

Synthesis, Surface Chemistry and Assembly of Plasmonic Nanoparticles towards New Optical Materials

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1. Background

1.1 Plasmonic Nanoparticles

My previous work focused on plasmonic nanoparticles, and in particular gold nanoparticles (AuNP), which can be considered one of the most prominent materials in this class. The intense research on these particles in the past decades is mainly motivated by their strong interactions with light. These interactions lead to a strong focusing of light on the nanoscale, in other words strong enhancements of the electromagnetic field close to the nanoparticles' surface which are often referred to as hot-spots. Additionally, AuNP are excellent absorbers and this absorption can result in energetic electrons, so-called hot electrons, and leads to very efficient local heating. These properties can be utilized in surface-enhanced spectroscopies like surface-enhanced Raman scattering (SERS) and surface-enhanced infrared absorption (SEIRA),¹ plasmonic heating,² catalysis³ and sensing⁴. The functionalization of AuNP surfaces with ligands is well-developed and AuNP are usually non-toxic (although this depends on the details of the geometry and surface chemistry) and therefore they have been studied intensely in the context of nanomedicine and nanobiotechnology.⁵

The research efforts addressing AuNP can be categorized as follows. *Colloidal chemistry* addresses the synthesis of AuNP and their functionalization. Today various shapes, even very complex ones, and a broad size range of AuNP can be synthesized in excellent quality. Open questions include reproducibility, scalability and the understanding of mechanistic details. The surface chemistry of gold is well understood and most prominently the reliable thiol-gold chemistry is used to synthesize even complex coatings with mixtures of different molecules often in a straightforward and reliable manner. Ongoing challenges are the confident structural and quantitative analysis of surface coatings and the control of complex compositions. It is also still of great interest how coatings affect and modulate the properties and interactions of nanomaterials in general, in particular in complex environments as in biological systems, but also in the context of colloidal dynamics and structure formation.

The *physical properties of AuNP* are intensely studied in fundamental work and with respect to their desired application. Ultrafast spectroscopy is used to study absorption, carrier dynamics and plasmon relaxation depending on excitation conditions and structure of the AuNP. This has direct relevance for applications in plasmonic photocatalysis. The same holds true for thermal properties, which are also utilized in nanomedicine for photothermal therapies and in general for applications that benefit from efficient localized heating, for example thermocycling in PCR analyses. The research on plasmonic properties of AuNP and AuNP superstructures is still a huge and very active field with a broad range of topics ranging from sensing to sophisticated optical materials for light manipulation. The structures under study range from isolated nanostructures to large patterned films. In recent years the field of chiral plasmonics has gained significant momentum.

Finally, AuNP are used in thousands of studies that focus not on the AuNP itself, but rather utilize their properties for their according research goal. For example, the AuNP might be used as a sensor or label or tracer in the optical range as well as in the X-ray regime, as local heater, as carrier for targeting and drug molecules or as substrate for SERS.

Very roughly one might categorize the research on AuNP and AuNP superstructures accordingly:

- fundamental colloidal chemistry: synthesis and surface chemistry
- fundamental physics: physical properties
- applied nanoscience: applications utilizing AuNP

This summary covers more than ten years of interdisciplinary research that can be categorized along the same lines. Naturally, a strict separation is not always possible. After a brief repetition of the most important basic concepts the second chapter will present key advances in synthesis and surface functionalization of AuNP. The third chapter describes a selection of coherent small-angle X-ray scattering (SAXS) studies that elucidated the structure of AuNP conjugates and AuNP assemblies or utilized AuNP as tracer material. The fourth chapter summarizes projects on AuNP self-assembly and the properties of self-assembled superstructures of AuNP. Each of these chapters will provide a short introduction into the specific topic and then present the most important publications by the author. Finally, a conclusion and an overview of additional scientific activities is given in chapter 5.

1.2 Localized Surface Plasmon Resonances (LSPR)

Arguably the most intriguing property of AuNP is their interaction with light in the optical range. The two dominant contributions to these interactions are band transitions ($5d \rightarrow 6sp$, threshold: 2.4 eV; 516.6 nm) and the so-called localized surface plasmon resonance (LSPR), often also just referred to as plasmon resonance or plasmon. The LSPR is the resonant coherent and collective oscillation of the conduction band electrons (the “free electron gas”, a plasma) driven by an external electromagnetic field, i.e. light.⁶ Since the theory of plasmonic absorption in single particles and interparticle coupling is well-established and covered in literature (see e.g. Kelly et al.⁶ or Gosh et al.⁷) only the most important aspects for this work will be discussed here. The theory for plasmonic absorption of spherical particles was developed by Gustav Mie in the beginning of the 20th century and is therefore called Mie theory or Mie solution.⁸ It was later refined for non-spherical and ensembles of particles.⁷ The dipolar plasmonic absorption of a spherical metal nanoparticle can be described as:

$$\sigma_{ext}^{dip} = \frac{24\pi^2 R^3}{\lambda} \frac{\varepsilon_m^{\frac{3}{2}} \varepsilon_{im}(\lambda)}{(\varepsilon_{re}(\lambda) + 2\varepsilon_m)^2 + \varepsilon_{im}(\lambda)^2} \quad \text{eq. 1}$$

with the extinction coefficient for the dipolar absorption σ_{ext}^{dip} , the particle radius R , the wavelength λ , the real and imaginary part of the dielectric function of the material $\varepsilon_{re}(\lambda)$ and $\varepsilon_{im}(\lambda)$, and the dielectric function of the medium ε_m .

Herein, the key assumptions are that the frequency-dependence of ε_m can be neglected and the so-called quasistatic approximation that the electric field experienced by all conduction band electrons at a given point of time is uniform. This is the case when the nanoparticle is much smaller than the wavelength of the driving light field. For AuNP < 100 nm in diameter, the dipolar plasmonic absorption is the dominant mode, for larger ones quadrupolar and higher order modes become increasingly important. Also, retardation

effects have to be considered for larger particles because they are no longer negligibly small compared to the light wavelength and accordingly the electric field is no longer uniform throughout the AuNP at a given point of time. Similar considerations hold true for the scattering that is neglected in eq. 1, which is a good approximation for AuNP up to 50 nm in diameter.⁶ The simplified description of the plasmon as an oscillating dipole is helpful for understanding interparticle plasmonic coupling and several theoretical approaches for the description of the plasmonic properties of more complex structures are based on this dipole approximation.^{7,9} From eq. 1 the resonance condition $\varepsilon_{re}(\lambda) + 2\varepsilon_m = \min.$ can be appreciated. This resonance is unique for nanoscale plasmonic materials and leads to their specific colors differing from the bulk materials. From eq. 1 it is also clear that the exact energy of the resonances depends on the dielectric function of the medium. When molecules are bound to AuNP, this can change the dielectric environment and therefore shift the resonance measurably (usually about some nanometers in wavelength). This is the effect utilized in refractive-index (RI) based sensing with plasmonic nanoparticles (NP) but it is also useful for the characterization of nanoconjugates.^{4,10} Eq. 1 also illustrates that the absorption cross-section, but not the energy of the resonance depends on the size of the AuNP. Experimentally, a slight size-dependence is observed, which underlines the limitations of the approximation. For small AuNP (diameters < 20 nm) surface damping effects become increasingly important because the particle dimensions are smaller than the mean free path of electrons in Au (~ 35 nm). It is also discussed if the use of bulk properties (dielectric functions) is still valid for small NP. For larger AuNP (> 40 nm) scattering contributions become more important and also higher order modes and retardation effects as discussed earlier. Nevertheless, the size-dependence is not very pronounced for spherical AuNP ($\Delta\lambda \sim 20$ nm for diameters of 10 nm vs. 60 nm diameter in water). In contrast, the behavior can be completely different for anisotropic AuNP. For instance, for gold nanorods (AuNR) a transversal and a longitudinal mode exist and the energy of the longitudinal mode strongly depends on the aspect ratio. Finally, the strongly distance-dependent interparticle plasmonic coupling has a strong effect on the plasmonic properties.⁷ For example, if AuNP aggregate, this can immediately be observed visually by a strong blueshift of transmission, according to a broadening and redshift of absorption. This behavior, like the RI sensitivity, is useful for both: colloidal characterization and AuNP-based sensing. Understanding the plasmonic properties of AuNP and their dependencies therefore provides a lot of valuable information on nanoparticle concentration, coating, aggregation state/colloidal stability and dispersion medium that can be obtained by straightforward UV/vis-spectroscopy. It is also the basis for understanding the other interesting properties of AuNP: near-field enhancement, hot electron generation and local heating.

The *localized near-field enhancement* of plasmonic nanostructures is a direct consequence of the local modulation of electron density due to their interaction with light. The Coulomb interactions between dislocated electron gas and positively charged Au-cores, which constitute the atomic lattice of the nanocrystal, provide the restoring force in the driven oscillation. The according (oscillating) near-fields can be modeled with various methods based on classical electrodynamics.¹¹⁻¹² For an isolated spherical AuNP under resonance condition, field enhancements of ~ 10-fold, can be calculated. The local field scales as d^{-3} , d being the distance to the nanoparticle surface.¹³ Bringing two plasmonic NP in proximity results in a stronger field enhancement due to plasmonic coupling, and, even more importantly, the region of maximum field enhancement is then located in the center between the two NP. This nanoscale volume of strongly enhanced near-field is called hot

spot. The field enhancement in the hot spot naturally depends on the material, size and shape of the constituting NP as well as strongly on their separation distance. Maximizing the field enhancement by decreasing the interparticle distance has its limits. For edge-to-edge distances below ~ 1 nm, electron tunneling becomes possible and increasingly dominant. The NP can no longer be considered as distinct plasmonic nanostructures if tunneling becomes dominant. Accordingly, the plasmonic interparticle coupling, including the resulting hot spot, breaks down. Any effect that depends on the electric field can therefore be modulated by plasmonic nanostructures creating hot spots. The most prominent example is Raman scattering, which is in itself a weak effect. The intensity of Raman scattering depends on the fourth power of the local field enhancements because both, incoming and scattered photon, are affected. By optimizing plasmonic nanostructures it is now possible to measure Raman scattering down to the single molecule level and SERS with plasmonic nanostructures has become a vibrant field of research.¹⁴ Another interesting application of hot spots is in plasmonic photocatalysis and this will be discussed in more detail later.

The quantized light-driven oscillation of the electron gas, the plasmon, can be described in good approximation as a driven damped harmonic oscillator. As discussed, the restoring forces are the attractive Coulomb forces between electrons and gold atoms/ions of the lattice. The origin of the damping is electron-phonon scattering which leads to Joule heating of the lattice.² The heat is transferred to the local environment (phonon-phonon coupling) and dissipated. Because AuNP are extremely good absorbers and absorbed energy is ultimately converted into heat, they are also very efficient local heaters. The local heating and local temperature profile for a given irradiance and NP geometry can be well-described quantitatively and accordingly structures can be optimized for efficient local heating.² This is interesting for catalysis, local melting, vaporization and photothermal therapies as mentioned, but can also be utilized in more sophisticated techniques like photoacoustic imaging, where local pressure-waves produced by plasmonic heating can improve the signal-to-noise ratio significantly.¹⁵

Another consequence of plasmonic resonances is the generation of so-called hot carriers, hot electrons and/or hot holes, respectively. The generation of hot carriers and their role in plasmonic photocatalysis has been a controversial topic in the last years.¹⁶⁻¹⁹ Experimentally, it is not trivial to prove the role of hot electrons in catalytic processes, because they are extremely short-lived (tens of femtoseconds) and entangled with other contributions. The photophysics of AuNP can be studied with optical ultrafast pump-probe spectroscopy which helped to gain a quantitative understanding of plasmon relaxation. After being activated by a resonant ultrashort pump, the plasmon relaxes in tens of femtoseconds by transferring its energy to the electron gas, thereby creating a non-equilibrium (energy-) distribution including the hot electrons. This distribution equilibrates (thermalizes) within hundreds of femtoseconds to a “warm” electron gas. Then, electron-phonon coupling takes place within picoseconds and ultimately the heat dissipates within many picoseconds. In own contributions in cooperation with Prof. Holger Lange (now University of Potsdam) the details of plasmon relaxation were addressed experimentally, studying the size-, excitation- and crystallinity- (mono- vs. polycrystalline) dependencies of the relaxation dynamics which reveal the electron-phonon coupling strength.²⁰⁻²¹ In additional cooperation with Dr. Matthias Graf and Prof. Manfred Eich (then Technical University Hamburg-Harburg) a combined photoelectrochemistry/spectroscopy-approach

was used to elucidate the different plasmonic contributions: non-thermal (hot electrons and holes) and thermalized carriers, near-field and local heating.²²

1.3 Synthesis and Surface Chemistry of AuNP

Although I have made significant contributions to the field of AuNP synthesis,²³ the theory of nanoparticle synthesis will not be covered here in great detail for several reasons: first, the synthesis was mostly developed in the PhD in is described in the dissertation in great detail, second: most mechanistic aspects are well-established and the open questions are not the focus of this work, and third, other particle syntheses used in the research presented herein are based on published protocols and only minor refinements were made for upscaling or improved reproducibility. Therefore, it should suffice to restrict the discussion here to the most crucial aspects. An excellent review covering the fundamental mechanisms and thermodynamic aspects of nanocrystal syntheses was provided by Thanh et al.²⁴ The majority of the work presented herein is based on two synthesis protocols: a refined Turkevich-method and a seeded-growth approach in the presence of cetyltrimethylammonium chloride (CTAC) or bromide (CTAB). Syntheses of anisotropic nanoparticles like gold nanorods (AuNR) are in principle adaptations of the seeded-growth approach with CTAB. All AuNP syntheses discussed herein use tetrachloroauric acid (HAuCl₄) as the metal precursor. Key parameters to describe a colloidal dispersion are the nanoparticle concentration (typically in the nanomolar (nM) range), the uniformity of the colloids and their dispersity; the relative standard deviation (or coefficient of variation, respectively) of their size distribution, typically expressed in %. The uniformity is not straightforward to quantify and usually used as a qualitative descriptor. For quasispherical NP, geometry parameters like a circularity can be defined to quantify the uniformity (cf. the discussion in Schulz et al.²³, Supporting Information).

The Turkevich-protocol uses sodium citrate in multiple roles: as reducing agent, stabilizer and pH-regulator. Deciphering the complex role of citrate in the synthesis was the key to obtain a protocol yielding a significantly improved reproducibility of the AuNP diameter, dispersity and uniformity of the AuNP as well as a better scalability.²³ This protocol is now established as a standard procedure to obtain AuNP with ~12 nm diameter.²⁵ Advantages of this protocol are its simplicity – experienced chemists can synthesize liters of 3-5 nM dispersion in less than an hour – and the coating ligand citrate, which is not only cheap and available, but also non-toxic and very easy to replace. Accordingly, these citrate-stabilized AuNP (AuNP@Citrate), are an excellent starting material for further functionalization by ligand exchange. The citrate ligand shell provides electrostatic stabilization but no steric stabilization, therefore the stability of the dispersions is limited. At low pH and at high ionic strengths the particles will aggregate because the repulsive electrostatic interactions are screened. Also, centrifugation of these AuNP@Citrate can lead to destabilization and is therefore not recommended if a dispersion without agglomerates or aggregates is desired. When stored at 7 °C (freezing will lead to irreversible aggregation) the as-synthesized dispersions can be stable for years.

AuNP@Citrate can be grown to larger diameters with the seeded-growth protocol presented by Bastùs et al.²⁶ This protocol is well established in the community, again because it is simple and reliable and because of the beneficial properties of the citrate ligand.²⁵ By using a sequence of dilution steps and precursor additions as well as a tailored concentration of the reducing agent and temperature to avoid secondary nucleation,

quasispherical AuNP@Citrate with diameters in the range 10-200 nm can be synthesized. However, the uniformity and dispersity, in particular for large diameters, can be limited. Typically, the dispersities range from 5-10 % for diameters > 40 nm. A different seeded-growth protocol was presented by Zheng et al.²⁷ This approach uses comparably small volumes (ml range), very high CTAB and CTAC concentrations and ascorbic acid as a reducing agent. CTAB and CTAC are cationic surfactants which form micelles in aqueous solution. In the synthesis they are used as a stabilizer, but the formation of a large number of uniform micelles (ensured by the high concentration) presumably also favors the uniform nucleation and growth of mostly single-crystalline spherical AuNP. A smooth uniform growth is additionally favored by a slow continuous addition of the Au-precursor solution with a syringe pump. This protocol is slightly more elaborate than the ones for AuNP@Citrate and it is more sensitive to synthesis parameters but it also has several advantages. Less steps are needed to obtain large particle diameters, because a growth in diameter Δd up to 50 nm is possible starting from 10 nm seeds compared to steps of $\Delta d \sim 2$ -4 nm for the Bastús protocol. The particle quality that can be obtained for AuNP@CTAC is also superior, the uniformity is excellent and for AuNP@CTAC > 40 nm, dispersities down to 1 % (corresponding to absolute standard deviations of < 1 nm) can be achieved, although they are typically in the range 2-4 % (corresponding to an absolute standard deviation of ~ 1 nm). The main disadvantage of the AuNP@CTAC protocol is the use of large amounts of CTAC, which is more expensive than citrate, cytotoxic, and much harder to replace from the AuNP surface than citrate. Although an upscaling of the protocol was achieved, as will be discussed later, the amounts of obtained AuNP are still much smaller than with the Bastús protocol.

It is therefore a matter of application and research context, which synthesis is preferable. If reliable and straightforward functionalization by ligand exchange is required, which is crucial for the design of complex ligand layers for instance, or if cell experiments are planned with the AuNP, AuNP@Citrate are usually the better choice. If the particle quality is beneficial or even crucial, for example in self-assembly experiments or in optical metamaterials with collective plasmon modes, AuNP@CTAC are preferable. Of note, the dispersity of AuNP@Citrate and AuNP@CTAC with diameters of 12 nm are comparable. The differences for these comparably small AuNP are that AuNP@CTAC are mostly monocrystalline and slightly more uniform, whereas AuNP@Citrate are polycrystalline and slightly less uniform. It is actually an interesting observation, that the sphericity seems to be better for monocrystalline AuNP in general.

The most common functionalization strategy for AuNP is based on thiol-gold binding chemistry.²⁸⁻³² Building upon the substantial body of research on so-called self-assembled monolayers (SAMs) of alkanethiolates on gold,³²⁻³³ a detailed understanding of the according structures and dynamics has been achieved. The pioneering research on SAMs has been done with macroscopic planar gold substrates, whereas nanoparticles exhibit a curved surface. However, curvature effects only play a major role for AuNP with diameters below 10 nm.³⁴ An important finding in the context of this work is that, depending on the number of methylene-units, *n*-alkanethiols can form quasicrystalline SAMs on gold surfaces, which are additionally stabilized due to attractive van-der-Waals interactions between the alkyl chains. For *n*-alkanethiols on Au(111) with *n* > 9, chemisorption contributes (126 ± 2) kJ/mol to the enthalpy of desorption, whereas physisorption contributes > 97 kJ/mol according to Lavrich et al.³⁵ 33 kJ/mol of the physisorption

contribution stems from the thiol-group, which means that the attractive interactions of the alkyl chains contribute about one third to the overall enthalpy of desorption, which is a substantial stabilizing effect. Notably, binding of both, thiols and disulfides, to gold is strong so that oxidation of thiols is usually not a concern.³⁶ In my PhD thesis I demonstrated that this effect is also relevant for polymer-coated AuNP.³⁴ To this end a straightforward esterification approach was adopted to synthesize poly(ethylene glycol)- (PEG-) based ligands with different terminating structures, that connect the PEG-polymer with the Au-binding thiol group via an ester bond. This connecting structure is referred to as spacer, and short alkyl chains ($n = 2$, here n refers to the number of methylene groups), long alkyl chains ($n = 10$) and aromatic spacers were compared. Since these results are fundamental for the research in the following years described in this work, the experimental approaches and theoretical background will be explained in more detail in the following.

First, the theoretical description of polymers grafted to surfaces can build upon classical polymer physics with notable foundational contributions from Flory and Alexander and de Gennes.³⁷ The so-called Flory radius R_F describes radius of gyration of a polymer coil in a solvent or a melt derived from random walk statistics of a freely-jointed chain.³⁸

$$R_F = \alpha N^\nu \quad \text{eq. 2}$$

Here α is the monomer length, N the number of monomers in the polymer and ν the Flory exponent or excluded volume parameter, respectively. In a bad solvent or a melt, the polymer chains collapse and the Flory exponent becomes smaller than 0.5, e.g. 1/3 is often used. $\nu = 0.5$ is the result for a simple random walk when the polymer chain behaves as an ideal chain. The according solvent conditions are called theta conditions and for specific polymers according theta solvents can be identified. For good solvents the Flory theory predicts $\nu = 3/5$, which is a surprisingly good estimate considering the simplicity of the model. The best estimate today based on much more complicated models and sets of experiments is $\nu \cong 0.588$.³⁹ Further refinement of the model can be achieved by using the Kuhn length of the specific polymer and the according number of Kuhn segments, which is a statistical “monomer size” (i.e. not the real monomer size) that accounts for stiffness and short-range interactions of the according polymer.^{37, 40} If the number of monomers and monomer length are known, the number of Kuhn segments N_b for a given Kuhn length b can be simply calculated as:

$$N_b = N \frac{\alpha}{b} \quad \text{eq. 3}$$

In other words, both αN and $b N_b$ give the contour length of the polymer, i.e. the length of the polymer in an *all-stretched* conformation. When polymers (and ligands in general) are grafted to a surface, the grafting density σ is an important parameter quantifying the number of polymers (or ligands) per area. Assuming a uniform (square) distribution of the polymers it can be related to the distance of grafting points D as $D = \sigma^{-1/2}$. Based on these parameters, the structure of the polymer coating can be described with the Alexander-de-Gennes formalism.⁴¹⁻⁴⁴ In good solvents at low grafting densities ($D \gg 2R_F$), lateral contact between the grafted polymers is negligible and they adopt a so-called mushroom

conformation, with a resulting shell thickness of $\sim R_F$. At high grafting densities, the polymers adopt a more stretched conformation to avoid polymer-polymer contacts and maximize polymer-solvent contacts. This conformation is called the brush conformation. These regimes are sometimes refined, e.g. into brush and dense brush when it seems necessary for the according research question.⁴⁵ For PEG it has been shown that the conformation of the PEG coating affects important pharmacokinetic properties like immune response and protein adsorption in general.⁴⁵⁻⁵² The hydrodynamic properties and conformations of surface-grafted PEG have also been simulated by elaborate force-field models, confirming the applicability of the Alexander-de-Gennes formalism.⁵³

Based on these considerations, one can conclude that grafting PEG-ligands via long alkylene spacers can lead to higher grafting densities due to the additional enthalpy of adsorption resulting from the contributions of the alkyl chains. This higher grafting density, in turn, can lead to a denser brush conformation of the PEG-shell that has been shown to be advantageous for reduced protein adsorption which is highly desirable for applications in nanomedicine. However, there are additional stabilizing effects, due to this “inner shell” or “hydrophobic shield” as it was termed by Sokolov et al.⁵⁴ These aspects are discussed in the PhD thesis of the author as well as in the according pioneering publication.³⁴ The thickness of the inner layer of alkyl chains can be estimated with ~ 0.7 nm for $n = 10$ based on the structural characterization of alkanethiol-SAMs.³² This inner hydrophobic layer can limit the diffusion of polar species – ions or molecules – to the Au-surface, thereby providing chemical stabilization against unwanted reactions of the Au-surface itself or unwanted displacement reactions at the surface. This was shown in the previous work with etching and displacement reactions. Cyanide etches gold, and when AuNP are exposed to cyanide solutions of sufficient concentration, they will be dissolved. This etching process can conveniently be monitored by UV/vis spectroscopy and plotting the intensity maximum of the plasmon resonance versus time, the etching kinetics can be analyzed.³⁴ If PEG-ligands with a long alkylene spacer ($n = 10$) are used for AuNP functionalization, the etching reaction is much slower because of the effect of the inner hydrophobic shield. As mentioned, such PEG ligands can be synthesized by straightforward esterification. Here, 11-mercaptoundecanoic acid (MUA) was reacted with methoxy-terminated PEG with a molecular weight of ~ 2000 g/mol, the according ligand is named PEGMUA throughout this work, and the molecular weight is indicated as PEGMUA2k. Accordingly, PEGMUA with a molecular weight of 5000 g/mol is named PEGMUA5k. Along the same lines, competitive displacement reactions with small dithiols can be monitored by UV/vis spectroscopy, because the displacement of the PEG-ligands in the presence of high salt concentrations leads to aggregation of the AuNP due to lack of steric stabilization. Again, PEGMUA coated AuNP (AuNP@PEGMUA) were much more stable than AuNP coated with PEG ligands featuring shorter spacers. If, however, a bidentate ligand, e.g. based on the esterification of PEG and lipoic acid $\rightarrow$ PEGLIP is used for functionalization, the AuNP@PEGLIP will be stabilized more efficiently against competitive ligand displacement than AuNP@PEGMUA. The reason is the chelating effect of the bidentate ligand: the probability that both thiol groups are displaced at the same time is small, but when only one thiol group is displaced, it stays in proximity to the AuNP-surface and is therefore likely to form a bond again. The chelate effect of ligands binding to NP-surfaces is an entropic effect and similar to the chelate effect known from coordination chemistry. On the other hand, lipoic acid, and accordingly PEGLIP, has a larger footprint (the inverse of the grafting density, σ^{-1}) and the according ligand layer is less dense than for PEGMUA.

Consequently, AuNP@PEGLIP are less stable against cyanide etching than AuNP@PEGMUA.³⁴

This example illustrates some of the complexities of NP-surface chemistry and stability and introduced the basic nomenclature used throughout this work. When discussing ligands and ligand shells on NP, one usually distinguishes the binding group, the spacer structure (which can be divided in several parts) and the terminal group, which can be used for further functionalization.^{28, 55} When discussing stabilizing ligands, different aspects of NP stability can be distinguished: the chemical stability, i.e. robustness against unwanted surface reactions as discussed, and the colloidal stability, i.e. the stability against aggregation or agglomeration (which is used here in the sense of reversible aggregation). Of note, also unwanted detachment of ligands, so-called ligand washing, can be a crucial aspect for NP stability. It is an equilibrium effect according to Le Chatelier's principle. When functionalized NP are washed (usually by centrifugation) to remove unbound excess ligand, this can shift the "on-off" equilibrium and "wash" off ligands from the NP surface. However, the thiol-gold bond is sufficiently strong, so that this effect usually doesn't play a role for thiol-functionalized AuNP in water. For instance, the stability of AuNP@PEGMUA against cyanide etching is not changed after excessive centrifugation (16 centrifugation steps), which confirms that the grafting density has not changed. This is actually a big advantage, because these NP can be obtained and stored in a pure dispersion without any free ligands or stabilizers. It has to be noted, that the ligand adsorption-desorption equilibrium, however, depends on the solvent, and accordingly the stability can be affected by phase transfer.⁵⁶⁻⁵⁷ Of course, various functionalization strategies exist, which are conceptually different from the direct binding approach discussed here, for example all kind of encapsulation strategies.^{28, 55, 58-60} These will not be discussed here, because they are not in the focus of this work.

Having discussed some aspects of ligand binding and molecular structure of ligand layers (small molecules and polymers) the most important effects of ligands or ligand layers on NPs' properties will be outlined. The fundamental aspect of stabilization was already mentioned. In nanomedicine and nanobiotechnology this aspect is crucial, because biological environments like blood, cytosol, tumor microenvironments etc., can be very challenging for colloidal stability due to high salt concentrations, the presence of various molecules (including small thiols and dithiols) which can competitively displace ligands and high protein concentrations. Protein adsorption is a central topic for the pharmacokinetic properties of nanomaterials, because it can lead to unwanted opsonization or change of properties.⁶¹ PEGylation is one well-established strategy, among others, to minimize unwanted protein adsorption.^{47-51, 54, 62-64} Protein adsorption and also experimental approaches to measure it will be an aspect of several publications presented herein. The solubility of NP is directly related to their colloidal stability. The ligand coating is the interface to the environment and dictates most chemical properties and interactions. Accordingly, if the coating ligand is soluble in a specific solvent, the same usually holds true for the NP coated with the according ligands and this is used in so-called phase transfer protocols.^{25, 65-67} The basic idea of NP phase-transfer is to transfer them into a desired solvent without compromising on their properties and stability and with maximum yield. For example colloidal quantum dots (QD) are often synthesized in organic solvents, so if they are to be used in aqueous buffers, they have to be phase-transferred and stabilized at the same time to avoid quenching of their luminescence by water.⁶⁸ Ligand coatings can be

complex, containing different molecular substructures with different solubilities. In PEGMUA, the alkylene spacer is hydrophobic and would be soluble in hydrophobic solvents, whereas the PEG-part is hydrophilic. This characteristic feature of amphiphilic molecules actually can be used for efficient encapsulation and stabilization of nanomaterials. For example, the encapsulation with amphiphilic copolymers has been very successful.^{58, 69-76} The fine-tuning of NP properties like polarity is also a critical consideration in the controlled assembly of NP in surface-coatings, cluster formation and self-assembly into superstructures.

1.4 Self-Assembly of Nanoparticles

Self-assembly of NP has been observed and discussed since the early days of nanoscience.⁷⁷⁻⁸⁹ Since then a wide range of superstructures has been demonstrated assembled from NP of different materials, sizes and shapes. A number of reviews are available on the topic.^{79, 90-92} I have also contributed a review with focus on, but not limited to, studies using electron microscopy or SAXS-based methods to study self-assembly in situ.⁹³ In principle, many aspects of the self-assembly of NP into ordered superstructures can be described in analogy to classical crystallization: the mechanism of structure formation as well as the resulting structures. Therefore, NP crystallization can also help to understand classical crystallization of atoms, ions and molecules because the time and length scales are more accessible for several analytical techniques.⁹⁴ This idea has been discussed e.g. by Mirkin's group, which contributed a substantial amount of work on the "programmable" assembly of oligomer-functionalized NP.⁹⁵⁻⁹⁹ They coined the analogy of NP and atoms and the DNA-oligomer ligands and chemical bonds. This bond analogy can also be expanded to hydrophobic ligands. If NP with a hydrophobic coating are dispersed in a non-polar solvent like toluene or hexane and this solvent evaporates, the ligand-mediated interparticle interactions become attractive at some point. Above a critical concentration (or volume fraction, respectively), nucleation and growth of crystallites sets in and the NP form a superstructure. The details of this crystallization process depend, often quite delicately, on the parameters of the experiment like evaporation rate, substrate, solvent and dispersion purity, partial pressure of solvent vapors, gravitational forces and so on. It has to be noted that even hard spheres, i.e. NP without any attractive interaction, can crystallize into well-ordered superstructures which can be described by optimization of packing density.⁹⁰ The - somehow counterintuitive - reason for this is entropy. In an ordered crystal with optimized packing density the individual constituents have maximized local degrees of freedom for rattling and vibrational motion. In contrast, in an arrested, jammed state, the overall disorder of the ensemble might suggest that these structures are entropically favored. However, the lack of degrees of freedom on the individual particle level outweighs the ensemble contribution to entropy and actually the well-ordered structures are entropically favored. In fact, strong attractive interparticle interactions are usually not desirable in self-assembly experiments, because they can prevent the reorganization and optimization of the lattice during crystallization, thereby leading to so-called arrested states which are disordered as in aggregation. Experimentally, all kind of structures, ranging from completely disordered aggregates to perfect crystals may be obtained and the key for obtaining desired structures is the balance of the interparticle interactions.¹⁰⁰ The simplest experimental approaches are drop-casting or slow evaporation of the solvent in a tilted vial, other approaches include non-solvent diffusion or the use of gravitational forces.⁹⁰ A remarkable approach for electrostatically induced self-assembly

was developed by the group of Rafal Klajn. They used small NP of different charges (controlled by their coating ligands) and added ammonium carbonate to the dispersions. The high ionic strength screened the electrostatic interactions and the NP were dispersed. Ammonium carbonate in water slowly decomposes into gaseous products: carbon dioxide and ammonia, which evaporate from the dispersion. This way the ionic strength is lowered continuously and the electrostatic interparticle interactions become more and more attractive. The crystallization of micrometer-sized binary NP supercrystals with a variety of crystal structures was achieved.¹⁰¹ This work shows that also other interactions than van der Waals (vdW) forces (as in the self-assembly of NP coated with hydrophobic ligands) or hydrogen bonding (as in the DNA-oligomer base-pairing-mediated assembly) can be utilized for well-ordered self-assembly, namely Coloumb forces here.

An important milestone in the self-assembly of thin crystalline films was the use of liquid subphases in the form of sessile droplets¹⁰² or in the Langmuir-Blodgett technique.¹⁰³⁻¹⁰⁶ The immiscible liquid subphase allows for a better mobility of the NP and even of the formed crystallites in contrast to a solid substrate where adhesion always plays a role. In the optimum scenario this allows for more rearrangements in the growing supercrystals leading to less defects and larger grain sizes. An impressive demonstration was published by the Murray group pioneering the experimental approach which is also central for the work on self-assembly described herein. They assembled binary supercrystals of small NP with hydrophobic alkylene-based coatings on diethylene glycol (DEG) and achieved a variety of crystal structures.¹⁰⁷ Later, the group of Alivisatos expanded this approach by using polystyrene-based ligands to coat the NP.¹⁰⁸ This way the interparticle spacing in the superstructures can be varied in a much wider range and more open lattices can be achieved. To tune the spacing, the molecular weight of the coating ligands is chosen appropriately. These polystyrene-based ligands are also fundamental for the work on NP self-assembly described here, and because they are thiol terminated, they will be abbreviated as PSSH. As for the ligand PEGMUA, the molecular weight will be indicated by a suffix: PSSH2k, PSSH5k and PSSH12k and the coated AuNP will be named AuNP@PSSH. The AuNP diameter will be indicated in the name as well, so e.g. AuNP with a diameter of 20 nm coated with a PSSH with an average molecular weight of ~2000 g/mol will be named AuNP20@PSSH2k. This nomenclature is also used in most publications discussed herein. The main difference of PSSH and PEGMUA is their polarity. PEGMUA is amphiphilic and yields a hydrophilic coating, AuNP@PEGMUA are dispersible in polar solvents like water and ethanol. PSSH is hydrophobic, and AuNP@PSSH are dispersible in non-polar solvents like toluene. Chloroform is a very good solvent that can disperse both AuNP@PEGMUA and AuNP@PSSH. It is often used in self-assembly experiments. The studies described so far were limited to comparably small NP (diameters < 15 nm). The grain sizes (sizes of single-crystalline coherent domains) are usually below 1 μm^2 . Various studies also addressed the assembly of larger PSSH-coated NP.⁹² As a tendency, in particular for simple 2D-hexagonal superlattices, the more or less homogeneously coated areas are much larger (up to cm^2), but the quality of the obtained structures is limited (e.g. no long-range order, limited particle uniformity and dispersity, many defects). The alternative to self-assembly to synthesize large areas with defined plasmonic structures is lithography. Lithographic approaches are in general more expensive and time-consuming but can produce well-defined structures reliably and reproducibly. However, lithographic approaches are limited in terms of resolution. With most sophisticated (and accordingly even more time and resource-consuming) approaches, lateral resolution of ~5 nm can be achieved today.¹⁰⁹

However, for strong plasmonic coupling e.g., even smaller interparticle gaps are desirable. Soft lithography and self-assembly can also be combined, as demonstrated for instance by the group of Liz-Marzán. They used a combination of polydimethylsiloxane (PDMS) stamping and self-assembly of spherical AuNP@PEG to produce 2D lattices of pyramids consisting of close-packed (fcc) AuNP@CTAC.¹¹⁰ The pyramidal supercrystals had a base of $\sim (2 \times 2) \mu\text{m}$ and a height of 1-1.5 μm and were arranged in square lattices with pitches in the μm range. Well-ordered structures over several mm^2 were obtained. It is noteworthy, that the same AuNP@PEG do not form supercrystals upon drying without template, just very small patches ($\sim 1 \mu\text{m}$ range or smaller) with 2D hexagonal order. This underlines that the confinement in the pyramidal voids of the template was essential for the formation of well-defined supercrystals in this approach. The templating approach was further refined and optimized by this group and others and large area plasmonic superlattices (here in the sense of supercrystals, individual plasmonic structures or assemblies arranged in a 2D superlattice) with lattice constants in the range from hundreds of nm to micrometers can be produced. In such structures the diffractive modes of the 2D superlattices and the plasmonic modes of the lattice constituents can couple to obtain exceptionally narrow resulting modes which can be utilized in optical metasurfaces, for plasmonic lasing and in sensing applications, e.g. based on SERS.¹¹¹⁻¹¹⁷ This is one example of hierarchical assembly, where a self-assembled structure is integrated into a another periodic structure. In their publication on templated assembly of pyramidal superstructures, Hanske et al. also present a synthesis approach for AuNP with large diameters ($> 20 \text{ nm}$) and discuss the importance of AuNP uniformity and dispersity for self-assembly. Self-assembly, the integration of self-assembled structures into devices and higher order structures and the properties and applications of self-assembled structures is an area of ongoing intense research. Fundamental research still explores the structural variety and addresses the mechanisms of structure formation both experimentally and theoretically, and the emerging properties which are possible.¹¹⁸⁻¹²⁹ Integration into devices and applications receives increasing interest.^{116, 130-132} Naturally, only a brief overview is provided here, rather than a comprehensive review. However, this discussion of some key developments, aspects, and examples aims to enhance the understanding of the context and significance of the work presented later.

1.5 Intermediate Summary

This first chapter explained the basic concepts of AuNP synthesis and surface chemistry, plasmonic properties and self-assembly which are important for the understanding of the research summarized in the following chapters. More detailed aspects will be introduced in the beginning of every chapter, along with the discussion of the general relevance and own contributions.

2. Expanding the concept of the amphiphilic PEG-ligand PEGMUA

2.1 Key publications and author contributions

Key publications:

- Schulz, F.; Friedrich, W.; Hoppe, K.; Vossmeier, T.; Weller, H.; Lange, H., Effective PEGylation of gold nanorods. *Nanoscale* **2016**, 8, 7296.¹³³
- Schulz, F.; Dahl, G. T.; Besztejan, S.; Schroer, M. A.; Lehmkuhler, F.; Grübel, G.; Vossmeier, T.; Lange, H., Ligand Layer Engineering To Control Stability and Interfacial Properties of Nanoparticles. *Langmuir* **2016**, 32 (31), 7897.¹³⁴
- Hoeg, F.; Schulz, J.; Graf, S.; Salah, D.; Chandralingam, S.; Maison, W.; Parak, W. J.; Schulz, F., Defined Coadsorption of Prostate Cancer Targeting Ligands and PEG on Gold Nanoparticles for Significantly Reduced Protein Adsorption in Cell Media. *Journal of Physical Chemistry C* **2022**, 126 (48), 20594.⁴⁷

I had a lead role in all of these projects (corresponding author), coining the idea and developing the AuNP or AuNR functionalization and characterization strategies. Students contributed to the investigations as part of research labs, bachelor theses and master theses under my supervision (G. Dahl, S. Graf, F. Hoeg, J. Schulz). W. Friedrich (AG Weller, University of Hamburgⁱ) led the syntheses of AuNR@CTAB. S. Besztejan did the cell experiments in the according publication. M. Schroer and F. Lehmkuhler (AG Grübel, Deutsches Elektronen-Synchrotron, DESY, Hamburg, Germany) led the SAXS experiments and the analysis of SAXS-data. S. Chandralingam (AG Maison, University of Hamburg) co-supervised F. Hoeg and led the synthesis of functional ligands and controls. T. Vossmeier, K. Hoppe, H. Lange, G. Grübel, W. Maison, W. J. Parak and H. Weller were involved in mentoring and supervision and fruitful discussions addressing the research design and methods as well as the publication process.

2.2 Background

Ongoing key challenges for many nanomaterials are scalability, reproducibility and stability. Additionally, the characterization of functionalized NP can be challenging, which addresses parameters like grafting density and exact composition (and structure) of the ligand coatings. In principle, many classical chemical characterization methods like NMR and IR-spectroscopy may be used, but they often face limitations due to the nanostructure itself, or due to the small amount of sample. In NMR the slow diffusion of NP (with diameters $> \sim 5$ nm) leads to a strong broadening of the peaks, significantly limiting the information obtained by NMR experiments.¹³⁵ For NP with small diameters such broadening effects can be used to distinguish bound and unbound ligand, quantify ligand exchange and study the structure of ligand layers,¹³⁶⁻¹³⁷ but for NP diameters > 10 nm the technique is of limited use without sophisticated experimentation. PEGMUA2k allows for a high grafting density of about 3 ligands per square nanometer, according to a footprint of 0.33 nm^2 . AuNP with a diameter of 12 nm exhibit a surface of 452 nm^2 assuming sphericity. Accordingly, one AuNP is coated by 1356 ligands on average. The typical AuNP concentration obtained by a Turkevich-based synthesis of AuNP@Citrate is in the range 3-

ⁱ all affiliations refer to the time when the research was conducted

5 nM and consequently, the nominal coating ligand concentration (assuming a purified sample without free ligands) is in the lower micromolar range, which is too low for many chemical standard characterization techniques. One strategy to circumvent this limitation is to concentrate the AuNP (or AuNR) to much higher concentrations. Since this is done by centrifugation, a purification effect (removal of unbound ligands) is achieved at the same time. Experimentally, this brings up the question of scalability, reproducibility and stability mentioned earlier. If the colloidal stability is insufficient, an effective concentration of the dispersion is not possible. Assuming for instance a yield of 60% in each centrifugation step, i.e. 40% of the NP are lost due to aggregation or irreversible adhesion to vessel walls, the overall yield after four centrifugation steps would be 13%. Of note, it becomes increasingly challenging with increasing NP concentrations to achieve a high yield in centrifugations, because agglomeration and aggregation become more likely, due to the higher pressure (proportional to the pellet's mass) acting on the pellet during the process. NP quality (dispersity and uniformity) also play a role. The calculation of grafting densities assumes sphericity (for AuNP) and is usually done with the arithmetic mean of the diameters. This is a good approximation as long as the diameters are normally distributed with a low dispersity and the uniformity is high, but with increasing dispersity and non-uniformity the errors can become considerable when the square (area-based calculations as for the grafting density) or cube (volume-based calculations as for NP concentration) values of the diameter are used. Standard characterization techniques for AuNP include transmission-electron-microscopy (TEM) for diameters, dispersity and uniformity of the NP-cores,⁷⁴ dynamic light scattering (DLS) for hydrodynamic diameters¹³⁸ and UV/vis-spectroscopy for AuNP concentration.¹³⁹⁻¹⁴⁰ From the difference of hydrodynamic diameters, d_h , and core diameters, d_{core} , the thickness of the coating can be determined, although one has to keep in mind that the resolution of DLS is limited. DLS reacts very sensitively to the presence of clusters, aggregates or agglomerates and contaminations in the sample, because the scattering intensity scales with d^6 . On the one hand, this provides a good method to test for the presence of aggregates in the sample, on the other hand it can be challenging to obtain meaningful results in the presence of aggregates. This, once more, underlines the role of colloidal stability, not only for applications but also for colloidal characterization, and similar considerations hold true for various characterization techniques and experiments. A good method to determine the grafting densities of polymers on NP is thermogravimetric analysis (TGA), however it requires comparably large sample amounts which are thermally decomposed during the measurement.¹⁴¹ Also the sample purity is crucial for TGA experiments. Although some studies attempt to distinguish bound and unbound ligands¹⁴² from the $m\%(T)$ (mass percent as a function of temperature) profiles, the accuracy of such approaches is limited for polymer-coated AuNP. Another powerful characterization method for NP is SAXS^{74, 143-144} which in particular usually provides superior statistics compared to TEM. A number of examples of advanced materials' characterization based on coherent-SAXS at synchrotron sources will be discussed in the next chapter. The indirect characterization of chemical and colloidal stability by using chemical reactivity and UV/vis-monitoring was introduced in the previous chapter. These methods were adopted for AuNP@PEG characterization in the PhD thesis of the author and also used in some of the studies presented here. Another experiment that was used is gel electrophoresis, which has proven to be very useful for the characterization of mixed ligand layers. Detailed discussions will be provided in the following.

2.3 Effective PEGylation of gold nanorods

Publication:

- [Schulz, F.; Friedrich, W.; Hoppe, K.; Vossmeier, T.; Weller, H.; Lange, H., Effective PEGylation of gold nanorods. *Nanoscale* **2016**, 8, 7296.](#)¹³³

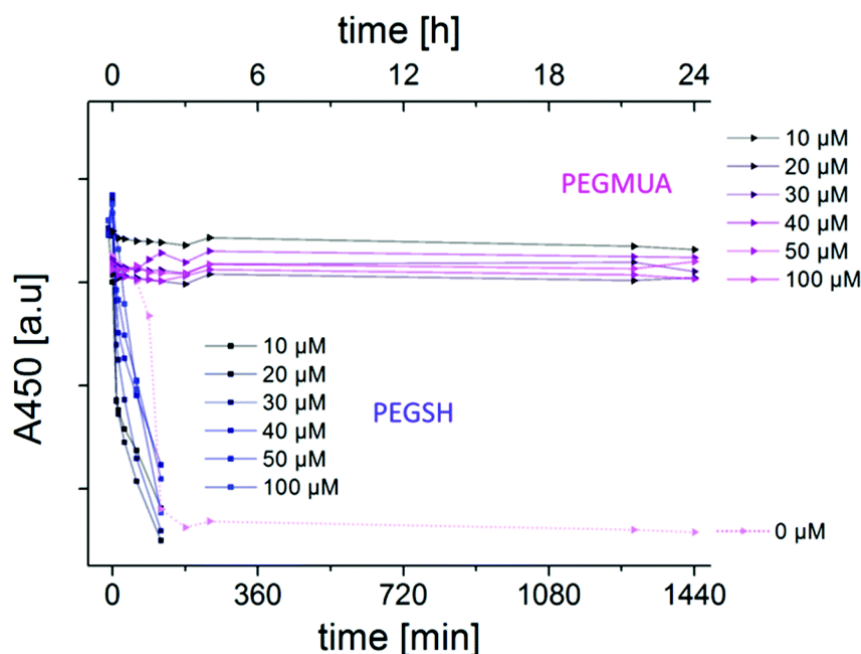


Figure 1. Absorbance at 450 nm (A450) of AuNR@PEGSH (blue squares) in the presence of 5 mM cyanide and of AuNR@PEGMUA (magenta triangles) in the presence of 25 mM cyanide versus the reaction time. The concentrations indicated by the color code correspond to the additions of the corresponding PEG-ligand in the functionalization. The preparative conditions were the same for both sets of samples. Lines are a guide to the eye. AuNR@PEGMUA were stable, even at the 5 times higher cyanide concentration, whereas AuNR@PEGSH were etched within a few hours.

AuNR are interesting for nanomedicine because of their tunable optical properties, including strong absorption in the NIR where the absorption of biological tissue is minimal. (so-called biological window)⁵ Since PEGylation is a well-established strategy to provide nanomaterials with biocompatibility and favorable pharmacokinetic properties, the PEGylation of AuNR is also desirable. The typical coating for AuNR is CTAB which is cytotoxic.¹⁴⁵⁻¹⁴⁶ This actually led to several wrong interpretations of cell experiments assuming a toxicity of the AuNR itself when in fact a toxic effect of CTAB was much more likely.¹⁴⁵ Because of the toxicity of CTAB, it is important to remove it quantitatively by the ligand exchange (e.g. with PEG) and subsequent purification before in vitro or in vivo experiments. However, CTAB is much harder to replace than citrate and it has proven to be quite difficult to achieve high grafting densities of PEG-ligands on AuNR.¹⁴⁷ A key study in this context was provided by Hore et al.¹⁴⁸ Based on SANS measurements they showed that the number of surface-bound PEG after a standard ligand exchange protocol was as low as 5–10 PEG-thiols, corresponding to a grafting density of $\sigma = 0.002 \pm 0.0005$ chains per nm², leaving the CTAB-layer virtually unchanged.¹³³ The study presented in this subchapter first aimed to develop and establish a cyanide etching/UV/vis monitoring based protocol to characterize the ligand exchange of AuNP@CTAB with PEGMUA. The basic idea is that the stability of AuNP@CTAB against cyanide etching is much lower than that of

AuNP@PEGMUA. It was demonstrated that the stability gradually increases with the amount of bound PEGMUA. With this method the conditions for ligand-exchange were studied and optimized and an efficient protocol was proposed. Additionally, a discussion of the role of PEGMUA structure in the ligand exchange is provided and it is proven that PEGMUA provides much better stability than PEG-thiol ligands without a MUA-spacer (Figure 1).

Significant experimental effort was devoted to the quantification of the grafting density based on quantitative NMR in combination with elementary analysis. These experiments also quantified the amount of silver, that is used in the synthesis of AuNR and is also cytotoxic. The relevance of this study lies in the fact that the highest reported grafting density of PEG on AuNR was achieved and experimentally confirmed. The determined value of $2.9\text{--}3.3\text{ nm}^{-2}$ is comparable to AuNP@Citrate after ligand exchange with PEGMUA2k. It is well in the very dense brush regime which is usually defined for grafting densities $> 1\text{ nm}^{-2}$ for PEG ligands of this molecular weight.⁴⁵ The presented ligand exchange protocol is straightforward and effective. The combination of advantageous ligand structure (PEGMUA) and chloroform extractions to remove CTAB efficiently leads to a protocol that is more reproducible, much faster (1-2 h vs. days), and four times less ligand consuming.



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Effective PEGylation of gold nanorods†

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Standard procedures to coat gold nanorods (AuNR) with poly(ethylene glycol) (PEG)-based ligands are not reliable and high PEG-grafting densities are not achieved. In this work, the ligand exchange of AuNR with PEGMUA, a tailored PEG-ligand bearing a C₁₀ alkylene spacer, is studied. PEGMUA provides AuNR with very high stability against oxidative etching with cyanide. This etching reaction is utilized to study the ligand exchange in detail. Ligand exchange is faster, less ligand consuming and more reproducible with assisting chloroform extraction. Compared to PEG ligands commonly used, PEGMUA provides much higher colloidal and chemical stability. Further analyses based on NMR-, IR- and UV/Vis-spectroscopy reveal that significantly higher PEG-grafting densities, up to ~3 nm⁻², are obtained with PEGMUA. This demonstrates how the molecular structure of the PEG ligand can be used to dramatically improve the ligand exchange and to synthesize PEGylated AuNR with high chemical and colloidal stability and high PEG grafting densities. Such AuNR are especially interesting for applications in nanomedicine.

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Introduction

To enable the wide use of gold nanorods (AuNR), the control of their surface chemistry is fundamental.^{1–6} However, the ligand exchange of cetyltrimethylammonium bromide (C₁₉H₄₂BrN, CTAB), the typical surfactant and stabilizer for AuNR, is much more challenging than *e.g.* the exchange of citrate on spherical gold nanoparticles (AuNP).^{7,8} This is a key challenge in the development of safe nanomedicines and AuNR-based probes for biological imaging because CTAB can be cytotoxic.⁹ CTAB forms a densely packed bilayer or admicelle at the AuNR surface that requires a sufficiently high concentration of unbound CTAB to stay intact and to provide effective stabilization of the AuNR.^{10–15} Especially the surface bound CTAB is difficult to remove and the surface chemistry is complicated by significant amounts of silver on the AuNR surface that is needed in their synthesis to promote anisotropic growth.^{6,16,17} Indrasekara *et al.* have recently reviewed the current strategies for AuNR ligand exchange and emphasized the challenge of analyzing the AuNR surface chemistry.⁷

Problems during the ligand exchange of CTAB stabilized AuNR, AuNR@CTAB, include concentration losses due to aggregation and incomplete exchange.^{6–8} Large amounts of residual CTAB result in a higher toxicity of the AuNR but also affect their stability.^{5,18–21} Polymers can be used as ligands to stabilize the AuNR and among these, poly(ethylene glycol)-(PEG-) thiols are probably the most important ones.^{6–8,22} PEG-ligands are well studied and established in nanomedicine.^{23,24} They can provide biocompatibility and stability and are readily available. Accordingly, many strategies have been studied that aim for functionalization of AuNR with PEG, PEGylation.^{22,25–29} However, although stabilization and reduced toxicity can be achieved, considerable amounts of CTAB often remain on the AuNR surface, AuNR-stability during ligand exchange and purification can be problematic and just low or moderate grafting densities of PEG are achieved.^{7,8,30} Recently, Hore *et al.* have used small angle neutron scattering (SANS) experiments to show that quantities as low as 5–10 PEG-thiols, corresponding to a grafting density of $\sigma = 0.002 \pm 0.0005$ chains per nm², are bound to the AuNR surface after a standard ligand exchange protocol, leaving the CTAB-layer virtually unchanged.¹² A higher grafting density of the PEG ligands would be highly desirable, not only for replacing more CTAB and increasing steric stabilization, but also because it is favorable in terms of immune response.³¹

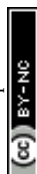
It is established that an effective ligand exchange of AuNR@CTAB critically depends on the effective removal of surface-bound and free CTAB.^{6–8,14} In the context of PEGylation, promising approaches include the use of additives,^{26,27} buffers²⁹ or ethanol²⁵ to assist the CTAB removal. Beyond PEGylation, chloroform³² and temperature³³ assisted strategies were

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†Electronic supplementary information (ESI) available: TEM and STEM-EDX analyses of AuNR, ATR-FTIR spectra of AuNR and ligands, UV/vis-spectroscopy based experiments addressing the ligand exchange and the stability of PEGylated AuNR, discussion of residual CTAB, NMR-spectra of etched PEGylated AuNR. See DOI: 10.1039/c6nr00607h



reported. In all these strategies, sufficient stabilization of the AuNR has to be ensured, *e.g.* by using stabilizers, 2-step procedures or sonication to prevent aggregation. Good results, even with quantitative CTAB removal,³⁴ were obtained especially with molecules that have a structure similar to CTAB, namely a long alkyl chain, at the surface-binding group.^{28,33,35} We thus investigated whether a structural similarity at the surface-binding group can also be utilized for the PEGylation of AuNR.

The incorporation of a C₁₀ alkylene spacer between the thiol-group and the PEG-moiety can dramatically increase the stability against oxidative etching with cyanide, as already observed for spherical gold nanoparticles (AuNP).³⁶ It is hypothesized that C₁₀-spacer favor a high grafting density *via* attractive hydrophobic interactions (in contrast to repulsive interactions of the hydrophilic PEG chains in water) and form an inner hydrophobic region in the ligand layer. This inner hydrophobic region constitutes an effective barrier against reactions at the particles' surface. Competitive displacement of PEG-ligands by small molecules, as *e.g.* present in plasma (as cysteine) and leading to opsonization and phagocytosis can be prevented by such a hydrophobic region as demonstrated by Larson *et al.*³⁷ These results show that rationally designing the molecular structure of PEG-ligands can be an effective tool to improve the stability and biocompatibility of nanomaterials. In the case of AuNR, CTAB provides very little stabilization against oxidative etching with cyanide (despite its postulated bilayer structure with hydrophobic interior). On the contrary, α -methoxypoly(ethylene glycol)- ω -(11-mercaptoundecanoate) (PEGMUA), a PEG-ligand with C₁₀-spacer, provides excellent stabilization in this respect as shown herein. Utilizing this large difference in stability of CTAB-coated and PEGMUA-coated AuNR against cyanide etching, the according ligand exchange was studied in detail.

We compared the displacement of CTAB by PEGMUA in water and the displacement in water assisted by extraction of CTAB with chloroform. The latter strategy was proposed for the first time by Takahashi *et al.* for the exchange of CTAB with phosphatidylcholine.³² The authors found that CTAB in solution was efficiently removed by chloroform extraction and that the amount of surface bound CTAB was reduced. To achieve a high grafting density, very high stabilization and good biocompatibility,^{37–39} PEGMUA is an excellent candidate for ligand exchange. Still, we show herein, that the ligand exchange can be improved effectively when combined with chloroform extraction. In additional experiments we compared the performance of PEGMUA to that of a commercially available PEG-ligand PEGSH, which has the same molecular weight as PEGMUA, but bears no alkylene spacer. PEGSH provided much less stabilization against oxidative etching and less colloidal stability than PEGMUA. ATR-FTIR-experiments strongly suggest that, using the same concentration and procedure, substantially more PEGMUA than PEGSH is ultimately bound to the AuNR. The grafting density of PEGMUA on AuNR was assessed with quantitative NMR and found to be sufficiently high to constitute a dense PEG-brush. Our experiments demonstrate that the use of PEGMUA significantly promotes the ligand

exchange reaction and enables highly efficient PEGylation. As a result, PEGylated AuNR with high chemical and mechanical stability and PEG grafting densities are obtained.

Experimental section

Materials

The AuNR syntheses, PEGMUA synthesis and subsequent experiments were performed with the following chemicals: ascorbic acid (99%, Sigma-Aldrich), cetyltrimethylammonium bromide (CTAB, 99%, Sigma-Aldrich), hydrochloric acid (HCl, 37%, Merck), nitric acid (70%, Merck), potassium cyanide (KCN, 96%, Sigma-Aldrich), silver nitrate (99%, Sigma-Aldrich), sodium borohydride (98%, Fluka), sodium oleate (97%, TCI), tetrachloroauric acid trihydrate (99%, Sigma-Aldrich), 11-mercaptoundecanoic acid (95%, Sigma-Aldrich), poly(ethylene glycol) monomethyl ether ($M_n \sim 2000$ g mol⁻¹, Sigma-Aldrich), α -methoxy- ω -mercaptopoly(ethylene glycol) ($M_n \sim 2000$ g mol⁻¹, Rapp Polymere GmbH, Tuebingen, Germany), iodoform (99%, Sigma-Aldrich). Ultrapure water (18.2 M Ω cm, Millipore) was used for all procedures. Solvents were used in p.a. grade. Chloroform-d₃ (CDCl₃, 99.8% with 0.03% tetramethylsilane, TMS) and deuterium oxide (D₂O, 99.9%) were from Deutero GmbH (Kastellaun, Germany).

UV/Vis-spectroscopy

Absorbance spectra were recorded using a Varian Cary 50, a Perkin Elmer Lambda 25 or a Perkin Elmer Lambda 1050 spectrometer. UV-micro-cuvettes sealed with lids (Plasti-brand®, Carl Roth GmbH, Karlsruhe Germany) were used for all experiments. AuNR-concentrations were determined by the method reported by Liz-Marzán's group using the absorbance at 400 nm.⁴⁰ The method is based on the idea that 0.5 mM gold causes an absorbance of 1.2 at 400 nm, regardless of the particles' shape if they are not too big.

ATR-FTIR spectroscopy

FTIR-spectra were recorded with a Varian 660 FTIR spectrophotometer equipped with a PIKE MIRacle™ ATR sampling accessory. Spectra of PEGMUA, CTAB and MUA were recorded from the pure solids pressed onto the crystal (diamond) with a high pressure clamp. To record spectra of AuNR samples, 2–3 μ l of the according solution were pipetted directly onto the crystal. After evaporation of the solvent (usually within 15–30 min) spectra were recorded. Afterwards the crystal was thoroughly cleaned with water and ethanol. 32 scans in absorbance mode with 4 cm⁻¹ resolution were recorded for each measurement.

¹H-NMR spectroscopy

¹H-NMR spectra were recorded using a Bruker AV2-400 NMR spectrometer operated at 400.13 MHz and 298 K.

Mass spectrometry

MALDI-TOF/TOF-measurements were done with a Bruker UltrafleXtreme mass spectrometer. 2,5-Dihydroxybenzoic acid



(20 mg ml⁻¹ in 30% acetonitrile, 0.1% trifluoroacetic acid, water) was used as a matrix for matrix assisted measurements. Concentrated and purified AuNR-samples were drop-casted onto a ground steel target for measurements and ionized by a smartbeam-II laser.

Graphit furnace atom absorption spectrometry (GF-AAS)

GF-AAS measurements were performed with a ContraA-700 AAS-spectrometer (Analytik Jena, Germany) at 242.795 nm (Au) or 328.068 nm (Ag). The limit of detection (LOD) was 10 µg l⁻¹. Measurements were performed in triplicates and the relative standard deviation of the mean was 1–5%.

Transmission electron microscopy, TEM

TEM measurements for standard characterization of the AuNR were performed using a Jeol JEM-1011 operating at 100 kV. Samples were prepared by drop casting ~10 µl AuNR solution onto carbon-coated copper grids. HR-STEM-EDX measurements were performed using a JOEL JEM 2200 FS (UHR) with a CESCOR and a CETCOR corrector and an JEOL JED-2300 T EDX with 50 mm² Si(Li) detector at 200 kV.

AuNR@CTAB synthesis

AuNR were synthesized based on the seeded-growth method developed by the Murray group using binary surfactant mixtures.⁴¹ It was essential for the success of the syntheses to store sodium borohydride under nitrogen atmosphere. The preparation was divided into a seed nucleation and a growth step. All glass devices were carefully cleaned with aqua regia [Caution! Aqua regia is hazardous. Handle with extreme care and dispose of unused material using methods appropriate for your workplace] and subsequently washed with ultrapure water before use. The basic synthetic protocol was as follows. For the growth solution, CTAB (7.00 g, 19.2 mmol) and sodium oleate (1.234 g, 4.053 mmol) were dissolved in water (250 ml) at 50 °C. After the solution had cooled down to 30 °C, AgNO₃ solution (18 ml, 4.0 mM) was added and the solution was kept undisturbed at 30 °C for 30 min. Subsequently, Au³⁺ solution (250 ml 1.0 mM) was added and the orange-yellow mixture was stirred until it was colorless for 90 min with 700 rpm at 30 °C. In between, the seed solution was prepared. A CTAB solution (5 ml, 0.2 M) was mixed with HAuCl₄ solution (5 ml, 0.5 mM) in a 20 ml glass vial. The yellowish solution was stirred with 1200 rpm and freshly prepared sodium borohydride solution (600 µl, 10 mM) filled up with water to 1.0 ml was quickly injected. After 2 min of stirring the solution was aged for 30 min while it changed its color from blue-grey to amber. To the now colorless growth solution HCl (2.1 ml 12 M) was admixed while stirring with 400 rpm. The speed was increased to 1200 rpm and ascorbic acid solution (1.25 ml, 64 mM) and after 30 s of stirring seed solution (0.8 ml) were added. The solution was stirred for another 30 s. Then the stirring was stopped and the growth solution was aged for 14 hours while it became brownish-pink. The AuNR were centrifuged for 30 min at 5000g and the supernatant replaced by water to yield

Table 1 AuNR batches used in this study

AuNR batch ^a	Variation	Length [nm]	Width [nm]	Aspect ratio
1	—	86.7 ± 7.6	29.0 ± 2.1	3.0 ± 0.4
2	—	85.7 ± 9.2	28.9 ± 2.4	3.0 ± 0.4
3	—	80.0 ± 12.8	26.1 ± 1.8	3.1 ± 0.4
4	900 µl NaBH (10 mM)	75.4 ± 7.4	21.4 ± 1.6	3.6 ± 0.4
5	200 rpm stirring ^a	80.1 ± 6.1	25.7 ± 1.7	3.1 ± 0.4
6	No stirring ^a	82.5 ± 8.0	26.3 ± 1.8	3.2 ± 0.4
7	12.0 ml AgNO ₃	81.6 ± 7.6	28.2 ± 2.3	2.9 ± 0.3

^a During NaBH addition.

a final volume of 160–170 ml per batch. Variations of the basic synthetic protocol are listed in Table 1.

PEGMUA synthesis

α-Methoxypoly(ethylene glycol)-ω-(11-mercaptoundecanoate) (PEGMUA) was synthesized by esterification of poly(ethylene glycol) monomethyl ether and 11-mercaptoundecanoic acid and characterized as described previously.^{36,42}

Ligand exchange with PEGMUA or PEGSH

Stock solutions of PEGMUA and PEGSH were 1 mM or 10 mM in water. For ligand exchange the according volumes were pipetted to AuNR solutions to the desired concentrations. In concentration rows all samples were adjusted to the same final volume, typically 1.000 ml, with water to ensure the same final AuNR-concentration. During the reaction time the mixtures were agitated by a magnetic stirrer or a lab shaker. For studies of the ligand exchange the reaction mixtures were incubated in centrifugal tubes, glass vials or directly in cuvettes. Large batches were synthesized in glass bottles.

Etching experiments

Stock solutions of KCN were 1.00 M in water, they were stored at -18 °C [KCN is hazardous. Handle with extreme care. All solutions should be stored in clearly labeled and tightly sealed containers. Dispose of unused material using methods appropriate for your workplace]. Because AuNR-solutions are typically acidic (pH ~ 3) it is important to ensure that the pH after KCN addition is sufficiently high (>11) to avoid formation of hydrogen cyanide (HCN, pK_a = 9.2). If necessary, this can be done *e.g.* by centrifugation of the AuNR and replacement of the supernatant by water to remove residual HCl from the AuNR synthesis and/or addition of base. KCN acts as a strong base and in our experiments KCN concentrations were sufficiently high to ensure a pH > 11. The etching reactions were started in a fumehood by pipetting the according volumes of KCN_{aq} into the cuvette containing the AuNR solution. The cuvette was then tightly sealed with a lid and vigorously shaken once. During the etching reactions the cuvettes were not agitated and kept at room temperature. Absorbance spectra were recorded at different reaction times of the etching reaction.



Chloroform extraction without centrifugation

AuNR@CTAB (20 ml, 3.0×10^{-2} nM, batch 1) were mixed with PEGMUA solution (50 μ l, 10 mM, $c_1(\text{PEGMUA}) = 25 \mu\text{M}$) in a separation funnel. The solution was extracted fivefold with chloroform (20 ml for each extraction). In the first separation steps an emulsion or mixed phase formed. This can be avoided by removing more CTAB before the extraction, e.g. by centrifugation. After 2–3 extractions the phases separated nicely. The AuNR concentration (3.0×10^{-2} nM) was not changed by the extraction.

Chloroform extraction with centrifugation

AuNR@CTAB (10 or 20 ml, $2\text{--}3 \times 10^{-2}$ nM, batch 1) were reacted for $t_1 = 22$ h with PEGMUA at the desired concentration. Then the mixtures were extracted fivefold with chloroform (10 ml for each extraction) using a separation funnel as described above. After extraction the solutions were purified by fourfold centrifugation (7000–9000g, 10–20 min) replacing the supernatants with water. The concentrations were then adjusted *via* the samples' absorbance to 9.3×10^{-3} nM.

Comparison of AuNR@PEGSH and AuNR@PEGMUA

AuNR (batch 1) were prepared as described for chloroform extraction with centrifugation. The AuNR were reacted with 50 μM PEG ligand before extraction ($t_1 = 22$ h) and with different concentrations of PEG ligand after extraction for $t_2 = 2$ h. For analysis AuNR@PEGSH were reacted with 5 mM KCN and AuNR@PEGMUA with 25 mM KCN and the etching reactions monitored as described.

Centrifugation-based strategies for CTAB removal

Ethanol-assisted: after $t_1 = 22$ h reaction with PEGMUA at the desired concentration ($c_1(\text{PEGMUA}) = 25$ or $50 \mu\text{M}$) the AuNR (batch 1) were washed by fourfold centrifugation (7000g, 10–20 min) replacing the supernatants by ethanol. After three or four washing steps the AuNR irreversibly adhered to the container walls (material: polypropylene). In a reproduction with different centrifuge tubes the purification was completed without significant losses, even though the material was the same. For subsequent etching experiments the concentrations were adjusted to 9.3×10^{-3} nM. Water-assisted: AuNR@CTAB (30 ml, 4×10^{-2} nM, batch 2) were centrifuged (7000g, 15 min) two rounds replacing the supernatants by water. The AuNR-concentration was adjusted to 0.037 nM and the solution divided into two parts. One part of the solution was extracted fivefold with chloroform (10 ml for each extraction), the other part of the solution was not treated any further. Both solutions, extracted and not extracted were reacted with different concentrations of PEGMUA and subsequently analyzed *via* their reaction with cyanide.

Chloroform extraction of AuNR@CTAB

AuNR@CTAB (10 ml, 3.8×10^{-2} nM, batch 6) were extracted fivefold with chloroform (10 ml for each extraction). 1 ml of the aqueous phase containing the AuNR were then transferred

into a cuvette and absorbance spectra recorded in 10 min intervals by running the spectrometer in cycle mode.

Highly concentrated AuNR@PEGMUA and AuNR@PEGSH samples for ATR-FTIR-analysis

PEG-ligand (10 mg, 5 μmol) was dissolved in water (180 ml) and with 800 rpm stirring, AuNR@CTAB (20 ml, $\sim 2.2 \times 10^{-1}$ nM, batch 1) were added. After 24 h reaction the solutions were extracted fivefold with chloroform (100 ml for each extraction). In the first extraction, phase separation took 10–15 min and a mixed phase/emulsion remained. In the second extraction, the phase separation took some minutes and again some mixed phase/emulsion remained. In the following extractions, no emulsion remained and the phases separated quickly, within seconds. Overall, 50 ml solution of each batch was lost due to emulsion formation and 150 ml AuNR-solution with unchanged concentration remained. The 50 ml mixed phases/emulsions were stored at 7 °C and the phase separation slowly proceeded. Thus it was possible to recover more of the AuNR-solutions. These were not combined with the fully extracted solutions, however, because their CTAB concentrations were expected to differ. The extracted solutions were adjusted with water to a total volume of 200 ml and a total concentration of 50 μM PEG-ligand. After reaction for 16 h with stirring, strong adhesion to the reaction vessel walls (material: glass) and stirring bar (PTFE) was observed for AuNR@PEGSH but not for AuNR@PEGMUA. AuNR@PEGSH could be resuspended by sonication. The solutions were then concentrated to 1.0 ml by repeated centrifugation (7000g, 60 min for volumes: 40 ml per tube, 20 min for small volumes: 1.5 ml per tube). In all centrifugation steps strong adhesion and possibly aggregation of AuNR@PEGSH was observed, that could be partly reversed by ultrasound treatment after each centrifugation step. AuNR@PEGMUA were not problematic in this respect but were also treated with ultrasound after each centrifugation step to improve the purification. The concentrated samples were purified by two additional centrifugation steps, replacing supernatants by water. The overall dilution was at least 1 : 320 000. AuNR@PEGMUA were 2.2×10^{-2} nM before extraction and 2.58 nM after concentration and purification. Because 50 ml were lost during the extraction and effectively 150 ml of the AuNR were concentrated, this corresponds to $\sim 20\%$ loss of AuNR due to concentration and purification. The AuNR-concentration directly before concentration could not be determined by UV/Vis spectroscopy because residual chloroform from the extraction affected the absorbance. AuNR@PEGSH were 2.4×10^{-2} nM before extraction and 8.4×10^{-1} nM after concentration and purification. Accordingly, $\sim 80\%$ of the AuNR were lost during concentration and purification. The concentrated and purified samples were analyzed by ATR-FTIR-spectroscopy.

Highly concentrated AuNR@PEGMUA sample for NMR- and ATR-FTIR-analysis

AuNR@CTAB (160 ml, 3.9×10^{-1} nM, batch 4) were centrifuged (7000g, 20 min) to reduce the CTAB concentration and the



supernatants (~150 ml) replaced by PEGMUA solution (27 μM). To determine the CTAB concentration of the starting material by quantitative NMR, the supernatants were collected. KCN (50 mg, 0.78 mmol) was added to the supernatants to dissolve residual AuNR@CTAB. After the solution had turned colorless, indicating complete dissolution of the residual AuNR, it was frozen and lyophilized. The residue was dissolved in CDCl_3 (5.000 ml) and the solution filtrated with a PTFE syringe filter (0.22 μm pore size). A part of the filtrate (700 μl) and iodoform in CDCl_3 (50 μl , 20.0 mg ml^{-1} $\rightarrow$ 1.0 mg, 2.5 μmol) were mixed in a NMR-tube for ^1H -NMR analysis. A CTAB-concentration of 22 mM was found, based on this analysis.

The reaction mixture (160 ml, AuNR@CTAB in the presence of 25 μM PEGMUA) was shaken for 2 days on a lab-shaker and then centrifuged (10 000g, 40 min). The combined pellets containing the AuNR (~12 ml) were filled up with water (to 20 ml). The supernatants containing unreacted PEGMUA (157 ml) were lyophilized without etching. The gold content was determined with GF-AAS to be 333 $\mu\text{g l}^{-1}$ ($c(\text{Ag}) = 51.6 \mu\text{g l}^{-1}$, 16% of $c(\text{Au})$) and was therefore negligible, *i.e.* no AuNR had remained in the supernatants. The residue of the lyophilized supernatants was solved in CDCl_3 and analyzed by ^1H -NMR analysis as described, but without syringe filtration, that was not necessary for this and all subsequent samples. ^1H -NMR analysis yielded 34–36 μM PEGMUA, 100–120 μM CTAB and 2.5–3.4 μM oleate in these supernatants. That the PEGMUA concentration was higher than theoretically expectable (max. ~25 μM) points out the limited accuracy of the method (probably most of all because of the molar mass distribution of PEGMUA) but also suggests that not much PEGMUA had bound to the AuNR before extraction. The AuNR solution (20 ml) from the combined pellets was extracted fivefold with chloroform (10 ml for each extraction). Then, PEGMUA was added (160.0 mg, $c_2(\text{PEGMUA}) = 4 \text{ mM}$) and the solution was shaken for $t_2 = 2 \text{ h}$. After subsequent addition of water (20 ml), the reaction mixture was then centrifuged (10 000g, 30 min) and concentrated to 5–6 ml. In the lyophilized supernatant 106–116 mg residue was obtained, that contained >97% PEGMUA (>85% thiol, <15% disulfide, based on ^1H -NMR analysis as described previously³⁶) corresponding to 2.6–2.9 mM of the 4 mM PEGMUA originally added after extraction. Accordingly, less than 90 μM CTAB was in this supernatant. The gold concentration was again negligible ($c(\text{Au}) = 1844 \mu\text{g l}^{-1}$, $c(\text{Ag}) = 39.6 \mu\text{g l}^{-1}$). For further purification, the concentrated AuNR@PEGMUA solution was centrifuged 4 more rounds (7000g, 30 min) replacing supernatants with water and with a minimum of 5 min sonication after each centrifugation. The dilution in each centrifugation was at least 1:25 and the overall dilution was at least $1:6 \times 10^6$. The volume of the final concentrated and purified solution was adjusted to ~4 ml. The AuNR-concentration was 16.7 nM based on UV/Vis spectroscopy and 16 nM based on GF-AAS ($c(\text{Au}) = 4.8 \times 10^6 \mu\text{g l}^{-1}$, $c(\text{Ag}) = 5.1 \times 10^4 \mu\text{g l}^{-1}$) assuming a cylindrical geometry with the parameters obtained by TEM-analysis.

NMR-analysis of concentrated AuNR@PEGMUA

Concentrated and purified AuNR (3.000 ml, 16 nM) were dissolved by adding KCN solution (1.000 ml, 1.00 M) followed by stirring overnight. The clear and colorless solution was then lyophilized and the residue dissolved in CDCl_3 (1.000 ml). 700 μl thereof and iodoform in CDCl_3 (50 μl , 20.0 mg ml^{-1}) were mixed in a NMR-tube for ^1H -NMR analysis. After complete evaporation of residual CDCl_3 the residues that were not dissolved in CDCl_3 were completely dissolved in D_2O (2.000 ml). 700 μl thereof and acetonitrile (50 μl , 1 vol% in D_2O) were mixed in a NMR-tube for ^1H -NMR analysis. In the CDCl_3 sample, 0.28 μmol PEGMUA were found and 0.175 μmol in the D_2O sample. This corresponds to 0.4 μmol in the 1.000 ml CDCl_3 and 0.5 μmol in the 2.000 ml D_2O and to a total concentration of 300 μM PEGMUA in the 3.000 ml concentrated AuNR-solution.

Results and discussion

First, we focus on the comparison of CTAB-ligand exchange by PEGMUA with and without assisting extraction with chloroform. To analyze the exchange, oxidative etching with cyanide is employed, utilizing the huge difference in stabilization of AuNR by CTAB and by PEGMUA. AuNR@CTAB are usually etched in less than a minute with 25 mM cyanide, whereas PEGMUA-coated AuNR, AuNR@PEGMUA, react for days and are still not completely dissolved by etching with this cyanide concentration.

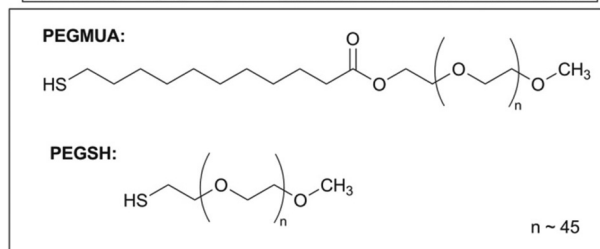
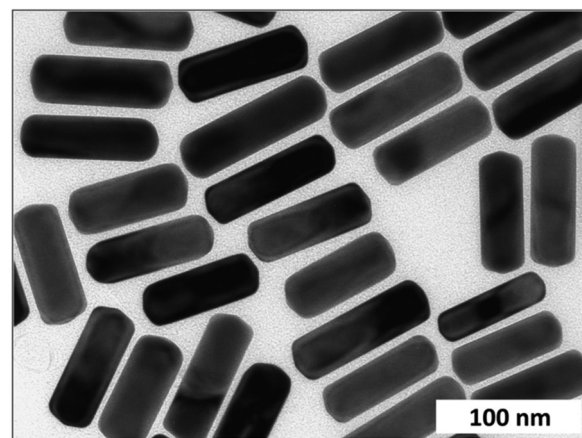


Fig. 1 Exemplary TEM measurement of AuNR and structures of PEG ligands used in this study.



Oxidative cyanide etching enables studying the CTAB/PEGMUA ligand exchange

To analyze the ligand exchange without extraction, AuNR@CTAB were incubated with different concentrations of PEGMUA for a fixed reaction/incubation time t_1 . AuNR@CTAB were prepared following the protocol of Ye *et al.* using CTAB at a reduced concentration together with sodium oleate to yield AuNR@CTAB with 70–90 nm length and 20–30 nm diameter (Fig. 1).⁴¹ The AuNR@CTAB dispersion was diluted by a factor of 10 to AuNR concentrations in the range 5–30 pM. The CTAB-concentration in these samples was ~ 2 mM. In general, variations of the CTAB concentration are due to the AuNR synthesis. Different sizes, aspect ratios and concentrations of the produced AuNR lead to varying amounts of adsorbed CTAB. Considering the strong impact of the CTAB concentration on ligand exchange kinetics¹⁴ this is hypothesized to be a reason for limited reproducibility of ligand exchange. In a first set of

experiments, AuNR@CTAB reacted with different concentrations of PEGMUA for 22 hours and were subsequently exposed to 50 mM potassium cyanide, KCN. Fig. 2 shows the absorbance spectra of these samples at different times after KCN addition. Without PEGMUA addition ($c = 0 \mu\text{M}$) the AuNR are completely etched within minutes and the absorbance after 12 minutes is ~ 0 . The formation of Au-cyanide complexes ($[\text{Au}(\text{CN})_2]^-$) is indicated by their spectral signature with maxima at ~ 213 , 230 and 240 nm.⁴³ In contrast, hardly any etching is observed for AuNR reacted with 300 μM PEGMUA. Depending on the PEGMUA concentration, the longitudinal localized surface plasmon resonance band (LSPRB) broadens and redshifts during the etching reaction.

For high PEGMUA concentrations (see *e.g.* 200 μM Fig. 2A) neither broadening nor strong shift was observed, but a gradual decrease of absorbance. These different optical responses during the etching reaction indicate different mechanisms of the etching processes. Similar to a simple

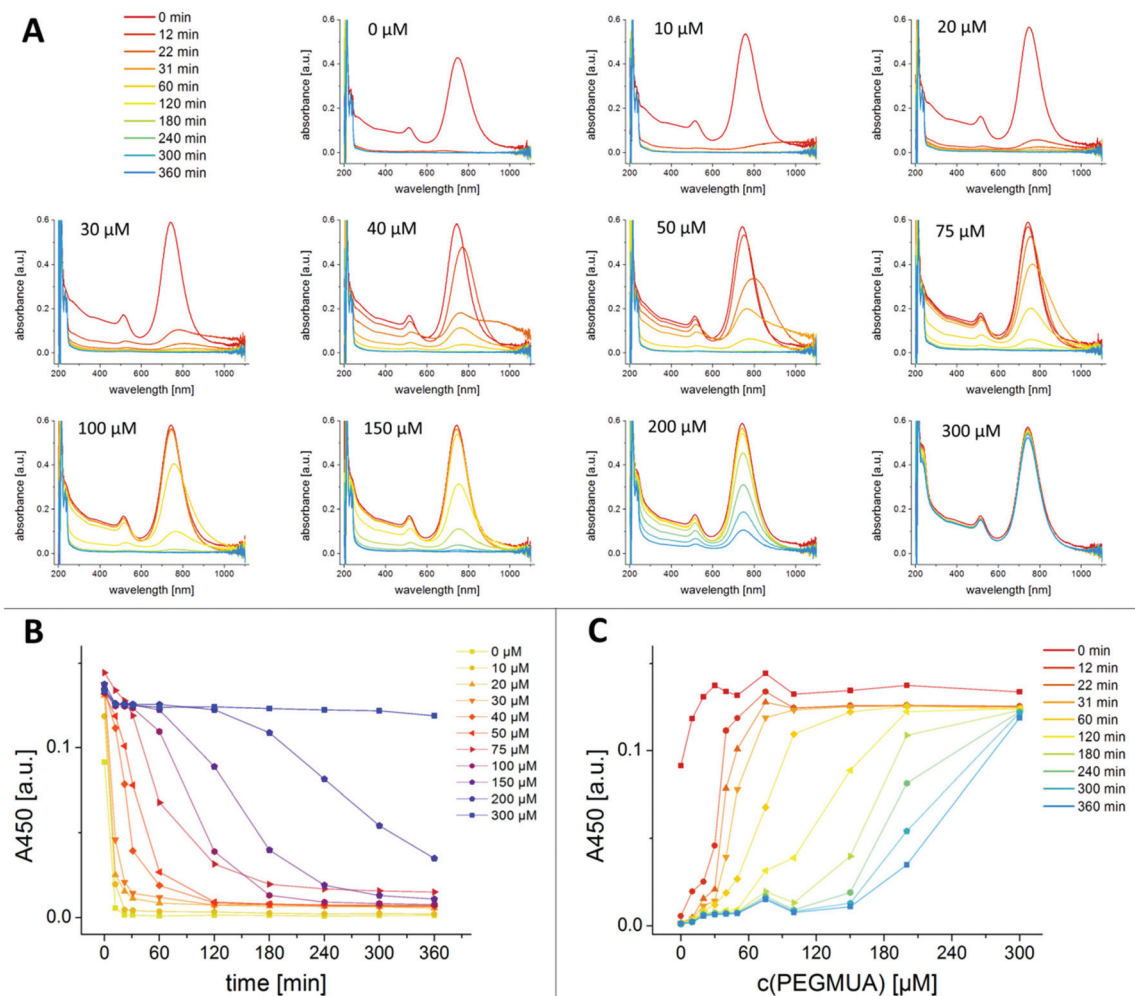


Fig. 2 Monitoring the oxidative etching of AuNR by UV/Vis-spectroscopy. AuNR were reacted 22 hours with different concentrations of PEGMUA as indicated. Then the samples were etched with 50 mM of KCN and absorbance spectra were recorded at different times during the etching reaction. 0 min corresponds to measurements directly after KCN addition. The absorbance at 450 nm (A_{450}), reflecting the stability of the sample, can be plotted *versus* the reaction time (B) or *versus* the PEGMUA concentration (C) to analyze the data. Lines are a guide to the eye.



model proposed earlier for spherical AuNP,³⁶ two extreme cases are conceivable: a situation where all AuNR are etched simultaneously at a similar rate or a particle-by-particle etching. Here, any morphological changes of the AuNR can be expected to result in shifts and broadening of the longitudinal absorption,^{44,45} especially on the ensemble level, when these changes are irregular, leading to a broadened morphological distribution of the sample. Additionally, aggregation processes have to be considered, that can also lead to broadening of the absorbance band.⁴⁶

Not only the ligand structure, but also the ligand coverage can be expected to affect the mechanism of the etching reaction. If no AuNR have a maximum coverage with PEGMUA, the etching rate will be in the same range for all AuNR in the sample. The rate will be comparably high because of the AuNRs' incomplete coverage with stabilizing PEGMUA. Accordingly, their morphological changes will be reflected in the optical properties of the sample, *i.e.* by broadening and shifts of the LSPRB, as observed for the samples treated with PEGMUA at lower concentrations. In contrast, if all AuNR have maximum coverage, and therefore maximum stabilization, by PEGMUA, the etching rates will be much higher for AuNR that are already etched locally, because the etching produces "weak spots" in their ligand layer. In consequence, a particle-by-particle etching occurs and morphological changes do not dominate the optical properties of the samples, as indicated by the uniform gradual decrease of absorbance observed in case of the samples treated with PEGMUA at high concentrations. TEM-analyses support this interpretation (Fig. S1†).

The kinetics of the etching reactions can be compared by plotting the absorbances at 450 nm (A_{450} , Fig. 2B) as an indicator of the samples' concentrations, which decrease during the etching reaction. This method is semiquantitative because the exact morphology and optical properties of the samples at a certain time point of the etching reaction are not known, but it still allows comparing the kinetics of the different samples reacting with KCN. The etching rate decreases with increasing PEGMUA concentration (Fig. 2B). For high PEGMUA excesses the reaction is not completed within the experiment duration. Another way to analyze the data is by plotting the absorbance (A_{450}) versus the PEGMUA concentration used for stabilization of the AuNR (Fig. 2C). The resulting curves resemble an isotherm because the stability (reflected by A_{450}) correlates with the PEGMUA coverage of the AuNR. The maximum absorbance shifts towards higher PEGMUA concentrations with increasing etching time. In these reactions no steady state is observed in the analyzed timeframe. The experiments demonstrate the high stabilization by PEGMUA that allows to utilize the reaction of coated AuNR with cyanide to study the ligand exchange. The stability of the AuNR, indicated by the absorbance at 450 nm, A_{450} , in Fig. 2B and C, clearly correlates with the PEGMUA concentration.

Ligand-exchange is more efficient when assisted by chloroform extraction

Previous work suggests that chloroform extraction can efficiently remove CTAB from AuNR samples but the ligand exchange

itself was not studied in detail.^{7,32,33} We were especially interested in comparing the ligand exchange assisted by extraction with chloroform to the ligand exchange without extraction. The extracted (extr) samples were mixed with 25 μ M PEGMUA immediately before fivefold extraction with chloroform in order to avoid destabilization during the extraction. In preliminary experiments we observed that AuNR@CTAB aggregate completely within a few hours after chloroform extraction (Fig. S2†). This confirms that CTAB is efficiently removed, at least down to a concentration below the critical concentration necessary for stabilization of the AuNR. This critical concentration was identified by Lee *et al.* to be in the range 0.2–0.5 mM.¹⁴ After extraction, different amounts of PEGMUA were added, followed by incubation for 22 hours or 1 hour. Fig. 3 shows the resulting $A_{450}(c(\text{PEGMUA}))$ -curves for these samples after 300 minutes of etching with cyanide. They are compared to samples that were not extracted (no extr) but just reacted for the indicated times (22 or 65 hours) with PEGMUA as described above. The experiments with 22 h reaction time were repeated to assess their reproducibility (Fig. 3, extr/no extr 22 h and 22 h (2)). Three conclusions can be drawn: the reproducibility of the ligand exchange seems to be much better for the extracted samples, the amount of PEGMUA needed for maximum stabilization is much lower and the reaction time needed for maximum stabilization is much shorter.

For the non-extracted samples the amount of PEGMUA for maximum stabilization is significantly lower when the AuNR@CTAB are reacted 65 hours compared to 22 hours reaction. We analyzed the long term stability of the PEGMUA ligand on AuNR by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) and found no signs of

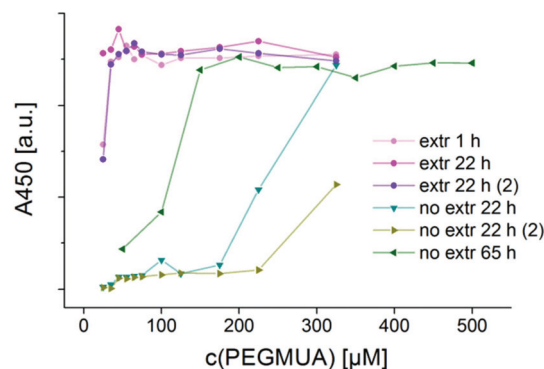


Fig. 3 Comparison of the absorbances at 450 nm (A_{450}) of different AuNR samples after 300 minutes reaction with 50 mM cyanide. All samples were prepared from the same batch of AuNR@CTAB (87 nm $\times$ 29 nm, batch 1) diluted to the same concentrations before reaction with different amounts of PEGMUA for different times as indicated. The reaction times for the extracted samples (extr, magenta) are the reaction times with PEGMUA after extraction with chloroform (immediately before chloroform extraction 25 μ M PEGMUA were added for stabilization). The concentrations $c(\text{PEGMUA})$ for the extracted samples are the sum of concentrations added before and after extraction to allow for comparison with the samples that were not extracted. Lines are a guide to the eye.



hydrolysis in the time frame of 12 days (Fig. S3, ESI†). Thus, the changes in reactivity cannot be attributed to changes of the PEGMUA ligand itself, *e.g.* due to hydrolysis. This (compare samples reacted 22 hours with PEGMUA in Fig. 3) indicates that the ligand exchange is extremely slow and that overnight reaction is not sufficient for maximum exchange. All samples were prepared from the same batch of AuNR@CTAB with the same dilution. Based on the synthetic protocol we know that the maximum possible concentration of free CTAB in these samples was 3.7 mM. This underlines that even at low concentrations, CTAB strongly hinders the ligand exchange of AuNR, confirming the results of Lee *et al.*¹⁴ In contrast, no difference in $c(\text{PEGMUA})$ for maximum stabilization was observed for the extracted AuNR-samples even when they were reacted for just 1 hour. Thus, the ligand exchange is much faster when the samples are extracted. Interestingly, the stabilization of the extracted samples is even then significantly higher when no PEGMUA is added after extraction (first points at $c(\text{PEGMUA}) = 25 \mu\text{M}$ in Fig. 3). The AuNR concentration $c(\text{AuNR})$ in these samples was $\sim 0.020 \text{ nM}$ and based on a cylindrical model with geometrical parameters obtained by TEM analysis, the PEGMUA concentration necessary for maximum coverage of all AuNR can be estimated. Even a footprint of just 0.2 nm^2 per PEGMUA molecule on the AuNR surface corresponding to a very high coverage, close to that of alkyl thiols on Au{111} surfaces^{47,48} would require less than $1 \mu\text{M}$ PEGMUA. In other words, the $25 \mu\text{M}$ PEGMUA added before chloroform extraction are at least 25 times the sufficient amount for maximum coverage of all AuNR. Keeping this in mind, the higher stability of the extracted samples with no PEGMUA added after extraction strongly indicates that some ligand exchange took place during the extraction procedure. This is possible because the ligand exchange is much faster and more efficient in the extracted samples.

Detailed study of the ligand exchange with assisting chloroform-extraction

To allow for direct comparison of extracted and non-extracted samples as described in the previous section, those samples were not purified by centrifugation, neither before, nor after extraction. The samples thus contained residual reagents from the AuNR-synthesis as silver nitrate (AgNO_3), hydrochloric acid (HCl) and others. In another set of experiments, details of the ligand exchange assisted by extraction were studied. For these experiments, lower AuNR-concentrations (9.3 pM) and a lower cyanide concentration (25 mM) for the etching reactions were used and the samples were purified after extraction by centrifugation to minimize matrix effects. The etching kinetics of these samples cannot be quantitatively compared to those shown in Fig. 3, because the AuNR concentrations and the matrix and pH are different. For comparable sets of etching experiments it is important to ensure the same initial AuNR concentration and a comparable matrix and pH in all samples.³⁶ Regarding the ligand exchange with extraction we focused on the following parameters: the concentration of PEGMUA before extraction $c_1(\text{PEGMUA})$, the PEGMUA concen-

tration after extraction $c_2(\text{PEGMUA})$ and the reaction time after extraction t_2 . Detailed descriptions and discussion of all experiments are provided as ESI (pages 4–8, Fig. S4–S9, ESI†). Fig. 4 shows an exemplary set of experiments. A steady state is reached after 1290 minutes (21.5 h) etching time. Afterwards, the plots A_{450} vs. $c(\text{PEGMUA})$ do not change significantly anymore in the higher concentration regime, even after a week (10 080 minutes). The minimum PEGMUA concentration for maximum stabilization can be determined; in the experiments shown it was $65 \mu\text{M}$ ($25 \mu\text{M}$ before extraction and $40 \mu\text{M}$ after extraction). We found that there is no benefit in using a large PEGMUA excess before extraction with chloroform because only small amounts will bind to the AuNR and unbound PEGMUA will be removed by the subsequent extraction. A reaction time after extraction, $t_2 = 30$ minutes was already sufficient for high stabilization. With $t_2 = 2$ hours the stabilization was just slightly better in some experiments. With reaction times $t_2 > 2$ hours no further increase in stabilization was observed, suggesting once more a fast ligand exchange after extraction. Our experiments consistently demonstrate, that PEGMUA provides a very high degree of stabilization for AuNR. When AuNR are extracted with chloroform to remove as much CTAB as possible, the ligand exchange is more reproducible, much faster (~ 1 – 2 hours vs. days), and about four times less ligand consuming.

Alternative strategies to remove CTAB include centrifugation-based purification with water or ethanol.^{6–8,25,26} Repeated centrifugation with water involves the risk of irreversible aggregation and/or adhesion of the AuNR. Even if the AuNR were not destabilized during centrifugations, 2.5 times more PEGMUA was required in order to obtain a stability comparable to an extracted sample (Fig. S10, ESI†). Centrifugation-

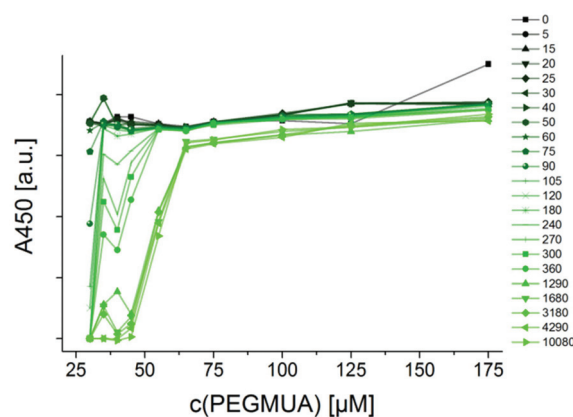


Fig. 4 Comparison of the absorbances at 450 nm (A_{450}) of AuNR samples after different times of reaction with 25 mM cyanide. The AuNR were reacted with $25 \mu\text{M}$ PEGMUA for 22 hours, extracted with chloroform and then again reacted with different PEGMUA concentrations $c_2(\text{PEGMUA})$ for 2 hours. $c(\text{PEGMUA})$ is the sum of PEGMUA concentrations added before and after extraction ($c_1(\text{PEGMUA}) + c_2(\text{PEGMUA})$). The numbers at the legend are the reaction times with cyanide in minutes. Outliers can result from aggregation/agglomeration, adhesion, sedimentation and turbidity. Lines are a guide to the eye.



based purification with ethanol after 22 hours reaction with 25 μM or 50 μM PEGMUA was only occasionally successful; irreversible adhesion to vessel walls during the centrifugation steps occurred in some cases. However, if no adhesion occurred, a high stabilization, comparable to chloroform-extracted samples, was obtained (Fig. S11, ESI†), indicating efficient removal of CTAB. It can be concluded that several strategies can be used to remove CTAB during ligand exchange. However, extraction with chloroform is a mild procedure because it involves no centrifugation during CTAB removal. In our experiments it was the most reliable and efficient procedure to assist ligand exchange of AuNR@CTAB.

Comparison of PEGMUA and PEGSH

As the etching experiments indicated that a high PEGMUA coverage was achieved when the ligand exchange was assisted by chloroform-extraction, we tested if this is also possible with a typical PEG-ligand of the same length, PEGSH. Such ligands are commercially available and used in the vast majority of studies with PEGylated AuNR. The difference between PEGMUA and PEGSH is the C_{10} alkylene-spacer, that connects the Au-surface binding thiol group with the PEG-moiety *via* an ester group in the case of PEGMUA. In case of PEGSH, the poly (ethylene glycol)-chain is simply terminated by a thiol group. This changes the adsorption because attractive van der Waals (vdW) forces between the alkylene-units favor adsorption and dense packing,^{48,49} whereas PEG-interactions are repulsive in water,^{50,51} leading to a decreasing coverage of PEG-thiols on gold with increasing PEG-length.⁵² Additionally, PEGMUA and similar ligands form an inner hydrophobic layer when coated on nanoparticles, that provides an effective barrier for polar molecules.^{36,37} In the case of AuNR, the CTAB bilayer represents a somewhat similar barrier that has to be overcome for ligand exchange.^{11,15} It can be hypothesized that CTAB-removal, *e.g.* by chloroform-extraction, induces collapse of and defect sites within the CTAB bilayers on the AuNR,¹⁴ thereby assisting ligand exchange. To test this strategy for PEGSH we prepared AuNR@PEGSH samples accordingly. The stability of these samples against oxidative etching was much lower and just 5 mM of cyanide was used for the etching experiments. Fig. 5 shows the absorbance at 450 nm (A_{450}) of AuNR@PEGSH in the presence of 5 mM cyanide in comparison to that of AuNR@PEGMUA, prepared with the same conditions, in the presence of 25 mM cyanide. All AuNR@PEGSH were completely etched within 2–3 hours with 5 mM cyanide. In contrast, at the fivefold cyanide concentration, AuNR@PEGMUA were significantly etched only if no PEGMUA was added after extraction (0 μM in Fig. 5) and even then, the sample was more stable than the AuNR@PEGSH samples. We tested longer reaction times t_2 for AuNR@PEGSH up to 24 hours, but no effect on the stabilization was observed (Fig. S12, ESI†). Also, in terms of PEGSH addition after extraction, no trend was observed, *i.e.* higher PEGSH concentrations did not lead to higher stabilization (Fig. 5). This observation supports the idea that the structures of the according ligand layers are significantly different and that PEGMUA provides a better stabili-

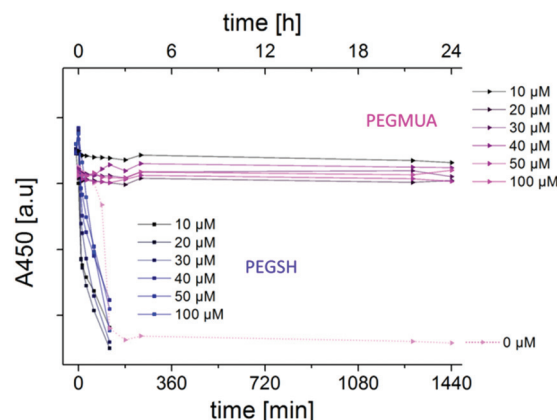


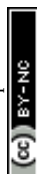
Fig. 5 Absorbance at 450 nm (A_{450}) of AuNR@PEGSH (blue squares) in the presence of 5 mM cyanide and of AuNR@PEGMUA (magenta triangles) in the presence of 25 mM cyanide *versus* the reaction time. The concentrations indicated by the color code correspond to the additions of the corresponding PEG-ligand after extraction. The preparative conditions were the same for both sets of samples ($t_1 = 22$ h reaction with $c_1 = 50$ μM PEG-ligand before extraction and $t_2 = 2$ h reaction after extraction). Lines are a guide to the eye.

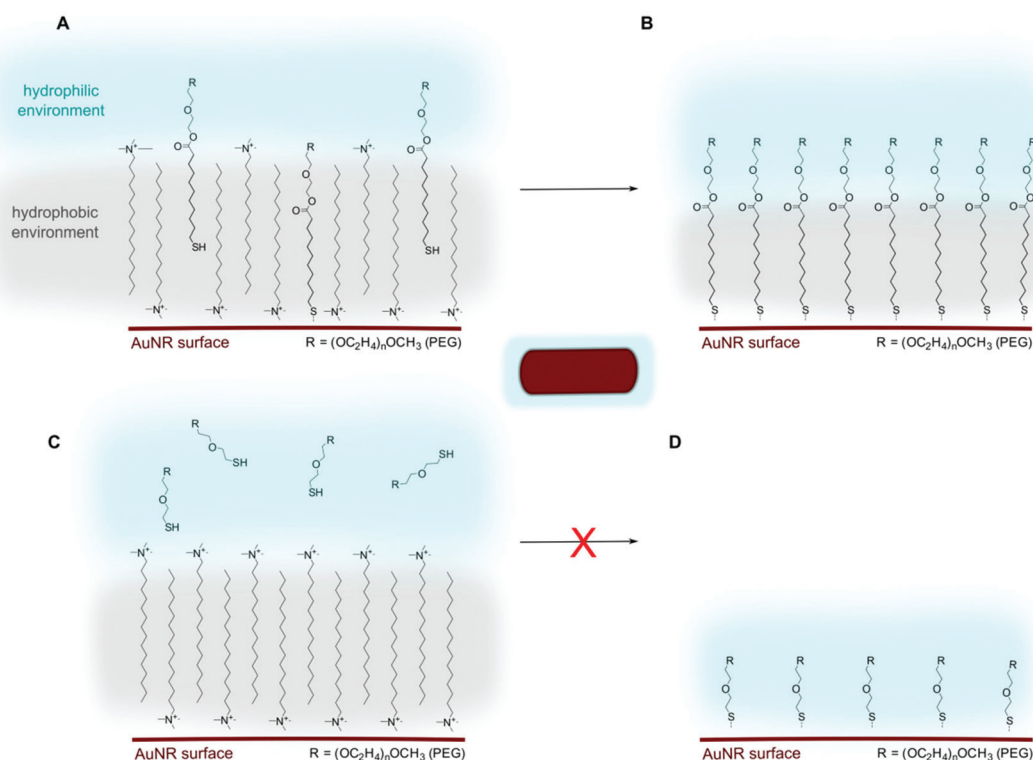
zation because of a higher grafting density and formation of an inner hydrophobic layer. A higher grafting density can be rationalized with the attractive vdW interactions discussed above. Another reason could be an improved penetration of the CTAB-layer by PEGMUA due to its amphiphilic character. This idea is illustrated in Scheme 1.

According to this idea, the molecular structure of PEGMUA improves not only the shielding effect of the final ligand layer but is also favorable for the ligand exchange itself. Either the PEGSH ligand layer provides no comparable stabilization after formation, or the ligand exchange is less effective with PEGSH, or both.

Estimating the PEGMUA coverage

The better stabilization of AuNR against oxidative etching with PEGMUA compared to PEGSH could be exclusively due to hydrophobic effects and not to a higher grafting density. To explain then, that the hydrophobic CTAB bilayer does not provide comparable stabilization one might point out attractive electrostatic interactions (of cyanide ions and hexadecyltrimethylammonium groups in CTAB) or the assumed dynamic nature of the CTAB bilayer. Therefore we tried to assess the grafting density of PEGMUA in comparison to PEGSH when coated with the same conditions onto AuNR. This is especially relevant for biomedical applications of AuNR because a high PEG grafting density is correlated with a lower immunogenicity.³¹ The PEG coverage can be described with its Flory radius R_F and the grafting distance D .⁵⁰ In the “mushroom” regime with $R_F/D \leq 1$ the PEG-chains are in a relaxed conformation at low coverage, in the brush-regime with $R_F/D \geq 1$ the PEG-chains adopt a more stretched conformation due to intermolecular repulsive interactions.^{23,31,50,51} At very high coverage with $R_F/D \geq 2.8$ the PEG-coating enters the “dense-



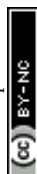


Scheme 1 Ligand exchange of PEGMUA versus PEGSH in a simplified picture. Due to its amphiphilic character, PEGMUA can penetrate the hydrophobic CTAB-layer more readily (A) to successively form a ligand layer that is stabilized by similar cooperative effects, driven by the stronger binding of thiol-groups to Au. For PEGSH as a polar molecule it is unfavorable to penetrate the CTAB-layer (C) and consequently no, or less, ligand exchange takes place (D). This simplified model does not take into account residual CTAB and the higher complexity of the AuNRs' surface chemistry including silver, bromide, oleate and reaction products remaining from their synthesis.

brush" regime that was found to be most favorable in terms of immune response and clearance.³¹ A dense PEG-brush on AuNR of dimensions used in this work requires at least several thousands of PEG-ligands per AuNR. In contrast, Hore *et al.* have recently presented small angle neutron scattering (SANS) data that indicate a coverage of just 6–10 PEGSH molecules (5 kDa) per AuNR using standard coating procedures.¹² Thus, new strategies to achieve higher PEG grafting densities on AuNR are highly desirable.

We prepared highly concentrated samples of AuNR@PEGSH and AuNR@PEGMUA to assess the number of PEG-molecules per AuNR by NMR and IR. About 80% of AuNR@PEGSH were lost during concentration due to irreversible adhesion to vessel walls and possibly aggregation, whereas in the case of AuNR@PEGMUA just ~20% were lost (Fig. S13, ESI†). This observation underlines once more the better stabilization by PEGMUA compared to PEGSH. The final samples, that were concentrated and purified, were analyzed by ATR-FTIR. Resulting spectra are shown in Fig. 6. The PEG molecules can be identified by their characteristic ether-vibrations (C–O stretch) at $\sim 1060\text{ cm}^{-1}$. In this region no CTAB vibrations are present. The IR-spectra of AuNR@PEGMUA and AuNR@PEGSH (Fig. 6C) strongly indicate that substantially more PEGMUA is bound to the AuNR than PEGSH. The amount of PEGMUA in the concentrated sample AuNR@

PEGMUA ($c = 2.6\text{ nM}$) was detectable by NMR but not sufficient for a quantitative analysis. Therefore, a higher amount of AuNR@PEGMUA was prepared, concentrated and purified. In every step of the procedure, supernatants were collected and lyophilized as detailed in the Experimental section. The gold and silver content was determined by elementary analysis and the PEGMUA, CTAB and oleate content was analyzed qualitatively and estimated by NMR. The amount of silver relative to gold in AuNR@PEGMUA samples decreased during the ligand exchange and purification from ~16% after the first reaction with PEGMUA before extraction to 2% after extraction and second reaction with PEGMUA. In the final purified sample the relative amount of silver was ~1%. This was confirmed by HR-STEM-EDX (high resolution-scanning transmission electron microscopy-energy dispersive X-ray spectroscopy) analysis that also confirmed that the silver is located at or near the AuNR surfaces (Fig. S14, S15 and Tables S1, S2, ESI†) in accordance with previous reports.⁵³ Silver can be the cause for unwanted toxicity in biological and biomedical applications of AuNR and complicates the surface chemistry of the AuNR. It is therefore desirable to remove as much silver as possible.²⁷ In the NMR analysis, CTAB could not be identified unambiguously in the final sample but matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) analyses suggested that residual



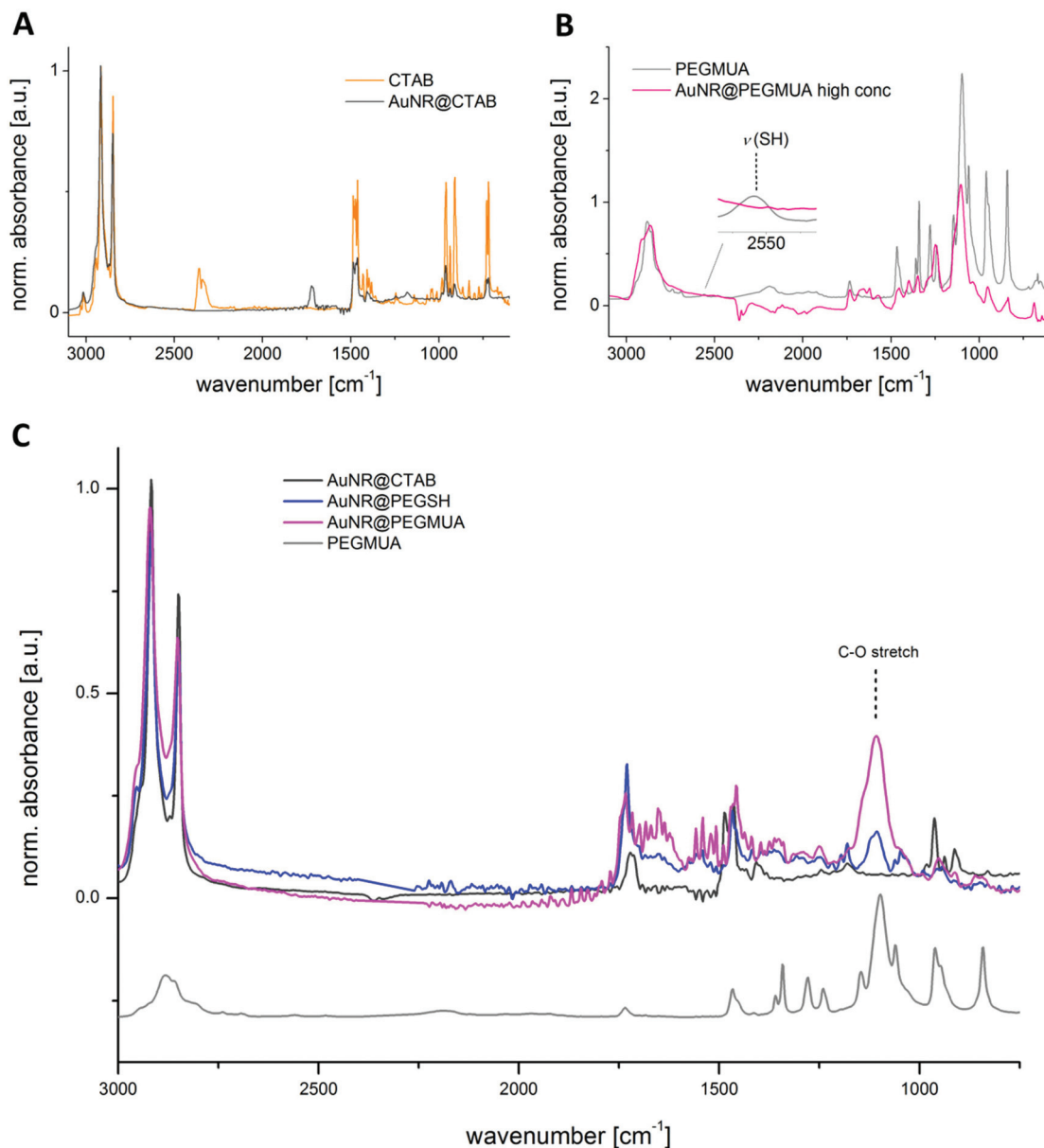


Fig. 6 ATR-FTIR-analysis of AuNR-samples. Comparison of AuNR@CTAB (grey line) with pure CTAB (orange line) (A), comparison of highly concentrated and purified AuNR@PEGMUA (pink line) with pure PEGMUA (grey line) (B) and comparison of AuNR@CTAB (dark grey line), AuNR@PEGSH (blue line) and AuNR@PEGMUA (magenta line) (C). The PEGMUA spectrum is also shown (grey line) and offset for clarity. The vibration at $\nu \sim 1700 \text{ cm}^{-1}$ in A stems from oleate in the AuNR@CTAB sample. The CO_2 vibration-related peaks were removed for most spectra and the backgrounds were accounted for with a second order polynomial and subtracted. Spectra were normalized at the asymmetric methylene vibrations ($\nu = 2918 \text{ cm}^{-1}$).

CTAB was present in all studied purified samples. A detailed discussion is provided as ESI (p. 13).[†]

The NMR analyses during the ligand exchange and purification confirmed that more PEGMUA reacts with the AuNR after extraction than before. After purification by repeated centrifugation the AuNR@PEGMUA were etched with 250 mM cyanide to ensure their complete dissolution and then lyophilized. The residues were dissolved in CDCl_3 and D_2O for NMR-analysis. The NMR samples were calibrated with iodoform (in CDCl_3) or acetonitril (in D_2O), the NMR-spectra are shown in

Fig. S16, ESI.[†] The analysis revealed that PEGMUA was hydrolyzed by the harsh etching conditions. Complementary MALDI-TOF analyses confirmed the presence of PEG and indicated that also partial oxidation of the PEG-chains might have had occurred during the etching. Quantifying the PEG content based on the NMR-data shown in Fig. S16[†] yielded a total PEG-concentration of $\sim 300 \mu\text{M}$. The AuNR concentration was determined by graphite-furnace atomic absorption spectrometry (GF-AAS) to be 16 nM and confirmed by UV/Vis-based determination as described by Liz-Marzán's group.⁴⁰ This esti-



mation suggests that indeed a very dense PEG-layer was obtained on the AuNR with a PEGMUA-footprint of 0.30–0.35 nm² corresponding to PEGMUA grafting densities of 2.9–3.3 nm⁻². The ATR-FTIR spectrum of these highly concentrated AuNR@PEGMUA (not etched) also confirms the presence of large amounts PEGMUA (Fig. 6B). Because of the higher concentration of the AuNR, more spectral features of the ligand can be identified and correlate very well with the spectral signature of PEGMUA. Also, in contrast to the free ligand PEGMUA, no thiol vibration at 2559 cm⁻¹ was observed. Even though the accuracy of the value for the grafting density is limited by the experimental conditions it can be concluded with high confidence that a footprint $\ll 1$ nm² and a PEG-layer in the dense brush regime was obtained. Thus, ~ 1650 times more PEG-chains per nm² are bound than in the study of Hore *et al.*, where similar AuNR were used,¹² and ~ 60 times more than in the study of Xia *et al.*, also using AuNR with similar dimensions.³⁰ In these studies, longer PEG-chains were used (5 kDa in contrast to 2 kDa used herein), but this alone cannot explain the huge difference in bound PEG. With advanced PEGylation strategies higher grafting densities can probably be achieved, but were not quantitatively determined in all studies. In the work of Kinnear *et al.* the PEG-grafting density was estimated based on TGA experiments to be 0.89 PEG nm⁻².²⁵ TGA-based determination of the PEG-grafting density can be problematic because CTAB was also present in the ligand layer, but the estimation should be still valid because the molecular mass of CTAB is much smaller than that of the PEG-thiol (5 kDa) used in their study.

The strategy of using PEGMUA, a PEG ligand with an optimized molecular structure, is complementary to promising strategies reported so far. In future work it should be tested if the combination of advanced PEGylation strategies and PEGMUA can improve even further the results of the ligand exchange.

Conclusion

In conclusion, our experiments consistently demonstrate that the PEGylation of AuNR can be dramatically improved by tailoring the molecular structure of the PEG-ligand. The mechanical and chemical stabilization is much higher and higher grafting densities are achieved when PEGMUA is used instead of PEGSH of the same length.

Because PEGMUA stabilizes AuNR against oxidative etching with cyanide very well, this etching reaction can be utilized as an analytical tool to study the ligand exchange. We observed that the ligand exchange is much faster and more reproducible, when CTAB is removed by chloroform extraction and that at least 50% less ligand is needed for maximum stabilization. Alternative strategies to reduce the amount of CTAB and assist the ligand exchange were less effective and less reliable. With extraction assisted ligand exchange, sufficiently high concentrations of AuNR@PEGMUA could be prepared and purified to allow quantitative NMR spectroscopy analysis. A grafting

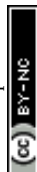
density in the order of 2.9–3.3 nm⁻² was estimated for PEGMUA on AuNR, corresponding to a footprint of 0.30–0.35 nm². In future studies it should be tested if quantitative CTAB and oleate removal is possible with advanced purification strategies. The toxicity of AuNR@PEGMUA has to be also addressed. For spherical AuNP coated with PEGMUA no toxicity was observed so far, neither *in vitro*^{37,39} nor *in vivo*.³⁸ This raises the hope that PEGMUA will enable to synthesize highly concentrated AuNR with very low or no toxicity with potential applications in biological and medical imaging and photothermal therapy.

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2.4 Ligand Layer Engineering To Control Stability and Interfacial Properties of Nanoparticles

Publication:

- [Schulz, F.; Dahl, G. T.; Besztejan, S.; Schroer, M. A.; Lehmkuhler, F.; Grübel, G.; Vossmeier, T.; Lange, H., Ligand Layer Engineering To Control Stability and Interfacial Properties of Nanoparticles. *Langmuir* **2016**, 32 \(31\), 7897.](#)¹³⁴

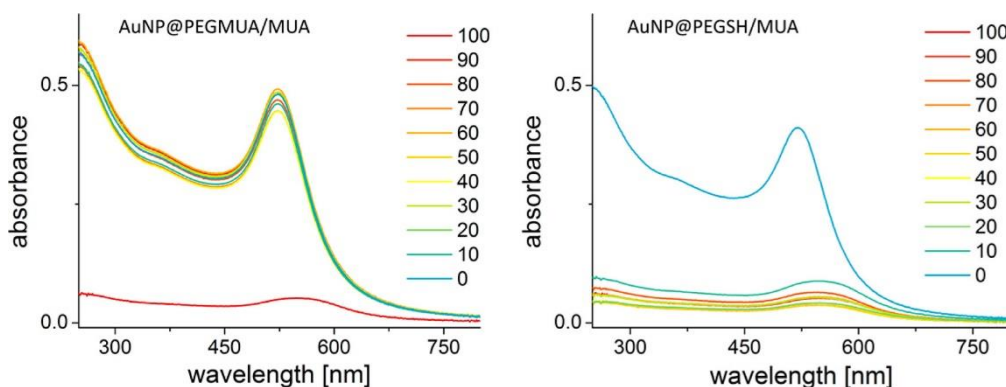


Figure 2. Absorbance spectra of AuNP conjugates after four centrifugations and redispersion ($20000g \times 20$ min at room temperature). The initial AuNP concentrations were ~ 5 nM, and the final concentrations of stable samples were ~ 150 nM. The concentrated samples were diluted for UV/vis measurements. AuNP coated with PEGMUA/MUA mixtures (left) were stable and showed no signs of aggregation, whereas AuNP coated with mixtures of PEGSH and MUA were all aggregated irreversibly. The mole percent of MUA used in the synthesis of the conjugates are indicated by the color code.

Based on the previous work on AuNP@Citrate synthesis²³ and functionalization with PEGMUA³⁴ the formation of mixed ligand layers was addressed in this study. In nanomedicine and nanobiotechnology not only an efficient stabilization (e.g. by PEGylation) but also further functionalization of the NP, for instance with targeting ligands or labels, is often desired. One common strategy to this end is the formation of mixed ligand layers. This raises the question of how well the formation of such mixed ligand layers can be controlled experimentally. Assuming that two thiols are added to AuNP@Citrate in a ratio of 1:1, the question remains if the molar ratio of these thiols bound to the AuNP surface after ligand exchange will be the same. In this study the reaction of AuNP@Citrate with mixtures of PEGMUA and MUA were used as a simple model system to address this question. The carboxylic acid groups of MUA are (partially) charged at the pH of the experiments whereas PEGMUA is formally neutral (methoxy-terminated), which means that the coadsorption of MUA modulates the surface charge of the AuNP-conjugates. This gradual increase of (negative) surface charge with increasing MUA-coadsorption could experimentally be verified clearly with gel electrophoresis experiments. Complementary IR-spectroscopy supported this finding. The data indicated that the molar ratio of PEGMUA and MUA in the mixed ligand layers could be well controlled. Control experiments with PEG revealed the important fact that this is not possible at all with mixtures of MUA and PEG (without MUA spacer). In such mixtures only MUA binds to the AuNP as revealed by stability tests based on UV/vis spectroscopy, Figure 2. Pure MUA coatings do not provide

sufficient colloidal stability for AuNP with 12 nm diameter for repeated centrifugations. AuNP@MUA aggregate more and more with every centrifugation step which can be verified with UV/vis spectroscopy. The remaining part of the study focusses on stability. It discusses the nuances of stability: colloidal vs. chemical stability and presents SAXS and etching data on the stability of the mixed-layer-conjugates. Finally, the interactions with cells and proteins are discussed. In summary the study demonstrates that the molecular structure is crucial in the controlled synthesis of mixed ligand layers and that with the molecular design of an alkylene spacer very stable colloids with tunable surface charge can be obtained. It is also shown that this surface charge affects the interaction with cells and the self-assembly of the AuNP. The data on colloidal stability indicate that the minimum molecular weight of the PEG for efficient colloidal stabilization is ~2000 g/mol. AuNP@PEGMUA2k are colloiddally stable even at extremely high concentrations in the micromolar range as proven by SAXS-measurements. Such high concentrations are necessary for several applications in nanomedicine.

Ligand Layer Engineering To Control Stability and Interfacial Properties of Nanoparticles

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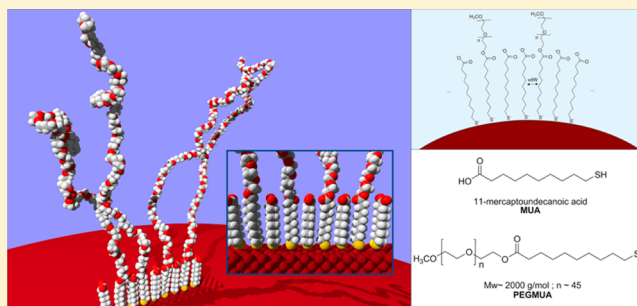
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S Supporting Information

ABSTRACT: The use of mixed ligand layers including poly(ethylene glycol)-based ligands for the functionalization of nanoparticles is a very popular strategy in the context of nanomedicine. However, it is challenging to control the composition of the ligand layer and maintain high colloidal and chemical stability of the conjugates. A high level of control and stability are crucial for reproducibility, upscaling, and safe application. In this study, gold nanoparticles with well-defined mixed ligand layers of α -methoxypoly(ethylene glycol)- ω -(11-mercaptopundecanoate) (PEGMUA) and 11-mercaptopundecanoic acid (MUA) were synthesized and characterized by ATR-FTIR spectroscopy and gel electrophoresis. The colloidal and chemical stability of the conjugates was tested by dynamic light scattering (DLS), small-angle X-ray scattering (SAXS), and UV/vis spectroscopy based experiments, and their interactions with cells were analyzed by elemental analysis. We demonstrate that the alkylene spacer in PEGMUA is the key feature for the controlled synthesis of mixed layer conjugates with very high colloidal and chemical stability and that a controlled synthesis is not possible using regular PEG ligands without the alkylene spacer. With the results of our stability tests, the molecular structure of the ligands can be clearly linked to the colloidal and chemical stabilization. We expect that the underlying design principle can be generalized to improve the level of control in nanoparticle surface chemistry.



INTRODUCTION

The ligand layer governs the interfacial properties of colloids and provides steric and/or electrostatic stabilization.^{1–7} An effective stabilization is crucial for the reproducibility, processability, safety, and thus applications of colloidal nanomaterials, e.g., in nanomedicine, plasmonics, catalysis, and energy conversion.^{8–11}

The stability of colloids encompasses their resistance to irreversible aggregation and/or disintegration of the ligand layer or the colloidal particle cores. Aggregation can be caused by high electrolyte concentrations in the case of electrostatically stabilized nanoparticles (NP), by mechanical compression (e.g., during centrifugation), or it can result from disintegration of the ligand layer by chemical decomposition, ligand stripping, or competitive displacement.^{7,12–14} Significant concentration losses can additionally result from irreversible adhesion of the NP to container or vessel walls. In complex biological media additional effects occur, the most prominent example being the so-called protein corona, i.e., additional layers of adsorbed proteins that affect e.g. the *in vivo* fate of the nanomaterial.^{5,15–17} Thus, as a rough guideline, one can differentiate

mechanical or physical stability, chemical stability, and interactions of the ligand layer with its environment. Theoretical models of colloidal stability at the nanoscale extend the classical DLVO theory to account for hydration forces, osmotic, entropic, and enthalpic effects.^{7,18} Such effects, which are dictated by the ligand layer, cannot be neglected in the nanometer size range.

To study the surface chemistry of colloids and the structure of ligand layers, gold nanoparticles (AuNP) serve as an ideal model system. Citrate-stabilized AuNP are stable under ambient conditions and can be reacted with thiols for straightforward ligand exchange and functionalization.^{9,10} In the plethora of ligand layers on AuNP that have been explored, monolayers of poly(ethylene glycol) (PEG) based ligands and mixed ligand layers including such PEG ligands play a prominent role.^{2,10,19–26} The so-called PEGylation allows for excellent stabilization of colloids and optimization of their

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interfacial properties, especially in the context of medical applications. Various studies explored the possibility of improving the performance of PEG ligand layers by structural variations of the PEG molecules.^{14,27–31} Alternative strategies have also emerged e.g. based on peptides or peptidols^{19,32–34} and on zwitterionic ligands^{35,36} or zwitterionic ligand layers, i.e., mixed layers from oppositely charged molecules.^{37,38}

Because the use of mixed ligand layers is a promising strategy for improving or enabling various technological or medical applications of nanoparticles, ongoing research aims at a more detailed understanding of the involved surface chemistry.^{39–41} The same holds true for the surface chemistry of PEG-thiols on AuNP.^{13,14,42–44} Several studies have pointed out that the grafting densities of typical PEG-thiol ligands on AuNP are significantly lower than those of small thiols.^{44–46} Thus, additional molecules can bind to vacant adsorption sites on the Au surface, a process sometimes termed as backfilling.^{42–44} A low grafting density also facilitates competitive displacement of ligands. Such displacement of PEG ligands on AuNP has been shown for dithiols, such as dithiothreitol (DTT),^{13,14,47,48} but also for monothiols, e.g., different alkanethiols,^{42,49,50} and for cysteine at physiological concentrations.⁵¹ As a result, the biorepulsive properties of the ligand layer and the provided stabilization are degraded. In recent studies, it has been shown that competitive displacement can be greatly reduced by using PEG ligands with hydrophobic spacers connecting the thiol group with the PEG moiety, e.g., α -methoxypoly(ethylene glycol)- ω -(11-mercaptopundecanoate) (PEGMUA).^{14,51} The use of hydrophobic spacers has been proven very effective for enhanced chemical stabilization and improved performance in biological media. In fact, for very small AuNP (~ 2 – 8 nm in diameter), so-called monolayer protected cluster, this design principle is well established and often perused using commercially available (1-mercaptopundec-11-yl)tetra(ethylene glycol) (EG₄MUA) or similar ligands (EG_{*x*}MUA), e.g., with six ethylene glycol units.^{4,11,52–54} However, for the stabilization and functionalization of larger AuNP (>10 nm) with PEG ligands this design principle is rarely taken into account.

In this study, we explore the stability and properties of mixed ligand layers composed of PEGMUA, a PEG-thiol with a hydrophobic spacer, and 11-mercaptopundecanoic acid (MUA). The main concept is that such mixed ligand layers enable the improved control of the ligand layer composition because the chemical structure of the adsorbates at the surface binding groups is similar. The concept is illustrated in Figure 1. Via the composition, properties like the surface charge of the AuNP-conjugates can thus be tuned precisely. As long as the mercaptopundecane spacer is used, well-stabilized colloids can be synthesized that resist multiple centrifugation steps at high forces (20000g) even at high MUA ratios, competitive ligand displacement, and cyanide etching at high concentrations. We demonstrate that comparable stability and control of the ligand layer composition cannot be achieved with mixtures of standard methoxy PEG thiols (bearing no hydrophobic spacer) and MUA. Thus, the design principle of a hydrophobic spacer not only increases the stabilization of colloidal nanoparticles but also enables a high level of control in the synthesis of AuNP with mixed self-assembled monolayers (SAMs) of PEG ligands and small thiols.

EXPERIMENTAL SECTION

Materials. Tetrachloroauric(III) acid ($\geq 99.9\%$ trace metal basis), trisodium citrate dihydrate ($\geq 99.0\%$), 11-mercaptopundecanoic acid

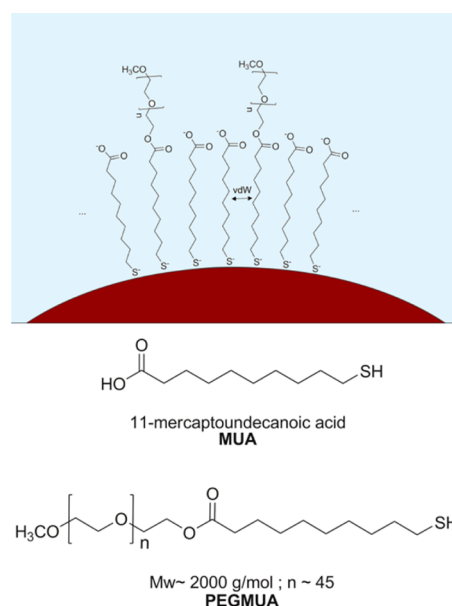


Figure 1. Illustration of a mixed ligand layer and structures of the according ligands. The structures of MUA and PEGMUA at the surface binding thiol group are the same and enable high grafting densities and stabilization of an inner hydrophobic layer by attractive van der Waals (vdW) interactions. The carboxylate groups of MUA affect the charge of the AuNP, depending on the pH. The hydrophilic PEG moieties provide steric stabilization and solubility in water and improve the biocompatibility.

(95%), sodium chloride ($\geq 99.0\%$), and dithiothreitol ($\geq 98\%$) were ordered from Sigma-Aldrich; ethanol absolute (100.0%) was obtained from VWR Chemicals. Ethylenediaminetetraacetic acid tetrasodium salt hydrate (EDTA) and citric acid monohydrate ($\geq 99.5\%$) were obtained from Merck. α -Methoxypoly(ethylene glycol)- ω -(11-mercaptopundecanoate) (PEGMUA, 2 or 5 kDa) was synthesized as described previously.¹⁴ α -Methoxypoly(ethylene glycol)- ω -(11-mercaptopundecanamide) (PEGMUA716, $M = 716$ g/mol, oligomer with 10 ethylene glycol units) was from Polypure (Polypure AS, Oslo, Norway). α -Methoxy- ω -mercaptopoly(ethylene glycol) (PEGSH, 2 kDa) was from Rapp Polymere (Tuebingen, Germany). Citrate-stabilized gold nanoparticles were synthesized and characterized as published earlier.⁵⁵ Their TEM characterization is provided as Supporting Information (Figure S1). Fetal calf serum (FCS), penicillin/streptomycin, and PBS were purchased from Lonza. Human serum was from Jackson ImmunoResearch (West Grove, PA). Ham's F12 and RPMI1640 media were purchased from Life Technologies (now Thermo Fischer Scientific). Cell culture flasks and multiwell plates were purchased from Sarstedt (Nuembrecht, Germany). Ultrapure water (18.2 M Ω cm, Millipore) was used for all procedures.

Functionalization of AuNP. AuNP with Mixed PEGMUA/MUA Ligand Layers. AuNP were functionalized by straightforward ligand displacement of citrate-stabilized AuNP using different mixtures of PEGMUA and MUA in an aqueous/ethanolic (1:1 vol) solution. Eleven PEGMUA/MUA mixtures were prepared by mixing different volumes of an aqueous solution of PEGMUA (1 mM) and an ethanolic solution of MUA (1 mM), yielding mixtures with the molar PEGMUA/MUA ratios of 100/0, 90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80, 10/90, and 0/100. For each mixture, the solvent imbalance was compensated by addition of respective amounts of water and ethanol. The total concentration of thiol ligands was 0.5 mM in 11.2 mL of water/ethanol (1:1 vol) in all final mixtures. All ligand mixtures were stirred thoroughly for ca. 10 min. To each mixture, 70 mL aqueous solutions of citrate-stabilized AuNP ($c(\text{AuNP}) \sim 5$ nM, $c(\text{citrate}) \sim 2$ mM, pH 5.5) were added at once and under constant stirring. After 10 min, the AuNP solutions were

subjected to centrifugation (20000g, 60 min), and the supernatant was discarded in order to remove free ligand molecules. Every sample was resuspended in 1.4 mL of water using a vortex mixer and further purified by three centrifugation steps (20000g, 15 min), each with subsequent replacement of 1.4 mL of the supernatant by water and resuspension. The final AuNP concentrations of the samples were in the range 120–180 nM. AuNP conjugates with other PEGMUA/MUA ratios (e.g., 25 and 75%) were prepared accordingly. All samples were stored at 7 °C, and no destabilization or indications of changed layer composition (e.g., due to hydrolysis of PEGMUA, tested by ATR-FTIR spectroscopy as described in the main text) were found after storage for >12 months. AuNP with mixed layers of PEGSH and MUA were prepared the same way, but aggregated during the centrifugation steps as described in the main text. AuNP coated with just PEGSH were stored under the same conditions as the AuNP@PEGMUA/MUA conjugates and were also stable for >12 months. AuNP concentrations were adjusted and determined by UV/vis spectroscopy as described by Haiss et al.⁵⁶

Characterization. ATR-FTIR Spectroscopy. FTIR spectra were recorded with a Varian 660 FTIR spectrophotometer equipped with a PIKE MIRacle ATR sampling accessory. Spectra of PEGMUA and MUA were recorded from the pure solids pressed onto the crystal (diamond) with a high pressure clamp. To record spectra of AuNP samples, 2–3 μL of the according concentrated solutions ($c(\text{AuNP}) \sim 1 \mu\text{M}$) were pipetted directly onto the crystal. After evaporation of the solvent (water, usually within 15–30 min) spectra were recorded. Afterward, the crystal was thoroughly cleaned with water and ethanol. Thirty-two scans in absorbance mode with 4 cm^{-1} resolution were recorded for each measurement.

UV/Vis Spectroscopy. Absorbance measurements were carried out using a PerkinElmer Lambda 25 or a Varian Cary 50 spectrometer. UV microcuvettes sealed with lids (Plastibrand, Carl Roth GmbH, Karlsruhe, Germany) were used for all experiments.

Transmission Electron Microscopy (TEM). TEM measurements were performed using a Jeol JEM-1011 instrument operating at 100 kV. For TEM sample preparation, 10 μL of sample solution was dropped onto a carbon-coated copper grid, which was placed on a glass slide, and left to dry for at least 24 h.

Gel Electrophoresis. For gel electrophoresis analysis, a 0.5% agarose gel was prepared by first dissolving 25 g of agarose in 500 mL of 1X TAE buffer solution (40 mM Tris, 40 mM acetic acid, 1 mM EDTA, in water). This was accelerated by heating the solution up to approximately 95 °C in a microwave oven. It was cooled down for 5 min at room temperature and poured into the gel box, which was equipped with a comb parallel to the electrodes. After complete polymerization, the comb was removed carefully and the box was filled with 1X TAE buffer, covering the entire gel and electrodes. 15 μL of each sample was mixed with 3 μL of gel loading dye (DNA Gel Loading Dye (6X) R0611, Thermo Scientific, USA) and transferred carefully into the respective pockets in the gel. The box was covered, and between the electrodes a direct voltage of 120 V was applied. After 200 min, the voltage was turned off. Photographic images of the gel were taken after 5, 50, and 200 min.

Small-Angle X-ray Scattering (SAXS). SAXS measurements were performed at beamline I22, Diamond Light Source, Didcot, UK. Two-dimensional scattering patterns were recorded with a Pilatus 2M detector at a sample–detector distance of 6.704 m using a $160 \times 300 (\nu \times h) \mu\text{m}$ X-ray beam with an X-ray energy of $E = 18 \text{ keV}$ (wavelength $\lambda = 0.68 \text{ \AA}$). The exposure time per SAXS pattern was 1 s. The SAXS patterns were azimuthally integrated, and the background signals from the buffer that were measured separately were subtracted from these. For the diluted suspensions, the SAXS signal is directly proportional to the particle form factor $P(Q)$. The form factor was modeled by that of polydisperse spheres.⁵⁷ Due to the low scattering contrast of the PEGMUA shell in water $\Delta\rho = 40 \text{ e}^-/\text{nm}^3$ compared to that of the gold core ($\Delta\rho = 4300 \text{ e}^-/\text{nm}^3$), the dominant scattering contribution arises from the latter one; i.e., the PEGMUA shell is not visible for the diluted suspensions.

Stability Tests. Stability against Cyanide Etching. For each etching experiment 800 μL of the AuNP sample was transferred into a

UV cuvette and mixed thoroughly with 200 μL of an aqueous KCN solution (1 M), yielding a KCN concentration of 0.2 M in the cuvette. The AuNP concentrations were 5–6 nM after mixing. The spectrometer was operated in cycle mode. Absorbance spectra in the range of 200–800 nm were recorded every 10 min for 18 h, collecting a total of 108 spectra per sample. During the measurement, the cuvettes remained sealed with a lid and were not shaken or stirred. The control experiments with PEGSH-coated AuNP, AuNP@PEGSH, were prepared accordingly, but the final KCN concentration was 0.1 M. All etching experiments were performed twice to test for reproducibility.

Stability against Competitive Adsorption of Dithiothreitol (DTT). 500 μL of each AuNP sample was transferred into a UV cuvette and mixed thoroughly with an aqueous solution containing DTT (2 M) and NaCl (0.8 M), yielding concentrations of DTT and NaCl in the mixture of 1 and 0.4 M, respectively. The AuNP concentrations were 5–6 nM after mixing. Absorbance spectra in the range of 200–800 nm were recorded in cycle mode every 2 min. For each experiment, 240 spectra were recorded over a period of 8 h, during which the cuvette was neither shaken nor stirred.

Internalization Assay. PC3 cells were cultivated in a 1:1 mixture of Ham's F12 and RPMI 1640, supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. LNCaP cells were cultivated in RPMI 1640 mixed with 20% fetal calf serum and 1% penicillin/streptomycin. For the experiments, PC3 cells were seeded with a cell density of 37 500 cells per well in a 24-well plate, while LNCaP cells were seeded with a density of 50 000 cells per well 24 h before the experiments. At the day of the experiments, the confluency was checked via light microscopy yielding approximately 70% confluency for both cell lines. The cells were washed two times with PBS, new media added, and the AuNP solutions were added to the cells to the desired final AuNP concentration. The cells were incubated with the AuNP for 1 and 24 h. After the incubation, the cells were washed twice so that there was no red color of the AuNP visible in the supernatant. Further, the cells were lysed with lysis buffer (0.1 M Tris-HCl pH 10, 0.1% Triton-X100) and prepared for element analysis. 200 μL of each sample was reacted with 200 μL of freshly prepared *aqua regia* overnight, then filled up to 1000 μL , and analyzed by GF-AAS to quantify the gold content.

Graphite Furnace Atom Absorption Spectrometry (GF-AAS). GF-AAS measurements were performed with a ContrAA-700 AAS spectrometer (Analytik Jena, Germany) at 242 and 795 nm. The limit of detection (LOD) was 10 $\mu\text{g/L}$. Measurements were performed in triplicates, and the relative standard deviation of the mean was 1–5%.

Toxicity Test Lactate Dehydrogenase (LDH) Activity Assay. To determine the toxicity of the AuNP, 7500 PC3 cells were seeded per well in a 96-well plate 24 h before the test. AuNP conjugates were added to the PC3 cells (final $c(\text{AuNP}) = 20 \text{ nM}$) and incubated for 1 and 24 h. Each sample was added to three different wells. The supernatants were collected after the different incubation times and stored at $-20 \text{ }^\circ\text{C}$ until the measurement. For the LDH assay the commercially available LDH Cytotoxicity Colorimetric Assay Kit II (Biovision Inc., USA) was used, and the samples and cells were prepared following the manufacturer's protocol. The analysis of the samples was performed photometrically at 450 nm using a plate reader (Tecan, Switzerland).

RESULTS

Synthesis and Characterization of Mixed Ligand Layers. A protocol for the highly reproducible synthesis of citrate-stabilized gold nanoparticles (AuNP) with very low dispersity (5–8%) and high uniformity was published recently.⁵⁵ Here, such AuNP were functionalized with mixtures of PEGMUA and MUA by simply mixing the reagents at room temperature (see the [Experimental Section](#)). The TEM characterization of AuNP used herein is provided as [Supporting Information](#), Figure S1.

The footprint of different thiols or, more general, different adsorbates, can differ strongly. While for MUA and other small alkyl thiols a footprint of 0.17 nm^2 is established,^{3,58} PEG molecules, thiolated DNA, peptides, and proteins can have much larger footprints up to several nm^2 per molecule. Therefore, the assessment of the ligand layer composition based on the composition of the reaction mixture of ligands before conjugation is difficult.⁵⁹ Will the ratio of the ligand reaction mixture transfer to the number ratio of ligands bound to the particle surface or the relative areas they occupy? Or will one ligand bind preferentially? Size, polarity, solubility, and steric demand of the ligands additionally affect the kinetics and enthalpy of their adsorption and therefore the composition of the mixed ligand layer.^{39,40,60,61} By using the same spacer for the adsorbed ligands, some of these complications are avoided. The footprint is more homogeneous, and the chemical structure near the thiol group is the same. This results in better control of the ligand layer's composition that is closer to the composition of the reaction mixture than for ligands with completely different footprints and affinities. It is known that for solutions containing two thiols adsorption of the thiol with the longer alkyl chain is preferred⁶⁰ a binary SAM is more homogeneous when the thiols have the same number of methylene units⁶¹ and that attractive van der Waals (vdW) interactions add significantly to the adsorption enthalpy.⁶² Therefore, a mixture of thiols with long alkyl chains, all of the same length, should be a good choice for the formation of stable and well-defined mixed ligand layers on AuNP. In the following sections we will show that this is indeed the case for mixtures of MUA and PEGMUA (2 kDa), even though their molecular masses differ by a factor of ~ 10 . We note that for AuNP with diameters $>4.4 \text{ nm}$ the structure of alkanethiolate SAMs on the gold surface has been shown to be similar to SAMs on planar gold surfaces; i.e., curvature effects are not expected to play a dominant role for the AuNP used in this study.⁶³

To directly analyze the mixed ligand layer compositions, we used Fourier transform infrared spectroscopy with attenuated total reflection (ATR-FTIR). Distinct modes of MUA and PEGMUA can be identified and utilized to quantify the relative ratios of the according ligands in the mixed ligand layer as shown in Figure 2. The data show a clear trend evidencing a decreasing PEG ratio in the ligand layer of the synthesized and purified conjugates with increasing MUA ratio used in the synthesis. This indicates that the composition of the ligand layer can be controlled by the experimental conditions.

The control of the conjugates' charge via ligand layer composition is demonstrated by gel electrophoresis experiments. The migration of the conjugates in an externally applied electric field is in complete accordance with their assumed ligand layer composition and surface charge (Figure 3), underlining the level of control in the synthesis. The AuNP with just MUA migrated much faster than all other conjugates, indicating a strong attenuation of electrophoretic mobility by the PEG moiety resulting from an increased hydrodynamic diameter. The electrophoretic mobility is affected by both the hydrodynamic diameter and the charge of the AuNP, and the effects cannot be separated. Considering that the change of AuNPs' hydrodynamic diameter, e.g. from 0 to 10% MUA content, should be very small but their migration differs significantly (compare Figure 3, bottom), we assume that the effect of charge is dominating in this experiment.

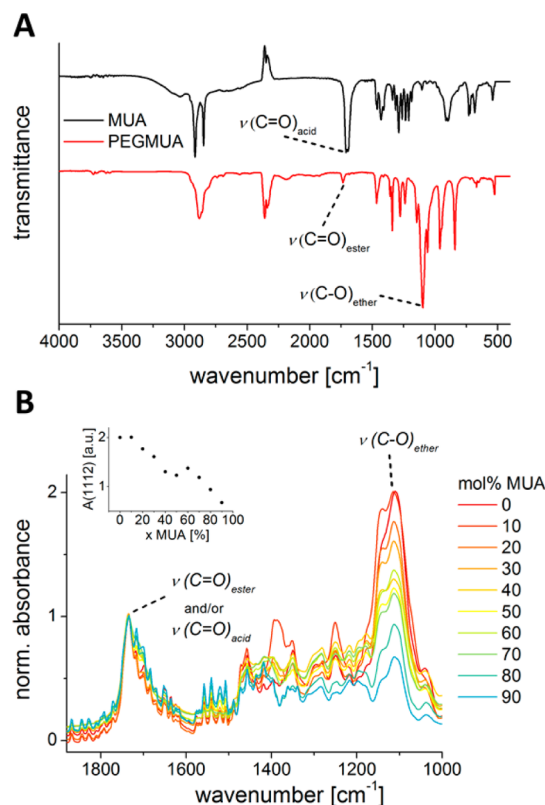


Figure 2. ATR-FTIR spectra of ligands MUA and PEGMUA (A) and AuNP conjugates with mixed ligand layers (B). A distinct spectral feature of MUA is the strong acidic C=O vibration at 1712 cm^{-1} . The close ester C=O vibration of PEGMUA at 1730 cm^{-1} is much weaker. For PEGMUA, the ether C–O vibrations at $\sim 1100 \text{ cm}^{-1}$ are characteristic (A). In the spectra of AuNP conjugates with different mixed ligand layers the backgrounds were accounted for with a second-order polynomial and subtracted. The spectra were then normalized at 1720 cm^{-1} to analyze the changing intensity ratio of the bands at 1720 and $\sim 1100 \text{ cm}^{-1}$ (B). The band at 1720 cm^{-1} is dominated by the acidic C=O vibration of MUA. The band at $\sim 1100 \text{ cm}^{-1}$, related to PEGMUA, decreases in intensity with increasing MUA ratio (assumed MUA mole percent based on the synthesis are indicated by the color code). The correlation of the relative intensity at 1112 cm^{-1} (maximum of the band at $\sim 1100 \text{ cm}^{-1}$) and the MUA mole percent of the MUA/PEGMUA reaction mixture is plotted in the inset. Part A is adapted with permission from ref 64.

The surface charge of colloids affects many of their properties and interactions. For example, the self-assembly of the AuNP conjugates was found to depend strongly on the presence of MUA in the ligand layer (Figure 4). Thus, precise control of the surface charge provides a tool not only for fundamental studies but possibly also for manipulating the assembly of nanoparticles.

In summary, the synthesis of mixed ligand layers of PEGMUA and MUA is straightforward and allows control of the AuNPs' surface charge. The layer composition affects the self-assembly of the AuNP and can more generally be expected to affect the interaction of the AuNP with different substrates, an aspect interesting in the context of nanofabrication.

Colloidal Stability. PEGMUA with a molecular weight of 2 kDa (or 5 kDa) provides AuNP with high stabilization against centrifugation. When aqueous solutions of AuNP@PEG-MUA2k (or -5k) are centrifuged at $20000g$ for 20 min, no signs of aggregation can be found. Thus, these conjugates can

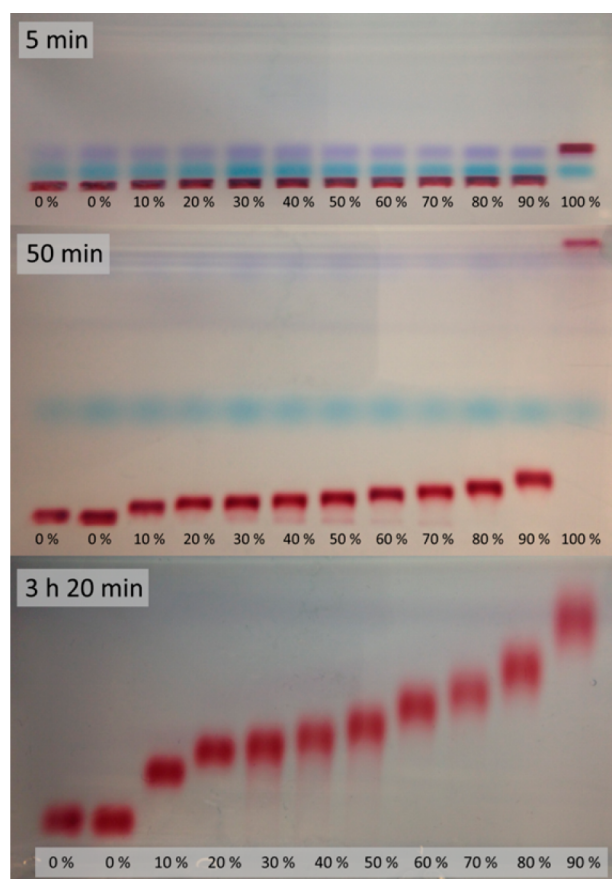


Figure 3. Migration of AuNP with different mixed ligand layers in an externally applied field (120 V) in a 0.5% (w/v) agarose gel. Blue and violet bands originate from the loading dyes bromophenol blue and xylene cyanol. The mole percent of the MUA/PEGMUA reaction mixture is indicated at each band. The AuNP with only MUA (100%) migrated much faster than all other conjugates and are not shown in the photograph after 3 h 20 min.

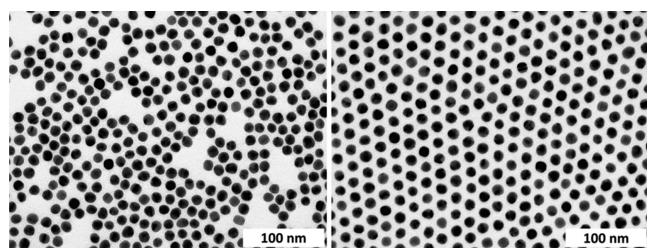


Figure 4. Transmission electron microscopy (TEM) images of AuNP conjugates with 75% (left) and 0% MUA (right). Purified aqueous solutions of the according AuNP conjugates were drop-casted onto carbon-coated copper grids and analyzed by TEM after drying at room temperature overnight. The self-assembly of the conjugates is strongly affected by their ligand layers' composition.

be purified and concentrated with negligible losses. This mechanical stability allows to achieve very high particle concentrations by centrifugation.

When comparing AuNP conjugates with mixed layers of PEGMUA and MUA, high stability against irreversible aggregation induced by centrifugal forces was observed for MUA ratios up to 90%. Significant aggregation was only observed for 100% MUA conjugates. Slight aggregation was observed for some samples with MUA ratios > 75%. Formation

of loose aggregates during centrifugation could be reversed by ultrasonication. At very high AuNP concentrations ($>1 \mu\text{M}$), however, the pellets of mixed layer conjugates are increasingly difficult to redisperse in comparison to the pure AuNP@PEGMUA conjugates. This underlines that the stability against centrifugation does not only depend on the parameters of centrifugation and the particle size but also on their concentration. At higher particle concentrations the compression of lower parts of the pellet becomes more severe due to its increasing mass.

In order to evaluate the colloidal integrity of concentrated AuNP solutions, small-angle X-ray scattering (SAXS) measurements were performed (Figure 5). The optical density of such concentrated samples is far too high for UV/vis spectroscopy, and SAXS provides a complementary technique to obtain structural information, in particular to determine the presence of aggregates in solution. At $c(\text{AuNP}) = 200 \text{ nM}$ no destabilization of conjugates with 25, 50, or 75% MUA ratio was observed. The experimental SAXS curves for all samples exhibit the same shape, i.e., position of the pronounced minima as well as similar forward scattering, for the whole Q -range determined, indicating their integrity. In more detail, the radii and polydispersities obtained by fitting the SAXS data were in excellent agreement with TEM analysis (Table S1), and no strong contributions at low Q -values were observed for all samples studied, indicating little or no aggregation. In conclusion, very high stability against irreversible aggregation can be obtained for mixed layer conjugates, even with MUA ratios as high as 75%. A difference in stabilization was observed only at very high particle concentrations ($>4 \mu\text{M}$ AuNP = 40 mg/mL Au) in that conjugates with 100% PEGMUA exhibited immaculate colloidal integrity, whereas for conjugates with mixed PEGMUA/MUA layers indications of aggregates were observed (Figure S2). The colloidal integrity of the main population, however, was unaffected.

The high colloidal stability of AuNP conjugates with PEGMUA/MUA mixtures is in stark contrast to that of AuNP coated with mixtures of a PEG-thiol of the same length but without C_{10} -spacer (α -methoxy- ω -mercaptopoly(ethylene glycol), 2 kDa, PEGSH) and MUA (Figure 6). After four centrifugations, all mixtures of PEGSH with MUA were aggregated irreversibly, visible by a broadening and red-shift of the plasmon band and decrease of absorbance. Redispersion of the aggregated samples by ultrasonication was impossible. Only AuNP coated with 100% PEGSH, i.e., when no MUA was added, were stable. In contrast, all AuNP coated with PEGMUA/MUA mixtures showed no signs of aggregation, and only AuNP coated with 100% MUA aggregated.

These observations demonstrate the remarkable difference in competitive adsorption behavior of PEGMUA and PEGSH. Several recent studies have shown that PEGSH ligands can be competitively displaced by small alkyl thiols. Especially Smith et al. have recently provided a detailed analysis based on quantitative NMR that demonstrated the ability of MUA to competitively displace PEGSH ligands.⁴⁴ This displacement or competitive adsorption took place regardless of whether MUA was added simultaneously with the PEGSH ligand or to AuNP that were already functionalized with PEGSH. The observation that even mixtures with just 10% MUA and 90% PEGSH do not provide colloidal stability for AuNP confirms that the adsorption of MUA must be kinetically and thermodynamically strongly favored over PEGSH adsorption. Thus, a controlled synthesis of mixed ligand layers with MUA and PEGSH is

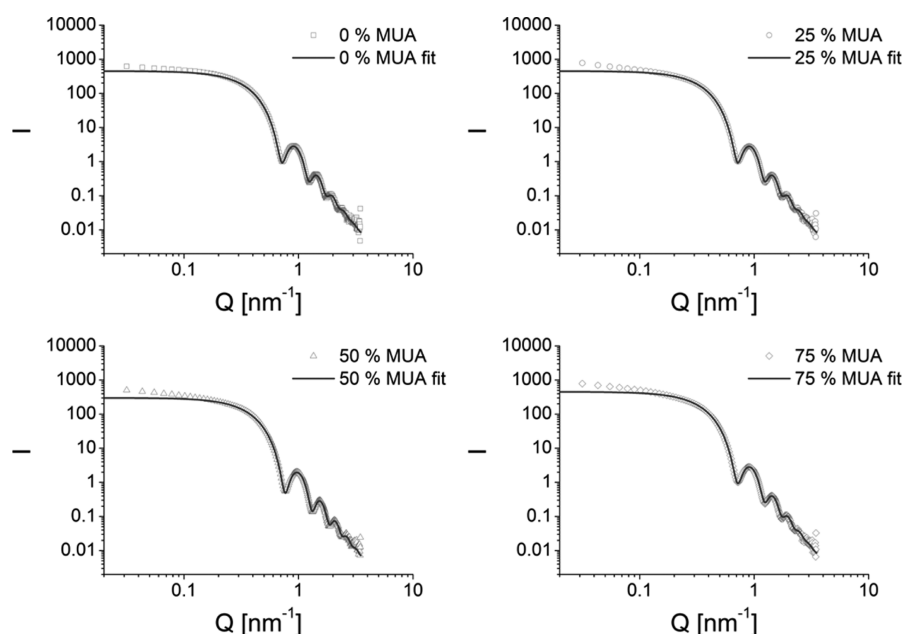


Figure 5. SAXS data of AuNP conjugate dispersions at high concentrations ($c(\text{AuNP}) = 200 \text{ nM}$) in water. Solid lines are fits to the data using the form factor of polydisperse spheres. Because of the low scattering contrast of the PEGMUA shell, only the Au core is visible. Aggregation would be visible by a strong increase of the scattering intensity at very low wave vector transfer Q .

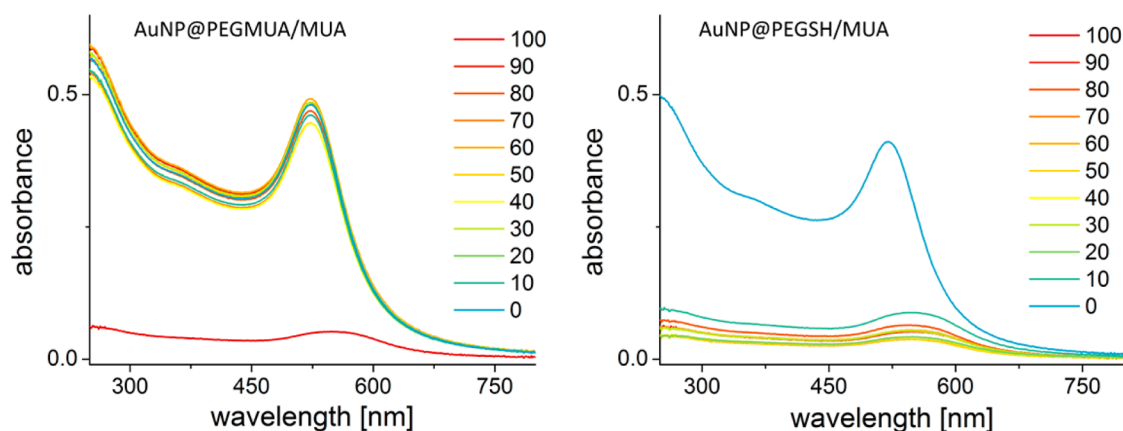


Figure 6. Absorbance spectra of AuNP conjugates after four centrifugations and redispersion ($20000g \times 20 \text{ min}$ at room temperature). The initial AuNP concentrations were $\sim 5 \text{ nM}$, and the final concentrations of stable samples were $\sim 150 \text{ nM}$. The concentrated samples were diluted for UV/vis measurements. AuNP coated with PEGMUA/MUA mixtures (left) were stable and showed no signs of aggregation, whereas AuNP coated with mixtures of PEGSH and MUA were all aggregated irreversibly. The mole percent of MUA used in the synthesis of the conjugates are indicated by the color code.

impossible. In contrast, PEGMUA/MUA mixtures provide colloidal stability even with just 10% PEGMUA. Tailoring the molecular structure of the PEG by introducing a C_{10} spacer completely changes the adsorption behavior to a much more balanced situation.

To test the steric effect of the PEG moiety on the colloidal stability, we compared AuNP functionalized with different ligands. AuNP stabilized with just MUA or PEGMUA with 716 Da (PEGMUA716) showed significant aggregation after centrifugation as indicated by DLS (Figure 7A). This resulted in significant concentration losses (85–90% in the case of MUA, 35–40% for PEGMUA716). In contrast, PEGMUA with 2000 or 5000 Da provided sufficient stabilization, and no indications of aggregates were observed (Figure 7B; see also SAXS results in Figure 5 and Figure S2). The DLS experiments confirm the increasing thickness of the ligand shell using

PEGMUA_{sk} instead of PEGMUA_{2k} and that PEGMUA716 is not providing sufficient steric stabilization to maintain colloidal stability during repeated centrifugation. The size distributions of AuNP@MUA and AuNP@PEGMUA716 are bimodal, indicating the presence of aggregates. They can therefore not be used to determine the thickness of the respective ligand shells.

Thus, the incorporation of PEGMUA with a minimum molecular weight in the range of 2000 Da into the mixed ligand layers is necessary for an enhanced stabilization. MUA or short EG_xMUAS alone do not provide comparable stabilization of AuNP in this size range ($>10 \text{ nm}$ diameter).

Chemical Stability. Oxidative etching with cyanide was used to probe the accessibility of the AuNP's surface. The experiments were performed as described and discussed previously.¹⁴ In that study we demonstrated that the stability

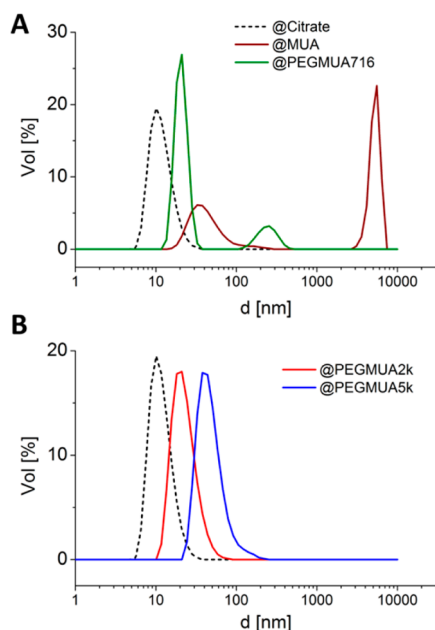


Figure 7. DLS measurements were performed to evaluate the mechanical stability of AuNP functionalized with different ligands as indicated by the color code. After four centrifugations ($20000g \times 20$ min at room temperature) and redispersion the AuNP were analyzed by DLS. DLS results of AuNP@Citrate particles (dashed lines) that were not centrifuged are shown for comparison. The starting AuNP concentrations of all samples were 3 nM. After the centrifugations, that are used to purify and concentrate the samples, the AuNP concentrations of AuNP@PEGMUA2k and AuNP@PEGMUA5k were 16 nM, AuNP@PEGMUA716 was 10 nM, and AuNP@MUA was 2 nM. Broad peaks and peaks at large hydrodynamic diameters in (A) indicate the presence of aggregates in the samples.

against oxidative etching dramatically increases with increasing length of a hydrophobic spacer. As a control experiment for our present study, we compared the stabilization provided by PEGMUA and by a commercial PEGSH ligand. As expected, the stabilization provided by PEGMUA is much higher: AuNP@PEGSH conjugates are etched completely within a few minutes, while only $\sim 20\%$ of the particle population of AuNP@PEGMUA conjugates are etched, even after several hours (Figure 8). The pronounced stability of the AuNP@PEGMUA conjugates is most likely provided by the inner hydrophobic layer, which is formed at the AuNP surface by the alkylene spacers. Also, due to hydrophobic interactions between the spacers, the PEGMUA ligands are probably more densely packed than PEGSH ligands (compare Figure 1) and, thus, provide additional stabilization. A high grafting density of PEGMUA ($\sim 3 \text{ nm}^{-2}$) in contrast to PEGSH was recently demonstrated with gold nanorods, strongly supporting this conclusion.⁶⁴

Comparing the oxidative etching of different mixed-layer MUA/PEGMUA-AuNP conjugates, a high stabilization was observed for all MUA ratios (Figure 8). This underlines that the PEG moieties in the ligand layer are not decisive for the conjugate's stability against oxidative etching but the molecular structure at the particle's surface. Despite the decrease in PEG density with increasing MUA ratio, the stability of the conjugates remains, and no clear correlation of the MUA fraction and stability against etching was observed. On the other hand, the results suggest that the packing density of PEGMUA is not much lower than that of MUA because

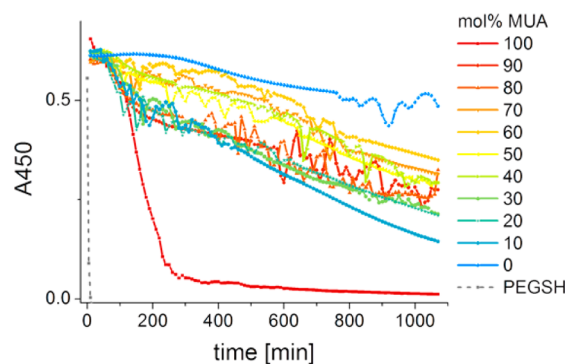


Figure 8. Stability of different AuNP conjugates in the presence of 200 mM KCN. The absorbance at 450 nm, A450, reflects the concentration of the AuNP that decreases during the etching reaction. The assumed mole percent MUA in PEGMUA/MUA mixed conjugates are indicated by the color code. The data for AuNP@PEGSH in the presence of 100 mM KCN are shown for comparison (gray squares, dashed line). AuNP coated with just MUA (100% MUA) is not stable in the presence of high electrolyte concentrations, and the decrease of their concentration is caused not only by etching but also by aggregation and sedimentation.

otherwise the stability should increase with the MUA ratio. The same effects were observed for the competitive displacement with DTT (Figure S3), confirming these conclusions. AuNP coated with just MUA are not as stable as AuNP coated with PEGMUA/MUA mixtures because their stabilization is mainly based on electrostatic repulsion provided by the carboxylate groups. This electrostatic repulsion is screened at high electrolyte concentrations leading to aggregation of the AuNP, similar to the well-known electrolyte-induced aggregation of citrate-stabilized AuNP. This behavior was also observed in both experiments—stability against oxidative etching and stability against competitive displacement with DTT (tested in the presence of 0.4 M NaCl)—and the explanation is the same.

It can be concluded that optimal stabilization requires both sufficient steric stabilization and a high grafting density of strongly bound ligands to form a hydrophobic inner shell. Coadsorption of sufficiently large PEG polymers provides steric stabilization against strong mechanical forces and at high electrolyte concentrations. MUA adsorption provides chemical stabilization against competitive displacement and other unwanted reactions at the particles' surface by forming an inner hydrophobic layer with high density. In PEGMUA both effects are combined, and we assume that the design principle can be generalized.

Stability in Serum and Interactions with Cells. Because the use of mixed ligand layers is a popular strategy especially in nanomedicine, we tested the stability of MUA/PEGMUA-AuNP conjugates in serum and their interaction with mammalian cells. In human serum, no destabilization of the conjugates was observed (tested for 10 days), neither at 37°C nor at room temperature (Figure S4). The uptake of the conjugates by prostate cancer PC3 cells was tested by incubation of the cells with the desired concentrations of AuNP conjugates. After 1 and 24 h incubation, the cells were washed, and their gold content was determined by elemental analysis as described in the Experimental Section. AuNP uptake slightly increased with increasing MUA ratio, but was very low ($<1\%$ of the gold added) for all samples (Figure 9). Similar results were obtained with A459 (lung cancer cells) and LNCaP (prostate cancer) cells (data not shown). The effect of AuNP

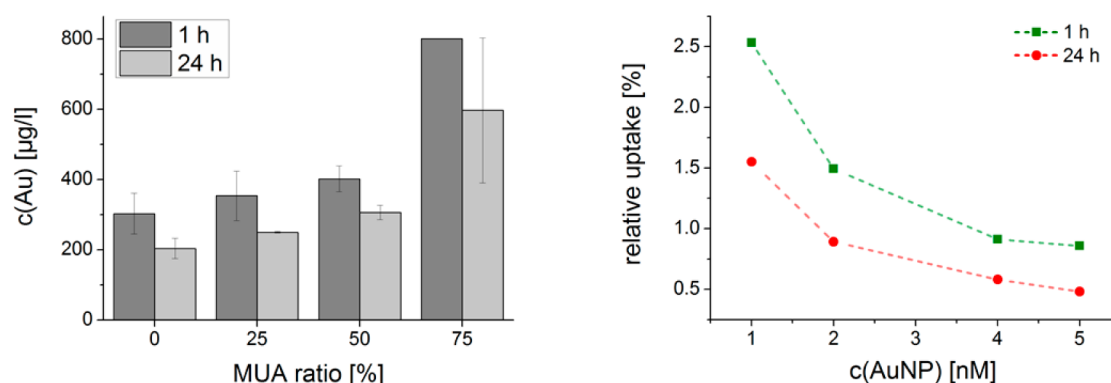


Figure 9. Left: uptake of AuNP ($c(\text{AuNP}) = 20 \text{ nM}$) with mixed ligand layers by PCR3 cells, 1 and 24 h after administration. The gold content was determined by elemental analysis as described in the [Experimental Section](#). The assumed (based on the synthesis conditions) MUA mole percent in the mixed ligand layer of PEGMUA and MUA are indicated. Right: relative uptake of AuNP@PEGMUA_MUA75 (assumed 75% MUA in the mixed ligand layer) for different AuNP concentrations and at different times after administration as indicated.

concentration was tested for AuNP conjugates with 75% MUA because these showed the highest uptake. To determine their relative uptake, the gold content of the medium was also analyzed. The relative uptake (gold content in the cells divided by the total gold content in cells and medium) decreased with increasing AuNP concentration, indicating low, unspecific uptake (Figure 9).

The uptake and cellular fate of AuNP are not completely understood yet.⁶⁵ PEG shells are known to reduce unspecific AuNP uptake, and thus, coadsorption of MUA could be an interesting tool to tailor and study the surface chemistry of bioconjugates. In our cell uptake experiments we found no signs of stress or toxicity (e.g., no changed morphology of the cells was observed with optical microscopy), even at high AuNP concentrations (tested up to 20 nM in the cell medium). Toxicity tests confirmed these findings (Figure S5). Colloids with stabilities that enable very high concentrations without inducing any toxicity are highly desirable for various applications in nanomedicine (e.g., CT-imaging and plasmonic photothermal therapy, PPTT).^{66–68} On the other hand, high stability is often compromised with increasing complexity of the system, e.g., by binding of functional molecules (peptides, DNA, antibodies, etc.) or labels. Finding the optimum balance of functionality and stability is usually a challenging task. The design proposed in this study represents a highly stable nanoparticle platform. Our experiments strongly indicate that the stability of bioconjugates with mixed ligand layers in general can be improved significantly by using PEGs and other ligands with hydrophobic alkylene spacers. These spacers (e.g., in form of MUA) can be coupled easily to PEG^{14,51} and biomolecules (PEGs, peptides, and oligonucleotides are also commercially available with undecanyl spacers, at least as custom synthesis). MUA in SAMs can be activated for EDC coupling and other coupling strategies,⁶⁹ possibly allowing for direct coupling strategies with AuNP@PEGMUA/MUA conjugates. Nieves et al. recently demonstrated that the incorporation of SH-MUAEG5-N3 into mixed ligand layers on AuNP enables the versatile functionalization, e.g., by introducing a maleimide group via click chemistry.⁷⁰ Such a ligand is also expected not to compromise the high stabilization of the mixed layers underlining the flexibility of the approach.

We have shown that functional peptides can be incorporated into mixed ligand layers with PEGMUA.⁷¹ Importantly, the lower number of conjugated peptides (because of the coadsorption of PEGMUA) did not result in decreased

functionality. In contrast, both the stability and functionality of the conjugates were improved with increasing PEGMUA ratio. The peptides in that previous study were not bound via a hydrophobic spacer, and we believe that by using such a spacer the control in the synthesis and stability of these mixed conjugates could be even further improved. In general, short functional peptides should be good candidates for the synthesis of functional mixed ligand layers as they typically have a similar size as PEGMUA with 2 or 5 kDa and via additional spacers (e.g., as oligoglycines) their presentation at the ligand shell surface can be tuned.

CONCLUSION

We have shown that by using a hydrophobic alkylene spacer comprising 10 methylene units (C_{10}), AuNP with tunable mixed ligand layers can be synthesized. The surface charge of these conjugates can be tuned without impairing their high stability against competitive displacement, oxidative etching, centrifugation, and against electrolyte concentration and protein adsorption in serum. In consequence, highly concentrated samples of these conjugates can be prepared with immaculate colloidal integrity, i.e., free of aggregates. Importantly, our study underlines that comparable stability of mixed ligand layer conjugates cannot be achieved by using PEG-thiol ligands without a hydrophobic spacer. Additional experiments demonstrated that the PEG part of the ligand layer is responsible for the colloidal stability, whereas the inner hydrophobic layer consisting of alkylene chains provides chemical stability. These findings are especially relevant for the synthesis of nanoconjugates for applications in medicine and bionanotechnology, where the use of mixed ligand layers including PEG-thiols as stabilizers is a popular strategy.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.langmuir.6b01704](https://doi.org/10.1021/acs.langmuir.6b01704).

TEM characterization of all AuNP batches, additional SAXS results, UV/vis monitoring of competitive ligand displacement with dithiothreitol, UV/vis spectra of AuNP conjugates in serum after different incubation times, results of lactate dehydrogenase (LDH) activity assay with different AuNP conjugates (PDF)

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Notes

The authors declare no competing financial interest.

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2.5 Defined Coadsorption of Prostate Cancer Targeting Ligands and PEG on Gold Nanoparticles for Significantly Reduced Protein Adsorption in Cell Media

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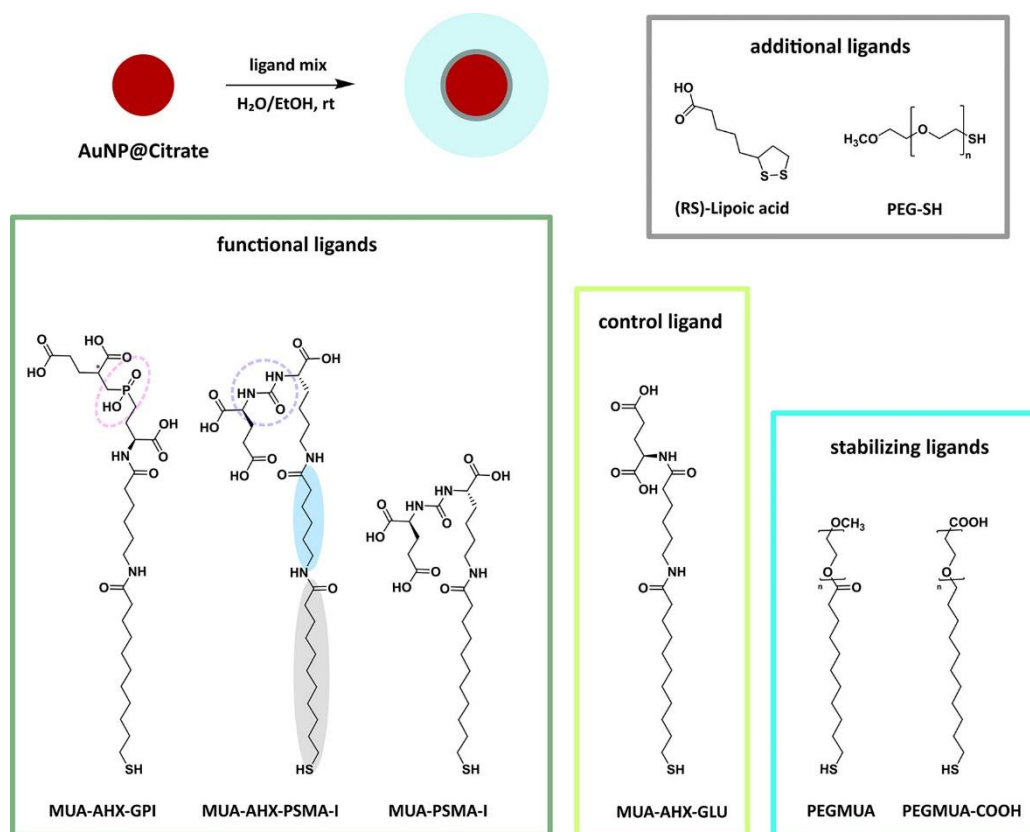


Figure 3. Overview of the different ligands used in this study. The functional ligands or targeting ligands for a prostate cancer related receptor. The functional, control and stabilizing ligands all feature the same structural unit, a MUA (11-mercaptopundecanoyl-) spacer which is highlighted in gray.

In this work the idea of mixed ligand layers was extended further. Whereas the work described in section 2.4 used a model system (PEGMUA/MUA mixtures), here designed ligands were used, which feature moieties for the targeting of the prostate-specific membrane antigen (PSMA), so-called PSMA-inhibitors, which are clinically established for the targeting of prostate cancer.¹⁴⁹⁻¹⁵³ The synthesis of these ligands was led by the Maison group (University of Hamburg) and published in a previous publication that focused on cell experiments and X-ray fluorescence imaging- (XFI-) based quantification of targeted AuNP in cells.¹⁵⁴ This study was based mainly on gel electrophoresis experiments, which were used for two purposes. Firstly, they allowed to confirm the control of ligand layer composition as discussed in the study before. Secondly, they were used to control for protein adsorption, that significantly changes the migration behavior of the NP in the gel. It could be shown that the ligand layer composition could be controlled for mixtures of all

functional ligands with PEGMUA. The proposed explanation for this was that all functional ligands featured a MUA-spacer (Figure 3) which ensures that the adsorption properties (enthalpy, molecular structure, footprint) are similar. It was also shown, that co-adsorption of PEGMUA always reduced protein adsorption significantly to an extent that it couldn't be measured with gel electrophoresis. In contrast, without PEGMUA co-adsorption a strong effect of protein adsorption was observed for all samples. This is an important finding, because it underlines that the use of a functional ligand alone can be counterproductive, because protein adsorption can “mask” the targeting ligand and completely change the NPs' properties in an undesirable way. In additional experiments the role of carboxylic acid groups and PEG length was addressed. The targeting ligands feature several carboxylic acid groups and it was therefore tested if coating of AuNP with PEGMUA terminated with a carboxylic acid group (PEGMUA-COOH) will increase protein adsorption. With optimization of the protocol well defined mixed-ligand layers of PEGMUA and PEGMUA-COOH could be synthesized and no indication of protein adsorption for neither of these mixed conjugates was observed. This shows that the carboxylic acid groups alone are not responsible for protein adsorption, or that PEG can efficiently reduce protein adsorption, even in the presence of carboxylic acid groups. A minor, but quite interesting and important aspect of this work is mentioned in the Supporting Information (Figure S4): the modulation of surface charge due to co-adsorption of charged ligands can be nicely studied with gel electrophoresis experiments but with ζ -potential measurements no effect is detected. The ζ -potential is often also used as a proxy for the surface charge of colloids, but this observation underlines that the ζ -potential does not *equal* the surface charge. In the conjugates with complex ligand layers studied herein, the surface charge is probably “masked” by the surrounding PEG ligands and does not affect the ζ -potential.

Defined Coadsorption of Prostate Cancer Targeting Ligands and PEG on Gold Nanoparticles for Significantly Reduced Protein Adsorption in Cell Media

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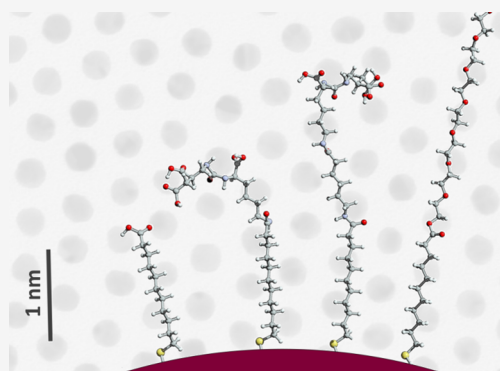


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Supporting Information

ABSTRACT: A study on the surface chemistry of gold nanoparticles (AuNPs) utilizing gel electrophoresis is presented. The molecular design of targeting and stabilizing PEG ligands allows one to combine them in mixed ligand layers with control of relative composition. For all tested targeting and control ligands, coadsorption of PEG significantly reduces protein adsorption and matrix effects of cell media as compared to conjugates without PEG. Introducing carboxylic acid groups into the PEG ligand layer does not lead to increased protein adsorption as shown with carboxylic acid terminated PEG ligands. The controlled adsorption of PEG ligands with different molecular weights is used to disentangle the effects of particle charge and hydrodynamic diameter. Taken together, this study provides directions for the functionalization of nanoparticles to obtain conjugates which are well-defined in terms of composition and colloidal stable in complex media. This is in particular relevant for targeting applications and cell experiments.



INTRODUCTION

Targeting of cancer with nanoparticles could be a viable tool to improve early diagnosis and therapeutic outcome.^{1–8} For radiotherapy gold nanoparticles (AuNPs) are discussed as a contrast agent providing additional radiosensitization effects.^{9–17} However, from the colloidal chemistry perspective, the various possible complex interactions of nanoparticles in cells and organisms are challenging the confident interpretation of experimental data and ultimately the reproducibility.^{18–24} In particular, the adsorption of proteins to nanoparticles, which in turn influences their interaction with cells, has been studied and discussed extensively and the term protein corona has been coined.^{25–36} The adsorption of proteins, i.e., protein corona formation, can significantly affect the stability and functionality of NPs in biological media.^{28,33,37,38} For instance, with regards to colloidal stability, it can provide a stabilizing as well as a destabilizing effect that results in agglomeration or aggregation of the NPs.³⁹ Aggregation in turn can have important consequences for cell experiments, for instance leading to a high uptake, that is, however, unspecific.^{40,41} The interactions of NPs with proteins is dictated by the NPs' surface chemistry, and consequently, a controlled surface chemistry is a prerequisite for controlled experiments and applications of NPs. In this context, poly(ethylene glycol) (PEG) is an established polymer ligand that reduces protein

interactions among other benefits.^{42–50} A typical approach when using PEG for the stabilization of nanodrugs is the terminal functionalization of the PEG ligands with a targeting ligand for improved overall stability of the NP-conjugate in biological media.⁵¹ Conceptually, this can alter the interaction with proteins because the pure PEG-interface is changed to a mixed interface: PEG and targeting ligand. Another approach is the preparation of mixed ligand layers. It has been shown how the chemical structure of the anchoring moiety can be used to control the mixture of PEG-ligands and small (but not functional) molecules.⁵² Here, we present the functionalization of gold nanoparticles (AuNPs) with clinically relevant targeting ligands, which are known to bind to the prostate specific membrane antigen (PSMA).^{11,12,53–59} To facilitate the controlled incorporation of these ligands into mixed ligand layers on AuNPs, they are equipped with a 11-mercaptopoundecanoyl spacer. With gel electrophoresis experiments we study

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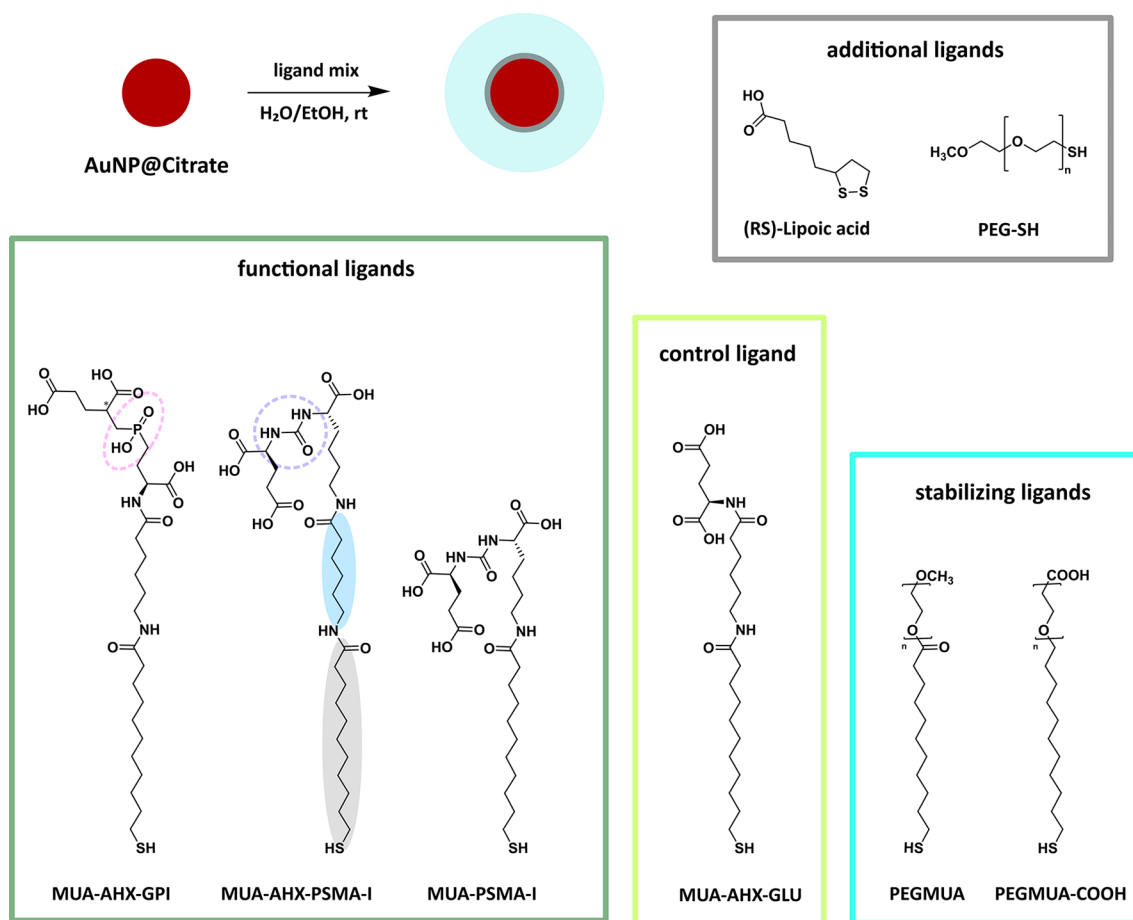


Figure 1. Overview of all ligands used in the study. For MUA-AHX-PSMA-I the MUA (11-mercaptopundecanoyl-) spacer is highlighted in gray and the AHX (6-aminohexanoyl) spacer in blue. PSMA-I features a urea (violet dashed circle) linked glutamate residue, mimicking the natural substrate of the PSMA catalytic cycle. GPI features a phosphinate (magenta dashed ellipse) linked glutamate residue.^{68,69} PEGMUA and PEGMUA-COOH had a molecular weight of ~2000 g/mol corresponding to $n \sim 45$ monomer units. In complementary experiments PEG-SH ligands without MUA spacer with different molecular weights (750 g/mol, 2000 g/mol, 5000 g/mol, 20 000 g/mol) were used. The sketch in the top left illustrates the functionalization and the according sizes to scale ($d(\text{AuNP}) = 12$ nm, $d(\text{AuNP} + \text{ligand shell}) = 28$ nm, thickness of “inner ligand shell” (gray) = 2 nm) as discussed in the main text.

the incorporation of these ligands into mixed ligand layers with PEG-ligands. The latter lead to significantly reduced protein interactions of the resulting nanoconjugates, and we find a clear correlation of ligand ratios in the syntheses and migration behavior in the gel electrophoresis experiments. In complementary experiments, we show that the terminal presentation of carboxylic acid groups in the PEG ligand layer does not affect its biorepulsive properties and try to experimentally disentangle the contribution of the hydrodynamic diameter to the migration behavior of the conjugates. Taken together these results outline a straightforward route to prepare and characterize well-defined functionalized AuNPs which are stable and show low levels of protein adsorption. With these protocols, large gold concentrations ($c(\text{AuNPs}) > 1 \mu\text{M}$ corresponding to $c(\text{Au}) > 10 \text{ mg/mL}$) can be prepared. These are desired for many experiments in nanomedicine, for instance computed tomography studies in phantoms and tissue and *in vivo* studies.

EXPERIMENTAL METHODS

Materials. All chemicals for the AuNP synthesis, ethanol (96%, techn. grade), Dulbecco's modified Eagle's medium (DMEM) without phenol red, Roswell Park Memorial

Institute (RPMI) 1640 Medium, fetal bovine serum (FBS), and (RS)-lipoic acid were ordered from commercial sources (Sigma-Aldrich, Merck, VWR, Roth) and used as received. Broad range agarose and 50× Tris-acetate-EDTA (TAE) buffer were purchased from Roth (Carl Roth, Germany). α -Methoxypoly(ethylene glycol)- ω -(11-mercaptopundecanoate) (PEGMUA ($M_w = 2156$ g/mol, $\text{PI} = 1.04$)) was synthesized as described before.⁶⁰ α -Methoxypoly(ethylene glycol)- ω -thiol ligands (PEG-SH, $M_w = 750, 2000, 5000$ and $20\,000$ g/mol) were purchased from Rapp Polymers (Tuebingen, Germany). α -(11-Mercaptopundecyl)poly(ethylene glycol)- ω -propanoic acid (PEGMUA-COOH, $M_w = 2000$ g/mol, $\text{PI} = 1.09$) was from Polymer Source (Dorval, Canada). Ligands with aminohexyl (AHX) spacer were synthesized via NHS-coupling chemistry as reported.^{40,61} MUA-PSMA-I was obtained by custom synthesis (ABX advanced biochemical compounds, Radeberg, Germany). Ultrapure water was used for all procedures and syntheses.

AuNP@Citrate Synthesis. Citrate-stabilized AuNPs in water, AuNP@Citrate, were synthesized and characterized with UV/vis absorption spectroscopy, transmission electron microscopy (TEM), and dynamic light scattering (DLS) as described previously.⁶² Their mean diameters were determined

with TEM-analysis (exemplary TEM measurements are provided as Supporting Information, Figure S1) and used to determine their concentrations $c(\text{AuNP})$ based on their absorbance at 450 nm or at 400 nm according to the methods described by Haiss et al.⁶³ and Scarabelli et al.⁶⁴ E.g. the extinction coefficient for $d = (12.0 \pm 0.6)$ nm AuNPs is $\epsilon = (1.2 \pm 0.2) \times 10^8 \text{ M}^{-1} \text{ cm}^{-1}$ and for $d = (12.6 \pm 0.8)$ nm $\epsilon = (1.4 \pm 0.3) \times 10^8 \text{ M}^{-1} \text{ cm}^{-1}$ assuming sphericity of the AuNPs.

Synthesis of Mixed Conjugates with Targeting Ligands. AuNP@Citrate with a mean gold core diameter of (12.6 ± 0.8) nm were used. Ligand stock solutions were prepared with $c = 1.0$ mM. PEGMUA was dissolved in water, and the targeting ligands MUA-AHX-PSMA-I, MUA-AHX-GLU, and MUA-AHX-GPI (cf. Figure 1) were dissolved in ethanol. To obtain the different nominal ratios of ligand:PEGMUA, the stock solutions were mixed in the according volume ratios and ethanol or water was added to yield volumes of 1600 μL with a 1:1 ratio of water to ethanol and a total ligand concentration of 0.5 mM. For MUA-AHX-PSMA-I, additionally a diluted approach was prepared with ten times less ligand, i.e. 50 μM ligands in the 1600 μL . PEGMUA ratios of 100% (solely PEGMUA), 75%, 50%, 25%, and 0% (solely targeting ligand) were prepared for each ligand. These are the nominal ratios, which are referred to throughout the text. The exact compositions of the resulting ligand layers on the AuNPs are not known. AuNP@Citrate (10 mL, $c(\text{AuNP}) = 1.3$ nM) were added to each ligand mixture which was stirred overnight. The mixtures were then purified and concentrated by repeated centrifugations (20 000 g, 30 min) to yield final AuNP concentrations in the range of 50–100 nM. 50 μL of these samples were incubated with cell medium (950 μL RPMI + FBS) overnight and then concentrated by centrifugation to ~ 50 μL once more for the electrophoresis experiments. Mixed conjugates with MUA-PSMA-I/PEGMUA were synthesized accordingly with another batch of AuNP@Citrate ($d = (11.6 \pm 0.6)$ nm) and using DMEM + FBS for the samples incubated with cell medium.

Synthesis of Mixed Layers of PEGMUA and PEGMUA-COOH. AuNP@Citrate in water ($d = (12.0 \pm 0.6)$ nm, $c(\text{AuNP}) = 3.7$ nM) were added to an aqueous solution of PEGMUA-COOH with a concentration corresponding to ~ 1 monolayer coverage (assuming for the calculation a footprint of 0.35 nm², similar to the one of PEGMUA determined in previous works^{65,66}) times the intended fraction of PEGMUA-COOH in the final conjugates: 0% (just PEGMUA), 25%, 50%, 75%, 90%, and 100% (just PEGMUA-COOH). After stirring the reaction for 1 h, the according amount (0%, 10%, 25%, 50%, 75%, or 100% of monolayer coverage) of PEGMUA in water was added and the mixture was stirred overnight (16 h). The dispersions were then concentrated and purified by at least three centrifugations (30 min, 20 000 g) to final concentrations of $c(\text{AuNP}) > 200$ nM.

Synthesis of Lipoic Acid/PEG-SH Functionalized AuNPs. AuNP@Citrate ($d = (12.6 \pm 0.8)$ nm, 10 mL, 1.3 nM) were reacted with aqueous (RS)-lipoic acid (800 μL , 1 mM) overnight under stirring. Then the conjugates were purified and concentrated by repeated centrifugation (30 min, 20 000 g) to ~ 50 μL . To prepare mixed ligand layers, aliquots of lipoic acid functionalized AuNPs were prepared accordingly. To each 50 μL aliquot was added 800 μL of water and 80 μL of the according PEG-SH solution ($c = 1$ mM, 750 Da, 2 kDa, 5 kDa, or 20 kDa), and the mixtures were placed on an orbital shaker for 60 min. Then, the mixtures were purified and

concentrated by repeated centrifugation (30 min, 20 000 g) to ~ 50 μL each.

Gel Electrophoresis Experiments. Gels were prepared with agarose (0.5 wt %) in TAE 1 $\times$. For the mixed conjugates with MUA-AHX-PSMA-I, MUA-AHX-GLU, and MUA-AHX-GPI and the mixed conjugates with lipoic acid, a 20 μL sample was mixed with 5 μL of glycerol in TAE (50% v/v) and pipetted into the wells of the gel submersed in the running buffer (TAE 1 $\times$) within the electrophoresis chamber. A constant voltage of 75 V was applied, and photographs were taken at different times as indicated in the main text. For all other samples, a 15 μL sample was mixed with 3 μL of loading buffer containing glycerol (30% v/v), TAE, bromophenol blue and xylene cyanol (DNA loading dye 6 $\times$, Carl Roth, Germany). The samples were pipetted into the wells of the gel (running buffer TAE 1 $\times$) and a voltage of 120 V was applied. The power supply used in these experiments had no constant voltage option, i.e., no measurement of effective voltage.

Complementary AuNP Characterization. TEM measurements were performed with a JEOL JEM-1011 at 100 keV, UV/vis absorption spectroscopy was done with a Cary 50 spectrophotometer and DLS measurements were done with a Zetasizer Nano ZS system (Malvern Instruments), which employs a He–Ne laser (4.0 mW, 633 nm). UV microcuvettes sealed with lids (Plastibrand, Carl Roth, Germany) were used for UV/vis absorption and DLS measurements. Folded capillary zeta cells (DTS 1070, Malvern Instruments, U.K.) were used for complementary ζ -potential measurements.

RESULTS AND DISCUSSION

Structure of the Nanoconjugates. An overview of the employed targeting ligands is presented in Figure 1, and a detailed description of their synthesis and characterization was presented recently.⁴⁰ The ligand α -methoxypoly(ethylene glycol)- ω -(11-mercaptoundecanoate) (PEGMUA) was synthesized by straightforward esterification as described previously.⁶⁰ All ligands used in the first set of experiments share the same anchoring structure, an 11-mercaptoundecanoyl residue, i.e., a C₁₀ spacer connecting the Au-binding thiol group with the rest of the respective molecule via an ester or an amide bond. It has been shown that such a spacer leads to an increased PEG grafting density and improves the chemical stability against competitive displacement of the ligands, which also leads to an improved stability in biological relevant media.^{52,60,65,67}

The PEG grafting density is directly related to the biorepulsive properties of PEG layers^{48,70–72} and a higher grafting density seems to be advantageous in terms of immune response.^{73,74} The inner hydrophobic region formed by the densely packed alkyl chains reduces displacement reactions at the AuNP surface⁶⁰ leading to strongly decreased opsonization and phagocytosis.⁶⁷ Most importantly for this study the MUA-spacer allowed for the controlled design of mixed ligand layers as tested previously with PEGMUA/MUA mixtures.⁵² With PEG-SH ligands without MUA spacer, no mixtures with MUA could be prepared. One goal of this study was to test the generality of this finding by testing the coadsorption of PEGMUA with different PSMA targeting ligands (Figure 1): MUA-PSMA-I, MUA-AHX-PSMA-I, and MUA-AHX-GPI. The PSMA, a very attractive tumor marker for drug delivery and radiotherapy, has multiple enzymatic roles including the cleavage of glutamic acid from *N*-acetylaspartylglutamate

(NAAG) or poly- γ -glutamated folates.^{69,75–78} The targeting ligands mimic the natural substrate NAAG and are known to inhibit PSMA in cell cultures with nanomolar affinity. As a control ligand, we synthesized MUA-AHX-GLU, which is simply glutamic acid attached to the spacers.

Citrate stabilized AuNPs (AuNP@Citrate) are available by reproducible, straightforward, and scalable protocols.⁶² Functionalizing these AuNP@Citrate with the according ligand mixtures can be achieved by simple mixing at room temperature. The sketch in Figure 1 (top left) also illustrates the geometry of the nanoconjugates to scale. The mean geometric diameter of the AuNPs is $d \sim 12$ nm (Figure S1), the length of the functional and control ligands is in the range 1–2 nm. Depending on the conformation the MUA spacer contributes ~ 1.2 nm to the ligand layer thickness.⁷⁹ In the sketch the dark gray part represents an inner layer of 2 nm thickness. The ligand PEGMUA used herein has an average molar mass of ~ 2000 g/mol corresponding to ~ 45 monomer units. This ligand forms a ligand shell in the dense brush conformation with 7–9 nm thickness, and coadsorption of small ligands (e.g., MUA) results in a reduced grafting density of PEG chains and consequently in some relaxation of the dense brush and a thickness of ~ 6 nm.⁶⁶ In the sketch, the PEG shell is represented as the blue circle with 28 nm diameter, i.e. a PEG layer with 8 nm thickness. This means that the targeting ligands are “buried” inside the PEG shell in the case of mixed ligand layers. Even though this can be expected to be advantageous in terms of protein adsorption, it could be a disadvantage for receptor binding. Due to radius of curvature effects “burying” however might not be quantitative. We speculate that an optimum balance exists regarding the presentation of the functional units within the ligand layer. By introducing additional spacers like AHX (oligo(ethylene glycol) spacers are also conceivable), this presentation can be tuned. Another noteworthy structural feature is that all targeting ligands have acidic groups like carboxylic and phosphinic acids, thus contributing a negative charge (at neutral and alkaline pH) to the conjugates, whereas the ligand PEGMUA has no charge. For an increasing amount of coadsorbed targeting ligands relative to PEGMUA, one therefore should expect an increase in negative charge and a slightly decreasing hydrodynamic diameter of the conjugates.

In a previous study, we prepared conjugates of all the targeting ligands and the control ligand without PEGMUA. Those conjugates were stable and could be concentrated in water and phosphate buffered saline (PBS), but in cell media + FBS, the colloidal stability of some was affected (stability trend: AuNP@MUA-AHX-GPI > @MUA-PSMA-I > @MUA-AHX-PSMA-I > @MUA-AHX-GLU), indicating unwanted protein interactions and salt effects. Cations like Ca^{2+} and Mg^{2+} , which are not present in PBS but are present in cell media and FBS, can significantly contribute to NP destabilization, even at comparably low concentrations (mM range).^{80,81} In cellular uptake studies with these conjugates, the results were inconclusive insofar high but unspecific uptake was observed, which indicates unwanted protein and salt interactions leading to agglomeration and sedimentation of the agglomerates onto the cells.⁴⁰ In conclusion on first sight, the conjugates with just targeting ligands seem to be advantageous in terms of size (a smaller size is usually considered desirable for improved clearance) and exposure of functional moieties. However, due to this high degree of exposure, or the absence of biorepulsive coadsorbents, in our

case, these advantages seem to be nullified by uncontrolled protein adsorption and salt effects leading to unspecific binding and low reproducibility in cell experiments. The second goal of this study was therefore to test if protein adsorption and salt effects can be reduced by PEGMUA coadsorption.

Gel Electrophoresis Experiments. Protein adsorption does not necessarily lead to agglomeration or aggregation of NPs and thus cannot be unambiguously monitored precisely with UV/vis absorption spectroscopy, which detects plasmonic coupling when AuNPs are in proximity⁸² and/or a decreasing absorbance due to sedimenting agglomerates or aggregates.⁸³ Changes in refractive index due to protein adsorption are small when ligands are already present.⁸² Dynamic light scattering (DLS) measurements of NP conjugates in cell media are also often difficult to interpret confidently, because proteins also contribute to the scattering and the complexity of the samples increases the chance of polydisperse distributions of hydrodynamic diameters (due to transient or permanent formation of high molecular weight protein aggregates or protein-NP agglomeration, adhesion to vessel walls, bubble formation, etc.).⁸⁴ To detect changes in the surface charge or hydrodynamic diameter of NPs or both, gel electrophoresis is an alternative method that provides various information in a straightforward experiment.^{52,72,85–97} It has to be noted that based on gel electrophoresis protein adsorption cannot be fully excluded because weakly adsorbed proteins can be stripped off during the experiment,⁹⁵ but it can reliably determine trends, in particular if the NP physicochemical characteristics like hydrodynamic diameter and surface charge are significantly altered. Because the mixed ligand layers studied herein consist of charged ligands (the targeting and control ligands) and neutral ligands (PEGMUA), the relative composition of the ligand layers can be easily determined with gel electrophoresis.⁵² The slight decrease in hydrodynamic diameter due to coadsorption of small ligands to PEGMUA contributes to the same trend in migration behavior as the increase in surface charge, namely an increase in electrophoretic mobility or drift velocity, respectively. With these considerations in mind, gel electrophoresis experiments were performed to characterize the conjugates. The purified AuNP conjugates with different ligand mixtures were incubated with water or medium (RPMI or DMEM) supplemented with FBS and pipetted into the wells of an agarose gel. Migration of the AuNP conjugates through the gel was induced by applying a voltage of 75 or 120 V. Several conclusions can be drawn from these experiments. First, we discuss the samples with mixed MUA-AHX-PSMA-I/ PEGMUA coatings (Figure 2). In water (Figure 2, parts a and b) AuNP@MUA-AHX-PSMA-I migrate much faster than the mixed conjugates containing PEGMUA, to an extent where they already passed the gel when the experiment was stopped. The according bands were well pronounced and discernible (Supporting Information, Figure S2). As discussed above, two factors define the mobility of the conjugates in the gel: their hydrodynamic size (NP core + ligand shell) and their net charge. MUA-AHX-PSMA-I is much smaller than PEGMUA, therefore the according ligand shell is thinner, and, in contrast to PEGMUA, is negatively charged at the pH of the experiment (pH = 8.3) due to deprotonation of the carboxylic acid groups in the molecules. Both factors contribute to the increased drift velocity of the pure AuNP@MUA-AHX-PSMA-I. The observation that the bands are sharp confirms uniform coating of all AuNPs in the sample. In contrast, the band of AuNP@MUA-AHX-PSMA-I incubated in cell medium (Figure

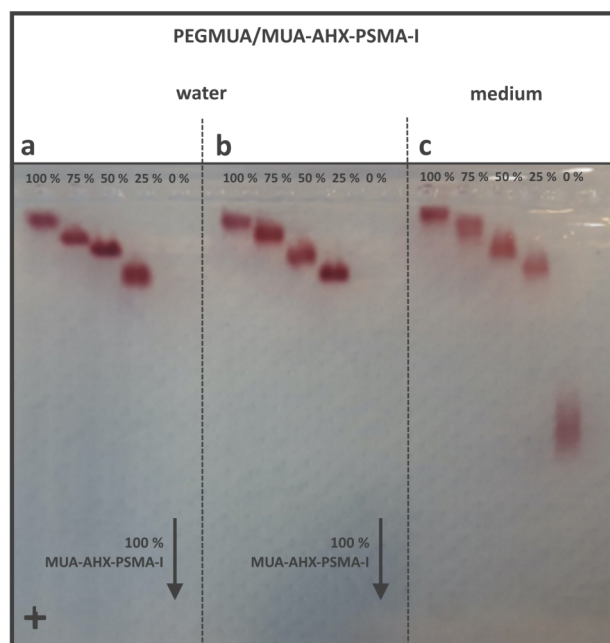


Figure 2. Electrophoresis of AuNP@PEGMUA/MUA-AHX-PSMA-I mixed conjugates after 2 h 38 min. The plus sign indicates the direction of migration and the plus pole of the electrophoresis chamber. In each set of samples the ratio of PEGMUA in the synthesis was 100%, 75%, 50%, 25% and 0% (just MUA-AHX-PSMA-I) from left to right. Conjugates prepared with diluted (a) and undiluted (b) ligand solutions stored in water and the same conjugates as in part b but stored in cell medium + FBS (c) are shown. In parts a and b, the conjugates with 100% MUA-AHX-PSMA-I cannot be seen because they migrated so fast that they already passed the gel at the end of the experiment. The same gel after 27 min is presented as [Supporting Information, Figure S2](#).

2c, right) is strongly smeared out, and the conjugates did not migrate as far as those incubated in water. This strongly indicates protein adsorption leading to an increase in the hydrodynamic diameter of the conjugates. These conjugates showed no signs of aggregation when stored in FBS, even for days, so strong salt effects leading to aggregation can be excluded (the adsorption of proteins does not necessarily lead to aggregation). Nevertheless, it cannot be ruled out that matrix components of the cell medium + FBS other than proteins contribute to the migration behavior. The possible interactions with the various molecular species and salts in the medium and the protein adsorption are not uniform for all AuNP-conjugates in the sample; therefore, the according bands are smeared out. Additionally, due to interactions with the agarose gel, part of the protein corona might detach during migration, leading to additional broadening of the band. Partial agglomeration under the conditions of the electrophoresis might also contribute to the broadening.

Comparing the pure MUA-AHX-PSMA-I conjugates with conjugates functionalized with PEGMUA/MUA-AHX-PSMA-I mixtures it is observed that all mixed conjugates migrate significantly slower, which, again, can be explained with the increased hydrodynamic diameter due to PEGMUA coadsorption, as well as decreased net charge of the particles. Furthermore, the bands of the mixed conjugates are not only well pronounced but follow the expected trend according to a decreasing net charge and a slightly increasing hydrodynamic diameter with increasing PEGMUA ratio. Similar behavior has been observed for AuNP conjugates with mixed ligand layers of PEGMUA and MUA.⁵² One can conclude that, with the experimental conditions, the relative ligand layer composition of the mixed conjugates can be well controlled. It should be noted, however, that a quantitative analysis based on gel electrophoresis is not possible; i.e., the absolute amounts of ligands are not known. The ligand excess has no effect on the

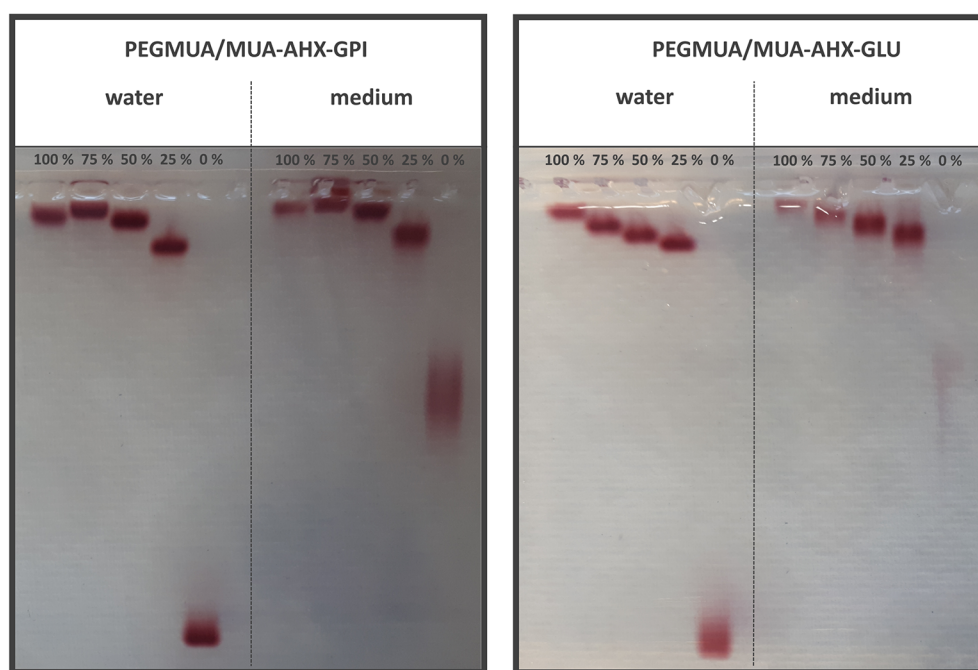


Figure 3. Electrophoresis of AuNP@PEGMUA/MUA-AHX-GPI and AuNP@PEGMUA/MUA-AHX-GLU mixed conjugates after 2 h. In each set of samples the ratio of PEGMUA in the synthesis was 100%, 75%, 50%, 25%, and 0% (just MUA-AHX-GPI or just MUA-AHX-GLU, respectively) from left to right. Conjugates stored in water and the same conjugates stored in cell medium + FBS are shown as indicated.

migration behavior (Figure 2, part a vs part b) indicating that the functionalization is robust even with low ligand excess (~ 4 times monolayer coverage total). For samples incubated in medium (Figure 2c), the migration behavior changed significantly for the AuNP@MUA-AHX-PSMA-I with no PEGMUA-coadsorption, but for mixed conjugates and pure PEGMUA-conjugates, no effect was observed. Even though protein adsorption cannot be completely ruled out based on these observations, they indicate that protein adsorption and salt effects are at least strongly reduced or protein NP interactions are weaker, or both.

In subsequent experiments, the same effect of PEGMUA-coadsorption was observed for all targeting and control ligands. In all cases, the migration behavior was in accordance with the PEGMUA:ligand ratio employed in the syntheses and no effect of protein adsorption or matrix effects could be observed for all samples with PEGMUA-coadsorption (Figure 3 and Supporting Information, Figure S3). In contrast, all conjugates without PEGMUA were strongly affected by incubation in medium + FBS, with similar effects as discussed: smeared-out lanes and much slower migration in the gel. The conjugates without PEGMUA can be observed in these experiments because they were stopped earlier (after 2 h). In the case of AuNP@MUA-AHX-GLU incubated in medium + FBS hardly any band could be observed due to destabilization. This destabilization is in agreement with the UV/vis absorption results presented previously.⁴⁰ For the MUA-AHX-GPI/PEGMUA conjugates an interesting behavior was observed. The mixed conjugates with higher PEGMUA ratios (50% and 75%) seemed to migrate slower than their analogues with other targeting ligands and in particular AuNP@MUA-AHX-GPI/PEGMUA with nominal 75% PEGMUA migrated slower than pure AuNP@PEGMUA. The reason for this is unclear, maybe the ligand MUA-AHX-GPI favors conformations of the coadsorbed PEGMUA which maximize attractive interactions with the gel (e.g., due to steric demands).

These results underline that PEG effectively reduces protein adsorption and matrix effects, salt effects in particular, even if coadsorbed in moderate ratios, and that the use of the MUA-spacer allows for coadsorption of the ligands for all tested targeting and control ligands. The observation that the bands of conjugates with mixed ligand layers do not smear out in gel electrophoresis experiments and that their migration behavior is in accordance with the ratios used in the syntheses indicates a good control of the composition, that is, however, not known quantitatively. Notably, the different charges of the conjugates can be clearly resolved with gel electrophoresis experiments in accordance with previous results⁵² but not with ζ -potential measurements (Supporting Information, Figure S4).

The Role of Carboxylic Acid Groups. We were additionally interested in the role of the conjugates' charge for protein adsorption and the effect of hydrodynamic radius on the migration in the electrophoresis experiments. To address the role of (exposed) carboxylic acid groups, we prepared mixed conjugates containing PEGMUA and PEGMUA-COOH (cf. Figure 1) in different ratios and incubated them in water or medium + FBS before gel electrophoresis. Interestingly, the synthesis of mixed conjugates was not as straightforward with these ligand mixtures as with the ones discussed before. In contrast to the mixed conjugates with PEGMUA and MUA-AHX-PSMA-I (cf. Figure 2), the absolute amount of ligands had a strong effect on the conjugate syntheses. PEGMUA seemed to bind stronger than

PEGMUA-COOH. Since the only differences of these ligands are the terminal carboxyl group and the different linkage of the MUA spacer (ester vs ether) one can speculate about the according roles of these structural features for the kinetics and packing behavior of the according molecular self-assembled monolayers (SAM),⁹⁸ but at this point the reason for the observed behavior is not clear. By optimizing the synthesis conditions, a good control of the conjugates' composition could be achieved, and we refer the interested reader to the Supporting Information for more details (Supporting Information, Figures S5–S7). In the electrophoresis experiments with AuNP@PEGMUA/PEGMUA-COOH mixed conjugates no strong effect of protein adsorption was observed (Figure 4),

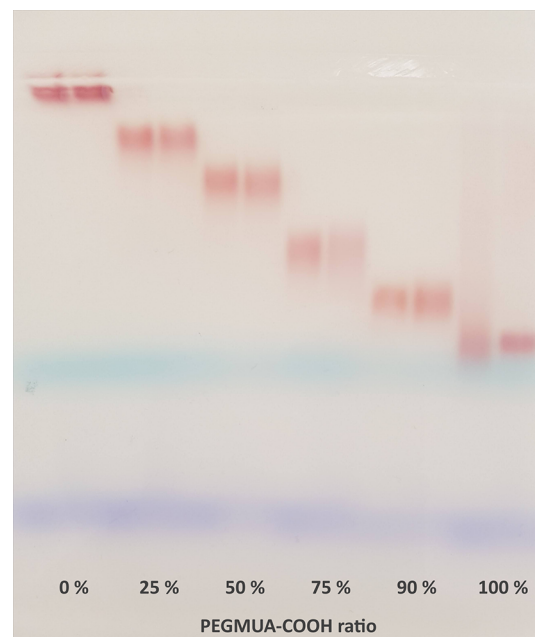


Figure 4. Gel electrophoresis for PEGMUA-COOH/PEGMUA conjugates with increasing ratio of PEGMUA-COOH as indicated after 2 h 20 min running time. For each ratio the left band corresponds to the sample incubated in water and the right band to the same sample incubated in medium + FBS. The cyan and violet bands are dyes (bromophenol blue and xylene cyanol) from the loading buffer.

regardless of the number of carboxylic acid groups, which dictated the migration in the gel. For these samples, the hydrodynamic radius is in the same range because the PEG ligands have the same molecular weight and the migration in the gel is primarily affected by the conjugates' charge. The stepwise increase in migration velocity with increasing number of carboxylic acid terminated ligands underlines the control of the ligand composition. This experiment indicates that the steric repulsion properties of PEG are not affected by the number of carboxylic acid groups, regardless of their presentation in the ligand layer: buried within the PEG layer or terminal. For the pure AuNP@PEGMUA-COOH conjugates some smearing of the band is observed, but for the sample in water. This could hint at PEGMUA-COOH being less stable under the conditions of the electrophoresis experiment. Assuming the footprint of PEGMUA-COOH to be 0.35 nm^2 , similar to PEGMUA,⁶⁶ ~ 1300 ligands are bound to one AuNP with 12 nm diameter. The majority of carboxylic acid groups are deprotonated at the pH of 8.3, so the charge of

the particles can be assessed with $\sim 1300\text{ e}^-$. The dye bromophenol blue according to the suppliers information migrates like DNA with 1150 base pairs in 0.5% agarose and 1 $\times$ TAE. Since each base pair (bp) corresponds to 2 charges (from the phosphodiester ions in the backbone) the charge would be $\sim 2300\text{ e}^-$. The hydrodynamic radius can be roughly assessed using $R_H \sim L^\nu \sim 33\text{ nm}$, for this number of base pairs, with L being the contour length of the DNA (assuming 0.34 nm per bp) and the excluded volume parameter $\nu = 0.588$.⁹⁹ The 100% PEGMUA-COOH conjugates migrate quite similar to the bromophenol dye in the electrophoresis experiment. One should not conclude that the contributions of hydrodynamic diameter and charge are scaling linearly, but this shows that both: hydrodynamic diameter and charge contribute significantly to the migration. In particular it indicates that the charge is significantly reduced by PEGMUA coadsorption in the experiments with mixed conjugates discussed before.

The Role of PEG Length. To elucidate the influence of hydrodynamic diameter on the migration in the gels, a set of samples was prepared with lipoic acid. Because this is a bidentate ligand, its competitive displacement by PEG-SH ligands can be considered to be negligible.^{60,100,101} Additionally, the footprint of lipoic acid is larger than that of the monodentate thiol ligands,¹⁰² leaving binding sites for subsequent coadsorption of thiols. One batch of lipoic acid functionalized AuNPs was prepared and aliquots of this batch reacted with PEG-SH ligands of different molecular weights, contributing differently to the hydrodynamic radius of the conjugate. This way the charge of the conjugates is kept constant (because the number of lipoic acid molecules is constant for all samples), but their hydrodynamic diameter is changed.

The experiment (Figure 5) demonstrates that the increase in hydrodynamic diameter due to the coadsorption of PEG-SH with 2 kDa leads to a significantly reduced migration in the gel,

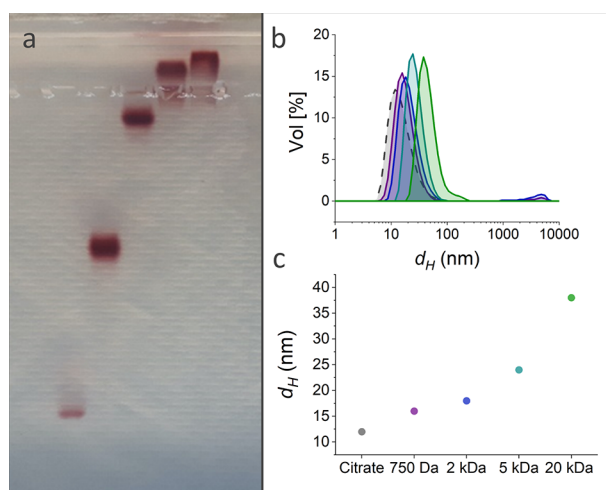


Figure 5. (a) Gel electrophoresis of conjugates with mixed layers of lipoic acid and PEG-SH after 2 h. From left to right: 100% lipoic acid; coadsorption of PEG with 750 Da, 2 kDa, 5 kDa, and 20 kDa. (b) Volume-weighted distributions of hydrodynamic diameters d_H measured by DLS: AuNP@Citrate (gray, dashed lines), @PEG with 750 Da (violet), 2 kDa (blue), 5 kDa (cyan), and 20 kDa (green). (c) Hydrodynamic diameters d_H corresponding to the maxima of the volume-weighted distributions in part b: 12, 16, 18, 24, and 38 nm.

similar to the mixed conjugates discussed before. In accordance with previous reports of experimental and theoretical results,^{87,103} the PEG shell does contribute not only to the hydrodynamic diameter but also to hydrodynamic drag and frictional coupling. Both increase with increasing molecular weight of the PEG-SH-ligands. Accordingly, the PEG molecular weight has a strong effect on the migration in the gel. In particular, the conjugates with large PEG-SH ligands (5 kDa and 20 kDa) even migrate toward the minus pole of the electrophoresis chamber, opposite to the expected direction. This behavior was also observed by Sperling et al. and Doane et al. and could be explained with the electroosmotic flow of the agarose gel ruling out a switch of the net charge to the opposite.^{87,97} Our results confirm this conclusion. From this experiment, it can be concluded that not necessarily large amounts of targeting ligands must have been replaced by PEGMUA in the experiments shown in Figure 2 and Figure 3, because the coadsorption of PEG-SH with 2 kDa alone already reduces the migration substantially.

CONCLUSION

In conclusion it has been shown that by using a 11-mercaptoundecanoyl spacer coadsorption of small targeting ligands and PEGMUA is possible, yielding stable conjugates in a simple process, namely simply mixing at room temperature in the desired ratio. Gel electrophoresis experiments underlined the tunable composition and that the coadsorption of PEGMUA strongly reduces matrix effects of cell media and FBS, which include NP–protein interactions and salt effects. In additional experiments, we tested the role of NP charge and hydrodynamic diameter. Both contribute to the migration behavior, but the contribution of PEG ligands seems to be particularly strong. Taken together, these experiments highlight that gel electrophoresis experiments are a valuable tool for the characterization of nanoconjugates, especially useful for studying mixed ligand layers but also for the assessment of protein adsorption. The scalable synthesis of mixed ligand AuNP-conjugates provides an attractive tool for fundamental studies of surface chemistry but might also be interesting for the development of contrast agents for improved early diagnosis and radiotherapy.

ASSOCIATED CONTENT

Data Availability Statement

The data that support the findings of this study are available upon request from the corresponding author.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jpcc.2c05415>.

EM of AuNPs, photographs of electrophoresis in Figure 2 after 27 min and of electrophoresis of MUA-PSMA-I/PEGMUA mixed conjugates, ζ -potential measurements of PEGMUA/MUA-AHX-PSMA-I and PEGMUA/MUA-AHX-GPI mixed conjugates and additional electrophoresis experiments for the optimization of mixed ligand layers of PEGMUA/PEGMUA-COOH on AuNPs (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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2.6 Further own work on the PEGMUA surface chemistry and surface chemistry in general

In my PhD time in the group of Prof. Horst Weller I was also involved in or supported projects with different nanomaterials. The PEGMUA coating was also successfully tested for QD⁶⁸ and I supported projects on the encapsulation of iron oxide NP.⁵⁹ In cooperation with the group of Prof. Melitta Schachner (then Institute of Molecular Neurobiology, Hamburg) I developed the functionalization of AuNP with small peptides based on the neural cell adhesion molecule L1 in mixed ligand layers with PEGMUA and the interactions of the conjugates with different types of neurons were tested, confirming stimulatory and neuroprotective effects.¹⁵⁵ In a follow-up project, the positive effect of AuNP@PEGMUA for the recovery from spinal cord injuries in mouse models was identified and studied in detail.¹⁵⁶ This sample system was also used later in cooperation with the group of Prof. Florian Grüner (Institute of Laser Physics, University of Hamburg) to demonstrate and benchmark XFI of AuNP in spinal cord samples.¹⁵⁷ AuNP@PEGMUA and their mixed conjugates were also successfully used for sensing applications. In cooperation with the group of Prof. Liao (Department of Chemical Engineering, National Taiwan University, Taiwan) a sensitive humidity sensor with fast response times was demonstrated. Here, the exact surface chemistry of the AuNP played a crucial role, and at the same time their excellent stabilization allowed to use them as a nano-ink in precise inkjet printing, underlining their potential for nanofabrication.¹⁵⁸ In cooperation with the group of Prof. Markus Fischer (Institute of Food Chemistry, University of Hamburg, Germany) their utility for aptamer-based lateral flow immunosensors was demonstrated.¹⁵⁹ Apart from these successful cooperations toward applications, a number of coherent SAXS beamtimes were dedicated to the fundamental properties of the PEGMUA and mixed coatings. The response to pressure was studied in detail, and these studies revealed how the interparticle interactions are tuned by this parameter but also allowed for a detailed characterization of mixed ligand layers.¹⁶⁰⁻¹⁶¹ The excellent stabilization provided by PEGMUA coatings are a crucial advantage for many coherent SAXS-based studies and the following section will discuss this and some of the follow-up studies.

In my time in the group of Prof. W. J. Parak (starting 2021) I also contributed to projects addressing surface chemistry beyond the PEGMUA approach. The encapsulation of nanomaterials with amphiphilic copolymer derivatives of poly(isobutylene-*alt*-maleic anhydride) (PMMA) was pioneered by Pellegrino, Parak et al.⁵⁸ Because this encapsulation strategy is based on hydrophobic interactions and not on a specific binding chemistry, a variety of nanomaterials can be encapsulated. Another advantage is that the straightforward anhydride chemistry of the PMMA backbone allows for versatile functionalization to create, for instance, defined surface charges including zwitterionic coatings,⁶⁹ or advanced labels which use the locally altered environment around the encapsulated nanomaterial as an advantage.⁶⁰ The physicochemical properties of coatings and how they affect protein adsorption (and cell interactions) is a key common interest of my research together with Prof. Parak. Besides the PMMA-based approaches we also use PEG-surface chemistry to study the role of surface charge in protein adsorption.¹⁶² In a pioneering study we demonstrated the effect of PEG-coated AuNP in dense protein solutions, and this will be detailed in the next chapter.

3. Particle Structure and Dynamics Studied with Coherent Small-Angle X-ray Scattering Methods

3.1 Key publications and author contributions

Key publications:

- Schroer, M. A.; Lehmkuhler, F.; Möller, J.; Lange, H.; Grübel, G.; Schulz, F., Pressure-Stimulated Supercrystal Formation in Nanoparticle Suspensions. *J. Phys. Chem. Lett.* **2018**, 9 (16), 4720.¹⁶³
- Schulz, F.; Westermeier, F.; Dallari, F.; Markmann, V.; Lange, H.; Grübel, G.; Lehmkuhler, F., Plasmonic Supercrystals with a Layered Structure Studied by a Combined TEM-SAXS-XCCA Approach. *Adv. Mater. Interfaces* **2020**, 7 (19), 2000919.¹⁶⁴
- Otto, F.; Dallari, F.; Westermeier, F.; Wieland, D. C. F.; Parak, W. J.; Lehmkuhler, F.; Schulz, F., The dynamics of PEG-coated nanoparticles in concentrated protein solutions up to the molecular crowding range. *Aggregate* **2024**, 5 (3), e483.¹⁶⁵

I shared the leading role (corresponding and first or last author) in all of these studies with Dr. Martin Schroer (then: Deutsches Elektronen-Synchrotron, DESY, Hamburg, Germany; now University Duisburg-Essen, Germany) and Dr. Felix Lehmkuhler (Deutsches Elektronen-Synchrotron, DESY, Hamburg, Germany), both experts for coherent SAXS. Together, we developed the ideas for the beamtimes and wrote the proposals. My responsibility and priority were the design, preparation and adaption of the sample systems before and during the beamtimes, which were conducted at the diamond light source (Didcot, UK), the European Synchrotron Radiation Facility (ESRF, Grenoble, France) or the DESY (Hamburg, Germany). Dr. Schroer and/or Dr. Lehmkuhler led the technical planning of the SAXS experiments and the data analysis. J. Möller (European X-Ray Free-Electron Laser Facility (XFEL), Hamburg, Germany), F. Dallari, V. Markmann (AG Grübel, DESY, Hamburg, Germany), F. Westermeier (beamline scientist at DESY, Hamburg, Germany) joined the according beamtimes, supporting the experiments, in particular data acquisition and analysis. F. Otto was a PhD student in the group of Prof. W. J. Parak under my supervision, and the according publication is a main outcome of his PhD thesis which he defended successfully in 2024. He contributed to both: sample design and synthesis, as well as data analysis, and he joined the according beamtime. W. J. Parak, G. Grübel and H. Lange were involved in mentoring and supervision and fruitful discussions addressing the research design and methods as well as the publication process. Dr. D. C. Wieland (Institute of Metallic Biomaterials, Helmholtz-Zentrum Hereon, Geesthacht, Germany) helped with complementary rheology experiments and their interpretation.

3.2 Background

The role of NP stability, dispersity, uniformity and purity was already discussed in the previous chapter. My work on AuNP synthesis and surface chemistry provided the groundwork for some of the studies presented here by developing the AuNP@PEGMUA system with excellent stability and purity. The strong scattering of AuNP due to their high electron density and Au having a comparably high atomic number Z is an additional

advantage for SAXS-based experiments. SAXS is a powerful characterization method for NP, the key parameter is the modulus of the scattering vector:

$$|\mathbf{q}| = q = \frac{4\pi}{\lambda} \sin\left(\frac{2\theta}{2}\right) \quad \text{eq. 4}$$

where 2θ is the scattering angle (reciprocal to the length scale of the scatterers) and λ the wavelength of the scattered (X-ray) light. The intensity of the scattered light in dependence of q , $I(q)$, can be described as the product of the so-called form factor $P(q)$ and the structure factor $S(q)$.¹⁴³⁻¹⁴⁴ The form factor depends on size, shape and dispersity of the scatterers and the structure factor describes their positional correlation. The studies summarized in this chapter represent three experiments based on coherent SAXS at synchrotron facilities: in situ SAXS, X-ray photon-correlation spectroscopy (XPCS), and thin-film scanning SAXS with X-ray cross-correlation analysis (XCCA). Reviews and early pioneering works are available from my cooperation partners from the Grübel group at DESY, in particular Felix Lehmkuhler, and XCCA is also covered in a review we wrote together.¹⁶⁶⁻¹⁶⁸ Briefly, XPCS allows to study the dynamics even of complex samples over the whole q -range provided by the detector. XCCA provides information on the local order of a sample and in combination with mapping experiments, statistical information can be extracted. The ever-increasing performance of the light sources in terms of brilliance and (coherent) flux and of detectors and X-ray optics allow for high temporal and spatial resolution and the generation of large amounts of valuable data. XPCS allows today to measure dynamics in the microsecond range, reaching the relevant regime for the diffusion of nanoparticles in water, and thin-film scanning SAXS experiments are possible with sub-micrometer beam sizes. The selection of studies in this section highlights the wealth of information on complex colloidal samples that can be obtained by these experiments at large scale facilities.

3.3 Pressure-Stimulated Supercrystal Formation in Nanoparticle Suspensions.

Publication:

- [Schroer, M. A.; Lehmkuhler, F.; Möller, J.; Lange, H.; Grübel, G.; Schulz, F., Pressure-Stimulated Supercrystal Formation in Nanoparticle Suspensions. *J. Phys. Chem. Lett.* **2018**, 9 \(16\), 4720.¹⁶³](#)

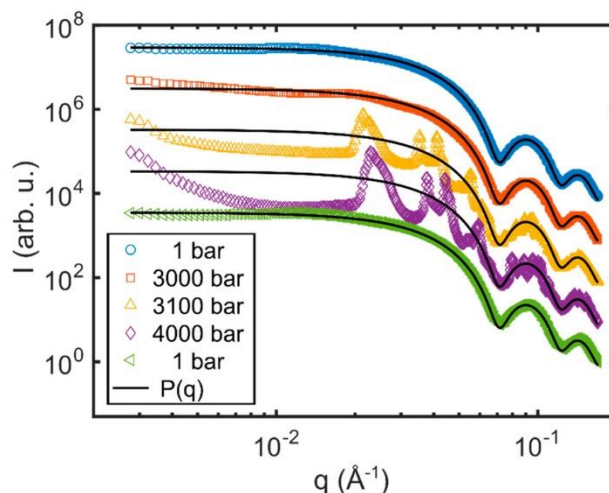


Figure 4. SAXS curves of a concentrated solution of AuNP@PEGMUA in the presence of 2 M CsCl at different pressures. Starting from a pressure of 3100 bar (yellow triangles), Bragg reflections are present. At decompression the reflections disappear (green triangles). Solid lines indicate the form factor fit.

This study was a follow-up on our previous work on pressure-induced changes of interparticle interactions in AuNP@PEGMUA samples.¹⁶⁰ The experiments were conducted at the beamline I22 of the Diamond Light Source (Didcot, UK) using a high pressure cell, that allows applying hydrostatic pressures up to 4000 bar to soft polycarbonate capillaries. The confident analysis of the data was favored by the immaculate colloidal stability, high uniformity and low dispersity of the AuNP@PEGMUA conjugates at very high particle concentrations. This leads to intense signals and well pronounced form factors, which was a benefit for confident fitting with elaborate models. The presence of aggregates would strongly affect the signal at low q thereby hampering the meaningful extraction of the structure factor $S(q)$ with the approach $S(q) = I(q)/P(q)$ mentioned earlier. Having observed a pressure-induced compression of the PEG-shell together with a change of interparticle interactions from repulsive to attractive, we were interested in how this behavior is affected by the presence of electrolytes. These are known to interact and affect the properties of PEG including its phase behavior like its lower critical solution temperature (LCST) and thereby also the structure formation of PEG-coated NP.¹⁶⁹⁻¹⁷¹ Because a key application of AuNP@PEG is in nanomedicine and nanobiotechnology, understanding their behavior in the presence of electrolytes is particularly interesting and important. The surprising finding in this study was, that applying high hydrostatic pressures to dispersions of AuNP@PEGMUA in the presence of high salt concentrations induces the formation of very well defined fcc supercrystals, Figure 4. This was rationalized with the combined effect of the salts and pressure, both decreasing the solubility of PEG.¹⁷² This was the first demonstration of pressure-induced supercrystal formation in dispersion.

Remarkably, the formation of very well-defined supercrystals was not only very fast (within seconds, and as shown in later time-resolved studies even within milliseconds when using pressure jumps¹⁷³) but also fully reversible. This observation and systematic parameter studies indicate a phase transition from the liquid to the crystalline phase. Once the supercrystals are formed, they are further compressed with increasing pressure and their compressibility depends mainly on the thickness of the PEGMUA-coating (dictated by the molecular weight of the PEGMUA ligand) but also on the salt type. Due to the reversibility the supercrystal formation, it is not useful for synthetic purposes as long as no way is found to fix their structure. However, the approach allows for tuning interparticle distances with subnanometer resolution by pressure in the formed supercrystals, and this is an interesting prospect for future studies correlating optical and SAXS-measurements at high pressures.

Pressure-Stimulated Supercrystal Formation in Nanoparticle Suspensions

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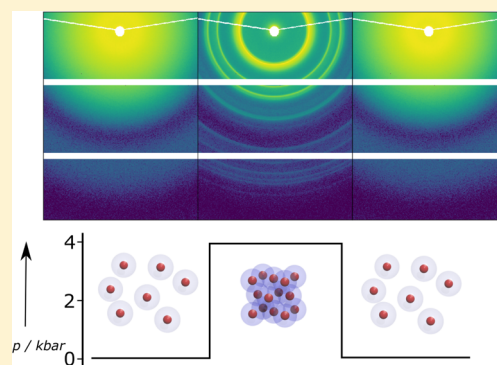
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Supporting Information

ABSTRACT: Nanoparticles can self-organize into “supercrystals” with many potential applications. Different paths can lead to nanoparticle self-organization into such periodic arrangements. An essential step is the transition from an amorphous state to the crystalline one. We investigate how pressure can induce a phase transition of a nanoparticle model system in water from the disordered liquid state to highly ordered supercrystals. We observe reversible pressure-induced supercrystal formation in concentrated solutions of gold nanoparticles by means of small-angle X-ray scattering. The supercrystal formation occurs only at high salt concentrations in the aqueous solution. The pressure dependence of the structural parameters of the resulting crystal lattices is determined. The observed transition can be reasoned with the combined effect of salt and pressure on the solubility of the organic PEG shell that passivates the nanoparticles.



The formation of nanoparticle (NP) supercrystals has been studied intensively throughout the last decades.^{1–5} The fact that NP supercrystals can exhibit tunable and collective properties that are different from the sum of their constituents is the main origin of interest in the crystallization processes.^{6–11} The transition of NP in solution into crystalline structures has already been realized in various ways.^{3,12–15} Self-assembly and crystallization of NPs is generally a complex process,^{16–21} usually based on the controlled evaporation or precipitation of the NP building blocks by the slow addition of a nonsolvent.^{3,22} These processes have to be slow to achieve homogeneous crystallization conditions for obtaining high-quality supercrystals. Applying pressure as a homogeneous method to induce various structural transformations and phase transitions in preordered NP assemblies has only recently been done.^{23–27} Wu et al. observed the pressure-induced annealing of gold nanoparticle (AuNP) assemblies into a crystalline phase with significantly fewer defects.²⁶ Podsiadlo et al. studied the stability and elasticity of layered PbS–NP supercrystals at high pressures.²⁷ In another recent study, using preordered heteroassemblies of NPs, pressure allowed defined structural transformations by interparticle fusion.²⁵ All of these studies, however, used already preordered NP structures.

On the basis of our recent work and the established effect of salt on the solubility of AuNPs coated with poly(ethylene glycol)- (PEG-) based ligands, we present that well-defined

crystallization of NPs by pressure changes can be observed not only in NP assemblies but also already from aqueous NP solutions. This crystallization is fast, largely reversible, and does not need the presence of preordered crystals. We studied the crystallization in situ by small-angle X-ray scattering (SAXS), varying the involved parameters: AuNP concentration, added salt type and concentration, and AuNP-stabilizing PEG-ligand length. The obtained pressure–salt concentration (p – c) phase diagram reveals that the crystallization is clearly a result of the combined effect of salt and pressure on the PEG coatings.

As a model system, we employed AuNPs ($R_{\text{core}} = \sim 6$ nm) coated with a PEG-based ligand that provides a high colloidal stability even at high particle concentrations. Two different ligand lengths (5 and 2 kDa) were used to prepare AuNP@PEG conjugates with final particle concentrations of 4 and 3 μM , corresponding to volume fractions of 6–10% (AuNP@PEG5k) and 1–3% (AuNP@PEG2k), respectively. Their syntheses were previously described.^{28–30} Upon evaporation of the solvent, AuNP@PEG can form extended solids with locally ordered domains.³¹ The addition of salt decreases the solubility of PEG in water, and at high salt concentrations,

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aggregation of the free polymer can occur depending on the type of salt.^{32–34} Zámbo et al. demonstrated that the lower critical solution temperature of AuNP@PEG can be lowered effectively by the addition of K_2SO_4 .³⁵ In addition, it was shown that AuNP@PEG in water can form face-centered cubic (fcc) superlattices at high concentrations of potassium carbonate, K_2CO_3 .^{36,37} Besides salt and temperature, pressure can also decrease the solubility of PEG.^{38,39} For AuNP@PEG, we observed a pressure-induced deswelling of the PEG-ligand layer affecting interparticle interactions.⁴⁰ On the basis of these observations and to explore a potential phase transition in solution, we prepared solutions of AuNP@PEG with different concentrations of NaCl, KCl, RbCl, and CsCl and studied their response to pressure by SAXS.

Figure 1 displays 2D SAXS patterns recorded for a concentrated suspension of AuNPs coated with 5 kDa PEG

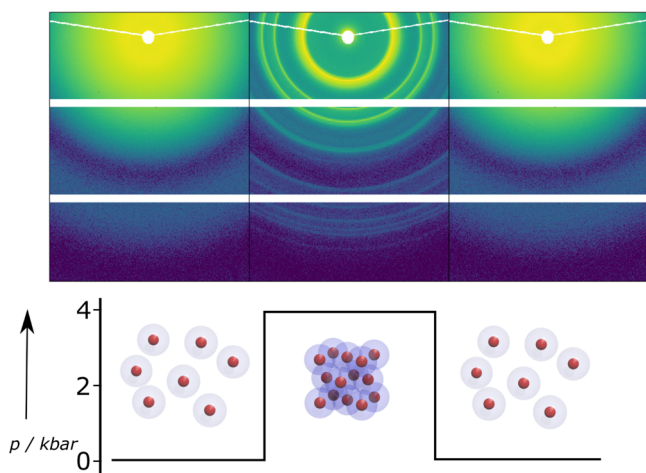


Figure 1. 2D SAXS patterns of PEG-coated AuNPs with 2 M CsCl added at different pressures. Left: 1 bar; middle: 4000 bar; right: after pressure release at 1 bar. The scheme at the bottom illustrates the structural assembly of the coated AuNPs at different pressures: At 1 bar, the particle ensemble is in an amorphous liquid state. Upon reaching the crystallization pressure, face-centered cubic crystallites are formed by the AuNPs. After pressure release, the AuNPs return to the liquid state.

in 2 M CsCl solution at 1 bar, at 4000 bar, and after pressure release. At 1 bar, the isotropic scattering pattern shows a pronounced forward scattering, indicative of an amorphous NP suspension in the liquid state. For larger scattering angles, annuli are present that stem from the form factor of the AuNPs. When increasing the pressure to 4000 bar, Debye–Scherrer rings appear in the SAXS pattern, demonstrating the pressure-induced formation of ordered supercrystals. After releasing the pressure, the SAXS pattern reverts to its original state.

This pressure-induced supercrystal formation takes place within a pressure range of $\Delta p < 100$ bar, as can be seen from the azimuthally integrated SAXS curves (Figure 2a). For pressures from 1 bar up to 3000 bar, the SAXS curves do not show any indication of diffraction peaks, confirming the absence of crystalline order within the suspensions.

The pronounced oscillation at larger q values stems from the form factor, $P(q)$, of the gold core with an average radius of $R_{\text{core}} = (6.25 \pm 0.05)$ nm, represented by solid lines. Only at small q are slight deviations to $P(q)$, indicating the presence of a structure factor for liquids. As previously shown for the

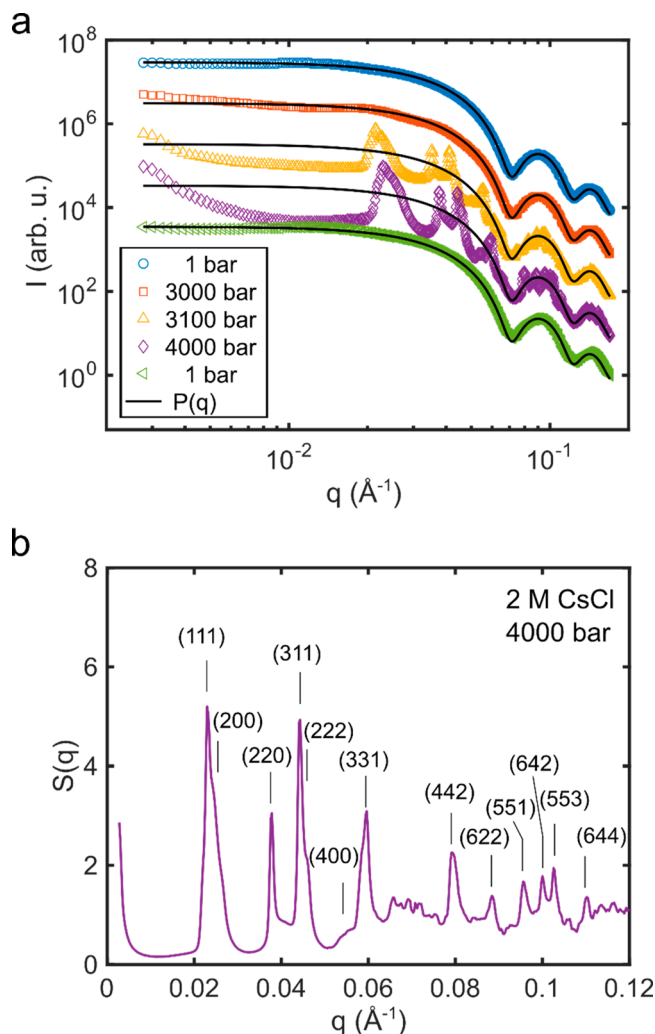


Figure 2. (a) SAXS curves of a concentrated solution of AuNP@PEG with 2 M CsCl at different pressures. Starting from a pressure of 3100 bar (yellow triangles), Bragg reflections are present. At decompression the reflections disappear (green triangles). Solid lines indicate the form factor fit. (b) Extracted structure factor for 2 M CsCl added to the suspension at 4000 bar. The reflections indicate a fcc structure.

AuNP@PEG system, the form factor basically represents the gold core, whereas the interparticle interactions are dominated by the PEG layer.^{40,41}

After increasing the pressure to 3100 bar, Bragg reflections appear. The reflections' positions shift to larger wave vectors q with increasing pressure. The supercrystal structure factor $S(q)$ reveals a large set of Bragg reflections (>14 in the presented q range) (Figure 2b). The underlying crystal structure can be identified as an fcc lattice, which is formed by the periodic arrangement of the AuNPs. The large number of higher order reflections indicates a high degree of crystallinity of the supercrystal, which is already present at the crystallization pressure of 3100 bar and does not significantly change with increasing pressure.

When reaching the crystallization pressure, supercrystals form within the whole suspension. The isotropic, continuous Debye–Scherrer rings indicate that these supercrystals are fully randomly orientated. After pressure release back to 1 bar, the reflections disappear, demonstrating a reversible transition.

The pressure response of AuNP@PEG with different chloride salts (NaCl, KCl, RbCl, CsCl) at different

concentrations shows a similar behavior (Figures S1–S3). Except for NaCl, pressure-induced crystallization sets in for salt concentrations from 1 M onward within the accessible pressure range from 1 to 4000 bar (see the SI, experimental section). An increase in salt concentration leads to a continuous decrease in the crystallization pressure, whereas the lattice structure and degree of crystallinity are independent of the salt type and concentration. The crystallization is a sharp and fast phase transition ($\Delta p < 100$ bar, $\Delta t < 30$ s) for all salt types studied. On the basis of these observations, a pressure–salt phase diagram was determined (Figure 3).

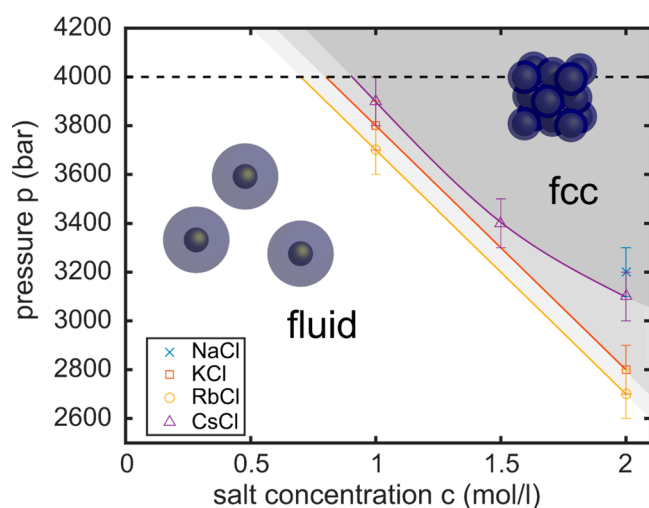


Figure 3. Pressure–salt concentration (p – c) phase diagram of AuNP@PEG. For low pressures, the particles are in the liquid state; beyond a critical pressure, fcc superlattices are formed. The error bars correspond to the pressure resolution of $\Delta p = 100$ bar. The transition depends on the salt concentration as well as on the salt type. Solid lines are guides for the eye.

The transition depends on the salt type. The lowest pressure for crystallization was found for RbCl, whereas for NaCl, no crystal formation was observed for a salt concentration lower than 2 M and pressures up to 4000 bar. Interpreting the crystallization as salt-induced precipitation, the trend $\text{Rb}^+ > \text{K}^+ > \text{Na}^+$ is similar to the Hofmeister series, but Cs^+ deviates significantly from this. This points to specifics of the PEG–cation interaction: The salting-out capability trend based on theoretical calculations proposed by Ren et al. follows the trend $\text{K}^+ > \text{Rb}^+ > \text{Cs}^+ > \text{Na}^+$, which is in better agreement with our experimental findings.³³ When mixing the AuNP@PEG with 1 M CaCl_2 no crystal formation was observed within the pressure range studied, whereas for the sulfate salt K_2SO_4 , crystallites with fcc structure, although less defined, were already formed under ambient conditions (Figures S4 and S5). Both observations are in accordance with expectations based on the Hofmeister series, which predicts a less pronounced salting-out capability for Ca^{2+} compared with alkali metal ions and a more pronounced one for sulfate compared with chloride.

The lattice constant a of the fcc superlattice was determined from the positions of the Bragg reflections as a function of pressure for all salt types studied at $c = 2$ M (Figure 4a), where crystals formed for all four salts. The lattice constant at the crystallization transition is found to be $a = (50.5 \pm 0.3)$ nm

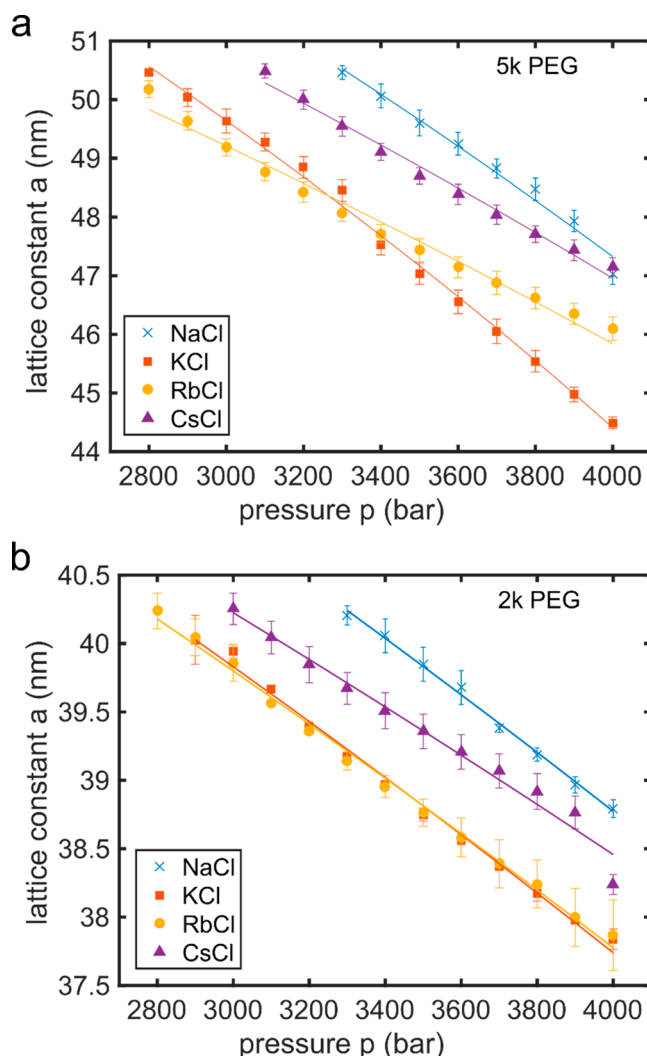


Figure 4. Effect of pressure on the formed supercrystals. Pressure dependence of the fcc lattice constant for all salt types at the highest salt concentration studied ($c = 2$ M). AuNP@PEG with (a) 5k PEG and (b) 2k PEG.

and is independent of the salt type and the crystallization pressure.

Assuming that the AuNP@PEG are closely packed in the initially formed crystallites, we can conclude from the corresponding interparticle distances $l = a(\sqrt{2})^{-1} = 35.7$ nm an effective AuNP@PEG radius of $R_{\text{eff}} \approx 18$ nm. The difference in the AuNP core radius can be attributed to an effective PEG shell thickness of $s \approx 12$ nm. This indicates, that the PEG shells in the crystallites are still in a swollen or strongly hydrated state upon formation (for a “dry” PEG-shell of this grafting density, PEG length s would be ~ 7 nm).⁴¹ Because of their large separation ($l/R_{\text{core}} \approx 6$) within the crystallites, attractive van der Waals interactions of the AuNP cores can be neglected.¹ The interaction potential and thus the formation of the supercrystals is dominated by the interactions of the PEG shells only.

A further increase in pressure leads to a continuous reduction of the lattice constant, which depends on the type of cation, again indicating cation specific M^+ –PEG interactions. This pressure dependence allows us to determine the effective compressibility of the lattice. The obtained values

with respect to $p = 4000$ bar (see Table 1) are larger than for water ($\sim 3.2 \times 10^{-5} \text{ bar}^{-1}$) (extrapolated from Fine et al.⁴²).

Table 1. Effective Compressibility of the Superlattices for Two PEG-Ligand Length and Different Salt Types (Concentration: 2 M)^a

	NaCl	KCl	RbCl	CsCl
2k PEG	16.8 ± 0.5	17.4 ± 0.4	16.8 ± 0.4	14.7 ± 0.8
5k PEG	32 ± 2	39.5 ± 0.8	23 ± 1	25 ± 1

^aValues in 10^{-5} bar^{-1} .

This indicates that the reduction of the lattice is dominated by the compression of the ligand layer, whereas the gold core is not affected by pressure, as evident by the absence of changes of the form factor.

For a PEG-ligand length of 2 kDa, we observed similar results (Figures S6 and S7). Only the resulting lattice constant is reduced as the initial ligand layer is thinner ($a = 40 \text{ nm}$; $s \approx 8 \text{ nm}$). The findings for the different ligand lengths strongly indicate a closed-packing of NPs with hydrated PEG shells. The effective compressibility for the 2 kDa ligand is smaller than that for the 5 kDa (Table 1), pointing to a less flexible and more densely packed ligand shell and resulting in a smaller degree of hydration in accordance with previous findings.⁴¹ In addition, the effect of salt is less pronounced for the shorter chain.

Our observations of a pressure-induced phase transition from AuNP suspensions into supercrystals can be reasoned with the following scenario. At the crystallization pressure, the extended, strongly hydrated PEG-ligand shell abruptly collapses into a less swollen, but still hydrated state. We attribute this sudden structural transition to a rapid change of the solvency of the PEG ligand in the aqueous solvent, similar to the well-known volume phase transition for thermogels.^{43,44} In this scenario, the preferred interactions between the less swollen but well-defined PEG-ligand shells of adjacent NPs are expected to be one of the driving forces to form highly ordered crystallites. A further increase in pressure continuously compresses the crystallites formed by further reducing the swollen shells. Without salt addition, no crystallization is observed in the pressure range studied. Thus the combined effect of pressure and salt, both decreasing the solubility of PEG in water, is essential for liquid–solid phase transition. The correct choice of parameters enables the formation of NP supercrystals in solution from a disordered, liquid state induced by hydrostatic pressure. Because PEGylation is an established method for the stabilization of nanomaterials, the demonstrated concept should be applicable for a variety of nanomaterials, including other sizes, shapes, and materials. Future studies, for example, based on pressure jumps, might reveal mechanistic insights into the supercrystal formation that will help us to understand crystallization processes and to utilize pressure for the synthesis of well-defined NP supercrystals.

EXPERIMENTAL METHODS

Experimental methods are available in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.8b02145.

Detailed description of sample preparation; description of SAXS experiment and data analysis; results for different chloride salt types and concentrations; results for other salt types; results for 2 kDa ligand; results for different particle concentration. (PDF)

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Notes

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3.4 Plasmonic Supercrystals with a Layered Structure Studied by a Combined TEM-SAXS-XCCA Approach

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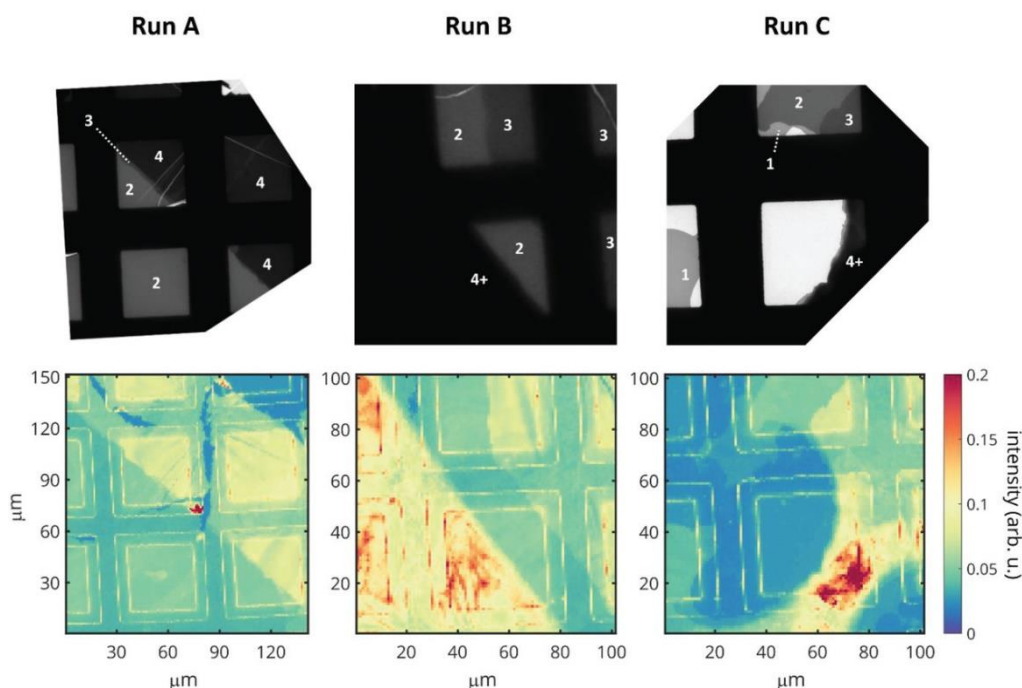


Figure 5. Direct comparison of TEM (top) and scanning SAXS intensity maps (bottom) of plasmonic supercrystals for different runs. The numbers in the TEM micrographs indicate the number of layers in the thin-film AuNP supercrystals.

Herein we studied the structure of well-defined thin-film AuNP supercrystals with 2D-hexagonal order with a correlated approach. The synthesis and properties of such supercrystals are described in detail in the next chapter. Important characterization techniques for such supercrystals include electron microscopy (TEM and SEM) and SAXS approaches, each offering unique advantages. TEM allows identification of minor variations (e.g. of shape) on the single particle level as well as point defects, whereas SAXS can provide a statistically robust structural analysis of both particle size and shape and superlattice structure. The samples studied had a defined layered structure ranging from mono- to multilayers. In our analysis we focused on mono-, bi- tri-, tetra- and multilayers. By a correlated TEM-SAXS (thin-film mapping SAXS) approach we were able to confidently correlate SAXS-intensity and sample thickness (i.e. number of layers, Figure 5) and this allowed a thickness-dependent analysis of the SAXS data revealing subnanometer variations of the lattice constant. The key for this approach was to identify the exact regions of interest that were mapped in the SAXS measurement in subsequent TEM measurements to confirm the local structure. XCCA then allowed to quantify orientational order in the films and to analyze domain orientations, thereby also allowing to confirm record sizes of single-crystalline domains in the samples. One motivation of the study was to establish a quantitative description of supercrystal quality which was demonstrated to be possible with

XCCA. The samples in this study were of very high quality allowing for a confident analysis. XCCA however, is also particularly powerful for much more disordered samples which feature only local or even hidden local order as discussed in previous studies.¹⁷⁴⁻¹⁷⁵

Plasmonic Supercrystals with a Layered Structure Studied by a Combined TEM-SAXS-XCCA Approach

Florian Schulz,^{*} Fabian Westermeier, Francesco Dallari, Verena Markmann, Holger Lange, Gerhard Grübel, and Felix Lehmkuhler^{*}

Supercrystals composed of plasmonic nanoparticles constitute a promising material platform for tailored light-matter interactions. The optimization of their optical properties requires precise syntheses and a detailed structural characterization that addresses not only the basic geometrical parameters but also the degree of order. Herein, plasmonic supercrystals with a well-defined layered structure are studied by a combined transmission electron microscopy, small-angle X-ray scattering and X-ray cross-correlation analysis (TEM-SAXS-XCCA) approach. It is demonstrated that scanning small-angle x-ray scattering (SAXS) data can unambiguously be assigned to the number of crystalline layers by comparison with complementary transmission electron microscopy (TEM) experiments on the same regions of interest. A small but significant increase of the lattice constant with increasing number of layers and a high degree of orientational order irrespective of the number of layers is found. This points to specifics of the supercrystal formation mechanism that could be utilized to improve the control of self-assembly for supercrystal geometries with subnanometer precision.

the crystalline structure within the assemblies is directly correlated with the emergence of coupled plasmon modes.^[4] For instance, dark interlayer plasmons can form in supercrystals with a defined layered structure.^[5] Higher order modes, however, can only be observed in highly defined crystals because dispersity and the lack of periodic order result in a broadening of the according resonances. While the dispersity and uniformity of the constituent NP can be stated in well-defined quantities, the periodic order within the supercrystal lattices is usually described more qualitatively with terms such as “well-ordered”, “highly-ordered”, and so on. Quantitative statements are mostly restricted to the overall dimensions of the crystals or the crystallite size. Since the current high standards of NP supercrystals allow for more detailed studies of light

1. Introduction

The ongoing research efforts in the field of nanoparticle (NP) supercrystals lead to ever improving synthetic protocols.^[1–3] Constituent nanoparticles with low dispersity and high uniformity allow for well-defined supercrystals with long-range periodic order, small number of defects and large crystallite size. In the case of plasmonic nanoparticles (e.g., Ag, Au)

matter coupling, a more detailed structural characterization is also desirable.

The most important characterization techniques electron microscopy and small-angle x-ray scattering (SAXS) provide complementary information in this respect. Electron microscopy experiments like transmission electron microscopy (TEM) allow intuitive assessments of particle uniformity and dispersity and their periodic arrangement as well as the density and nature of lattice defects. Analysis of TEM measurements yield values for NP dispersity, uniformity, nearest neighbor distances and crystallite/domain sizes. The statistics of TEM-measurements, however, are limited. The maximum domain size that can be depicted in a single measurement is restricted by the minimum magnification that allows discerning individual NP (e.g., < 200 μm^2 for NP diameters of 25 nm). Larger domains can be assessed by serial measurements but typically, a sampling approach is employed in contrast to a systematic mapping. Another limitation of TEM is its restriction to monolayer superlattices up to a few layers only. The number of layers can be identified by stepwise counting up to 5–6 layers depending on the specific sample, but further information like particle diameter and spacing, as well as crystal structure, are hard to obtain for multilayers. For thick multilayer crystals, the absorption of the material will prevent any meaningful analysis. As a complementary approach, SAXS at partially coherent photon sources is routinely used to determine the crystalline order of volume materials.^[6,7] Scanning SAXS experiments allow mapping of comparably large areas and detailed structural information can be obtained by X-ray cross-correlation analysis

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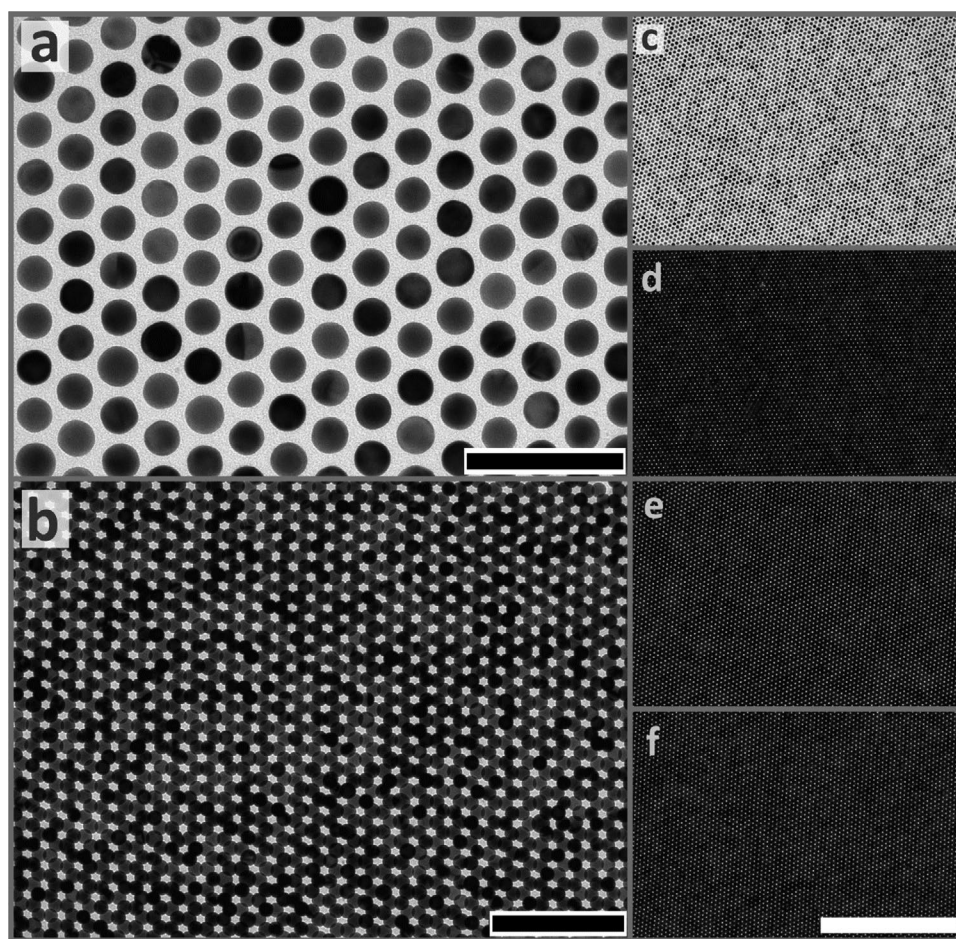


Figure 1. Exemplary TEM measurements of plasmonic supercrystals. a) Monolayer, b) bilayer with AB stacking, c) monolayer at lower magnification d) bilayer, e) trilayer, and f) tetralayer. (scalebar in (a) 100 nm, (b) 200 nm, (c–f) 1 μm).

(XCCA).^[8–12] Recently, the use of XCCA in structural studies of self-assembled nanoparticles has increased,^[8,13–15] including in-situ experiments of phase transitions.^[16] Form factor measurements in solution provide statistically robust data for particle diameter, shape and dispersity.^[7,17] However, slight deviations from ideal shapes or low uniformity/multiple shape variations challenge confident analysis of SAXS data. Furthermore, SAXS is not sensitive to the structure of specific single particles and thus certain types of defects like point defects will be left unnoticed. As a simplified statement EM provides a confident microscopic picture down to the single particle level, whereas SAXS provides statistically more robust structural data. In this study we use a correlated SAXS-TEM approach to study plasmonic supercrystals of gold nanoparticles (AuNP). We synthesize AuNP supercrystals with a defined layered structure, ranging from mono- to multilayer. By performing microfocus scanning SAXS experiments on AuNP supercrystal samples on TEM grids we are able to identify the same regions of interest with both methods and directly compare TEM and SAXS measurements. We analyze SAXS measurements on mono-, bi-, tri-, tetra-, and multilayers with XCCA and discuss quantitative parameters such as domain size, intensity, lattice parameters and orientational order and how they depend on the number of layers.

We observe a slight increase of the narrowly distributed lattice parameters with the number of layers, large single crystalline domains up to thousands of square micrometers and a high degree of orientational order independent of the number of layers. The observed structural features can help to understand the formation mechanism of the superlattices.

2. Results

Plasmonic supercrystals were synthesized by self-assembly of polystyrene-coated AuNP (AuNP@PSSH) at a toluene-diethylene glycol (DEG) liquid-liquid interface as described recently.^[18] The synthesis protocol (see Section 5.) yields well-defined supercrystals with crystallite sizes $> 1000 \mu\text{m}^2$, few defects and a layered structure (Figure 1). Two reference samples consisting of either mostly monolayers or mostly bi- and multilayers were prepared. Table 1 lists the basic geometrical parameters obtained by TEM and SAXS: particle diameter (d_{core}) and dispersity (rsd, relative standard deviation of the mean diameter for TEM and the dispersity Δd given by the width of the Schulz–Zimm distribution in SAXS^[7]) and the lattice constants a . The values obtained by SAXS and TEM are in

Table 1. Geometrical parameters of the two samples obtained by TEM and SAXS.

	TEM			SAXS		
	d_{core} [nm]	rsd	a [nm]	d_{core} [nm]	Δd	a [nm]
Monolayer sample	23.4	4.3%	31.4	24.2	5.8%	32.4
Multilayer sample	23.4	4.3%	30.8	24.2	5.8%	32.8 ^{a)}

^{a)}depends on the number of layers as discussed below.

good agreement. The lattice constants a of the two individually prepared samples deviate just slightly (in fact, as we will show later, a was the same ± 0.1 nm for monolayers in both samples), confirming the reproducibility of the protocol that utilizes the molar mass of the PSSH ligand to control the interparticle distances in the supercrystals.

The standard deviations of the lattice parameters a averaged over all SAXS measurements (hits) are well below 1%, underlining the crystalline long-range order of the samples. The domain sizes obtained by TEM exceed several μm (see Figure 1) so that microfocus SAXS measurements on defined numbers of layers are possible. We mapped selected regions of interest (ROI) with characteristic microstructures on TEM grids. In subsequent TEM-experiments, these regions could be identified and the according microstructure analyzed (Figure 2). This way, we can confidently assign the number of layers to the SAXS

measurements. An overview scan is shown in the Supporting Information (Figure S1, Supporting Information). We focus on three ROI which include distinct mono- to multilayer areas and are therefore suitable for our discussion of the local structure as a function of film thickness. For thin samples as probed in this study, the scattering intensity at large q ($q > 0.56 \text{ nm}^{-1}$) is directly proportional to the thickness of the sample. Histograms of $I_h \equiv I(q > 0.56 \text{ nm}^{-1})$ then allow to determine the number of layers (Figure 3).

The TEM measurements and scanning SAXS intensity maps in Figure 2 clearly show the underlying TEM grid. The grid edges produce strong streaks in the scattering patterns raising the measured intensity, and consequently clearly highlight the grid position in the SAXS maps. Furthermore, the intensity appears to be reduced on the copper grid because of absorption of X-rays by copper. Therefore, we only used the areas inside

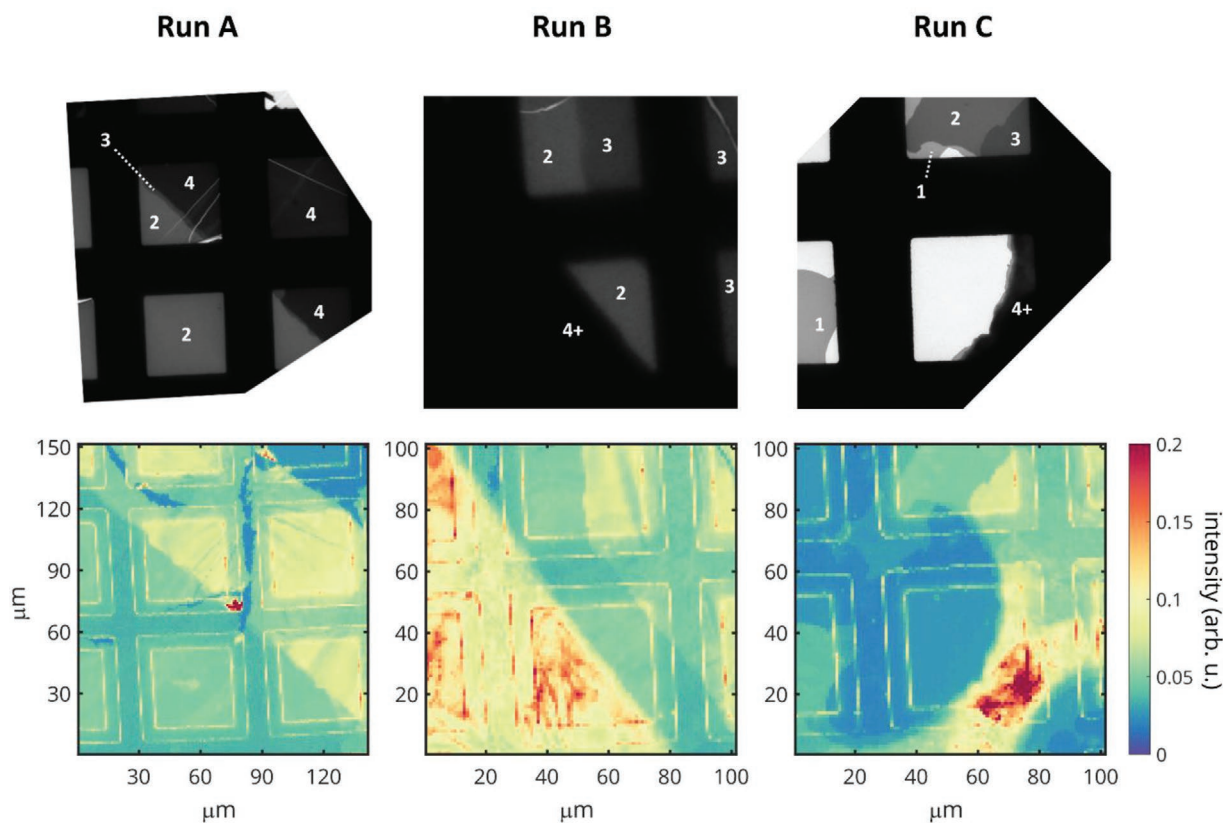


Figure 2. Direct comparison of TEM (top) and scanning SAXS intensity maps (bottom) of plasmonic supercrystals for all three runs. The SAXS intensity has been averaged for $q > 0.56 \text{ nm}^{-1}$. The according ROI were identified in subsequent TEM measurements to confirm the number of layers as indicated (top). Up to four layers can be confidently assigned with TEM as shown in Figure 1, thicker regions more than four layers (compare dark regions in run B and C indicated by 4+) can often not be confidently assigned because of the strong absorption. Note that even subtle features like small cracks can be discerned in the SAXS intensity maps. However, due to sample mounting and transfer slight deviations in the microstructure can occur.

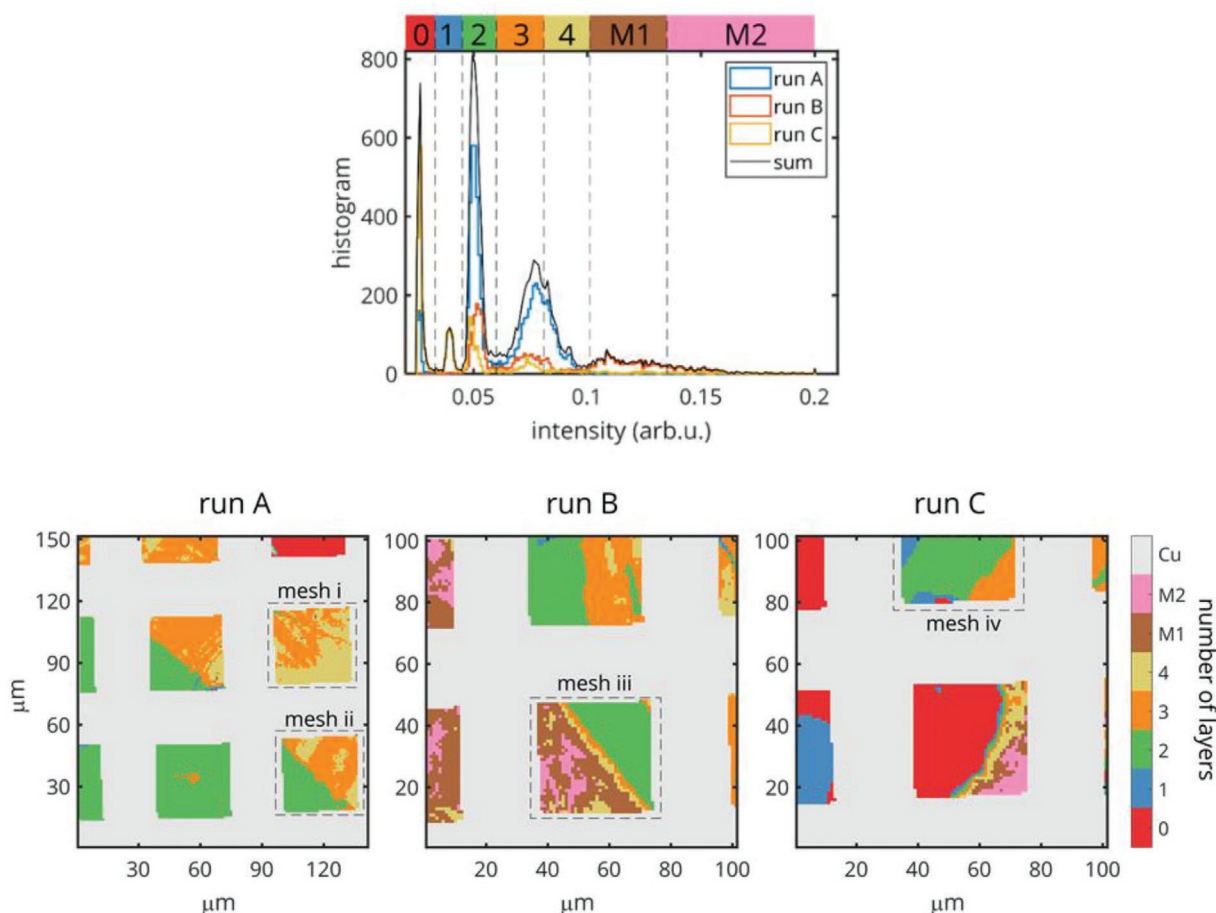


Figure 3. Correlation of intensity and film thickness. Top: Intensity histogram of the three runs studied for $q > 0.56 \text{ nm}^{-1}$. The dashed vertical lines mark the limits to differentiate the number of layers by the scattered intensity as indicated on top. Note that three and four layers overlap. More than five layers cannot be distinguished unambiguously so that M1 and M2 are arbitrarily divided at $I_h = 0.135$. Bottom: The number of layers as defined in the histogram has been assigned for each spot of the scanning intensity maps shown in Figure 2, grey areas represent the copper grid. The labeled meshes i–iv will be discussed in Figure 6.

the mesh (nominal mesh dimensions: $(38.5 \mu\text{m})^2$ mesh, $25 \mu\text{m}$ bridge width) for the determination of the number of layers by the histogram in Figure 3 (top). The corresponding maps of number of layers are shown in Figure 3 (bottom), where the gray regions reflect the masked copper grid. Apart from the large tetralayer in run A, the assignment based on the SAXS intensity is in excellent agreement with the one based on TEM analysis. An accurate and statistically robust determination of structural parameters like the lattice constant a can hardly be obtained by TEM analysis, especially for more than one layer. With the confident assignment of the number of layers by our combined approach we can now take a closer look at the SAXS results as a function of number of AuNP layers.

2.1. Lattice Constant a

Azimuthally integrated SAXS intensities $I(q)$ have been averaged over all patterns and are shown in Figure 4a for run B. Independent from the number of layers, Bragg reflections dominate the intensity curves. All reflections can be indexed

according to a 2D hexagonal lattice. With increasing number of layers, the Bragg reflections shift slightly towards smaller q , suggesting an increase of the lattice constant. Thus, we calculated the lattice constant from the position of the $\{10\}$ reflection for all sample spots separately, and averaged it afterwards. The results are shown in Figure 4b. The lattice constant increases by 1.4% from 32.4 nm for monolayers to 32.8 nm for multilayers (M1 and M2) where it appears to saturate.

2.2. Orientational Order

Similar to the SAXS analysis, the orientational order was quantified by Fourier coefficients of the intensity $\hat{I}_\ell$ (see Experimental Section for definitions). A perfect 2D hexagonal lattice resulting in strong Bragg reflections would yield $\hat{I}_6 = 1$. Disorder as well as a different symmetry is reflected by $0 < \hat{I}_6 \ll 1$. In experiments, setup parameters such as beam size and detection noise, as well as the width of the Bragg reflections can additionally reduce I_ℓ values. In our case we obtain a maximum of $(\hat{I}_6) \approx 0.96$ (Figure S2, Supporting Information). Therefore,

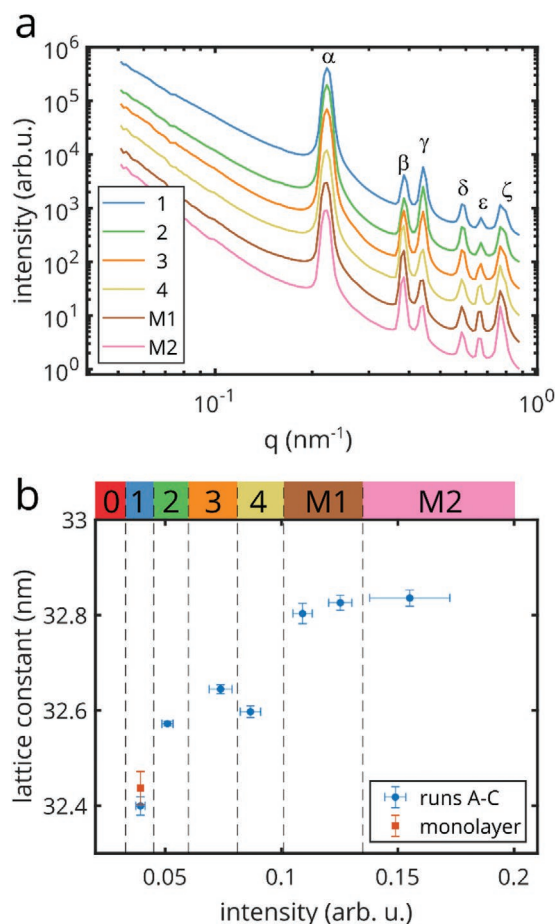


Figure 4. a) $I(q)$ averaged for the different number of layers of run B. Data has been offset vertically for clarity. The Bragg reflections correspond to a 2D hexagonal lattice: $\alpha - \{10\}$, $\beta - \{11\}$, $\gamma - \{20\}$, $\delta - \{21\}$, $\epsilon - \{30\}$, $\zeta - \{22\}$. b) Lattice constant obtained from fits to the Bragg reflections averaged over data from all runs. The horizontal error bars reflect the standard deviation over the corresponding intensity values. The value for the monolayer sample is also shown.

maxima close to 0.95 in **Figure 5** reflect a very high degree of sixfold order. Because of limitations due to low intensity, in particular scattering from monolayer regions, we concentrate the analysis on the $\{10\}$ Bragg reflection. As this reflection shows a strong six-fold symmetry, we limit the discussion to $\ell = 6$.

The resulting maps of the degree of sixfold order are shown in **Figure 5**. The top part depicts the amplitude of sixfold order $|\hat{I}_6|$. In contrast to the intensity maps, the copper grid is less pronounced in these maps. This can be understood by the normalization of the correlation function which is calculated from relative intensity fluctuations and is thus less sensitive to absolute intensity variations.^[12] Cracks of the film and layer steps become visible by this approach, for example, the diagonal lines for runs A and B from top left to bottom right. Beside the amplitude of orientational order, XCCA enables access to the patch size via the orientation angle ω_k related to the phase of the Fourier coefficients (Figure S3, Supporting Information).^[8,15] Connected areas with the same ω_k represent a perfect lattice without measurable defects. This is shown in the bottom part of **Figure 5**. Although ω_k is calculated for the

whole map including empty spots where it does not provide a meaningful measure, areas with continuous layers of AuNP seem to show large domain sizes, well beyond 20–30 μm in 1D. To take a closer look into the domain sizes and to neglect potential effects from the copper grid, we take a closer look into the meshes highlighted in **Figure 3**. Velocity plots of these meshes are shown in **Figure 6**.

The velocity plots highlight in particular changes of orientation at layer steps, such as in mesh ii from bi- to tri- and tetralayers, and the gradual increase from bilayers to multilayers in mesh iii. The transition from bi- to trilayers in mesh iv results in a change of the orientation, however, this cannot strictly be observed for mono-bilayer steps. Most interestingly, mesh i consisting of tri- and tetralayers (or one large tetralayer according to TEM) shows only very weak modulations of ω_k . This indicates that the local order and its orientations extends over the whole mesh of approximately $(38.5 \mu\text{m})^2$ in size. Taking a closer look on **Figure 5**, domains of same order appear to extend even further over different meshes, covering areas of up to $60 \mu\text{m} \times 60 \mu\text{m}$ (run A). We note that our analysis is focused on heterogeneous regions including different numbers of layers and not on large regions with uniform thickness (i.e., number of layers). Such large uniform areas are regularly observed in TEM (cf. **Figure S1**, Supporting Information) and accordingly even larger single crystalline domains are conceivable. For smaller AuNP and NP of different materials large 3D supercrystals are well-established.^[19] For 2D crystalline superlattices, monolayer domain sizes of some square micrometers and up to 20 μm in 1D, as observed with EM, have been reported.^[1,20–22] Thus, even considering the larger particle size used herein, our XCCA analysis indicates record scale 2D single crystalline domains.

Finally, we discuss the effect of film thickness on the degree of orientational order. While the lattice constant was found to depend on the number of layers, the question arises if this can also be observed for $|\hat{I}_6|$. Increase of the degree of local order as well its variation has been reported for heterogeneously ordered films of AuNP.^[8] The amplitudes of the Fourier coefficients $|\hat{I}_\ell|$ averaged over the particular number of layers are plotted in **Figure 7**.

Besides $\ell = 6$, we show results for $\ell = 2$ as an example for even coefficients that do not reflect the dominating symmetry. Scattering patterns from supercrystals are typically centrosymmetric, that is, $I(q) = I(-q)$ which is known as Friedel's law. Therefore we also show $\ell = 5$ which is thus a measure of background and noise contribution. Obviously, the sixfold order dominates. Most importantly, there is no dependence on the number of layers. Only for mono- to trilayers we observe an increase of $|\hat{I}_2|$ which is connected to the increase of intensity with the number of layers and thus the visibility of Friedel's law. We find similar results for other even symmetries that are not multiples of six. The constant sixfold local symmetry as a function of the number of layers suggests that the self-assembled films show a well-developed local order already for monolayers that does not develop significantly with a growing number of layers.

3. Discussion

Self-assembly of small nanoparticles with short ligands has been studied for many years now. However, experimental

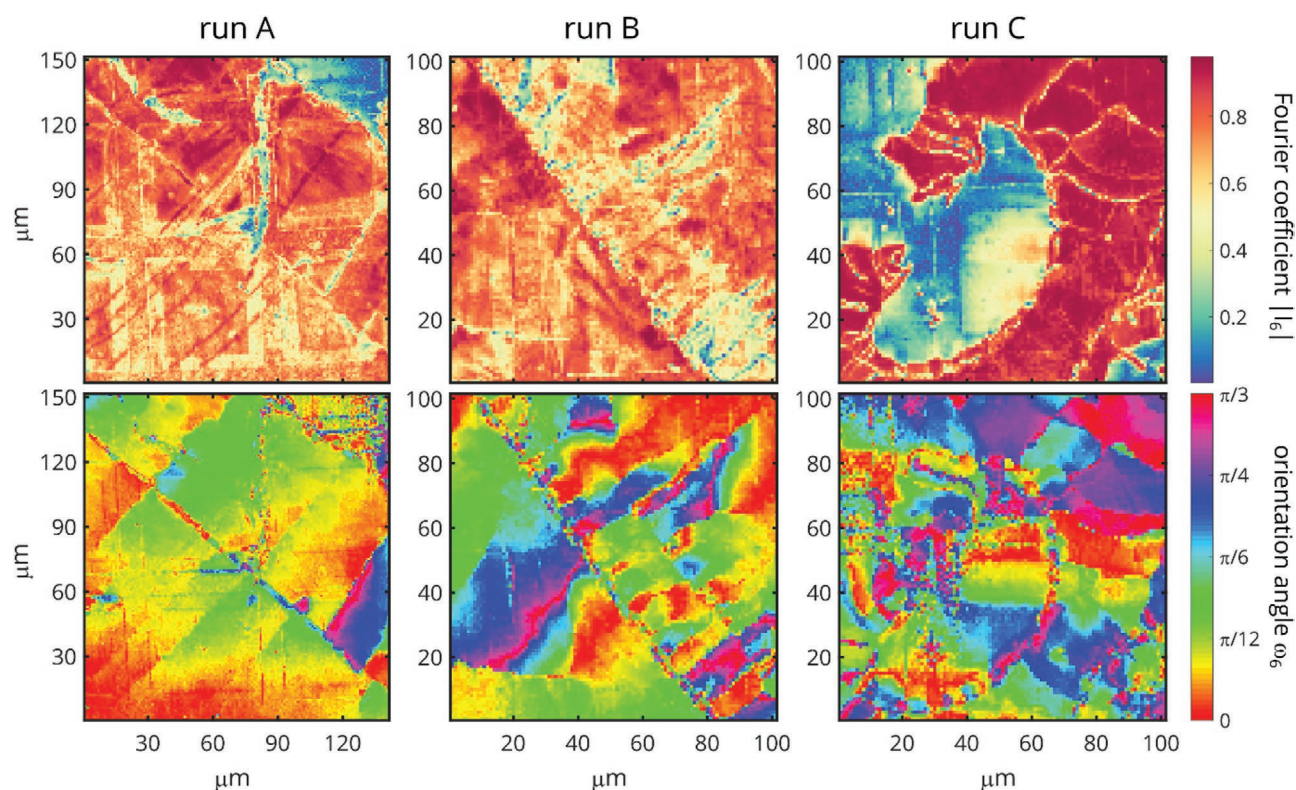


Figure 5. Orientational order. Top: Map of sixfold order $|l_6|$ for the three runs. Bottom: Orientation angle α_6 .

control of self-assembly is still challenging because of complex processes involving various parameters and forces over many length and time scales.^[1,2,23] The same holds true for larger AuNP, coated with polymer ligands.^[22] To understand the self-assembly of such systems, two perspectives are helpful: the ensemble and the pairwise particle-particle interaction.^[23] In a simplified view on the ensemble level, the assembly can be understood as an optimization of a hard-sphere (considering the whole AuNP-core/polymer-shell system as one hard sphere) packing, that reduces the overall free energy by increasing the free volume entropy (maximized local degrees of freedom for each constituent outweighs the reduced configurational entropy of the ensemble).^[1] This approach is established, for example, for predicting the structural variety of binary nanoparticle superlattices.^[24–26] Following this picture, the crystallinity of the AuNP supercrystals indicates that a thermodynamic minimum is reached. The closest packing of the “hard-spheres” (AuNP + polymer shell) results in a non-closed packing of the AuNP-cores, that can be tuned via the thickness of the polymer shell (i.e., molecular mass of the ligand), a major advantage of polymers compared to small molecular ligands.^[18,21,22,26,27] The particle-particle interactions in such systems are dominated by the polymer shell. Because the system is nonpolar, electrostatic contributions can be neglected, leaving Van-der-Waals attractive forces between the AuNP-cores and repulsive forces upon compression of the polymer shells at decreasing interparticle distances.^[2,23] However, the interparticle ligand-ligand interactions itself change during the self-assembly process. Being dispersed in the solvent toluene, a good solvent

for polystyrene, the interaction of polymer segments with solvent molecules is favored over polymer–polymer interactions.^[28] Accordingly, the AuNP@PSSH are well dispersed in toluene and do not aggregate. In the final formed superlattices, no toluene is present, and ligand-ligand interactions are favored over interactions with air or the polar liquid subphase, resulting in coherent films (thin films would also form from bare polystyrene-ligand solutions without particles).^[18] Because the attractive ligand-ligand interactions are rather weak (no hydrogen-bridges, chemical cross-linking etc.), the resulting superlattice films are also fragile and brittle. Thus, the interparticle ligand-ligand interactions gradually change, during slow evaporation of the solvent, from repulsive to weakly attractive. The weak interactions are the key for reaching a thermodynamic minimum during assembly, because they allow for structural rearrangements in the forming lattices.^[2,22] The other factor in this respect is the liquid subphase providing mobility for the arranging particles.^[24] The viscosity of the liquid subphase affects the 2D-Brownian motion of the floating particles and forming superlattice segments at the liquid-liquid and the liquid-air interface and therefore the kinetics of the assembly. The kinetics are related to the formation of defects, which can be understood as local minima of the free energy landscape often termed kinetic trap states.^[2] From these considerations we can conclude that the synthesis protocol provides a thermodynamically and kinetically well-balanced system, that allows the drying AuNP@PSSH to reach a thermodynamic minimum in the form of crystalline superlattices. The layered crystalline structure is thermodynamically favored and the particle-particle

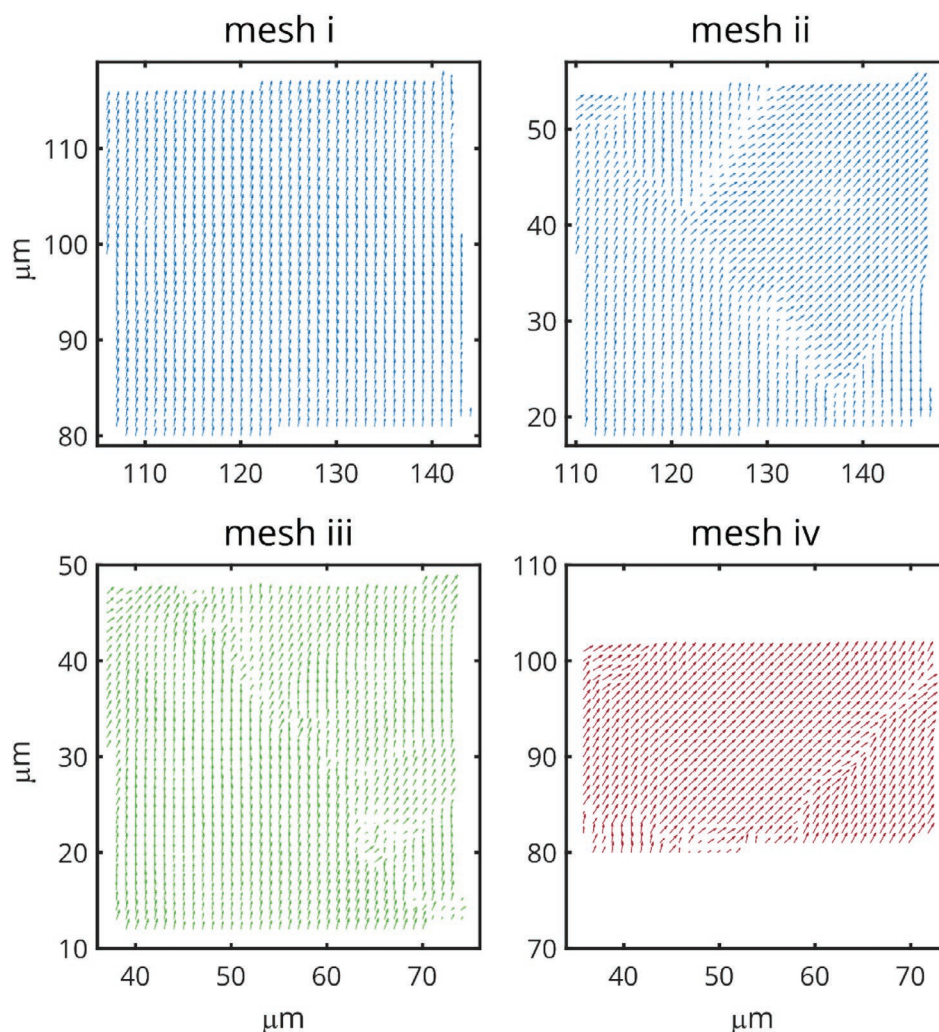


Figure 6. Velocity plots of the four meshes labeled in Figure 3. The length of the arrows is given by $|\hat{l}_6|$, its direction by ω_6 .

interactions are sufficiently weak to allow rearrangements. In particular, single particles, or even small islands, on a monolayer (same for bi- and multilayers) are practically never

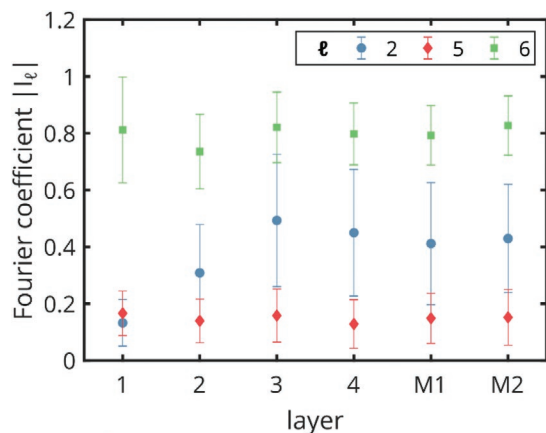


Figure 7. Mean Fourier coefficients $|\hat{l}_\ell|$ for $\ell = 2, 5, 6$, as a function of number of layers for the three runs. The error bars reflect the rms error.

observed. The number of layers cannot be perfectly controlled today, but it is related to the number of particles in the drying solution in a given geometry. This number can be controlled via the particle concentration or via the volume.

Previous experiments indicate that the evaporation time affects the lattice constant a . For a fast evaporation we observed slightly larger a compared to a slow evaporation (Figure S4, Supporting Information), suggesting a contraction of the AuNP@PSSH lattice in the late stage of assembly to maximize the increasingly attractive ligand-ligand interactions. After complete evaporation of the solvent, further contraction is not possible. No change of the lattice constant was observed for aging dry samples. Because the observed increase of a is a small subnanometer effect, it has to be interpreted with care. One could speculate that the contraction of the lattice at the late stage of assembly is possible for monolayers with all particles being mobile on the liquid subphase as illustrated in Figure 8. In contrast, once a second layer has formed, further contraction is limited. Assuming that thicker crystals with more layers start growing at an earlier point of the assembly process, the lattice contraction would be stopped earlier. In consequence the

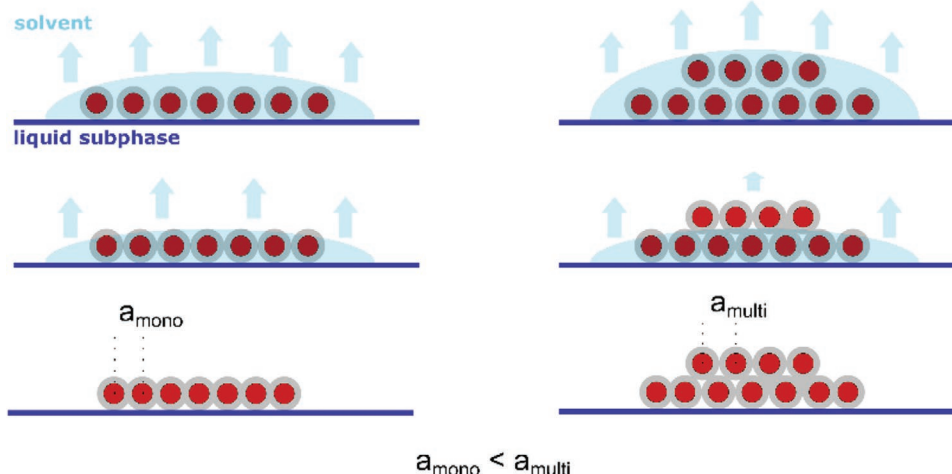


Figure 8. Illustration of the final stage of self-assembly: mono- versus bilayer (not to scale).

lattice constant would gradually increase with the number of layers as indicated by our results shown in Figure 4b. No peak broadening was observed for an increasing number of layers (Figure 4a) indicating that the lattice constants within domains of a given number of layers are constant.

4. Conclusion

The combination of TEM, SAXS, and XCCA enables a detailed and confident structural characterization of plasmonic supercrystals, combining statistically robust structural data from SAXS with a detailed microscopic picture obtained by TEM analysis and XCCA. Supported by direct comparison of TEM measurements and SAXS-maps, we show that the correlation of SAXS intensity and sample thickness can be used to categorize intensity maps and analyze the SAXS-results as a function of layer number. We find a subnanometer but significant increase of the lattice constant with increasing number of layers, record-scale single-crystalline domains up to thousands of square μm and a high degree of orientational order irrespective of the number of layers. The structural details obtained in this study will help to understand and further optimize the self-assembly process of AuNP@PSSH, yielding well-defined plasmonic supercrystals.

5. Experimental Section

Plasmonic Supercrystals Synthesis: Supercrystals were synthesized with Polystyrene-thiol (PSSH) functionalized AuNP ($d_{\text{core}} = 23.4 \text{ nm}$, $M_n(\text{PSSH}) = 11\,400 \text{ g mol}^{-1}$) by self-assembly at a toluene-diethylene glycol (DEG) liquid-liquid interface as described recently.^[18] By adjusting the volume of the AuNP@PSSH solution (7.3 nm) in toluene that was evaporated on the liquid subphase DEG, one sample with mostly monolayers (300 μL) and one sample with mostly bi- and multilayers (500 μL) was prepared.

Transmission Electron Microscopy: TEM measurements were performed using a Jeol JEM-1011 instrument operating at 100 kV. Samples of self-assembled AuNP@PSSH films were carefully skimmed off with a carbon coated copper grid held by a tweezer. The grid was then dried in vacuum for at least 1 h. TEM measurements were analyzed with ImageJ (version 1.50i) to determine the parameters summarized in Table 1. The indicated

mean values were obtained from analyses of at least three images from different spots on the samples. To identify the regions of interest mapped in the SAXS experiments, overview maps (Figure S1, Supporting Information) were used to identify characteristic structural features.

Coherent SAXS: The coherent SAXS experiment were performed at beamline P10 of PETRA III (DESY, Hamburg, Germany) at an X-ray energy of 8.2 keV. The samples were placed in the in house sample chamber that has been evacuated afterwards. The scattering patterns were measured using an Eiger X4M detector with 2167×2070 pixels with a pixel size of $(75 \mu\text{m})^2$. This detector was placed 5 m downstream the sample using an evacuated flight path. In order to obtain micrometer scanning resolution, the beam was focused by a stack of compound refractive lenses to a size of $1.2 \mu\text{m} \times 1.0 \mu\text{m}$ (horizontal $\times$ vertical) at the sample position.^[29] To scan the sample, we used piezo stages and step sizes of $1 \mu\text{m}$ in horizontal and vertical direction. Runs were performed covering regions of interest of $140 \mu\text{m} \times 150 \mu\text{m}$ (run A) or $100 \mu\text{m} \times 100 \mu\text{m}$ (runs B and C), respectively. The exposure time was 1 s per sample spot. The mean particle diameter and dispersity was determined from a form factor sample (Figure S5, Supporting Information), that is, AuNP@PSSH in toluene, because for the supercrystals the $I(q)$ are dominated by the Bragg reflections preventing form factor fitting.

X-ray Cross Correlation Analysis: The XCCA method allows to access orientational order information via angular correlation functions from 2D scattering patterns. In standard SAXS experiments this information is typically lost due to the averaging over the azimuthal angle:

$$I(q) = |I(q)| = \langle I(q, \varphi) \rangle_\varphi \quad (1)$$

where

$$q = |q| = \frac{4\pi}{\lambda} \sin\left(\frac{\theta}{2}\right) \quad (2)$$

denotes the modulus of the wave vector transfer, λ the wavelength of the X-rays and θ the scattering angle. In XCCA, the correlation function probing orientational order is given by^[9]

$$C(q, \Delta) = \frac{\langle I(q, \varphi) I(q, \varphi + \Delta) \rangle_\varphi - \langle I(q, \varphi) \rangle_\varphi^2}{(\langle I(q, \varphi) \rangle_\varphi)^2} \quad (3)$$

Orientalional order can be quantified by the Fourier coefficients of C of order l with respect to the angle difference Δ ^[11,12] which is connected by the Wiener–Khinchin theorem to the Fourier coefficients of the intensity

$$\hat{C}_l(q) \propto |\hat{I}_l(q)|^2 \quad (4)$$

For thin films in particular, XCCA enables quantification of the extent of sample orientation by the phase Ω_ℓ of the Fourier coefficients

$$\hat{I}_\ell(q) = |\hat{I}_\ell(q)| \exp(i\Omega_\ell(q)) \quad (5)$$

In this way, domain size of few μm are reported for self-assembled films of gold nanoparticles^[8] and PbS nanocrystals.^[14] The orientation of the sixfold symmetry in the scattering pattern and thus the probed sample area is given by

$$\omega_6 = \frac{\Omega_6}{6} \quad (6)$$

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

nanomaterials, small-angle X-ray scattering, self-assembly, supercrystals, X-ray cross-correlation analysis

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3.5 The dynamics of PEG-coated nanoparticles in concentrated protein solutions up to the molecular crowding range

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This study is an example of applying XPCS to biologically relevant systems. As discussed, XPCS allows to measure sample dynamics and structure of colloidal systems at the same time. The sample dynamics are obtained by temporal autocorrelations of individual 2D-detector images (so-called speckle patterns) measured at high repetition rates. The principle is similar to dynamic light scattering (DLS) that uses visible light, but XPCS additionally allows for analysis of the $I(q)$ providing structural information and the q - (i.e. length scale-) dependent analysis of dynamics. Simple dynamics for spherical particles are diffusive and can be described by their Stokes-Einstein diffusion constant. The parameters are temperature, viscosity (which is also temperature dependent) and particle diameter. In nanobiotechnology protein adsorption is a very important topic because it can completely change the behavior of a nanomaterial. Because protein adsorption increases the particle diameter it can be measured by correlation methods based on particle dynamics like DLS, fluorescence correlation spectroscopy (FCS) and XPCS. In previous work in the Parak group, XPCS had been already explored in this direction, but issues with sample stability imposed some limitations, in particular at high protein concentrations.¹⁷⁶ By using the AuNP@PEGMUA system, these stability issues could be overcome, even at very high protein concentrations mimicking the macromolecular crowding inside cells (200-400 mg/ml). Optical measurements (like DLS and FCS) are not feasible at such high protein concentrations because of their strong scattering. With XPCS we were able to measure AuNP@PEGMUA dynamics in a broad range of protein concentrations up to the molecular crowding range. We confirmed that bovine serum albumin (BSA, the most abundant serum protein) can be used to mimic the dynamics of complex protein mixtures in serum.

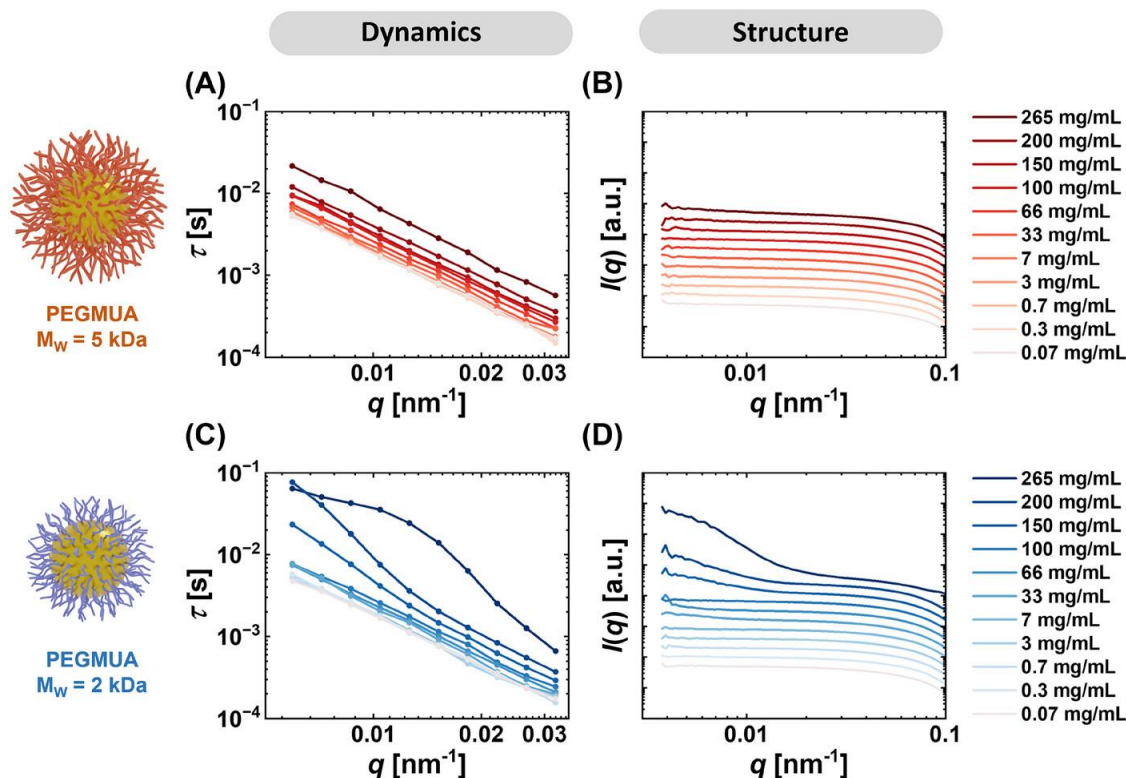


Figure 6. Colloidal stability of nanoparticles (NPs) with different (PEGMUA) ligand lengths for varying bovine serum albumin (BSA) concentrations. q -dependency of the relaxation time τ and SAXS scattering intensity as a function of q of NPs coated with PEGMUA5k (A and B) and PEGMUA2k (C and D) for a BSA concentration range of 0.07–265 mg/mL. The measurements reveal that at the highest protein concentrations only PEGMUA5k provides full colloidal stability.

At very high protein concentrations an unusual behavior was observed, namely that the AuNP@PEGMUA dynamics were still diffusive (underlining their excellent stabilization) but much faster than expected when carefully correcting for viscosity at such high protein concentrations. In other words, the nanoscopic viscosity strongly deviated from the experimentally confirmed bulk viscosity. These findings are relevant for nanomedicine because they underline that not only the properties of nanomaterials can be changed by their biological environment but also the local properties of the environment can be changed by the nanomaterials. When this is not appropriately considered, severe misinterpretations can result when studying the behavior of nanomaterials in cells, tissue and organisms. Analysis of structure at high protein concentrations also revealed that the stability of AuNP@PEGMUA2k was affected, but the one of AuNP@PEGMUA5k was not, Figure 6. This underlines that even if nanomaterials are stable in buffers, the situation can change in real biological environments, e.g. with high protein concentrations. Understanding the dynamics of nanomaterials in biological systems is crucial for their safe application and XPCS is an interesting method, which is yet at the beginning of being explored in this direction. Revealing the unexpected NP dynamics and mismatch of local and global properties in this study is among the pioneering demonstrations of the capabilities of XPCS in this context.

RESEARCH ARTICLE

The dynamics of PEG-coated nanoparticles in concentrated protein solutions up to the molecular crowding range

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Abstract

Polymer-coated nanoparticles are widely studied in the context of nanomedicine and it is therefore of utmost importance to understand not only how their structure but also how their colloidal dynamics are affected by physiologically relevant conditions. A characteristic feature of the cytosol of cells is the very high concentration of proteins among other matrix components, often termed macromolecular crowding. Here, the structure and colloidal dynamics of poly(ethylene glycol) (PEG)-coated gold nanoparticles in the presence of bovine serum albumin (BSA) concentrations ranging from 0 to 265 mg/mL are studied with X-ray photon correlation spectroscopy. For protein–nanoparticle mixtures with high BSA concentrations, comparable to intracellular levels, a significant deviation of the apparent viscosity from expectations for pure BSA solutions is found. The findings strongly indicate that the nanoscopic viscous properties of the dense protein solutions are significantly affected by the nanoparticles. At these high concentrations, the colloidal stability of the samples depends on the molecular weight of the coating PEG–ligand, whereas at lower concentrations no differences are observed.

KEYWORDS

aggregation, nanoparticles, PEG, polymers, proteins, XPCS

1 | INTRODUCTION

One major field envisioned for nanoparticle (NP)-based applications is medicine and in this context the behavior of NPs in biological media is the subject of intense research efforts.^[1–7]

When NPs are introduced into the body, they encounter various physiological environments with different physico-chemical conditions, including pH, ionic strength, and local molecular composition, but also viscosity. Of particular significance is crowded and protein-rich fluids, such as blood, interstitial fluids, and intracellular fluids, which are present in the cytosol (though NPs most of the time will not be free in the cytosol but rather are trapped in endosomal vesicles) (Figure 1A).^[8–10] The protein concentration in the cytosol can reach values of up to 400 mg/mL, and for this condition, the term macromolecular crowding has been

established.^[8,11–16] The relevance of macromolecular crowding for dynamics and binding properties in cells has received increasing attention within the last two decades. Macromolecular crowding affects the diffusive properties, stability, and folding of proteins. While many attempts to understand the impact of crowding have been of theoretical nature due to the cellular complexity,^[17,18] among the experimental studies, fluorescence correlation spectroscopy (FCS),^[19–21] nuclear magnetic resonance spectroscopy,^[22–26] and neutron scattering^[27] have been used to study protein dynamics and protein–protein interactions in crowded environments. In particular, in situ FCS and single-particle tracking studies in living cells revealed anomalous and spatially heterogeneous dynamics with indications of different sub-diffusive mechanisms.^[20,28,29] More recently, X-ray photon correlation spectroscopy (XPCS) has emerged as a powerful technique to study complex dynamics of soft matter

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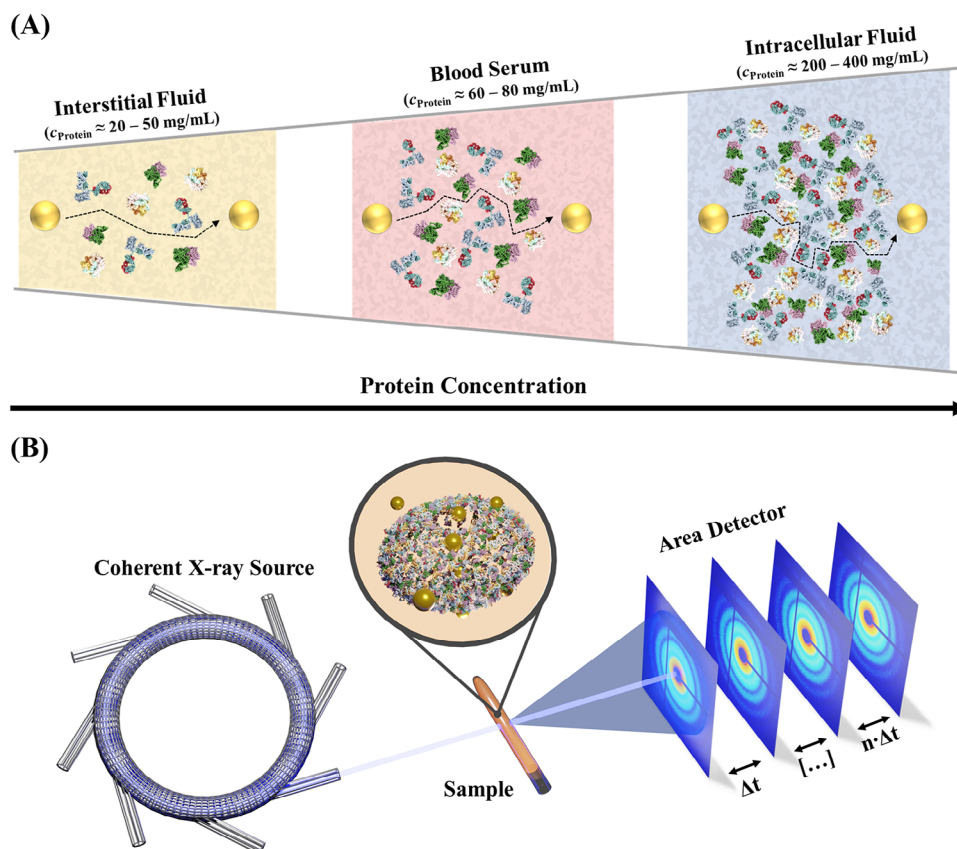


FIGURE 1 (A) Schematic representation of protein concentrations in different physiological fluids. (B) Scheme of the X-ray photon correlation spectroscopy setup.

systems.^[30] In XPCS experiments, (partially) coherent X-rays generated by a synchrotron radiation source create fluctuating speckle patterns on an area detector upon interacting with a dynamic sample, which are then correlated over time. A schematic of an XPCS measurement is visualized in Figure 1B. Recently, XPCS has proven to be a powerful tool for studies of protein dynamics.^[31–35] For instance, Reiser et al. monitored antibody protein dynamics at sub- μs temporal resolution on molecular length scales using megahertz XPCS measurements at the European X-ray free-electron laser (XFEL).^[32] Typically, NPs can be used as tracers in XPCS studies or be the subject under study itself.^[36–40] In contrast to FCS studies, fluorescence properties are not required in XPCS experiments and via their surface chemistry the interactions of NPs with their environment can be tuned to achieve, for instance, minimized protein adsorption or specific targeting.^[6,41–45] In XPCS experiments, the sample dynamics are measured over a range of scattering vectors q , corresponding to a range of length scales, and also a very broad window of experimental characteristic times can be covered.^[30,46] As an X-ray technique, it allows for probing sample dynamics in native environments without modifications of sample properties. Because gold nanoparticle (AuNP) are strong scatterers due to their electron density, they are excellent tracer particles for X-ray-based experiments in general. In XPCS experiments, their strong scattering intensity enables to measure dynamics of comparably low concentrations with high temporal resolution. In contrast to dynamic light scattering (DLS), XPCS is not affected by multiple scattering and thus allows probing the single-particle dynamics. Addressing the dynamics of

NPs in biological fluids, Unni et al. investigated the rotational and translational diffusion of poly(ethylene glycol) (PEG)-coated cobalt ferrite NPs in synovial fluid and hyaluronic acid.^[47] An XPCS study of the agglomeration behavior of AuNPs and their colloidal dynamics in human whole blood was presented recently.^[48] An experimental challenge in such studies can be the colloidal stability of the NPs. Protein abundances have a crucial influence in this context.^[49,50] NPs exposed to low protein concentrations can already aggregate, for instance, driven by unfolding of adsorbed proteins.^[51] Even when the NPs are stable at moderate protein concentrations, the situation can completely change in dense protein solutions.^[52,53] Agglomeration or aggregation of the NPs can then impede a meaningful analysis of colloidal structure and dynamics. In this study, we therefore probed AuNPs with a tailored PEG coating with high grafting density, that is known to exhibit excellent colloidal and chemical stability as well as minimum protein adsorption.^[54–57] The structure and dynamics of these AuNP@PEG were measured in a range of bovine serum albumin (BSA) concentrations up to 4000 μM ($\sim 265 \text{ mg/mL}$). Albumin is the most abundant serum protein and therefore well suited to mimic physiological conditions, which we confirm by comparing to measurements in human serum. It is also commonly used as a model for macromolecular crowding.^[27,58,59] We observed an excellent stability of AuNP@PEG with different PEG-shell thickness up to BSA concentrations of $\sim 100 \text{ mg/mL}$. At higher concentrations, entering the range of intracellular levels and thus the regime of macromolecular crowding, the stability depends on the PEG-shell thickness. The particle dynamics stay diffusive, but significantly deviate from expectations based on

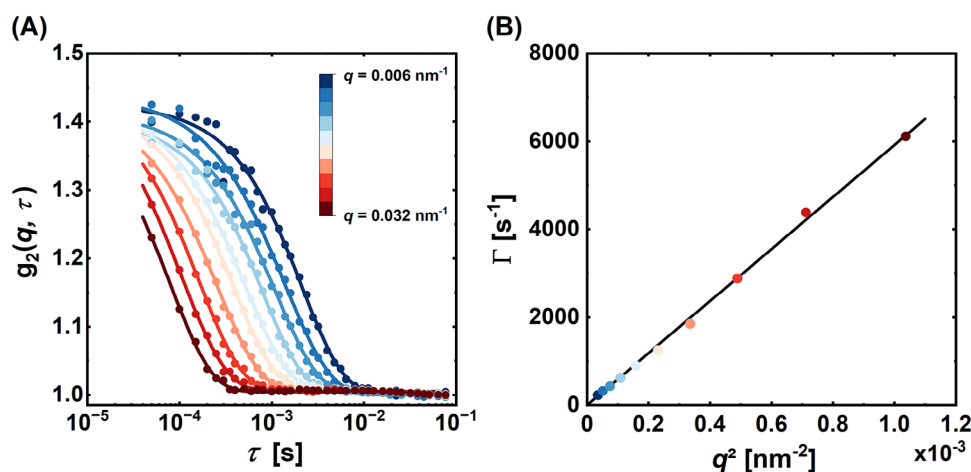


FIGURE 2 X-ray photon correlation spectroscopy (XPCS) measurements of aqueous gold nanoparticles (AuNPs). (A) Averaged autocorrelation functions of 10 individual XPCS runs as a function of the lag time τ for 10 binned q -values ranging from 0.006 to 0.032 nm^{-1} . (B) Relaxation rates Γ of the averaged autocorrelation functions as a function of squared momentum transfer q^2 . Displayed solid lines represent fits to the data.

established viscosity models. The particle diffusion is faster than expected, which cannot be explained with structural changes (such as a collapse of the PEG shells). Our observations therefore point at a NP-specific contribution to the viscous properties of the dense protein solutions, namely a significantly decreased nanoscopic viscosity experienced by the PEG-coated NPs. These findings are in particular relevant in the context of nanomedicine, where polymer-coated NPs are commonly employed. Understanding that and how local properties can be affected by such nanomaterials is not only crucial to avoid misinterpretations in studies of dynamics in complex environments but also in analyzing and predicting biodistribution, pharmacokinetic properties, clearance, and local doses.

2 | RESULTS AND DISCUSSION

2.1 | Preparation, characterization, and XPCS measurements of PEG-coated AuNP

The physicochemical properties of NPs in physiological environments can be strongly affected by protein adsorption, resulting in altered pharmacokinetic properties.^[6,45,60–62] PEG-based coatings, among others, have been demonstrated to minimize unwanted protein adsorption and are therefore often referred to as “stealth” or antifouling coatings for NPs.^[42,44] In this study, AuNPs (core diameter $d_c \approx 50$ nm) were conjugated with α -methoxypoly(ethylene glycol)- ω -(11-mercaptopundecanoate) (PEGMUA), a PEG ligand with a hydrophobic 11-mercaptopundecanoic acid (MUA)-based spacer. This spacer enables a high grafting density of PEG polymers on the NPs and provides very high colloidal and chemical stabilization as well as minimized protein adsorption.^[54,55,57] PEGMUA with molecular weights (M_w) ~ 2 kDa (PEGMUA2k) and ~ 5 kDa (PEGMUA5k) were used, corresponding to shell thicknesses of ~ 8 and ~ 12 nm and grafting densities of ~ 3 and ~ 1.7 nm^{-2} , respectively.^[56] At such high grafting densities, >1 nm^{-2} , the conformation of the ligand layer is usually described as a dense brush that is considered ideal in terms of minimized protein adsorption.^[63] The ζ -potential of these

methoxy-terminated PEG coatings with no formal charge is slightly negative (-11.7 ± 0.6 mV).^[57] The characterization of the PEGMUA-coated AuNP by transmission electron microscopy, ultraviolet/visible spectroscopy and DLS is summarized in Figure S1.

Exemplary autocorrelation functions in the q -range of 0.006 and 0.032 nm^{-1} as obtained by XPCS are shown in Figure 2A for AuNPs coated with PEGMUA5k (AuNP@PEGMUA5k) in water. To provide statistical significance and to detect potential outlier measurements, each displayed g_2 -function is the result of 10 individual XPCS measurements on different positions of the same sample. An example for this procedure is provided as Figure S3. By applying an exponential fit to the autocorrelation functions (Methods, Equation 6) and fitting the so-obtained relaxation rates Γ , a diffusion coefficient $D_0 = 5.92 \times 10^{-12}$ m/s is determined. Here, the linear dependence $\Gamma \propto q^2$ is characteristic for free diffusion. Comparing XPCS and DLS results, the hydrodynamic diameters d_H obtained with the Stokes–Einstein equation (Equation 7) are in good agreement ($d_{H,XPCS} = 72.8 \pm 1.7$ nm; $d_{H,DLS} = 68.1 \pm 2.3$ nm). The hydrodynamic diameters of the other samples were in a similar range as presented in Supporting Note S2 and Table S1. The initial discussion focuses on AuNPs conjugated with the longer ligand PEGMUA5k, unless specified otherwise. In all measurements, it was ensured that radiation damage does not affect the sample dynamics (for details, we refer to Supporting Note S5 and Figures S4–S7).

Optical methods such as DLS, FCS, and nanoparticle tracking analysis (NTA) are commonly used to study the size and diffusive properties of NPs and their interactions with proteins.^[43,45,62,64]

Due to the significant scattering of proteins, DLS and NTA are restricted in terms of accessible protein concentrations. This is exemplified in Figure 3A for DLS by comparing measurements of AuNP@PEGMUA5k in water, in BSA solutions at high concentrations ($c_{\text{protein}} = 100$ mg/mL), and in bovine serum ($c_{\text{protein}} = 45$ mg/mL), where the two latter ones are impeded in accurate size determination. Scattering contributions of proteins result in a size broadening and a split of the size distributions. AuNP in BSA even present a size shift due to multiple scattering, highlighting the concentration

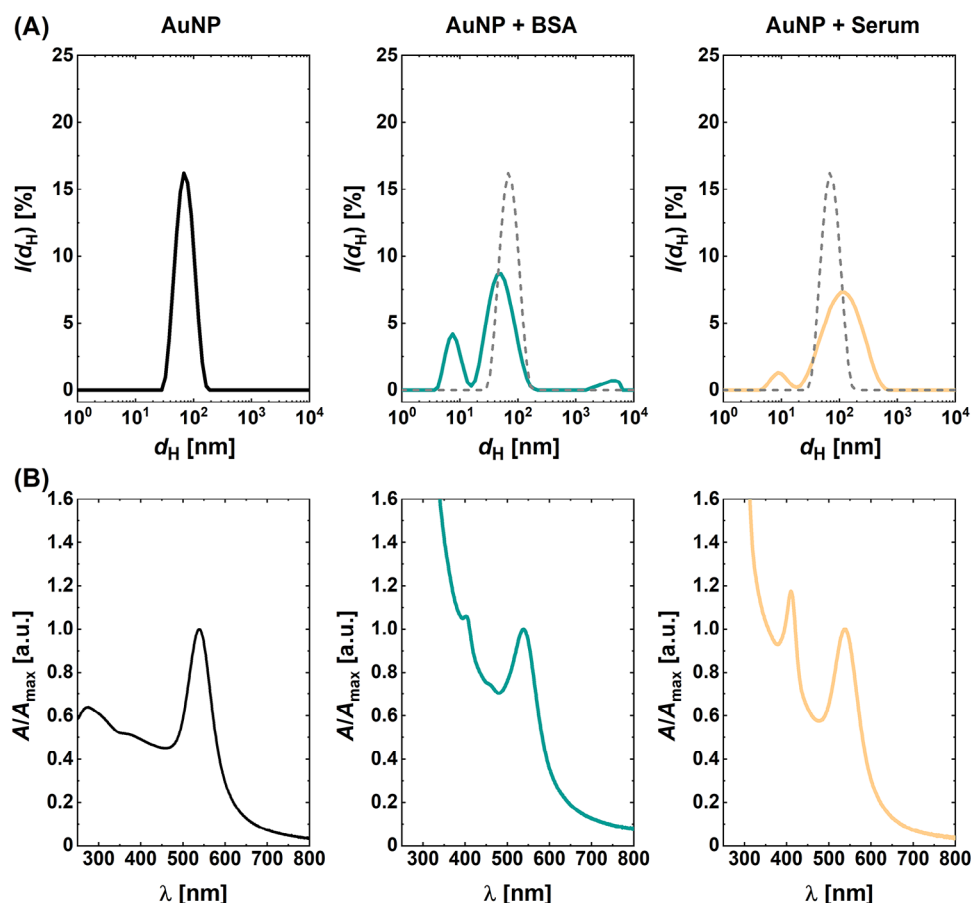


FIGURE 3 Limitations of dynamic light scattering (DLS) in dense biological fluids. (A) Intensity-weighted distributions of hydrodynamic diameters obtained by DLS: α -methoxypoly(ethylene glycol)- ω -(11-mercaptopundecanoate) (PEGMUA)-coated gold nanoparticles (AuNPs) in water, bovine serum albumin (BSA) solution ($c_{\text{protein}} = 100$ mg/mL), and bovine serum ($c_{\text{protein}} = 45$ mg/mL), respectively. (B) Corresponding normalized ultraviolet/visible (UV/vis) spectra. The pronounced peak in serum at $\lambda = 410$ nm corresponds to hemoglobin.

limitations of DLS.^[65] Absorption spectra of the same samples (Figure 3B) underline that the AuNPs are not aggregated; aggregation would lead to a broadening and shift of the plasmon resonance band at ~ 535 nm due to plasmonic coupling.^[61] The spectra of the protein solutions are the sum of characteristic AuNP and protein contributions to the absorbance without any indications of agglomeration or aggregation. The DLS measurements of AuNPs in protein solutions, however, do not provide reliable hydrodynamic diameters, because of the background noise caused by the large amounts of scattering proteins and thus multiple scattering processes. FCS and fluorescence cross-correlation spectroscopy circumvent some of these issues by utilizing fluorescence instead of light scattering. These methods have been used to study intracellular dynamics and protein–NP interactions.^[20,28,61,66]

2.2 | The role of viscosity in analyzing dynamics

The dynamics of AuNP@PEGMUA conjugates were measured with XPCS in solutions with different BSA and bovine serum concentrations. The NP concentrations were kept constant in each set of experiments and the pH was buffered (phosphate buffered saline, PBS) at 7.4. A BSA concentration range of 0–265 mg/mL was investigated in order to represent different physiologically relevant conditions (cf.

Figure 1). As potential protein-induced aggregation and protein corona formation are dynamic processes, all NP samples were incubated for the same time (2 h) in the according protein solutions before XPCS measurements were recorded. Diffusivities of AuNP@PEGMUA as a function of increasing BSA concentrations are shown in Figure 4A. For BSA concentrations larger than 10 mg/mL, the diffusivities decrease because of the increasing viscosity of the solution. Various models have been developed in order to describe the viscosity in dense protein solutions.^[67–71] The viscosity model by Ross and Minton was developed for concentrated protein solutions and has been confirmed experimentally.^[58,70] The modeled viscosity η_{th} is described by

$$\eta_{\text{th}} = \eta_0 \exp \left(\frac{\eta_{\text{in}} c_{\text{protein}}}{1 - \frac{k}{v} \eta_{\text{in}} c_{\text{protein}}} \right) \quad (1)$$

Here, η_0 denotes the viscosity of the initial medium (PBS), η_{in} is the intrinsic viscosity of the protein ($\eta_{\text{in, BSA}} = 4.13 \times 10^{-3}$ mL/mg for pH 7, value from supplier), c_{protein} is the protein concentration, k is a crowding factor, and v is a shape factor for non-spherical particles. The model accounts for excluded volume effects and their influence on the solution viscosity. The accuracy of this approximation varies, depending on the specific protein and experimental conditions, for example, the pH value.^[58] Yadav et al. determined a reasonable value of $k/v = 0.45$ for BSA^[58] based on prior

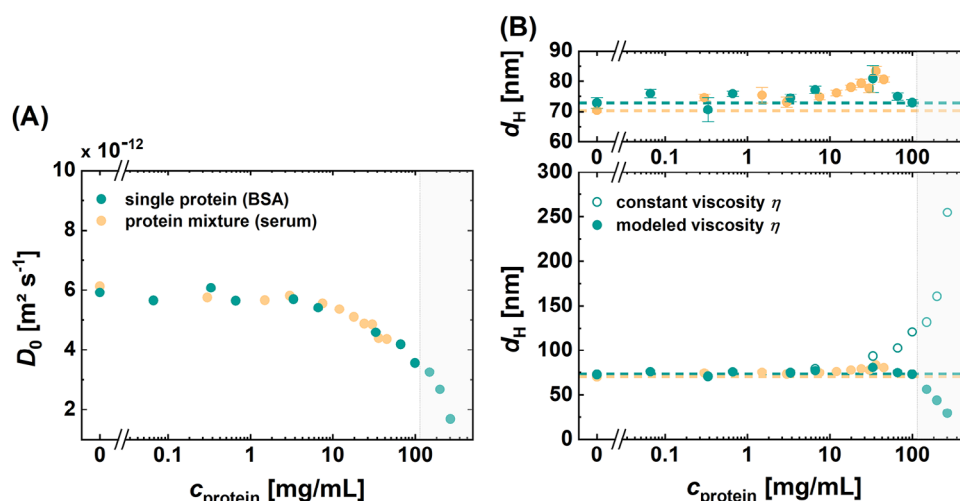


FIGURE 4 Evolution of nanoparticle (NP) dynamics and hydrodynamic diameter with increasing bovine serum albumin (BSA) (teal) and bovine serum (orange) concentration. (A) Diffusion coefficients of AuNP@PEGMUA5k as a function of protein concentration. (B) Calculated hydrodynamic diameters d_H as a function of protein concentration. Empty data points represent d_H values calculated with constant viscosity and filled dots with modeled viscosity obtained by the Ross and Minton equation. The protein concentration range, where the viscosity model does not agree with the data anymore is highlighted in gray. The dashed lines mark the NP sizes in aqueous condition. The top part of (B) shows a tighter y-axis scaling of the range of $c_{\text{protein}} = 0\text{--}100$ mg/mL for a better visualization of the size fluctuations. AuNP, gold nanoparticles; PEGMUA, α -methoxypoly(ethylene glycol)- ω -(11-mercaptopundecanoate).

studies on hemoglobin^[70] and IgG₁,^[71] which was used in the following.

The effective hydrodynamic diameters d_H were calculated with the Stokes–Einstein equation (Equation 7) using the diffusion coefficients D_0 determined from the XPCS measurements and the viscosities from the Ross–Minton model (Equation 1). In the BSA concentration range up to 100 mg/mL , we find no indications of protein-induced aggregation or agglomeration but good agreement of the hydrodynamic diameters with the corresponding values in water as shown in Figure 4B. Exact values are listed in Supporting Note S3 and Table S2. Only a slight size fluctuation ($\Delta d_{H,\text{average}} \approx 2.4$ nm) was observed. This accuracy is not sufficient to confidently rule out any BSA adsorption, for example, in sub-monolayer coverages, but we find no trends that suggest an adsorption isotherm as would be characteristic for protein adsorption.^[61] Taken together, we conclude that protein-induced aggregation of NPs is unlikely to play a major role and that no indications of significant protein adsorption to the AuNP@PEGMUA were observed in the BSA concentration range up to 100 mg/mL . These findings underline the excellent stabilization and bio-repulsive properties provided by the PEGMUA coatings.

For NPs exposed to bovine serum, obtained diffusion coefficients and hydrodynamic diameters are in excellent agreement with the BSA measurements, including their dependence on protein concentration. This underlines once more the stabilization of the samples and robust surface chemistry (two different batches of AuNP@PEGMUA are compared), but also shows that the viscosity model can be generalized to some extent to more complex protein mixtures. Only when comparing the deviation of measurements in serum and in water it seems that the viscosity correction is slightly better in the BSA case compared to the measurements in serum. To illustrate the increasing effect of viscosity on size determination at high protein concentrations, Figure 4B (bottom) shows the calculated hydrodynamic diameters when assuming a constant viscosity.

We conclude that the AuNP@PEGMUA conjugates are well stabilized and that their dynamics in protein solutions up to ~ 100 mg/mL can be well described by free diffusion according to the Stokes–Einstein model when accounting carefully for the changing viscosity at higher protein concentrations. This protein concentration (~ 100 mg/mL) is well above the total protein concentration in serum, however, in the cytosol of cells protein concentrations can be significantly higher and the regime of macromolecular crowding is reached.^[17,72] In the data presented in Figure 4B (bottom), it can be appreciated clearly, that in the regime of high BSA concentrations (> 100 mg/mL), the calculated hydrodynamic diameters strongly deviate from the expectation, even with the viscosity correction. The sample dynamics at high BSA concentrations are therefore analyzed in more detail in the following section.

2.3 | Dynamics in the regime of macromolecular crowding

Hydrodynamic diameters differ strongly from the expected size for the three highest BSA concentrations that have been measured. The smaller d_H values suggest significant faster dynamics than expected in this crowded environment. In contrast to reports on intracellular dynamics of proteins and particles with superdiffusive behavior due to active intracellular transport,^[73–76] we find no indications of anomalous dynamics. All investigated AuNP@PEGMUA samples in BSA and bovine serum undergo free diffusion even at the highest BSA concentration. This is expectable, as for active intracellular transport motor proteins and corresponding “tracks”, such as the kinesin–microtubule system are needed. A comprehensive analysis of the diffusive behavior can be found in Supporting Note S7. A more likely explanation lies in the applied viscosity model by Ross and Minton. Figure 5 illustrates the deviations of viscosities experienced by the NPs from the Ross and Minton model for both

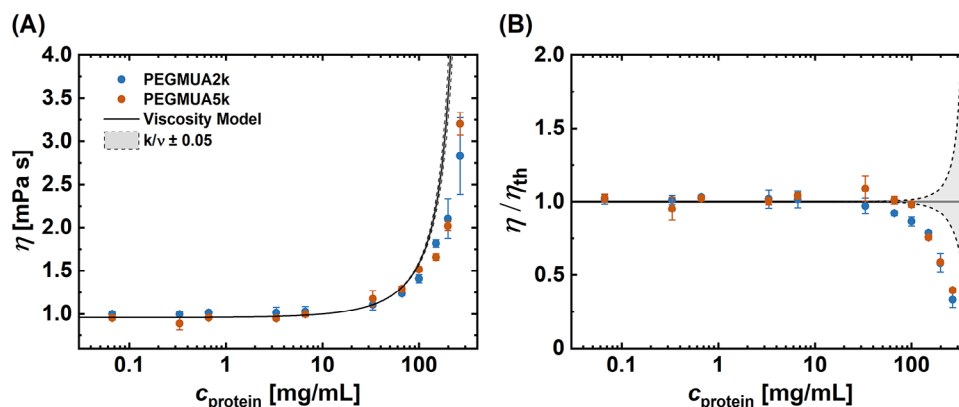


FIGURE 5 Comparison of the viscosity model by Ross and Minton with viscosities determined experimentally assuming a constant hydrodynamic diameter of the nanoparticle (NP). (A) Dependence of the viscosity η on the protein concentration for NPs coated with PEGMUA2k (blue) and PEGMUA5k (red) and the values predicted by the applied viscosity model (black line). The effect of varying k/v by ± 0.05 is indicated by the gray shading. (B) Same data but with the relative viscosity η/η_{th} . PEGMUA, α -methoxypoly(ethylene glycol)- ω -(11-mercaptopundecanoate).

PEGMUA2k and PEGMUA5k. Nanoscale viscosities η were calculated by assuming a constant particle size d_H and using the diffusion coefficient D_0 as determined from the XPCS measurements. For concentrations exceeding 100 mg/mL, nanoscopic viscosities are notably lower as predicted, even though the model is generally suitable for protein densities in that range and was experimentally confirmed.^[58,67,70,71] As a semi-empirical model, the viscosity determination relies on the experimentally determined crowding and shape factor k/v . To investigate the potential influence of k/v on the theoretical predictions, its value was varied by ± 0.05 . The impact of k/v on the model accuracy is presented in Figure 5B by the gray shading. To illustrate the margin due to the variation of k/v more clearly, normalized viscosity values η/η_{th} are used. Despite the wide range covered by the variation of k/v and its increasing relevance at high BSA concentrations, the faster NP dynamics still do not align with the model's predictions. In complementary microrheology experiments, we tested the macroscopic viscosity of BSA solutions with and without AuNP@PEGMUA. The results were in agreement with the model and notably we observed no significant difference of the viscosities in the presence of AuNP@PEGMUA in these experiments (Supporting Note S6 and Figure S8).

As discussed by Yadav et al., when analyzing the viscosity behavior of BSA solutions at high concentrations, intermolecular forces cannot be neglected.^[58] Hence, one explanation for the reduced nanoscopic viscosity might be the repulsive properties of the NP's PEG coating leading to a disruption of transient networks formed by the BSA molecules. These transient networks increase the diffusive resistance and accordingly the local viscosity decreases when they are disrupted.^[23,77] Unni et al. observed a similar discrepancy in the viscosity of NPs dispersed in synovial fluid and hyaluronic acid.^[47] In their study, macroscopic shear viscosity of synovial fluid determined by rheology strongly deviated from the nanoscopic viscosity experienced by PEG-coated cobalt ferrite NPs. Thus, we can confirm their important findings for the most abundant serum protein BSA, where the effect is much weaker compared to hyaluronic acid—the major component of synovial fluid that determines its viscous properties—but still significant. For the strongly network-forming glycosaminoglycan hyaluronic acid, the nanoscale viscosities in the presence of PEG-coated NPs were sev-

eral orders of magnitude smaller than expected for solutions above 1 mg/mL. For the BSA solutions at high concentrations (>100 mg/mL) studied herein, we find a factor in the range 1–3. Even though this effect is much more subtle, it is still significant and has to be considered in analyses of intracellular dynamics and transport of NPs.

In the final section, the role of PEG-coating thickness in terms of colloidal stability will be discussed.

2.4 | The role of PEGMUA-coating thickness

The influence of PEG-coating thickness on the colloidal stability was investigated by comparing the dynamics of AuNPs coated with different PEGMUA ligand lengths. PEGMUA2k ($M_w \sim 2000$ g/mol) and PEGMUA5k ($M_w \sim 5000$ g/mol) coatings were studied, corresponding to shell thicknesses s in the range 6–9 nm (2k) and 12–14 nm (5k) according to previous studies.^[54–56] AuNP@PEGMUA2k exhibited some indications of structural changes at high protein concentrations, whereas AuNP@PEGMUA5k seemed to be colloidally stable in the whole measured BSA concentration range. Figure 6 summarizes the measurements, with the q -dependence of τ representing the dynamics and the $I(q)$ representing the structure of the samples. For AuNP@PEGMUA2k at high BSA concentrations, the upturn of the $I(q)$ at low q (Figure 6D) and the non-linear q -dependence of τ (Figure 6C) indicate structural changes of the samples, such as agglomeration or aggregation, leading to changed dynamics. A closer look at the q -dependency of the relaxation times τ for the investigated q -range reveals length-scale dependent behavior deviating from homogeneous dynamics in the system (Figures 6C, S9, and S10). The calculated hydrodynamic diameters for AuNP@PEGMUA2k in comparison to AuNP@PEGMUA5k are shown in Figure S2. In contrast, NPs that are conjugated with PEGMUA5k exhibit a consistent and homogeneous diffusion behavior, indicating excellent colloidal stability across the entire range of protein concentrations studied. This underlines the importance of thorough experimental testing of colloidal stability in this context. Even when the colloidal stability is immaculate at comparably high protein concentrations up to 100 mg/mL, it might still be reduced at protein concentrations which are

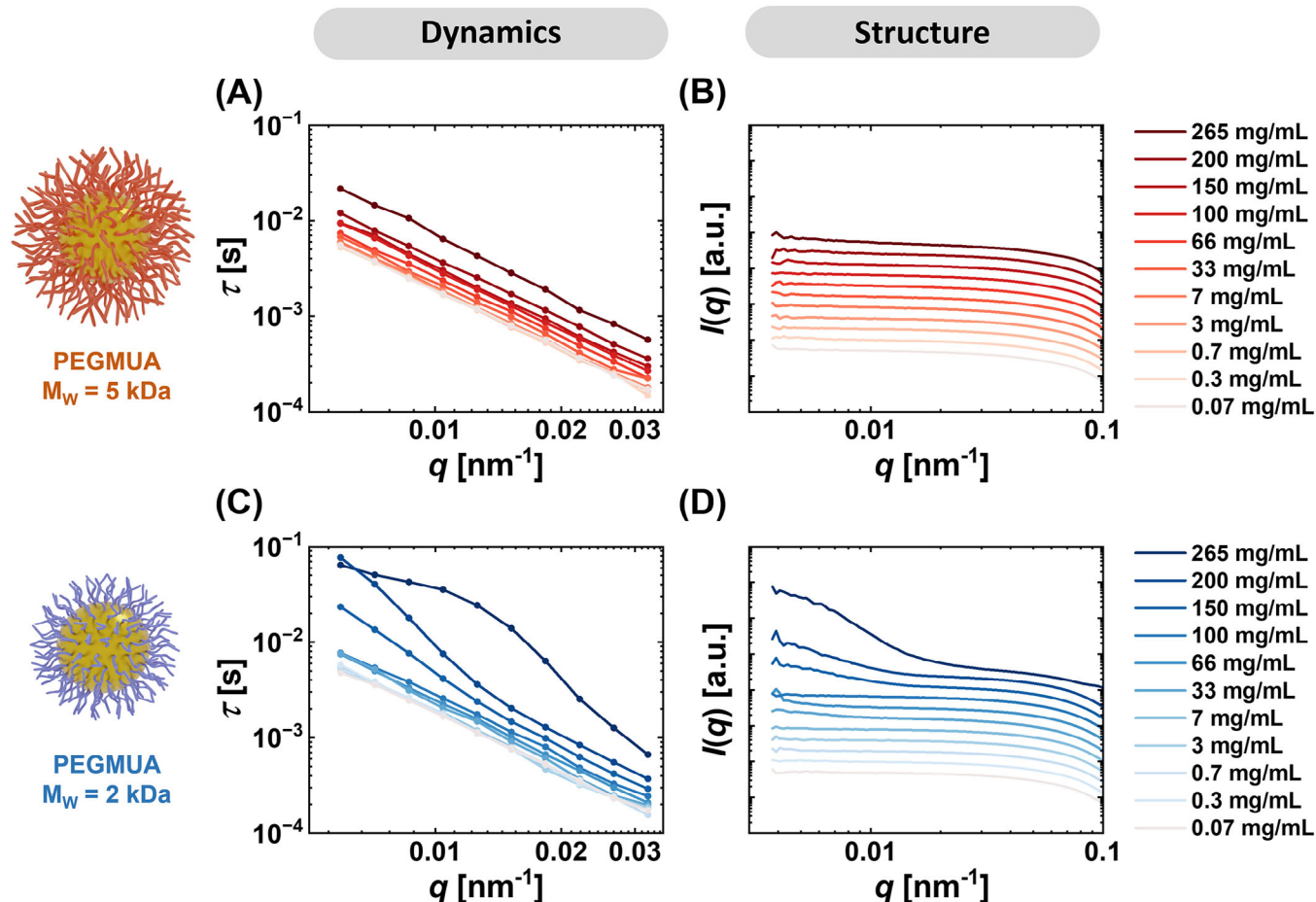


FIGURE 6 Colloidal stability of nanoparticles (NPs) with different α -methoxypoly(ethylene glycol)- ω -(11-mercaptoundecanoate) (PEGMUA) ligand lengths for varying bovine serum albumin (BSA) concentrations. q -dependency of the relaxation time τ and small-angle X-ray scattering (SAXS) scattering intensity as a function of the scattering vector q of NPs coated with PEGMUA5k (A and B) and PEGMUA2k (C and D) for a BSA concentration range of 0.07–265 mg/mL.

even higher (>100 mg/mL), but nonetheless physiologically very relevant, because they represent intracellular levels.

3 | CONCLUSIONS

In conclusion, we find an excellent colloidal stability for AuNP@PEGMUA5k in physiologically relevant high protein concentrations representing intracellular levels. AuNP@PEGMUA2k also exhibit good colloidal stability, which is, however, slightly impaired at BSA concentrations >100 mg/mL. Studying the colloidal dynamics of AuNP@PEGMUA5k over a large range of BSA concentrations with XPCS, we find viscosities at high BSA concentrations that significantly differ from expectations based on well-established models and complementary rheology experiments. This might be explained with the bio-repulsive properties of the PEG coatings leading to a nanoscopic viscosity that differs from the macroscopic viscosity of BSA solutions. Our findings are relevant for nanomedicine and NP-based in vitro studies, where PEG coatings, and other polymer coatings in general, are commonly employed. Biological systems are very complex, not only in terms of structure but also in terms of dynamics, and a thorough understanding of the diffusive properties of nanomaterials is crucial for their safe and successful application. XPCS studies can make valuable contributions in this respect, because

they allow to correlate structure and dynamics over large and relevant spatial and temporal ranges.

4 | EXPERIMENTAL

4.1 | Materials

BSA ($\geq 96\%$), tetrachloroauric(III) acid trihydrate ($\geq 99.9\%$ trace metal basis) and sodium citrate dihydrate ($>99.0\%$) were purchased from Sigma Aldrich. PBS (w/o Mg, Ca) was purchased from Cytiva. Bovine serum (lyophilized powder) was purchased from United States Biological. For bovine serum, the total protein concentration was determined by the commercial supplier by a Bradford assay with an IgG standard curve to be 60 mg/mL. PEGMUA (2 and 5 kDa) was synthesized and characterized as described previously.^[54]

4.2 | Synthesis and characterization of AuNPs

AuNPs were synthesized according to a seeded-growth method presented by Bastús et al.^[78] Details of the synthesis, functionalization and characterization are provided as Supporting Note S1.

4.3 | Sample preparation

In situ coherent X-ray scattering measurements were performed in quartz capillaries ($\varnothing = 1$ mm, wall thickness = 0.01 mm). BSA stock solutions were prepared using PBS. To assure equivalent conditions, NP concentrations were kept constant ($c_{\text{NP}} = 10$ nM) and NPs exposed to BSA and bovine serum were incubated for 2 h before the respective measurements. Control experiments with identical measurement conditions were carried out for each investigated BSA and bovine serum concentrations without the addition of NPs.

4.4 | Scattering experiments at ID02 beamline

XPCS experiments were performed at ID02 at the European Synchrotron Radiation Facility, Grenoble, France. An ultra-small-angle X-ray scattering geometry was used with a sample-to-detector distance of 30.7 m allowing access to a q -range of 0.0038–0.11 nm⁻¹. The X-ray photon energy was set to 12.2 keV. The partially coherent X-ray beam was focused by compound refractive lenses to a beam size of $40 \times 25 \mu\text{m}^2$. Two-dimensional scattering patterns were recorded with an Eiger2x4M (pixel size $75 \times 75 \mu\text{m}^2$) and speckle patterns were obtained with an Eiger500k detector (pixel size $75 \times 75 \mu\text{m}^2$) at a maximum framing rate of 23 kHz. Preliminary exposures were performed to determine the ideal measurement conditions. Measurements followed a strict procedure to exclude radiation-induced alteration of the sample. Initially, five single shot exposures with varying durations (0.002, 0.01, 0.05, 0.1, and 0.25 s) were carried out, followed by 10 individual series of 2000 diffraction patterns with a single exposure time of 0.02 ms and a detector deadtime of 0.03 ms after exposures. In between measurements, the sample was spatially displaced to prevent repeated exposures at the same capillary position. Each sample was investigated at different photon flux by implementing Zr- or Mo-based beam attenuators.

4.5 | X-ray photon correlation spectroscopy analysis

In XPCS experiments, the dynamics of the sample are obtained via the intensity–intensity correlation function

$$g_2(q, \tau) = \frac{\langle I(q, t) \cdot I(q, t + \tau) \rangle}{\langle I(q, t) \rangle^2}, \quad (2)$$

with the intensity $I(q, t)$ measured typically by a 2D detector at time t and wave vector transfer q . The average is taken for pixels within the same q bin of modulus

$$q = \frac{4\pi}{\lambda} \sin \frac{2\theta}{2}, \quad (3)$$

where 2θ denotes the scattering angle and λ denotes the wavelength. Via the Siegert relation, the correlation function g_2 is connected to the intermediate scattering function

$g_1(q, \tau)$ via

$$g_2(q, \tau) = 1 + \beta(q) |g_1(q, \tau)|^2. \quad (4)$$

Here, β is the so-called speckle contrast that depends on the coherence properties of the radiation and the experimental geometry. For many systems, it is expressed by a Kohlrausch–Williams–Watts (KWW) function as

$$g_1(q, \tau) = \exp [-(\Gamma(q) \cdot \tau)^\gamma] \quad (5)$$

and thus

$$g_2(q, \tau) = 1 + \beta \exp [-2(\Gamma \cdot \tau)^\gamma]. \quad (6)$$

The KWW exponent γ is a measure of the distribution of relaxation times in the studied sample volume. Diffusion processes are characterized by $\gamma = 1$. Furthermore, the relaxation rate Γ or the relaxation time $\tau_0 = 1/\Gamma$ shows a particular q -dependence that is usually given by a power law $\Gamma \propto q^p$. A square dependence ($p = 2$) is found for diffusive dynamics, while sub- and super-diffusion are expressed by $p > 2$ and $p < 2$, respectively. In case of free self-diffusion, XPCS allows measuring the Stokes–Einstein diffusion constant

$$D_0 = \frac{k_B T}{3\pi\eta d_H} \quad (7)$$

directly via $\Gamma = D_0 q^2$. Moreover, as a real-time probe the access to heterogeneous dynamics is possible by XPCS. Further information is given in [Supporting Notes S4 and S5](#).

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

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SUPPORTING INFORMATION

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3.6 Further work at large scale synchrotron and free-electron laser (FEL) facilities

Our work on pressure-induced supercrystal formation led to follow-up beamtimes addressing the kinetics of this process with time-resolved SAXS measurements after defined pressure jumps.¹⁷³ We also tested the crystallization of PEGMUA-coated gold nanorods (AuNR@PEGMUA) and found a pressure-induced lateral 2D-hexagonal ordering along the short axes, confirming that the approach is not limited to spherical NP.¹⁷⁷ The XCCA studies on the structure of dried AuNP@PEGMUA films with heterogeneous local order were already mentioned in section 3.4.¹⁷⁴⁻¹⁷⁵ XCCA beamtimes on more complex samples (anisotropic NP, binary NP mixtures) have been conducted but data analysis is ongoing and results have not been published yet.

Beyond the comparably simple, mostly diffusive, dynamics discussed in the previous section, XPCS has unique strength in measuring complex dynamics as in gelation processes and phase transitions and correlating them with structure. Due to their strong scattering, AuNP are excellent tracer particles for studying complex colloidal gelation dynamics, which we have shown in several studies. When dispersed in glycerol:water mixtures (70:30 v/v), AuNP@PEGMUA show a thermoreversible gelation behavior and we studied the evolution of gel dynamics and aging in several beamtimes revealing heterogeneous and anisotropic dynamics, a three-step gelation process and a strong dependency on the coating thickness.¹⁷⁸⁻¹⁸⁰ In FEL experiments at the European XFEL in Hamburg we observed that nonlinear heating due to the intense X-ray pulses can lead to superheated water states where the Stokes–Einstein relation for nanoparticle diffusion at short times is violated.¹⁸¹ I also supported beamtimes focusing on method development for XPCS experiments.¹⁸²

A completely different method that has not been discussed yet is single-particle-imaging (SPI) at FEL facilities. I supported several beamtimes at the European XFEL led by Dr. Kartik Ayyer (Max Planck Institut for the Structure and Dynamics of Matter, Hamburg) demonstrating e.g. 3D-diffraction imaging of anisotropic AuNP with record resolution in the 3D reconstructions based on millions of individual diffraction patterns of single particles.¹⁸³ A main motivation for these studies is the development of efficient computational data purification methods that will ultimately allow for SPI of proteins, but the method is also powerful for analyzing heterogeneous structural ensembles in a statistically meaningful manner.¹⁸³⁻¹⁸⁴ In a time-resolved SPI experiment at FLASH, (DESY, Hamburg) led by Prof. H. Lange, it was possible to record the “breathing” oscillations of spherical AuNP on a picosecond timescale after an optical pump.¹⁸⁵

Time-resolved and thin-film scanning SAXS/WAXS, XPCS, XCCA and SPI at synchrotron and FEL facilities are main ongoing research directions. Apart from that we also use XFI in the context of nanomedicine as mentioned earlier and I had a minor supporting role in a method-oriented publication on anomalous SAXS.^{154, 157, 186} I contributed to a comprehensive review that covers promising X-ray based techniques in the context of nanobiotechnology.¹⁸⁷

4. Self-Assembly of Gold Nanoparticles

4.1 Key publications and author contributions

Key publications:

- Schulz, F.; Pavelka, O.; Lehmkuhler, F.; Westermeier, F.; Okamura, Y.; Mueller, N. S.; Reich, S.; Lange, H., Structural order in plasmonic superlattices. *Nat. Commun.* **2020**, *11* (1), 3821-(1-9).¹⁸⁸
- Mueller, N. S.; Okamura, Y.; Vieira, B. G. M.; Juergensen, S.; Lange, H.; Barros, E. B.; Schulz, F.; Reich, S., Deep strong light-matter coupling in plasmonic nanoparticle crystals. *Nature* **2020**, *583* (7818), 780-784.¹⁸⁹
- Herran, M.; Juergensen, S.; Kessens, M.; Hoeing, D.; Köppen, A.; Sousa-Castillo, A.; Parak, W. J.; Lange, H.; Reich, S.; Schulz, F.; Cortés, E., Plasmonic bimetallic two-dimensional supercrystals for H₂ generation. *Nat. Catal.* **2023**, *6* (12), 1205-1214.¹⁹⁰

The first publication focusses on the synthesis of AuNP supercrystals developed by me over several years (first author and corresponding author), the second one describes a study led by the group of Prof. Stephanie Reich (Freie Universität Berlin, Germany) who quantitatively described the emergent polaritonic properties of such supercrystals supported by simulations and spatially resolved spectroscopy. The last study results from a cooperation with Prof. Emiliano Cortés (Ludwig-Maximilians-Universität München, Germany) where we developed the idea of utilizing binary supercrystals for plasmonic photocatalysis together (corresponding authors). O. Pavelka pursued part of his PhD thesis in the group of H. Lange (then University of Hamburg) under my supervision, working on the synthesis of AuNP and AuNP supercrystals. F. Lehmkuhler and F. Westermeier (DESY, Hamburg) led the SAXS experiments and analysis. Y. Okamura, N. S. Mueller, M. Kessens and S. Juergensen conducted the micro-absorbance spectroscopy measurements and analyzed the spectroscopy data under supervision of S. Reich, B.G.M. Vieira and E.B. Barros, together with N.S. Mueller and S. Reich, developed the theory to describe the optical properties quantitatively. M. Herran and A. Sousa-Castillo synthesized catalytic nanoparticles (platinum and palladium) and conducted quantitative photocatalysis experiments. D. Hoeing conducted and analyzed transient absorption spectroscopy experiments under supervision of H. Lange. A. Köppen contributed with HR-TEM sample characterization. H. Lange, W. Parak, E. Cortés, S. Reich, E.B. Barros and me supervised the different aspects of the projects with leading roles as mentioned.

4.2 Background

A short general background on NP self-assembly was provided in section 1.4 and the nomenclature introduced there will be used in the following. For instance, AuNP40@PSSH12k indicates AuNP with ~40 nm diameter coated with polystyrene-thiol ligands with a molecular weight around 12000 g/mol. A major motivation of research on NP assembly are the novel structures and the emergent properties of the self-assembled materials. Truly emergent properties resulting from strong coupling and coherent collective interactions have rarely been demonstrated experimentally as discussed in recent reviews.^{93, 128} In the case of plasmonic NP, collective coupled plasmon modes can emerge from their assemblies,¹⁹¹⁻¹⁹³ but robust methods to assemble large AuNP into well-defined

superlattices over large areas have been rare, in particular towards 3D systems. A promising route towards well-defined self-assembly was the evaporation-induced assembly of NP dispersions on a liquid subphase pioneered by the Murray group for small NP with small hydrocarbon-based ligands¹⁰⁷ and later extended to small NP with PSSH ligands by Xingchen Ye in the group of Alivisatos.¹⁰⁸ In early studies I explored how bigger AuNP can be functionalized with PSSH ligands and demonstrated their potential for well-ordered self-assembly.⁶⁵ These early works led to the cooperation with the Reich group, in particular Niclas S. Mueller, and the first observation and discussion of collective plasmonic modes in the material.¹⁹⁴ From the synthesis point of view, the key parameters are the NP quality (low dispersity and high uniformity) and their efficient coating and stabilization. The self-assembly routes discussed here are all based on NP dispersions in organic solvents (toluene or chloroform) whereas the common syntheses for large AuNP take place in water. Accordingly, the functionalization of the AuNP with PSSH requires a phase transfer at the same time, which has to be optimized to ensure colloidal stability and sufficient yields. After our first observations of collective plasmonic modes, we were interested in supercrystals of larger AuNP to achieve even stronger coupling. At the same time it became clear that the classical route for the synthesis of large quantities of spherical AuNP based on sodium citrate as a reducing agent and stabilizer yielded large amounts of AuNP but with some limitations in terms of dispersity and uniformity.¹⁹⁵ Alternative synthesis routes were available in the literature but those were limited in scale and imposed restrictions regarding the surface chemistry.¹⁹⁶ As discussed, a significant advantage of citrate-stabilized AuNP, AuNP@Citrate, is the straightforward ligand exchange because citrate binds weakly to gold. The alternative synthesis approach uses CTAC (cetyltrimethylammonium chloride) as a stabilizing ligand which is much harder to replace, similar as discussed for CTAB on AuNR in section 2.3. Therefore, to prepare a suitable starting material for self-assembly, the AuNP@CTAC synthesis had to be upscaled and a protocol for ligand exchange had to be developed. Then the self-assembly itself could be tested and was optimized systematically towards a robust protocol.

4.3 Structural order in plasmonic superlattices

Publication:

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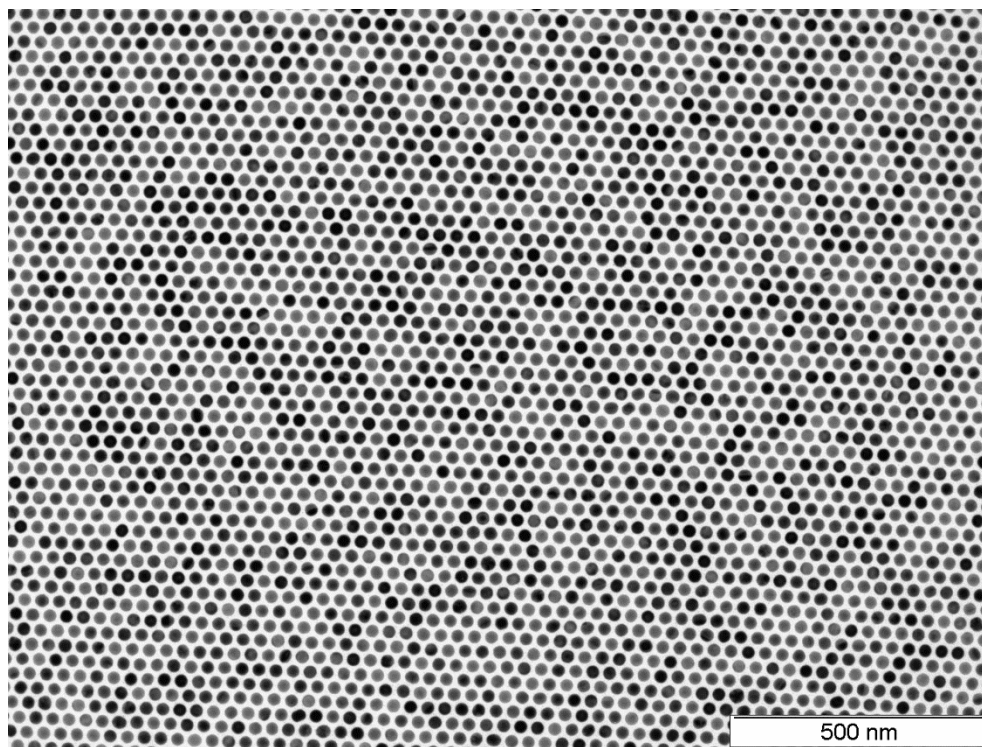


Figure 7. Defect-free monolayer area of self-assembled AuNP25@PSSH5k.

In this publication we describe the self-assembly of AuNP@PSSH, the quantitative description of their quality and their optical properties. Based on an upscaled version of an established AuNP@CTAC synthesis and a phase transfer ligand exchange with different PSSH ligands (2000 g/mol, 5000 g/mol and 12000 g/mol; PSSH2k, PSSH5k and PSSH12k) we were able to demonstrate large-scale thin-film plasmonic supercrystals of unprecedented quality and scale. The approach was tested for AuNP diameters varying from 25 nm to 60 nm and we identified the necessary ligand size for efficient stabilization for each of these diameters. Increasing diameters require increasing molecular weights of the ligand for efficient stabilization. We describe in detail the critical experimental parameters to ensure an excellent AuNP quality (dispersities of 2-3% and high uniformities), efficient ligand exchange and, most importantly, thorough purification of the final product, i.e. quantitative removal of excess PSSH ligands and remaining CTAC which has been proven critical for reproducible and well-defined self-assembly. The formed thin-film supercrystals feature a 2D-hexagonal order, Figure 7, a very small number of defects, large domain sizes and span up to tens of thousands of square microns. The supercrystals were analyzed with thin-film scanning SAXS, providing thousands of individual scattering patterns for a statistically robust analysis of structural quality. The narrow distributions of the lattice constants confirmed the exceptional crystalline quality, and their small standard deviation well below 1 nm even allowed to clearly discern sub-nanometer differences in interparticle

spacing for different coating thicknesses (PSSH2k vs. PSSH5k). Advantages of the approach include the tunability of interparticle spacings (gaps) simply via the molecular weight of the PSSH ligands and the possibility of transferring the thin-film supercrystals to various substrates from the liquid subphase. The supercrystals feature a well-defined layered structure starting from monolayers up to thick films with > 30 layers which still present well-defined flat surfaces. Due to the size of the AuNP and the domains, the number of layers can easily be identified with optical microscopy in transmission and with micro-absorbance spectroscopy the optical properties can be identified for defined numbers of layers. In the publication we show and discuss exemplarily how polaritonic modes (hybrid modes of collective plasmonic excitations and photons) are activated for each additional layer and that they depend on the structure of the supercrystals (AuNP diameter and gaps). Importantly, we also demonstrate that the structural quality is critical for the observation of such modes by comparing a high-quality sample prepared from AuNP@CTAC and a sample with similar geometry but prepared with less uniform and more disperse AuNP@Citrate as a starting material. This study demonstrates that a structural precision can be achieved with colloidal self-assembly that can compete with lithography approaches while achieving gaps below 5 nm which are essential for strong plasmonic coupling.

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Structural order in plasmonic superlattices

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The assembly of plasmonic nanoparticles into ordered 2D- and 3D-superlattices could pave the way towards new tailored materials for plasmonic sensing, photocatalysis and manipulation of light on the nanoscale. The properties of such materials strongly depend on their geometry, and accordingly straightforward protocols to obtain precise plasmonic superlattices are highly desirable. Here, we synthesize large areas of crystalline mono-, bi- and multilayers of gold nanoparticles >20 nm with a small number of defects. The superlattices can be described as hexagonal crystals with standard deviations of the lattice parameter below 1%. The periodic arrangement within the superlattices leads to new well-defined collective plasmon-polariton modes. The general level of achieved superlattice quality will be of benefit for a broad range of applications, ranging from fundamental studies of light-matter interaction to optical metamaterials and substrates for surface-enhanced spectroscopies.

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Ordered arrays and superlattices of nanoparticles (NPs) have a huge potential for new materials with tuneable electrical, optical, and mechanical properties^{1–13}. Common strategies to produce such structures include lithography-based nanofabrication^{14–16}, and self-assembly of NPs functionalized with polymers¹⁰, DNA^{17–22}, or dendritic molecules^{23,24}. The ability to control both, the structure of the NP building blocks and the geometry of the superstructure might lead to designer materials with, for example, topological states and Dirac minibands in the case of semiconductor NPs^{25,26}. However, the transition from localized to the required delocalized electrons is often hindered by the lack of periodic order, size variations and, most importantly, the insufficient degree of electronic coupling between the NPs²⁷. This can in part be circumvented by coupling the electromagnetic (dipole) fields instead of electrons. Then, scale and order of the superlattice can lead to new collective properties^{28–30}. For example, the superfluorescence from periodically coupled excitonic dipoles of perovskite NPs was recently demonstrated³¹. In similar ways, plasmonic dipoles of metal NPs interact via dipole–dipole interaction^{30,32–34}. This creates a continuum of collective plasmonic states where the electrons of the metal NP crystal remain localized. According to recent experiments, the coupling strength of such periodically coupled plasmons to photons can become comparable to the photon energy³⁵. The optical properties are expected to be widely tuneable through crystal symmetry and geometry^{35–37}.

A prerequisite for the formation of well-defined coupled modes is the uniformity of the constituents and a high degree of order on relevant scales (optical wavelength). The coupling strength increases with decreasing interparticle spacings, gaps <10 nm are favorable^{5,35,38}. Upon today, it has been difficult to combine a high particle uniformity (size and shape) with a high degree of periodic order and large crystalline areas in the desired geometry. For small NPs (diameters <15 nm), a huge variety of mesoscale superlattices have been reported^{6,10–12,39–51}. However, most reports of these NP superlattices focus on the structural and electronic properties. The few optical studies exploiting the periodic order of plasmonic components, e.g. use lithographic structures as waveguides, where plasmons couple over much longer distances^{34,52}. The reason for the lack of reports on periodic plasmon coupling is that there exists no robust protocol to assemble large plasmonic NPs like gold NPs (AuNPs) into large-scale crystalline superlattices. The plasmonic dipole strength, which is relevant for the periodic coupling, scales with particle size, yielding small AuNPs less attractive for periodic dipole field coupling. For example, to observe well-defined plasmon polaritons, the minimum AuNP diameter is ~25 nm^{36,53,54}.

In this work, we present a robust protocol for assembling large (diameter >20 nm) AuNPs into crystalline superlattices up to >0.01 mm² for monolayers and even larger crystalline bilayer and multilayer with interparticle gaps in the range of 1–8 nm and standard deviations of the lattice constant of below 1%. This degree of order allows well-defined collective plasmon-polariton modes to emerge: we compare the film properties with the ones of less ordered, conventional films of comparable scales and demonstrate how order leads to the formation of new polaritonic excitations.

Results

AuNP synthesis and functionalization. Periodically coupled plasmonic modes require a continuous lattice of uniform NPs. NP size and shape deviations are disadvantageous for two reasons: they result in local variations of the (size-dependent) plasmonic properties and reduce the periodicity of the NP superlattice. To reproducibly obtain superlattices with interparticle gaps below 10 nm,

our strategy therefore was to synthesize AuNPs with optimized dispersity and uniformity using the cetyltrimethylammonium chloride (CTAC)-based seeded growth strategy presented by Zheng et al.⁵⁵. The AuNPs were functionalized with polystyrene-thiol ligands (PSSH) that have sufficiently low molecular weights (~2000, 5000, and 12,000 g mol^{−1}; PSSH2k, PSSH5k, and PSSH12k) to allow for small interparticle gaps. At the same time, they still provide the stabilization that is necessary for high particle concentrations and stable assemblies in the desired diameter range. In a previous study we tested the potential of PSSH-promoted self-assembly with citrate-stabilized AuNPs (AuNP@Citrate) with dispersities (rsd) of ~8%. Despite some promising results, the limitations discussed above were encountered⁵⁶. AuNP@CTAC have a more challenging surface chemistry and are obtained in lower yields than AuNP@Citrate but exhibit superior uniformity and lower dispersity. The CTAC-based protocol was scaled up by a factor of 10 to obtain sufficient final concentrations. Phase-transfer-based ligand exchange as described for AuNP@Citrate does not work for AuNP@CTAC⁵⁶. Therefore, AuNP@CTAC were functionalized with PSSH by a ligand exchange in THF, transferred to toluene, purified and concentrated for self-assembly experiments. The ligand exchange and the colloidal stability of the functionalized AuNPs were monitored by UV/Vis spectroscopy.

AuNP superlattice films were prepared by evaporation of AuNP solutions in toluene on a liquid subphase: diethylene glycol (DEG), as presented by Dong et al. for smaller NPs⁵⁷. A scheme and resulting exemplary superlattice films are shown in Fig. 1. The straightforward protocol starts by pipetting of the AuNPs in toluene onto the DEG subphase in a teflon or high density polyethylene (HDPE) well (Fig. 1a). The well is then covered with a cover slip and left undisturbed. Superlattice films usually form within 16–24 h (Fig. 1b) and can then be transferred onto arbitrary substrates like transmission electron microscopy (TEM) grids, glass slides, or silicon wafers. The superlattices can also form freestanding films (~40 μm × 40 μm, Supplementary Fig. 1).

All AuNP samples formed well-ordered AuNP superlattices, differing in the number of layers ranging from monolayer to multilayer (>20), number of defects, crystallite size and the crystal mosaicity (Fig. 1c–g and Supplementary Figs. 2–11). For all superlattice films, defect-free monolayers in the μm² range were routinely observed (Fig. 1 and Supplementary Figs. 2–11) up to monolayers with long-range periodic order over areas >10,000 μm². Even larger areas were obtained for crystalline bilayers (Fig. 1f and Supplementary Figs. 2–4, 6, 10, 11). Table 1 lists the studied samples with the concentrations *c* used for superlattice preparation and the interparticle edge-to-edge distances (gaps) in the formed superlattices determined by TEM and small-angle x-ray scattering (SAXS).

The gaps obtained by SAXS deviate up to a few nm from those obtained by TEM analysis. This observation points at the limits of the TEM analysis accuracy, especially for the larger AuNP which are slightly less uniform (cf. Supplementary Figs. 2–11)⁵⁸. Consequently, complementary SAXS measurements for comparison are recommendable for the characterization of superlattices in general. AuNP superlattices covering large areas have been reported in several pioneering studies and recent reviews are available^{10,39–47,59,60}, but the crystalline order presented herein is unprecedented to the best of our knowledge. With increasing number of layers, the superlattices can be described as well-defined AuNP-supercrystals (Fig. 1g and Supplementary Fig. 12). The number of layers cannot be controlled perfectly, but as a tendency slow evaporation and an increasing number of particles in the drying solution seem to favor higher fractions of multilayer superlattices. Accordingly, a fast evaporation and low AuNP concentrations favor the formation of monolayers. Quantitative statements are not possible because the exact fractions of the

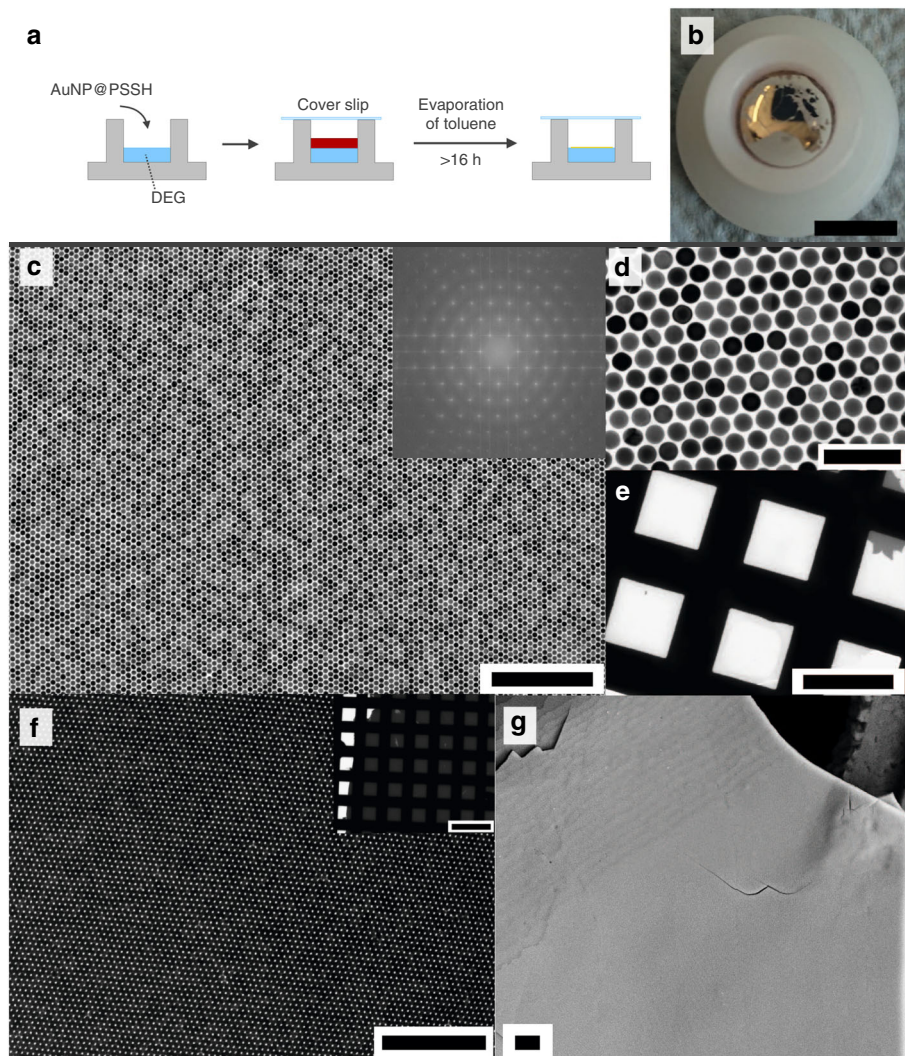


Fig. 1 AuNP superlattice synthesis. **a** Scheme of superlattice preparation. First, 100 µl of AuNP@PSSH (sample details in Table 1) in toluene are pipetted onto a liquid subphase (DEG), then the well is covered with a glass slip to slow down the evaporation of the toluene. Superlattice films typically form within 16–24 h. **b** Photograph of a formed superlattice, scalebar 1 cm. **c, d** TEM measurements at different magnifications of a monolayer area in superlattices formed from AuNP25@PSSH2k (**c**: scalebar 500 nm, **d**: scalebar 100 nm). The inset in **c** shows the FFT of the digital image. **e** Low magnification of the same crystalline monolayer area covering >0.01 mm² of the TEM grid (scalebar: 50 µm). The small darker gray area in the top right corner is a bilayer. **f** Bilayer with few defects formed from AuNP25@PSSH5k (scalebar 500 nm). The inset shows an extended bilayer area (scalebar 100 µm). **g** SEM measurements of a crystalline multilayer superlattice with 22–23 layers formed from AuNP35@PSSH12k (scalebar 2 µm).

Table 1 AuNP samples used for film formation and resulting interparticle distances (gap).					
Sample name	AuNP diameter (nm) ^a	Ligand	c(AuNP) (nM)	Gap (TEM) (nm) ^b	Gap (SAXS) ^c
AuNP25@PSSH2k	(25.1 ± 0.7)	PSSH2k	6.3	3.0	3.8 nm
AuNP25@PSSH5k	(25.1 ± 0.7)	PSSH5k	29.6	3.3	4.3 nm
AuNP25@PSSH12k	(25.1 ± 0.7)	PSSH12k	30.0	7.7	NA
AuNP35@PSSH5k	(37.3 ± 1.2)	PSSH5k	1.6	5.1	NA
AuNP35@PSSH12k	(37.3 ± 1.2)	PSSH12k	1.6	5.2	6.3 nm
AuNP50@PSSH5k	(50.5 ± 1.1)	PSSH5k	0.7	1.3	3.6 nm
AuNP50@PSSH12k	(50.5 ± 1.1)	PSSH12k	0.7	2.7	NA
AuNP60@PSSH2k	(63.4 ± 1.8)	PSSH2k	0.3	1.2	NA
AuNP60@PSSH5k	(63.4 ± 1.8)	PSSH5k	0.3	2.2	NA
AuNP60@PSSH12k	(63.4 ± 1.8)	PSSH12k	0.3	1.3	4.3 nm

^aCore diameter by TEM, with standard deviation of the mean (N > 200).
^bBy nearest neighbor analysis of TEM measurements.
^cObtained by analysis of SAXS measurements as discussed in the text.

different layer numbers within one sample are not accessible. A detailed description of the synthesis and relevant parameters is provided in the “Methods” section.

An important advantage of using grafted polymers for film formation is that they allow controlling the interparticle distance by changing their molecular weight^{10–12,39,43,45,56,61}. However, although the PSSH ligand length was found to affect the interparticle distances (gaps), a clear tendency was not always observed and the correlation was not linear. For PSSH12k a correlation of gap distance with particle diameter was observed that points to the increasing attractive van-der-Waals-potentials between the AuNP-cores (Supplementary Fig. 13)⁶². However, with PSSH5k the trend was not that clear, indicating that additional parameters of the crystallization affect the gaps. For PSSH2k just two samples can be compared due to the limited stabilization provided by this small ligand. In the range of particle diameters and ligands tested, the gap spacings varied from 1 to 8 nm (Table 1, Supplementary Fig. 13), an ideal range for periodic plasmonic coupling that has been challenging to achieve with high precision⁵.

For a quantitative measure of the superlattice crystallinity, scanning microfocus SAXS measurements (beamsize $2.8\ \mu\text{m} \times 1.7\ \mu\text{m}$ ($h \times v$)) were performed on selected AuNP superlattice films (Fig. 2) in transmission mode. We measured SAXS patterns on hundreds of different spots on each sample including thin (monolayer and bilayer) and thick regions (multilayers). An exemplary intensity map is shown in Fig. 2a, where each pixel represents the measured intensity in a certain q -range of a single SAXS measurement. The 2D-scattering pattern and azimuthally averaged $I(q)$ (Fig. 2b) exhibit pronounced Bragg peaks of a six-fold order which can be well described with scattering from a 2D-hexagonal lattice. This confirms the periodic order and allows extracting the lattice constant a . The distributions $P(a)$ of the lattice constant a for different samples are shown in Fig. 2c. The exceptionally narrow distributions of a underline the robust and long-range periodic order within the superlattices. In comparison, the distribution for an AuNP@Citrate-based superlattice (30 nm, functionalized with PSSH5k) is 1.5–6 times broader (Fig. 2d, gray dashed line). Remarkably, the small lattice constant difference (~ 0.5 nm) of AuNP25@PSSH2k and -5 k due to different ligand lengths can be clearly discerned (Fig. 2c) and agrees well with the analysis of TEM measurements (Table 1).

The achieved large superlattice areas in combination with the possible deposition on transparent substrates enable the investigation of the plasmonic properties by optical microscopy and spectroscopy. The number of layers can be clearly distinguished in optical transmittance (Fig. 3a, Supplementary Fig. 14) and the layer-number-dependent optical properties can be measured with spectroscopy.

Figure 3b shows the optical absorbance of a monolayer, bilayer, and trilayer of periodically arranged AuNPs. Pronounced absorbance peaks appear in the NIR spectral range from the direct optical excitation of plasmon polaritons that form standing waves in the supercrystals (see insets)^{35,36,63}. With each additional layer a new polaritonic mode is activated, starting from a single mode for a bilayer^{35,36}. The excitation of plasmon-polaritons leads to pronounced reflectance dips and transmittance maxima in the optical spectra (Fig. 3c). A polaritonic stop band prevents light from entering the supercrystal in the visible spectral range and light is mostly reflected³⁵. The reflectance decreases for excitation energies larger than 2 eV because of the interband transitions of gold. The optical spectra are in good agreement with finite-difference time-domain (FDTD) simulations (compare solid and dashed lines in Fig. 3c), demonstrating the control of the structural parameters in the synthesis process. The number, energy, and spectral width of the polaritonic modes depends sensitively on the interparticle

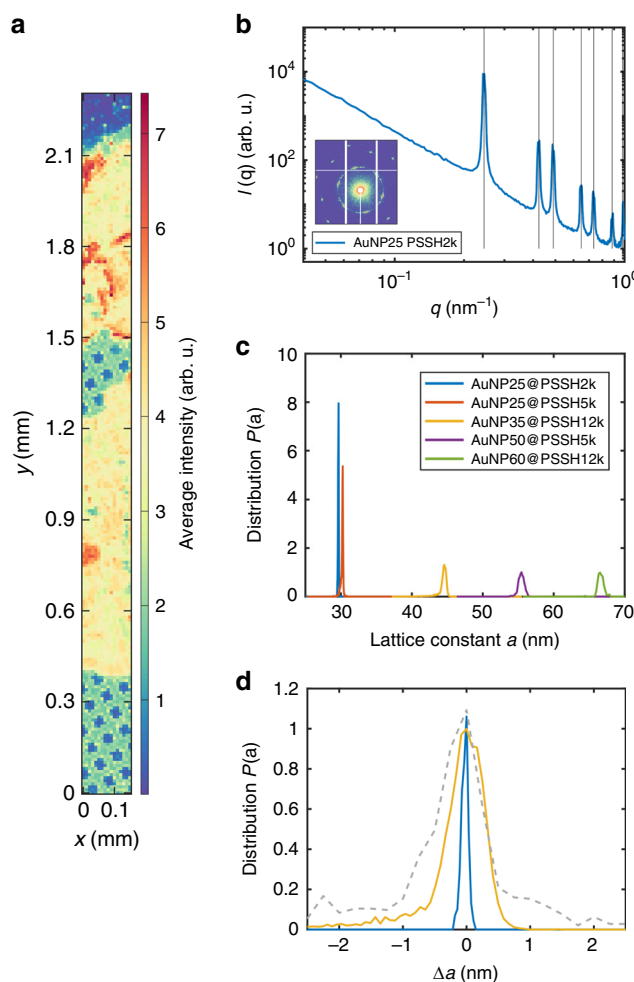


Fig. 2 SAXS investigation of AuNP superlattices. **a** Exemplary intensity map (AuNP25@PSSH2k) obtained by scanning SAXS with $10\ \mu\text{m}$ step width where each pixel corresponds to one SAXS measurement. The structure of the copper grid can be discerned. **b** Exemplary $I(q)$ of a single SAXS pattern and 2D scattering pattern (logarithmic intensity scale) for AuNP25@PSSH2k. Vertical black lines are expected peak positions for a 2D-hexagonal lattice. **c** Fit results for the lattice constant a (assuming a 2D-hexagonal lattice) averaged over all measurements (>700 for each sample) on an absolute a -scale. **d** Exemplary fit results (same color code as in **c**) on a normalized $P(a)$ and a -scale ($\Delta a = a - \langle a \rangle$) compared to results for an AuNP@Citrate-based superlattice (gray dashed line, $d \sim 30$ nm, functionalized with PSSH5k).

distance, number of layers, AuNP shape, diameter, and lattice type^{35,36,53,54}. In consequence, an accurate control of all these parameters enables the design of tailored optical materials. Figure 4a shows the optical reflectance of supercrystals with different nanoparticle diameters. The polariton energies red shift with increasing nanoparticle diameter because of an increase in light-matter coupling strength³⁵. Furthermore, the reflection dips become more pronounced because of a smaller wavelength mismatch of the light inside and outside the supercrystal. The strong dependence of the polariton energy on the nanoparticle diameter and other structural parameters implies that already slight variations within the crystal will lead to a spectral broadening or even disappearance of the polaritonic modes³⁶. Figure 4b shows a comparison of two superlattices of similar geometry ($d \sim 30$ nm, gaps ~ 3 – 4 nm): one prepared starting with AuNP@Citrate and the other one with AuNP@CTAC. Both were functionalized with PSSH5k, i.e. the only difference is the quality of the core AuNP.

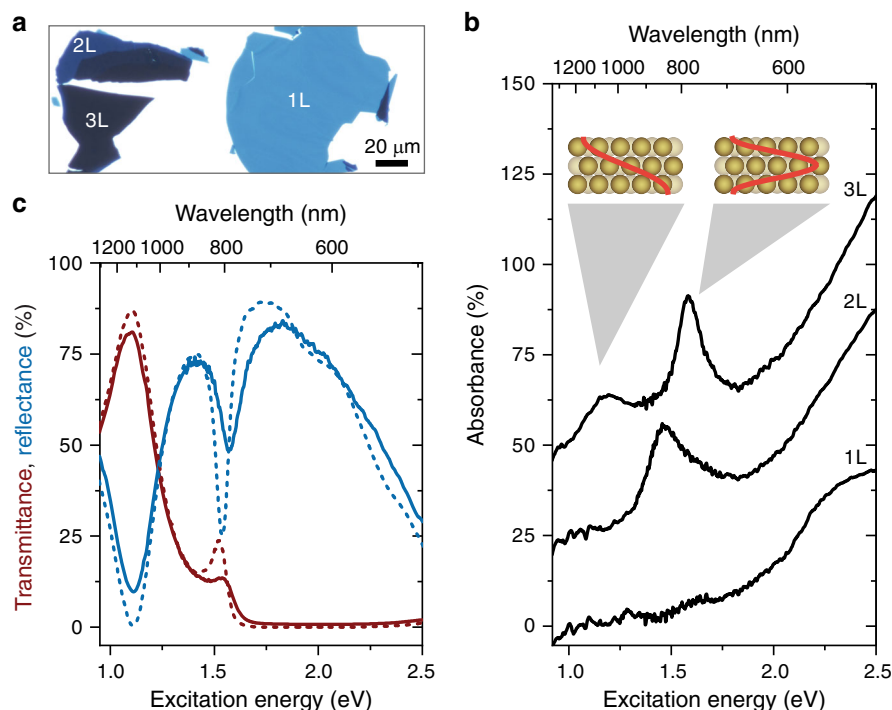


Fig. 3 Optical properties of AuNP superlattices. **a** Optical transmittance microscope image of a AuNP50@PSSH5k superlattice deposited on a glass substrate. The number of nanoparticle layers is clearly visible from the optical contrast and given by the labels. **b** Measured absorbance spectra of a AuNP50@PSSH5k monolayer (1L), bilayer (2L), and trilayer (3L). For 2L and 3L, pronounced absorbance maxima arise from the direct optical excitation of plasmon-polaritons that form standing waves in the supercrystal (see inset for 3L). With each additional layer an additional polaritonic mode is activated. The spectra are offset by 25% for clarity. **c** Measured reflectance (blue) and transmittance (red) spectra for the same AuNP trilayer as in **a** (solid lines). Simulated spectra are shown for comparison (dashed lines).

The different uniformity and crystallinity of the two lattices is clearly visible in the spectra (Fig. 4b) and in microscope images (Fig. 4c, d). The polaritonic modes broaden or even vanish for a decreasing particle and crystalline quality.

The large transmittance of light and small interparticle gaps lead to a strong near-field intensity enhancement at the gaps between the NPs throughout the entire supercrystal^{4,54,63}. In combination with the tunability of the polaritonic excitations, this makes plasmonic supercrystals a promising platform for applications in sensing and spectroscopy^{4,64,65}. For example, the dominant polariton mode of the trilayer in Fig. 3c matches the wavelength 785 nm of a typical laser used for Raman spectroscopy. The comparison of the Raman scattering of the bulk ligand (PSSH5k) and surface-enhanced Raman-scattering (SERS) of the ligand within the superlattice hot spots yields an enhancement factor of $\sim 3 \times 10^4$ per molecule (Supplementary Fig. 15 and Supplementary Note 1). This stresses the potential of defined AuNP superlattices for SERS and related techniques like surface-enhanced infrared-absorption (SEIRA) spectroscopy in addition to the design of polaritonic materials.

Discussion

Large AuNP-supercrystals with hexagonal order ranging from 2D to 3D, i.e. monolayer to multilayer, were synthesized with straightforward protocols. By varying particle diameter and concentration, as well as ligand length and evaporation time, large AuNP-supercrystal sheets were synthesized with varying number of layers and interparticle distances in the range of 1–8 nm. Since PSSH-thiol ligands are available with almost arbitrary molecular weights, larger gaps should be possible as well, but for strong plasmonic coupling small gaps are preferable. The obtained large scales in combination with the high degree of periodic order allow

the formation of periodically coupled plasmon-polariton modes, pointing towards applications in optical metamaterials and surface-enhanced spectroscopies. More complex superlattices (e.g. binary and including NP of different materials) can be envisaged depending on the desired application. A key point for utilizing the protocol for different NP materials and shapes will be the particle quality and a controlled surface chemistry. A complete ligand exchange and thorough purification should provide stable dispersions of the according nanomaterial in suitable solvents without additional stabilizers and at sufficiently high concentrations. The correlations of geometry and electronic and/or mechanical properties would be interesting to address in future studies. Wang et al. recently presented organic nano-floating-gate memory devices based on self-assembled AuNP-films, which greatly benefit from defined ordered structures compared to amorphous films⁴¹. This can reasonably be expected to be the case for many other microelectronic devices. Thus, the potential for fundamental studies with well-defined superlattice structures is quite broad. Our protocol expands the available range of precise geometries for such studies. It demonstrates that self-assembled structures obtained by wet-chemistry can compete with lithographic precision and control, and probably also provide sufficiently large areas for various devices and applications in the near future.

Methods

Materials. Tetrachloroauric(III) acid ($\geq 99.9\%$ trace metals basis), hexadecyltrimethylammonium bromide (CTAB, $\geq 98\%$) and chloride (CTAC, $\geq 98\%$), L-ascorbic acid (reagent grade) and sodium borohydride ($\geq 98\%$) were from Sigma-Aldrich (USA). Toluene ($\geq 99.5\%$), tetrahydrofuran ($\geq 99.5\%$), and ethanol (denat., $>96\%$) were from VWR (USA). DEG (reagent grade) was from Merck (Germany). Thiolated polystyrenes (PSSH, PSSH2k: $M_n = 2000 \text{ g mol}^{-1}$, $M_w = 2300 \text{ g mol}^{-1}$; PSSH5k: $M_n = 5300 \text{ g mol}^{-1}$, $M_w = 5800 \text{ g mol}^{-1}$; PSSH10k: $M_n = 11,500 \text{ g mol}^{-1}$,

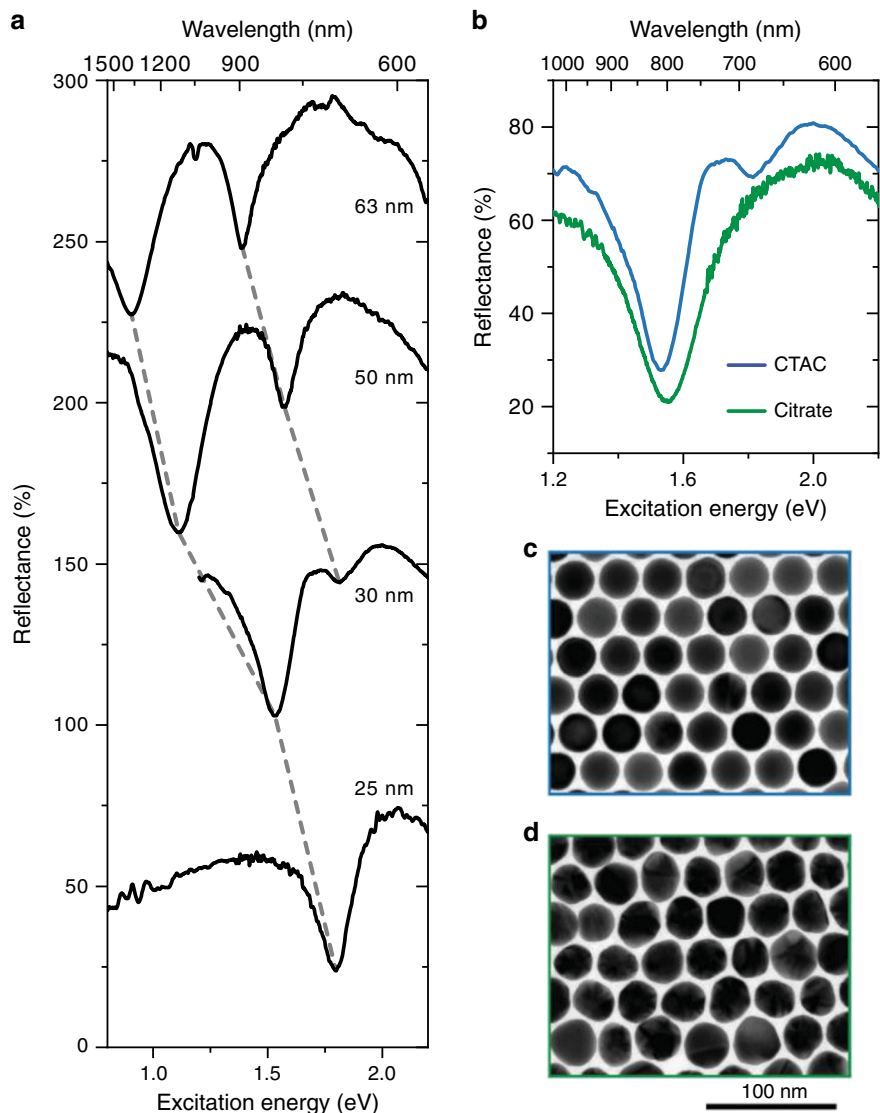


Fig. 4 Role of structural parameters and order for the properties of plasmon-polaritons. **a** Reflectance spectra of AuNP trilayers with different NP diameters (i.e. AuNP25@PSSH5k, AuNP30@PSSH5k, AuNP50@PSSH5k, AuNP60@PSSH12k—see labels). Reflectance dips that belong to the same polaritonic mode are connected by dashed lines. The spectra are offset by 75% for clarity. **b** Comparison of the reflectance spectra of 30 nm AuNP trilayer supercrystals synthesized from AuNP@CTAC (blue line) and AuNP@Citrate (green line), i.e. different core particles were coated with PSSH5k. The main reflection dip in the spectrum of the AuNP@Citrate-based superlattice is significantly broadened and the reflection dip at higher energy is not discernable. Both spectra were recorded on trilayers with the same experimental settings and were reproducible for trilayers throughout the sample. **c** TEM image of an AuNP@CTAC-based monolayer ($d = (29.7 \pm 0.9)$ nm, gaps ~3 nm). **d** TEM image of an AuNP@Citrate-based monolayer ($d = (31.8 \pm 2.4)$ nm, gaps 3–4 nm).

Table 2 Parameters of AuNP@CTAC seeds used for AuNP@CTAC syntheses.			
Sample	V_{seeds} (μl)	d_{seeds} (nm)	c_{seeds} (nM)
AuNP25@CTAC	750	9.5 ± 0.5	37
AuNP35@CTAC	100	9.1 ± 0.4	43
AuNP50@CTAC	75	11.1 ± 0.7	21
AuNP60@CTAC	45	11.1 ± 0.7	21

$M_w = 12,400 \text{ g mol}^{-1}$) were from Polymer Source (Canada). All reagents were used without further treatment.

AuNP@CTAC synthesis. Gold nanoparticles (AuNP) were synthesized based on the protocol presented by Zheng et al.⁵⁵ that was upscaled to achieve sufficiently

high AuNP concentrations. All solutions were in ultrapure water form (18.2 Ω). For the initial CTAB-stabilized seeds, sodium borohydride (NaBH_4 , 600 μl, 10 mM) was quickly injected into a mixture of CTAB (9.9 ml, 100 mM) and tetrachloroauric (III) acid (HAuCl_4 , 100 μl, 25 mM) under rapid stirring (1000 rpm) and then stirred at 300 rpm for 3 min. Afterwards, the reaction mixture was left for 3 h without agitation.

First growth step: 50 μl of these CTAB-seeds were mixed with ascorbic acid (1.5 ml, 100 mM) and CTAC (2.0 ml, 200 mM) at 600 rpm, followed by a one-shot injection of HAuCl_4 (2.0 ml, 0.5 mM). After 15 min stirring (300 rpm), the AuNP were purified by centrifugation ($20,000 \times g \times 20 \text{ min}$) and the pellet redispersed in CTAC (1.0 ml, 20 mM). For an upscaled version of this first growth step, all volumes were scaled up by a factor of 10 without any other changes to the protocol. These AuNP@CTAC were used as seeds to synthesize all AuNP@CTAC with larger diameters. To this end the AuNP@CTAC were purified by two additional centrifugation steps, without changing the AuNP and CTAC concentrations, i.e. the pellets were redispersed in 20 mM CTAC.

Second growth step: CTAC (20 ml, 100 mM) and the desired volume of AuNP@CTAC seeds (in 20 mM CTAC) were mixed and treated with ultrasound for 10 min. The mixture was then stirred (400 rpm) in a water bath at 30 °C, ascorbic acid (1.3 ml, 10 mM) was added and after one minute the addition of

HAuCl₄ (20 ml, 0.5 mM) with a syringe pump at 20 ml h⁻¹ was started. After the addition completed, the mixture was stirred (400 rpm) for another 10 min at 30 °C and then centrifuged, and the AuNP@CTAC pellet redispersed in the same volume of water. The parameters of the AuNP@CTAC seeds (added volume V_{seeds} , diameter d_{seeds} , and seed concentration c_{seeds}) used for the different samples are summarized in Table 2.

Ligand exchange. The direct ligand exchange protocol which we used previously for AuNP@Citrate⁵⁶ could not be applied in the case of AuNP@CTAC and a modified approach had to be used. In a typical procedure 10 ml of the AuNP@CTAC solution were centrifuged twice in order to remove excess CTAC. The volume of the solution was reduced to <100 µl after the second centrifugation round and such concentrated particles were then added dropwise into a vigorously stirred (1000 rpm) solution of the respective PSSH-ligand in THF (2 ml, 0.5 mM). The resultant mixture was left to be stirred at 300 rpm for at least 2 additional hours (usually overnight) to allow for a complete replacement of CTAC by PSSH. The THF solution of AuNP@PSSH was then dried in a rotary evaporator and the particles were redispersed in toluene. Finally, two centrifugation rounds were used to remove excess PSSH ligands from the solution and to increase the particle concentration for film preparation. Residual CTAC can adversely affect ordered film formation and should be removed by repeated washing of the toluene phase with water if necessary. To this end, equal volumes of water and ethanol are added to yield a 1:1:1 mixture with the AuNP@PSSH in toluene. After thorough mixing and phase separation, the toluene phase containing the AuNP@PSSH is removed and the procedure repeated. If the phases do not separate properly, sodium chloride can be added. The final toluene solution is centrifuged twice and adjusted with toluene to the desired final concentration. UV/Vis spectroscopy measurements were used to evaluate the concentration losses in nanoparticle solutions due to the ligand exchange procedure. AuNP with diameters >25 nm were not reliably stabilized (i.e. irreversible aggregation during centrifugation steps was not prevented by the polymer shell) with PSSH2k, although one batch of stable AuNP60@PSSH2k was obtained with comparably low yield. With PSSH12k, yields (comparing the amount of initial AuNP@CTAC with the final purified AuNP@PSSH) from 65% to >95% were obtained, with PSSH5k: 40–60%. For AuNP with a diameter of 25 nm yields >90% were obtained with all three ligands.

Self-assembly of AuNP@PSSH at the liquid-liquid interface. The method introduced by Dong et al. was used for self-assembly of AuNP@PSSH⁵⁷. In all experiments 100 µl of the AuNP@PSSH in toluene (prepared according to the above described ligand exchange protocol) were pipetted onto 300 µl of DEG in a HDPE well (inner diameter ~1.1 cm, max. vol = 1.0 ml). The well was covered with a glass slide in order to reduce the evaporation rate of toluene. The waiting times were at least 12 h, after which toluene was evaporated and a golden film had formed on the surface of the DEG phase. The particle concentrations used for film formation of different AuNP@PSSH solutions are presented in Table 1. Because of the fragility of the films and associated challenges with the film transfer, the area fraction of the different crystals (monolayer, bilayer, trilayer, and multilayer) has to be interpreted with care. As a tendency we observed that with increasing ligand length thicker crystals, i.e. more layers, were obtained, but evaporation time also plays a role. Fast evaporation (without a lid) leads preferably to monolayers with more defects, slow evaporation favors thicker crystals. A smaller number of defects upon slow evaporation was also described by Gu et al. for self-assembled monolayers of small AuNP@PSSH ($d = 5$ nm)¹¹. A longer evaporation time can be achieved by covering the well with a glass slip as described and additionally by increasing the volume of AuNP@PSSH solution. The AuNP concentration did not sensitively affect the outcome of the self-assembly experiments. As a tendency, higher particle numbers lead to thicker crystals. The particles number can be increased via the concentration or the volume of drying solution. The optimum concentration to reliably obtain self-assembled AuNP films was estimated with the theoretical concentration needed for a full monolayer coverage of the available area. Even with much higher concentrations well-ordered films including monolayer and bilayer were obtained. On the other hand, too low concentrations prevent the formation of extended well-ordered films. The concentrations used herein range from 0.3 nM for AuNP60 up to 30 nM for AuNP25. Considering the 2D hexagonal structure of the films, the area of one AuNP@PSSH in the films is $A_{\text{AuNP}} = (d_{\text{AuNP}} + \text{gap})^2 \sin(\pi/3)$, so for instance to cover 1.0 cm² with a monolayer of 35 nm AuNP with 5 nm gaps, 7.2×10^{10} AuNP would be required, corresponding to 100 µl of a 1.2 nM solution. Accordingly, guide values for suitable AuNP concentrations can be calculated.

Transmission electron microscopy. TEM measurements were performed using a Jeol JEM-1011 instrument operating at 100 kV. Samples of self-assembled AuNP@PSSH films were carefully skimmed off with a carbon-coated copper grid held by a tweezer. The grid was then dried in vacuum for at least 1 h. Quantitative analyses of AuNP@CTAC size distributions and nearest-neighbor distances of AuNP@PSSH superlattices based on TEM measurements were performed with ImageJ (version 1.50i). The indicated mean values are obtained from analyses of at least three images from different spots on the samples.

UV/vis spectroscopy. Absorbance measurements were carried out using a Varian Cary 50 spectrometer. Quartz cuvettes (Hellma QS, Hellma, Germany) were used. The AuNP concentrations were estimated with the absorbance at 400 and 450 nm as described by Haiss et al. and Scarabelli et al.^{66,67}.

Small-angle X-ray scattering. The SAXS experiments were performed at beamline P10 at PETRA III (DESY, Hamburg). An X-ray photon energy of $E = 8$ keV was used. The X-ray beam was focused by compound refractive lenses⁶⁸ to $2.8 \mu\text{m} \times 1.7 \mu\text{m}$ ($h \times v$). The detector (Dectris Eiger X 4M) was placed 5 m downstream of the sample, covering a q -range from 0.014 to 1.15 nm^{-1} . The samples were mounted in a vacuum chamber connected to an evacuated flight path. SAXS patterns were taken from different regions of the samples in scanning mode. Therein, scanning steps of 5–10 µm step size were typically chosen in horizontal and vertical directions normal to the X-ray path. At least 2000 individual patterns were measured from each sample in this way, obtaining >700 hits that were used for further analysis (cf. Fig. 2a: some measurements just cover TEM-grid without sample). From the patterns, the lattice constants a were obtained from the position of the indexed Bragg reflections. The distributions $P(a)$ were calculated from the histograms of a . The interparticle distances (gaps) based on SAXS (cf. Table 1) were determined by subtracting the AuNP diameters from particle form factor fits from the lattice parameters a .

Optical microscopy. The optical spectra of the gold nanoparticle trilayer were recorded with a home-built micro-absorbance spectrometer. Details of the setup can be found in ref.³⁶. A supercontinuum laser (Fianium, SC-400-4) was used as a white light source. The light was guided through a linear polarizer into an inverted optical microscope (Olympus, IX71) and focused with an $\times 100$ objective (Leica HCX PL Fluotar $\times 100$) onto the sample. We used objectives with a numerical aperture of $NA = 0.9$ to focus light to a diffraction-limited spot on the sample. This corresponds to a maximum angle of incidence of 64° outside the supercrystal. The maximum angle of incidence inside the supercrystal is with 5 – 13° , much smaller and close to normal incidence because of the large effective index of refraction, $n_{\text{eff}} = 4$ – 10 , of the supercrystals³⁵. The laser power was kept below 200 µW. The AuNP films were transferred to a glass substrate for optical characterization. The layer number was identified through the optical contrast in transmission (cf. Supplementary Fig. 14). The sample was moved in x -direction and y -direction with a combination of a motorized stage and a piezo stage. The transmitted light was collected with a second $\times 100$ objective (Olympus MPlan FL N BD $\times 100$) and coupled through a fiber (Ocean Optics, QP600-2-UV-BX for 450–900 nm and BIF600-VIS-NIR for 900–1600 nm) into a spectrometer (Avantes, AvaSpec 3648 for 450–900 nm and AvaSpec NIR512 for 900–1600 nm). In a subsequent measurement, the back-reflected light was separated from the incoming light beam with a beam splitter (Thorlabs, BSW26R for 450–900 nm and BSW29R for 900–1600 nm) and detected with the same combination of fibers and spectrometers as for the transmitted light. The spectra of the VIS and NIR ranges were pinned together.

Finite-difference in the time-domain (FDTD) simulations. For the FDTD simulations in Fig. 3 the commercial software package Lumerical FDTD Solutions was used as described recently^{36,54}. Spherical NPs with 50 nm diameter were packed into a hexagonal lattice with 1.6 nm interparticle gaps. Three layers were stacked in the abc sequence of an fcc crystal to construct a trilayer. A mesh override region with 0.5 nm cells was used to resolve the geometry of the supercrystal. The dielectric function of gold was modeled by fitting experimental data from Olmon et al.⁶⁹ and the polystyrene ligand molecules were modeled as a dielectric medium with refractive index $n = 1.4$ filling the space between the NPs. The FDTD simulation region was chosen as the unit cell of the supercrystal with periodic boundary conditions along x and y . Perfectly matched layers were used along z . Light was injected along z with a broadband plane wave source. The transmittance and reflectance were recorded with power monitors behind the nanoparticle layers and behind the source.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

F.S. developed the synthesis protocol. F.S. and O.P. synthesized AuNP and AuNP superlattices, F.S. did the TEM measurements, F.S. and O.P. analyzed the TEM data, F.S., F.L., and F.W. performed the SAXS measurements, F.L. and F.W. analyzed the SAXS data, N.S.M. and Y.O. did the optical measurements and calculations including SERS. F.S., H.L., and S.R. supervised the project. All authors discussed the results and implications and commented on the manuscript at all stages. F.S. and H.L. wrote the manuscript with contributions of all authors. All authors have given approval to the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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4.4 Deep strong light-matter coupling in plasmonic nanoparticle crystals

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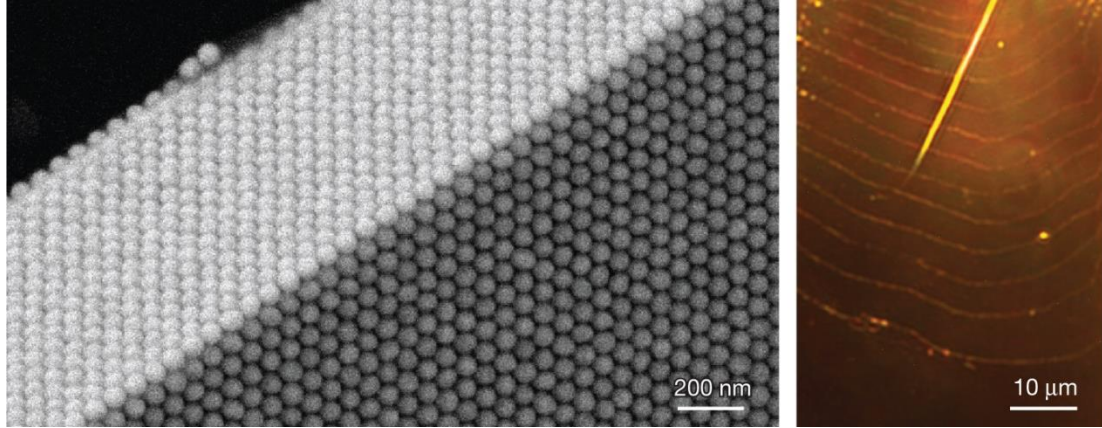


Figure 8. SEM micrograph and darkfield microscopy of the samples used in the study ($d(\text{AuNP}) = 60 \text{ nm}$).

The structure and quality of thin-film plasmonic supercrystals that was achieved allowed for a systematic study of their layer-dependent optical properties. With optical transmission for thin areas and darkfield microscopy for thicker parts of the sample, the number of layers could be identified even for multilayers (Figure 8) and a full optical characterization was obtained by micro-absorbance spectroscopy (absorption, transmission and reflection). A microscopic model was developed to describe the collective plasmonic excitations in a fcc crystal of AuNP and their interaction with light was described with a Hopfield Hamiltonian that yields hybridized light-matter polariton states $\omega_{pp}(\mathbf{k})$ (ω being the frequency and $\mathbf{k}$ the wave vector). The plasmon polariton dispersion, i.e. the dependence of ω_{pp} on $\mathbf{k}$ along a certain direction in the fcc crystal, can then be calculated for different geometries. Due to the strong interaction of AuNP with light, a level anticrossing with a large Rabi frequency, Ω_R , results, that increases with packing density of the AuNP $(d/(d+a))^3$ where d is the diameter and a is the gap size (interparticle edge-to-edge distance). Ω_R quantifies the light-matter coupling strength and can be normalized with the energy of the collective plasmon mode ω_{pl} to obtain the normalized coupling strength $\eta = \Omega_R/\omega_{pl}$. A normalized coupling strength $\eta > 1$ thus indicates that the energy of light-matter coupling is higher than the excitation itself and the calculations indicate that AuNP supercrystals enter this so-called deep strong coupling regime for $a/d < 0.1$. Our well-defined and densely packed AuNP crystals achieve such high packing densities and, importantly, allow to experimentally determine all required parameters to calculate the lower polariton branch of the plasmon polariton dispersion. The energy (frequency) of the polaritonic modes were obtained from the layer-dependent micro-absorbance spectroscopy measurements and their wavelengths λ_{pp} could be obtained because each mode j can be assigned to a standing wave in the supercrystals with $j = 2h/\lambda_{pp}$ with h being the known height of the crystal. With systematic measurements on different supercrystals the model could be confirmed and a normalized coupling strength of up to 1.83 was observed, which is by far the highest one

ever reported. The highlights of these study are not only that this record deep strong coupling was demonstrated, but also that it is robust at room temperature because the absolute value of Ω_R is so high (3.35 eV for AuNP with $d = 60$ nm and $a = 1$ nm). Upon that, AuNP supercrystals provide a versatile platform because the coupling strength can be tuned by their geometry (AuNP diameter d and gaps a via the PSSH ligands). Deep strong coupling can fundamentally change the properties of a material and is therefore receiving great interest in quantum electrodynamics, nanophotonics and nonlinear optics.¹⁹⁷ It might also be utilized for quantum technologies, analytics and polaritonic chemistry.

Deep strong light–matter coupling in plasmonic nanoparticle crystals

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In the regime of deep strong light–matter coupling, the coupling strength exceeds the transition energies of the material^{1–3}, fundamentally changing its properties^{4,5}; for example, the ground state of the system contains virtual photons and the internal electromagnetic field gets redistributed by photon self-interaction^{1,6}. So far, no electronic excitation of a material has shown such strong coupling to free-space photons. Here we show that three-dimensional crystals of plasmonic nanoparticles can realize deep strong coupling under ambient conditions, if the particles are ten times larger than the interparticle gaps. The experimental Rabi frequencies (1.9 to 3.3 electronvolts) of face-centred cubic crystals of gold nanoparticles with diameters between 25 and 60 nanometres exceed their plasmon energy by up to 180 per cent. We show that the continuum of photons and plasmons hybridizes into polaritons that violate the rotating-wave approximation. The coupling leads to a breakdown of the Purcell effect—the increase of radiative damping through light–matter coupling—and increases the radiative polariton lifetime. The results indicate that metallic and semiconducting nanoparticles can be used as building blocks for an entire class of materials with extreme light–matter interaction, which will find application in nonlinear optics, the search for cooperative effects and ground states, polariton chemistry and quantum technology^{4,5}.

Light is usually understood as an external perturbation to materials, and electromagnetic modes are neglected in material modelling. This approach works well in most cases because the interaction between light and materials is weak. With increasing light–matter interaction, however, we reach a regime in which the electromagnetic states fundamentally change the physical and chemical properties of a material^{4,5,7}. This interaction can be quantified by the Rabi frequency Ω_R , which describes how a two-level system periodically exchanges energy with a photon field^{4,5}. Quantum optics defines distinct coupling regimes depending on how Ω_R compares to the excitation frequency and lifetime. The classification highlights the relative contributions to light–matter coupling^{4,5}: when Ω_R becomes comparable to the excitation frequency ω_e (ultrastrong coupling, $\Omega_R \geq 0.1\omega_e$), the counter-rotating terms of the light–matter Hamiltonian become sizable, creating virtual excitations in the ground state of the system^{1,6,8}. In the ultimate limit of light–matter coupling, the interaction frequency Ω_R exceeds the energy of the excited state ω_e (refs. ^{4,5}). In this deep strong coupling regime, light and matter are expected to decouple owing to the self-interaction energy of the photons, which leads to a breakdown of the Purcell effect⁶. Surprisingly, the regime of deep strong coupling is perturbative, although with switched roles compared to weak coupling: the light–matter interaction dominates the energy scale and the material excitations are reduced to perturbations⁹. Light–matter coupling proceeds through the vacuum fluctuations of the electromagnetic field, which means

that in the deep strong coupling regime high-field processes of nonlinear optics will occur at low photon flux with unit efficiency¹⁰. Deep strong coupling is expected to result in nonlinear processes, such as the collective emission of a single photon by multiple atoms¹¹, and to modify the rate of chemical reactions and reaction coordinates^{7,12}. Another exciting application is protocols for quantum information processing, for example, for ultrafast quantum computation and protected qubits^{5,13,14}.

Ultrastrong and deep strong light–matter coupling have been realized on various molecular and solid-state platforms^{4,5}. The coupling regime in which the counter-rotating and diamagnetic terms become dominant ($\Omega_R \geq 0.5\omega_e$), however, has been restricted to Landau states and superconducting circuits^{4,5}. With few exceptions¹⁵, their Rabi splitting was of the order of microelectronvolt to millielectronvolt and only emerged at cryogenic temperatures. Such devices couple discrete photonic and materials states. Although the deep strong coupling of an artificial atom to an electromagnetic continuum was realized recently¹⁶, a system that couples a continuum of material excitations to free-space photons in the strongest regimes is still lacking. Continuous systems will expand the realm and applicability of deep strong light–matter coupling, for example, for broadband cloaking and to replace dielectric metamaterials^{17,18}.

Here we propose and realize three-dimensional crystals of plasmonic nanoparticles for deep strong light–matter coupling under ambient conditions. The collective transverse plasmon mode of

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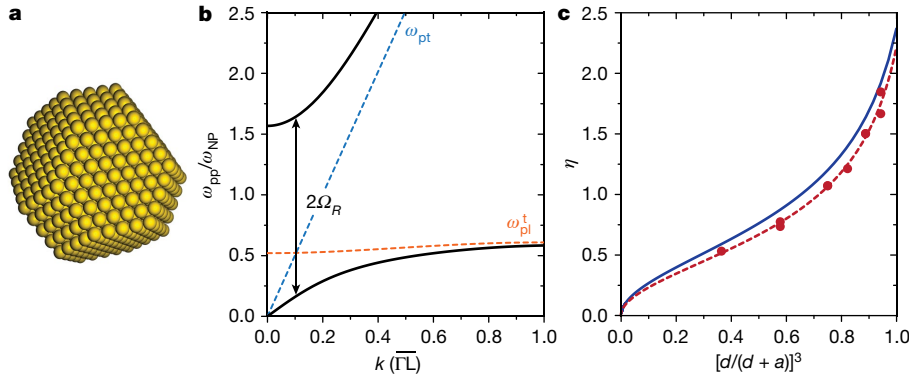


Fig. 1 | Plasmon polaritons in metal nanoparticle fcc crystals. **a**, fcc crystal of plasmonic nanoparticles with diameter $d = 10\text{--}100\text{ nm}$. **b**, Plasmon polariton dispersion (black lines) along ΓL for a Drude metal with $d = 30\text{ nm}$ and $a = 2\text{ nm}$. The orange dashed line shows the transverse plasmon dispersion, $\omega_{\text{pl}}^{\text{t}}(k)$, and the blue dashed line shows the photon dispersion, $\omega_{\text{pt}}(k)$. Light–matter

coupling results in a level anticrossing with a large Rabi frequency of $\Omega_{\text{R}} = 1.4\omega_{\text{pt}}^{\text{t}}$. **c**, Normalized coupling strength $\eta = \Omega_{\text{R}}/\omega_{\text{pl}}^{\text{t}}$ as a function of d and a , within the point-dipole approximation for a Drude metal (blue solid line) and from finite-difference time-domain simulations of gold ($\epsilon_{\text{m}} = 1.96$; circles; the dashed line is a guide to the eye); see Methods and Supplementary Table 1.

the crystal hybridizes with the continuum of photonic states into three-dimensional polaritons with Ω_{R} approaching twice the plasmon frequency.

Plasmon polaritons in nanoparticle crystals

Metal nanoparticles absorb and scatter light through their localized surface plasmons, the collective oscillation of free electrons^{19,20}. Their high oscillator strength makes them excellent building blocks for materials that strongly interact with light^{21–23}. In an artificial crystal of metal nanoparticles (Fig. 1a) the dipole plasmons ω_{np} couple through the Coulomb interaction, creating a continuum of collective plasmonic states^{22,24} (see Methods and Extended Data Fig. 1). We introduce retardation through a propagating light field $\omega_{\text{pt}}(\mathbf{k})$, where $\mathbf{k}$ is the wave vector. $\omega_{\text{pt}}(\mathbf{k})$ couples to the transverse plasmon $\omega_{\text{pl}}^{\text{t}}(\mathbf{k})$ (refs.^{12,21,24}), which is a unique state in nanoparticle crystals without a counterpart in bulk metals. Within a quantum mechanical formalism, the interaction may be described by a Hopfield Hamiltonian that yields hybridized light–matter polariton states $\omega_{\text{pp}}(\mathbf{k})$ (ref.²⁵, Fig. 1b),

$$\omega_{\text{pp}}^4(\mathbf{k}) - \omega_{\text{pp}}^2(\mathbf{k})[\omega_{\text{pt}}^2(\mathbf{k}) + \omega_{\text{pl}}^2(\mathbf{k}) + 4\Omega_{\text{R}}^2] + \omega_{\text{pt}}^2(\mathbf{k})\omega_{\text{pl}}^2(\mathbf{k}) = 0, \quad (1)$$

where $\omega_{\text{pl}}^{\text{t}} \equiv \omega_{\text{pl}}$; $\omega_{\text{pt}} = c|\mathbf{k}|/n_{\text{eff}}$, c is the speed of light in vacuum and n_{eff} is the mean refraction index if we replace the metal by vacuum; see Supplementary Table 1. An alternative approach models the plasmonic crystal through a classical oscillator with a giant oscillator strength; see Methods.

The polariton dispersion consists of an upper and a lower branch arising from an anti-crossing of the photon and plasmon dispersions (Fig. 1b). We calculate the Rabi splitting $2\Omega_{\text{R}}$ between the two branches with a microscopic model; see Methods. For a face-centred cubic (fcc) nanoparticle crystal with particle diameter $d = 30\text{ nm}$ and interparticle gap $a = 2\text{ nm}$, light–matter interaction results in a giant Rabi splitting (Fig. 1b). The normalized coupling constant $\eta = \Omega_{\text{R}}/\omega_{\text{pl}}^{\text{t}} = 1.4$ is clearly in the deep strong regime. η depends on the packing density and the crystal structure. For an fcc crystal of spherical nanoparticles we obtain an expression for η that depends on d and a ; see Fig. 1c and Methods. The function agrees very well with numerical simulations of η using the experimental dielectric function of gold (circles in Fig. 1c). fcc crystals of gold nanoparticles enter the deep strong coupling regime for $a/d < 0.1$. Prior fabrication protocols for plasmonic nanoparticle crystals were restricted to small crystallites^{26,27} or low packing densities ($a/d \gg 0.1$)^{23,28–30}, which has prevented the observation of deep strong coupling in such crystals.

Gold nanoparticle crystals

We synthesized densely packed fcc crystals of large gold nanoparticles ($d = 25\text{--}59\text{ nm}$) with small gaps ($a \approx 1\text{--}4\text{ nm}$) using polystyrene as the nanoparticle ligand; see Methods. Polystyrene-stabilized nanoparticles assemble into hexagonally packed monolayers at a liquid–liquid interface (Fig. 2a)^{31–33}. By reducing the reaction rate during self-assembly and increasing the nanoparticle concentration, we synthesized nanoparticle fcc crystals (Fig. 2b, c). Scanning electron microscopy (SEM) images show micrometre-sized crystals with little imperfection and excellent three-dimensional order (Fig. 2, Extended Data Fig. 2). We found the number of crystal layers N for $N \leq 5$ using optical microscopy in transmission mode (Fig. 2d) and by counting the scattering edges in dark-field images for thicker crystals (Fig. 2e)^{33,34}.

The optical response of the nanoparticle crystals demonstrates the presence of plasmon polaritons with deep strong light–matter coupling (Fig. 3). A series of polariton peaks stems from the absorption of photons in the infrared and lower visible energy range ($<1.7\text{ eV}$; Fig. 3a), before light gets reflected above the polariton band edge ($>1.7\text{ eV}$; Fig. 3c). At 2.3 eV the interband transitions of gold set in and absorb any incoming light^{19,20}. To quantify light–matter coupling, we extract the polariton dispersion from the optical spectra. The extrema in the spectra arise from open cavity boundary conditions (Fig. 3b). Each determines a pair of polariton frequency ω_{pp} and wavelength λ_{pp} ; see Methods. The measured lower polariton branches of five nanoparticle crystals with $d = 25\text{--}59\text{ nm}$ (circles in Fig. 3d) show the characteristic bending for large η . We fit the dispersions using the Hopfield Hamiltonian, treating Ω_{R} and $\omega_{\text{pl}}^{\text{t}}$ as free parameters (lines in Fig. 3d). Four of the five nanoparticle crystals are well in the deep strong coupling regime $\eta > 1$; see Table 1. The upper polariton branches are inaccessible optically because they start above the onset of the gold interband transitions; see $\omega_{\text{pl}}^{\text{t}}$ in Table 1. This branch should be observable in aluminium nanoparticle crystals using ultraviolet excitation (Extended Data Fig. 3).

Deep strong coupling in plasmonic nanoparticle crystals stands out owing to its exceptional normalized coupling strength and the large absolute values of the Rabi frequency (Fig. 3e). The highest normalized coupling (1.83) for the 59-nm nanoparticles greatly exceeds the values reported in optimized systems of Landau polaritons² (1.43) and superconducting circuits³ (1.34). The large absolute Rabi frequency ensures that our system is simultaneously in the deep strong ($\Omega_{\text{R}} > \omega_{\text{pl}}^{\text{t}}$) and the strong coupling regime; that is, Ω_{R} exceeds any losses in the material³⁵. We estimate the cooperativity factor as $C \approx 4\Omega_{\text{R}}^2/\gamma_{\text{pp}}^2 = 5,500$ from the width of the polariton peaks $\hbar\gamma_{\text{pp}} = 90\text{ meV}$ ($\hbar$, reduced Planck constant; $d = 59\text{ nm}$; see below)^{5,8}. The Rabi frequency may be tailored by varying

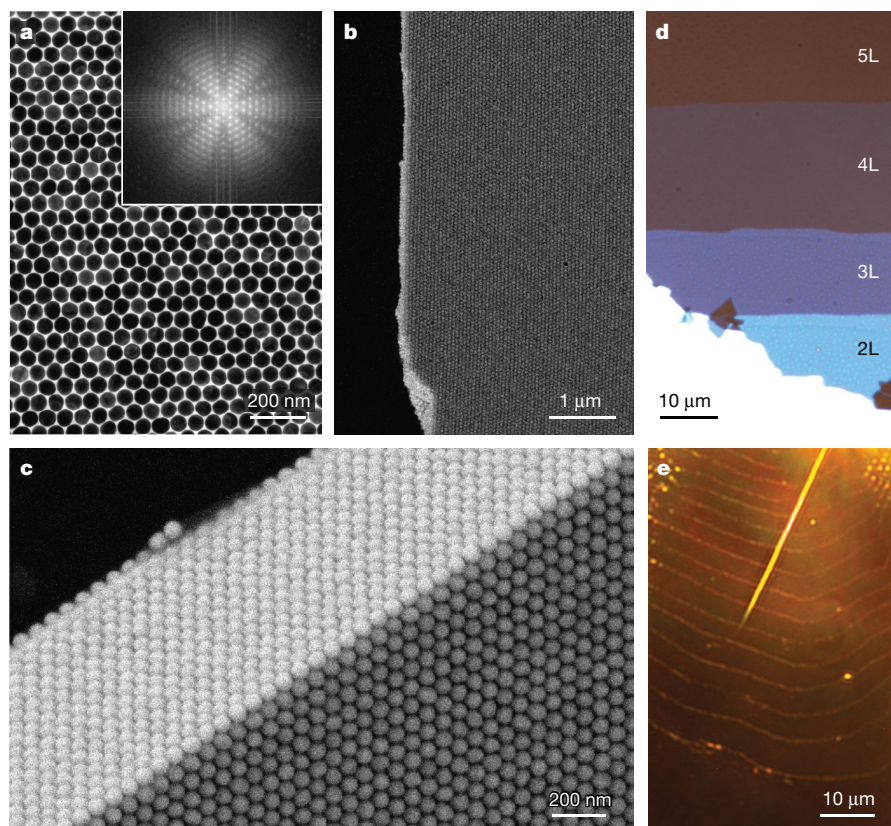


Fig. 2 | Synthesis of gold nanoparticle crystals. **a**, Transmission electron microscopy (TEM) image of a nanoparticle monolayer; the inset shows a Fourier transformed image. **b**, SEM image of a crystal with size $>6\ \mu\text{m}$. **c**, Edge view of a nanoparticle crystal showing two (111) faces of an fcc crystal. **d**, Transmission optical microscopy image of $N=2-5$ nanoparticle layers (L). **e**, Dark-field microscopy image of up to $N=23$ layers. The bright lines are layer edges that scatter light. The crystals have nanoparticle diameters of $d=59\ \text{nm}$ (**a-c**) and $d=30\ \text{nm}$ (**d,e**).

the crystal parameters, which allows us to obtain $\eta=0.95-1.83$. A further increase in packing density will push the coupling towards the upper bound $\eta=2.2$ predicted in Fig. 1c. Exceeding this limit will be possible by changing the dielectric background, optimizing the oscillator strength per nanoparticle, and perhaps by using other crystal structures.

Light-matter coupling strongly reduces the phase v_{ph} and group velocity v_{gr} of light inside the nanoparticle crystal (Table 1). The reduction in wavelength by an order of magnitude implies an enhancement of 10^3 for dipole and 10^5 for quadrupole transitions that get excited by the polariton near field compared to far-field excitation³⁶. Crystals with embedded molecules may show forbidden transitions through the shrinking of light³⁶. The group and phase velocities in the crystals depend sensitively on d , a and on the dielectric constant ε_{m} , making such crystals potential materials for manipulating light at the nanoscale. Plasmonic nanoparticle crystals may borrow concepts from photonic crystals and realize them with a smaller footprint owing to the drastically reduced wavelength¹⁸.

Purcell effect in deep strong coupling

Among the effects that have been predicted for deep strong light-matter coupling is the breakdown of the Purcell effect⁶. The Purcell effect is the increase of radiative damping through light-matter coupling, $\gamma_{\text{rad}} \propto \Omega_{\text{R}}^2$ for $\Omega_{\text{R}} \ll \omega_{\text{e}}$ (here, $\omega_{\text{pl}}^{\text{t}}$)^{37,38}. It has a key role in engineering optical emission, for example, for near-field enhanced spectroscopy, nonlinear optics and lasing⁷. The Hopfield Hamiltonian provides insight into which terms of light-matter coupling decrease the radiative damping for $\eta > 1$ (ref.⁶). To demonstrate this, we first analyse the contribution of the counter-rotating terms and the photon self-interaction energy to the polariton dispersion^{4,5}. A two-mode version of the Hopfield Hamiltonian is²⁵ (see Supplementary Information)

$$\mathcal{H} = \hbar\omega_{\text{pt}}(k)\hat{a}^{\dagger}(k)\hat{a}(k) + \hbar\omega_{\text{pl}}^{\text{t}}(k)\hat{b}^{\dagger}(k)\hat{b}(k) + \mathcal{H}_{\text{int}} + \mathcal{H}_{A^2}, \quad (2)$$

with the interaction Hamiltonian

$$\mathcal{H}_{\text{int}} = i\hbar\Omega_{\text{R}}\sqrt{\frac{\omega_{\text{pl}}^{\text{t}}(k)}{\omega_{\text{pt}}(k)}} [\hat{a}^{\dagger}(-k) + \hat{a}(k)][\hat{b}(-k) - \hat{b}^{\dagger}(k)] \quad (3)$$

and the photon self-interaction (A^2 term)

$$\mathcal{H}_{A^2} = \frac{\hbar\Omega_{\text{R}}^2}{\omega_{\text{pt}}(k)} [\hat{a}^{\dagger}(-k) + \hat{a}(k)][\hat{a}^{\dagger}(k) + \hat{a}(-k)]. \quad (4)$$

$\hat{a}^{\dagger}(k)$ and $\hat{a}(k)$ are the creation and annihilation operators, respectively, of a photon with $\omega_{\text{pt}}(k) = ck/n_{\text{eff}}$, and $\hat{b}^{\dagger}(k)$ and $\hat{b}(k)$ are those of the collective transverse plasmon $\omega_{\text{pl}}^{\text{t}}(k)$ (taken as a constant). A fit with the full Hamiltonian shows excellent agreement with the measurements (Figs. 3d, 4a). When forcing all counter-rotating terms ($\hat{a}^{\dagger}\hat{b}^{\dagger}$, $\hat{a}\hat{b}$, $\hat{a}^{\dagger}\hat{a}^{\dagger}$ and $\hat{a}\hat{a}$) to zero, the polariton energy becomes finite for $k \rightarrow 0$ and increases by the vacuum Bloch-Siegert shift, $|\Delta_{\text{VBS}}| = 100-900\ \text{meV}$ (orange line in Fig. 4a and Extended Data Fig. 4a)⁸. Even when treating Ω_{R} and $\omega_{\text{pl}}^{\text{t}}$ as free parameters, it is impossible to fit the data with a dispersion that neglects the counter-rotating contributions (see Extended Data Fig. 4b): the best fit produces optical resonances (orange arrows in Fig. 4b) that disagree strongly with the measured peak positions. We obtain a similar disagreement (Fig. 4a, b) without the A^2 term (Dicke model; pink line) and for the rotating-wave approximation (RWA; blue line). The counter-rotating contributions to light-matter coupling populate the ground state of the system with virtual excitations¹. The population exceeds unity for small wave vectors (Extended Data Fig. 5). The virtual photons may be converted into real states when turning off light-matter coupling non-adiabatically^{1,4,39}.

The Purcell effect depends on the density of electromagnetic states and their spatial overlap with the excited states of the material^{6,37}. The strong dielectric contrast of metals and dielectrics confines the electric field to the particle gaps for $\omega_{\text{pt}} < \omega_{\text{pl}}^{\text{t}}$. To minimize the large photon self-interaction energy, the A^2 term rearranges the electric field and

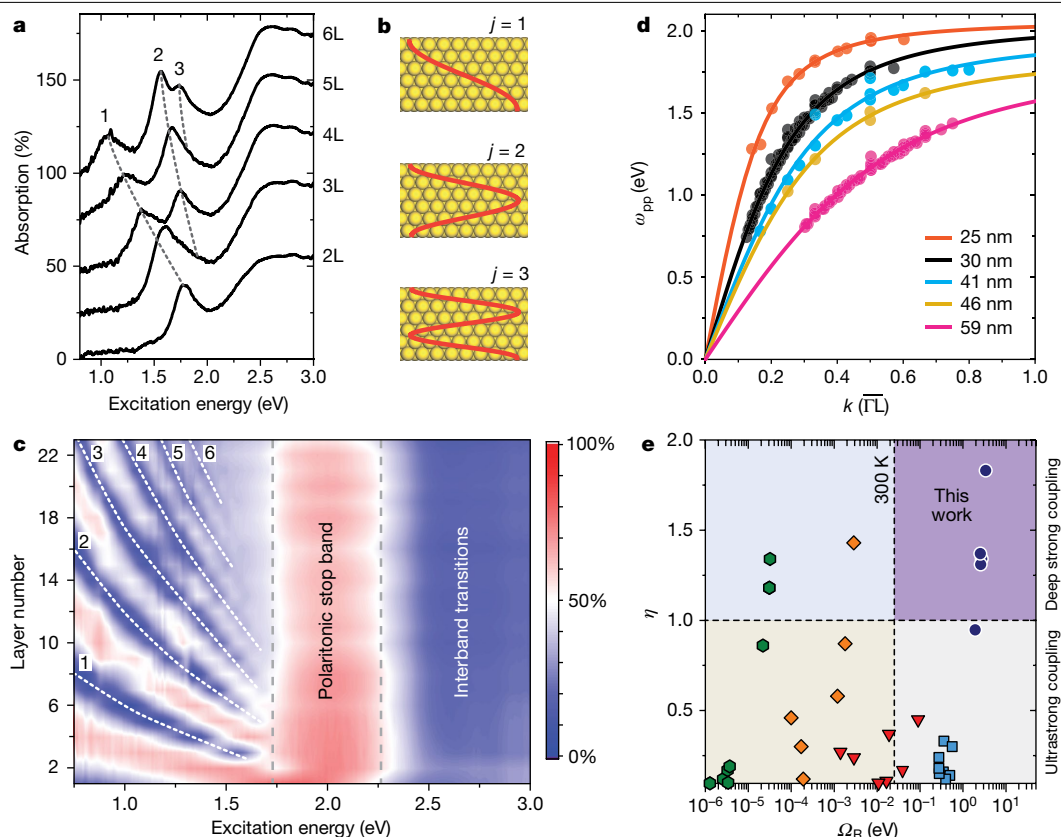


Fig. 3 | Deep strong light-matter coupling in nanoparticle crystals.

a, b, Absorption spectra of a 30-nm gold nanoparticle crystal with two to six layers, offset by 25% increments (**a**). The peaks correspond to standing waves inside the crystal, as sketched in **b**. **c**, Reflectance as a function of excitation energy and layer number. Dashed lines and labels indicate $j = 2h/\lambda_{pp}$ (h , crystal height). Grey dashed lines indicate the onsets of the polaritonic stop band and the interband transitions. **d**, Measured lower polariton branch for crystals with $d = 25$ nm (orange circles), 30 nm (black), 41 nm (blue), 46 nm (yellow) and 59 nm

(pink). The solid lines are fits with equation (1) assuming that the coupling strength is constant with layer number (see discussion in Methods). The fitting parameters are listed in Table 1. **e**, Comparison of η and Ω_R values measured in this work (circles) with measurements from superconducting circuits (hexagons), Landau polaritons (diamonds), intersubband polaritons (triangles) and organic molecules (squares) taken from the studies in table 1 of ref. ⁴. The horizontal dashed line marks the onset of deep strong light-matter coupling; the vertical dashed line shows the thermal energy at room temperature.

partly pushes it into the metal⁶. This shifts the plasmon resonance to higher energies, inhibiting the superradiant phase transition expected within the Dicke model^{4,5}. Most strikingly, we observe a decrease in radiative damping, which follows the scaling predicted from the breakdown of the Purcell effect⁶ (Fig. 4c). The full-width at half-maximum (FWHM) of the polariton peak decreases by a factor of 2 when increasing η from 0.95 to 1.83. The drop in emission is accompanied by a raise in integrated absorption intensity (10%; Fig. 4c) owing to the stronger overlap between the electric field and the metal electrons. The

calculated optical absorption fully captures this counter-intuitive behaviour, showing an increasing polariton absorption combined with a narrowing line width in the lower polariton branch (Extended Data Fig. 6). The Purcell effect in plasmonic nanoparticle crystals depends on the polariton wave vector, the plasmon energy and the crystal thickness, in addition to the Rabi frequency, which opens up ways to engineer the radiative decay for a target application.

Discussion

Deep strong light-matter coupling emerged naturally for the transverse plasmons in fcc nanoparticle crystals with very high normalized coupling strength. The large Rabi frequency made the phenomena robust against thermal excitation and the polaritons were observable at room temperature. Quantum optics sets an inspiring framework for studying the properties of plasmonic crystals; the theory predicts physical phenomena, such as the vanishing Purcell effect, and quantum effects, such as squeezed light and virtual excitations (see Extended Data Fig. 5 and ref. ⁴⁰). A classical way of modelling the optical properties of the crystals is to treat the crystals as an artificial material with a giant oscillator strength of $G > 10$ (see Methods). Nanoparticle crystals are a versatile platform for engineering light-matter interaction. They can be synthesized from nanoparticles of various shapes, from other metals and from doped semiconductors. Mixed crystals of metals and quantum dots will simultaneously couple excitons and plasmons to photons. Embedding molecules in a material with deep strong coupling

Table 1 | Measured light-matter coupling

d (nm)	a (nm)	η	Ω_R (eV)	ω_{pl}^t (eV)	ω_{pl}^l (eV)	$\lambda_{pp}(0.8\bar{\Gamma})$ (nm)	v_{ph}/c	v_{gr}/c
25	3	0.95	1.94	2.05	4.39	58	0.09	3×10^{-3}
30	4	1.34	2.72	2.04	5.80	69	0.11	9×10^{-3}
41	3	1.31	2.57	1.96	5.49	90	0.13	1.7×10^{-2}
46	2	1.4	2.5	1.8	5.4	100	0.1	2×10^{-2}
59	1	1.83	3.35	1.83	6.94	123	0.14	4.7×10^{-2}

The Rabi frequency Ω_R , transverse plasmon frequency ω_{pl}^t and normalized coupling strength η were measured on five nanoparticle crystals. d and a were estimated from TEM images. ω_{pl}^l is the longitudinal plasmon frequency calculated from the fits. The table shows the wavelength λ_{pp} at $k = 0.8\bar{\Gamma}$ together with the phase velocity $v_{ph}/c = \lambda_{pp}/\lambda_0$ (λ_0 is the wavelength of the light in vacuum) and group velocity $v_{gr}/c = (1/c)(\partial\omega_{pp}/\partial k)$ at that wavelength. The uncertainty is ± 2 in the last digit, except for d ; see Methods.

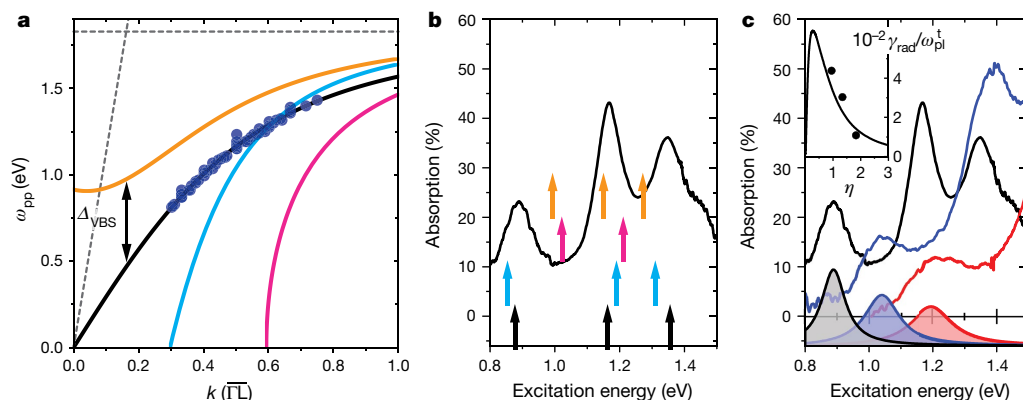


Fig. 4 | Counter-rotating and photon self-interaction terms in deep strong coupling. **a**, Polariton dispersion ($d = 59$ nm) measured (blue circles) and calculated with the Hopfield model (black line), without the counter-rotating contributions (orange line), Dicke model (pink line) and the RWA, neglecting both the counter-rotating and the A^2 terms (blue line). The arrow indicates the vacuum Bloch–Siegert shift ($\Delta_{\text{VBS}} = 510$ meV) under resonance conditions, $\omega_{\text{pt}} = \omega_{\text{pl}}$. The dashed lines indicate the bare photon and plasmon dispersion. **b**, Absorption spectrum for $d = 59$ nm and $N = 6$ layers compared to the peak positions predicted by the Hopfield model (black arrows), without counter-rotating contributions (orange arrows), the Dicke model (pink arrows)

and RWA (blue arrows). The predictions were made from the best fits of the models to the experimental data (Extended Data Fig. 4b). **c**, Increase in the intensity and decrease in the line width of the absorption peak due to the A^2 term. Absorption spectra (lines) and fits (shaded peaks) to the $j = 2$ peak for 300-nm-thick crystals of $d = 59$ nm (black; FWHM of 90 meV), $d = 30$ nm (blue; 132 meV) and $d = 25$ nm (red; 160 meV) nanoparticles. Inset, symbols show the radiative damping γ_{rad} obtained after subtracting an intrinsic contribution of $\hbar\gamma_i = 70$ meV (gold, 1 eV; see Methods), and the line is the behaviour predicted for $\gamma_i = \gamma_{\text{NP}} = 0.1\omega_{\text{pl}}$ (γ_{NP} is the radiative line width of an isolated nanoparticle)⁶.

will tailor their excited-state dynamics and may realize vibrational strong coupling. The potential applications of our work range from fundamental studies of quantum electrodynamics for quantum technologies, nanophotonics and nonlinear optics to analytics in chemistry and life sciences.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-020-2508-1>.

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Methods

Synthesis of gold nanoparticle crystals

Gold nanoparticles with diameters of $d = 30$ nm, 41 nm and 46 nm were synthesized with the seeded growth protocol proposed by Bastús et al.⁴¹ and functionalized with thiol-terminated polystyrene (molar weight $M_n = 5,300$ g mol⁻¹; Polymer Source) using a phase-transfer protocol that some of us reported recently³¹. Gold nanoparticles with $d = 25$ nm and 59 nm were synthesized with the seeded growth protocol of Zheng et al.⁴² scaled up by a factor of 10. Then, 10 ml of cetyltrimethylammonium chloride-stabilized nanoparticles dissolved in water was purified and concentrated by repeated centrifugation ($d = 25$ nm: at 5,000g for 12 min; $d = 59$ nm: at 2,000g for 12 min) to 100 μ l and injected into a solution of thiol-terminated polystyrene (25 nm: $M_n = 5,300$ g mol⁻¹; 59 nm: $M_n = 11,500$ g mol⁻¹; Polymer Source) in tetrahydrofuran (0.5 mM, 2 ml). After reacting overnight, the solvent was removed under reduced pressure in a rotary evaporator, and the residue was solved in 2.0 ml toluene, purified and concentrated to a gold nanoparticle concentration of 30 nM (25 nm) and 1.4 nM (59 nm) by repeated centrifugation.

The synthesis of the gold nanoparticle crystals was performed with the method described by Murray and co-workers⁴³: self-assembly during evaporation of toluene on a liquid subphase, namely, 300 μ l diethylene glycol. The self-assembly was carried out in small wells (high-density polyethylene) that were covered with glass slides. We used 300 μ l of 30-nm (3.5 nM) and 41-nm (0.3 nM) gold nanoparticles in toluene, 250 μ l of 46-nm nanoparticles (2.1 nM), and 100 μ l of 25-nm (30 nM) and 59-nm (1.4 nM) nanoparticles. After complete evaporation of the solvent (usually after 24–48 h) the formed crystals were carefully skimmed off with carbon-coated TEM grids for TEM characterization or transferred onto glass slides (0.13–0.16 mm thickness; Carl Roth) for optical spectroscopy.

Electron microscopy

TEM and SEM were carried out to characterize the samples on various length scales. TEM measurements were performed using a Jeol JEM-1011 instrument operating at 100 kV. The particle diameters and nearest-neighbour distances were determined with the software ImageJ 1.50i. Extended Data Fig. 7 shows an exemplary analysis for a monolayer of 59-nm nanoparticles. The nanoparticle diameters determined by TEM and their standard deviations were 59 ± 2 nm, as obtained by analysing $N_{NP} = 216$ particles. For the other particles we obtained: 25 ± 1 nm (25-nm nanoparticles; $N_{NP} = 366$), 30 ± 3 nm (30-nm nanoparticles; $N_{NP} = 455$), 41 ± 3 nm (41-nm nanoparticles; $N_{NP} = 651$) and 46 ± 4 nm (46-nm nanoparticles; $N_{NP} = 228$). The interparticle distances were found from a nearest-neighbour analysis, yielding $a = 3$ nm for the 25-nm nanoparticles, 4 nm for the 30-nm nanoparticles, 3 nm for the 41-nm nanoparticles, 2 nm for the 46-nm nanoparticles and 1 nm for the 59-nm nanoparticles. The accuracy of a was limited by variations in diameter and deviations of the shape from the ideal sphere. The interparticle distance a was evaluated for nanoparticle monolayers; distances may vary for thicker slabs, which we will study in the future using scattering techniques.

SEM images of the nanoparticle crystal were taken with a Pioneer Two system (Raith). We used an acceleration voltage of 7 kV and set the aperture size to 7.5 μ m. The working distance was adjusted to 6.7 mm. To detect the ejected electrons, a standard secondary-electron detector was used. The images were recorded on TEM grids, which were attached to a silicon substrate with silver glue.

Optical microscopy and spectroscopy

We used optical microscopy to assess the quality of the synthesized crystals and as a pre-characterization tool for spectroscopy. Measuring the polariton spectra and dispersions shown in Fig. 3 required precise control over the number of stacked layers. We identified sample areas

with step-wise increasing thickness that were ideal for our measurements. For thin crystals with fewer than six layers, we determined the thickness from the optical contrast in transmittance (Fig. 2d). For thicker crystals, we used the scattering of light from the layer edges to determine the number of stacked layers. These were visible as lines in optical reflectance and dark-field images (Fig. 2e). We identified sample areas with step-wise increasing height up to $h = 0.60$ μ m (23 layers) in a crystal with $d = 30$ nm and $h = 0.84$ μ m (17 layers) in a crystal with $d = 59$ nm. The samples also contained much thicker crystals, but we needed an accurate measure of h to determine the polariton dispersion.

The optical spectra of gold nanoparticle crystals were measured with a micro-absorbance spectrometer. A detailed description of the setup is provided in ref.³³. A supercontinuum laser (Fianium, SC-400-4) acts as the light source over a broad spectral range (450–2,400 nm). An optical microscope (Olympus, IX71) with home-made modifications focused the light onto the sample with a Leica HCX PL Fluotar 100 $\times$ objective (numerical aperture, NA = 0.9). Owing to the large refraction index of the nanoparticle crystals, $n_{pp} = 4$ –10 (see Table 1), the effective NA inside the material, $NA_{eff} = NA/n_{pp}$, was in the range $NA_{eff} = 0.09$ –0.23. The sample position was changed using a motorized xy stage combined with a piezo stage (PI, P-542.2CD). The position of the laser spot on the sample was identified with a microscope camera in transmission or reflection mode. The laser power at the sample was kept below 100 μ W.

The transmitted light was collected with an Olympus MPlan FL N BD 100 $\times$ objective (NA = 0.9). An optical fibre (Ocean Optics, QP600-2-UV-BX for 450–900 nm and BIF600-VIS-NIR for 900–1,600 nm) was used to guide the light to a spectrometer (Avantes, AvaSpec 3648 for 450–900 nm and AvaSpec NIR512 for 900–1,600 nm), where it was dispersed and detected. As a reference for the transmission spectra we used the transmittance through the substrate at a position without nanoparticle crystals. The reflected light was separated from the incoming light by a beam splitter (Thorlabs, BSW26R for 450–900 nm and BSW29R for 900–1,600 nm) and detected with the same fibres and spectrometers as for the transmitted light. The reflections from a silver mirror (Thorlabs, PF10-03-P01) served as a reference for the reflection measurements. The transmittance T and reflectance R spectra in the two spectral ranges were combined and the absorption was calculated as $A = 100\% - T - R$.

The absorption spectra were fitted with a cubic background and a series of Lorentzians to obtain the polariton frequencies. The crystal forms an open cavity for polaritons, creating standing waves with wavelength $\lambda_{pp} = 2h/j$, where j is an integer (Fig. 3b)^{21–23,33}. The wave vectors in units of the Γ L distance, λ_{pp} (0.8 Γ L), are given by $k_{pp} = 2\pi/\lambda_{pp} = (j/N)\Gamma$ L, where N is the number of layers. In Fig. 3c we highlight polaritons with $j = 1$ –6 that were used to relate the measured polariton energy with its wavelength λ_{pp} ; in that way, we obtained the experimental polariton dispersions shown by the symbols in Fig. 3d.

Finite-difference time-domain simulations

We used the commercial finite-difference time-domain (FDTD) simulation package Lumerical FDTD Solutions. We constructed the unit cell of a hexagonal close-packed monolayer of metallic nanoparticles with diameters d , interparticle gap sizes a and periodic boundary conditions along x and y . To obtain thin slabs of an fcc crystal, we stacked nanoparticle layers along z in ABC stacking.

We used a mesh-override region with 0.5 nm mesh size for $a > 1.5$ nm and $a/3$ mesh size for smaller gaps. The gold nanoparticles were modelled with the experimental dielectric function of gold from ref.⁴⁴. For simulations with the Drude model, we used the dielectric function $\epsilon(\omega) = 1 - \omega_p^2/(\omega^2 + i\omega\gamma_p)$ with $\hbar\omega_p = 9.07$ eV and $\hbar\gamma_p = 40$ meV. We modelled the surrounding medium with a dielectric constant of $\epsilon_m = 1.96$ mimicking the experiment, where the gold nanoparticles were surrounded with polystyrene molecules³³.

To calculate the optical response of the crystal slab, we illuminated the structure with a broadband plane electromagnetic wave source along z with linear polarization along x . We verified that the direction of polarization in the x - y plane has no influence on the calculated far-field spectra. The transmitted T and reflected R light were recorded with two-dimensional frequency-domain power monitors and the absorption was calculated as $A = 100\% - T - R$.

We calculated the normalized coupling strength for gold nanoparticle crystals shown in Fig. 1c from simulated absorption spectra of gold nanoparticle films that consisted of six nanoparticle layers; see Extended Data Fig. 8. To simulate crystals with different coupling strength η , we either varied the nanoparticle diameters in the range $d = 10$ – 100 nm for fixed $a = 2$ nm, or varied the gap size in the range $a = 1$ – 20 nm for fixed $d = 50$ nm. The simulated absorption spectra were analysed as described for the experimental data to obtain the simulated polariton dispersion. For spectra in which the absorption peaks strongly overlapped, we analysed the internal current distribution as explained in ref. ³³. The polariton energies and wavelengths yield the polariton dispersions that are shown in Extended Data Fig. 8 as data points. We find the normalized coupling strength $\eta = \Omega_R/\omega_{\text{pl}}^t$ from a fit with the Hopfield model in equation (1). The resulting η values are the data points shown in Fig. 1c.

Hopfield and Lorentz models

The Hopfield model, introduced with equations (2)–(4), represents a quantum mechanical formalism used to describe the interaction of light and a material excitation²⁵. An alternative approach models the excitations through a classical Lorentz oscillator (or a series of oscillators)⁴⁵. The Lorentz model yields a dielectric function $\varepsilon_L(\omega)$ that is determined by the excitation frequency ω_{pl} , its line width γ , reduced oscillator strength G , and the high-frequency dielectric constant ε_∞ .

$$\varepsilon_L(\omega) = \varepsilon_\infty + G \frac{\omega_{\text{pl}}^2}{\omega_{\text{pl}}^2 - \omega^2 - i\gamma\omega}. \quad (5)$$

This is exactly the dielectric function of the Hopfield model $\varepsilon_H(\omega)$ as obtained by substituting the dispersion relations $c^2|\mathbf{k}|^2 = \varepsilon_H\omega_{\text{pp}}^2$ for the polariton and $c^2|\mathbf{k}|^2 = n_{\text{eff}}^2\omega_{\text{pt}}^2$ for the photon into equation (1) and setting $\omega \equiv \omega_{\text{pp}}$ (refs. ^{24,25}). We include intrinsic losses by a complex frequency component $i\gamma\omega$.

Comparing the two dielectric functions ε_L and ε_H allows us to determine $G = 4n_{\text{eff}}^2\Omega_R^2/\omega_{\text{pl}}^2 = 4n_{\text{eff}}^2\eta^2$ and $\varepsilon_\infty = n_{\text{eff}}^2$. The reduced coupling strength is related to the oscillator strength of the transition. This useful connection simplifies screening for materials and metamaterials in the ultrastrong and deep strong coupling regimes. For example, excitons in semiconductors typically have $G \approx 10^{-4}$ – 10^{-3} (ref. ⁴⁵); ZnO, a semiconductor with very high oscillator strength, reaches $G = 0.01$ (ref. ⁴⁶). Plasmonic metamaterials modelled with the effective-medium theory have much higher oscillator strength, for example, $G = 2.7$ in ref. ⁴⁷. However, all materials fall at least an order of magnitude short of the maximum value of $G = 16.6$ that we observed.

The dielectric function allows us to calculate transmission and reflection spectra for thin slabs of plasmonic crystals that fully reproduce our optical spectra. The plasmonic crystal can be treated as an open cavity in which radiative damping occurs through cavity losses²², in addition to the intrinsic loss γ . To calculate the optical spectra, we treat the plasmonic crystal as a metamaterial of effective thickness h with dielectric function $\varepsilon_L(\omega)$ (or $\varepsilon_H(\omega)$) and permeability $\mu_{\text{pp}} = 1$. The optical transmittance and reflectance at normal incidence are then given by⁴⁸

$$T = \left| \frac{4n_{\text{pp}}e^{ihn_{\text{pp}}\omega/c}}{(1+n_{\text{pp}})^2 - (1-n_{\text{pp}})^2e^{2ihn_{\text{pp}}\omega/c}} \right|^2 \quad (6)$$

and

$$R = \left| \frac{(1-n_{\text{pp}}^2)(1-e^{2ihn_{\text{pp}}\omega/c})}{(1+n_{\text{pp}})^2 - (1-n_{\text{pp}})^2e^{2ihn_{\text{pp}}\omega/c}} \right|^2, \quad (7)$$

where $n_{\text{pp}} = \sqrt{\varepsilon_L} = \sqrt{\varepsilon_H}$. The model was used to calculate the optical absorption spectra in Extended Data Fig. 6 as $A = 100\% - T - R$.

Whereas the Hopfield and Lorentz models are equally suited to describe our experimental observations, the Hopfield Hamiltonian gives insight into the physical processes of light–matter coupling that the Lorentz model lacks²⁵. For example, the Hopfield Hamiltonian allows us to calculate the light and matter fraction of polaritons, the ground-state energy of the system and the population with virtual excitations¹. It also sets the framework for studying quantum effects in nanoparticle crystals, for example, squeezed light, polariton condensation and the generation of correlated photons from the quantum vacuum^{1,4,7,39,40}.

Microscopic model for collective plasmons

The plasmon polaritons of the nanoparticle crystals in Fig. 1 were calculated with the microscopic model of Lamowski et al.²⁴. We stress that all quantitative results reported in the paper, as well as the data analysis, were based on the Hopfield Hamiltonian, using ω_{pl}^t and Ω_R as free parameters. The microscopic model describes how polaritons emerge in nanoparticle crystals, starting from the dipole excitation of each sphere. In the following, we summarize the main features and findings of the microscopic description; see ref. ²⁴ for a full account.

The model considers an infinite three-dimensional crystal of plasmonic nanoparticles. The particles are described as spheres with diameter d , interparticle gaps a and the dielectric function $\varepsilon(\omega) = \varepsilon_\infty - \omega_p^2/\omega^2$ of a Drude metal, where $\hbar\omega_p = 9.07$ eV is the plasma frequency and ε_∞ the dielectric screening by the electrons of the metal. We discuss the applicability of all approximations made in this microscopic model below. The nanoparticle dipole plasmon with frequency

$$\omega_{\text{NP}} = \frac{\omega_p}{\sqrt{\varepsilon_\infty + 2\varepsilon_m}} \quad (8)$$

is described as a point dipole in the centre of a sphere with dielectric constant ε_∞ . This sphere is surrounded by a medium with dielectric constant ε_m . The Coulomb interaction between the plasmonic dipoles in the crystal creates collective plasmonic states described by a plasmonic band structure^{22,24} (see Extended Data Fig. 1)

$$\omega_{\text{pl}}(\mathbf{k}) = \omega_{\text{NP}}\sqrt{1 + g f(\mathbf{k})}, \quad (9)$$

with coupling factor

$$g = \frac{3}{4} \frac{3\varepsilon_m}{\varepsilon_\infty + 2\varepsilon_m} \left(\frac{d}{d+a} \right)^3 F_0, \quad (10)$$

where F_0 is the metal fill factor for vanishing gap size (see Supplementary Table 1). $f(\mathbf{k})$ is a dimensionless factor that depends on the crystal structure and plasmon polarization. It is calculated from the lattice sums of the plasmonic dipoles²⁴. The plasmonic band structure consists of transverse, $\omega_{\text{pl}}^t(\mathbf{k})$, and longitudinal, $\omega_{\text{pl}}^l(\mathbf{k})$, branches. The transverse plasmons $\omega_{\text{pl}}^t(\mathbf{k})$ couple strongly to light $\omega_{\text{pt}}(\mathbf{k})$, resulting in hybridized plasmon polaritons $\omega_{\text{pp}}(\mathbf{k})$, as described in the main text.

The coupling factor g may be related to the coupling of the transverse plasmon to the retarded photon field given by the Rabi frequency $\Omega_R = \omega_{\text{NP}}\sqrt{g}$. At first sight this appears surprising, but it results from the simple fact that both types of coupling (plasmon–plasmon and plasmon–photon) proceed through the dipole interaction.

From the above model we derive an analytical expression for the normalized coupling constant

$$\eta = \frac{\Omega_R}{\omega_{pl}^t} = \left[\frac{\varepsilon_\infty + 2\varepsilon_m}{3\varepsilon_m} \frac{4}{3F_0} \left(\frac{d+a}{d} \right)^3 + f_t(\mathbf{k}) \right]^{-1/2}, \quad (11)$$

which is plotted in Fig. 1c. $f_t(\mathbf{k})$ is the dispersion function $f(\mathbf{k})$ of the transverse plasmon branch.

The point-dipole approximation is valid for any ε_∞ and ε_m if $a \geq d/2$ or any d and a if $\varepsilon_\infty = \varepsilon_m = 1$ ($d \geq 10$ nm and $a \geq 1$ nm, because we neglect quantum size effects and electron tunnelling between particles). In our modelling we used $a \ll d$ and $\varepsilon_m > 1$ (1.96). To verify the quality of the point-dipole approximation under these conditions, we compared the polariton dispersions predicted by the analytical model with FDTD simulations. Extended Data Fig. 9a shows the dispersion for $d = 30$ nm, $a = 2$ nm, $\varepsilon_\infty = 1$ and $\varepsilon_m = 1.96$, as used in Fig. 1. The analytical model agrees well with the numerical simulations, confirming that the point-dipole approximation may be employed for this range of parameters. We note that no free parameters were used in preparing Extended Data Fig. 9; we simply plotted two independent calculations on top of each other. Close to the Γ point, the energy of the upper polariton branch is underestimated by the analytical theory. The discrepancy disappears when changing ε_m to 1 (Extended Data Fig. 9b). For completeness, Extended Data Fig. 9c presents the perfect agreement of the analytical dispersion with FDTD data for $a > d/2$ ($d = 30$ nm, $a = 20$ nm, $\varepsilon_\infty = 1$ and $\varepsilon_m = 1.96$).

A second aspect to consider is material losses. Although the analytical theory neglects losses, it accurately reproduces the polariton frequencies from FDTD simulations, in which intrinsic and radiative losses are included. Losses do not affect the Rabi splitting and normalized coupling strength, but they manifest in the spectral width of the peaks in the transmission, reflection and absorption spectra³⁵. Losses can be included in the polariton dispersion with a complex frequency component, as shown in Methods section ‘Hopfield and Lorentz models’.

Effect of crystal thickness on coupling strength

The experimental polariton dispersions in Fig. 3d were obtained from the optical far-field spectra of plasmonic crystals with varying thickness. When fitting the experimental data with the dispersion from the Hopfield Hamiltonian, we assumed that the coupling strength is independent of the crystal thickness. In this section we use simulations to show that the normalized coupling strength decreases by less than 10% when decreasing the thickness from 20 to 2 nanoparticle layers. Larger differences (16%) were observed in the experimental data, which we attribute to a decrease in the interparticle gap sizes with increasing crystal thickness.

Extended Data Figure 10a shows the simulated polariton dispersion for a gold nanoparticle crystal with $d = 50$ nm and $a = 2$ nm. The data points were obtained from fits of the absorption peaks in the simulated spectra and are colour-coded by the number of nanoparticle layers. The black line is a fit of the data points for 20 layers with the Hopfield dispersion (equation (1)), from which we obtain $\eta = 1.59$, $\hbar\Omega_R = 2.84$ eV and $\hbar\omega_{pl}^t = 1.79$ eV. Whereas the data points for 10 layers nicely track the fit for 20 layers, we observe a systematic shift of the data points to higher energies for smaller layer numbers. To analyse the coupling strength for smaller layer numbers, we reduce the number of fitting parameters from two (Ω_R and ω_{pl}^t) to one (Ω_R) by setting $\omega_{pl}^t = \sqrt{\omega_{NP}^2 + \Omega_R^2 f_t}$, using $\hbar\omega_{NP} = 2.2$ eV from the Mie theory and $f_t = -0.21$ from the fit for 20 layers. The normalized coupling strength drops with decreasing layer number, reaching $\eta = 1.45$ (8% decrease) for a bilayer (see inset of Extended Data Fig. 10a).

We repeated this analysis for the experimental data of the gold nanoparticle crystals with $d = 41$ nm (Extended Data Fig. 10b). The deviation

of the data points from the Hopfield fit was strongest for this dataset (compare blue circles and blue line in Fig. 3d). The black line in Extended Data Fig. 10b is a fit of the data points for five to seven layers. Similar to the simulations in Extended Data Fig. 10a, the data points for smaller layer numbers systematically shift to larger energies. The normalized coupling strength remains almost constant for four to seven layers ($\eta = 1.33$ for four layers and $\eta = 1.37$ for seven layers) and drops to $\eta = 1.14$ for a bilayer (see inset of Extended Data Fig. 10b). The decrease of the normalized coupling strength in the experiment (16%) is larger than in the simulations (8%). We believe that this is due to a decrease of the interparticle gap size with increasing thickness of the gold nanoparticle crystals used in our experiments.

The reduction in coupling strength for small layer numbers leads to an underestimation of η in Table 1, especially for the gold nanoparticle crystals with $d = 46$ nm where only 2–4 layers were analysed. For the gold nanoparticle crystals with $d = 41$ nm the coupling strength is $\eta = 1.37$ for seven layers, whereas a fit of the data points for all layers yields $\eta = 1.31$. The deviation is less than 5%. We conclude that, except for the $d = 46$ nm gold nanoparticle crystals, the uncertainty of η in Table 1 remains of the order of 10^{-2} .

Data availability

The measured and simulated optical spectra that support the current study are available at <https://doi.org/10.17169/refubium-26819>. Source data are provided with this paper.

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Author contributions S.R. and N.S.M. conceived and designed the research project. The gold nanoparticle crystals were synthesized by F.S. and H.L.; the characterization and optical experiments were performed by N.S.M., Y.O., F.S. and S.J. The analytical theory and FDTD simulations were developed by N.S.M., B.G.M.V., E.B.B. and S.R. The data were analysed by N.S.M., F.S. and S.R.; S.R. and N.S.M. wrote the initial draft of the manuscript, on which all co-authors commented.

Competing interests The authors declare no competing interests.

Additional information

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4.5 Plasmonic bimetallic two-dimensional supercrystals for H₂ generation.

Publication:

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In this study we utilized the concept of plasmonic supercrystals for plasmonic photocatalysis. One consequence of the deep strong light matter coupling described in the previous section is the decoupling of light and matter. At the polariton modes the electromagnetic field is located nearly exclusively in between the AuNP and not inside them, leading to high transmission and low ohmic losses. Due to the crystal geometry a high density of hot spots is created. In plasmonic photocatalysis the relative contribution of different effects is discussed: local heating, enhanced near-fields (as in hot spots) and energetic carriers like hot electrons which are created by plasmon relaxation. Together with Holger Lange I had explored carrier dynamics in AuNP with ultrafast spectroscopy also in this context during my PostDoc time in his group.²¹⁻²² A challenge in plasmonic catalysis is to design the plasmonic photocatalysts in a way that minimizes plasmonic losses.

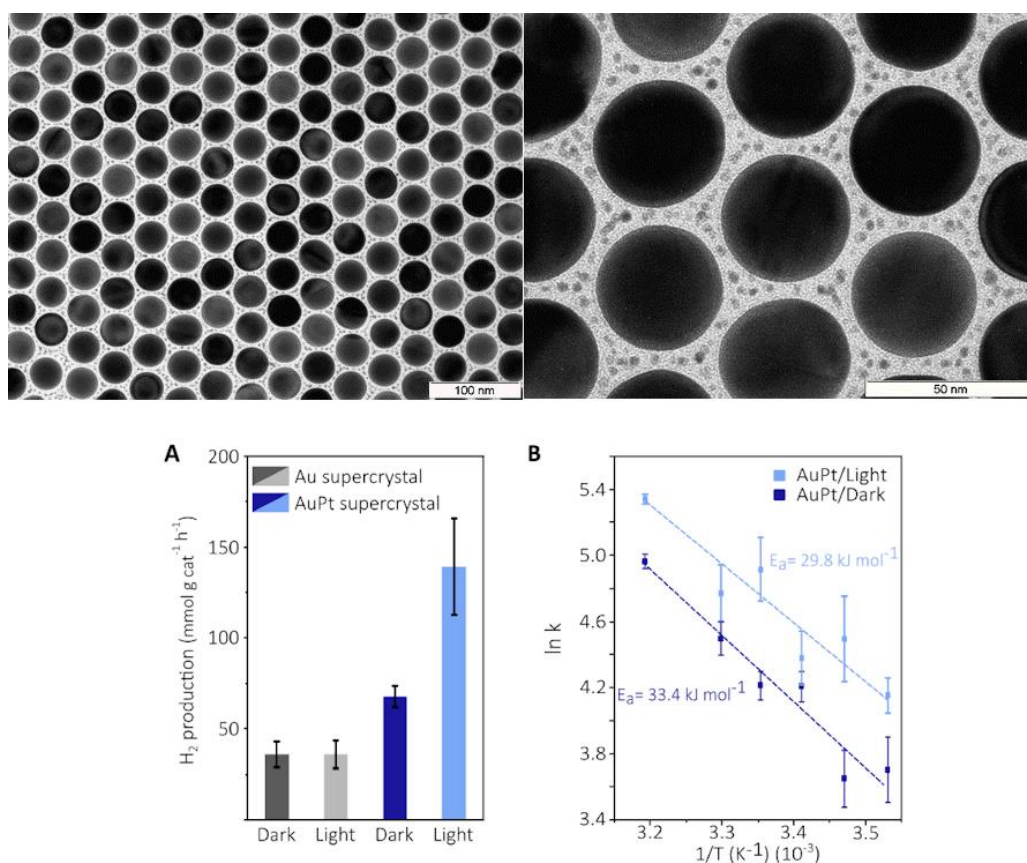


Figure 9. Exemplary TEM micrographs of the bimetallic metasurfaces used in this study and the analysis of their photocatalytic activity. The small platinum NP (PtNP, $d = 2.3$ nm) are located in the gaps of the 2D-hexagonal superlattice formed by the AuNP ($d = 22$ nm). For AuNP supercrystals no strong effect of illumination on catalytic activity was observed, but for the bimetallic supercrystals a twofold increase in hydrogen production was observed for formic acid

decomposition in water (A). Temperature-dependent measurements confirmed a change of activation energy due to the illumination (B).

Due to the strong absorption (and sometimes scattering) of plasmonic NP, the penetration depth of light into a dispersion can be limited, thus reducing catalytic efficiency. The other experimental challenge is the confident analysis of the different contributions mentioned above. Thin-film plasmonic supercrystals offer distinct advantages in this context: their geometry can be used to tailor and optimize light interactions and it also allows for a confident mechanistic analysis. The idea is to use a 2D-photocatalytic metasurface instead of a dispersion or a powder. To this end, we prepared well-defined bimetallic thin-film supercrystals consisting of AuNP ($d = 22$ nm) for strong light interactions throughout the optical range (plasmonic material) and small platinum NP (PtNP, $d = 2.3$ nm) as catalytically active material. We then quantitatively analyzed the photocatalytic formic acid decomposition with these materials leading to hydrogen formation. We observed a record high photocatalytic activity for this reaction with a plasmonic catalyst. However, stability and scalability still have to be addressed. We showed that the concept can also be used for other metals like palladium and that the geometry of the metasurfaces can be tuned (AuNP and catalytic NP diameter, gaps and loading of the catalytic NP). In the supercrystals, the AuNP still form a well-defined 2D-hexagonal structure and the smaller catalytic NP are positioned in the gaps between the AuNP but without contact to the AuNP. In this geometry the catalytic NP are intrinsically positioned within the hot spots created by the plasmonic lattice. Based on a quantitative optical characterization, simulations and transient absorption spectroscopy, together with wavelength dependent measurements of photocatalytic activity, we were able to confidently assign the high photocatalytic production to field enhancement effects which increase the absorption of the PtNP in the optical range. In these structures, thermal effects and hot electron transfer from AuNP to PtNP did not contribute dominantly to catalytic activity. This work demonstrates a boosted chemical reactivity emerging from collective effects and paves the way for the exploration of a completely new class of photocatalyst architectures.

Plasmonic bimetallic two-dimensional supercrystals for H₂ generation

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Sunlight-driven H₂ generation is a central technology to tackle our impending carbon-based energy collapse. Colloidal photocatalysts consisting of plasmonic and catalytic nanoparticles are promising for H₂ production at solar irradiances, but their performance is hindered by absorption and multiscattering events. Here we present a two-dimensional bimetallic catalyst by incorporating platinum nanoparticles into a well-defined supercrystal of gold nanoparticles. The bimetallic supercrystal exhibited an H₂ generation rate of 139 mmol g_{cat}⁻¹ h⁻¹ via formic acid dehydrogenation under visible light illumination and solar irradiance. This configuration makes it possible to study the interaction between the two metallic materials and the influence of this in catalysis. We observe a correlation between the intensity of the electric field in the hotspots and the boosted catalytic activity of platinum nanoparticles, while identifying a minor role of heat and gold-to-platinum charge transfer in the enhancement. Our results demonstrate the benefits of two-dimensional configurations with optimized architecture for liquid-phase photocatalysis.

Molecular hydrogen (H₂) will inevitably be a key player in the quest for clean and sustainable energy. Its high exothermic combustion makes it a suitable replacement for carbon-based fuels, thereby reducing the carbon footprint¹. Similar to what occurs in nature, capturing the energy from sunlight in H₂ bonds is a promising strategy to store and manipulate solar energy in further processes, and materials that facilitate this energy transduction are of great interest^{2–4}. In this regard, subwavelength metallic nanoparticles (NPs) offer unique possibilities for photocatalysis driven by localized surface plasmon resonances. Plasmonic metals such as gold, aluminium, silver, magnesium and copper display these resonances in the visible range of the electromagnetic spectrum, enabling them to efficiently absorb light in the spectral region of maximum solar irradiance⁵. This light–matter interaction results in intensified electric fields at the NP surface, highly energetic holes and electrons (hot carriers), and local

heat. Despite these outstanding optical properties, the plasmonic metals used so far exhibit comparatively low catalytic activities^{6,7}. Therefore, to take advantage of these remarkable optical properties in chemical transformations, plasmonic NPs were integrated into multicomponent nanostructures to produce photocatalysts with better performance with respect to the individual constituents^{8,9}. In particular, bimetallic structures consisting of catalytic and plasmonic metals combine strong absorbance of visible light and high reactivity^{10–12}. The term ‘antenna–reactor’ was coined for configurations in which the plasmonic and catalytic NP were placed in close proximity, but was also extended to arrangements in which the active materials either form islands or are dispersed as single atoms at the surface of the plasmonic antenna. This configuration presents the highest efficiency in converting light into reaction products compared with other configurations^{13–17}.

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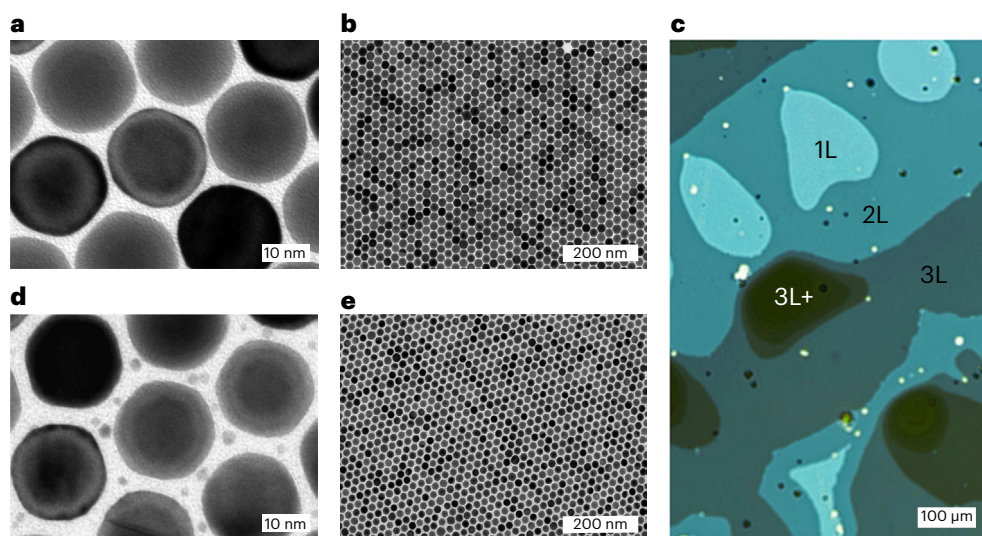


Fig. 1 | Microscopic characterization. **a, b**, TEM images of Au supercrystal made of 22 nm AuNPs at two different magnifications. The interparticle gap of the NPs is ~2 nm. **c**, Representative transmission microscopy image of the supercrystal. The different blue hues indicate different numbers of particle

layers. 1L, monolayer; 2L, bilayer; 3L, trilayer; 3L+, multilayer. **d, e**, TEM images of a bimetallic 2D AuPt supercrystal at two different magnifications. PtNPs (~3 nm) are hosted at the interparticle gap (~3.5 nm) between the 22 nm AuNPs. No interface is created between the different metals.

Despite the enhanced activity of these bimetallic photocatalysts, their performance in liquid environments faces several experimental challenges. For instance, concentrated solutions suffer from multiple scattering and absorption events, impeding the simultaneous activation of all nanostructures present by reducing the light penetration into the reactor down to hundreds of micrometres (~500 μm)¹⁸. This restricts the working range for catalyst concentration in solution and increases the challenge of manufacturing suitably engineered reactors. In addition, ligands providing stability to colloidal NPs can undergo photochemical desorption^{19,20}, resulting in uncontrolled aggregation of nanostructures leading to altered optical properties, sedimentation of the aggregates and less available surface for catalytic activity. Moving from colloidal suspensions to a film configuration is an appealing strategy to circumvent the limitations due to light penetration and aggregation. This approach allows for the collective excitation of every individual NP in a relatively fixed position, thus presenting a solution to the experimental limitations described above^{21–23}. In this regard, plasmonic two-dimensional (2D) supercrystals^{24–26}, devices in which the photoresponse is governed by the periodic organization of individual components in a plane^{27,28}, promote the transition from 3D to quasi-2D materials, aiming for better exploitation of the benefits of bimetallic photocatalysts. In this context, 2D expresses the expansion of the crystals in two dimensions. It is not limited to monolayers, but rather implies a defined number of layers in a given domain²⁹.

The proximity of the individual NPs in the 2D periodic array results in the formation of plasmonic hotspots—spatial regions characterized by enhanced electric fields—at the interparticle gaps^{30,31}. The intense electric fields sustained in these hotspots boost the otherwise weak absorption of the catalytic centre in the visible range^{32–34}, and stand out as an important factor to be modulated in the elusive interplay between the thermally driven and electric-field-driven photoactivity of plasmonic bimetallic catalysts^{15,35–39}. Thus, creating a large number of hotspots in densely packed supercrystals, which is unlikely to be achieved in colloidal solution or disordered catalysts, is expected to maximize the use of active metals for catalytic applications.

We achieved plasmonic structures by assembling individual gold nanoparticles (AuNPs) and platinum nanoparticles (PtNPs), obtaining highly ordered bimetallic supercrystals by a colloidal approach^{24,25,30}.

This fabrication methodology yielded 2D bimetallic photocatalysts in which AuNPs were assembled into a periodic hexagonal array and PtNPs were placed in the interparticle gaps of the AuNPs, scaling the preferred antenna–reactor configuration up to the mm^2 regime. Here we show that, when operated under white-light illumination and sunlight irradiance, the rationally designed bimetallic structures generate higher H_2 production than existing top performers for formic acid dehydrogenation. The remarkable activity was achieved with very low platinum loadings. The well-defined structure of the photocatalyst made it possible to spectrally resolve the plasmonic effects. Wavelength-dependent experiments and electrodynamic simulations revealed that the electric field enhancement in hotspots dictates the photoactivation of the PtNPs, which is also supported by transient absorption spectroscopy. Charge injection from gold to platinum, and photothermal contributions, were confirmed to play a minor role in photocatalytic enhancement. The results show that bimetallic 2D supercrystals are promising candidates for photocatalytic applications.

Results

Synthesis and characterization of 2D supercrystals

Figure 1 presents the microscopic characterization of the synthesized plasmonic supercrystals. The monometallic 2D supercrystal (Au supercrystal) that serves as reference/control sample was fabricated according to a previously reported method in which individual AuNPs are used as building blocks²⁵. The precise control of the synthesis of AuNPs, their surface chemistry and the self-assembly step resulted in a well-packed hexagonal array of 22 nm AuNPs with an edge-to-edge distance of ~2 nm (Fig. 1a). No crystalline arrangement other than hexagonal close packing of the large AuNPs was found in the sample when investigating different areas by transmission electron microscopy (TEM). The homogeneity of the structure over a large area is shown in the TEM image in Fig. 1b. These layers can extend up to a few square millimetres (Fig. 1c). In addition to monolayers, areas with multilayers also emerge during the crystallization process (Fig. 1c). The multilayers maintain the same crystalline phase²⁵, but result in different optical properties. With optical microscopy in transmission mode, we were able to identify the different layer numbers due to contrast difference, indicating the different sample thicknesses^{24,40}. One example of the Au supercrystal in transmittance is depicted in Fig. 1c. The lighter the blue,

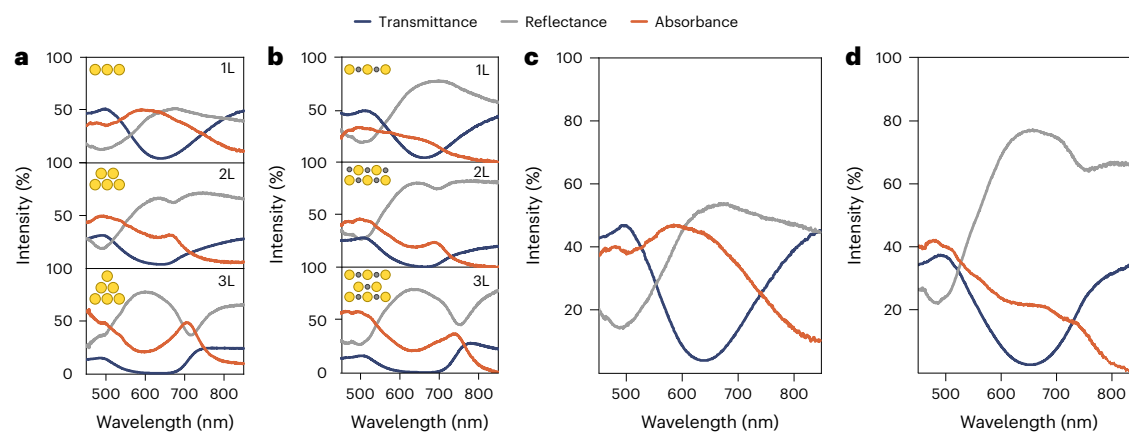


Fig. 2 | Optical characterization of the supercrystals. a, b, Experimental layer-dependent reflectance, transmittance and absorbance spectra of the pure Au supercrystal (**a**) and the bimetallic AuPt supercrystal (**b**). **c, d,** Weighted

reflectance, transmittance and absorbance spectra of the pure Au supercrystal (**c**) and the bimetallic AuPt supercrystal (**d**) from analysis of the composition of the different layer numbers.

the thinner the supercrystal. The unambiguous colour contrast was utilized to measure the areas of the different layer numbers.

The colloidal approach to supercrystal fabrication was extended to bimetallic structures comprised of AuNPs and PtNPs. Through the same preparation methodology as used for Au supercrystals, we managed to assemble both 22 nm AuNPs and ~3 nm PtNPs simultaneously, yielding a bimetallic supercrystal (AuPt supercrystal). The integration of platinum, a catalytically highly active metal, realized a supercrystal with combined optical and catalytic properties. In this composition the AuNPs dominate the optical behaviour, whereas the PtNPs act as the active site for chemical reactions. The homogeneity and periodicity of the supercrystal was not affected by the presence of PtNPs (Fig. 1e). A higher-magnification TEM image revealed that during the assembly step the PtNPs were placed at the interparticle gaps between the AuNPs, which increased the gap size to 3.5 nm (Fig. 1d and Supplementary Figure 1). At this point, it is important to emphasize that the small PtNPs were located in the interstices (gaps) of the 2D hexagonal array of AuNPs and no direct contact exists between the two components of the bimetallic AuPt supercrystal. This structure is acknowledged to be an antenna-reactor configuration. The crystallinity of both AuNPs and PtNPs and the material distribution were confirmed by high-resolution TEM and energy-dispersive X-ray spectroscopy (Supplementary Figure 2).

The tunability of physical parameters, such as AuNP size, PtNP loading, interparticle gap size and number of layers, was confirmed by assembling larger particles with longer thiolated polystyrene (PSSH) ligands, which yielded similar results (Supplementary Fig 3). The assembly methodology was also proven to work for different catalytic metals, as shown by replacing PtNPs with palladium NPs (Supplementary Figure 4). Hence, the developed approach enables the creation of bimetallic supercrystals with a desired antenna-reactor combination, spanning several square millimetres consistently, while providing a broad range of tunable physical parameters. For a detailed explanation of the manufacturing process, see Methods.

To investigate the optical properties of the indium tin oxide (ITO)-supported Au and AuPt supercrystals, microtransmission and reflection measurements were performed on both supercrystals within the spectral range of 450–850 nm. The spectra were taken in both cases on monolayers, bilayers and trilayers (1L, 2L, 3L). The absorbance (A) was calculated afterwards as the difference between the transmitted (T) and reflected (R) light (Methods). The resulting spectra for the pure gold supercrystals are shown in Fig. 2a and the spectra for the bimetallic sample in Fig. 2b. The obtained experimental spectra are in good agreement with the finite-difference time-domain (FDTD) simulations of both supercrystal structures (Supplementary Figure 5).

Table 1 | Compositional analysis by optical microscopy for Au and AuPt supercrystals

Sample	Monolayer (1L)	Trilayer (3L)	Multilayer (3L+)
Au supercrystal	81%	16%	3%
AuPt supercrystal	61%	9%	30%

For the Au and AuPt supercrystals, the monolayer spectrum is dominated by interband transitions of the gold³¹. For bilayers and trilayers, the characteristic polaritonic modes that occur due to deep strong light-matter coupling start to appear between 650 and 750 nm (ref. 24). The energetic position of these polaritonic resonances depends on the film thickness, the diameter of the particles, the gap size and the dielectric environment^{24,30}. Therefore, the small redshift of the bimetallic spectra with respect to the resonances presented by the monometallic supercrystal can be explained by the larger gap size, and the changes in the refractive index of the background are due to the presence of PtNPs. These interesting optical properties presented by the supercrystals are intricately related to the photocatalytic activity of the bimetallic structures.

As mentioned above, the colour contrast of the supercrystal layers imaged with an optical microscope in transmission mode made it possible to estimate the size of the domains. Thus, it was possible to calculate the overall optical response of the samples by weighting the experimental response of the individual layers by the different areas. It is important to know the ratios of the various layers because during the catalysis experiments a large area of the sample will be illuminated containing different numbers of layers. The details of the sample composition are given in Table 1. The study revealed that monolayers with ~80% coverage for the control sample and ~60% for the AuPt supercrystal make up the main part of the samples. The bimetallic sample also has a notable amount of trilayers and multilayers (~30%), whereas the monometallic sample only consists of ~3% multilayers.

The weighted optical behaviour of the control and AuPt sample are depicted in Fig. 2c,d. As expected, due to the large contribution of monolayers the overall response of the ITO-supported supercrystals was dominated by the interband transitions of the gold. In contrast to the pure Au supercrystal, the optical response of the AuPt sample shows an influence of the multilayers. Two shoulders are clearly visible in the absorbance spectrum at 671 and 732 nm due to the presence of the polaritonic modes in the bilayers and trilayers. Once the optical response of the samples was determined, they were tested as catalysts for formic acid dehydrogenation.

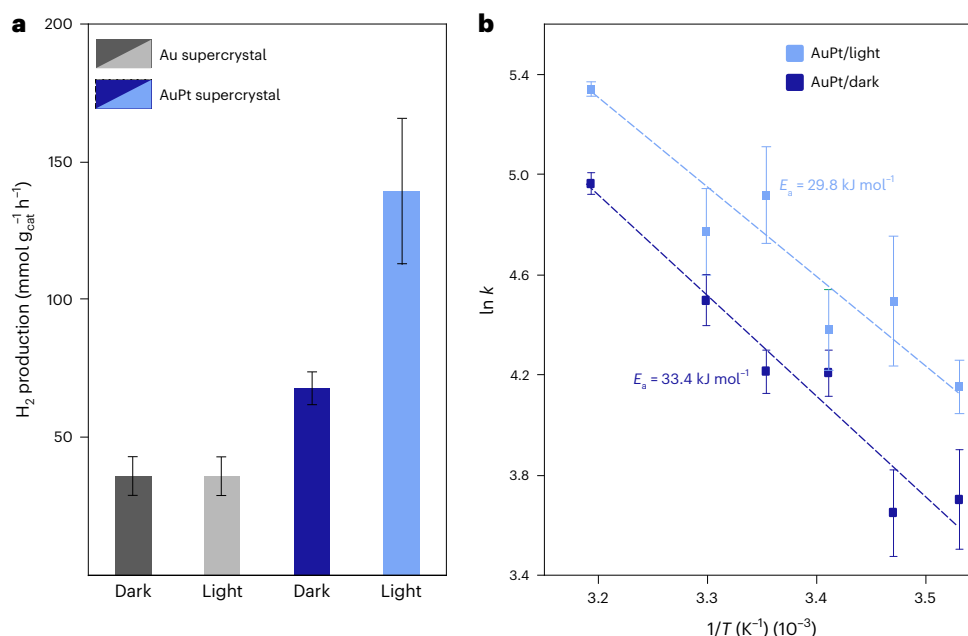


Fig. 3 | Photocatalysis performance on formic acid conditions. a, H₂ generation rate normalized by the total mass of catalyst in both conditions, dark and light, for Au and AuPt supercrystals. **b**, Arrhenius plot for AuPt supercrystal. E_a was reduced from 33.4 kJ mol⁻¹ to 29.8 kJ mol⁻¹ upon white-light illumination.

Irradiance used in all experiments, ~110 mW cm⁻², $T = 25^\circ\text{C}$. Data are presented as the mean values of at least three independent measurements. Error bars represent the s.d.

Photocatalysis

The photocatalytic power of the resulting supercrystals was investigated by testing their performance on formic acid decomposition without any further additives. The probe reaction was chosen based on the lower activity of gold versus platinum in this context, and also because it is a carbon-neutral H₂ carrier^{1,41,42}. The experiments were conducted in the absence (dark) and presence of light and repeated at least three times each to ensure reproducibility. It has been shown that the probe redox reaction can follow two different pathways, namely, dehydrogenation or dehydration. The former produces H₂ and CO₂ and is favoured thermodynamically ($\Delta G = -48.4$ kJ mol⁻¹), whereas the latter produces H₂O and CO ($\Delta G = -28.5$ kJ mol⁻¹)¹. CO was not detected throughout our experiments, excluding dehydration. For these studies a broadband lamp, equipped with an ultraviolet long-pass filter ($\lambda > 400$ nm) and an infrared filter, was used as a source of light, to work exclusively in the visible range of the spectrum and rule out heat generated from solvent absorption. An external cryostat set at 25 °C was used to control the temperature during the reaction. The experiment was set up for the reactor to be illuminated with ~110 mW cm⁻², which corresponds to sunlight irradiance. The stirring conditions helped to reduce any temperature gradient⁴³. Further details on the experimental set-up and conditions are given in Methods.

Figure 3a shows the performance of ITO-supported gold and AuPt supercrystals when tested for formic acid decomposition. The mass of the catalysts was obtained by inductively coupled plasma mass spectrometry (ICP-MS), while the preservation of the optical properties was monitored by optical spectroscopy (Supplementary Figs. 8 and 9). Details of the normalization are provided in Supplementary Fig. 6 and Supplementary Table 5. The Au supercrystal presented an activity of 36 mmol g_{cat}⁻¹ h⁻¹ and a negligible enhancement upon illumination. Conversely, the inclusion of small amounts of PtNPs in the supercrystal increased the performance of the binary AuPt supercrystal up to 67 mmol g_{cat}⁻¹ h⁻¹. When illuminated with white light, the performance was increased by approximately a factor of two, reaching 139 mmol g_{cat}⁻¹ h⁻¹. The improved performance of platinum under illumination in the experiment suggests that the interaction of the

incoming light with the gold array results in energized platinum, useful for catalytic purposes. Indeed, the AuPt supercrystal appears to be the best plasmonic performer when formic acid is used as the H₂ carrier⁸. Additionally, to assess the boost in PtNP performance, the activity of the AuPt supercrystal was only normalized by the platinum mass and compared with a platinum control (Supplementary Fig. 7), evidencing its better use when incorporated into the 2D supercrystal (Supplementary Fig. 10).

Temperature-dependent experiments were conducted to evaluate the activation barrier (E_a)¹⁶. We first investigated the temperature-dependent activity of the Au supercrystal versus the AuPt supercrystal over the 10–40 °C temperature range, without illumination. As expected, the bimetallic sample always presented a larger activity (Supplementary Fig. 11a). While the activation barrier for the Au supercrystal was 40.7 kJ mol⁻¹ (Supplementary Fig. 11b), the one for the AuPt supercrystal was 33.4 kJ mol⁻¹ (blue curve in Fig. 3b), evidencing the superior activity due to the platinum. The same temperature-dependent study was carried out again, but now comparing the activity of AuPt supercrystal in dark and light over the same temperature range. Interestingly, we found that the H₂ production was always enhanced across the range upon light assistance, although the enhancement factor was not constant. Despite this, the use of white light for photocatalysis resulted in a 10% decrease in the activation barrier for the Pt–FA interface (Fig. 3b and Supplementary Fig. 12). All these experimental results highlight the synergy between the optical properties of the hexagonal array of 22 nm AuNPs and the interstitial PtNPs for sunlight-driven H₂ production. A similar trend was observed for the same system with 39 nm AuNPs (Supplementary Fig. 13). In the next section, we intended to disentangle the plasmonic contributions to the improved performance of AuPt with respect to H₂ generation.

Mechanistic insight

The superior photocatalytic activity of the AuPt clearly suggests that PtNPs benefit from the optical properties of the supercrystal. Several possible contributions are discussed for plasmonic photocatalysis with mono- or bimetallic systems: enhanced near fields, thermalized

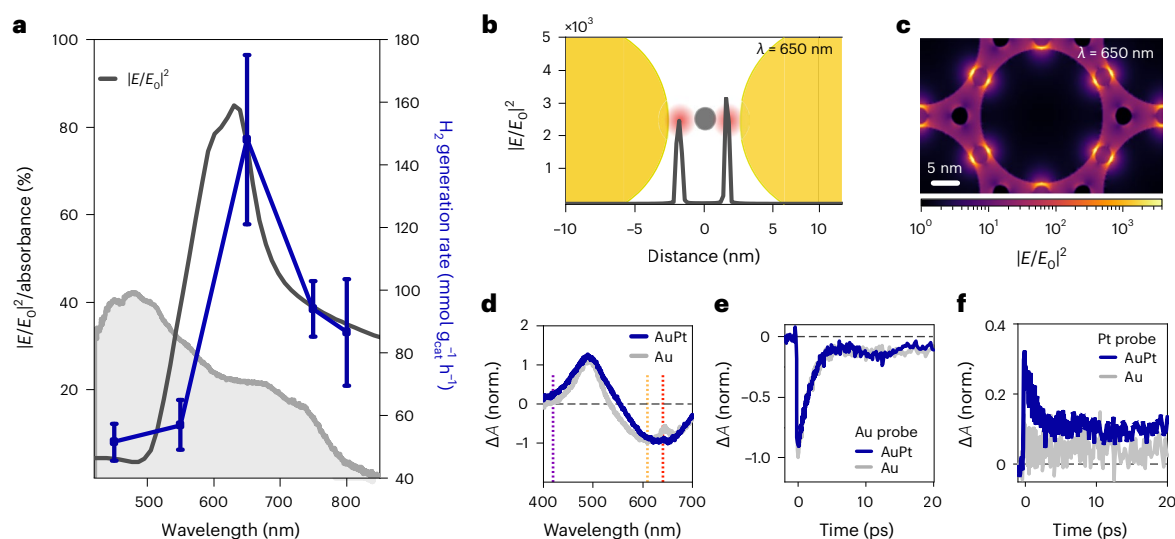


Fig. 4 | Mechanistic insight of AuPt supercrystal performance on formic acid decomposition. **a**, Wavelength-dependent H_2 production (blue) plotted simultaneously with the electric (E) field strength (black) in the hotspots within the visible range and the weighted absorbance (grey). The photoactivity of the films peaks at 650 nm. Data are presented as the mean values of at least three independent measurements. Error bars represent the s.d. **b**, Weighted electric field intensity in between two AuNPs with a PtNP located in the centre of the gap. The asymmetry of the electric field to the left and right side of the PtNP is due to numerical inaccuracy. **c**, Weighted electric field intensity map of a bimetallic

supercrystal. **d**, Transient absorption (ΔA) spectra of Au and AuPt supercrystals when pumped at $\lambda = 650$ nm. Orange and red dashed lines, gold probing wavelengths; purple dashed line, platinum probing wavelength. **e**, Kinetics in AuNPs on Au and AuPt supercrystals at the probing wavelengths. $\lambda_{\text{pump}} = 650$ nm. No differences are observed in plasmon decay, suggesting no modification in hot electron dynamics upon platinum inclusion. **f**, Kinetics at $\lambda_{\text{probe}} = 420$ nm. $\lambda_{\text{pump}} = 650$ nm. Additional contrast is presented in the bimetallic supercrystal, indicating platinum activation.

hot carriers, non-thermalized hot carriers and local heating^{44–47}. Hence, unravelling the dominant energy pathway improving PtNP performance at the interparticle gap of the gold array is crucial to fully exploit the advantages of such structures. A set of experiments was conducted to gain insight into the PtNP activation due to the interaction between the incoming light and the supercrystal. Figure 4 summarizes our efforts to elucidate the plasmonic contributions to the increased reaction rates.

First, wavelength-dependent catalytic experiments were performed on the bimetallic supercrystal, which made it possible to discriminate the photocatalyst response in different spectral regions⁴⁸. For these tests, the broadband lamp was equipped with bandpass filters centred at 450, 550, 650, 750 and 800 nm, with a bandwidth of 20 nm. The power (5 mW) was corrected for each wavelength to conduct the experiments with a constant irradiance and to match the power used previously with unfiltered light. The resulting wavelength-dependent H_2 generation rates are shown in Fig. 4a, plotted together with the simulation of the enhanced electric field intensity at the hotspot for the topmost gold layer within the same spectral range, and the weighted absorbance. The relevance of hotspots in bimetallic plasmonic structures for photocatalysis has been proven for different systems^{15,35}. Only the top layer of each domain was considered in the simulations, as this is where the catalysis mainly occurs, as we will discuss in the following paragraph. The average electric field enhancement at the hotspots was calculated by FDTD simulations (Methods). The simulations were evaluated in the centre of an interparticle gap between two gold particles because this is the location where we expect the PtNPs to exhibit the highest photocatalytic activity due to the strongest hotspots existing there.

As can be observed, the photoactivity of the AuPt supercrystal correlates with the electric field enhancement: the reaction rate peaks at the enhancement maximum. Based on this result, it can be inferred that at around 650 nm, the plasmonic components are funnelling more energy to the PtNPs, enhancing their absorption, and thus increasing the generation of excited carriers at the active metal which contribute to the redox reactions^{9,15,35}. This manifests as an improved performance

in catalysis at that wavelength. Concomitantly, the maximized reaction rate does not coincide with the maximum absorbance (Fig. 2). This is an indication that the experimentally observed enhancement upon illumination is not primarily influenced by heat generated upon absorption by the supercrystals, but rather that the electric field enhancement facilitates the reaction. If heat had caused the rate increase, a larger enhancement would have been expected at shorter wavelengths, where the absorbance is higher (Fig. 4a)⁴⁹. The sample fabrication process yields 2D supercrystals with monolayer, bilayer and multilayer domains (Table 1). Therefore, it is worth investigating how the electric field enhancement depends on the number of layers. We simulated the spatial distribution of the electric field enhancement in the third dimension for different numbers of layers illuminated at 600 nm excitation wavelength (Supplementary Figure 14), the spectral range where we saw the highest catalytic activity. Consistent with Mueller et al.³⁰, it is almost exclusively the topmost layer that is plasmonically activated, giving a strong electric field that boosts the catalytic process. This is due to the plasmonic stop band, which unlike the polaritonic modes, does not allow the light to enter the supercrystal^{24,30}, maximizing the field enhancement at the topmost layer.

Additional optical simulations were performed to screen the electric field enhancement across the gap (in the x,y plane) when exciting the AuPt supercrystal in and out of the field enhancement maximum at 550, 650 and 800 nm (Supplementary Figure 15), covering the wavelength range used in photocatalytic experiments. While the presence of PtNPs led to an electric field intensity enhancement of 10^3 times, a superior confinement is achieved at 650 nm (Fig. 4b). The intensity of the weighted electric field at 650 nm was almost three times larger than the intensity at the other wavelengths (Fig. 4a and Supplementary Figure 15). Figure 4c depicts the lattice unit cell of the FDTD simulations, showing the PtNPs in the strong hotspots of the AuNPs where they are responsible for the enhancement and therefore for the high photocatalytic activity. It is important to emphasize that Fig. 4a was simulated without PtNPs in the gap, while Fig. 4b,c was calculated with PtNPs in the gaps between the AuNPs, leading to different electric field

enhancements. However, both kinds of simulation show the strong enhancement at 650 nm and the same enhancement ratio between different wavelengths. The electric field enhancement simulations are in line with the experimental data. Interestingly, the H_2 generation rate at 800 nm was 1.6 times larger than at 550 nm, even though the absorbance at lower energy was negligible compared to the one at 550 nm (Fig. 2). This further supports our interpretation that heat is not the main plasmonic contribution to the enhanced performance of PtNPs.

Studying the plasmon relaxation of the excited sample makes it possible to gain an insight into the interaction between the constituents of the samples, and to precisely evaluate tunnelling from the plasmonic to the catalytic component driven by the enhanced electric field^{50,51}. To this end, the electronic dynamics of AuPt and Au supercrystals, and the platinum control, were investigated by transient absorption spectroscopy. These results complemented the bulk wavelength-dependent experiments and optical simulations.

The resulting normalized transient absorption spectra of both Au and AuPt supercrystals, when pumped at the largest photoactivity ($\lambda = 650$ nm), are shown in Fig. 4d. The red ($\lambda = 610$ nm) and orange ($\lambda = 640$ nm) dashed lines correspond to the probing wavelengths for the kinetics in the Au and AuPt supercrystals, respectively. These wavelengths were selected as they correspond to the center wavelength of the plasmon-related resonance (bleach) in each system, and thereby give access to information about the relaxation of excited carriers in gold⁵². It is worth mentioning that the emergence of polaritonic modes does not affect the electron dynamics in each individual AuNP^{52,53}. The dynamics at the plasmon bleach are displayed in Fig. 4e. Essentially no changes in the dynamics were observed, suggesting the absence of meaningful gold-to-platinum charge transfer when exciting the optically active layers in the wavelength regime under investigation⁵⁴. The similarities in the kinetics also make it possible to neglect the charge transfer through the conductive ITO substrate. The immediate onset of additional contrast in the platinum spectral region of the hybrid material points towards a direct excitation of platinum when the system is excited at 650 nm (Fig. 4f). Plain PtNPs show no contrast under these excitation conditions (Supplementary Figs. 16 and 17). The fact that this larger contrast is only observed for the AuPt supercrystal at wavelengths where stronger fields act on the PtNPs is an indication of enhanced absorption processes due to the strongly confined electric field. This probably corresponds to increased d -to- s transition rates within platinum^{32,33}. Even when pumping the 2D supercrystals at 400 and 500 nm, the spectral region at which interband transitions dominate (Supplementary Fig. 18), no notable differences between the materials and no strong indication of platinum excitation is observed. This further supports the hypothesis that the electric fields in the hotspots drive the reaction.

The evidence presented in this section sheds light on the mechanisms for harnessing light to boost the performance of plasmonic-catalytic nanoreactors. Our findings indicate that by incorporating PtNPs into the interparticle gaps of hexagonally arrayed 22 nm AuNPs, it is possible to turn a catalyst with weak visible light absorption into an active sunlight-type photocatalyst via enhanced excitation. From our experiments, we have effectively determined that the improved photoactivity is a consequence of the interaction of strongly confined electromagnetic fields (hotspots) with interstitial PtNPs, whereas plasmonically generated heat, and charge injection from gold to platinum, appear in comparison to have minimal effect on the reaction rate enhancement. As such, and according to our pump–probe results, the main contribution of the plasmon in this hybrid structure is the activation of the catalytic sites (platinum) via the plasmonic (gold) enhanced near-fields. The increased performance of PtNPs upon illumination can be attributed to the generation of excited carriers (holes and electrons) participating in the redox cycle of formic acid dehydrogenation. The light-enhanced reaction rate is primarily due to the cleavage of the C–H bond, which represents the rate-limiting step of the reaction^{11,15,46,55}.

These results highlight that accurately positioning catalytic metals in optical hotspots is a crucial parameter to modulate in these types of bimetallic photocatalysts. Various parameters, ranging from interparticle distance, constituent size or even the number of layers, can be modified to achieve greater electric fields at the hotspots, which in turn will impact the performance of these optocatalytic structures.

With these established design rules, efforts will be directed towards inexpensive and sustainable devices, which can be achieved with both Earth-abundant plasmonic (that is, copper, aluminium, magnesium) and catalytic (that is, iron) metals^{17,56}, and the development of supports preserving the achieved supercrystal structure (that is, mesoporous silica). In addition to formic acid dehydrogenation, we anticipate that the underlying physical principles described in our work will be exploited towards other important chemical transformations, such as CO_2 reduction or carbon–carbon coupling^{57,58}.

Conclusions

In this study, we introduce a plasmonic bimetallic supercrystal in which we achieved the desired antenna–reactor configuration from colloidal suspension. The size of the supercrystal can be extended up to several square millimetres while maintaining the configuration, and monolayer, bilayer and multilayer domains can be achieved. We prove the synergistic effect of plasmonic-catalytic components in supercrystals by observing a two-fold increase in the rate of generation of H_2 in the formic acid dehydrogenation reaction at solar irradiances. By using well-defined supercrystals, we were able to distinguish the plasmonic contributions to the catalytic enhancement. Our findings reveal that the performance of the PtNPs is primarily dictated by the intensity of the electromagnetic fields at the hotspots, whereas thermal contributions and charge injection from AuNP into the catalytic centre have a lower impact on the reaction enhancement.

Methods

Materials

Tetrachloroauric(III) acid ($\geq 99.9\%$ trace metals basis), hexadecyltrimethylammonium bromide ($\geq 98\%$) and chloride ($\geq 98\%$), L-ascorbic acid (reagent grade), chloroplatinic acid solution (8 wt% in H_2O), sodium citrate trihydrate ($\geq 99\%$), sodium borohydride ($\geq 98\%$) and ITO-coated glass slides (surface resistivity, $8\text{--}12\ \Omega\ \text{cm}^{-2}$) were from Sigma-Aldrich. Toluene ($\geq 99.5\%$), tetrahydrofuran ($\geq 99.5\%$) and ethanol (denatured, $>96\%$) were from VWR. Diethylene glycol (DEG, reagent grade) was from Merck. PSSHs (PSSH2k: $M_N = 2,000\ \text{g mol}^{-1}$, $M_W = 2,300\ \text{g mol}^{-1}$; PSSH5k: $M_N = 5,300\ \text{g mol}^{-1}$, $M_W = 5,800\ \text{g mol}^{-1}$; PSSH10k: $M_N = 11,500\ \text{g mol}^{-1}$, $M_W = 12,400\ \text{g mol}^{-1}$) were from Polymer Source. All reagents were used without further treatment. H_2 standards (100, 500 and 1,000 ppm) were purchased from Linde Gas. Ultrapure water with a resistivity of $18\ \text{M}\Omega\cdot\text{cm}$ was used in all experiments.

Synthesis of AuNPs

AuNPs were synthesized based on the seeded growth protocol from Zheng et al.⁵⁹ and functionalized with PSSH as described previously²⁵. Several batches were prepared: AuNPs with a core diameter of $\langle D \rangle = 22.0 \pm 1.0$ nm and a particle concentration of $c = 9.5\ \text{nM}$ were functionalized with PSSH2k; AuNPs with $\langle D \rangle = 39.0 \pm 1.0$ nm and $c = 2.4\ \text{nM}$ and AuNPs with $\langle D \rangle = 38.7 \pm 0.9$ nm and $c = 2.0\ \text{nM}$ were functionalized with PSSH5k; and AuNPs with $\langle D \rangle = 35.5 \pm 1.0$ nm and $c = 2.6\ \text{nM}$ were functionalized with PSSH5k and with PSSH2k.

Synthesis of PtNPs

PtNPs with diameters of 2.7 ± 0.6 nm were synthesized as follows: 500 μl of H_2PtCl_6 0.05 M were added to 8.6 ml of ultrapure water (stirred at 500 r.p.m.), succeeded by the addition of 500 μl of sodium citrate 0.1 M, which served as the stabilizing agent. The platinum precursor was reduced upon rapid addition of 500 μl of NaBH_4 0.015 M, evidenced

by the dark brown colour of the final solution. Finally, the solution was left undisturbed for 10 min⁶⁰.

The as-synthesized PtNPs were functionalized with PSSH2k by phase transfer as described in a previous report⁶¹. In detail, the aqueous PtNP dispersion (1,000 μ l) was mixed with PSSH2k (1,100 μ l, 0.18 mM) in toluene and ethanol (1,000 μ l). After vigorous shaking, phase separation was facilitated by addition of a few milligrammes of sodium chloride. The toluene phase with the PtNPs was then carefully removed and used for self-assembly experiments without further purification. The PtNPs had a diameter of 2.7 ± 0.6 nm and the platinum concentration in the final dispersion in toluene was 9.6 mg l^{-1} , corresponding to 65 nM particle concentration.

Self-assembly

Self-assembly of pure and mixed samples of polystyrene-functionalized NPs took place on DEG as a liquid subphase as described previously^{25,62}. Different volume ratios of AuNP@PSSH and PtNP@PSSH2k were pipetted onto the DEG and the sample cell was covered with a glass cover-slip to slow down the evaporation of the toluene. After 16–24 h crystalline films had formed, which were carefully skimmed off with TEM grids or transferred to ITO substrates for catalysis experiments. Crystalline films with 2D hexagonal symmetry and incorporated PtNPs formed under all tested conditions (Supplementary Figs. 1 and 3). The samples differed in platinum loading and the resulting interparticle spacing. For the controls, just AuNP@PSSH or just PtNP@PSSH2k were used accordingly.

TEM

The TEM measurements for standard characterization were performed using a JEOL JEM-1011 instrument operating at 100 kV. Samples of self-assembled films were carefully skimmed off with a carbon-coated copper grid held by a tweezer. The grid was then dried in vacuum for at least 1 h. Quantitative analyses of size distributions and nearest-neighbour distances based on TEM measurements were performed with ImageJ v.1.50i. The indicated mean values are obtained from analyses of at least three images from different spots on the samples.

Layer identification and area calculation

The identification of the layer number and the size calculation of the different layers was done with an optical microscope (Nikon Eclipse LV 100) equipped with a microscope camera (Nikon DS-Ri2). The different contrasts in the transmission images enabled identification of the layers. Subsequently, the layer sizes were measured using Nikon NIS-Elements software.

Optical spectroscopy

A home-built microabsorbance set-up was used to measure the optical spectra of both Au supercrystals and AuPt supercrystals. A supercontinuum laser (NKT-FIU15) with a broad spectral range was used as the light source. The light was guided to an inverse microscope (Olympus IX71) equipped with a 100 $\times$ objective (numerical aperture, 0.9) that focuses the light onto the sample, and a second 100 $\times$ objective (numerical aperture, 0.8) to collect the transmitted light (T). Via an optical fibre the collected light was guided to the spectrometer (Avantes AvaSpec 3648). For the reflectance measurements the reflected light (R) was guided through two beam-splitters to a collector lens that was connected via an optical fibre with the Avantes spectrometer. Afterwards, the absorbance (A) was calculated by $A = 100\% - R - T$.

FDTD simulations

For the FDTD simulations the commercial Lumerical FDTD Solutions software package was used as described in refs. 24,31,40. Spherical AuNPs with a diameter of 22 nm and interparticle gaps of 2 nm were packed into a hexagonal lattice. To simulate the different crystal layers one to three layers were stacked in the ABC sequence of an fcc crystal.

The cell size of the mesh was set to 0.2 nm to accurately model the geometry of the supercrystal. The dielectric function of gold was determined by fitting the experimental data from Olmon et al.⁶³. For the ligand molecules (polystyrene), a dielectric medium with refractive index $n = 1.4$ was assumed to fill the space between the gold particles. The lattice unit cell of the crystal was chosen as the FDTD simulation region with periodic boundary conditions. A broadband plane-wave source along the z direction was used as excitation source. The reflectance (R) was recorded with a monitor behind the source while the transmittance (T) was measured with a monitor behind the supercrystal. The absorbance (A) was subsequently calculated by $A = 1 - R - T$. For the AuPt simulations, 3 nm platinum particles were added in the gaps of the AuNPs and the gap size was changed to 3.5 nm. The dielectric function of the platinum was determined by fitting the experimental data from Palik⁶⁴. All the other parameters were kept the same. To simulate unpolarized light, every simulation was performed twice, once with x -polarized light and once with y -polarized light. Subsequently, the mean value was taken to represent unpolarized light. For the simulations of the electric field enhancement, the simulated spectra of the topmost layer for 1L, 2L and 3L were summed up and weighted by the percentage of the area (Table 1) to simulate the case of the photocatalysis experiments.

Catalysis

The catalytic experiments were performed in a clean 15 ml reactor containing a flat quartz window (diameter, 1.22 cm) and a rubber septum for sampling. The samples (supercrystals on ITO) were glued to a sample holder facing the quartz window, ensuring a complete and homogeneous illumination. The reactor is fitted with a water jacket where water was pumped in and out, allowing accurate control of the temperature for the duration of the experiments using an external cryostat ($\pm 0.5^\circ\text{C}$). The reaction mixture was stirred at 550 r.p.m. using a magnetic bar. The catalysis time was set to 1 h and a renewed degassed reaction mixture (N_2 for 30 min) was used in all the experiments. The reactor was thoroughly rinsed for every new experiment. For illumination, a commercial broadband arc lamp (Newport, 66921) was used; the ultraviolet and infrared contributions were filtered out by a long-pass filter ($\lambda > 400 \text{ nm}$) and with an infrared liquid filter (6123NS), respectively. The power was determined using a commercial thermal detector (Thorlabs, S401C). For gas detection, we used a gas chromatograph (Perkin Elmer, Clarus 590 GC) equipped with both a flame-ionization detector to detect organic traces and a thermal conductivity detector for H_2 detection. In addition, the flame-ionization detector was coupled to an H_2 generator (Perkin Elmer, NM plus H2 100).

ICP-MS

Elemental mass concentrations were measured with an Agilent 7700 ICP-MS set-up. The calibration curves were prepared with corresponding elemental standards (Carl Roth). Film samples on ITO were dissolved overnight in 200 μ l freshly prepared aqua regia and then diluted to 4,000 μ l with 2% hydrochloric acid. To determine the PtNP concentration, 50 μ l of the dispersion was dried, dissolved in 240 μ l aqua regia overnight and then diluted to 2,500 μ l with 2% hydrochloric acid. The elemental concentrations in these solutions were determined and used to calculate the according masses.

Transient absorption measurements

Transient absorption data were obtained using an Ultrafast Systems Helios transient absorption spectrometer. The system was seeded with 800 nm, 35 fs laser pulses generated by a mode-locked titanium:sapphire oscillator (SpectraPhysics, MaiTai) and amplified by chirped pulse amplification (SpectraPhysics, Spitfire Ace) operating at 1 kHz. The output of the amplifier was split in two arms: one arm was used to generate pump pulses of varying wavelengths, either using an optical parametric amplifier (Light Conversion, TOPAS Prime) or by simple frequency doubling in a beta barium borate crystal. Every

second one of these pump pulses was blocked by a chopper operating at 500 Hz before they were focused onto the sample, which was fixed in space using a 3D translation stage. The spot diameter of the pump pulses was 500 μm ($I = I_{\text{max}} \times 1/e^2$). Using a neutral density filter, the fluence of the pump was adjusted to 150 $\mu\text{J cm}^{-2}$. The second arm was led through a delay stage and then used for white-light generation in a CaF crystal. The generated white light was then focused onto the sample with a spot diameter of 250 μm ($1/e^2$). Spectra were recorded for varying delay times in transmission with an average time of 1 s. Differential spectra were obtained by subtracting the pumped and unpumped spectra for each delay time. Afterwards, the differential spectra were corrected for chirp and pump scattering and normalized to the bleach signal at the time of maximum contrast. Single dA versus delay time kinetics were extracted for fixed probe wavelengths.

Data availability

All data in the manuscript and Supplementary Information are stored in Zenodo: <https://doi.org/10.5281/zenodo.8354800>

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Author contributions

Idea and coordination: F.S. and E.C. Colloidal synthesis: M.H., A.S.-C. and F.S. Colloidal assembly: F.S. Photocatalytic experiments: M.H. Electron microscopy analysis: F.S. and A.K. Optical spectroscopy and optical microscopy characterization: S.J., M.K. and S.R. Transient absorption spectroscopy: D.H. and H.L. FDTD simulations: S.J. Data analysis and discussion: all authors. Supervision and funding: E.C., S.R., W.J.P., H.L. and F.S. Figures: M.H. and S.J. Writing: M.H. and S.J. Reviewing and editing: all authors.

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Competing interests

M.H., F.S. and E.C. are listed as co-inventors of a European patent application, ‘Embedding of catalytically active nanoparticles into superstructures of plasmonic nanoparticles to enhance the photocatalytic activity’ (EP22186109.9). The patent describes the fabrication of bimetallic supercrystals and the photocatalytic performance presented in this manuscript. The remaining authors declare no competing interests

Additional information

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4.6 Further work on self-assembly

Studies on the self-assembly of AuNP and the properties of the resulting plasmonic supercrystals have been presented in a number of additional publications. The XCCA study exploring their structure in detail was already discussed (section 3.4).¹⁶⁴ From the synthesis point of view we discussed and demonstrated how the gap size in the supercrystals can be controlled¹⁹⁸ and how fluorophores can be integrated into the supercrystals.¹⁹⁹ Together with Holger Lange and in cooperation with the Reich group we explored the hot electron generation in supercrystals and their utility for surface-enhanced spectroscopy.^{194, 200-202} In new cooperations with Dr. Cyrille Hamon (Université Paris-Saclay) and Dr. James K. Utterback we explore supercrystals of anisotropic NP and demonstrated that their geometry leads to anisotropic thermal transport.²⁰³ In the future, more geometries will be explored leading to novel optical near- and far-field properties.

5. Closing remarks and overview of scientific activities

The presentation of my previous work covered the most important directions of my research since my PhD thesis in the group Prof. Horst Weller in 2014. Starting from my PostDoc time in the group of Prof. Holger Lange I always worked independently and acquired funding covering my own position and research. Together, we also worked on ultrafast carrier dynamics in AuNP leading to a number of publications that were not discussed in detail in this summary.²⁰⁻²² Since starting my PhD I was involved in teaching and supervision in exercises, laboratory courses, research labs and bachelor theses. In my time in the Lange group, I supervised and co-supervised Master and PhD theses additionally as well as PostDocs along with my regular teaching duties. In my time in the group of W. J. Parak I took more responsibility, leading my own team and being involved in the supervision of 10-14 PhD students on a regular base which includes regular seminars, leading role in lab management and supervision of lab safety, and individual discussions of their projects and career advice and mentoring. I also do lectures regularly and continue to teach in the framework of laboratory courses and exercises. The teaching duties include evaluation of exams and theses as assessor and committee member on all levels including in external international panels (bachelor-, master- and PhD-defenses). Since my research activities were located in both: chemistry and physics institutes and groups, my teaching scope is also quite broad spanning from chemistry to physics with focus on physical chemistry, nanoscience and basic and experimental physics. As outlined in the previous chapters my research has been highly interdisciplinary and cooperative throughout, including cooperations spanning from medicine to experimental and theoretical physics. I always worked independently on my own projects acquiring necessary funding and applying for beamtimes at synchrotron and FEL sources (28 beamtimes, 6 as PI or leader), organized and contributed to workshops, seminars and conferences. With increasing national and international visibility, I also had more requests for invited contributions and was requested as reviewer and assessor for peer-reviewed publications and funding proposals. Most of the collaborations outlined in the previous chapters are ongoing and have always been productive. My research led to > 60 publications (h-index: 25) including small and large reviews and a patent (EU and US). An overview of all these activities is provided in the following. A publication list of own publications is provided as individual document.

5.1 Beamtimes at synchrotron and free-electron laser facilities

Since 04/2015

Awarded and participated in 28 beamtimes at synchrotron and free-electron laser (FEL) facilities. 3 at Diamond Light Source (Didcot, UK), 4 at the European Synchrotron Radiation Facility (ESRF, Grenoble, France), 14 at PETRA III (Synchrotron) and 1 at FLASH (Free-Electron Laser, FEL) both at Deutsches Elektronen-Synchrotron (DESY, Hamburg, Germany), 5 at European XFEL (Hamburg, Germany) and 1 at the National Synchrotron Light Source II (NSLS II, Long Island, New York, USA). Here, only the beamtimes with PI or leader role are listed.

11-12/2019

Proposal: *“Studying structural diversity in overlapping nanoparticle superlattices to understand emerging plasmonic properties”* (I-20190171)

Place: PETRA III, DESY

08/2020

Proposal: *“Local order in overlapping nanocrystal superlattices and its role in plasmonic properties”* (I-20200619)

Place: PETRA III, DESY

06/2022

Proposal: *“In-situ nanoparticle supercrystal formation in vertical scattering geometry”* (I-20211357)

Place: PETRA III, DESY

06-07/2023

Proposal: *“Using a new vertical scattering geometry to understand the self-assembly of binary nanoparticle mixtures”* (I-20221388)

Place: PETRA III, DESY

04/2024

Proposal: *“Correlating atomic and superlattice in nanoparticle supercrystals of diverse building blocks”* (I-20231419)

Place: PETRA III, DESY

06/2024

Proposal: *“Time-resolved amyloid-beta aggregation”* (ID: 5747)

Place: European XFEL

5.2 Patents

07/2022

“Embedding of catalytically active nanoparticles into superstructures of plasmonic nanoparticles to enhance the photocatalytic activity” (EU: EP4309784A1, US: US20240024862A1, published 01/2024, status: pending)

5.3 Third-party funding

04/2016-04/2019

German Research Foundation (DFG) funding – equipment allowance (Sachbeihilfe):

“Flexible Synthesis of Stable Gold Nanoparticle Clusters”

grant ID (*Fördernummer*) 290481307

Own function: Project manager and sole submitter

Overall funding: EUR 273.000 €

With this project my position and independent research addressing surface chemistry and controlled assembly of nanoparticles was funded in this time period.

08/2021-08/2023

PIER seed project:

“Nanoparticle supercrystal formation studied with coherent X-ray scattering”

grant ID (*Fördernummer*) PIF-2021-02

Own function: Project manager and main applicant

Overall funding: EUR 34.850 €

This seed project provided funding for a collaborative project (University of Hamburg and DESY) addressing self-assembly of nanoparticles studied with coherent X-ray scattering

since 2022

German Research Foundation (DFG) funding – equipment allowance (*Sachbeihilfe*):

“Polaritons in binary nanoparticle superlattices”

grant ID (*Fördernummer*) 504656879

Own function: Co-Investigator

Overall funding: EUR 480.000 €

Co-Investigator role in this collaborative project with Prof. Holger Lange (University of Potsdam) and Prof. Stephanie Reich (FU Berlin) addressing the light-matter interactions of plasmonic nanoparticle supercrystals.

Awards and Scholarships

05-08/2007

Scholarship of the German Academic Exchange Service (DAAD),

2014

Doctoral thesis award of the Department of Chemistry, University of Hamburg

5.4 Ad-hoc reviewer

since 2011

> 80 reviews for: ACS Applied Nano Materials, ACS Nano, Advanced Healthcare Materials, Advanced Materials Interfaces, Analytical and Bioanalytical Chemistry, Analytical Chemistry, Angewandte Chemie/Angewandte Chemie International Edition, Applied Materials Today, Beilstein Journal of Nanotechnology, Cancers, Chemical Communications, Chemical Physics Letters, Chemical Science, Chemistry, Chemistry - A European Journal, Chemistry of Materials, ChemNanoMat, Crystals, International Journal of Molecular Sciences, International Journal of Nanomedicine, Inorganics, Journal of Applied Physics, Journal of Materials Chemistry B, Journal of Materials Science, Journal of Microscopy, Journal of Nanobiotechnology, Journal of Physical Chemistry, Macromolecular Chemistry and Physics, Macromolecules, Materials, Materials Today Advances, Metals, Micro & Nano Letters, Molecules, Nano Letters, Nanomaterials, Nanoscale, Nanoscale Horizons, Nature Communications, New Journal of Chemistry, Particle & Particle Systems Characterization, Pharmaceuticals, PLOS One, RSC Advances, Scientific Reports, Seminars in Immunology, Sensors, Small, Small Structures, Zeitschrift für Physikalische Chemie

5.5 Organization of workshops

2019-2022

Organizing team of three workshops “Light-Matter Coupling with Plasmonic Platforms” in Wittenberge (27.-28.3.2019), Online (16.-17.11.2020) and in Hamburg (9.-10.3.2022)

5.6 Cooperations

a non-exhaustive selection of ongoing collaborations includes:

since 2014

Partner: **Dr. Felix Lehmkuhler**, Deutsches Elektronen-Synchrotron (DESY), Hamburg, Germany

Topic: Advanced coherent X-ray scattering experiments (X-ray photon correlation spectroscopy (XPCS), X-ray cross-correlation analysis (XCCA)) to study structure and dynamics of nanomaterials

since 2018

Partner: **Prof. Dr. Stephanie Reich**, Freie Universität Berlin, Berlin, Germany

Topic: Light-matter coupling in well-ordered plasmonic systems

since 2019

Partner: **Dr. Kartik Ayyer**, Max-Planck-Institut für Struktur und Dynamik der Materie, Hamburg, Germany

Topic: Single-particle 3D diffractive imaging with free-electron lasers

since 2020

Partner: **Prof. Dr. Holger Lange**, Universität Potsdam, Potsdam, Germany

Topic: After working with Prof. Lange as a Postdoc in his group in the excellence cluster CUI at the University of Hamburg from 2014-2020 we continued our joint work on carrier dynamics in plasmonic systems

since 2021

Partner: **Prof. Dr. Emiliano Cortés**, Ludwig-Maximilians Universität, München, Germany

Topic: Bimetallic plasmonic supercrystals for plasmonic photocatalysis

since 2023

Partner: **Dr. Cyrille Hamon**, Université Paris-Saclay, Orsay, France

Topic: Superstructures of anisotropic nanoparticles

since 2023

Partner: **Dr. James K. Utterback**, Sorbonne Université, Paris, France

Topic: Thermal transport in self-assembled nanoparticle supercrystals

5.7 Invited contributions – conferences, seminars and lectures

- 1) ACS Fall Meeting 2023, 13.-17.8.2023, San Francisco, USA, oral invited, *Phase transitions of polymer-stabilized gold nanoparticles studied with X-ray photon correlation spectroscopy*
- 2) MRS Fall Meeting 2023, 26.11.-1.12.2023, Boston, USA, oral invited, *Role of the Ligand Structure Around Nanomaterials for Chemical Reactions*
- 3) PIERS 2024, 21.4.-25.4.2024, Chengdu, China, oral invited, *Thin Film Plasmonic Supercrystals*
- 4) Cavity control of quantum materials, 14.5.-17.5.2024, Hamburg, Germany, oral invited, *Thin Film Plasmonic Supercrystals*
- 5) GDR NINO Webinar 2024 “Inorganic Nanostructures by solution chemistry”, 4.7.2024, Online, oral invited, *Thin Film Plasmonic Supercrystals as Polaritonic Materials*
- 6) Seminar at Chalmers University of Technology, 12.11.2024, Gothenburg, Sweden, oral invited, *Thin Film Plasmonic Supercrystals as New Optical Materials*
- 7) Seminar at University of Potsdam, 8.11.2024, Potsdam, Germany, oral invited, *Thin Film Plasmonic Supercrystals*

- 8) Seminar at the Institute of Condensed Matter Chemistry of Bordeaux (ICMBC-CNRS), 20.11.2024, Bordeaux, France, oral invited, *New optical materials based on self-assembled plasmonic nanoparticles*
- 9) International Lecture Series on Nanophotonics – Winter Semester 2024/2025 – 4.12.2024, Ludwig-Maximilians-Universität München, Germany, Online, oral invited, *Thin-Film Plasmonic Supercrystals by Colloidal Self-Assembly*
- 10) SPIE WEST 2025, 25.-30.1.2025, San Francisco, USA, oral invited, *Nanoparticles as tracers in X-ray photon correlation spectroscopy studies of biological systems*
- 11) MATSUS Fall 2025, 20.-24.10.2025, Valencia, Spain, oral invited, *Thin-Film Plasmonic Supercrystals*

5.8 Conference contributions

Oral presentations

- 1) E-MRS Spring Meeting 2013, 27.-31.5.2013, Strasbourg, France, *Stable gold nanoparticle-L1-peptide conjugates for the treatment of nervous system injury*
- 2) E-MRS Spring Meeting 2015, 11.-15.5.2015, Lille, France, *Improved Turkevich Synthesis and Effective Stabilization of Gold Nanoparticles*
- 3) 5th International Conference on Multifunctional, Hybrid and Nanomaterials, 6.-10.3.2017, Lisbon, Portugal, *Advanced PEGylation of gold nanoparticles*
- 4) NaNaX - Nanoscience with Nanocrystals (NANAX8), 3.-7.7.2017, Braga, Portugal, *Surface chemistry of PEGylated gold nanoparticles*
- 5) Gold 2018, 15.-18.7.2018, Paris, France, *Self-Assembly of Polymer-Coated Gold Nanoparticles*
- 6) nanoGe Spring Meeting 2021, 9.-12.3.2021, Online, *Synthesis and characterization of quasicrystalline plasmonic superlattices*
- 7) Gold 2022, 17.-20.7.2022, Quebec City, Canada, *Synthesis, characterization and optical properties of plasmonic supercrystals*
- 8) E-MRS Spring Meeting 2024, 27.-31.5.2024, Strasbourg, France, *Thin Film Plasmonic Supercrystals as Polaritonic Materials*
- 9) Gold 2025, 11.-14.5.2025, San Sebastian, Spain, *Thin Film Plasmonic Supercrystals as Polaritonic Materials*

Poster presentations

- 1) Bunsentagung 2011, 2.-4.6.2011, Berlin, Germany, *Conjugation of L1-peptides to Gold nanoparticles and characterization of the conjugates*
- 2) Bunsentagung 2013, 9.-11.5.2013, Karlsruhe, Germany, *Advanced PEGylation of Gold Nanoparticles*
- 3) NaNaX - Nanoscience with Nanocrystals (NANAX7), 4.-8.4.2016, Marburg, Germany, *Advanced PEGylation of Gold Nanoparticles*
- 4) International Conference on Self-Assembly in Confined Spaces, San Sebastian, Spain, 25.-27.10.2016, *Advanced PEGylation of Gold Nanoparticles*
- 5) NanaX - Nanoscience with Nanocrystals (NANAX10), 3.-7.7.2023, Klosterneuburg, Austria, *Thin Film Plasmonic Supercrystals*

5.9 Contributions to scientific workshops (selected)

Oral presentations

- 1) CUI (excellence cluster CUI: Advanced Imaging of Matter) Annual Meeting 2019 – 13.-15.11.2019, Hohwacht, Germany, oral, *Gold nanoparticles - order and dynamics*
- 2) Workshop: Light-Matter Coupling with Plasmonic Platforms, Online, 16.-17.11.2020, oral, a) (Tutorial) *Nanoparticle synthesis and functionalization: concepts and strategies* b) *Synthesis of high quality plasmonic superlattices*
- 3) Schwerpunktprogramm ‚ESSENCE‘ (SPP-1857) – Online-Workshop: 26.11.2021, oral, *Nanoplasmonics and DNA-Origami*
- 4) Plasmonics Workshop, Hamburg, 9.-10.3.2022, Hamburg, Germany, oral, *nanoparticle supercrystal syntheses strategies*
- 5) EUGLOH Matchmaking Workshop, 22.-23.2.2024, oral, *Importance of Ligands and Surface Chemistry in Nanomaterials*

Poster presentations

- 1) DESY (Deutsches Elektronen-Synchrotron) Research Course 2016 – Soft Matter in the Light of Modern X-ray Sources 2.-4.3.2016, *Gold Nanoparticles Synthesis, Surface Chemistry and Assembly*
- 2) CUI Annual Meeting 2017 – 11.-13.10.2017, Hohwacht, Germany, *Gold Nanoparticles Surface Chemistry and Assembly*

5.10 Overview of offered courses (labs not included)

(SWS: Semesterwochenstunden/teaching hours per week during semester)

Course	Participants	SWS
winter semester 2014/15		
Exercises in physical chemistry I	17	1
Exercises in physical chemistry and mathematics	27	1
summer semester 2015		
Exercises in physical chemistry II	23	1
Exercises in physical chemistry II	25	1
winter semester 2015/16		
Exercises in physical chemistry I	26	1
Exercises in physical chemistry I	26	1
Exercises in physical chemistry I	22	1
Exercises in physical chemistry and mathematics	26	1
summer semester 2016		
Exercises in physical chemistry II	26	1
Exercises in physical chemistry II	10	1

winter semester 2016/17		
Exercises in physical chemistry I	30	1
Exercises in physical chemistry I	30	1
Exercises in physical chemistry I	22	1
Exercises in physical chemistry and mathematics	22	1
summer semester 2017		
Exercises in physical chemistry II	20	1
Exercises in physical chemistry II	22	1
winter semester 2017/18		
Exercises in physical chemistry I	23	1
Exercises in physical chemistry I	23	1
Exercises in physical chemistry I	12	1
Exercises in physical chemistry and mathematics	25	1
summer semester 2018		
Lecture: nanochemistry I	78	2
Exercises in nanochemistry I	17	1
winter semester 2018/19		
Lecture: physical chemistry and mathematics	190	3
Exercises in physical chemistry and mathematics	25	1
Exercises in physical chemistry and mathematics	25	1
Exercises in physical chemistry and mathematics	16	1
winter semester 2019/20		
Seminar on physics for pharmacists	21	1
Seminar on physics for pharmacists	24	1
winter semester 2020/21		
Seminar on physics for pharmacists	22	1
Seminar on physics for pharmacists	19	1
Lecture: Methods in nanobiotechnology I	30	2
summer semester 2021		
Lecture: Methods in nanobiotechnology II	30	2
Exercises in methods in nanobiotechnology II	30	2
Exercises in physics for nanoscience students B	8	2
winter semester 2021/22		
Lecture: Methods in nanobiotechnology I	30	2
Exercises in methods in nanobiotechnology I	30	2

Exercises in physics for nanoscience students A	8	2
summer semester 2022		
Lecture: Methods in nanobiotechnology II	30	2
Exercises in methods in nanobiotechnology II	30	2
Exercises in Physico-Chemistry III	8	2
winter semester 2022/23		
Lecture: Methods in nanobiotechnology I	43	2
Exercises in methods in nanobiotechnology I	43	2
Exercises in physics for nanoscience students A	8	2
summer semester 2023		
Lecture: Methods in nanobiotechnology II	32	2
Exercises in methods in nanobiotechnology II	32	2
Exercises in physics for nanoscience students B	6	2
winter semester 2023/24		
Lecture: Methods in nanobiotechnology I	54	2
Exercises in methods in nanobiotechnology I	54	2
Exercises in physics for nanoscience students A	20	2
summer semester 2024		
Lecture: Methods in nanobiotechnology II	48	2
Exercises in methods in nanobiotechnology II	48	2
winter semester 2024/25		
Lecture: Methods in nanobiotechnology I	36	2
Exercises in methods in nanobiotechnology I	36	2
Exercises in physics for nanoscience students A	20	2

5.11 Supervisions of labs

since 2011

Supervisor in the advanced physical chemistry lab, laboratory course I for students of natural sciences (physics lab for beginners), advanced physics lab and 22 research labs (4-6 weeks each) in the master modules: soft (nano-) matter, nanochemistry, nanotechnology, nanobiology, regenerative energy conversion and optical spectroscopy of nanomaterials

5.12 Supervision / assessment of qualifying respectively final theses

since 2014

Supervision/co-supervision of ten bachelor theses, ten master theses (4 second assessor, 2 ongoing), 12 doctoral theses (ten ongoing).
Committee member of five PhD-defenses. Second assessor of four PhD-theses.

Committee:

Jordie Piella Bagaria, Universitat Autònoma de Barcelona, *Advanced Synthesis and Characterization of Noble Metal Nanoparticles*, 08.11.2018 (chairman)

Daniel Brito der Araújo, Universidade Federal do Ceará, *SURFACE INTERACTIONS IN MOS2: A STUDY ON THE EFFECTS OF CONTACT ON A 2D SEMICONDUCTOR*, 10.07.2023

Charline Stahlhut, Universität Hamburg, *Creation of Novel Composites with Distinct Silver Nanoclusters*, 05.03.2024

Juan Jesús Barrios Capuchino, Universität Hamburg, *Self-assembly of gold nanoparticles into supercrystals, hybrid systems, and binary superstructures*, 2025

Kai-Wei Lin, Universität Hamburg, *Alternating Magnetic Field Triggered Nanoplatform in Biomedical Application of Drug Delivery*, 2025

2nd Assessor:

Maria Pozzi, Universität Hamburg, *Calcium Release Upon Photothermal Heating of Gold Nanostars in Jurkat T Cells*, 2024

Bo Peng, Universität Hamburg, *Labelling of cells with different materials for cell magnetic sorting and X-ray fluorescence imaging studies*, 2024

Hessam Hosseinkazemi, Universität Hamburg, *Cell Imprinted In Vitro Models for Normal and Cancer Cell Migration and Separation*, 2024

Juan Jesús Barrios Capuchino, Universität Hamburg, *Self-assembly of gold nanoparticles into supercrystals, hybrid systems, and binary superstructures*, 2024

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6.2 Eidesstattliche Versicherung / Declaration on oath

Hiermit versichere ich an Eides statt, die vorliegende Habilitationsschrift mit dem Titel

Synthesis, Surface Chemistry and Assembly of Plasmonic Nanoparticles towards New Optical Materials

selbst verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt zu haben. Sofern im Zuge der Erstellung der vorliegenden Habilitationsschrift generative Künstliche Intelligenz (gKI) basierte elektronische Hilfsmittel verwendet wurden, versichere ich, dass meine eigene Leistung im Vordergrund stand und dass eine vollständige Dokumentation aller verwendeten Hilfsmittel gemäß der Guten wissenschaftlichen Praxis vorliegt. Ich trage die Verantwortung für eventuell durch die gKI generierte fehlerhafte oder verzerrte Inhalte, fehlerhafte Referenzen, Verstöße gegen das Datenschutz- und Urheberrecht oder Plagiate. Erklärungen über meinen Beitrag zu allen gemeinschaftlich verfassten wissenschaftlichen Publikationen finden sich in der Habilitationsschrift.

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Dr. Florian Schulz