follows:

Chapter 1 – 4: Research background and experimental techniques.
Chapter 5: Modular AuNP synthesis decoupling core size and surface functionality.
Chapter 6: Comparative study of characterising the size distribution of AuNPs.
Chapter 7: SAXS study of AuNP colloidal stability and molecular interaction in solution.
Chapter 8: QCM-D monitoring of AuNP interaction with small molecules.

The fundamental aspects of molecular forces and self-assembly are introduced in Chapter 1, followed by a summary of their impacts on the behaviour of biotic and abiotic colloidal systems. Proteins and AuNPs are compared and their resemblance is discussed for further implementation in bio-nanotechnology. Chapter 2 is dedicated to the introduction of the state-of-art AuNP synthesis, functionalisation and applications. The research aims of this thesis are outlined in Chapter 3. Chapter 4 summarises the methodology of the material synthesis and characterisation techniques that were employed in the course of this research.

The experimental results of my doctoral research are discussed in Chapter 5 to 8. A modular synthetic AuNP platform is established in Chapter 5 to provide a novel solution of comparative fine-tuning of the ligand shell composition. With a revised synthesis protocol using oleylamine, monodisperse AuNPs of various sizes were prepared in a simple and reproducible protocol. Subsequent thiol-for-oleylamine ligand-exchange process demonstrated a great versatility for preparing thiol-capped AuNPs with targeted surface functionality. The advanced characterisation of AuNP size distribution is explored in Chapter 6. A comparative study offers comprehensive feedback between conventional direct electron microscopy imaging and less studied X-ray scattering and analytical centrifugation.

Results on AuNP colloidal stability and molecular interaction are presented in Chapter 7. In aim to shed light on protein behaviour, the solvation, clustering as well as long-range ordering of amphiphilic AuNPs were investigated by small-angle X-ray scattering (SAXS),
uncovering the significant hydrotropic impact of small molecules in modulating NP-NP interaction. The interplay of AuNPs and small molecules on flat surfaces is discussed in Chapter 8. Following the immobilisation of AuNPs on thiolated gold surfaces, boronate ester formation between salicylic acid derivatives in solution and boronic acids on AuNPs with prescribed ligand shells was studied via quartz crystal microbalance with dissipation monitoring (QCM-D), revealing a drastic effect of both ligand architecture and concentration of the Lewis base on the interaction strength and enabling quantitative analysis of the respective binding affinities.

Lastly, conclusions and possible further studies are summarised in Chapter 9.
Chapter 1

Colloidal systems: from proteins to nanoparticles

Colloidal science and relevant interfacial phenomena are central to numerous research questions across chemistry, biology and materials science. This chapter aims to introduce the basic principles of molecular interaction and how they are of crucial prevalence in both biotic macromolecules and synthetic nanoparticle systems.

1.1 Nanoscale molecular forces

Nanoscale molecular forces involve intramolecular covalent bonding and intermolecular non-covalent association. Tab. 1.1 summarises relevant molecular forces ubiquitous among biological and chemical environments.[1] Both intramolecular and intermolecular associations are thermodynamic processes and thereby can be related the Gibbs free energy:

\[ G(p, T) = U + pV - TS = H - TS, \tag{1.1} \]

where \( p, T \) and \( V \) are the pressure, temperature and volume of the system. \( U, H = U + pV \) and \( S \) are the internal energy, enthalpy and entropy, respectively. For an isothermal and isobaric system at a constant volume, the Gibbs free energy can be described by:

\[ \Delta G = \Delta H - T \Delta S, \tag{1.2} \]

where the enthalpic part (\( \Delta H \)) represents the average potential energy of interaction whilst the entropic part (\( \Delta S \)) measures the orderness of intermolecular correlations.[2] From this aspect, covalent bonds are spontaneous associations due to the strong propensity for sharing electron pairs of participating elements. This dynamic process is driven largely by \( \Delta H \) with values in the range of 300–1500 \( k_B T \) (\( k_B \) is the Boltzmann constant for \( T = 298 \text{ K} \)) which outweighs the contribution of entropy \( \Delta S \).[3] Conversely, the interplay between \( \Delta H \)
and $\Delta S$ rises to prominence in intermolecular interaction as a result of the weak enthalpic contribution.\[4\]

### Table 1.1: Nanoscale molecular forces and their relative strength.

<table>
<thead>
<tr>
<th>Molecular force</th>
<th>Relative strength</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intramolecular</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Covalent bonding</td>
<td>Strong</td>
<td>Boronate ester</td>
</tr>
<tr>
<td>Electrostatic: ion-ion</td>
<td>Strong</td>
<td>NaCl crystallisation</td>
</tr>
<tr>
<td><strong>Intermolecular</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electrostatic: ion-ion</td>
<td>Strong-moderate</td>
<td>Charge repulsion in proteins</td>
</tr>
<tr>
<td>Hydrogen bonding</td>
<td>Moderate</td>
<td>Solvation in $H_2O$</td>
</tr>
<tr>
<td>Electrostatic: ion-dipole</td>
<td>Moderate</td>
<td>Ion hydration</td>
</tr>
<tr>
<td>Electrostatic: dipole-dipole</td>
<td>Moderate-weak</td>
<td>$HCl$ polarity</td>
</tr>
<tr>
<td>Other van der Waals force</td>
<td>Weak</td>
<td>Noble gases</td>
</tr>
<tr>
<td>Aromatic interaction</td>
<td>Moderate</td>
<td>Phenylalanine-ligand recognition</td>
</tr>
<tr>
<td>Hydrophobic force</td>
<td>Moderate-weak</td>
<td>Protein folding</td>
</tr>
</tbody>
</table>

#### 1.1.1 Electrostatic forces

Electrostatic forces arise from the surface charge distribution in colloidal systems. Surface charges are generated with the adsorption of ionic species or the dissociation of ionisable groups on surfaces. When dissolving charged colloids in solution, the solvated electrolytes form an electric double layer (EDL) to compensate the charge distribution around the colloids. The EDL consists of a dense shell (Stern layer) due to the adsorption of counterions and a wide diffuse layer of opposite charges via the electrostatic interaction, resulting in local concentration increase of counterions and decrease of coions (Fig. 1.1). The local density of counterions $\rho$ affects the electrostatic potential $\varphi$, which can be described with the expected Boltzmann distribution.\[3\]

$$\rho = \rho_0 \exp(-ze\varphi/k_B T), \quad (1.3)$$

where $z$ is the charge number of the counterion. Meanwhile, the net charge density also follows the well-known Poisson equation:

$$ze\rho = -\varepsilon_0 \varepsilon \nabla^2 \varphi, \quad (1.4)$$

where $\varepsilon_0$ and $\varepsilon$ are the absolute dielectric constant (permittivity) of free space and the relative dielectric constant of the solvent. Combining Eq. 1.3 and Eq. 1.4 yields the non-linear Poisson-Boltzmann equation:
∇^2 \varphi = -(ze\rho_0/\epsilon_0\epsilon)\exp(-ze\varphi/k_BT). \tag{1.5}

When considering the contribution of coions in a solution with electrolytes,

\rho = e(c_+ - c_-) = -2ze_c_s\sinh(e\varphi/k_BT), \tag{1.6}

where \(c_s\) is the concentration of electrolytes. Therefore, Eq. 1.5 can be written as following equation:\cite{5}

∇^2 \varphi = \frac{2ze_c_s}{\epsilon_0\epsilon}\sinh\left(\frac{e\varphi}{k_BT}\right). \tag{1.7}

As a partial differential equation, the boundary conditions, e.g., the nature of particles acquire charges via the dissociation of counterions, needs to be specified. In line with other electrostatic systems, the potential at the particle/solution interface must be continuous.\cite{6}

With the introduction of the dimensionless electrostatic potential \(\psi = e\varphi/k_BT\), this equation can be simplified as

∇^2 \psi = \frac{2ze_c_s}{\epsilon_0\epsilon k_BT} \sinh \psi = \kappa^2 \sinh \psi, \tag{1.8}

where \(\kappa^{-1} = \sqrt{2ze^2c_s/\epsilon_0\epsilon k_BT}\) is the so-called Debye length, which represents the scale of electrostatic interactions. For monovalent NaCl concentration of 1 and 0.001 M, \(\kappa^{-1}\) is estimated to be 0.3 and 9.6 nm, respectively.\cite{3, 5, 7}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1_1.png}
\caption{Schematic representation of the ion distribution near a negatively charged surface: accumulation of counterions and depletion of coions. OHP: Outer Helmholtz Plane. Adapted with permission from ref\cite{3}. Copyright 2011 Elsevier Inc.}
\end{figure}

At the molecular level, the ion distribution inside the EDL has been studied by either Monte Carlo (MC) or molecular dynamics (MD) simulations based on ion-ion interactions.
Commonly used models are the restricted primitive model (RPM) and the mean spherical approximation (MSA). Both models treat colloids as hard spheres. The more simplified RPM relies on an average uniform electrostatic interaction whilst MSA applies linearisation of the electrostatic field across the whole range.\[6\] For nanoscale interactions in particular, an improvement to MSA was established by incorporating a hypernetted-chain (HNC) model, which includes non-linear electrostatics as well as short-range intermolecular forces.\[8\]

The knowledge of the EDL and the boundary conditions serves as the foundation for the determination of the interaction potential between charged particles. While it is often problematic to perform valid integration over all contributing species, the so-called Derjaguin approximation provides useful solutions to the interparticle interaction of various particle shapes and configurations. It assumes that the interaction forces per unit area of curved surfaces are approximated as those of infinite parallel plates, separated by a distance of $h'$.\[6\] For two spheres with radii $a_1$ and $a_2$, the interaction potential can be calculated as

$$U = \frac{2\pi a_1 a_2}{a_1 + a_2} \int_h^\infty u(h')dh',$$

where $h$ is the distance between the charged particle surfaces. The exact form of function $u$ relies on the boundary conditions. For instance, the interaction potential of two particles of same size $a$ and with constant potential $\varphi$ at the interface can be give as

$$U = 2\pi \varepsilon_0 \varepsilon a^2 \ln[1 + \exp(\kappa h)].$$

The Derjaguin approximation is generally in line with the non-linear Poission-Boltzmann equation for relatively small separations, i.e. $2 < \kappa a < 5$. In diluted systems, the linear-superposition approximation (LSA), which treats the potential of the entire system as a sum of the potentials of isolated particles, provides a better estimation for particles that are far apart.\[6, 9\] Moreover, it is also important to mention the analytical approximations for inhomogeneous systems. One commonly used functional form of the interaction potential is the so-called Yukawa potential or screened Coulombic potential:

$$\varphi_i(d) = \frac{Q_i^\infty}{4\pi \varepsilon_0 \varepsilon (1 + \kappa a_i)} \frac{\exp[-\kappa(d-a)]}{d}.$$  

Here, $Q_i^\infty$ is the renormalised charge on the sphere $i$ at infinite separation and $d$ is the actual separation distance.\[10\]
Although both the Derjaguin approximation and the LSA are useful in many cases, they have rather restricted reliability when interpreting interactions at smaller separations. When \( \kappa a \) or \( \kappa h \approx 1 \), the overlap of the EDL of the particles can result in the ion redistribution around the entire particle, in contrast with the local redistribution that occurs at large separations. Furthermore, the pairwise principle applied in both methods is no longer valid with the emergence of the many-body effects under such conditions. For example, the introduction of a third particle may screen the interaction between the existing particle pair with energetically favourable contributions.

Overall, nanoscale electrostatic repulsive interaction is highly robust and can be controlled precisely over its main driving factors: the colloidal surface charge and the EDL determined by the colloidal surface charges and the ionic strength of the media. A number of charged headgroups, such as carboxylate, sulfonate and ammonium, have been employed for surface functionalisation on different metallic and inorganic surfaces. Meanwhile, the EDL can be altered by simply adjusting salt concentration for varying the Debye length \( \kappa^{-1} \). Alternatively, fine-tuning may also be achieved by utilising different counterions at similar charge density.

### 1.1.2 Van der Waals forces

Van der Waals forces, as known as intermolecular forces, are prevalent in nanoscale interactions. These forces play a key role in a range of research fields, including structural biology, supramolecular chemistry and interfacial science. Van der Waals forces arise from fluctuations in the electron density. Unlike long-range Coulomb electrostatic forces, van der Waals forces are much weaker (0.2 to 10 \( k_B T \)) and limited within short ranges. However, the attractive interaction induced by van der Waals forces is still considerable, especially in the absence of other more dominant forces and often leads to aggregation and precipitation in solution.

Van der Waals forces are generally categorised into three forms: a) dipole–dipole interactions (Keesom force), b) permanent dipole-induced dipole interactions (Debye force), and c) spontaneous dipole–induced dipole interactions (London dispersion force). All three forces scale exponentially with separation distance \( d \) between interacting elements. A first-order approximation of van der Waals force can be given as

\[
 u_{\text{vdW}}(d) = -\frac{C_{\text{vdW}}}{d^6},
\]  

(1.12)
Chapter 1. Colloidal systems: from proteins to nanoparticles

where \( C_{vdW} \) is a constant derived from the sum of Keesom, Debye and London dispersion forces. For two spheres of radii \( a_1 \) and \( a_2 \) at a centre-to-centre distance \( d \), a pairwise summation of \( u_{vdW} \) throughout the volumes of the two spheres gives the overall interaction force:\[5\]

\[
U_{vdW}(d) = \frac{A}{3} \left[ \frac{a_1 a_2}{d^2 - (a_1 + a_2)^2} + \frac{a_1 a_2}{d^2 - (a_1 - a_2)^2} + \frac{1}{2} \ln \left( \frac{d^2 - (a_1 + a_2)^2}{d^2 - (a_1 - a_2)^2} \right) \right].
\] (1.13)

where \( A \) is the Hamaker coefficient calculated from Hamaker integral approximation. Particularly, when \( d \gg a_1, a_2 \), Eq. 1.13 can be simplified as following equation,

\[
U_{vdW}(d) = -\frac{A a_1 a_2}{6d(a_1 + a_2)}.
\] (1.14)

Recent studies have pointed out the limitation of the Hamaker approximation considering van der Waals forces are generally non-additive. Similar to the case of electrostatic interactions, the pairwise summation of interacting forces between two entities does not provide an accurate estimation because of the many-body effects.[5, 13] In other words, the presence of nearby entities are attributed to alter the overall force field between the pair interaction and often results in a reduction of the overall interaction strength.[3] Adaptions have been developed based on quantum field theory, most notably the continuum Dzyaloshinskii–Lifshitz–Pitaevskii theory and the discrete coupled-dipole method.[13–15] They permit more accurate estimations with the consideration of many-body effects that are integral for nanoscale interactions.

1.1.3 Hydrophobic forces

Together with hydrogen bonding, the hydrophobic force is an important intermolecular effect associated with the interaction with water. The term hydrophobic describes the water-fearing property of certain substances (hydrophobes) like oil. Hydrophobes are composed of non-polar molecules, such as hydrocarbons, alkanes, fluorocarbons and inert gases, which are generally incapable of forming hydrogen bonds.[16] Hydrophobic forces are related to the loss of entropy attributed to the coordination of water molecules whose hydrogen bonding is disrupted around hydrophobes.[3, 17] In this context, the solvation free energy \( \Delta G_s \) represents the reversible work of solvent molecules to reorganise and solvate the hydrophobe. \( \Delta G_s \) scales linearly with the overall excluded volume for small solutes whilst it grows linearly with solvated surface area for large solutes whose volume to surface ratio \( > 1 \) (Fig. 1.2).[2]. As a result of \( \Delta G_s \) minimisation, the hydrophobic interaction
is attributed to the spontaneous organisation of hydrophobes into clusters or aggregates. In a more recent review, Whitesides and co-workers suggested that the hydrophobic effect is strictly "water-centric" and of both entropic and enthalpic contributions, which are attributed to the change in the free energy of the networks of hydrogen bonds.[18] The hydrophobic effect is ubiquitous among biological and chemical events and leads to a number of fundamental interfacial phenomena. Fig. 1.3 displays a collection of observed effects of hydrophobic interaction both in solution and at the water interface, in addition to a range of self-assembly events including the formation of bilayers, micelles and vesicles, protein folding as well as the gating of ion channels.[1, 19, 20]

Although hydrophobic interactions are of high relevance for current research questions in chemistry and nanobiotechnology, the quantitative description of hydrophobic forces remains under debate.[21] The complexity of this problem is largely due to the passive nature of the interaction potentials when involving many-body contributions, which poses immense challenges for direct experimental measurements. The first experimental attempt based on direct force measurements was carried out by Israelachvili and Pashley. They observed the long-range hydrophobic interaction between two mica cylinders modified with a hydrophobic monolayer of the cationic surfactant cetyltrimethylammonium bromide (CTAB). In the range of 1 – 10 nm, the measured forces followed an exponential decay function with separation distance.[22] However, it has become increasingly clear that original experimental set-up did not encompass the full complexity of the hydrophobic
force. To accumulate more experimental evidence, subsequent studies have employed an atomic force microscopy (AFM) together with a surface force apparatus for measurements between macroscopic surfaces. A notable example is the imaging of nanoscopic bubbles on silanised silicon wafer surfaces via AFM reported by Higashitani and co-workers.[23]

There are many aspects to be explored for a unified description to quantify hydrophobic forces. One of the crucial challenges is the understanding of the length-scale and the scaling dependence of the hydrophobic force. Various theoretical considerations have been proposed to address this intensively debated topic. Recent studies suggested separate
treatments for differently sized systems, following the scaling trend of $G_s$. On the other hand, based on recent experimental results describing force–distance relationships, it has been proposed that the strong attractive hydrophobic force has different separation ranges. For a long range force at a separation distance $d$ ranging from $\sim 20$ to $\sim 1$ nm, it roughly follows an exponential increase with decreasing separation distance due to an enhanced Hamaker constant associated with water polarisation. When $d$ falls below 1 nm, the short range hydrophobic force increases even more steeply due to potential water structuring effects associated with surface-induced charges and hydrogen bonding.[19, 21]

### 1.1.4 Colloidal stability

Colloidal stability in solution relies on the interplay of electrostatic repulsion, van der Waals attraction and other interaction forces. The balance between these forces remains central to colloidal science. The classical Derjaguin-Landau-Verwey-Overbeek (DLVO) theory provides a simple solution for separate additive contribution from electrostatic ($V_{el}$) and van der Waals ($V_{vdW}$) interaction potentials, as a function of the centre-to-centre distance $d$: [25–27]

$$V(d) = V_{el}(d) + V_{vdW}(d). \quad (1.15)$$

The graphical representation of the DLVO theory shown in Fig. 1.4 highlights the summation of the two interaction forces as well as the formation of an energy barrier, known as the aggregation barrier. Thus, the height of the aggregation barrier is a direct indication of the stability of the colloidal system. The aggregation barrier can be increased either by higher surface charge density and lower concentration of electrolytes, or smaller interaction constants for $V_{vdW}$.

The DLVO theory allows accurate description of the colloidal behaviour at macro- and microscales, including several successful studies for nanoparticles (NPs) above 50 nm.[28, 29] However, recent studies raised concerns over its failure in predicting nanoscale interactions. For instance, Kotov and co-workers summarised in a recent review the divergence of the DLVO theory and the non-additivity in the interactions between NPs.[30] Generally, the application of the DLVO theory for nanoscale systems is limited by the exclusion of solvation forces and the linear simplification of interaction potentials. The exclusion of solvation forces arises from two invalid assumptions on interaction dimensions for nanoscale colloids, especially for those with dimensions below 20 nm (Fig. 1.5). The first assumption requires that solvent molecules and solvated ions are much smaller than dispersed colloids and thus, their influence on the surface charge density of colloids is
negligible. Another failed assumption is the separation distance \( d \) to be much larger than the dimension of the colloids. As a result, both assumptions exclude the impacts of other non-DLVO forces, which can be monotonically repulsive, monotonically attractive or oscillatory. These non-DLVO forces, such as solvation forces, can be even stronger than the electrostatic and van der Waals forces, especially at a short separation.[3] In aim for a better approximation of nanoscale colloids in complex media, a key research focus has been centred on formulating the extended DLVO theory (xDLVO) by incorporating additional terms describing other interaction forces such as hydrophobic \( (V_{\text{hd}}) \) or other potentials \( (V_{\text{o}}) \).[27]

\[
V(d) = V_{\text{el}}(d) + V_{\text{vdW}}(d) + V_{\text{hd}}(d) + V_{\text{o}}(d).
\] (1.16)

Furthermore, DLVO applies the additive linear superposition of the electrostatic potential from isolated colloids, which excludes the consideration of non-linear effects and the surface boundary conditions described in Eq. 1.8.[31] A comprehensive model for predicting the colloidal stability at the nanoscale remains challenging and a series of simulations studies MD, MC and dissipative particle dynamics are being tested before further experimental validation.[30]
1.2 Molecular interaction in nature: proteins

Proteins are some of the most abundant macromolecules in biological systems. With an estimate of $10^4$ variations, they are involved in almost all biological events. As a nanoscale colloidal system, proteins are amphiphilic entities with complex surface morphology. The principles of molecular forces and self-assembly govern their formation, function, transportation and degradation.[32, 33] Researchers have just started to understand the intricate biomolecular interactions, which dictate protein behaviour and evolution in the dynamic biological environment.

1.2.1 Protein folding

Protein folding is ubiquitous in biology and it plays a vital role in the structure formation of proteins. Proteins are composed of 20 proteinogenic amino acids as elementary building
blocks. Fig. 1.6 represents the hierarchical protein structure formation process. The complex protein folding involves multiple molecular self-assembly processes, driven by solvent-mediated interactions (e.g., hydrogen bonding, hydrophobic and depletion forces) to minimise the exposed non-polar surface.[34] The hydrophobic part of amino acids acts as the initiation site to form a linear sequence, known as polypeptide chain, and serves the primary structure of proteins with both hydrophobic and hydrophilic components.[35] In aqueous environment, this amphiphilic entity favours lowest free-energy state by minimising the exposure of its hydrophobic parts. Associated with hydrogen bonds, the primary structure thus tends to fold into local structural "motifs" such as helices and sheets. To achieve global energy minimisation, 3D tertiary and quarternary structures are formed from these local structures by the association of their side-chain groups.[4]

As a consequence, common globular proteins maintain a compact core structure formed by interacting secondary structures within a highly hydrophilic surface. However, the surface morphology varies significantly for different proteins. One of the most common phenomena is the exposure of hydrophobic residues. These residues tend to form patch-like domains, as shown in the experimental findings.[36, 37] Furthermore, Jones and Thornton evaluated various protein-protein interaction sites and set up fundamental criteria for hydrophobic patch determination.[38] The contact of these patches in biological environments has profound impacts on the behaviour of proteins. Importantly, the presence of hydrophobic patches dictates specific protein functions and a series of intermolecular interactions, including hydration and protein-protein interaction.[2] For example, Snyder et al. revealed the vital role of hydrophobic interaction sites in molecular recognition from a study using arylsulfonamides and carbonic anhydrase as a model system.[39] The MD simulation by Zhou et al. suggested the interaction between hydrophobic patches also initiate the hydrophobic collapse for complex multidomain proteins such as the BphC enzyme.[40]

1.2.2 Protein misfolding and aggregation

As mentioned above, a polypeptide chain is required to undergo several energy-minimisation arrangements to arrive at its final conformation. As stated in Anfinsen’s dogma, this final conformation is determined by the amino acid sequence and is therefore referred as the native conformation.[41] However, the search for the native conformation involves global optimisation. The paths for protein folding can be illustrated with a energy level landscape (Fig. 1.7). To guide this seemingly random process, nature has set up a series of biochemical controls and even employed the so-called molecular chaperones for correcting faulty proteins with exposed hydrophobic stretches. Still, the malfunctioning of the cellular machinery occurs with occasional genetic mutations.[42] As proposed by Gregersen et
Protein misfolding leads to the exposure of the hydrophobic patches, which are in turn susceptible to interact with other faulty proteins either intra- or extracellularly. For example, rapid protein aggregation results in highly ordered fibrillar aggregates, also known as the amyloids. A broad range of human diseases, including Alzheimer’s and Parkinson’s diseases, are revealed to be associated with proteins in the amyloid state.[44, 45]

1.3 Gold nanoparticles as synthetic colloidal model system

Nanomaterials, on the scale of 1 – 100 nm, demonstrate enticing properties, which differ from both those of conventional bulk materials and the individual constituents. The size and shape dependent interfacial phenomena are among the most commonly studied research topics of the past decades.[46]

As one of the major synthetic colloidal systems, metal NPs are widely employed in many applications with biological systems due to their highly controllable physicochemical properties. One of the key aspects of NPs lies in the greatly amplified surface to volume
ratio compared to their bulk counterparts.\cite{47} For example, a 3 nm sized palladium NP has more than 50% surface atoms whilst the ratio for a 1 cm$^3$ gold cube is below $10^{-7}$.\cite{48} In some extreme cases, atoms forming the structure can all be considered to be interfacial.\cite{49} The excess free energy from surface atoms induces rapid aggregation, whilst at the same time, the vastly amplified surface area permits the interaction with other material systems.

The particular type of NPs described in this thesis is of a hybrid nature, with a gold core between 1 – 10 nm and a self-assembled coating layer of organic ligands. This material system features following characteristics: 1) the localised surface plasmon resonance (LSPR), induced by the resonant oscillation of free electrons on AuNP surfaces, leads to size and shape dependent light scattering and absorption; 2) Au cations (Au$^{1+}$ or Au$^{3+}$) have a low electrochemical potential and AuNPs can be easily synthesised by a simple reduction of metal complexes; 3) the size and surface functionality can be flexibly tailored by adjusting reaction conditions, such as temperature, reactant concentrations, strength of reducing agents and types of surface ligands; 4) the robustness of Au-ligand bonds enables further surface functionalisation by place-exchange reactions; 5) functionalised AuNPs have remarkable chemical stability and biocompatibility and are suitable for various biochemical applications.\cite{50}

### 1.3.1 The ligand shell: self-assembled thiolate monolayer

To stabilise NPs, various organic and inorganic adsorbates have been developed to act as electrostatic or physical barriers. The self-assembled monolayer (SAM) of organic ligands provides a facile solution for wet chemistry synthesis. The ligand shell not only protects the NP surface by lowering the free energy, but also dictates its surface properties.
The SAM is a fluid organic assembly formed spontaneously on the flat or curved metal surfaces in a well-packed semicrystalline structure by molecular constituents from solution or the gas phase.[49] Although SAMs are typically only thin layers with a thickness between 1 – 3 nm, the integration of SAM onto NPs has multiple advantages in addition to serving as physical barriers.[52] For example, by changing the functionality of the SAM, one can induce or modify the interaction between not only NPs but also NPs and other biotic and abiotic components such as small molecules, proteins and cell membranes.[53]

The functionality of the SAM arises from the functional headgroups of the ligand molecules. In a typical example of simple linear ligands, one end is terminated by headgroups which have specific chemical affinity towards metal surfaces. The most common example is the spontaneous adsorption of alkanethiols on Au surfaces due to the formation of the robust Au-sulfur bond, facilitated by van der Waals interactions between the molecules. The interpretation of Au-sulfur bond is an ongoing research topic that has extensively evolved in the past decade. Notably, the co-existence of several bonding motifs suggests the multitude dynamic interplay on the gold-thiolate interface.[51] A variety of Au-sulfur bonding schemes, such as disulfide (RSSR), monothiolate (RSH), RS-Au-SR complex and thioether (RSR), are active during SAM formation (Fig. 1.8).[54, 55] Distinct interfacial behaviour of thiolates has been reported in relation to the curvature of the surface, including the thiolate structure and mobility. Recent studies suggest the dominance of the sandwich-like RS-Au-SR motifs on a curved surface, formed by bridging one gold atom in a formal oxidation state of +1.[56]

![Figure 1.9: Schematic representation of SAM on the flat and curved surfaces: (a) homo-ligand on a flat surface, (b) homo-ligand on a curved surface and (c) binary-ligand on a curved surface. The grey areas represent free volume of the ligand tails. Adapted with permission from ref[57]. Copyright 2013 Randy Carney.](image)

The other end of the ligand is terminated by, in most cases, a functional group such as methyls, phenyls, alcohols, acids, sulfonates, amines.[58] This functional group plays an essential role in the chemical properties of the stabilised NPs and governs a range of surface and solution characteristics, including solvation, wetting and charge interactions.[59] Additionally, it can be utilised as an intermediate platform for further modification. Apart from the availability of thiol ligands with different functionalities, another key aspect
in the SAM is the incorporation of ligand mixtures in aim to achieve multiple and ligand morphology-related functionalities.[60] Contrary to SAMs composed of one type of ligand (homo-ligand), the structure and behaviour of SAMs from a binary (or ternary and greater) mixture of thiolates may involve a thermodynamically driven phase separation process.[49] Generally, the mixing behaviour of the binary ligand blend is governed by the minimisation of the Gibbs free energy through the interplay of enthalpic and entropic terms. On flat surfaces, the enthalpic loss from interface minimisation outweighs the entropic gain from microphase separation, which leads to macroscopic phase separation.[61, 62] Pioneered by the groups of Whitesides and Weiss, the simultaneous self-assembly of two types of ligands resulted in surface compositions that were different from those in feed solutions.[63] Stranick et al. revealed the existence of phase separated nano-sized domains with scanning tunnelling microscopy (STM).[64] This thermodynamic trade-off also leads to size dependence of domains on many ligand properties, such as chain length, steric hindrance as well as chemical functionality.

![Figure 1.10: Scheme of binary ligand mixture patterns organised on a curved surface: (a) Janus, (b) randomly mixed, (c) stripe-like. Adapted with permission from ref[65]. Copyright 2017 Springer-Verlag.](image)

In contrast, different phase separation patterns may occur on a curved surface such as those of NPs. This is due to the free volume depicted by the grey areas in Fig. 1.9. NP-related geometric factors, such as surface curvature and ligand ratio, length and bulkiness, all have profound impacts on the conformational entropy, which is in turn affects the phase separation process. As a result, local microphase separations are energetically favourable under many conditions.[66, 67] Geometric constraints are particularly exaggerated with a size mismatch between the thiol ligands. The extra entropic gain for the longer ligands when surrounded by shorter ones favours random mixing or partial phase separation, driving an optimisation of the relative ligand position, also termed as surface morphology of the ligand shell (Fig. 1.10).[65] Notable experimental demonstrations of this microphasic phenomenon were presented by Stellacci and co-workers for binary thiolate mixtures on AuNPs with a core size around 4 nm.[66] In follow-up studies, a series of surface organisation patterns such as random, patchy, Janus as well as stripe-like were evidenced.
by a range of characterising techniques including STM,[68, 69] nuclear magnetic resonance (NMR),[66] small-angle neutron scattering (SANS),[70, 71] and mass spectrometry (MS).[72] In addition, simulation studies by the groups of Glotzer and Alexander-Katz provided theoretical confirmations.[67, 73] Importantly, it has been found that the interfacial energy of the NPs is affected by their spatial ligand distribution. As shown in a detailed experimental and computational study, a non-monotonic wetting dependence can be generated at the molecular level through the self-assembly of ligand mixtures on NPs, contributed by both the formation of cavities at the liquid-solid interface and the confinement of solvent molecules over attractive domains.[74]

1.3.2 Gold nanoparticles as synthetic protein analogue

The diversity and complexity of protein surface morphology serves as the basis of many biological processes such as protein folding and protein aggregation. In the past decades, scientific advances in chemistry, biology and nanotechnology have deepened our understanding of protein behaviour. Interfacial phenomena are of particular relevance due to their profound impact on various pathological conditions. From a molecular chemistry perspective, proteins and functionalised NPs share a plethora of structural similarities. Significantly, they are amphiphilic colloidal systems with distinct interfacial properties at similar nanoscopic length scale.[75, 76] The similarities between NPs and proteins serve as main motivation of this doctoral research. Besides the ability to synthesise NP of various sizes and shapes, the versatile functionalisation of NPs with SAMs offers a rich resource to explore structure-property relationships, thus promoting a better understanding of many unresolved questions around proteins. Building on tailored NP surface structures, the opportunities lie in the systematic evaluation of the NP interaction with small molecules as well as NP-NP interaction.
Chapter 2

Gold nanoparticles: synthesis, functionality and applications

2.1 Synthesis of gold nanoparticles

Generally, the synthesis of AuNPs can be categorised into two systems: 1) the "bottom up" approach which generates AuNPs from individual molecules via a chemical or biological reduction and 2) the "top down" approach which produces AuNPs by breaking down a bulk material. While some suitable top-down routes exist (e.g., nanolithography and wet chemical etching), the bottom-up approach offers a more versatile platform in terms of size control, size range and functionalisation.[77]

The bottom-up synthesis of AuNPs dates back to 1850s, when Sir Michael Faraday demonstrated the reduction of tetrachloroaurotates into "minuscule particles" by reducing solvated gold salt with surface capping agents for aggregate prevention.[78] As shown in Fig. 2.1, two major physical chemistry processes, nucleation and growth, are involved in this simple experiment. Following the reduction of gold salts into gold atoms in solution, the collision of gold atoms triggers particle nucleation when they are solvated above their equilibrium concentration. Once the enthalpic gain from creating a bulk crystal exceeds the energy required for creating a solid surface, a nucleus reaches the critical radius and remains stable in solution. Supplied with gold atoms from ongoing precursor reduction, the subsequent growth of stable nuclei yields AuNPs with increasing sizes until the free atoms are consumed.[79] NP growth and NP aggregation share the same driving force, i.e. the reduction in surface energy in solution. Therefore, the surface capping agents are required for the protection of AuNPs.

2.1.1 One-step direct synthesis

While a plethora of synthetic protocols exist, concepts for AuNP synthesis can be related to a number of landmark developments. One important route is the citrate method introduced by Turkevich in 1951, which led to the synthesis of monodisperse AuNPs based on a seed-mediated growth mechanism.[79, 80] Further modifications were presented by Frens
in 1973 to allow size variation by controlled nucleation.[81] The citrate method, in principle, offers facile synthesis of water-soluble AuNPs in the range of 10 – 50 nm and has been widely adopted ever since. However, this approach suffers from a number of limitations, such as the weak reducing power of sodium citrate that requires for high dilutions and consequently a low production rate, as well as the difficult access to monodisperse AuNPs below 10 nm in diameter. To this end, Puntes and co-workers recently obtained 5 nm monodisperse citrate AuNPs with the addition of tannic acid.[82, 83]. In contrast to this in situ method in which the final core size is already determined at an early stage upon the completion of nucleation, the seed-growth method separates the chemical reduction into nucleation and successive growth and has been widely used in the synthesis of AuNPs with larger diameters (>30 nm).[79] Extending the AuNP size step by step, this procedure is favoured when size and shape of the AuNPs need to be carefully controlled.[77] For example, it has been reported the sequential seed-growth method can yield AuNPs up to 200 nm.[84]. Another cornerstone is the Brust-Schiffrin biphasic method with the introduction of thiolates for stabilising AuNPs.[85, 86] The general principle of the Brust-Schiffrin method is presented in Fig. 2.2, in which the surfactant tetracylammonium bromide (TOAB) enables the transfer of the gold precursor from the aqueous phase to an organic phase (toluene), followed by subsequent reduction by sodium borohydride (NaBH₄) in the presence of thiol ligands.[77] This approach allows the preparation of AuNPs below 5 nm with a densely packed thiol ligand shell.

Sub-10 nm monodisperse AuNPs are promising candidates as biomedical agents as well as building blocks to construct multidimensional supra-structures.[87] While the Brust-Schiffrin method was widely pursued in the bottom-up synthesis of AuNPs for more than a decade, limitations remained over the lack of size and surface morphology control. [77] Another milestone for AuNP synthesis was reported in 2006 by Zheng and Stucky. They utilised t-butylamine-borane complex (tBAB) as an organic-soluble reducing agent, which enabled the synthesis of thiolate-stabilised AuNPs with all reactants mixed in a
one-phase solution.\textsuperscript{88} The resulting homogeneous reduction and nucleation permitted the production of AuNPs of size $\sim$5 nm with a size dispersity below 10%.

The aforementioned one-step methods are widely employed for preparing monodisperse AuNPs with size and shape variations. However, it is important to address a number of limitations. Firstly, these methods (apart from the citrate method) take place in non-polar organic solvents and produce AuNPs stabilised with hydrophobic ligand shells, which inhibits the use in water-based biomedical applications. Secondly, although one-step methods are highly robust for incorporating different ligands, these reactions involve many degrees of complexity and are highly parameter sensitive. A small variation in certain experimental variables can lead to dramatic changes in the AuNP size and distribution.\textsuperscript{89} Thiol ligands, in particular, affect both the nucleation and the growth processes by altering the size and reactivity of the nuclei. Therefore, synthetic protocols for varying the composition of the SAM often lead to a variation of the NP core, making comparative and systematic studies difficult.\textsuperscript{90} To serve the purpose of utilising AuNPs in complex biological environments, flexibility in the interfacial design of AuNP is required. From this perspective, an one-step approach is no longer suited for studies which require tailored surface functionality in combination with precise control over core size and ligand shell composition.

### 2.1.2 Ligand-exchange synthesis

The Murray place-exchange approach entails replacing the initially anchored thiol ligands with alternative thiol ligands (Fig. 2.3(a)).\textsuperscript{87, 91} It offers a viable route for the post-functionalisation of AuNPs, especially for the incorporation of ligand mixtures. Furthermore, hydrophobic AuNPs can be transferred from organic solvents into aqueous solutions by introducing a polar ligand.\textsuperscript{92} The kinetics and evolution of the ligand shell morphology in thiol-for-thiol exchange reactions was recently studied in detail.\textsuperscript{93, 94}
Nonetheless, the place-exchange reaction remains a lengthy process due to the strong binding affinity of the initial capping layer. The reaction rate of the substitution further depends on the chain length and steric bulkiness of thiols as well as the surface charge of the AuNPs.[59] Furthermore, the etching of the gold cores due to the large excess of incoming thiols cannot be avoided and often leads to unexpected size reduction.[95] As a result, the implementation of thiol-for-thiol method is plagued by the slow reaction as well as inhomogeneous replacement.[96]

**Figure 2.3:** General scheme of ligand-exchange synthesis: (a) Thiol-for-thiol place-exchange method and (b) Thiol-for-agent ligand-exchange method (phosphine as an example). Adapted with permission from ref[87]. Copyright 2012 American Chemical Society.

An alternative to the thiol-for-thiol substitution is the synthesis of AuNPs with a more labile intermediate capping agent to facilitate replacement with a thiol ligand (Fig. 2.3(b)). The use of intermediate agents such as citrate,[96–98] phosphine[99–101], pyridine[102–104], and amine[105–107] allows to separate the synthesis of AuNPs into two steps: 1) formation of AuNPs stabilised by intermediate capping agents; 2) formation of AuNPs with tailored ligand shells via a thiol-for-agent ligand-exchange procedure. Based on the early work by Schmid,[108] the group of Hutchison developed the use of phosphine-based labile capping ligands.[99] Notably, a broad scope of thiol ligand-exchange reactions was reported by replacing triphenylphosphine (PPh$_3$) on the surface of 1.5 nm AuNPs at room temperature.[100] Both organic-soluble and water-soluble AuNPs, with a variety of thiol ligand shells, were prepared either in dichloromethane (DCM) or a DCM-H$_2$O biphasic solution. This powerful ligand-exchange method offers a generic and effective approach to prepare AuNPs with specific ligand capping under mild conditions. The possibility in preparing ultrasmall water-soluble AuNPs opened new doors for the synthesis of soluble and stable nanoscale colloids in physiological aqueous environments.[109] Similarly, 4-(N,N-dimethylamino)pyridine (DMAP) was introduced by the group of Lennox as another capping agent for the ligand-exchange synthesis of AuNPs. As gold exhibits a higher
Chapter 2. Gold nanoparticles: synthesis, functionality and application

affinity for thiols than labile pyridine, the thiol-for-DMAP exchange reaction was able to efficiently engage a variety of functionalised thiols.[103] For example, Milette et al. reported the synthesis of AuNPs capped with thiolated liquid crystal (LC) mesogens. The use of DMAP enabled facile fine-tuning of subsequent thiol compositions.[104]

Another group of reagents that is widely used in AuNP synthesis is alkylamine, which acts as both reducing and capping agent. Similar to the citrate method, amines are weak reductants and thereby trigger the formation of AuNPs.[105] The affinity of the amine group towards gold renders the AuNP surface passivated by the free amines in solution.[110, 111] It is important to point out that the strength of amine-gold association sits between those formed by citrates and thiols. Therefore, amines offer great potential for both replacing the citrate capping and subsequent displacement by thiols on the AuNP surface. For example, the addition of hexylamine, nonylamine or dodecylamine induced the phase transfer of citrate-AuNPs into non-polar solvents.[112] Dioctylamine (DOA) can act as agent for AuNPs of various sizes to serve for subsequent ligand replacement with functionalised thiols.[106] Based on the same principle, oleylamine (OAm) provides effective protection for AuNPs. One noteworthy example is the burst nucleation method which renders the size tuning in the range of 1–10 nm in a solvent mixture of OAm and tetralin. The addition of extra reducing agent tBAB facilitates the nucleation and thereby improves the sphericness of yielded AuNPs.[113] In spite of these advantages, the ligand exchange using OAm capped AuNPs remains largely unexplored. One reason for this may be due to the complex role of OAm in AuNP synthesis, which is widely agreed to be three-fold, i.e. as surfactant, solvent, and reducing agent.[114, 115]

2.2 Surface functionality of nanoparticles

In nature, many processes, such as cell penetration and biotransformation, are primarily determined by specific external stimuli at biological interfaces.[116] Host-guest interaction facilitates the selective binding event between ligand and target analyte based on molecular recognition.[117] Complex host–guest supramolecular systems, such as nanocarriers and artificial molecular machines, have been developed for molecular recognition as well as the delivery of drugs and other therapeutic materials.[118–120] As a versatile model system in nanochemistry, SAM-AuNPs exhibit comparable sizes to those of biological entities, such as proteins, nucleic acids and many cellular sub-structures.[121] Detailed control over the core size and ligand shell of SAM-AuNPs is imperative to convey biochemical functionalities and model interfacial phenomena at bio-nano interfaces.[74, 122]
2.2.1 Surface functionality design

As outlined in Chapter 1, AuNPs stabilised with a SAM, typically composed of thiolate ligands, have been extensively studied due to the strong interaction between gold and sulfur.\[51, 123\] The SAM ligand shell dictates many AuNP properties, such as solubility, chemical reactivity and binding affinity.\[100\] Particular functional groups engender specific molecular interaction on the bio-nano interface.\[92\] With the immense library of thiolate ligands, it is possible to tether a plethora of surface functionalities on SAM-AuNPs.\[58, 124\] Fig. 2.4 summarises the commonly adopted chemical modification methods and corresponding functional ligands. In brief, the following routes for functionalisation are highlighted: 1) SAM-AuNPs terminated with target functional groups (\textit{i.e.} alcohol, phenol, carboxylic acid, amine) \textit{via} place-exchange reaction (step h, g, s); 2) SAM-AuNPs conjugated with biomolecules (\textit{e.g.}, protein, peptide, antibody and nucleotide) \textit{via} peptide bonding (step j, l); 3) SAM-AuNPs terminated with biotins \textit{via} biotinylation (step l, q).\[53\] These modifications have a profound impact on the properties and applications of AuNPs, as shown in Fig. 2.5. The modulation of AuNP surface chemistry can be accessed with control over ligand properties such as charge and hydrophobicity. The conjugation with biological entities also permits the surface interaction with small molecules,
bio-macromolecules or other external stimuli. The modular approach shown in Fig. 2.4 presents flexibility in nanomaterial design and significantly expands the utilisation of tailored AuNPs as biomarkers, drug delivery vehicles, or therapeutics for specific extra- and intracellular activities.[125, 126]

Moreover, it is also possible to harness multiple functionalities by incorporating mixed-ligand shell on AuNPs. This provides a route to multivalent binding and contrasting hydrophobicity and interfacial energy on the AuNP surface. For example, the ligand mixture of a naphthalene-terminated thiol ligand and a hexanethiol facilitated the binding of polycyclic aromatic hydrocarbons on AuNPs through supramolecular receptors.[127] Enhancement of AuNP hydrophilicity was also observed at the air-water interface by varying the composition of hydroxyl-thiols in the ligand shells.[128]

![Figure 2.5: Surface functionalities of AuNPs.](image)

**Figure 2.5**: Surface functionalities of AuNPs. With a myriad of thiol ligands and bioconjugation protocols, AuNPs with specific functionalities can be prepared from a modular synthetic platform. *Adapted with permission from ref[125]. Copyright 2011 The Royal Society of Chemistry.*

### 2.2.2 Principles of AuNP solubility and colloidal stability

The colloidal stability of NP dispersions is generally associated with electrostatic and steric forces in aqueous or organic solutions.[12] One of the most enticing aspects of the ligand-exchange method is the possibility to tune the solvation of AuNPs. The displacement of surface-bound ligands with opposing polarity is often followed by the phase transfer of AuNPs. This interfacial phenomenon has been reported in many recent studies, one of which showed the spontaneous transfer of 12 nm citrate-AuNPs from H₂O to DCM upon the addition of thiolated poly(ethylene glycol) (PEG) due to a significant gain in entropy of PEG in DCM. This was facilitated by the use of a common solvent methanol (MeOH) miscible with both H₂O and DCM.[129] Similarly, a two-step method to transfer
citrate-AuNPs between H$_2$O and toluene was developed. The replacement of citrate by hydrophobic octadecylamine (ODA) triggered the transfer of AuNPs into toluene while a reverse transfer was enabled by rapid cationising ODA-AuNPs with positively charged (11-mercaptoundecyl)trimethylammonium bromide (MUTAB).[130] Therefore, the ligand replacement with a polar thiol ligand offers a facile synthetic procedure to prepare water-soluble amphiphilic AuNPs. Two synthetic strategies have been developed using: 1) a binary mixed-ligand shell, typically through the combination of charged thiolates and linear alkanethiols; 2) a homo-ligand shell composed of multivalent thiolates. Meanwhile, two groups of charged thiolates, namely terminations with negatively charged sulfonate or positively charged trimethylammonium (TMA), are commonly employed for mediating ionic associations with biological entities.

![Hydration layer](image.png)

**Figure 2.6:** Schematic representation of a hydration layer formed around a liposome. Adapted with permission from ref[131]. Copyright 2006 Springer-Verlag.

When hydrophilic or amphiphilic AuNPs are dissolved in an aqueous solution, the reorganisation and re-orientation of surrounding H$_2$O molecules generates a repulsive force, the hydration force, which is a short-range non-DLVO solvation force. With a decay length estimated to be 0.2 — 1.1 nm, the hydration force, associated with the formation of a hydration layer (Fig. 2.6), induces an effective solute-solvent interaction that is strongly influenced by the interfacial energy of the solutes as well the effects of different electrolytes.[132] The divergence in relative efficiencies of cation and anion binding is known as the specific ion effect or the Hofmeister effect.[133, 134] In relation, the presence of hydration layer plays a role in stabilising colloids at high ionic strengths. The detailed mechanism is still to be uncovered but generally it is associated with the binding of hydrated cationic electrolytes.[3] This particular effect was observed in a study of AuNPs stabilised with thyminethiol, which demonstrated excellent colloidal stability at extreme basic conditions by weak electrostatic and strong hydration repulsion. Importantly,
the strength of the hydration repulsion was maintained in concentrated NaCl and KCl solutions but weakened in the presence of LiCl and CsCl.[135]

Apart from the influence by AuNP surface functionality and dispersion solvent, the colloidal stability of AuNPs can also be controlled by varying internal solution variables, such as pH, electrolyte concentration and other inorganic salts. Another research area of interest is the control of colloidal stability of AuNPs with external stimuli, such as light, heat and electric field.[136] This strategy, based on the responsiveness of ligand molecules, has been studied extensively, leading to switchable and reversible supramolecular self-assembly.[137] The applications of this self-assembly principle in constructing hierarchical suprastructures will be discussed in details in Section 2.3.2.

2.2.3 Solvation and colloidal behaviour in biological environments

Biological media is an immensely complex environment, containing electrolytes, small molecules, proteins, lipids. The solubility and stability of AuNPs are important prerequisites to maintain their functions in various media in order to directly relate their in vitro and in vivo properties.[138, 139] The contact with biological solutions, such as body fluids or cell culture media, can alter the dispersion state of water-soluble NPs.[140] The interaction with biological components often leads to AuNP clustering or aggregation.[141] As a universal colloidal phenomenon, aggregation refers to the particle-particle adherence, which results in the formation of irregular clusters with extended dimensions. Fundamentally, it is in relation to high ionic strength and abundance of macromolecules like proteins in the complex physiological environments. High ionic strength enhances electrostatic screening and thereby induces aggregation by NP-NP interaction. Due to excessive surface energy on AuNPs, biological proteins tend to adhere and form a protein corona on the surface of NPs via NP-protein interaction. On one hand, this biomolecular corona plays an active role in defining the biological interactions of NPs and their translocation across biological barriers.[140] For example, the abundance of clusterin in the protein corona demonstrated the inhibition of non-specific cellular uptake.[142] On the other hand, the adsorption of proteins, dominated by human serum albumin and fibrinogen, can disrupt the colloidal stability of NPs by altering their mobility and surface functionality.[143] Colloidal aggregation of NPs can lead to shorter circulation half-life in blood and a more rapid clearance by the reticuloendothelial system (RES) of our human body, on top of ineffective biodistribution and enhanced in vivo toxicity.[144] Thus, it poses severe challenges for the utilisation of AuNPs in biomedical applications.

Common in vitro experimental strategies are presented in Fig. 2.7. An estimation of the colloidal interaction, based on the xDLVO theory with the additional steric repulsion
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**Figure 2.7:** Colloidal behaviours of AuNPs. (a) Van der Waals forces originating from permanent or induced dipoles within AuNPs can result in attractive interaction and subsequent aggregation. (b) Adsorption of macromolecules (e.g., hydrophilic proteins and polymers) promotes AuNP stabilisation by providing steric shielding to prevent NP-NP interaction. (c) Electrostatic stability is attributed to surface charges on AuNPs, which results in the formation of an EDL, composed of a strongly associated Stern layer and an outer diffuse layer. (d) Prediction of interaction potential composed of different stabilisation procedures. The xDLVO theory represents the direct summation of individual contributions. Adapted with permission from ref[144]. Copyright 2015 The Royal Society of Chemistry.

term, gives a good representation of each contribution. In principle, the stabilisation of AuNPs can be achieved by promoting electrostatic repulsion, and steric repulsion or a combination thereof, *i.e.* electrosteric repulsion. Surface charges of AuNPs induce electrostatic repulsion between individual AuNPs, whose effectiveness is directly related to charge density. For instance, dispersed AuNPs functionalised with multivalent tris(sodium-<em>m</em>-sulfonatophenyl)phosphine (TPPTS) exhibited colloidal stability in a number of protein-rich cell culture media.[145] However, the effect of electrostatic stabilisation is strongly dependent on the ionic strength of the solution, which dictates the radial size and strength of the EDL. In media with high ion strength, most of electrostatic interactions are screened by the presence of electrolytes. In contrast, the stabilisation by surface polymer coating is typically the consequence of an increase of steric hindrance, which inhibits biological protein adsorption. By attaching a layer of hydrophilic polymers, steric repulsion arises with the repulsive osmotic force due to the unfavourable entropy associated with compressing the polymer chains, especially when the outer most polymer segments of two AuNPs overlap.[132] The polymer coating also lowers the surface energy of AuNPs and thereby
prevents bound or adsorbed proteins forming a protein corona. Both natural and synthetic polymers, such as PEG, alginate, chitosan and poly(vinyl pyrrolidone) (PVP), are used for this purpose.[139] For example, PEG polymer chains and thiolated PEG ligands can improve the stability and biodistribution of AuNPs.[146] Also, PEG functionalisation leads to the reduction of cytotoxicity, especially for AuNPs below 5 nm.[147] This is in direct contrast to AuNPs with low steric hindrance, which is in line with the highest cytotoxicity reported in the 1 – 2 nm size range.[148] Alternatively, electrosteric repulsion was also studied by using a binary mixture of thiolated PEG and mercaptopropionic acid (MPA) ligands, which led to excellent in vitro stability at varying pH values and high salt concentrations.[149]

### 2.3 Applications of nanoparticles

#### 2.3.1 Interactions with biological systems

To interact with biological entities, SAM-AuNPs are required to display well-defined interfacial and wetting properties. The interplay between hydrophobicity and surface charges is crucial for the colloidal behaviour.[124, 150] For example, the immune activation of splenocyte cells exhibited strong dependence on the hydrophobicity of AuNPs.[151] Through surface functionalisation of multivalent thiolates, amphiphilic AuNPs also demonstrated excellent affinities towards cell membranes as well as cytosolic components, such as proteins and enzymes, after cell internalisation.[120, 152]

![Chemical structure and simulation snapshot of a MUS-OT AuNP](image)

**Figure 2.8:** Chemical structure and simulation snapshot of a MUS-OT AuNP. Adapted with permission from ref[125]. Copyright 2014 Macmillan Publishers Limited.

A myriad of work has been reported based on the mixed-ligand combination of 11-mercapto-1-undecanesulfonate (MUS) and 1-octanethiol (OT), whose structures are shown in Fig. 2.8. Notably, Verma and co-workers presented cell-membrane penetration via the non-endocytic membrane fusion and suggested that the surface morphology affects cell
uptake.\cite{153} This non-disruptive intracellular behaviour was later evidenced by the experiments of lipid bilayer membrane fusion in addition to atomistic MD simulations.\cite{154, 155} MUS-OT AuNPs were also used for interacting with bacterial and the structural heterogeneity induced by replacing OT with its branched version altered the EDL interface and manifested lower \textit{Escherichia coli} adsorption, highlighting the importance of SAM surface morphology in determining AuNP colloidal stability.\cite{156} In a more recent study, the multivalent anchoring of MUS-OT AuNPs propagated the design of novel virucidal assays that are effective against a broad range of human pathogens. By mimicking heparan sulfate proteoglycans (HSPG), the target of viral attachment ligands, MUS-OT AuNPs maintained their virucidal efficacy at nanomolar concentration.\cite{157}

Alternatively, ligands with the positively charged TMA group served as the backbone of many multifunctional homo-ligand systems. Taking advantage of the flexible post functionalisation of the place-exchange method, Rotello and co-workers reported a wide range of experiments utilising cationic AuNPs. An early study validated that TMA-AuNPs exhibit a pronounced strong toxicity as a result of their interaction with cell membranes.\cite{158} To mitigate this cytotoxicity during intracellular activation, an improved design was reported by using an intermediated threading agent for the endosomal release of diaminohexane-terminated AuNPs through competitive interactions.\cite{119} The size dependence of AuNPs on cellular uptake was also reported in a comparative study, in which divergent interplay patterns were observed between 2, 4, 6 nm AuNPs.\cite{159} The strong association between cationic AuNPs and cell membranes was also harvested in antimicrobial applications. AuNPs with a cationic and hydrophobic homo-ligand shell demonstrated outstanding suppression for a range of multi-drug-resistant bacteria.\cite{160} Furthermore, when functionalised with cationic amino acids, AuNPs exhibit considerable deoxyribonucleic acid (DNA) binding affinities and serve as effective transfection vectors for gene delivery.\cite{161} In a recent study, arginine-functionalised AuNPs facilitated effective cytosolic protein delivery via the cell membrane fusion of NP–protein supramolecular assemblies.\cite{162}

While the cytotoxicity may be mediated via size variation and surface modification, it is the accumulative behaviour of AuNPs that makes them eventually an unfavourable material system for \textit{in vivo} applications.\cite{163–165} For example, Ma \textit{et al.} reported the accumulation of internalised AuNPs in lysosomes, which inhibited lysosome degradation capacity through alkalisation of lysosomal pH and induced autophagosome accumulation.\cite{166} This non-biodegradable nature of AuNPs addresses challenges in further clearance from the human body.\cite{167, 168} Moreover, AuNPs may also exhibit immunotoxicity and trigger a series of unfavoured immune responses, as shown in Fig. 2.9.\cite{169} Consequently, the development of therapeutic nanomaterials has shifted towards more biodegradable material systems, such as magnetic NPs (iron oxide based) and polymersomes (polymer based).\cite{170–173}
Nevertheless, the ease of rational surface functionalisation makes AuNPs an important model system for proof-of-concept studies, whose findings can ultimately inform the material design of other platforms.

2.3.2 Multi-dimensional structuring via self-assembly

Beyond interaction at the colloidal interface lies the multi-scale self-assembly in supramolecular nanochemistry. Directed self-assembly provides new tools for a tailored bottom-up hierarchical material design.[136] SAM-AuNPs, in particular, are an ideal nanoscale system offering interchangeable building blocks for fabricating multi-dimensional nanostructures or even microscopic crystals.[174] To integrate simple colloidal building blocks within complex supramolecular networks, a broad variety of self-assembly strategies has been demonstrated, leveraging on control over AuNP surface functionality, solution chemistry, external stimuli or a combination thereof.

Different AuNP functionalities have been studied for molecular self-assembly. A number of studies demonstrated the advantage of electrostatic repulsion in facilitating the organisation of nanoscale components into ordered supra-structures. Notably, Kotov and co-workers reported the self-assembly of CdTe quantum dots functionalised with charged ligands. With negatively charged thioglycolic acid, CdTe nanocrystals assembled into 1D chains while positively charged 2-(dimethylamino)ethanethiol induced free-floating 2D sheets.[175–177]
Walker et al. focused on the assembly of gold nanotriangles functionalised with TMA, which assembled into large-area monolayers of closed-packed arrays.[178] Alternatively, the strong attraction between oppositely-charged nanoscale colloids was identified as a novel pathway for large-scale self-assembly and constructing dynamic aggregates or superlattices. Unlike microscale conditions where oppositely-charged colloids undergo continuous precipitation, charged AuNPs would only precipitate when the total positive charges compensate the negative charges, also known as the point of electroneutrality.[6, 179] This effect was extensively studied by the group of Grzybowski and various complex structures were constructed by electrostatic assembly, including patch-like coatings by depositing densely packed films composed of negatively- and positively-charged NPs and diamond-like micro-sized crystals through the assembly of oppositely-charged binary AuNPs.[180, 181] Furthermore, the polymer-mediated strategy pioneered by the Rotello group also demonstrated the possibility of extending nanostructures to macroscopic scale.[182] By stabilising AuNPs with thymine terminated-thiol ligands, the assembly between AuNPs and polymers was immediately observed as a consequence of the interaction between the thymine groups and triazines via site-selective hydrogen bonding. Another promising route towards building block directed self-assembly involves the tailored functionalisation of AuNPs to achieve anisotropy, e.g., by changing the NP shape for localised structuring, or by introducing patch- or Janus-type NP morphologies.[183, 184] For example, the reversible and controllable self-assembly of Au-organosilica Janus NPs was realised with varying surface charges in a recent study.[185]

As discussed in Section 2.2.2, the solution environment dictates the colloidal behaviour of AuNPs. Various chemical stimuli including solvent, pH, gaseous signals and metal ions will affect the interplay between electrostatic and solvation forces.[137] The ordering of
Chapter 2. Gold nanoparticles: synthesis, functionality and application

AuNPs stabilised with polystyrene (PS) is a well studied example for the solvent-induced effect. Notably, the precipitation of PS-AuNPs induced distinct LSPR coupling when H₂O was added as a non-solvent into the tetrahydrofuran (THF) solution. As presented in Fig. 2.10, this clustering event was modulated by the hydrophobic interaction and the cluster size could be controlled by PS chain length and the addition of a polymeric surfactant PS-b-PAA, comprising of a hydrophobic PS and a hydrophilic poly(acrylic acid) (PAA) block.[186] Conversely, the self-assembly of AuNPs can also be achieved by the inhibition of colloidal electrostatic repulsion. The control over pH allows reversible protonation and deprotonation of AuNPs stabilised with weakly-charged thiol ligands and thereby induced dynamic clustering and dissociation. A number of pH-responsive self-assembly effects have been reported with thiolates whose pKₐ values are close to neutral pH, such as 2-fluoro-4-mercaptophenol and poly[2-(diethylamino)ethyl methacrylate].[187, 188] Similarly, the protonation of amine-functionalised AuNPs by purging with CO₂ has been employed to trigger the disassembly of AuNP aggregates in aqueous solutions. The process can be switched by introducing N₂ and thereby lowering the surface polarity of AuNPs.[189] Furthermore, the binding of divalent metal ions offers another facile approach to link AuNPs functionalised with carboxylate groups. The assembly is also reversible and the dissociation of AuNP clusters is triggered by the subsequent addition of a stronger chelator, such as alkaline ethylenediaminetetraacetic acid.[190] Based on this mechanism, the chelation of metal ions provides a powerful sensing tool for the detection of heavy metals. Please refer to Section 2.3.3 for detailed discussion.

Alternative routes to AuNP self-assembly involve external factors such as light, heat as well as electric field. The use of light has been extensively studied due to the advent of organic chromophores, which undergo reversible molecular structural modification and colour change upon light irradiation.[132] Notable examples include the AuNP functionalisation using thiols terminated with azobenzene or spiropyran. Zhao et al. reported the UV-induced switching between a dissolved state of azobenzene-AuNPs in apolar solvents and the assembly into ordered aggregates through isomeric transition from the stable trans state to the more polar cis state upon UV irradiation (Fig. 2.11(a)). This self-assembly behaviour was mostly driven by the dipole–dipole attraction between cis-azobenzene moities and resulted in close-packed supracrystals, as shown in Fig. 2.11(b). Furthermore, cavities (nanopores) formed within the crystalline structure could be used as specific nanoreactors to host designated chemical reactions of trapped polar molecules (Fig. 2.11(c)).[191] Although chromophore-stabilised AuNPs generally display hydrophobicity and thereby are poorly dispersed in H₂O, the incorporation of polar ligands, such as thiolated PEG, oligo(ethylene) glycol, charged carboxylates or TMA ligands, provided excellent solubility in aqueous solutions.[192, 193] The simplicity of light-switchable AuNP
assembly and disassembly also offers applications in combination with other stimuli for multi-fold responsive self-assembly systems. As exemplified in a study using AuNPs functionalised with a mixture of azobenzene and ethylenediamine ligands, reversible clustering occurred in toluene upon exposure to UV light or CO$_2$.[194] The temperature-induced assembly behaviour of AuNPs decorated with thermotropic liquid crystal (LC) ligands or thermoresponsive polymers has been described in many recent studies.[195, 196] However, thermoresponsive systems are generally not water-soluble and less controllable, which hinders their potentials for biomedical applications. In contrast, the impact of an external electric field, although largely unexplored, may provide novel interfacial assembly routes, as exemplified by the recent realisation of an electrochemical nanoplasmonic platform at the water-organic interface.[197]
2.3.3 Nanoparticle-based biochemical sensing

With a better understanding of nanomaterial structure-function interplay, we have seen a remarkable growth in relevant applications, especially in nanochemical and biomedical sciences. Among them, one the most active fields of research is NP-based chemical or biological sensing. For detection with high sensitivity and specificity, SAM-NPs are ideal candidates due to their precise bottom-up surface control on the sub-10 nm scale, distinct surface-to-volume ratio and excellent *in vitro* compatibility when using appropriate ligands.[87]

Generally speaking, two functional components are featured in a sensing device: a recognition element which binds with the target analyte in a selective manner and a transducer component which signals the binding event in a readable form. The efficacy of the recognition process strongly relates to the response time, signal-to-noise (S/N) ratio, selectivity, and limit of detection (LOD). Sensor design thus involves the development of novel materials which improve both the recognition and signalling processes. In this regard, SAM-AuNPs are ideal candidates for combining the distinct physical properties of the gold core and the customisable functionalities of the ligand shell.

AuNPs have been used as important colorimetric reporters due to their high extinction coefficients, especially when they aggregate and induce interparticle surface plasmon coupling. This behaviour provides a practical platform for absorption-based colorimetric sensing of various analytes with different chemical properties. In recent years, considerable progress has been made using AuNP-based colorimetric sensing to detect heavy metal ions, such as divalent lead, mercury, and cadmium, which pose significant public health hazards and environment concerns. One particular example is using AuNPs stabilised with 11-mercaptoundecanoic acid (MUA) as extinction dyes to signal otherwise spectroscopically silent heavy-metal ion contaminants in H$_2$O via the ion-chelation induced aggregation process.[198] Along similar pathways, a plasmonic assay with excellent selectivity to mercury ions has been reported, where the target induced aggregation of AuNPs based on coordination chemistry, leading to an instant red-to-blue colour change.[199] Alternatively, a fluorescence mercury sensing scheme was proposed using fluorescent AuNPs encapsulated and stabilised by polyamidoamine.[200] Another important sensing analyte are lithium ions, which are prevalent in the chemical and industrial fields. Its detection has also been reported with an optical readout through selective binding to a phenanthroline derivative that was anchored on the AuNP surface.[201]

Food nanotechnology has recently attracted increasing interest with the growing importance of food packaging and safety. Through improved surface engineering, AuNP assays
have been able to signal the presence of gasses, aromas, chemical contaminants and pathogens, which is vitally useful not only for the manufacturers to have better quality control but also for the customers to avoid food-borne illnesses.[202] For example, the colorimetric detection of melamine, a plant metabolite of cyromazine pesticides in raw milk, was reported with a LOD of 6 ppb using crown-ether-thiolated AuNPs.[203] Also, AuNPs were used in surface-enhanced Raman scattering (SERS) sensing of perchlorate, an environmental water pollutant, at the nanomolar level.[204]

Besides in colorimetric detection of chemical agents, AuNPs have found a number of applications in biomedical sensing in recent years. Advantages of using AuNPs in those fields are particularly related to the capping shell. The multitude of ligand-based functionalisation routes enables bioconjugation of various sorts and thus the ability to construct numerous target biomolecular sensing mechanisms.[205] Transduction for AuNP biosensing is typically based on colorimetry, SPR and fluorescence.[87] Fig. 2.12 summarises major biochemical sensing interactions and underlying mechanisms.

Similar to the case in chemical sensing, AuNPs first found applications in colorimetric biosensors via extinction upon aggregation. Mirkin’s group pioneered the concept by reporting the detection of polynucleotides with mercaptoalkyloligonucleotide-modified AuNPs.[206] An instant colour change was induced in the formation of a polymeric network of NPs and the nucleotides. Based on the same concept, a general design of colorimetric sensors was proposed based on the disassembly of AuNP aggregates linked by DNA aptamers, which enables fast and simple detection of adenosine and cocaine.[207] Xia et al. developed the sensing strategy employing AuNPs and aptamers into the detection of various DNA, small molecules and proteins with the addition of a specific conjugated polyelectrolyte which triggered AuNP aggregation when interacting with complimentary single-stranded DNA.[208]

AuNPs were also used as amplifiers in biosensing due to their unique SPR behaviour. The mechanism involves the propagation of surface plasmons along an interface where the wave vector of plasmons is dependent on the dielectric constants of the medium.[209] The signal is extremely sensitive to the surrounding environment and distinct optical features can be detected upon interaction with different stimuli.[210] As a consequence, the label-free detection of proteins, nucleic acids, and other biomolecules may be achieved in real time.[211] One notable example is the use of optical sensing to study biomolecular interaction based on the change in the absorbance spectrum of AuNPs on glass substrates. With a concentration-dependent binding relationship, this spectrophotometric sensor was able to detect streptavidin at a LOD of 16 nM with a dynamic range of 1 – 30 µg ml\(^{-1}\).[212] SPR detection has also been explored in cancer diagnostics. For instance, cellular imaging
was realised by the strong resonant light scattering of AuNP aggregated within cells with conjugated antibodies.\cite{213} Based on plasmon coupling effect, DNA hybridisation events can also be detected by monitoring the distances between single pairs of AuNPs.\cite{214}

In addition to SPR sensing, AuNPs have been studied as fluorescent probes. Fluorescent behaviour is presiding in most of the biological systems and thereby fluorescence-based sensors are particularly useful in analyte quantification and cellular imaging.\cite{205} For all fluorescence based sensing techniques, fluorescent quenching and resonance energy transfer (RET) are important aspects that need to be addressed. As ideal fluorescent probes, AuNPs efficiently quench adsorbed fluorophores and generate signal patterns.
that track analytes or changes in complex mixtures.\cite{215} This array-based mechanism has been utilised for the detection of proteins, pathogens and mammalian cells.\cite{87} One remarkable example is the quantification of protein targets with AuNP-fluorescent polymer conjugates. Distinct fluorescence response was observed when the presence of proteins disrupts the NP–polymer interaction.\cite{216} Further improvement of the protein detection in biofluids can also be achieved by introducing an enzyme amplifier.\cite{217} Similarly, the identification of pathogens was realised with a non-covalent conjugates of AuNPs and poly(p-phenylene ethynylene). A variety of bacteria were tested in which the functional NPs and the fluorescent polymer generated pronounced fluorescence response.\cite{218} The RET process, on the other hand, exploits the extraordinary high molar extinction coefficients and broad energy bandwidth of AuNPs, which serve as excellent fluorescence quenchers.\cite{87} Notable examples include the sensing assay based on the fluorescence RET between quantum dots and AuNPs, which inhibits specific biomolecular interactions.\cite{219} AuNP-based RET probes can also detect and quantify intracellular analytes. Considering messenger ribonucleic acid (mRNA) in cells as an example, the AuNP-aptamer complex was able to monitor physiologically relevant changes such as the concentration levels of adenosine triphosphate (ATP).\cite{220}

Lastly, it is important to mention AuNP biosensors based on other advanced physical phenomena, such as SERS and QCM-D. Because of the quantised inelastic scattering of photons, Raman scattering is sensitive to different vibrational modes and can thus identify target molecules.\cite{87} AuNPs are widely used to amplify the Raman scattering intensity by surface enhancement. One example is the SERS-based multiplexed detection of DNA and RNA target using AuNP probes labelled with oligonucleotides and Raman-active dyes.\cite{221} Similarly, protein detection was reported in a AuNP-based SERS study of protein-DNA interactions.\cite{222} As an alternative platform, QCM-D enables the monitoring of surface mass change arising from analyte binding events. AuNPs are normally used as a "mass enhancer" to amplify the frequency changes, the incorporation of which led to the realisation of various QCM-based biosensing systems. Several studies have proven the feasibility to detect oligonucleotides and proteins.\cite{87} For example, QCM was utilised in detecting a 33-base oligonucleotide with the amplification of streptavidin-AuNP conjugates.\cite{223} A more detailed experimental description and discussion on QCM-D can be found in Chapter 8.
Chapter 3

Statement of research aims

This chapter outlines the research aims of this doctoral thesis, centering on the synthesis, colloidal behaviour and molecular recognition of AuNPs.

3.1 Thesis scope: synthesis of AuNPs

In spite of the success in preparing SAM-AuNPs of certain sizes and with specific functionalities, a unified platform is lacking to enable the AuNP preparation of bespoke size and surface functionalisation. Meanwhile, in emerging fields related to supramolecular chemistry and bio-nanotechnology, SAM-AuNPs are required with accurate size and surface morphology control as well as solubility in aqueous solutions. For most applications that are based on specific molecular interaction, it is the NP ligand shell that plays the decisive role, and thus, its chemical nature requires careful verification. The implementation of a direct one-step SAM-NP synthesis is restricted by not only the incompatibility of some ligands with the reaction conditions, but also the strong dependence of the core size on the ligands used during synthesis, which eventually causes comparability and optimisation issues.

It has been reported that OAm offers facile and reliable reduction and protection of sub-10 nm AuNP. The core size and monodispersity can be tuned with reaction variables such as temperature, concentration and solvent choice. However, further studies and applications using this method are rather limited, especially the exploration of subsequent functionalisation via ligand exchange is severely lacking. For this reason, one main scope of my research was to develop a modular synthetic approach for SAM-AuNP preparation based on synthesis in OAm and subsequent thiol-for-OAm ligand exchange. This two-step approach promised to produce different subsets of SAM-NPs with the same population of well defined gold cores stemming from the identical batch. Other aspects such as size tuning, size distribution analysis and large-scale production were also of interest. Chapter 5 is dedicated to the discussion of this modular synthetic approach and Chapter 6 focuses on a comparative study of size distribution analysis. This AuNP synthetic platform is of fundamental importance to this doctoral study. It serves as foundation for subsequent systematic studies on AuNP molecular interactions employing a variety of thiolate ligands.
3.2 Thesis scope: colloidal behaviour of AuNPs

AuNP surface is vital to colloidal stability and molecular interactions, which are in turn central to bio-nano interfacial sciences. SAM-AuNPs have been serving as a versatile scaffold to induce intermolecular interactions, commonly including Coulomb electrostatic forces, hydrophobic and hydration effects, as well as van der Waals forces. Despite the considerable progress, many questions on the nanoscale colloidal stability remain unsolved. I attribute the limiting factors to be three-fold: material design, characterisation methods and theoretical considerations. Firstly, synthetic colloidal systems are an ideal candidate for modelling \textit{in vitro} and \textit{in vivo} behaviour of biomolecules. However, there is a lack of versatile NP design strategy to cater for the complex chemical landscape of the bio-nano interface. Secondly, the complex nature of biological environment further hinders accurate experimental assessment of colloidal stability in different material systems. There is an urgent need for developing improved characterisation methods to probe various colloidal effects in a facile and accurate manner. Thirdly, in aim for accurate analysis of experimental data for comprehensive insights, it requires comparative validations for the recently developed theoretical considerations on colloidal interaction potential.

Building on the foundation of the OAm ligand-exchange method, I aim to address these challenges using a simplified model system based on amphiphilic AuNPs. Chapter 7 is dedicated to this study, for which amphiphilic AuNPs with varying ligand composition are synthesised by tuning the ratio of polar and non-polar ligands. Consequently, one major scope of this thesis is to explore the SAXS profiles of AuNP solutions. The distinct scattering length density of the gold core is exploited in the extreme small angle range, which offers important insights on medium- and long-range molecular interactions as well as the impact of non-DLVO forces.

3.3 Thesis scope: molecular recognition of AuNPs

AuNPs serve as an excellent platform to study molecular interactions at various interfaces of biological relevance and to develop advanced stimuli-responsive multifunctional devices in biomedical applications. The detection of chemical and biological entities has vastly benefited from the distinct physical properties and diverse surface functionalities of AuNPs. However, the challenge of engineering sensing functionality with high sensitivity and specificity still prevents the widespread application of many simple, rapid, and low-cost point-of-care sensing strategies. Therefore, a more precise control over NP functionalisation to enable specific interaction and selective recognition of target analytes is pending.
further investigations. Biomedical sensing, in particular, poses great difficulties due to the complex nature of biological systems. The importance of AuNP functionalisation is again emphasised as it directly affects the particular chemistry at the interface where the NP-analyte interaction takes place. The sensitivity is influenced by a variety of factors including the analyte, the recognition partner, and the transduction process. To detect specific target analyte, the key is to optimise material design, induce specific recognition and achieve high-throughput detection with both fundamental and clinical importance.

In order to provide SAM-AuNPs suitable for novel sensing solutions, the screening of the affinity and selectivity of specifically designed ligand shells towards molecular targets is instrumental. Therefore, another scope of this doctoral research is the utilisation of QCM-D, which offers, in principle, a viable route for label-free *in situ* monitoring of molecular adsorption at interfaces. Particularly, I aim to quantitatively study the interaction with small molecules, which remained unexplored due to either an insufficient interfacial area or limited binding affinity in previous studies. This attempt is detailed in Chapter 8.
Chapter 4

Experimental and analytical techniques

This chapter introduces the methodology of relevant synthesis and characterisation of functionalised AuNPs, as well as two major analytical techniques employed for this doctoral work: SAXS and QCM-D.

Disclosure: Section 3.1 is a lightly adapted version of the experimental section in the publication: A versatile AuNP synthetic platform for decoupled control of size and surface composition. Yang Y, Serrano L A, Guldin S. Langmuir, 2018, 34(23), 6820-6826.[224]

4.1 Nanoparticle synthesis

Unless otherwise stated, all commercially available chemicals were purchased from Sigma Aldrich. Reagents of ACS grade or above were used without further purification. All glassware used in AuNPs reactions was cleaned with fresh aqua regia (HCl:HNO₃, 3:1 v:v).

4.1.1 Oleylamine ligand exchange

The preparation of AuNPs intermittently stabilised by OAm is the main synthetic platform for the studies described in this thesis. This two-step process involves the preparation of OAm-AuNPs and subsequent functionalisation by thiol exchange, as demonstrated in Fig. 4.1. A generic OAm-AuNP synthesis started with preparing the precursor at room temperature (∼22 °C) by dissolving 0.5 mmol hydrogen tetrachloroaurate (III) hydrate (HAuCl₄·3H₂O, 95%) in a 40 ml solvent mixture of OAm (C18 content: 80%–90%, Acros Organics) and n-octane (97%) (1:1 v:v). The solution was mixed and sonicated under Ar flow for 10 min before stirring at a designated reaction temperature. The balance of balloon pressure generated the Ar flow in a multi-neck round-bottom flask. The temperature was normally controlled by a thermoprobe of the heating mantle. In some experiments, the reaction temperature was precisely controlled by using a 100 ml jacketed flask with a temperature-controlled circulating bath (GR150-R2, Grant Instruments) The reducing solution was prepared by dissolving 0.5 mmol tBAB (97%) in 1 ml OAm and 1 ml octane. Intensive mixing was required before the injection into the precursor solution under vigorous stirring.
The mixture was left reacting in Ar atmosphere at the specified reaction temperature for 2 h before 30 ml of ethanol (EtOH) was added to quench the reaction. The AuNPs were collected by centrifugation at 5000 g for 10 min and then re-dispersed in DCM. The obtained samples were dried in a vacuum desiccator after repeated centrifugal washing in EtOH and MeOH.

AuNPs stabilised by a SAM were prepared by a subsequent ligand-exchange reaction using the OAm-AuNPs obtained from previous step. The general procedure for this thiol-for-OAm ligand-exchange process started by dissolving 0.1 mmol of the designated thiol mixture in 10 ml of DCM by vigorous stirring at room temperature for 10 min. For the ligand exchange using amphiphilic ligands (e.g., MUS and MUTAB) for preparing homo-ligand AuNPs, solvent mixtures such as two-phase H₂O-DCM and one-phase MeOH-DCM were also used. Subsequently, 30 mg of the OAm-capped AuNPs in 6 ml DCM solution were injected and the solution was allowed to react at room temperature for 6 – 24 h depending on the ligand mixture. To quench the ligand-exchange procedure of organic-soluble AuNPs, the solution was firstly evaporated in a rotary evaporator. Further repetitive washing with MeOH or acetone was able to remove the excess free ligands in solution. In the case of targeting water-soluble AuNPs, acetone was directly added to terminate the reaction. The functionalised AuNPs were collected by centrifugation at 5000 g for 10 min. To remove unbound water-soluble ligands, repetitive washing in H₂O were implemented with Amicon Ultra centrifugal filters (15 ml, 10,000 NMWL, Merck Millipore) at 5000 g for 10 min before final washing in acetone and subsequent vacuum drying.

**4.1.2 One-phase method**

This is a modified version from a published protocol suitable for both homo-ligand or mixed-ligand AuNPs.[88] Here is a detailed example of preparing homo-ligand AuNPs.
stabilised by 1-octanethiol (OT, 98%). Generally, the organic precursor solution was prepared by mixing 0.5 mmol chloro(triphenylphosphine)gold(I) (95%) with 2 mmol OT in 20 ml chloroform at 80 °C for 10 min. Subsequent reduction was initiated with the addition of 5 mmol tBAB dissolved in chloroform, left stirring at the same temperature for 1 h. The solution was cooled down to 40 °C before the addition of 30 ml MeOH. The AuNPs were collected after repetitive centrifugal washing in acetone and MeOH before subsequent vacuum drying.

4.2 Nanoparticle characterisation

SAM-AuNPs are a highly complex material system. Although a variety of advanced techniques has been explored extensively, its characterisation is still an evolving field at the frontier of nanoscience and nanotechnology.[225] Herein, I aim to introduce the major characterisation methods employed in the course of this doctoral research.

4.2.1 Ultraviolet-Visible absorption spectroscopy

Ultraviolet-Visible (UV-Vis) absorption spectroscopy is widely used in AuNP research as the signature SPR peak provides an initial characterisation step for AuNP dispersions.[226] The shift and bandwidth of the peak offers an overview of important features, including size, aspect ratio, solubility and aggregation, as shown in Fig. 4.2(a),(b). Moreover, the absorbance of light by a sample can be correlated to the sample concentration $c$ scaled by the molar extinction coefficient $\varepsilon$ (Fig. 4.2(c)), as established by the Beer–Lambert law:[227, 228]

$$A = \varepsilon cl, \quad (4.1)$$

where $l$ is the length of optical path. The relationship enables the accurate quantification of AuNPs, which can be highly useful when sample has an insufficient weighing quantity.

In my experiments, UV-Vis absorption results were recorded using a fibre-based set-up that combined a stabilised tungsten-halogen light source (SLS201/M, Thorlabs) a cuvette holder (qpod 2e, Quantum Northwest) and a high sensitivity spectrometer (QE-Pro, Ocean Optics). The sample solutions were measured in quartz cuvettes and the respective background signal was compensated with reference subtraction.
Figure 4.2: Size effects on the surface plasmon absorption of AuNPs. (a) The absorption maximum peak red-shifts with increasing particle size; (b) the bandwidth increases with decreasing nanoparticle radius in the intrinsic size region and also with increasing radius in the extrinsic size region as predicted by the Mie theory; (c) linear relationship between AuNP extinction coefficients at respective plasmon absorption peak position with respect to their volume on a double logarithmic scale. Adapted with permission from ref[227]. Copyright 1999 American Chemical Society.

4.2.2 Imaging techniques

4.2.2.1 Transmission electron microscopy

Transmission electron microscopy (TEM) is one of the major microscopy techniques to characterise NP systems due to its powerful imaging capabilities. Comparing to basic light microscopy, the use of electrons with lower wavelength allows a remarkable increase in resolution, making it possible to visualize objects on the order of a few angstroms (Å, \(10^{-10}\) m). Importantly, the low-density organic SAM does not provide sufficient contrast to the electron beam, and thereby TEM predominantly provides visualisation of the gold cores. Information on characteristics, such as core size distribution and aspect ratio can be obtained directly from TEM images. Apart from the limited sampling size which may not be statistically representative, another disadvantage of conventional TEM is the requirement for vacuum condition. AuNPs need to be dried on the sample grid rather than stay in their native dispersed state. Please refer to Chapter 6 for more in-depth discussion. The recent
advent of cryogenic TEM (cryo-TEM) and liquid-phase TEM provides solutions to this drawback. Cryo-TEM enables the imaging of aqueous solution dispersions by freezing the samples in vitreous ice, while liquid-phase TEM provides in situ imaging of the sample solution by incorporating a closed liquid cell, which normally has a transparent window with a thickness of $\sim 100 \mu$m. As a result, both the direct imaging of both in situ and ex situ colloidal interactions has been realised.[229–231]

In my TEM studies, samples were prepared by drop-casting a well-dissolved diluted AuNP solution with an optical density (OD) of $\sim 0.5$ over a fresh carbon-film coated copper grid (400 mesh, EM Resolutions). For solutions at lower concentrations, the sample preparation was also carried out by dipping the fresh grid into the solution. The sample grids were left to dry completely in ambient conditions. A high-resolution 200 keV TEM system (JEM-2100, JEOL) were used to characterise the AuNP samples. The size distribution was determined by automated image analysis of the respective AuNP populations (count rates typically $> 1000$) using the particle analysis function of software ImageJ with a batch-processing macro plug-in developed by Dr Marie Müller in the group of Prof Francesco Stellacci at EPFL, Switzerland. The size and circularity threshold were set as $> 1 \text{ nm}^2$ and $> 0.6$, respectively.

4.2.2.2 Scanning tunnelling microscopy

Using a physical probe to form images by rastering across surfaces, scanning probe microscopy enables the simultaneous imaging of several interactions between the sample and tip. STM traces the variations in quantum tunnelling as a function of the height of a microscopic metal tip above the surface of a crystal. With its ultra-high sub-nanometre resolution, STM has gradually become an important tool for the studies of SAM-NPs, especially for the understanding of molecular arrangement of the ligand shell.[232]. STM was utilised in this doctoral study to investigate the immobilisation of AuNPs on QCM sensor surfaces, with the aim to provide information on the spatial distribution of AuNPs, including their surface coverage pattern with respect to layering and interdigitation.

Herein, the STM characterisation was carried out by Dr Nikolaos Nianias in the group of Prof Francesco Stellacci at EPFL, Switzerland. QCM sensors immobilised with AuNPs were used directly as STM samples. STM imaging was performed at room temperature in ambient air with two STM set-ups: 1) an Easyscan-2 (Nanosurf) on a vibration damping table and 2) a Multimode Scanning Probe Microscopy with E scanner (Veeco) situated in an acoustic chamber. Mechanically-cut Pt-Ir STM tips were used. Set point currents were in the range of 40 – 400 pA with a voltage bias of 400 – 900 mV. Integral gains varied from 0.7 to 0.5 and proportional gains from 0.5 to 0.2. The imaging and analysis was
carried out after allowing the system to stabilise over night to avoid further drifts. Image processing was performed with the software Gwyddion.

### 4.2.3 $^1$H Nuclear magnetic resonance

NMR is widely regarded one of the most versatile and informative spectroscopic techniques. It is based on the phenomenon that occurs when a nucleus with a magnetic moment is immersed in a static magnetic field and subsequently exposed to a second oscillating magnetic field. Chemical shifts, which contain compositional information, are induced by the varying electron density at each nucleus on the resulting magnetic field. Moreover, the interplay of neighbouring nuclei give rise to spin-spin coupling. Combining both effects, NMR can thus evaluate molecular composition and chemical structure as well as sample purity for basic studies.[233] For SAM-NPs, in particular, solution phase NMR is widely used for the structural and compositional elucidation of molecules in the ligand shell.[96, 234, 235] In principle, the $^1$H NMR footprints of the thiol ligands are quenched upon binding, resulting in signal broadening, in contrast to the pronounced signature peaks of the unbound ligands.[234] Based on this phenomenon, it is feasible to distinguish and quantify between bound and unbound ligands by NMR and thus elucidate the ligand composition in the SAM.

In a routine practice, each batch of SAM-AuNPs was re-dissolved in designated deuterated solvents and $^1$H NMR was used to verify the complete removal of the unbound ligands during centrifugal washing. For clean samples, the subsequent addition of I$_2$ enabled to strip off the bound ligands into the solution before a further $^1$H NMR measurement which eventually provided valuable information of molecular composition of the ligand shell. The $^1$H NMR results relevant to this thesis were collected in a Avance III (400 MHz, Bruker). An approximate amount of 5 mg from each batch of AuNPs were dissolved in 500 µl deuterated solvents, followed by ligand stripping with the addition of 10 mg I$_2$.

### 4.2.4 Thermogravimetric analysis

Thermogravimetric analysis (TGA) is another key technique to characterise the ligand shell of AuNPs. TGA measures the thermal stability of the sample by monitoring temperature-induced sample transitions with accurate weight measurement under inert or reactive gas flow. The decrease in weight is generally attributed to the transition and degradation of compounds into gas phases. For AuNPs, the application is straightforward because the volatile organic ligand shell decomposes at $\sim$200 °C whilst the gold core maintains its stability up to $\sim$600 °C.[236] Therefore, the fraction of the thermally decomposed
organic ligand components can be accurately measured by the determination of the weight loss occurring upon heating a AuNP sample in dry powder form. A direct comparison establishes the weight of the organic ligand shell compared to the residual gold core. Importantly, when combined with size information by TEM and molecular composition by NMR, a full picture of the ligand shell can be presented, including grafting density of the ligands as well as the molecular ratio for binary ligand mixtures. Both values are information of vital importance for evaluating AuNP colloidal stability as well as potential conjugations.

In this study, an approximate amount of 5 mg from each AuNP sample was placed on an aluminium sample pan before the analysis in a thermogravimetric analyser (TA Instruments, TGA 550) under N₂ flow. The experimental procedure entailed heating from room temperature to 550 °C at a ramp rate of 20 °C min⁻¹.

### 4.2.5 Analytical ultracentrifugation

Analytical ultracentrifugation (AUC) is a sedimentation-based technique developed primarily to study the hydrodynamic behaviour of colloidal systems such as proteins and NPs. It is based on the mass transport in a liquid medium induced by an applied force field. The technique relies on the interplay of sedimentation induced by the centrifugal force and
diffusion due to the Brownian motion. With ultra-high rotor speeds of up to 60,000 rpm, AUC is able to resolve nanoscale colloids below 1 nm in diameter. In addition, AUC is also compatible with a range of detectors, such as single wavelength absorption, interference and fluorescence.[238] Depending on pre-existing knowledge of certain structural parameters, this technique can yield quantitative information on the size, shape, molecular weight, density or surface heterogeneity of various colloids in solution.[239, 240] Typical AUC measurements include the analysis of sedimentation velocity (SV) and sedimentation equilibrium (SE), as shown in Fig. 4.3. The major difference is a moderate centrifugal force that is applied in the latter case to achieve the equilibrium between sedimentation and back diffusion. The sedimentation and diffusion of a solute under centrifugation at angular velocity $\omega$ follows following equations:[241]

$$s = \frac{DM(1 - \rho_P \rho_S)}{RT},$$ (4.2)

$$\frac{\partial c}{\partial t} = D[(\frac{\partial^2 c}{\partial r^2}) + \frac{1}{r}(\frac{\partial c}{\partial r})] - s\omega^2[r(\frac{\partial c}{\partial r}) + 2c].$$ (4.3)

Eq. 4.2 is the so-called Sveberg equation, in which $D$ is the diffusion constant and $M$ is the molecular weight of the particle. $\rho_P$ and $\rho_S$ are the particle density and the solution density, and $\eta_S$ is the solution viscosity. Eq. 4.3 is the Lamm equation where $c$ is the solute concentration, evolving with time $t$ and radius $r$. Approximate solutions to Eq. 4.3 can be obtained from recorded experimental data via numerical finite element modelling. This fitting procedure generates a 1D sedimentation coefficient distribution $c(s)$, which could be further extended to a 2D sedimentation-correlation $c(s, D)$. The hydrodynamic radius $d_H$ of spherical NPs can then be calculated using Eq. 6.2 by converting $c(s)$ or $c(s, D)$ into a particle size distribution combining Eq. 4.2 and the Stokes-Einstein relation (Eq. 4.4):[237]

$$D = \frac{k_B T}{d_H 3\pi \eta_S},$$ (4.4)

$$d_H = \frac{k_B T}{D 3\pi \eta_S} = \frac{1}{\sqrt{\frac{18\eta_S s}{\rho_P - \rho_S}}.}$$ (4.5)

I note that the validity of the Stokes-Einstein relation requires the colloids to be perfect spheres. In the case of SAM-AuNPs, the calculation can be extended with an overall density model, please refer to Chapter 6 for detailed discussion.

The AUC results in this thesis were obtained in collaboration with Suyyang Liao in the group of Prof Francesco Stellacci at EPFL, Switzerland. The measurements were performed
using an Optima XL-A Ultracentrifuge (Beckman Coulter) with rotating speeds ranging from 6,000 to 40,000 rpm. Absorbance data were measured at 400 nm. Experimental data were analysed in the software SEDFIT, which generated individual sedimentation coefficient distribution $c(s)$ based on a numerical finite element method.\textsuperscript{239} Weighted average sedimentation and diffusion coefficients were calculated using a previously published custom made MATLAB code.\textsuperscript{241}

4.3 Analytical techniques for the study of interparticle interactions

In order to obtain information on the interaction of AuNPs with other entities (AuNPs, small molecules), two major advanced analytical techniques were explored: 1) transmission SAXS was used primarily for characterising the colloidal stability of amphiphilic SAM-AuNPs and probing NP-NP interactions as well as their interactions with small molecules; 2) QCM-D was employed in the development of a screening platform for the quantitative determination of AuNP interaction with small molecules. Herein, I introduce underlying scientific principles and recent developments of both techniques.

4.3.1 Small-angle X-ray scattering in solution

SAXS is a well-established tool to examine various structural information at the nanoscale. Generally, it is based on the principle of elastic scattering. When X-ray photons get in contact with electrons of illuminated sample, the scattering behaviour reflects the fluctuations of electron density.\textsuperscript{242} The exploration of this technique dates back to the landmark theoretical work by Guinier in the 1938, in which the main concepts were established to extract size and shape information. SAXS has since widely been utilised in structural studies of metal alloys and colloids. Another important development has been the emergence of synchrotron sources in the 1970s, whose ultra-high beam intensities enable to resolve organic entities with lower electron density, such as polymers and biological macromolecules in solution. Further studies employing indirect Fourier transforms gave rise to the calculation of real space distance distribution functions.\textsuperscript{243, 244} Solution-based SAXS also enables analysis for the inner structure of disordered systems and is still one of the only methods to provide direct structural information on systems with random density distribution or inhomogeneities of colloid size and shape.\textsuperscript{245} Since the 1990s, SAXS has become increasingly popular for resolving biological macromolecules due to the advent of \textit{ab initio} modelling, whose algorithm applies automated modeling of densely
packed beads in a constrained search volume. It thus affords the reconstruction of 3D macromolecular structures with unprecedented complexity.[242]

4.3.1.1 Experimental setup

Fig. 4.4(a) depicts the typical experimental setup for a solution-based transmission SAXS measurement. The scattering profile of the sample solution is collected on a 2D detector after being illuminated by a monochromatic focused X-ray beam at wavelength $\lambda$. Generally, a radial integration treatment is processed for the isotropic scattering data to obtain the scattering intensity $I$, as a function of the incident angle $2\theta$. The momentum transfer $q$ (Fig. 4.4(b)). $q$ can be calculated as

$$
q = \frac{4\pi \sin \theta}{\lambda}.
$$

The resulting 1D relationship of $I(q)$ is a direct measurement of dissolved scatterers, including solvent molecules and salts used in the buffer. It is therefore important to subtract the contribution of the background solution, obtained by a blank scan under identical experimental conditions. The true representation of the scattering pattern from the sample can then be revealed, as shown for a sample of bovine serum albumin (BSA) in Fig. 4.4(c). In addition, the scattering intensity $I$ can be normalised on an absolute scale, i.e. by the flux density of incident beam, using the absolute intensity of reference standard such as water. In this regard, solution-based SAXS measurements offer facile measurement of solutions under various conditions, such as concentration, temperature or buffer conditions. Another advantage of SAXS lies in the coverage of vast length scale ($1 – 100$ nm) that can be used for structural interpretation. By the varying sample-to-detector distance, the resolving q-range, and thus length scale of interest, can be easily adjusted.

In this study, SAXS measurements were performed using a Ganesha 300XL (SAXSLAB) at 20 °C under vacuum. The X-ray source was a high brilliance microfocus Cu-source (wavelength: 1.5418 Å). The SAXS data were recorded on a Pilatus 300K solid-state photon-counting detector with a 2 mm beam stop. Various modes for sample-to-detector distance were used: medium angle (MA, q-range of 0.015 to 0.65 Å$^{-1}$), small angle (SA, q-range of 0.007 to 0.25 Å$^{-1}$) and extreme small angle (ESA, q-range of 0.0035 to 0.18 Å$^{-1}$). AuNP sample solutions and corresponding buffers were measured in 1 mm (I.D.) borosilicate glass capillaries (Capillary Tube Supplies Ltd).
4.3.1.2 Data interpretation

The SAXS data interpretation is an active field of research even after decades of exploration. A number of theoretical fitting treatments and simulation models have been developed for different material systems and research questions. Herein, I limit the discussion to the interpretation of our particular colloidal system, namely a solution of AuNPs, which is a solution composed of monodisperse spherical scatterers with high electron density. The electron density of the SAM ligand shell is negligible compared to that of the gold core, and thereby mainly the gold cores contribute to the SAXS profiles.

Guinier, Porod, and invariant analysis
To check the data quality before further fitting analysis, a coarse inspection is implemented focusing on the asymptotic behaviour of each SAXS curve after background subtraction.[246] For monodisperse samples, one of the most useful parameter that can be extracted from the measured scattering data is the radius of gyration $R_g$ from the so-called Guinier plot, $\ln[dI(q)]$ versus $q^2$, as formulated by Guinier in 1938:

$$dI(q) = I(0)\exp\left(-\frac{1}{3}R_g^2q^2\right).$$ \hfill (4.7)

A linear approximation with the Guinier plot is valid for low $q$ values for which $qR_g < 1.3$, as known as the Guinier region. The linearity is a good indication of the monodispersity of the sample. The slope of this plot can be used to calculate $R_g$ and its intersection represents the forward scattering intensity $I(0)$. Moreover, the Guinier region provides a straightforward indication of interparticle interactions. In general, attraction and nonspecific aggregation give rise to a sharp increase of $dI(q)$ towards low $q$ values and thereby result in an overestimation of both $R_g$ and $I(0)$. Conversely, the decrease of low-angle scattering intensity is attributed to repulsive interparticle interaction.[242]

Another important overall parameter is the hydrated volume of the scatterer $V_p$ that can be computed from the Porod equations. Contrary to the Guinier region, the Porod approximation, also known as Porod’s law, describes the scattering asymptotic behaviour of solid spheres with sharp interfaces (non-fractal form) in the high $q$ range, in which the scattering intensity follows a power law decay of $q^{-4}$:[246]

$$Q = \lim_{q \to \infty} q^4dI(q).$$ \hfill (4.8)

Here $Q$ is the Porod invariant. Considering $V_p$ as the mean square volume of the particle, it can be derived as follows:

$$V_p = \frac{2\pi^2 I(0)}{Q},$$ \hfill (4.9)

$$Q = \int_0^\infty q^2I(q)\,dq.$$ \hfill (4.10)

With these equations, one can estimate the size and molecular mass as well as particle volume concentrations of the scatterer in a homogeneous solution.[242]

**Form factor and structure factor analysis**
Following the confirmation of these overall parameters, more detailed structural information can be extracted with the overall fitting of scattering curves. Generally, the scattering profile of SAM-AuNPs in solution yields from two major contributions, i.e. 1) intrinsic shape information (form factor \(P(q)\)), and 2) extrinsic NP-NP interaction information (structure factor \(S(q)\)). A simulated the SAXS profile of monodisperse is presented in Fig. 4.6. By comparing the momentum transfer \(q\) and the corresponding separation distance \(d\), the simulated SAXS profile of a AuNP solution can also be separated into two regions, representing specific structural features of the NP system, as shown in Fig. 4.5. The scattering in the high-resolution MA region (MAXS mode) contains the size, shape and morphology information (form peaks) and is thereby determined largely by the form factor \(P(q)\), whilst the low-angle ESA region (ESAXS mode) is dominated by the particle-particle interactions. A comparison can be made between the log-log plots of ideal non-interacting AuNPs (Fig. 4.5(a)) and AuNPs with strong NP-NP interaction (Fig. 4.5(b)). The divergence is particularly evident in the ESA region while the form factors in the MA region are almost...
identical. Similar to diluted samples, non-interacting AuNPs give rise to flat and featureless scattering curves in the ESA region, as a result of ideal gas like dispersions. In comparison, inter-particle interactions result in specific scattering patterns: 1) concentration-dependent peak for repulsive interactions; 2) sharp increase at low angles for attractive interaction. These features are in direct relation to the structure factor $S(q)$.

![Figure 4.6: Simulated representation of SAXS profiles of monodisperse spheres. The size of this colloidal system is 40 nm in diameter (10% polydispersity), following a Schultz-Zimm distribution. The blue solid curve represents the absolute intensity while the contribution of form factor $P(q)$ and structure factor $S(q)$ are shown as dashed curves. Adapted with permission from ref[246]. Copyright 2016 American Chemical Society.](image)

In theoretical considerations, the recorded scattering profile of monodisperse spheres after background subtraction can be decoupled as the product of $P(q)$ and $S(q)$:[247]

$$dI(q) = KP(q)S(q). \quad (4.11)$$

Here $K$ is a scaling parameter. A simulated SAXS profile of monodisperse spheres is shown in Fig. 4.6. In the MA region, $P(q)$ is well matched with the absolute intensity due to $S(q)$ being invariant and equal to 1. For diluted solutions or non-interacting systems, the value of $S(q)$ is equal to 1 in all $q$ ranges and thereby the overall scattering pattern relates to the form factor as follows:

$$dI(q) = KP(q) = NI_0\Delta\rho^2V_p^2P(q), \quad (4.12)$$

where $I_0$ is the energy density of the incident beam, $\Delta\rho = \Delta\rho_p - \Delta\rho_0$ is the difference of electron density between scatterers and buffers and $V_p$ is the volume of the scatterer. For NP analysis, the form factor $P(q)$ gives crucial morphological information including size, shape as well as size distribution. As shown in Fig. 4.7, randomly oriented spheres give
rise to signature scattering patterns, which are also varying as a function of polydispersity $(\sigma/R)$. With increasing polydispersity, $P(q)$ oscillations tend to smear out and result in featureless SAXS profiles.[246]

![Simulated representation of SAXS profiles of non-interacting spheres.](image)

**Figure 4.7:** Simulated representation of SAXS profiles of non-interacting spheres. (a) 2D scattering image, (b) 1D form factor $P(q)$ curves for randomly oriented spheres, (c) form factor $P(q)$ curves at varying polydispersity $(\sigma/R)$. The size of this colloidal system is 20 nm in diameter. Adapted with permission from ref[246]. Copyright 2016 American Chemical Society.

The comprehensive analysis of $P(q)$ has been extensively developed in past decades and meanwhile serves as a reliable tool in characterising a variety of colloidal systems. In comparison, the analysis of structure factor $S(q)$ remains unexplored. It is mainly due to the lack of 1) suitable colloidal systems and 2) a unified theoretical approach to solve the complex interaction potentials. From a qualitative aspect, the structure factor $S(q)$ can be extracted from Eq. 4.11 through $P(q)$ analysis and serve to probe colloidal stability and interparticle interactions. For example, a scattering peak in the ESA region, which resolves long distance scattering patterns, typically represents ordered and periodic space arrangement of the scatterers. The peak position of this so-called Bragg peak relates to the interparticle distance with the Bragg’s law.[247]
Chapter 4. Experimental and analytical techniques

\[ d_{\text{int}} = \frac{2\pi}{q_{\text{peak}}}. \]  (4.13)

Furthermore, the \( S(q) \) and the overall scattering profiles can also be used to compute interactions potentials. Depending on the model of interaction forces, they allow quantitative analysis of interaction forces as well as corresponding decay lengths, which can offer important insights into the colloidal stability of NPs or biomacromolecules. I refer to Chapter 7 for in-depth discussions.

4.3.2 Quartz crystal microbalance with dissipation monitoring

As mentioned in Chapter 2, QCM-D is a powerful tool for probing colloidal interactions by label-free monitoring of binding or release events. It enables real-time determination of areal mass changes with ultra-high sensitivity, typically below 10 ng cm\(^{-2}\). Moreover, the viscoelastic properties of the adsorbed layer can be directly extracted from QCM-D data, which is particularly valuable for the interplay at the bio-nano interface. It has thus provided important information for biochemical sensor development such as the detection of oligonucleotides, proteins, antigens and pathogens.\[248] AuNPs, with much higher surface area and larger molecular mass compared to the analyte, have been generally applied as "mass enhancers" to amplify the response of frequency drifts. In this study, SAM-AuNPs will be introduced to the system to act as inducers of binding events.

4.3.2.1 Working principles

The working principle of QCM-D is based on the so-called converse piezoelectric effect. As shown in Fig. 4.8(a), the quartz crystal sensor is made conductive with a thin-film metal coating for the incorporation into oscillator circuits. The quartz crystal deforms and oscillates upon the application of an alternating electric potential and corresponding resonance frequency is determined by the mass and viscous loading. Different analyses can be made based on this principle. The working mode for QCM-D is the so-called "ring-down" scheme, which monitors the decaying mechanical oscillations while the external voltage is turned off intermittently. The recorded signal yields two important parameters: the resonance frequency \( f_n \) and the dissipation \( D_n \) (Fig. 4.8(b)). Alternatively, the so-called impedance analysis investigates the polarization at the crystal surface as a function of the frequency of the applied voltage. This mode results in \( f_n \) and a new parameter, the bandwidth \( \Gamma_n \), which can also be related to \( D_n \) with \( 2\Gamma_n = D_n f_n \), as seen in Fig. 4.8(c).\[249]
4.3.2.2 Experimental setup

The main components for a QCM-D instrument consist of a temperature-controlled flow module, an oscillation circuit controller as well as a peristaltic pump, as presented in Fig. 4.9.
In this thesis, a Q-Sense QCM-D Analyser (Biolin Scientific) was used together with an Ismatec IPC-N 4 peristaltic pump (Cole-Parmer). All measurements were carried out at a controlled temperature of 20 °C and the pumping rate was in the range 10–400 µl min⁻¹ with tubing of an inner diameter of 0.64 mm. Flow rates were applied as follows: 200 µl min⁻¹ for H₂O or MeOH, 100 µl min⁻¹ for buffer, 10–50 µl min⁻¹ for thiols, AuNPs and binding partners. Gold-coated Q Sensors (4.95 MHz, AT-cut), purchased from Biolin Scientific and Quartz Pro, served as the quartz crystal sensors used in the measurements. Prior to use, the sensors were immersed in a base piranha solution (H₂O₂, NH₃, and ultrapure Type 1 H₂O in a volume ratio of 1:1:5) at 80 °C for 10 min before rinsing thoroughly with ultrapure Type 1 H₂O and ethanol with N₂ drying. The experimental data were collected and analysed with the software QSoft 401 and QTools 301 developed by Biolin Scientific.

### 4.3.2.3 Data interpretation

Based on the ultra-sensitive piezoelectric effect, the application of QCM-D as a microbalance based is attributed to the linear relationship between the changes of resonance frequency and the changes of resonator mass. This relationship is given by the Sauerbrey equation as follows:[250]

\[
\Delta m = -C \times \Delta f_n, \tag{4.14}
\]

\[
\Delta f_n = \frac{\Delta f_n^{Abs}}{n}. \tag{4.15}
\]

Here \( \Delta m \) is the mass change per unit area, \( C \) is a constant, equal to 17.5 ng (cm² Hz)⁻¹ for 4.95 MHz Q Sensors, \( \Delta f_n^{Abs} \) is the absolute frequency shift of \( n \)th overtone and \( n \) is the overtone number. As a result, the mass change due to adsorption or desorption on quartz crystal surface can be calculated from the frequency shift. It is important to point out that this calculation is only valid when the attached layer is rigid and the dissipation shift of the crystal is negligible, i.e. \( \Delta \Gamma_n \ll -\Delta f_n \). In contrast, when a soft and thick layer is deposited, the dissipation, which is the damping of the crystal oscillation, needs to be taken into account. In this case, the QCM-D data relate to the viscoelastic properties of the adsorbed layer. The shift of \( D_n \) can be fitted with a variety of theoretical approaches, such as the viscoelastic model (Kelvin-Voigt or Maxwell), intrinsic viscosity fitting and the hydrodynamic finite element method.[249, 251–253]
Chapter 5

Gold nanoparticles: synthetic platform for decoupled control of size and surface composition

Disclosure: This chapter is a slightly modified version of the publication: A versatile AuNP synthetic platform for decoupled control of size and surface composition. Yang Y, Serrano L A, Guldin S. Langmuir, 2018, 34(23), 6820-6826.[224]

5.1 Motivation

As outlined in the introductory chapters, the precision engineering of functionalised AuNPs is instrumental in many applications. While a plethora of protocols exist for the synthesis of sub-10 nm AuNPs, the independent control over size and surface composition remains restricted. The motivation of this chapter is therefore to provide a more systematic, versatile and modular approach for the synthesis of SAM-AuNPs via a two-step ligand-exchange method using OAm.

5.2 Introduction

The chemical nature of the stabilising ligand shell structure is decisive for many NP properties, including solubility, chemical reactivity and binding affinity.[74, 128, 254–256] The demand for decoupled control over AuNP size and surface functionalities poses challenges to existing preparation protocols, because most are parameter-sensitive wet-chemistry syntheses. For example, the classic one-step synthesis is extremely sensitive to reaction conditions and thereby appears incapacitated in reproducibility. Typically, adapting the synthetic protocol to target specific ligand functionalisation also affects the AuNP size distribution, thus rendering comparative studies on the effect of size and surface functionality rather unattainable. This hinders systematic studies of AuNP structure-function relationships and optimisation of crucial design parameters.[88, 89]
As discussed in Chapter 2, the two-step thiol-for-agent ligand-exchange approach offers a viable route to decouple gold core preparation from ligand shell functionalisation.[257, 258] While a number of methods using different intermediate agents have been successfully implemented in certain protocols, their general applicability is plagued by drawbacks such as: 1) the available AuNP size range and size tunability, 2) the uniformity in size and shape of the AuNP core, 3) the stability and exchangeability of the intermediate capping agent, 4) the yield and the scalability of the reaction and 5) the practicality of the procedure. For example, reliable thiol-for-thiol exchange is time-consuming, with most protocols requiring daylong procedures.[94] Due to the strong association between the capping agent and the gold core, it is generally challenging to achieve complete and homogeneous substitution. Typically, replacement thiols are introduced in large excess which raises concerns over partial etching of the AuNP core.[259, 260] For citrate-based AuNPs the available size range is typically 10 – 150 nm,[261] though some protocols enable to tune the mean size below this range.[262] Contrastly, the diameter of AuNPs prepared from phosphine-based protocols are limited below 2 nm. For DMAP-based, DOA-based and TOAB-based AuNPs, the available uniformity and tunability of the AuNP core is somewhat restricted.[104, 106, 263] Moreover, it remains a challenge to achieve satisfactory yield and compatibility with large-scale synthesis when the intermediate AuNP complex is unstable.[77, 101]

The use of OAm in AuNP synthesis has attracted considerable interest due to its ability to play multi-fold roles as surfactant, solvent and reducing agent.[114] Similar to other phosphines, OAm passivates the gold surface via coordination to a gold adatom (Fig. 5.1).[264] OAm alone is able to reduce gold precursor and stabilize AuNPs at elevated temperature (> 80 °C) in small-scale syntheses.[265–268] Recently, the introduction of additional reducing agent tBAB enabled the facile production of smaller AuNPs with narrow size distribution and size tunability near room temperature.[113, 269] The potential of further surface functionalisation by ligand exchange was recently validated for alkylthiol,[270] biphenylthiol,[271], azothiol[272] and charged thiol mixtures.[273]

In this chapter, a two-step synthetic protocol is examined for the independent control over AuNP core and ligand shell, based on the use of OAm as intermediate capping agent. I studied the applicability of this approach to produce thiol-capped AuNPs via thiol-for-OAm ligand replacement and established a successful procedure to tune the core size as well as the capping-layer composition within a broad library of thiol ligands and mixtures thereof.
5.3 Experimental

The syntheses and characterisations were carried out following the protocols described in Chapter 4.

5.3.1 Size and dispersity of oleylamine-nanoparticles

Various batches of OAm-AuNPs were prepared in octane to study the effect of reaction temperature in the range of 1 – 50 °C. The effect of reducing agent ratio was investigated at 15 °C when 0.125, 0.25 and 0.5 mmol tBAB were used to reduce 0.25 mmol HAuCl₄ in 40 ml solution mixture of octane and OAm. The scalability of this method was also studied at 15 °C when 0.25 and 1.0 mmol HAuCl₄ were used in 20 and 80 ml solution mixture of octane and OAm, respectively. The obtained AuNPs were re-dispersed in DCM for TEM size analysis.

5.3.2 Thiol-for-oleylamine ligand exchange

To prepare homo-ligand AuNPs with different solvation properties, various batches using following thiol ligands were prepared in the same protocol: 1-hexanethiol (HT, 97%), OT (97%), 11-mercapto-1-undecanol (MUO, 97%), 2-phenylethanethiol (PET, 98%), 4’-(12-mercaptododecyloxy)biphenyl-4-carbonitrile (MDDCBO), mercaptohexyl naphthalenylmethyl thioether (MNT), MUA (95%) and MUS. MDDCBO and MNT ligands were synthesised in the group by Dr Luis Serrano, as reported. The MUS ligand was synthesised by Dr Paulo Jacob Silva in the group of Prof Francesco Stellacci at EPFL, Switzerland, following published protocols. UV-Vis absorption spectra of the obtained
AuNPs were measured for the solubility test in different solvents. To compare the ligand density of thiol-protected AuNPs prepared by the two-step OAm ligand-exchange method with classic one-step synthesis, OT-capped AuNPs were obtained following the one-phase method. After vacuum drying, an approximate amount of 5 mg from each batch were analysed by TGA.

Mixed-ligand AuNPs were prepared by mixing 0.2 mmol of the thiol ligand mixture at given feed ratio with 30 mg of the OAm-capped AuNPs in DCM. Functionalised AuNPs with following ligand mixtures (molar feed ratio): MUO-PET (50%-50%), MNT-HT (60%-40%), MDDCBO-HT (60%-40%) and MUS-OT (60%-40%). For MUS-OT composition variation, following prescriptions were introduced: 80%-20%, 60%-40%, 40%-60%, 20%-80%. In addition to TGA characterisation, $^1$H NMR results were collected both before and after AuNP ligand stripping in designated deuterated solvents with excessive I$_2$.

### 5.4 Results and discussion

The synthetic platform based on AuNP synthesis with OAm as intermediate capping agent and subsequent thiol-for-OAm ligand-exchange is illustrated in Fig. 5.2. While the reaction temperature is used as the sole parameter to tune the AuNP core size in the initial synthesis, subsequent ligand exchange involving thiols in minimal excess allows to
produce thiol-capped AuNPs with ionic, hydrophilic, amphiphilic and hydrophobic ligand functionalisation as well as mixtures thereof.

5.4.1 Size and dispersity of oleylamine-nanoparticles

The synthesis of OAm-capped AuNPs was revisited using the burst nucleation method based on the reduction of HAuCl₄ by tBAB. Of particular interest in this study was to elucidate the interplay of tBAB and the multi-fold role of OAm in the synthetic process, acting as surfactant, solvent and reducing agent.

To investigate the effect of reducing agent, 0.125, 0.25 and 0.5 mmol tBAB were added to 0.25 mmol HAuCl₄ solution at 15 °C and yielded OAm-AuNPs with core size of 4.5±0.9, 3.8±0.3 and 3.9±0.5 nm, respectively. The results are summarised in Fig. 5.3. The reduction of 0.25 mmol HAuCl₄ requires a minimal feed of 0.25 mmol tBAB (1:1) from a stereochemistry perspective and thereby the polydisperse distribution obtained at reduced tBAB usage suggests the incomplete reduction of HAuCl₄, inhibiting homogeneous growth. On the other hand, the excessive tBAB may have accelerated the reduction process and at the same time hindered effective diffusion of gold nuclei in solution. As a result, an ideal reducing ratio of tBAB was identified at 1:1 (molar ratio of tBAB:HAuCl₄) for preparing monodisperse AuNPs. It is important to note that the synthesis of OAm-AuNPs is a parameter-sensitive process. A further examination of the effect of various parameters
was beyond the scope of this thesis. Please note that a detailed parameter screening based on Design of Experiments (DoE) was recently carried out in collaboration with Niamh Mac Fhionnlaoich.[276]

A representative TEM image and size distribution histogram of AuNPs stemming from synthesis at different reaction temperatures is shown in Fig. 5.4. The resulting size distribution as a function of reaction temperature is summarised in Fig. 5.5. A clear effect of the reaction temperature variation on the resulting AuNP size can be observed. For example, at a reaction temperature at 1°C, AuNPs with an average size of 6.3±0.7 nm were obtained, which reduced to 5.0±0.6 nm for 10°C, to 4.0±0.6 nm for 20°C, to 3.0±0.5 nm for 30°C and to 2.2±0.4 nm for 40°C. An increase in the reaction temperature to 50°C did not lead to a further reduction of the average diameter (2.2±0.4 nm). These results are in line with the reported value using OAm-octane mixtures and show a similar trend to the results in OAm-tetralin mixtures.[89, 113] Thus, by only controlling one single experimental variable, the reaction temperature, the average size of the AuNPs was systematically tuned in the range of 2 to 7 nm. Moreover, this approach proved to obtain excellent scalability evidenced by the consistent results varying the batch size by a factor of 4. At 15°C, the size distribution for 0.25 mmol, 0.5 mmol and 1.0 mmol HAuCl₄ batch size were determined at 4.6±0.5, 4.6±0.6 and 4.7±0.6 nm, respectively (Fig. 5.6). This simple tunability over such a broad size range renders the OAm-based AuNP synthesis an ideal base for the subsequent functionalisation via thiol-for-OAm substitution.

### 5.4.2 Thiol-for-oleylamine ligand exchange

The preparation of SAM-AuNPs via thiol-for-OAm ligand exchange is motivated by establishing a facile and straightforward process of producing target size AuNP cores that can then serve for further investigation of the ligand shell. Another important reason is the fact that most alkanethiols or aromatic thiols are either hydrophobic or amphiphilic, which makes them compatible with the same solvent system, here DCM, that also dissolves OAm-capped AuNPs.

#### 5.4.2.1 Ligand packing density

In order to compare the ligand shell of OT-capped AuNPs after thiol-for-OAm exchange (OAm-OT AuNP) with those obtained directly by the one-phase method (OT AuNP), batches were prepared, cleaned by centrifugal precipitation in excessive acetone for each method and dried thoroughly by rotary evaporation and subsequently in a vacuum desiccator overnight. From TEM images, the average sizes of OAm-OT and OT AuNPs
Figure 5.4: Representative TEM images and size distribution histograms of OAm-AuNP for different reaction temperatures.
were analysed to be 4.9±0.4 nm and 4.4±0.4 nm in diameter, respectively. The TGA results of those samples are presented in Fig. 5.7. The weight loss observed in the range below 600 °C can be exclusively related to the decomposition of the organic ligand shell as most of the organic component is converted into H₂O or CO₂ under N₂ flow. This conversion was predominantly observed at around 200 °C for OT ligands. Combining the mass loss determined by TGA and the average size from TEM analysis, the ligand density of OAm-OT AuNPs and OT AuNPs was determined to be 4.5 nm⁻² and 4.7 nm⁻² using a simplified spherical model (Tab. 5.1)[277] The similarity in the ligand packing between the two synthetic routes suggests that the OAm method is an ideal alternative to the established one-step one-phase method with distinct advantages. While the reaction temperature is key in the one-phase method, size control over the full reported range typically requires further adjustment of the reaction solvent mixture.[88] More importantly, the type of thiol ligand plays an important role for the observed size distribution of the AuNP core, making it particularly difficult to independently vary both parameters in systematic studies.[100]
5.4.2.2 Variation of AuNP thiol-capping and resulting solubility

Various thiol-capped AuNPs from an extensive library of ligands were successfully prepared via the presented thiol-for-OAm ligand-exchange approach. An overview of the studied ligands and the resulting AuNP solubility after functionalisation is shown in Table 5.2. Notably, during the ligand exchange of thiols with polar end groups, such as MUO and MUA, the colour of the solution turned from purple to grey in the first two minutes of the reaction. Further aggregation of the NPs led the precipitation in DCM, suggesting a rapid substitution of ligand shells with higher polarity. A re-dispersion of the precipitated NPs was observed with the addition of MeOH. UV-Vis absorption spectroscopy was used to evaluate the AuNP solvation by tracking their respective plasmon resonance peak (Fig. 5.8). The simple but versatile ligand-exchange step enabled the functionalisation of thiol-capped AuNPs that could be dissolved in solvents ranging from non-polar solvents (hexane, toluene, chloroform) to polar aprotic (DCM) and polar protic solvents (H2O, MeOH, EtOH).

Furthermore, the complete thiol replacement was also evidenced by the disappearance
of the OAm characteristic footprint (peak at 0.8–0.9 ppm) from $^1$H NMR, as presented in Fig. 5.9. An overview for the obtained yield for both AuNP synthesis in OAm (Step 1) and thiol-for-OAm AuNP functionalisation (Step 2) is shown in Tab. 5.3. After centrifugal precipitation and vacuum drying, a reaction yield of 94.9% was retrieved for Step 1, and 91.6% and 87.8% for subsequent functionalisation with MUA and MUO, respectively.

AuNPs with various binary mixtures of thiol ligands were also studied by introducing a prescribed feed composition to OAm-AuNPs with a core size of 4.9±0.4 nm during the thiol-for-OAm ligand exchange. An overview of the different mixtures can be found in Tab. 5.4 with UV-Vis results presented in Fig. 5.8(i). Similar to homo-ligand AuNPs, a variety of

![UV-Vis spectra of AuNPs in various solvents.](image-url)
Table 5.2: Thiol ligands used for homo-ligand AuNPs.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Chemical structure</th>
<th>Resulting AuNP solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT</td>
<td><img src="ht.png" alt="Chemical Structure" /></td>
<td>DCM, Chloroform</td>
</tr>
<tr>
<td>OT</td>
<td><img src="ot.png" alt="Chemical Structure" /></td>
<td>Toluene, DCM, Hexane, Chloroform</td>
</tr>
<tr>
<td>MUO</td>
<td><img src="muo.png" alt="Chemical Structure" /></td>
<td>MeOH, EtOH</td>
</tr>
<tr>
<td>PET</td>
<td><img src="pet.png" alt="Chemical Structure" /></td>
<td>Toluene, THF</td>
</tr>
<tr>
<td>MDDCBO</td>
<td><img src="mddcbo.png" alt="Chemical Structure" /></td>
<td>5CB</td>
</tr>
<tr>
<td>MNT</td>
<td><img src="mnt.png" alt="Chemical Structure" /></td>
<td>DCM, Toluene</td>
</tr>
<tr>
<td>MUA</td>
<td><img src="mua.png" alt="Chemical Structure" /></td>
<td>H₂O (basified)</td>
</tr>
<tr>
<td>MUS</td>
<td><img src="mus.png" alt="Chemical Structure" /></td>
<td>H₂O</td>
</tr>
</tbody>
</table>

Table 5.3: Calculated reaction yield.

<table>
<thead>
<tr>
<th>Step</th>
<th>In (gold) mg</th>
<th>Out (gold) mg</th>
<th>TGA core content (mg)</th>
<th>Out (gold) mg</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAm capping</td>
<td>108.5</td>
<td>112.6</td>
<td>91.4%</td>
<td>102.9</td>
<td>94.9%</td>
</tr>
<tr>
<td>MUA-for-OAm</td>
<td>27.4</td>
<td>27.7</td>
<td>90.5%</td>
<td>25.1</td>
<td>91.6%</td>
</tr>
<tr>
<td>MUO-for-OAm</td>
<td>27.4</td>
<td>26.5</td>
<td>90.4%</td>
<td>24.0</td>
<td>87.8%</td>
</tr>
</tbody>
</table>

Solvation behaviour were achieved by tuning the polarity of the ligand mixtures. Most distinct is the effect of a binary ligand mixture on solvation for a combination of MDDCBO and HT. While the homo-ligand AuNPs stabilised with MDDCBO (or HT) showed very limited solubility in the liquid crystal (LC) 4-Cyano-4'-pentylbiphenyl (5CB, isotropic phase), the binary ligand mixture of MDDCBO and HT manifested greatly improved solubility. This can be explained by the introduction of the short ligands as spacing agents.\[104\]

Table 5.4: Thiol ligand mixtures used for mixed-ligand AuNPs.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Feed ratio</th>
<th>Resulting AuNP solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUS-OT</td>
<td>80%-20%</td>
<td>H₂O</td>
</tr>
<tr>
<td>MUO-PET</td>
<td>50%-50%</td>
<td>MeOH</td>
</tr>
<tr>
<td>MNT-HT</td>
<td>60%-40%</td>
<td>Chloroform</td>
</tr>
<tr>
<td>MDDCBO-HT</td>
<td>60%-40%</td>
<td>5CB</td>
</tr>
</tbody>
</table>
5.4.2.3 Ligand composition variation

In order to establish a detailed relationship between the feed ratio of the binary ligand mixture in the thiol-for-OAm exchange step and the resulting ligand composition on the surface of the AuNPs, sample batches were prepared from six different molecular ratios of MUS and OT. The same OAm-protected AuNPs were used as base material throughout this study, with a core size of $3.7 \pm 0.4$ nm in diameter. Similar to the water-soluble batch with MUA shell, a rapid AuNP precipitation was observed during the ligand exchange in the batches with MUS. After the first washing step in acetone, the AuNPs showed a pronounced solubility in $H_2O$, which facilitated further purification with Amicon Ultra centrifugal filters in $H_2O$. The purified AuNPs after ligand exchange were re-dissolved in a mixture of $D_2O$ and MeOD (batches with MUS) and $CDCl_3$ (the batch with OT only) for the
purpose of AuNP (and I₂) solvation. The ¹H NMR spectra of these solutions maintained only peaks from the solvents or impurities (as exemplified in Fig. 5.10), which suggest that the vast majority of the thiol ligands were attached to the AuNP surface. [278] The addition of I₂ in excess gave rise to the distinct and characteristic peaks by the desorption of the thiols from the AuNP surface. By comparing the integration data of peaks contributed by MUS (1.6 – 1.9 ppm, 2.6 – 2.9 ppm) and OT (0.8 – 0.9 ppm, 1.6 – 1.9 ppm, 2.6 – 2.9 ppm) individually, the yield composition was determined and compared with the original feed ratio, as presented in Figure 5.11. [155] Interestingly, the resulting surface composition scaled in proportion with the feed ratio variation, demonstrating that the ligand composition can be precisely controlled with the herein presented approach. These results are in clear contrast to thiol-for-thiol place-exchange reactions, where the difference in thiol affinity towards the gold surface poses a challenge for the consistent variation and tunability of the effective binary ligand composition on the AuNPs. [93, 94, 96] In the present approach, the competition for surface adsorption is between thiol and OAm rather than thiol and thiol. The composition variation was further supported by the TGA results shown in Fig. 5.12. Based on the calculations for the molar ratio of MUS and OT on the AuNP surface from NMR analysis, the partial packing density for each ligand component was calculated for the different MUS-OT batches, as shown in Tab. 5.5.

It is important to note that for reliable and reproducible functionalisation with a consistent
relationship between ligand feed in solution and ligand yield on the AuNPs, the presented route relies on the solubility of the respective target thiols in a common solvent with the OAm-protected AuNPs, here DCM. A vast library of thiol ligands are either hydrophobic or amphiphilic and thus compatible with the approach. Indeed, most of the commonly used thiol ligands exhibit a hydrophobic alkyl backbone with a functional (and often polar) end group. By contrast, hydrophilic thiols with poor solubility in DCM required functionalisation via a two-phase process with H$_2$O, which resulted in less precise control over the ligand composition. Additionally, the above shown tuning of ligand shell composition by feed ratio relies on a non-preferential co-adsorption of both thiol ligands in a binary mixture. Recent work by Klajn and co-workers elucidated the effect of electrostatic interactions between different ligand types on the final composition of the ligand shell.[273] For a mixture of a positively charged viologen-based ligand and a zwitterionic sulfobetaine ligand, a variation of the ligand feed ratio of a factor of 5000 only changed the effective ligand composition on the AuNPs by a factor of 3.
Chapter 5. **Gold nanoparticles: decoupled control of size and surface composition**

### Figure 5.12: TGA spectra of MUS-OT AuNPs with various ligand compositions.

### Table 5.5: MUS-OT AuNPs ligand density.

<table>
<thead>
<tr>
<th>Feed ratio MUS-OT</th>
<th>Yield ratio MUS-OT</th>
<th>Total organics</th>
<th>MUS density nm⁻²</th>
<th>OT density nm⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%-0%</td>
<td>–</td>
<td>11.6%</td>
<td>3.2</td>
<td>–</td>
</tr>
<tr>
<td>80%-20%</td>
<td>77.3%-22.7%</td>
<td>10.6%</td>
<td>2.5</td>
<td>0.7</td>
</tr>
<tr>
<td>60%-40%</td>
<td>56.7%-43.3%</td>
<td>12.0%</td>
<td>2.4</td>
<td>1.8</td>
</tr>
<tr>
<td>40%-60%</td>
<td>43.0%-57.0%</td>
<td>12.7%</td>
<td>2.1</td>
<td>2.8</td>
</tr>
<tr>
<td>20%-80%</td>
<td>23.9%-76.1%</td>
<td>11.4%</td>
<td>1.2</td>
<td>3.8</td>
</tr>
<tr>
<td>0%-100%</td>
<td>–</td>
<td>10.0%</td>
<td>–</td>
<td>5.3</td>
</tr>
</tbody>
</table>

### 5.5 Conclusions

The presented synthetic platform for AuNP synthesis enabled the decoupling of core size refinement and ligand shell variation in thiol-capped AuNPs. Following a modified synthesis with OAm as intermediate protecting capping agent, the core size was tuned in the range of 2–7 nm with low dispersity by varying the reaction temperature. Subsequently, the functionalisation of the OAm-AuNPs with a wide choice of thiol ligands and binary mixtures thereof was achieved, resulting in tunable solubility ranging from H₂O and alcohols to apolar organic solvents and LCs. Moreover, the thiol-for-OAm ligand-exchange method proved to be as efficient as the conventional one-step method in preparing AuNPs with dense ligand packing. Other notable advantages of this approach include the excellent scalability for large-scale synthesis, the ligand exchange occurring rapidly and at room temperature as
well as simplified purification and collection of AuNPs. As exemplified in MUS-OT protected
AuNPs, a consistent relationship was established between the thiol ligand mixture in the
feed solution and the resulting composition on the AuNP, demonstrating the ability to
fine-tune surface properties. With the precise control over core size and ligand shell
functionalities, this synthetic platform constitutes a valuable tool to fabricate thiol-protected
AuNPs with rational design optimisation for target applications.
Chapter 6

Advanced characterisation of nanoparticle size distribution

This chapter presents a comparative study of sub-10 nm AuNP size distribution between conventional TEM imaging and two alternative methods: Monte Carlo fitting of SAXS and hydrodynamic analysis of AUC.


6.1 Motivation

The accurate determination of the size distribution of sub-10 nm AuNPs plays a crucial role in their biomedical applications. Although TEM is capable of resolving AuNPs down to sub-nm scale, the reliable representation of non-uniform size distribution remains plagued by a lack of statistical significance, predominantly due to suboptimal sample preparation procedures and user bias in image acquisition and analysis. Recent advances in SAXS and AUC allow the estimation of AuNP sizing through form-free methods: the Monte Carlo (MC) fitting of SAXS data offers the access to a direct representation of the core size distribution while AUC sedimentation analysis provides information of the hydrodynamic size distribution. The motivation of this chapter is to explore these new tools for characterising AuNPs with different size distribution patterns in comparison to TEM imaging analysis. The work described in this chapter was carried out in collaboration with Suiyang Liao and Dr Zhi Luo in the group of Prof Francesco Stellacci at EPFL, Switzerland.

6.2 Introduction

In biological environments, the efficacy of ultrasmall NPs demonstrates significant dependence on both size and dispersity. For example, the cutoff of efficient renal clearance is below 10 nm and thereby NPs with broad size distributions can impair the biocompatibility
Chapter 6. Advanced characterisation of nanoparticle size distribution

of the system.[279] For AuNPs, in particular, various important physicochemical properties are closely dependent on size and thus the overall size distribution of the population. AuNP populations with mean sizes ranging from 2 – 10 nm manifested drastic difference not only concerning in vitro colloidal stability but also their intracellular and antimicrobial properties.[148, 159, 280, 281] Consequently, the quantitative assessment of the AuNP size distribution in a given sample is an indispensable routine that needs particular attention. To date, the most commonly implemented technique is TEM imaging, combined with a plethora of software-based image analysis methods. As a direct imaging method, TEM is a convenient tool to study both size and shape in ultra-high resolution. However, due to the nature of this labour-intensive technique, a statistical representation of AuNP size is hindered by both suboptimal sample preparation and user bias in subsequent image acquisition and analysis.[225, 246, 282] Considering the rather limited sample size, it is challenging to obtain reproducible results, especially for non-monodisperse or multimodal samples. Moreover, the drop casting for TEM sample preparation often results in drying artefacts which vastly complicates image analysis.[283, 284] To address these issues, a number of bulk-scale quantification techniques for nanostructures have been developed as alternatives to TEM imaging. For instance, dynamic light scattering (DLS) is widely used for its easy access and simple protocol. Whilst DLS is able to probe the hydrodynamic size information of colloidal systems at both microscopic and nanoscopic scales, it tends to give artefacts for non-monodisperse samples due to the size-dependent scattering cross-section. Thus, smaller NPs are usually overshadowed by larger NPs or aggregates.[282, 285] In addition, the interference of multiple scattering events can also impair the accuracy of DLS results. For AuNPs below 20 nm, it is extremely challenging to obtain reliable results with DLS.[286]

While less utilised in the NP community, SAXS is advancing as an effective ensemble method to characterise colloidal size distributions due to its well-matched resolution. With a momentum transfer $q$ of $0.06 – 6.3$ nm$^{-1}$, SAXS measurements are able to cover the NP size distribution ranging from 1 – 100 nm in diameter. Moreover, gold has a scattering length density (SLD) of $125 \times 10^{-6}$ Å$^{-2}$ for Cu-source X-ray while the SLDs for H$_2$O and alkanethiols are below $10 \times 10^{-6}$ Å$^{-2}$. This distinct feature of gold gives rise to significant contrast and excellent signal-to-noise ratio for resulting AuNP SAXS profiles. Contrary to TEM analysis, SAXS is an indirect method and requires data correction and fitting for reconstructing the size distribution from measured data.[287] In principle, the size distribution of a NP sample can be directly calculated with an assumed functional form but limited to samples with dispersity based on i.e. lognormal, Gaussian, Boltzmann or Schultz-Zimm distribution.[246] Various attempts have been made to explore form-free solutions. Notably, a conventional form-free regularisation approach, derived from indirect Fourier transform,
has been widely adopted to probe colloidal systems in solution. The process involves form
factor pattern matching and optimisation with least-squares methods.[243, 288, 289] For
example, SAXS was recently used to characterise sub-5 nm hybrid core-shell silica NPs
capped with a PEG corona. Compared to DLS and fluorescence correlation spectroscopy,
SAXS demonstrated compelling advantages by resolving not only the core size distribution
but also the molecular mass dispersity of the polymer shell with quantitative modelling
using a core-shell sphere form factor.[290] Similarly, the maximum entropy method can
also be employed to estimate size distributions by pattern matching.[246] Despite these
successful examples, the parametric regularisation methods are sensitive to prior infor-
mation such as the maximum diameter, which may occur artificial oscillations.[291] To
this end, a MC method, based on model-free trial-and-error sampling, was proposed for
unbiased NP size distribution analysis.[292, 293] Although this iterative process requires
substantial computing power, MC fitting has rather simple theoretical structure since it as-
sumes the scattered intensity is approximated by the sum of elementary components.[294]
Pioneered by Martelli and Di Nunzio, this approach was validated for non-interacting hard
sphere ensembles, which relied on the "dilute solution" assumption. In a comparative
study, they confirmed that the MC fitting algorithm exhibits a similar retrieving power for
non-interacting spheres when compared to the established indirect Fourier transform,
structure interference and maximum entropy methods.[292] However, this method was
limited to spherical systems, which severely restricted its further application. An improved
algorithm was developed by Pauw et al. by compensating the effect of size and shape on
the scaling of the form factors, which extended the use in polydisperse ensembles with
unknown shapes.[293] Moreover, the recent development of the user-friendly software
McSAS has further broadened the scope of this approach.[295] For example, Pauw et
al. compared the parametric fitting method and the MC approach for determining the
size of a unimodal 5 nm AgNP from 45 SAXS datasets collected in 22 laboratories. Both
fitting routes yielded consistent and similar results, highlighting the validity and reliability
of employing the MC approach.[296] Similarly, Maes et al. confirmed the consistency
between the presupposed model fitting method and the MC approach in retrieving the
size distribution of unimodal PbS nanocrystals in the range of 3 – 10 nm.[297] In addition,
the MC method was also validated in elucidating the size distribution of other nanosized
objects, including MgZn alloy rods,[298] and 100 nm AuNPs.[299] Despite the success in
comparison to conventional SAXS fitting schemes, there is still a lack of comprehensive
investigations utilising the MC-SAXS method, especially for characterising non-uniform
nanostructures below 10 nm as well as cross comparison with other characterisation
techniques.
Another emerging characterisation technique is the sedimentation-based AUC. In principle, AUC measures the sedimentation and diffusion transport of NPs in solution. Both processes are affected by the compositional and solvation properties. As mentioned in Chapter 4, SV measurements allow the determination of size, density and shape for nanosized colloidal systems. For AuNPs stabilised with a thiol ligand shell, AUC offers straightforward evaluation of hydrodynamic parameters in solution, which can be used for extracting size and shape information of both the gold core and the ligand shell.[240, 241] In spite of being a simple and versatile technique, the utilisation of AUC is still highly underrepresented in NP research. One of the main reasons is attributed to the difficulty in determining the effective NP density for core-shell hybrid colloids, since a solvation layer in addition to the ligand shell can pose significant influence. This challenge was addressed by Carney et al. with a 2D evaluation of sedimentation and diffusion coefficients, which enabled the direct estimation of the size, density and molecular weight distributions of NPs.[241] Further developments shared a focus on modern algorithms for the analysis of core-shell properties as well as polydisperse systems.[300, 301] However, the AUC representation of non-monodisperse or multimodal NPs remains largely unexplored.

While research interests are growing in using both advanced techniques, the cross validation between classical methods and modern approaches is severely lacking. Herein, I present a comparative study of quasi-monodisperse, polydisperse and bimodal thiol-capped AuNPs of 2 – 7 nm between TEM, SAXS and AUC. Experimental data are presented with the discussion on data analysis, followed by a side-by-side comparison of the size distribution of unimodal and bimodal samples obtained from data analysis of these three techniques. I aim to shed light on the general applicability and further exploitation of the less utilised MC-SAXS and AUC-SV methods, while also providing critical identification of their limitations.

6.3 Experimental

Following the two-step OAm-AuNP ligand exchange synthesis as described in Chapter 5, homo-ligand AuNPs stabilised by MUS were prepared from four batches of differently-sized OAm-AuNP. A biphasic solution of DCM-H$_2$O (1:1, v:v) was used for ligand exchange. The yielded MUS-AuNP samples were labelled as MUS-NP1–4 (Tab. 6.1). The core size of the four OAm-AuNP batches ranged from 2 – 7 nm, together with varying degrees of size dispersities. Two bimodal samples were prepared by mixing MUS-NP1 and MUS-NP4 at weight ratios of 1:1 and 1:5, labelled as MUS-B1 and MUS-B2, respectively (Tab. 6.2).
### Table 6.1: Sample information of employed unimodal MUS-AuNPs.

<table>
<thead>
<tr>
<th>Sample label</th>
<th>OAm-AuNP synthesis temperature °C</th>
<th>Core size by TEM nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUS-NP1</td>
<td>40</td>
<td>2.8±0.4</td>
</tr>
<tr>
<td>MUS-NP2</td>
<td>20</td>
<td>4.0±0.5</td>
</tr>
<tr>
<td>MUS-NP3</td>
<td>10</td>
<td>5.1±0.6</td>
</tr>
<tr>
<td>MUS-NP4</td>
<td>5</td>
<td>5.6±0.6</td>
</tr>
</tbody>
</table>

### Table 6.2: Sample information of employed bimodal MUS-AuNPs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Unimodal sample</th>
<th>Mixing ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUS-B1</td>
<td>MUS-NP1:MUS-NP4</td>
<td>1:1 wt</td>
</tr>
<tr>
<td>MUS-B2</td>
<td>MUS-NP1:MUS-NP4</td>
<td>1:5 wt</td>
</tr>
</tbody>
</table>

Standard TEM imaging was performed for all AuNP samples. To assess the reproducibility of TEM analysis, three individual sample grids were prepared from each of MUS-NP1 and MUS-NP4 sample solutions. Subsequent TEM imaging was performed in three separate experimental sessions.

#### 6.3.1 SAXS experiments and Monte Carlo fitting

Samples for solution-based SAXS were prepared in 10 mM NaCl aqueous solution at a concentration of 5 mg/ml for MUS-NP1–4 and 10 mg ml\(^{-1}\) for MUS-B1–B2. The SAXS profiles were collected with the medium angle mode (MA, q-range: 0.015 to 0.65 Å\(^{-1}\)) with a measurement time of 1 h. After subtracting the scattering from the 10 mM NaCl buffer solution, the SAXS data were fed into the software McSAS (version 1.3) for size distribution analysis by MC fitting.\[295\] The implemented algorithm assumes the overall scattered intensity is approximated by the sum of elementary components, which are the Rayleigh scattering functions of homogeneous hard spheres in dilute solutions. Fig 6.1 highlights the main processes of the parameter optimisation in McSAS. Each random set of parameters \(P\), which consists of the constant background term \(b\) and the scaling factor \(A\), corresponds to a fixed number of spheres \(n_s\). The respective scattering intensity \(I\) is calculated as the following equation:

\[
I(q) = b + A \sum_{k=1}^{n_s} |F_{s,k}(qR_k)|^2 \left(\frac{4}{3}\pi\right)^2 R_k^{(6-p_c)}.
\]  

(6.1)

Here, \(R_k\) is the radius for sphere \(k\) and \(F_{s,k}(qR_k)\) is the Rayleigh form factor. \(p_c\) is a factor biasing the volume weighting of the contributions and adjustable in the range of 1 – 6.\[293\]
The calculated $I$ is then compared with the measured data. A least-squares residual minimisation procedure is applied as a measure of chi squared $\chi^2$ in aim to reduce the discrepancies between these two scattering profiles. In my MC analysis, the scattering feature size limits were estimated to be $0.6 - 5.0\,\text{nm}$ based on the measured $q$ range.

The fitting of each SAXS dataset consisted of 10 individual repetitions using strict fitting criteria, namely a convergence criterion of $\chi^2 < 1$ and a minimum uncertainty estimate of $2\%$. Sphere model was chosen as the fit model and the $\Delta\text{SLD} (= \text{SLD}_{\text{Au}} - \text{SLD}_{\text{H}_2\text{O}})$ was input as $115.5 \times 10^{-6}\,\text{Å}^{-2}$. Number-weighted histograms were generated in the post-fit analysis. The number of bins was set to be 100 in a size range of $1.8 - 9.0\,\text{nm}$ in diameter.

\begin{figure}[h]
\centering
\includegraphics[width=0.6\textwidth]{figure6.1.png}
\caption{The main process of the software McSAS for parameter optimisation. Each cycle is made to replace one of the model contributions to improve the agreement between the fitted model and measured data. Adapted with permission from ref\cite{295}. Copyright 2015 International Union of Crystallography.}
\end{figure}

### 6.3.2 AUC-SV experiments

The AUC-SV measurements were performed using diluted SAXS sample solutions (with 10 mM NaCl). Concentrations were adjusted to lie in the range $0.2 - 0.4\,\text{mg/ml}$, which corresponded to $0.5 - 1.0\,\text{OD}$, as confirmed by a pre-scan of UV-Vis absorption at 400 nm for each individual sample before the SV measurement. The recorded AUC data were processed in the software SEDFIT to derive approximate solutions to the Lamm equation (Eq. 4.3) via a numerical finite element method.\cite{239} As described in Chapter 4, this fitting process generated a smooth sedimentation coefficient distribution $c(s)$ for each sample. Extended 2D analysis was performed to calculate the weighted average sedimentation
and diffusion coefficients based on average effective density using a previously published custom made MATLAB code.[241] The AUC-SV measurements and subsequent 1D and 2D analysis were carried out in collaboration with Suiyang Liao. For size distribution analysis, the sedimentation coefficient $s$ was related to the hydrodynamic radius $d_H$ using the Stokes-Einstein equation:[241]

$$d_H = \sqrt{\frac{18 \eta_S s}{\rho_{\text{eff}} - \rho_S}}.$$  (6.2)

where $\rho_{\text{eff}}$ and $\rho_S$ are the particle density and the solution density, and $\eta_S$ is the solution viscosity. This relationship is valid for perfect sphere in dilute solutions. In an ideal scenario, the diameter of a hard sphere equals to $d_H$ at infinite dilution.[285] Alternatively, the hydrodynamic size and shape of the employed MUS-AuNPs can be represented using a spherical core-shell model. As shown in Fig 6.2, the hydrodynamic radius of the employed MUS-AuNPs consists of a rigid gold core and a soft shell formed by the MUS ligand capping and a solvation layer. $d$ is the core diameter while $l$ is the shell thickness. Consequently, $\rho_{\text{eff}}$ can be calculated in following equations:

$$d_H = d + 2l,$$  (6.3)

$$\rho_{\text{eff}} = \frac{M_{\text{core}} + M_{\text{shell}}}{(4/3)\pi(d_H/2)^3} = \frac{\rho_{\text{Au}}(4/3)\pi(d/2)^3 + \rho_l(4/3)\pi[(d/2 + l)^3 - (d/2)^3]}{(4/3)\pi(d/2 + l)^3}.$$  (6.4)

Combining Eq. 6.2 and Eq. 6.4, the $d_H$ vs. $c(s)$ relationship was calculated for each sample. Furthermore, considering the measurement was represented by absorbance, $c(s)$ data were corrected with an extinction coefficient variation to compensate the influence of Rayleigh scattering and absorption on the particle size distribution.[302] A previously
Chapter 6. Advanced characterisation of nanoparticle size distribution

reported correlation between AuNP core size and extinction coefficient $\varepsilon$ was used for this calculation:[303]

$$\ln \varepsilon = 3.321 \ln d + 10.805.$$  \hspace{1cm} (6.5)

6.4 Results and discussion

6.4.1 TEM imaging

The TEM size distribution histograms of sample MUS-NP1 and MUS-NP4 are shown in Fig 6.3. The histograms were analysed from TEM images of three different TEM experiments with separate sample preparation. The count rates for each individual analysis were $> 2000$. Tab 6.3 summarises the statistical information of each analysis. As seen in these results, while the position of the main peaks in separate histograms remained unchanged, a discrepancy of the relative intensity and overall distribution pattern was observed for both AuNP samples. Especially for the minority populations deviated from the main peak, a substantial divergence was obtained across measurements. This was directly reflected in the fluctuating values of standard deviation and dispersity in Tab 6.3. As a consequence, although the average sizes obtained from separate measurements were well matched, the divergent standard deviation and corresponding polydispersity
results hindered an accurate representation of the size distribution. These findings are in line with earlier studies, emphasising on the inherent limitations of TEM size analysis, which can be attributed to the irreproducible sampling process.[287, 304]

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Average diameter, nm</th>
<th>Standard deviation, nm</th>
<th>Dispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUS-NP1-1</td>
<td>2.6</td>
<td>0.3</td>
<td>9.6%</td>
</tr>
<tr>
<td>MUS-NP1-2</td>
<td>2.9</td>
<td>0.5</td>
<td>17.7%</td>
</tr>
<tr>
<td>MUS-NP1-3</td>
<td>2.8</td>
<td>0.4</td>
<td>15.9%</td>
</tr>
<tr>
<td>MUS-NP4-1</td>
<td>5.6</td>
<td>0.8</td>
<td>14.4%</td>
</tr>
<tr>
<td>MUS-NP4-2</td>
<td>5.7</td>
<td>0.5</td>
<td>8.5%</td>
</tr>
<tr>
<td>MUS-NP4-3</td>
<td>5.4</td>
<td>0.7</td>
<td>12.0%</td>
</tr>
</tbody>
</table>

### 6.4.2 MC-SAXS analysis

![SAXS profiles and MC fitting (log-log plots)](image)

**Figure 6.4:** SAXS profiles and MC fitting (log-log plots) of (a) MUS-NP1, (b) MUS-NP2, (c) MUS-NP3 and (d) MUS-NP4.

The SAXS profiles of both unimodal and bimodal samples were measured in aqueous solution. For a better estimation of the form factor of individual AuNPs, 10 mM NaCl was added to increase the solution ionic strength, which could inhibit the electrostatic interaction between AuNPs induced by the negatively charged MUS and thereby eliminate the
contribution of structure factor. Please refer to Chapter 7 for detailed discussion. The SAXS curves after background subtraction are presented as log-log plots in Fig 6.4 and Fig 6.5, together with the summary in Tab 6.4 of statistical information of 10 number-weighted output distributions generated from independent MC fitting repetitions for each sample. Qualitatively, both unimodal and bimodal measured curves demonstrated pronounced form peaks in the high \( q \) region and featureless flat profiles for \( q < 1 \text{ nm}^{-1} \). In unimodal samples, the peak position of the form peak in the high \( q \) region was at 3.2, 2.8, 2.2 and 1.9 nm\(^{-1}\) for MUS-NP1 – NP4, respectively. This clear shift towards lower angles corresponded to a size increase of the scatterer, which was in line with the TEM results. Meanwhile, the bimodal curves followed closely the pattern overlapping of individual form peaks observed in unimodal samples.

**TABLE 6.4:** Discrepancies among 10 number-weighted output distributions generated from independent MC fitting repetitions for each sample.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean, nm</th>
<th>Deviation of mean</th>
<th>Variance(^{1/2}), nm</th>
<th>Deviation of variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUS-NP1</td>
<td>3.1</td>
<td>1.0%</td>
<td>0.5</td>
<td>5.6%</td>
</tr>
<tr>
<td>MUS-NP2</td>
<td>4.0</td>
<td>0.4%</td>
<td>0.4</td>
<td>9.3%</td>
</tr>
<tr>
<td>MUS-NP3</td>
<td>5.2</td>
<td>0.5%</td>
<td>0.3</td>
<td>3.7%</td>
</tr>
<tr>
<td>MUS-NP4</td>
<td>5.6</td>
<td>1.6%</td>
<td>0.8</td>
<td>9.7%</td>
</tr>
<tr>
<td>MUS-B1</td>
<td>3.5</td>
<td>2.4%</td>
<td>0.9</td>
<td>5.3%</td>
</tr>
<tr>
<td>MUS-B2</td>
<td>4.4</td>
<td>1.5%</td>
<td>1.1</td>
<td>2.4%</td>
</tr>
</tbody>
</table>

Both the unimodal and bimodal fitting results indicated that effective scattering occurred from individual AuNPs with non-interacting contributions. This was subject to three major factors: 1) the distinct SLD of gold enabled pronounced form factors in diluted solutions, 2) the ionic repulsion by MUS maintained excellent colloidal stability and prevented NP clustering or aggregation, 3) the addition of 10 mM NaCl gave rise to charge screening which disrupted medium- and long-range NP-NP ionic interaction. It is important to note
that these features were key prerequisites for the implementation of MC fitting analysis. This was evidenced by the closely matched fitting curves generated from MC modelling as well as the negligible discrepancies between the independent MC fitting repetitions (Tab 6.4). These results are consistent with the herein presented SAXS method being capable of retrieving the size distribution of non-monodisperse and bimodal AuNP solutions by robust MC modelling that requires minimal external information.

6.4.3 AUC-SV analysis

The sedimentation of NPs relates to their size, shape and density and thus, the sedimentation coefficient distribution is a direct indication of size distribution. Herein, the procedure
Figure 6.7: Sedimentation and diffusion analysis of MUS-B2 (1:5 \text{wt}). Two plots correspond to the integral 1D sedimentation coefficient distribution and the 2D sedimentation-diffusion correlation. These data were analysed by Suiyang Liao.

of an AUC-SV measurement alongside numerical finite element fitting in SEDFIT enabled to retrieve the 1D sedimentation coefficient distribution $c(s)$ and 2D correlation between $s$ and $D$. The results are shown in Fig 6.6 for MUS-NP1 and MUS-NP4 and in Fig 6.7 for MUS-B2, respectively. In unimodal samples, MUS-NP1 gave rise to two peaks in the 1D plot, with the primary peak at 48 S (Svedberg unit, equals to $10^{-13}$ s) and the secondary peak at 77 S. Both peaks were also retrieved in the 2D plot with matched peak position and relative intensity. By contrast, although the 1D analysis of MUS-NP4 resolved a dominant peak at 204 S and a much reduced peak at 141 S, the secondary peak was diminished after 2D analysis, which required experimental data of higher signal-to-noise ratio. On the other hand, two peaks were well resolved at 58 S and 198 S in both 1D and 2D plots on the bimodal sample MUS-B2. Since MUS-B2 was a mixture of MUS-NP1 and MUS-NP4 (1:5 \text{wt}), both peaks can be assigned to the primary peaks in unimodal solutions. Meanwhile, no further distribution feature was extracted from the 2D analysis, suggesting a potential loss of information such as the secondary peaks that were present in unimodal solutions. In line with a previous study by Walter \textit{et al.}, this insufficient resolution can be attributed to the regularisation process and the simplified treatment of applying a global average density in the 2D sedimentation-diffusion analysis, which ignored the size dependence of $\rho_{\text{eff}}$.\[301\] These results confirmed that the hydrodynamical size information of a sample solution can be characterised by AUC-SV measurement via the fitting analysis of the size-dependent 1D sedimentation coefficient distribution, 2D sedimentation-diffusion correlation, or a combination of thereof. However, care must be taken since these fitting methods exhibit discriminations in different size ranges and may give divergent size distributions for underrepresenting secondary populations.
Considering the observed limitations of the implemented fitting methods, the results of the 1D sedimentation coefficient distribution were used to calculate the size distribution, instead of the further processed 2D sedimentation-diffusion correlation data, which failed to take into account the size dependence of $\rho_{\text{eff}}$. Based on the spherical core-shell model which consists of a rigid gold core and a soft shell formed by the ligand capping and a solvation layer, the sedimentation coefficient distribution was converted into the distribution of the hydrodynamic Stokes’ diameter $d_H$ using Eq. 6.2 and Eq. 6.4, as exemplified by the plots for MUS-NP4 and MUS-B2 in Fig 6.8(a),(b). For this calculation of employed MUS-AuNP systems, bulk values were used for $\rho_{\text{Au}}$ and $\rho_l$ at 19.32 and 1.03 g cm$^{-3}$, respectively.[241, 305] The thickness of the soft shell $l$ was set to be 1.3 nm according to published results on an identical NP system.[240] Accounting for the Rayleigh scattering and absorption,[302] the size dependence of NP extinction coefficient and corrected distribution plots for MUS-NP4 and MUS-B2 are summarised in Fig 6.8(c) and (d). Finally, the core size distribution of measured AuNP solution was obtained by subtracting $2 \times l$ from $d_H$. It is important to note that retrieving core size distribution of SAM-AuNPs using the implemented 1D approach is subject to the approximation of the thickness of the ligand capping layer and the solvation layer. Herein, a fixed value for $l$ was used to account for both contributions, which was indeed a simplified condition that can entail systematic deviations. Nonetheless, the immense density contrast between the gold core and the soft
shell layer still renders this method transferable to other NP systems which have defined shape and smooth surface morphology.

### 6.4.4 Comparative studies

![Size distribution analysis by TEM (red), SAXS (blue) and AUC (green):](image)

(a)–(c) MUS-NP1, (d)–(f) MUS-NP2, (g)–(i) MUS-NP3, (j)–(l) MUS-NP4. To represent core size distribution, $2 \times l$ were subtracted for the AUC results after normalisation with corresponding extinction coefficients.

Fig 6.9 and Fig 6.10 summarises the size distribution histograms obtained from TEM imaging, MC-SAXS and AUC-SV for unimodal and bimodal samples. Among all unimodal and bimodal samples containing MUS-AuNP ranging from $2 – 7 \text{ nm}$, all three methods provided similar estimation of the quasi-monodisperse sample MUS-NP3, while varying results were obtained for the other samples. The following discussion aims to examine the advantages and limitations of each techniques systematically.

It was observed in all samples that TEM histograms were more likely to incur inconsistency when compared to those of SAXS and AUC. Also, the size distribution observed in TEM results entailed a higher polydispersity, as evidenced in MUS-NP2–4. Both effects may be related to the fact that 1) TEM is a local imaging technique, which carries biased statistical representation by examining only a small fraction of the whole sample population; 2) TEM relied on the inspection of single particles and the labour-intensive workflow of sample preparation, imaging and data analysis can entail errors from user bias.[225, ...]
Figure 6.10: Size distribution analysis by TEM (red), SAXS (blue) and AUC (green): (a)–(c) MUS-B1 (1:1 \text{wt}) (d)–(f) MUS-B2 (1:5 \text{wt}). To represent core size distribution, \(2 \times l\) were subtracted for the AUC results after normalisation with corresponding extinction coefficients.

Also, the conventional measures of mean and median distribution value are not suitable for representing a non-uniform size distribution. As pointed out by Scheibelhofer et al., the implementation of a non-parametric \(\chi^2\)-homogeneity test could provide more reliable information when comparing different size distributions. TEM results also underestimated the size of the smaller-sized population in bimodal samples. This can be explained by the size dependence of imaging contrast. The increasing contrast of the larger-sized population overshadows those of smaller-sized population during imaging. This effect can be further translated to size reduction during image analysis because the outer part of the smaller sphere with poor contrast is typically filtered when applying an overall threshold in the software ImageJ. Furthermore, it is important to mention that although we have seen a growing utilisation of \textit{in situ} liquid-phase TEM and cryo-TEM, the commonly used standard \textit{ex situ} TEM requires the removal of the suspending liquid after the drop-cast sample preparation. This process is typically realised by vacuum or ambient drying, which often alters the dispersion state of sample materials and introduces artifacts obscuring accurate measurement. All these factors generate discrepancies...
for representing the size distribution, which can impair comparable and reproducible data analysis.[287] In spite of these drawbacks, TEM still offers a number of advantages including accessibility and ease of data analysis, thus providing a rapid characterisation of AuNP core sizes and shapes, with a semi-quantitative estimation of the degree of homogeneity.[283]

By contrast, both SAXS and AUC are ensemble methods which provide collective data in solution that can be used for detailed statistical analysis of in situ colloidal features. Due to the distinct X-ray SLD of gold core and the similar SLD values between alkanethiols and solvent molecules in a SAM-AuNP sample, SAXS permits the selective characterisation of the gold core while excluding the influence of the ligand shell and the solvation layer. Compared to the results by TEM and AUC, the histograms by MC-SAXS featured more detailed distribution patterns in both unimodal and bimodal samples. This can be related to the adequate resolving power of SAXS measurement and the implementation of the unbiased MC modelling, which extends the use of SAXS for non-monodisperse NP systems. It is important to note that the average size in SAXS histograms was slightly larger than those of TEM and AUC for MUS-NP1 and both bimodal samples, which contained NPs below 3 nm. These results are in line with the findings by Maes et al. on PbS nanocrystals with a unimodal distribution. They observed 1) an increase in average size from 2.84 to 3.16 nm for an identical SAXS profile using classical log-normal model-based fitting and the MC method; 2) this discrepancy reduced for samples above 4 nm.[297] This limitation of the MC method for sub-3 nm NPs can be attributed to a number of factors: 1) as described by the Porod’s Law, the scattering intensity decreases rapidly at the high $q$ region (corresponding to smaller size), which entails non-negligible systematic data noise; 2) molecules in solution (e.g., salt and solvent) share similar length scales to ultra-small NPs and the scattering of these molecules can not be effectively cancelled by solvent subtraction.[246] Similar feature was reported by Maes et al. and they claimed that the scattering of unbound lead oleate molecules resulted in a secondary population at 2.3 nm in their MC fitting results.[297] Consequently, the presented MC-SAXS is generally not suitable for characterising the ultra-small AuNPs below 3 nm. Moreover, considering the limit of sample-to-detector distance in SAXS instrumentation as well as the reduced beam flux at the low $q$ region, this MC-SAXS method may not provide accurate estimation of NPs whose diameter above 100 nm without the use of ultra-small angle X-ray scattering (USAXS).

AUC-SV analysis, on the other hand, is a strictly in situ process that examines the hydrodynamic behaviour of the measured material system. In my experiment, the analysis of 1D sedimentation results using the simplified core-shell model resulted in sharp distribution profiles, observed by the pronounced primary peak in both unimodal and bimodal samples.
Consequently, this underrepresentation of minor populations led to an overestimation of the homogeneity when compared to SAXS and TEM. This can be explained by the constrained resolution of both AUC measurements and subsequent data fitting. For example, although diluted sample solutions with salt stabilisation were used to minimise the charge effects, the concentration dependence of $d_H$ could not be completely excluded without the extrapolation to infinite dilution.[285] Moreover, the process of extrapolating core size from hydrodynamic size information of 1D sedimentation analysis can incur systematic errors, the influence of which can be quite significant especially for non-spherical samples or materials with extended soft shell structures. For such complex systems, more advanced instrumentation, such as multiwavelength detector, and fitting schemes, such as the Custom Grid spectrum analysis, are required to facilitate the implementation of AUC.[238, 300, 301]

### 6.5 Conclusions

To summarise this chapter, a comparative study of sub-10 nm AuNP size distribution characterised by TEM, SAXS and AUC is presented. The modular platform of AuNP synthesis established in Chapter 5 enabled the preparation of water-soluble MUS-AuNPs with varying size dispersities including unimodal and bimodal distributions. All three methods provided consistent assessment of quasi-monodisperse AuNPs with an average core diameter of 5 nm. The conventional TEM imaging method permitted facile characterisation of AuNPs with various size distributions, but it suffered from poor reproducibility and a lack of statistical significance. SAXS measurement, combined with subsequent model-free MC fitting, enabled reliable estimation of non-monodisperse size distributions with comprehensive statistical analysis. However, this method is not suitable for ultra-small NPs below 3 nm due to the reduced scattering intensity and the interference of small molecules. The less utilised AUC-SV method allowed comparable estimation of both unimodal and bimodal samples. However, its relatively low resolution resulted in the underrepresentation of the minor populations, whose accurate representation is vital in many research questions. This study offers valuable insights in state-of-art AuNP characterisation methods and may be transferred to guide the size distribution analysis on other material systems.
Chapter 7

Probing colloidal stability and molecular interactions of nanoparticles by SAXS


7.1 Motivation

Polar and hydrophobic interactions dominate most biophysical processes with ionic functional groups and non-polar domains commonly displayed by biological entities. However, the intriguing behaviour of nanoscale macromolecules extends beyond the current understanding of colloidal systems. NPs can meanwhile be tailored to exhibit protein-like features, most notably with respect to their complex surface structures and their relative sizes to surrounding molecules in solution. The lack of experimental insights on colloidal behaviour and in vitro interactions of NPs hinders further validations of in vitro and in vivo biomolecular interactions. The motivation of this chapter is to utilise SAXS to study the complex colloidal behaviour of protein-mimetic NPs, with a particular focus on the effect of small molecules. The work described in this chapter was in collaboration with Dr Zhi Luo in the group of Prof Francesco Stellacci at EPFL, Switzerland.

7.2 Introduction

As outlined in Chapter 1, the interactions between macromolecules in aqueous solutions are complex phenomena that govern many important chemical and biological processes. For example, the intermolecular interactions of proteins dictate their spatio-temporal distributions, such as cluster formation, aggregation and liquid-liquid phase separation, which are vital in many cellular functions as well as pathological conditions.[307–309] However,

* authors contributed equally to this work.
the direct assessment of structure-function relationships in proteins is extremely challenging due to their chemical and geometrical complexity. For this reason, synthetic NPs may serve as model system to mimic proteins in aim for unveiling in vivo mechanisms.[121] In particular, AuNPs, when stabilised with a binary mixture of charged and hydrophobic ligands, can exhibit a strong resemblance to globular proteins.[75] From a classical colloidal perspective, the regulation of intracellular protein interactions remains unfathomable: although the percentage of non-polar residues on protein surfaces may be as high as 70%, the cellular concentrations of proteins are stably maintained at extremely high level, i.e. up to 40% in volume fraction.[18, 310] In comparison, most of the abiotic colloidal systems, such as NPs, tend to precipitate at such high concentration due to various attractive forces and electrolyte screening of Coulomb repulsion.[311]

One of the most important forces in water-based chemical and biological environments is the hydrophobic interaction. Typical examples include lipid bilayer organisation and protein folding.[307] This attractive intermolecular force originates from the alteration of $H_2O$ around the solute surface.[2] In other words, the hydrophobic interaction relies on the interplay between multiple solvation energies. For large solutes, both hydrogen bonds and dispersion interactions between solutes and $H_2O$ come into effect and the enthalpy plays a dominating role. Conversely, the hydrophobic interaction for nanoscale small solutes is associated with the entropy loss of $H_2O$ molecules for surrounding apolar entities, thus hindering the formation of hydrogen bonds.[30] Although the hydrophobic force is vital at the bio-nano interface, its underlying principles have not been fully understood.[18]

A number of recent studies demonstrated the modulation of nanoscale hydrophobic interaction on functionalised surfaces. For example, Abbott and co-workers reported on the impact of nanoscale chemical heterogeneity on the hydrophobicity of a surface via chemical force microscopy measurements. It was suggested that hydrophobicity is not an intrinsic property of any given non-polar domain but instead can be effectively modulated by functional groups located as far away as 1 nm.[312] Moreover, the influence of sub-nanoscale hydrophobicity on modulating polar interactions was reported by Aida and co-workers. By altering the distance of hydrophobes from 1.2 to 0.2 nm, ionic interactions of the polar head group were significantly suppressed.[310] In spite of these advances, the solution-based modulation of nanoscale colloidal interactions, which is more relevant to biological systems, remains largely unexplored. Two major obstacles include the lack of 1) an amphiphilic colloidal system whose surface hydrophobicity can be tuned continuously and 2) a powerful characterising tool to probe molecular interactions at different ranges of separation distance in solution.

Growing evidence has suggested that small amphiphilic molecules play an important role on the deaggregation and stability of proteins.[313, 314] In fact, a number of small
solutes maintain high physiological concentrations, up to the order of 10 mM in some cases.[315] As a result, the solvation environment of proteins is inevitably affected by these molecules. Among biological small molecules, it is widely accepted that osmolytes, while adjusting the pressure and stress of the cytoplasm, contribute to stabilising protein conformations through preferential solvation or exclusion near the protein surfaces.[316, 317] This uncommon phenomenon gives rise to the concept of certain small molecules serving as biological hydrotropes, which are effective in solubilising hydrophobic molecules in aqueous solutions by their structuring in H2O.[318, 319] Notably, it is recently reported that ATPs not only act as the primary energy carrier, but also regulate the solubility of proteins, demonstrating exceptional hydrotropic effects.[315] Although the effects of various types of small molecules on specific proteins have been reported in various studies, there is a lack of systematic investigations to address the universality of this intriguing phenomenon.[314, 320–322] Several questions remain to be answered, among which are 1) how do small molecules affect the interplay of hydrophobicity and surface charges on proteins, and 2) how does an altered solvation in turn affect the self-assembly behaviour of proteins.

The modular synthetic platform presented in Chapter 5 for independent control over core size and ligand composition brings new possibilities by offering both nanoscale colloids with tunable ligand shells and strong X-ray scatterers for reliable SAXS analysis at small angles. The motivation of this chapter was to utilise amphiphilic AuNPs as a protein-mimetic model system to investigate systematically colloidal stability, molecular interaction as well as the effect of small molecules. The composition of the hydrophobic and charged components were fine tuned while the gold core size remained identical. This permitted comparative studies of modulating Coulomb electrostatic and hydrophobic interactions, electrolyte charge screening, preferential solvation and stabilisation by amphiphilic small molecules.

7.3 Experimental

7.3.1 Nanoparticle synthesis

Based on the modular AuNP synthesis established in Chapter 5, amphiphilic AuNPs were prepared. The ligand compositions of each batch of mixed-ligand AuNPs were determined individually by 1H NMR analysis after the ligand exchange. Please refer to Chapter 4 for detailed experimental procedures. Homo-ligand AuNPs stabilised with anionic MUS ligand and mixed-ligand AuNPs with varying MUS-OT ligand compositions were synthesised, as summarised Tab. 7.2. Please note that sample MUS-OT1 was synthesised by Dr
Zhi Luo using a modified one-phase approach. Complementary cryo-TEM analysis was performed by Dr Pelin Güven in the group of Prof Francesco Stellacci at EPFL, Switzerland. Cryo-TEM samples were prepared using a Vitrobot Marc IV (FEI) and imaged under a Tecnai F20 Cryo electron microscope (FEI). Cationic thiol ligands MUTAB and 11-amino-1-undecanethiol hydrochloride (AUT) were used to prepare cationic amphiphilic AuNPs. In combination with OT and 1-hexadecanethiol (HDT), three series of mixed-ligand AuNPs, i.e. MUTAB-OT, MUTAB-HDT and AUT-OT were synthesised from an identical OAm-AuNP batch, as shown in Tab. 7.3. Please note that the $^1$H NMR analysis of AuNPs with HDT were carried out in deuterated acetone.

### Table 7.2: Sample information of employed AuNPs with MUS.

<table>
<thead>
<tr>
<th>Sample label</th>
<th>Size by TEM</th>
<th>Ligand exchange solvent</th>
<th>Feed ratio</th>
<th>Ratio by NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUS1</td>
<td>4.1±0.5</td>
<td>DCM-H$_2$O</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MUS2</td>
<td>3.7±0.4</td>
<td>DCM-H$_2$O</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MUS-OT1</td>
<td>3.8±0.6</td>
<td>-</td>
<td>-</td>
<td>66:34</td>
</tr>
<tr>
<td>MUS-OT2</td>
<td>3.7±0.4</td>
<td>DCM-H$_2$O</td>
<td>80:20</td>
<td>88:12</td>
</tr>
<tr>
<td>MUS-OT3</td>
<td>3.7±0.4</td>
<td>DCM-H$_2$O</td>
<td>60:40</td>
<td>76:24</td>
</tr>
<tr>
<td>MUS-OT4</td>
<td>3.7±0.4</td>
<td>DCM-H$_2$O</td>
<td>40:60</td>
<td>67:33</td>
</tr>
<tr>
<td>MUS-OT5</td>
<td>3.7±0.4</td>
<td>DCM</td>
<td>60:40</td>
<td>57:43</td>
</tr>
<tr>
<td>MUS-OT6</td>
<td>4.1±0.5</td>
<td>DCM</td>
<td>65:35</td>
<td>60:40</td>
</tr>
</tbody>
</table>

#### 7.3.2 SAXS data collection and analysis

The prepared AuNPs were dissolved in ultrapure Type 1 H$_2$O. The addition of NH$_4$Cl, EtOH, proline, glycerol, urea, triethylamine (TEA), acetic acid (AcA) or butyric acid (BuA) were explored in both anionic and cationic AuNP aqueous solutions.
TABLE 7.3: Sample information of employed cationic AuNPs.

<table>
<thead>
<tr>
<th>Sample label</th>
<th>Size by TEM (nm)</th>
<th>Ligand mixture</th>
<th>Feed ratio A:B</th>
<th>Ratio by NMR A:B</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUT-OT1</td>
<td>4.1±0.5</td>
<td>AUT-OT</td>
<td>90:10</td>
<td>93:7</td>
</tr>
<tr>
<td>AUT-OT2</td>
<td>4.1±0.5</td>
<td>AUT-OT</td>
<td>80:20</td>
<td>85:15</td>
</tr>
<tr>
<td>AUT-OT3</td>
<td>4.1±0.5</td>
<td>AUT-OT</td>
<td>60:40</td>
<td>73:27</td>
</tr>
<tr>
<td>AUT-OT4</td>
<td>4.1±0.5</td>
<td>AUT-OT</td>
<td>40:60</td>
<td>65:35</td>
</tr>
<tr>
<td>AUT-OT5</td>
<td>4.1±0.5</td>
<td>AUT-OT</td>
<td>20:80</td>
<td>38:62</td>
</tr>
<tr>
<td>MUTAB-OT1</td>
<td>4.1±0.5</td>
<td>MUTAB-OT</td>
<td>60:40</td>
<td>57:43</td>
</tr>
<tr>
<td>MUTAB-OT2</td>
<td>4.1±0.5</td>
<td>MUTAB-OT</td>
<td>40:60</td>
<td>42:58</td>
</tr>
<tr>
<td>MUTAB-OT3</td>
<td>4.1±0.5</td>
<td>MUTAB-OT</td>
<td>20:80</td>
<td>16:84</td>
</tr>
<tr>
<td>MUTAB-HDT1</td>
<td>4.1±0.5</td>
<td>MUTAB-HDT</td>
<td>80:20</td>
<td>84:16</td>
</tr>
<tr>
<td>MUTAB-HDT2</td>
<td>4.1±0.5</td>
<td>MUTAB-HDT</td>
<td>20:80</td>
<td>39:61</td>
</tr>
<tr>
<td>MUTAB-HDT3</td>
<td>3.1±0.5</td>
<td>MUTAB-HDT</td>
<td>60:40</td>
<td>65:35</td>
</tr>
</tbody>
</table>

For each SAXS sample, 60 µl of the AuNP sample solution was loaded into a 1 mm (I.D.) borosilicate glass capillary. The measurement for each sample consisted of two separate experiments: medium angle (MA, q-range of 0.015 to 0.65 Å\(^{-1}\)) scan for 30 min and extreme small angle (ESA, q-range of 0.0035 to 0.18 Å\(^{-1}\)) scan for 60 min. The prolonged ESA scan was to compensate the reduced beam flux at low angles.

For data analysis, an overall SAXS profile across the q-range of 0.0035 to 0.65 Å\(^{-1}\) was obtained by merging the MA and ESA data in the program Primus/qt of the software package ATSAS (version 2.8.4).[323] The first few data points at the lowest q values were removed to avoid artefacts of the beam stop. The background subtraction was processed for the merged data before further fitting and analysis.

To extract the form factors from measured SAXS profiles that served for the subsequent determination of structure factors, the monodisperse hard sphere model in the software SasView (version 4.1) was used for curve fitting.[324] The fitted curves served as form factors for the determination of structure factors. SAXS profiles of MUS AuNPs were fitted with the hard sphere repulsive model based on the Hayter mean-spherical approximation (Hayter-MSA) in the software SasView and the Rogers-Young (RY) closure in the software SasFit (version 0.94.11).[325] The interaction potential calculation was carried out for the SAXS profiles of MUS-OT AuNPs using a two-term Yukawa potential in the software SAXSutilities and the xDLVO model in SasFit.[325–327]
7.4 Results and discussion

SAXS was used as the primary characterisation tool in this study. Please refer to Chapter 4 for in-depth discussion on the interpretation of experimental results.

7.4.1 Electrostatic repulsion and screening in aqueous solutions

To explore the possibility of probing long-range molecular interactions by SAXS, the Coulomb electrostatic repulsion of homo-ligand MUS AuNPs (sample MUS1 and MUS2) of various concentrations was examined in aqueous solution. Due to the anionic sulfonate head groups, MUS1 AuNPs were highly soluble in H$_2$O and concentrated solutions, up to 100 mg ml$^{-1}$, were prepared as stock solutions. As shown in the log-log plots of Fig. 7.1(a), an increase in AuNP concentration not only resulted in elevated scattering intensity of the form peak at high angles, but also gave rise to a distinct structure peak in the low $q$ region. Apart from the increased peak scattering intensities, the structure pattern also exhibited a shift of peak position in concentrated solutions. Using the Bragg’s equation (Eq. 4.13 in Chapter 4), the shift of structure peak from 0.022 to 0.028 Å$^{-1}$ corresponded to a change of separation distance from 28.6 to 22.4 nm for 20 and 40 mg ml$^{-1}$ AuNP solutions, respectively. This structure peak is a direct indication of long-range collective ordering of AuNPs, as a result of strong intermolecular repulsion induced by the negatively charged MUS, dominating over the Brownian motion. Due to the strong repulsive nature, the SAXS profile of 40 mg ml$^{-1}$ MUS AuNPs can be fitted with one-term numerical models developed for screened Coulomb potential. Both the hard sphere Hayter-MSA model (SasView) and the thermodynamically self-consistent RY closure (SasFit) were used for this study. As presented in Fig. 7.1, the experimental data and theoretical fittings closely matched. Moreover, the electrolyte concentration was fitted to be 3.15 mM, suggesting a long Debye screening length of 5.4 nm. This further supports the dominance of Coulomb repulsions at long separation distance.

On the other hand, the addition of 2.2 mg ml$^{-1}$ (40 mM equivalent) NH$_4$Cl, which corresponds to a Debye screening length of 1.5 nm, to 20 mg ml$^{-1}$ MUS2 AuNPs resulted in a featureless low-angle scattering profile (Fig. 7.1(b)). Herein, the charge screening, triggered by the increased ionic strength, disrupted the electrostatic interaction between MUS AuNPs. Since MUS AuNPs possess minimum surface hydrophobicity, charge screening did not result in NP precipitation, but instead induced random dispersions. Consequently, the MUS AuNP system was able to maintain its colloidal stability, similarly to a non-interacting ideal gas system. In other words, the scattering profiles across the whole $q$ range was determined solely by $P(q)$ while $S(q) \sim 1$. This result suggests that Coulomb electrostatic
Figure 7.1: Solution-based SAXS profiles of MUS1 in H$_2$O. (a) Concentration dependence of the long-range ordering induced by Coulomb repulsion in 5–40 mg ml$^{-1}$ MUS AuNP solutions; (b) 20 mg ml$^{-1}$ MUS2 AuNP solution with the addition of 2.2 mg ml$^{-1}$ (40 mM equivalent) NH$_4$Cl to induce electrolyte screening of the electrostatic interactions.

Figure 7.2: Fitting of SAXS profiles of MUS1 in H$_2$O. (a) Curve fitting with the hard sphere repulsive model Hayter-MSA in the software SasView. (b) Curve fitting and (c) structure factor using the Rogers–Young (RY) closure in the software SasFit.
interaction can be screened with the addition of electrolyte. It is important to note that biological media consist of various electrolytes and thus maintains a high ionic strength. In such environments, electrostatic repulsive forces are effectively screened and thereby can be excluded for sustaining the colloidal stability of biomacromolecules.

7.4.2 Modulating hydrophobic interactions

![Graph showing scattering intensity vs. q (Å⁻¹) for different AUT-OT compositions](image)

**Figure 7.3:** Effect of non-polar components on AUT-OT AuNPs. Solution-based SAXS profiles of 20 mg ml⁻¹ AUT-OT1–5 in H₂O. All samples share an identical core size of 4.1±0.5 nm and the OT composition, determined by ¹H NMR analysis, is shown in the plot.

Amphiphilic AuNPs with different AUT-OT, MUTAB-OT and MUTAB-HDT ligand mixtures were successfully prepared from a single batch of 4.1±0.5 nm OAm-AuNPs via thiol-for-OAm ligand exchange. Consequently, these samples shared identical form factors and resulted in overlapping scattering profiles in the MA region, as shown in Fig. 7.3 and Fig. 7.4. This is crucially important for a direct comparison of the structure factors in the ESA region. As demonstrated in the solution-based SAXS profiles of 20 mg ml⁻¹ AUT-OT1–5 AuNPs (Fig. 7.3), the increasing OT content gave rise to the sharp increase of scattering intensity at the ESA region and a shoulder pattern at ∼0.1 Å⁻¹, while diminishing the structure peak observed in samples with 7% and 15% OT. The sharp scattering peak at ∼0.1 Å⁻¹ and the significantly reduced intensity of the form peak of the 62% OT sample is consistent with the formation of AuNP clusters and aggregates. By increasing the OT content, this nanoscale colloidal system was gradually transformed from a repulsive
regime to an attractive regime. These results suggest the rise of attractive hydrophobic force when introducing non-polar OT ligands into the ligand shell of cationic AUT AuNPs. Since the ligand density of SAM-AuNPs remained stable when prepared from the OAm approach, an increase of OT content, which increased surface hydrophobicity, resulted in less surface charge density and hence weaker Coulomb repulsive force through the reduced AUT ligand density. As suggested by the previous study by Moglianetti et al. on similar NP system, the pK_a values are mostly irrelevant to OT content. Unlike previous studies centred on clustering or agglomeration driven by short-range forces, it is important to note that the utilisation of solution-based SAXS in this study enables precise assessment of medium- and long-range NP-NP interaction, which is of crucial importance in dictating nanoscale colloidal stability.

The effect of both ligand components was subsequently studied by comparing AuNPs with three different ligand mixtures using sample AUT-OT1, AUT-OT5, MUTAB-OT1–3 and MUTAB-HDT1–2. Fig. 7.4 summarises the SAXS profiles of these AuNPs with excessive and minimal hydrophobic components, respectively. At 58% OT content, the structure peak pattern and downward scattering intensity in the ESA region indicated the repulsive NP-NP interaction between MUTAB-OT AuNPs. This was in drastic contrast to the SAXS profiles of MUTAB-HDT (61%) and AUT-OT (62%), in which distinct clustering peak and upward ESA curves were already present, suggesting strong attractive NP-NP interactions.
It is important to note only at an elevated OT content of 84%, a diminished structure peak and an upward ESA curve were observed for MUTAB-OT AuNPs, which implied reduced repulsive forces. On the other hand, the identical structure peak in the SAXS profiles of MUTAB-OT (43%) and MUTAB-HDT (16%) indicated the dominance of repulsive interaction in both samples. Contrastly, AUT-OT AuNPs with 7% OT content did not give rise to a similar structure peak while the flattened ESA curve evidenced weaker repulsive interactions. In these results, the repulsive forces can be attributed to the ionic interactions between cationic ligands while the attraction between AuNPs was a direct consequence of the hydrophobic interaction between the non-polar ligands. Therefore, this comparative study entails intriguing insights in two separate aspects. When comparing MUTAB-OT and MUTAB-HDT, the divergence in hydrophobicity can be attributed to the relative height of non-polar ligand to MUTAB, similar to the indirect assessment on flat SAM surfaces by Aida and co-workers as mentioned in Section 7.2.[310] Herein, we accomplished the direct representation of this hydrophobic modulation on highly curved AuNPs surfaces. On the other hand, the direct comparison between MUTAB-OT and AUT-OT suggests the impact of the polar end group. The enhanced Coulomb repulsion in MUTAB-OT AuNPs can be explained by a higher surface charge density and the bulk structure of the TMA headgroup, which provided steric hindrance for hydrophobic interaction. These results are in line with the findings by Laaksonen et al., which demonstrated that the adsorbed quaternary ammonium ions on AuNPs acted as a steric barrier and increased the distance of closest approach between AuNPs.[12]

7.4.3 Preferential solvation induced by ethanol

Based on results of cationic MUTAB and AUT, respectively, the introduction of a hydrophobic co-ligand had a distinct effect on anionic MUS AuNPs (sample MUS-OT1). As presented in Fig. 7.5(a), direct cryo-TEM imaging showed a randomly distributed dispersion behaviour with the presence of small clusters. This finding was in line with solution-based SAXS studies shown in Fig. 7.5(c). A sharp increase of scattering intensity (instead of structure peaks) in the ESA region, together with a pronounced shoulder pattern at $\sim 0.1 \text{ Å}^{-1}$, was observed for MUS-OT AuNPs with an increase in concentration: 5, 10, 20 and 40 mg ml$^{-1}$. These features provided a direct indication for partial cluster formation at a short separation distance, suggesting the dominance of attractive NP-NP interaction. This shift from repulsive interaction to attractive interaction can be attributed to rise of surface hydrophobicity with the addition of OT, which at the same time leads to reduced surface charge density.
Chapter 7. Nanoparticle colloidal stability and molecular interactions

EtOH was introduced into the MUS-OT AuNP solution as a co-solvent. As shown in the cryo-TEM image (Fig. 7.5(b)), MUS-OT AuNPs were individually separated and well distributed in an ordered state. In complement, the SAXS profiles in Fig. 7.5(d) demonstrated a distinct concentration-dependent structure peak at $\sim 0.02 \text{ Å}^{-1}$ as well as diminished shoulder feature at $\sim 0.1 \text{ Å}^{-1}$. These SAXS findings are in line with TEM observations on the long-range ordering of AuNPs and the evanescence of AuNP clusters, suggesting an intriguing transition of the dispersion state of AuNPs upon the addition of 10% vol EtOH. This phenomenon can be explained by the resurgence of Coulomb repulsive force which causes AuNP deaggregation as well as long-range structure ordering by overriding the Brownian motion. Therefore, the addition of EtOH as a co-solvent can be used to induce the solvation of amphiphilic AuNPs by effectively shielding the hydrophobic interaction.
This effect is line with the SANS study by Spinozzi and co-workers, in which the co-solvent EtOH induced the preferential solvation of lysozyme in EtOH-H$_2$O mixtures by accumulation at the protein surface.[329]

**Figure 7.6:** Solution-based SAXS study of hydrophobic shielding of EtOH on MUS-OT AuNPs. (a)–(c) SAXS profiles and (d)–(f) interaction energy of 20 mg ml$^{-1}$ MUS2 and MUS-OT2–5 in H$_2$O, 10$\%$$_{vol}$ and 20$\%$$_{vol}$ EtOH aqueous solutions fitted with the two-term Yukawa potential with fractal contributions. The samples were prepared from five batches of AuNPs with identical core size of 3.7 nm and varying OT content of 0$, 12$, 24$, 33$ and 43$, as determined by $^1$H NMR analysis.

The effect of EtOH was further examined in a systematic study for AuNPs with varying ligand compositions. The versatile OAm ligand exchange synthesis permitted the continuous tuning of OT contents at 0, 12, 24, 33 and 43 for five batches of AuNPs (sample MUS2 and MUS-OT2–5) with identical core size of 3.7 nm, which resulted in identical SAXS form factors and overlapping scattering profiles in the MA region. Consequently, it became possible to directly compare each individual structure factor in the ESA region. Fig. 7.6 summarises the SAXS profiles of sample MUS2 and MUS-OT2–5 in H$_2$O, 10$\%$$_{vol}$ and 20$\%$$_{vol}$ EtOH aqueous solutions. As presented in Fig. 7.6(a) for the measurements in H$_2$O, the samples with 0 and 12 OT gave rise to overlapping SAXS curves with distinct structure peak, suggesting the dominance of strong intermolecular repulsion. With increasing OT content, the intensity of this structure peak was gradually lessened and eventually
diminished for the 43% OT sample, together with a clear shift of the peak position towards lower angles. Meanwhile, a shoulder pattern at $\sim 0.1 \text{ Å}^{-1}$ emerged in the 33% and 43% OT samples, indicating a partial cluster formation. The results are in line with the finding on AUT-OT AuNPs and can be attributed to the interplay between the Coulomb repulsion and hydrophobic attraction, induced by the charged MUS and hydrophobic OT components, respectively. On the other hand, the addition of EtOH altered the dispersion of MUS-OT AuNPs and the effect was particularly significant for samples with higher OT content. As shown in Fig. 7.6(b), the 24% OT sample exhibited a overlapping structure peak with the 0% and 12% OT samples, suggesting an increase of Coulomb repulsion in 10%$_{\text{vol}}$ EtOH. The emergence of structure peaks of the 33% OT sample and the reduced upward slope of the 43% OT sample suggest the transformation from the attraction-dominant dispersion in $\text{H}_2\text{O}$ to a partial repulsion state in 10%$_{\text{vol}}$ EtOH. This effect was further enhanced in 20%$_{\text{vol}}$ EtOH aqueous solutions, as shown in Fig. 7.6(c). Samples with 0%, 12% and 24% OT shared an identical SAXS profile which also showed close resemblance to that of the 33% OT sample. Moreover, the emergence of the structure peak and the diminished clustering shoulder pattern in the 43% OT sample indicated strong repulsive interaction due to the suppression of hydrophobic force.

The interaction energy fitting of the measured SAXS profiles using a two-term Yukawa potential model in the software SAXSutilities is shown in Fig. 7.6(d)–(f). Firstly, a clear transition from repulsive potential to attractive potential with increasing OT content was observed in Fig. 7.6(d). Secondly, Fig. 7.6(e),(f) demonstrated a clear shift of attractive systems to the repulsive regime with the addition of EtOH. These results provide further proof of the influence of non-polar components and EtOH. It is important to mention that the SAXS structure fitting for such complex system is still an active field of research. This two-term Yukawa potential model with fractal contribution provided satisfactory fitting results. However, it was limited in representing strongly repulsive samples, in which fractals could not be excluded.[330]

Both experimental and fitting results suggest that the presence of EtOH induced the preferential solvation of amphiphilic AuNPs. This is in line with previous studies in which EtOH can act as both a co-solvent and a hydrotrope.[319, 331] By acting as a shield to attractive hydrophobic interaction, EtOH altered the ionic-hydrophobic interplay and triggered transformation into a repulsive regime. This intriguing effect not only offers a facile approach to improve colloidal stability of various NP systems, but also carries profound implications for the regulation of cellular proteins with substantial surface hydrophobicity.
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7.4.4 Amphiphilic small molecules as hydrotropes

Based on the EtOH study, a series of small molecules were mixed with sample MUS2 and MUS-OT2–5 to assess their intermolecular interactions. The effect of the amino acid proline is presented in Fig. 7.7. Compared to the results in pure H₂O (Fig. 7.6(a)), the addition of 230 mg ml⁻¹ (10% vol equivalent) proline gave rise to the structure peak of 24% and 33% OT samples and reduced the upwards slope of 43% OT sample, suggesting a similar suppression of hydrophobic attraction to the effect of adding 10%vol EtOH. This is in line with previously reported protein studies which identified proline as a biological hydrotrope.[316, 322] Considering the amphiphilic nature of both mixed-ligand AuNPs and prolines as well as the specificity of this hydrophobic shielding effect, one may hypothesise the working mechanism to be the surface adsorption driven by the attraction between the hydrophobic components of both sides.[319, 332]

To align with a more holistic model, attempts were made to fit the scattering profiles of 24% OT AuNPs using the xDLVO theory. As mentioned in Chapter 1, xDLVO includes a
hydrophobic term \( V_{hd} \) in an exponential decay function in addition to the Coulomb repulsion term \( V_{el} \) and the van der Waals term \( V_{vdW} \), as given in following equations:

\[
V_{xDLVO}(r) = \begin{cases} 
\infty, & \text{if } r \leq \sigma; \\
V_{el}(r) + V_{vdW}(r) + V_{hd}(r), & \text{if } r > \sigma.
\end{cases}
\]  

(7.1)

\[
V_{el}(r) = k_B T L_B Z^2 \frac{1}{(1 + \kappa \sigma / 2)^2} \frac{\exp[-\kappa (r - \sigma)]}{r},
\]

(7.2)

\[
V_{vdW}(r) = -k_B T A_H \frac{1}{12} \left[ \frac{\sigma^2}{r^2 - \sigma^2} + \frac{\sigma^2}{r^2} + 2\ln\left(1 - \frac{\sigma^2}{r^2}\right) \right],
\]

(7.3)

\[
V_{hd}(r) = -H \exp[-(r - \sigma)/D].
\]

(7.4)

Here, \( \sigma \) is the particle size and \( r \) is the separation distance, \( L_B \) is the Bjerrum length, \( Z \) is the effective macroion valency. \( A_H \) is the Hamaker constant while \( H = V_{hd,max} \) is the hydrophobic interaction strength. \( \kappa \) is the Debye screening wave vector and \( D \) is the decay length of the hydrophobic interaction.[325,333]

The fitting of this three-term model was performed by manually adjusting fitting parameters in the software SasFit. The results are presented in Fig. 7.8 and Tab. 7.4. Both the calculated interaction potentials and extrapolated interaction parameters provide further proof that the EtOH solubilisation and proline stabilisation effects display a resemblance to each other. More precisely, the presence of EtOH and proline led to a significant reduction of hydrophobic interaction strength: from 58.5 \( k_B T \) in pure H\(_2\)O to 10 and 15 \( k_B T \) in 10\% vol EtOH and 10\% vol proline aqueous solutions, respectively. Meanwhile, the surface charge density increased from 36 \( e^- \) in H\(_2\)O to 51 \( e^- \) in both mixtures, which was even higher than the value of MUS AuNPs in H\(_2\)O. This variation of surface charge density provides crucial evidence to the surface adsorption mechanism of amphiphilic small molecules, as proposed in Fig. 7.7(a). Compared to the interaction energy fitting with the two-term Yukawa potential, xDLVO offered better fitting data with improved chemical considerations and enabled directly extrapolation of surface charge density and hydrophobic decay length. Although the fitting process in the software SasFit remains manual with automation procedures currently under development, it carries great potential for a better understanding of hydrophobic interactions.

The effect of positively charged TEA on MUS-OT AuNPs in solution was also probed by SAXS. Fig. 7.9 summarises a systematic study on the effect of different small molecules on
Chapter 7. Nanoparticle colloidal stability and molecular interactions

**Figure 7.8**: Interaction potential analysis using the xDLVO model. (a) Overall summation of interaction energies, (b) Coulomb repulsion potential and (c) the hydrophobic and van der Waals attraction potential extracted from the structure factors of four separate samples: 0% and 24% OT MUS-OT AuNPs in H₂O, 24% OT MUS-OT AuNPs in 10%vol EtOH and 230 mg ml⁻¹ (10%vol equivalent) proline aqueous solutions. This analysis was carried out by Dr Zhi Luo.

**Table 7.4**: Interaction parameters obtained from xDLVO analysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>OT%</th>
<th>Condition</th>
<th>Surface charge, e⁻</th>
<th>Debye length, nm</th>
<th>$V_{\text{hd, max}}$ kT</th>
<th>$V_{\text{hd decay}}$ length, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUS2</td>
<td>0%</td>
<td>H₂O</td>
<td>47</td>
<td>4.5</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>MUS-OT3</td>
<td>24%</td>
<td>H₂O</td>
<td>36</td>
<td>4.2</td>
<td>58.5</td>
<td>2.4</td>
</tr>
<tr>
<td>MUS-OT3</td>
<td>24%</td>
<td>10%EtOH</td>
<td>51</td>
<td>4.0</td>
<td>10.0</td>
<td>2.4</td>
</tr>
<tr>
<td>MUS-OT3</td>
<td>24%</td>
<td>10%Proline</td>
<td>51</td>
<td>4.5</td>
<td>15.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Sample MUS-OT6 (40% OT AuNPs with a core size of 3.8±0.5 nm). In particular, the SAXS curve of MUS-OT added with 120 mM TEA exhibited a non-interacting profile, evidenced by the flat ESA curve and diminished shoulder pattern at $\sim$0.1 Å⁻¹, which demonstrated the pronounced hydrotropic behaviour of TEA in solubilising amphiphilic AuNPs. Compared to the individual effect of charge screening by NH₄Cl and the hydrophobic shielding by EtOH, TEA induced both effects simultaneously. By contrast, hydrophilic small molecules such as urea and glycerol, posed no influence on MUS-OT AuNPs, as shown in Fig. 7.9(c). These results provide further experimental evidence for the aforementioned surface adsorption hypothesis. In particular, the effect of TEA may be attributed to a multivalent binding scheme between AuNPs and TEA, involving the charge-charge Coulomb attraction and hydrophobic interaction, which results in enhanced surface adsorption of TEA and thus more effective shielding for hydrophobic attraction between AuNPs. Meanwhile, the charge screening effect of TEA is consistent with the surface adsorption of oppositely charged TEA that cancels out partially or wholly the surface charges of AuNPs, in combination with an increase in ionic strength of the solution.
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The findings of anionic MUS-OT AuNPs were also compared to cationic AuNP sample MUTAB-HDT3. As shown in Fig. 7.10(a), the addition of EtOH induced the preferential solvation of AuNPs, indicating a similar hydrophobic shielding effect to the case of MUS-OT AuNPs. The effect of organic acids including AcA and BuA is presented in Fig. 7.10(b). The flat ESA curve of MUTAB-HDT AuNPs suggests a combined effect of electrostatic screening and hydrophobic shielding with the addition of 20% \text{vol} BuA, similar to the

Figure 7.9: Overview of the effect of small molecules on MUS-OT AuNPs. Solution-based SAXS profiles of 20 mg ml$^{-1}$ MUS-OT6 with 40% OT: (a) in H$_2$O, electrostatic screening with 120 mM NH$_4$Cl, hydrophobic shielding with 20%$_{\text{vol}}$ EtOH (similar to proline); (b) cooperative effect with 120 mM NH$_4$Cl and 20%$_{\text{vol}}$ EtOH, electrostatic screening and hydrophobic shielding with 120 mM TEA; (c) minimal impacts with hydrophilic glycerol and urea.

Figure 7.10: Overview of the effect of small molecules on cationic AuNPs. Solution-based SAXS profiles of 20 mg ml$^{-1}$ MUTAB-HDT3: (a) in H$_2$O, hydrophobic shielding with 20%$_{\text{vol}}$ EtOH; (b) electrostatic screening and hydrophobic shielding with 20%$_{\text{vol}}$ AcA and 20%$_{\text{vol}}$ BuA. The AuNPs were prepared from 3.1$\pm$0.5 nm OAm-AuNPs and the HDT content was determined to be 35% by $^1$H NMR.
impact of TEA on MUS-OT AuNPs. In comparison, the effect of the less hydrophobic AcA was much weaker. This may be due to small molecules with extended hydrophobic domains being able to induce more effective surface interaction with amphiphilic AuNPs, as suggested by Bauduin et al. in the study of the hydrotropic effect affected by the volume of the hydrophobic parts of different hydrotropes.[334] Once again, these findings provided additional proof to the surface adsorption hypothesis of amphiphilic small molecules in solubilising AuNPs. This hydrophobic tails permit the adsorption on the hydrophobic patches of AuNPs and thereby act as shields to the attractive hydrophobic interaction between AuNPs. This enticing phenomenon can be directly related to the role of biological hydrotropes in regulating amphiphilic macromolecules.[45]

7.5 Conclusions

In this chapter, amphiphilic AuNPs with varying polar and hydrophobic components were used as model systems to investigate the colloidal stability and molecular interaction in aqueous solutions. X-ray scattering at extreme small angles was successfully integrated and proved to be a powerful tool for probing medium- and long-range intermolecular interactions. While anionic and cationic AuNPs were homogeneously dispersed in solution, an increasing surface hydrophobicity transformed the NP-NP interactions from an electrostatic repulsive regime to a hydrophobic attractive regime. The addition of small molecules demonstrated remarkable impact to reverse such trend. Notably, EtOH and proline were able to induce effective hydrotropic stabilisation of MUS-OT AuNPs by shielding hydrophobic attraction, which facilitated the Coulomb repulsive interaction. For charged amphiphilic molecules such as TEA and BuA, an additional electrostatic component came into play, resembling the effect of inorganic salts in screening the Coulomb interaction of AuNPs. In summary, amphiphilic small molecules can tune the solution assembly behaviour of amphiphilic AuNPs through a combination of both electrostatic and hydrophobic interactions. This phenomenon is universal for both positively and negatively charged AuNPs and can be explained by the surface adsorption of small molecules. This study provides valuable insights on the cellular stability of amphiphilic proteins in a complex biological media, which is in abundance of electrolytes and small biomolecules.
Chapter 8

QCM-D monitoring of nanoparticle interactions with small molecules

Disclosure: This chapter is an adapted version of the publication: Probing the interaction of nanoparticles with small molecules in real time via quartz crystal microbalance monitoring. Yang Y, Poss G, Weng Y, Qi R, Zheng H, Nianias N, Kay E R, Guldin S. Nanoscale, 2019, 11, 11107-11113.[335]

8.1 Motivation

As outlined in Chapter 2, the immense library of NP surface functionality offers a broad variety of structural motifs for specific molecular interactions. In spite of extensive studies in the field of molecular recognition and nanomaterial-based sensing, the in situ monitoring of binding between NPs and small molecules remains a challenge. This hinders subsequent development of chemical and biological sensors with high sensitivity and specificity. In this chapter, the aim was to explore the detection of NP-based binding events utilising a QCM-D, which enables not only the stepwise in situ quantification of mass uptake and release but also the in-depth analysis of binding kinetics during surface interactions. Upon immobilisation of SAM-AuNPs on QCM sensor surface via dithiol linkers, this platform can provide quantitative characterisation of selective binding with analytes of low molecular weight. In a case study, the dynamic covalent binding process was assessed between AuNPs functionalised with thiolated boronic acids (BAs) and cis-diols or salicylic acid (SA) derivatives to establish a route for rational screening of AuNP candidates for molecular recognition. The work described in this chapter was carried out in collaboration with Guillaume Poss in the group of Dr Euan Kay at University of St Andrews, UK and Dr Nikolaos Nianias in the group of Prof Francesco Stellacci at EPFL, Switzerland.

8.2 Introduction

Thiol-based surface stabilisation of SAM-AuNPs offers a variety of surface functionalities for the selective interaction with antibodies, peptides, proteins, drugs and other small
molecules.[53, 59, 205, 336, 337] The self-assembly of ligand shells with biotic and abiotic molecules enables bottom-up morphology control at the nanoscale to study the structure-property-function interplay with biological entities.[140, 338, 339] In analogy to biomolecular recognition prevalent in many biological processes,[117, 340] AuNPs have been developed for molecular recognition as well as drug delivery, alongside other synthetic supramolecular systems, such as nanocarriers and artificial molecular machines.[116, 341, 342]

The screening of the affinity and selectivity of tailored AuNP ligand shells towards molecular targets is essential for any of the above mentioned applications. A number of characterisation techniques have been developed based on different transduction principles, such as optical, acoustic and calorimetric read-out.[343] Molecular labelling, as often used in fluorescence and Raman-based spectroscopy, restricts the choice of materials and may also interfere with the target study.[344, 345] A label-free alternative is based on SPR, which provides excellent sensitivity and responsiveness but the signal interference by non-specific binding often renders the analysis rather challenging.[346, 347] Thermal calorimetry methods offer established routes for the determination of binding constants,[348] but limitations exist regarding their low throughput and the requirement of large sample quantities. A number of suitable methodologies based on NMR spectroscopy have recently emerged to study NP-analyte interaction, including specifically designed diffusion filter and magnetisation transfer methods.[235, 349–351] While it is meanwhile possible to verify detailed binding mechanisms and identify multiple analytes by NMR, challenges remain, such as the requirement for relatively high sample concentrations and the availability of a suitable instrumentation infrastructure.

Similar to SPR, QCM-D offers a viable route for label-free monitoring of molecular adsorption at interfaces in real time.[352–354] The established relationship between the change in the resonant frequency and adsorbed wet-mass enables accurate quantification of binding events. This principle has been implemented to probe various molecular interactions, in which AuNPs play two major roles. The incorporation of AuNPs has been subject to the amplification of the frequency response as "mass enhancers" since AuNPs have much higher surface area and larger molecular mass comparing to common target molecules.[248, 355, 356] SAM-AuNPs with matching surface functionality have also been used as "binding inducers" for recognition of macromolecules, such as proteins, nucleic acids, lipid bilayers, as well as multilayer solvent vapours.[155, 357–360] While it is possible to detect mass changes as low as 1 ng cm$^{-2}$, the detection of molecules with low molecular weight remains challenging, often related to either an insufficient interfacial area (due to a low grafting density), limited binding affinity, or a combination thereof.
One particular type of molecular interaction, that has been applied in a variety of NP-based recognition systems, is the binding of NP-bound BAs with diol containing biomolecules or other dihydroxy compounds, including nucleosides, saccharides, glycans and glycoproteins, all of which are of significant biological relevance.[361–365] Governed by a reversible covalent binding scheme, the interaction between BA and dihydroxy functional groups induces the formation of boronate esters, which is typically affected by the presence of Lewis bases.[366–368] Various strategies have been explored for achieving stable complexation at near-neutral pH values, including adding electron-withdrawing groups (e.g., nitro, fluoro and sulfonyl) and intramolecular tetracoordinated B-N bonds (known as Wulff-type BAs).[369–372] As building blocks of aforementioned biomacromolecules, catechols and SAs have been used as primary candidates to gain a better understanding of underlying binding mechanism. Unlike aerobically unstable catechols, SAs are oxidatively inert and thus ideally suited for selective dynamic covalent interactions with BAs.[373, 374] From measurements using $^{19}$F and $^1$H NMR spectroscopy, binding strength of SAs and catechols with BAs were found to be highly sensitive to experimental variables, including concentration of bases, as well as the molecular architecture of both BA ligands and their target binding partners.[349, 373] It is therefore crucial to develop a comparative screening method with simplicity and versatility for the study of simple BA binding partners, which is fundamental for the development of novel sensing schemes for applications in proteomics and metabolomics.

In this chapter, a methodical case study is presented for the screening of BA ligands, binding partners and base concentration utilising multichannel QCM-D as a suitable powerful tool for the in situ monitoring of AuNP-analyte interactions. By stepwise immobilisation of AuNPs and subsequent exposure to binding partners, I aim to validate the ability of QCM-D to characterise uptake and release of small molecular weight binding partners and report on a thorough analysis of binding kinetics obtained for different ligand architectures, target binding partners and Lewis base concentrations.

8.3 Experimental

8.3.1 Material fabrication

Fig. 8.1 summarises the phenylboronic acid (PBA) ligands and SA binding partners used in this chapter. The PBA ligands and PBA-AuNPs were prepared and characterised by Guillaume Poss.[373] AuNPs stabilised by MUO were prepared via the OAm ligand-exchange approach and its characterisation is described in Chapter 5. Commercially
available 4-fluorosalicylic acid (FSA) and 3,5-dinitrosalicylic acid (DNSA) were used as binding partners.

### Table 8.1: Sample information of employed AuNPs.

<table>
<thead>
<tr>
<th>Sample label</th>
<th>Ligand</th>
<th>Synthesis method</th>
<th>Size (nm)</th>
<th>Organic content</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBA1-NP</td>
<td>PBA1</td>
<td>One-phase</td>
<td>3.6±0.6</td>
<td>15.5%</td>
</tr>
<tr>
<td>PBA2-NP</td>
<td>PBA2</td>
<td>One-phase</td>
<td>3.0±0.5</td>
<td>21.0%</td>
</tr>
<tr>
<td>MUO-NP</td>
<td>MUO</td>
<td>Thiol-for-OAm</td>
<td>3.7±0.4</td>
<td>9.6%</td>
</tr>
</tbody>
</table>

#### 8.3.2 QCM-D monitoring and characterisation

QCM-D was employed as the primary tool to probe the covalent binding of NP-tethered PBAs and SAs in MeOH mediated by the Lewis base N-methylmorpholine (NMM). The instrumentation and data analysis information of my QCM-D study is detailed in Chapter 4. Fig. 8.2 summarises the stepwise experimental protocol of the QCM-D monitoring method described in this chapter, including three majors steps: 1) functionalisation of the QCM sensor surface by dithiols, 2) AuNP immobilisation, and 3) exposure to binding partners. In details, this *in situ* process was started with the flow of pure MeOH for conditioning the sample module and baseline stabilisation. All overtones of frequency and dissipation were offset before the flow of 40 mM 1,8-octanedithiol (diOT) MeOH solution for 40–60 min. When the frequency shift curve reached a plateau, MeOH buffer rinsing was applied for 10 min to remove excessive diOT molecules. Subsequently, the thiolated sensor surface was exposed to 0.1 mg ml$^{-1}$ AuNP MeOH solution for 30–60 min before a similar MeOH rinsing interval was repeated afterwards. Before the introduction of binding partners, the
sensor surface was conditioned with NMM buffer solution for 10 min. The flow of SA MeOH solution added with NMM was kept for 30 – 60 min before extended rinsing with NMM buffer solution and pure MeOH. Various concentration of SA and NMM were used in this step.

The prepared sensors immobilised with AuNPs were analysed directly by STM, which was carried out by Dr Nikolaos Nianias. Please refer to Chapter 4 for detailed instrumentation protocols.

![Schematic representation of the QCM monitoring process.](image)

**Figure 8.2:** Schematic representation of the QCM monitoring process.

### 8.4 Results and discussion

#### 8.4.1 DNSA binding with specificity

Fig. 8.3 and Fig. 8.4 summarise the frequency and dissipation monitoring of the three-step process probing the binding of NP-bound PBA1 and DNSA. Prior to the assessment of analyte binding, reusable QCM sensors (C2 and C3) were prepared by grafting monolayer stabilised AuNPs onto dithiol functionalised surfaces. C4 was used as control sample by flowing 10 mM MeOH solution of PBA1 in the disulfide form (dPBA1). As shown in Fig. 8.3(a), a decrease in frequency was observed with the flow of diOT, in line with the attachment of the diOT molecules. In comparison, the dissipation signal saw an increase during the process (Fig. 8.4(a)), which can be attributed to a change in the bulk solution across the sensor surface. This is further supported by the fact that the dissipation shift returned to zero while the frequency shift stabilised at a certain level after rinsing with MeOH at 100 μl min⁻¹, suggesting both the removal of the loosely attached diOT molecules but also the formation of a rigid layer on the sensor surface. Consequently, the validity of the Sauerbrey equation allowed calculation of the areal mass (AM) change on the surface upon adsorption/desorption from the frequency shift (Fig. 8.3(b)).[252] With an AM change
Chapter 8. QCM-D monitoring of nanoparticle interactions

Figure 8.3: Stepwise QCM monitoring results. Frequency shift and corresponding AM change (5th overtone): (a),(b) grafting of diOT onto gold surface; (c),(d) immobilisation of AuNPs and (e),(f) detection of 10 mM DNSA in MeOH with 10 mM NMM.

At 137 and 151 ng cm\(^{-2}\) for C2 and C3, similar diOT grafting densities of 4.6 and 5.1 nm\(^{-2}\), respectively, were achieved. Note that in comparison, the grafting of dPBA1 onto the sensor surface remained limited, with a mass change of 20 ng cm\(^{-2}\).

Following the dithiol functionalisation step, the C2 and C3 sensors were then exposed to MeOH solutions of AuNPs at a flow rate of 10 \(\mu\)l min\(^{-1}\). C2 was exposed to MUO-AuNPs (core size 3.7 nm in diameter) as a control, whilst PBA1-NPs (core size 3.6 nm in diameter) were flowed into C3. A significant decrease in frequency was recorded for both channels during the first 15 min and no further adsorption was detected after \(\sim\)60 min constant flow (Fig. 8.3(c)). Again, no significant dissipation shift was observed (Fig. 8.4(b)) during the
process, indicating the formation of a rigid layer. Limited desorption was observed after the MeOH rinsing step, suggesting the robust immobilisation of AuNPs, with AM change of 2120 and 2109 ng cm$^{-2}$ in each channel, respectively (Fig. 8.3(d)). Meanwhile, C4 control channel was flowed constantly with MeOH at flow rates matching those used with C2 and C3; negligible background drift was observed. In consideration of AuNP core size and ligand coverage, the AuNP density was determined to be approximately 3.60E+12 and 3.78E+12 cm$^{-2}$ for MUO-NPs and PBA1-NPs, respectively (Tab. 8.2).

<table>
<thead>
<tr>
<th>NP</th>
<th>NP core size, nm</th>
<th>Weight of NP core, ng</th>
<th>Organic content</th>
<th>Weight of NP, ng</th>
<th>AM change, ng cm$^{-2}$</th>
<th>Grafting density, cm$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUO</td>
<td>3.7</td>
<td>5.12E-10</td>
<td>13.0%</td>
<td>5.89E-10</td>
<td>2120</td>
<td>3.60E+12</td>
</tr>
<tr>
<td>PBA1</td>
<td>3.6</td>
<td>4.72E-10</td>
<td>15.5%</td>
<td>5.59E-10</td>
<td>2109</td>
<td>3.78E+12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NP</th>
<th>Ligand length, nm</th>
<th>NP size, nm</th>
<th>Grafting density of close packing, cm$^{-2}$</th>
<th>Packing density</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUO</td>
<td>1.53</td>
<td>6.76</td>
<td>2.53E+12</td>
<td>1.42</td>
</tr>
<tr>
<td>PBA1</td>
<td>2.09</td>
<td>7.78</td>
<td>1.91E+12</td>
<td>1.98</td>
</tr>
</tbody>
</table>

The coverage of AuNPs was further assessed by STM imaging, as shown in Fig. 8.5. Sensor surfaces of MUO-NPs (C2) and PBA1-NPs (C3) were resolved at sub-10 nm scale. In agreement with the QCM-D results, surfaces were fully covered by AuNPs. The AuNPs were also observed to be immobile against STM tips when imaging, suggesting a firm attachment of AuNPs to the sensor surface via dithiol linkers. The packing density was also extrapolated from the QCM data. For hard spheres with diameter of $D = D_{core} + 2L$. 

\[ D = D_{core} + 2L \]
Chapter 8. QCM-D monitoring of nanoparticle interactions

Figure 8.5: STM images of AuNPs immobilised on thiolated sensor surfaces: (a),(b) MUO-NPs, (c) PBA1-NPs, (d) fresh sensor. These images were acquired by Dr Nikolaos Nianias.

\[(L:\text{ligand length, as predicted in Fig. 8.6}),\text{this would lead to a packing density of 1.42 and 1.98 (Tab. 8.3). I relate the values}\geq 0.907 (\text{the expected number from a hexagonally packed monolayer})\text{to three factors: 1) As shown in Fig. 8.5(a),(b), the centre-to-centre distance between MUO-NPs was measured at 5.9 \text{nm, i.e. below \(D\). Hence, interdigitation is likely to occur on the substrate, thus increasing the packing sensity of the AuNPs. 2) The significant surface roughness increased the available contact area for grafting. 3) The size dispersity of AuNPs was likely to allow a more efficient packing than for spheres all of the same size. Taking into account the lateral dimension of the AuNP ligand shell, this result suggests a near-unity grafting density onto the sensor surface. Whilst the AuNP immobilisation on gold surfaces built on previous studies,}[375–377]\text{the herein reported method promoted a significantly more efficient and robust AuNP grafting, which in return provided a larger interfacial area for assessment of AuNP-analyte interactions.}

To assess the reactivity of the prepared sensor surface, 10 mM DNSA in 10 mM NMM MeOH solution was introduced to the system after conditioning and equilibration with the
buffer solution of 10 mM NMM in MeOH. As shown in Fig. 8.3(e),(f), the adsorption of DNSA onto the sensor with PBA1-AuNPs (C3) led to a drastic frequency shift of 11 Hz and an AM change of 192 ng cm$^{-2}$. This compared to negligible changes for control sensors C1 (bare gold) and C4 (dPBA1). Significantly, no substantial frequency shift was observed for the C2 sensor with MUO-NPs, suggesting the signal observed for C3 is the result of a specific dynamic covalent interaction with the NP-bound BAs. In approximation, the net AM change can be obtained by subtracting the contribution of non-specific physical adsorption and bulk solution obtained in control channels C1 and C4. Upon PBA-SA binding, the effective molecular weight of adsorbed species can be calculated as following,

$$MW_{eff} = MW_{SA} - 2MW_{H_2O} + MW_{NMM}$$  \hspace{1cm} (8.1)

which takes into account the H$_2$O release and NMM association upon binding.\[373\] The density of bound DNSA on C3 was thus determined to be 2.75 nm$^{-2}$, showing a binding ratio of an average of 73 DNSA molecules per NP. This is particularly encouraging since there were approximately 156 PBA1 ligands on each PBA1-NP surface (calculated from the TGA results in Tab. 8.2), with a significant proportion expected to be inaccessible, being orientated towards the sensor surface. I attribute this high binding ratio of 47%, in combination with the large interfacial area created by the high density AuNP surface grafting, to enable the monitoring of low molecular weight species, previously inaccessible by QCM-D.
8.4.2 Variable screening of PBA-NPs and SA binding

Following the protocol described above, two sets of three fresh sensors were grafted with diOT and then immobilized with PBA1-NP or PBA2-NP, respectively. With the AM change for PBA1-NPs and PBA2-NPs measured at 2077±38 and 1418±40 ng cm$^{-2}$, similar AuNP coverages were achieved for the sensors in the same group (Fig. 8.7). The grafting density of NP immobilisation as well as PBA ligand density calculation is shown in Tab. 8.4.

The binding of two SAs (FSA or DNSA) to each of the PBA-functionalised NPs was assessed at three NMM base concentrations (0.05, 5 and 500 mM) in MeOH. AM change (Tab. 8.4) for each boronate ester complex was calculated from the recorded frequency

![Graphs showing QCM-D monitoring results of sensor preparation](image)

**Figure 8.7:** Stepwise QCM monitoring results of sensor preparation. Three sensors were prepared simultaneously. AM change (5th overtone): (a) diOT grafting and (b) subsequent immobilisation of PBA1-NPs, (c) diOT grafting and (d) subsequent immobilisation of PBA2-NPs.

**Table 8.4:** Sensor ligand density calculation for PBA1-NPs and PBA2-NPs.

<table>
<thead>
<tr>
<th>NP</th>
<th>Weight of NP, ng</th>
<th>NP ligand density, nm$^{-2}$</th>
<th>AM change, ng cm$^{-2}$</th>
<th>Grafting density, cm$^{-2}$</th>
<th>Sensor ligand density, nm$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBA1</td>
<td>5.59E-10</td>
<td>3.8</td>
<td>2077±38</td>
<td>3.79E+12</td>
<td>5.82</td>
</tr>
<tr>
<td>PBA2</td>
<td>3.46E-10</td>
<td>4.7</td>
<td>1418±40</td>
<td>4.22E+12</td>
<td>5.49</td>
</tr>
</tbody>
</table>
Figure 8.8: Variable screening of PBA-AuNP and SA binding. Frequency shift (7th overtone) at NMM base concentration of 0.05, 5 and 500 mM: PBA1-NPs with 5 mM of (a) FSA and (b) DNSA; PBA2-NPs with 5 mM of (c) FSA and (d) DNSA.

Table 8.5: Observed AM change at varying experimental conditions.

<table>
<thead>
<tr>
<th>Sensor type</th>
<th>AM change, ng cm(^{-2})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration of NMM, mM</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>PBA1-NP/FSA</td>
<td>44</td>
</tr>
<tr>
<td>PBA1-NP/DNSA</td>
<td>20</td>
</tr>
<tr>
<td>PBA2-NP/FSA</td>
<td>32</td>
</tr>
<tr>
<td>PBA2-NP/DNSA</td>
<td>20</td>
</tr>
</tbody>
</table>

shift (Fig. 8.8), evidencing a strong dependence on base concentration. At minimal base concentration (0.05 mM NMM), the frequency shift was largely subject to non-specific physical adsorption and bulk solution change. The negligible boronate ester formation confirmed the requirement of a basic environment for stabilising boronate ester complexes. Significantly improved binding affinity was observed for all four complexes when increasing the base concentration to a 1:1 molar ratio of NMM (5 mM) to binding partners. Meanwhile a reduced complex stability was observed at the higher NMM concentration of 500 mM, where the binding affinity for all four complexes was reduced compared to the values.
at 5 mM. These results are in direct agreement with studies of structurally analogous species in solution by quantitative NMR spectroscopy, which showed that a low base concentration (in the range of 1:1 molar ratio) was effective to induce strong boronate ester formation with SAs, contrary to the ultra-high concentrations (> 100 molar equivalents) required to maximise binding with catechols.\[349, 373\] In relation to the low molecular weights of simple SAs, one limitation of the proposed platform is the detection at low concentrations by QCM-D, being a mass-based technique. The frequency shift of the 7\textsuperscript{th} overtone was used for calculation to avoid noisy baselines and I managed to assess the binding strength comfortably at analyte concentration of 5 mM with tolerable experimental errors. I anticipate the limit of detection to be further decreased by alternative binding partners of larger molecular weight.

**Figure 8.9:** Control baseline of non-interacting MUO-NPs. AM change (7\textsuperscript{th} overtone): (a) 5 mM FSA and (b) 5 mM DNSA in 5 mM NMM MeOH solution.

**Figure 8.10:** Binding ratio of SA to PBA calculated from net AM change of 5 mM SA at 0.05, 5 and 500 mM NMM. The error estimation is based on control subtraction and sensor ligand density variation.

To account for physical adsorption and the change of the bulk solution, \textit{i.e.} to estimate the net AM change from boronate ester formation, the control baseline was carefully chosen as the AM change when flowing 5 mM FSA or DNSA on sensors immobilised with
non-interacting MUO-NPs (Fig. 8.9). The impact of NMM was compensated by frequency and dissipation offset for each individual measurement. The ratio of bound SA to total amount of PBA receptors was thus calculated, with the effective molecular weight $\text{MW}_{\text{eff}}$ (from Eq. 8.1) for FSA and DNSA at 221.2 and 293.2 g mol$^{-1}$, respectively, as summarised in Fig. 8.10. Direct comparison of the four complexes at 5 mM NMM reveals that PBA1-NP/FSA yielded the strongest association under these conditions with a net binding of $3.54 \pm 0.51$ FSA molecules per nm$^2$, corresponding to 61% of the surface-immobilised BAs. The quantitative binding is consistent with results from NMR studies on closely analogous model compounds.[373] Also in line with solution-phase studies is the observation of weaker association exhibited by PBA2 complexes compared to each analogous PBA1 complex, which is a consequence of the lower Lewis acidity at the boron of PBA2 bearing an electron-donating amido group.[370, 373, 378] Under the same conditions, PBA1-NP/DNSA achieved a 32% binding ratio (falling to 8% for PBA2-NP/DNSA). For acidic binding partners such as SAs, boronate complex formation is maximised at an optimal concentration of base.[373] Each of the complexes studied exhibited reduced stability at the high base concentration of 500 mM, with the more acidic DNSA binding partner most severely affected. Once more, these findings reflect the behaviour expected on the basis of solution-phase studies.[373, 378]

**Figure 8.11:** Variable screening of PBA-AuNP and SA binding. AM change ($7^{th}$ overtone) at NMM base concentration of 0.05, 5 and 500 mM: PBA1-NPs with 5 mM of (a) FSA and (b) DNSA; PBA2-NPs with 5 mM of (c) FSA and (d) DNSA.
When rinsing with NMM MeOH solution, the release of both SAs was observed, resulting from the dissociation of the boronate esters, thus proving the reversible nature of this dynamic covalent condensation-hydrolysis reaction.\[368\] After extended washing for 1 h, all pre-bound SA molecules were released as indicated by the net frequency shift returning to the same resonant frequency as during NMM MeOH equilibration (Fig. 8.11). Quantitative information on the the binding kinetics of boronate ester formation can be assessed by AM change over time with following equations,\[379–381\]

\[
[PBA] + [SA] \xrightarrow{k_{\text{on}} / k_{\text{off}}} \text{Complex}, \tag{8.2}
\]

\[
\Delta m_t = \Delta m_{\text{max}} [1 - \exp(-t/\tau)], \tag{8.3}
\]

\[
\tau^{-1} = k_{\text{on}} \times [SA] + k_{\text{off}}, \tag{8.4}
\]

where \(k_{\text{on}}\) and \(k_{\text{off}}\) are the association and dissociation rate constants, \(\Delta m_{\text{max}}\) is the maximum mass change and \(\tau\) is the relaxation time.

During rinsing, in particular, \([SA] = 0\), Eq. 8.3 can be written as following.

\[
\tau_{\text{off}}^{-1} = k_{\text{off}} \tag{8.5}
\]

Therefore, the apparent binding constant \(K_a\) can be calculated from following equation.

\[
K_a = \frac{k_{\text{on}}}{k_{\text{off}}} = \frac{\tau_{\text{on}}^{-1} - \tau_{\text{off}}^{-1}}{[SA] \times \tau_{\text{off}}^{-1}} \tag{8.6}
\]

With local fitting of the dissociation and then the association process, the binding parameters for the experiments at 5 and 500 mM NMM solutions were modelled with exponential decay function as detailed in Eq. 8.3. The obtained relaxation time \(\tau\) for both association and dissociation was used to calculate association and dissociation rate constants (\(k_{\text{on}}\) and \(k_{\text{off}}\)) as well as apparent binding constant \(K_a\). The fitting curves are shown in Fig. 8.12 and fitting results are summarised in Tab. 8.6. On top of the kinetic information provided by \(\tau\) values, the binding constants further evidenced the observed effects of NMM concentration as well as the structure of both PBA and SA components. Boronate complex stability depends on the structure of each binding partner, as well as the concentration of base. The absolute complex stability, optimum base concentration, and sensitivity to
Figure 8.12: Kinetic analysis of AM change (7th overtone) at 5 and 500 mM NMM: PBA1-NPs with 5 mM of (a) FSA and (b) DNSA; PBA2-NPs with 5 mM of (c) FSA and (d) DNSA.

changes away from these conditions all depend on the relative and absolute acidities of the two binding partners. To fully map this multi-dimensional variable space and optimise boronate complex formation, traditional methods such as NMR spectroscopy consequently present a significantly time-consuming and costly challenge, which becomes even greater when examining NP-bound systems. Furthermore, it is not commonly possible to extract information on binding kinetics for such labile molecular interactions from NMR studies. Potentiometric methods have traditionally been used to characterise these aspects of boronate esters, whilst such approaches are non-trivial to apply to NP systems and are not well-suited to studies in non-aqueous solvents. By contrast, analysis by QCM-D is intrinsically surface based, with reusable AuNP-grafted sensor surfaces that are readily reactivated simply by extended rinsing. Moreover, the proposed QCM-D method requires small sample quantity, i.e. 20–50 µg AuNPs is sufficient for grafting a sensor surface. QCM-D thus presents a parallel and sample-efficient method for rapid screening and optimising complex formation for variations in both structural and experimental variables, while at the same time extracting detailed kinetic and thermodynamic information.
### Table 8.6: Apparent binding parameters calculated from AM change of 5 mM SA at 5 and 500 mM NMM.

<table>
<thead>
<tr>
<th>Sensor type</th>
<th>Binding parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau_{\text{off}}^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$s$^{-1}$</td>
</tr>
<tr>
<td>5 mM NMM</td>
<td></td>
</tr>
<tr>
<td>PBA1-NP/FSA</td>
<td>0.82</td>
</tr>
<tr>
<td>PBA1-NP/DNSA</td>
<td>0.20</td>
</tr>
<tr>
<td>PBA2-NP/FSA</td>
<td>2.29</td>
</tr>
<tr>
<td>PBA2-NP/DNSA</td>
<td>1.39</td>
</tr>
<tr>
<td>500 mM NMM</td>
<td></td>
</tr>
<tr>
<td>PBA1-NP/FSA</td>
<td>0.62</td>
</tr>
<tr>
<td>PBA1-NP/DNSA</td>
<td>1.52</td>
</tr>
<tr>
<td>PBA2-NP/FSA</td>
<td>2.42</td>
</tr>
<tr>
<td>PBA2-NP/DNSA</td>
<td>4.10</td>
</tr>
</tbody>
</table>

### 8.5 Conclusions

This chapter describes an effective route to study NP-analyte surface interactions in real time using a multichannel QCM-D platform. The method consists of essentially three steps, namely the functionalisation of gold coated quartz crystal sensors with dithiol linkers, the grafting of thiol-stabilised AuNPs with functional end groups and the exposure to the binding partners of interest. We employ this approach for the study of boronate ester formation induced by the binding of SAs to AuNPs with BA ligands. Both variables, the molecular architecture of the BA ligands and the concentration of Lewis bases, led to significant changes to the equilibrium binding ratio. Furthermore, the frequency shift pattern was used to model the kinetics during association and dissociation events. Important characteristics including relaxation time as well association/dissociation rate constants could be calculated from this analysis. In contrast to previous QCM-D studies focusing on macromolecules in the kDa molecular weight range or the use of NPs as signal amplification labels, the proposed method is able to resolve frequency shifts induced by the binding of immobilised AuNPs to analytes in solution, with molecular weight as low as 100–300 Da. This represents an ideal characterisation platform to rationally screen and select AuNP candidates for molecular recognition, adding unique opportunities to the existing tool box for AuNP-analyte interaction.
Chapter 9

Conclusions and outlook

The aim of this doctoral thesis was to explore the use of functionalised AuNPs as a model system to study nanoscale colloidal stability and molecular interaction with small molecules. A modular AuNP synthetic platform was established in Chapter 5. This two-step approach is based on the synthesis of AuNPs stabilised with OAm followed by the subsequent functionalisation with target thiol ligands. The synthesis of OAm-capped AuNPs enabled fine tuning of the core size in the range of 2–7 nm by varying the reaction temperature. The subsequent thiol-for-OAm ligand-exchange from a vast library of thiol ligands allows a reliable generation of thiol-capped AuNPs with target surface functionality and detailed control of mixed-ligand composition. The decoupled control over the AuNP core and ligand shell provides a powerful toolbox for the methodical screening of optimal design parameters and facile preparation of AuNPs with target properties, the implementation of which was used for the fundamental understanding of particle size distribution analysis (Chapter 6), colloidal stability and interaction with small molecules in solution (Chapter 7) and molecular recognition on AuNP surfaces (Chapter 8). The present work was limited to the synthesis with amphiphilic or hydrophobic thiol ligands, but in principle hydrophilic ligands such as thiolated peptides could also be incorporated in a biphasic solution such as DCM-H$_2$O or a monophasic mixture, here DCM-MeOH. Further work is needed to validate the aspect of hydrophilic ligand and investigate the impact of the experimental conditions during ligand exchange (e.g., type of solvents, reaction time or temperature) on final ligand compositions. Another research direction is resolution of ligand-exchange kinetics and the evolution of the ligand shell morphology on mixed-ligand AuNPs when varying ligand compositions, which can be assessed by recently established characterisation techniques utilising SANS and MS with matrix-assisted laser desorption/ionisation (MALDI-MS).[71, 72, 94, 382]

A comparative study of AuNP size distribution was discussed in Chapter 6. TEM, SAXS and AUC were employed to characterise 2–7 nm quasi-monodisperse, polydisperse and bimodal MUS-AuNPs. The conventional TEM imaging method permitted facile characterisation of AuNPs with various size distributions, but it suffered from poor reproducibility and a lack of statistical significance for representing non-uniform samples. Solution SAXS measurement, combined with subsequent model-free MC fitting, enabled accurate estimation of non-monodisperse size distributions with comprehensive statistical analysis for NPs above
3 nm due to the reduced scattering intensity and the interference of small molecules. The AUC SV method allowed comparable estimation of both unimodal and bimodal samples but incurred underrepresentation of the minor populations in non-monodisperse systems. This study offers valuable insights in state-of-art AuNP characterisation by identifying the limitations of each technique in direct comparison. To improve the reproducibility and data interpretation of TEM analysis, further research should prioritise the implementation of non-parametric measures such as the $\chi^2$-homogeneity test.[306] Another direction of future work is to streamline the data interpretation of AUC for the size and shape information of SAM-AuNPs. So far, the widespread application of this powerful technique is limited by convoluted fitting methods including the Custom Grid spectrum analysis.[225, 301]

The findings of the colloidal behaviour of amphiphilic AuNPs presented in Chapter 7 are of high relevance for the fundamental understanding of molecular interactions. The utilisation of X-ray scattering at extreme small angles served as a powerful tool for probing medium- and long-range intermolecular interactions of amphiphilic AuNPs with varying polar and hydrophobic components, which dictates AuNP surface hydrophobicity. Direct experimental proof suggested that tuning this intrinsic property could induce the transformation of the NP system from an electrostatic repulsive regime to a hydrophobic attractive regime. Furthermore, the addition of amphiphilic hydrotropes can alter the AuNP assembly behaviour through a combination of both electrostatic and hydrophobic interactions. A hypothesis based on the multivalent interactions was proposed to explain this phenomenon, which may be prevalent among amphiphilic biotic entities. Further research is needed for the validation of this hypothesis. One direction is the integration of SANS to map the footprints of small molecules on AuNP surface and validate the solvation pattern of AuNPs with the addition of a co-solvent.[329] Alternatively, the herein presented results on AuNPs should make further impacts in guiding in vitro and in vivo protein studies, which involved a complex medium in abundance of electrolytes and small biomolecules.[383]

Chapter 8 described the in situ monitoring method of probing NP-analyte surface interactions via QCM-D. Unlike previous QCM-D studies focusing on macromolecules in the kDa molecular weight range or the use of NPs as signal amplification labels, the proposed method was able to resolve frequency shifts induced by the binding analytes with molecular weight as low as 100–300 Da. In a three-step procedure, functionalised AuNPs with BA ligands were immobilised on thiolated quartz crystal sensor surfaces before the exposure to SA binding partners, which resulted in the formation of boronate esters. The quantitative assessment and kinetic analysis of experimental results suggested the impact of both variables, the molecular architecture of the BA ligands and the concentration of Lewis bases, during association and dissociation events. This characterisation platform is ideally
suited to screen experimental conditions and select AuNP candidates for specific molecular recognition of interest. One direction for future research includes the implementation of this platform in the development of multivalent binding schemes and study competitive binding in complex media, which is of crucial importance for advanced biomedical applications.[354, 384]
Published outcome of doctoral research

[12] "Measuring and regulating hydrophobic interactions between amphiphilic macro-


[10] "A comparative study of sub-10 nm AuNP size distribution using TEM, SAXS and

[9] "DoE-It-Yourself: a case study for implementing Design of Experiments into nanoparticle

[8] "Reversible microscale assembly of nanoparticles driven by the phase transition of

[7] "Multidimensional characterization of mixed ligand nanoparticles using small angle

[6] "Probing the interaction of nanoparticles with small molecules in real time via quartz

[5] "Supramolecular binding of small molecules on self-assembled monolayer protected
gold nanoparticles" - Boccardo S, Marson D, Yang Y, Trzcinski J, Guldin S, Posocco P. NanoMed 2018, hdl.handle.net/11368/2934585

[4] "Chapter 11 - Noble metal nanoparticles with anisotropy in shape and surface function-
ality for biomedical applications" - Marson D, Yang Y, Guldin S, Posocco P. Anisotropic Particle Assemblies, Elsevier, 2018, 313-333

[3] "A versatile AuNP synthetic platform for decoupled control of size and surface composi-

[2] "Phase behaviour and applications of a binary liquid mixture of methanol and a ther-

[1] "pH-Mediated molecular differentiation for fluorimetric quantification of chemotherapeu-

* authors contributed equally to this work.
Bibliography


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Bibliography


Bibliography


Bibliography


Bibliography


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