Ligand Design and Bioconjugation of Colloidal Gold Nanoparticles**

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The binding of inorganic nanoparticles to biological molecules has created a rapidly expanding field of research that deals with numerous aspects of biosensors, medical diagnostics, and drug delivery up to fundamental investigations on the sector of self-organizing nanoelectronics.[1] An example is the labeling of biological substrates with noble metal nanoparticles or fluorescent semiconductor particles.[2,3] To prepare such conjugates from nanoparticles and biomolecules, the surface chemistry of the nanoparticles must be such that the stabilizing ligands are fixed to the nanoparticle and possess terminal functional groups that are available for biochemical coupling reactions if required. The binding to the particle surface is frequently done through thiol groups.[4–6] In the case of gold nanoparticles, thiolated DNA quasi serve as ligands.[7–10] In most cases, however, a simple thiol bond to the particle surface is not sufficient to accomplish a permanent linkage. Instead an equilibrium is established with dynamic ligand exchange. To avoid this, a shell of silica is often grown on the particle itself by means of the sol–gel technique[11–14] and the linkage groups pointing outwards are added as functionalized alkoxysilanes during the polycondensation process.[15,16] The result is relatively compact silica shells and a tight coating of the surface with coupling groups.

In an ideally designed surface the number of linkage groups should be adjustable and inert functional groups should be added which determine the solubility and the surface potential of the particles. The linkage groups of the ligands should adhere to the crystallite surface as much as possible and the ligands should be covalently linked together to completely avoid exchange. The crystallite surface should be tightly covered to terminate all free valence states. The thickness of the total linked ligand shell should preferably be small to keep the mass fraction of the nanoparticle core as high as possible.

Herein we describe how to synthesize such an optimized ligand shell and explain the coupling to biotin and the conjugative effect of the particles bound to biotin with avidin. Owing to the high chemical resistivity we initially developed this synthesis for gold nanoparticles; however, preliminary investigations revealed that semiconductor nanoparticles can be synthesized in an analogous way.

The schematic structure of the ligand shell is shown in Figure 1. First gold nanoparticles were prepared by reduction

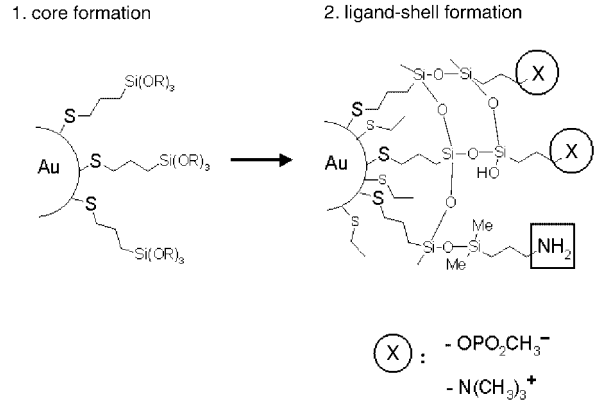
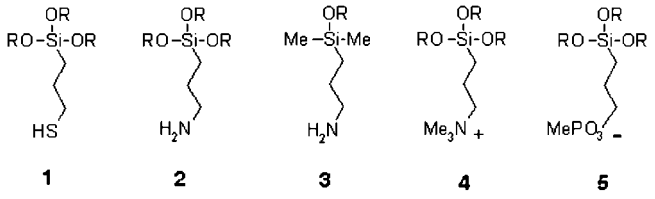


Figure 1. Schematic composition of the ligand shell.

of $KAuCl_4$ with $NaBH_4$ in propanol in the presence of the ligand 3-sulfanylpropyltrimethoxysilane (**1**). We obtained



particles with an average diameter of about 2.2 nm and a standard deviation of 21 %. The thiol group of ligand **1** binds very tightly to the particle surface and the trimethoxysilyl group allows further functionalization and cross linkage of the ligands. If the polycondensation necessary took place in the presence of excessive aminopropyltrimethoxysilane (**2**), it was followed by relatively quick coagulation. From this we conclude that the trialkoxysilyl groups of the ligands **1** and **2** have formed extensive interparticular networks. We were able to avoid this coagulation step by carrying out polycondensation in the presence of 3-aminopropyldimethylethoxysilane (**3**). Ligand **3** has only one condensable alkoxy group and therefore has a terminating effect during the polycondensation process. This results in a precipitation of hydrophilic colloids which readily redissolve in H_2O. After washing with propanol, the particles can be redispersed in H_2O. Figure 2 shows a transmission electron microscopy (TEM) image with the appropriate histogram. Crystalline, well-separated particles can be discerned, their minimum distance is about 1.5 nm which corresponds to the length of twice the sum of ligands **1** and **3**. The cross-linked ligands presumably prevent the external

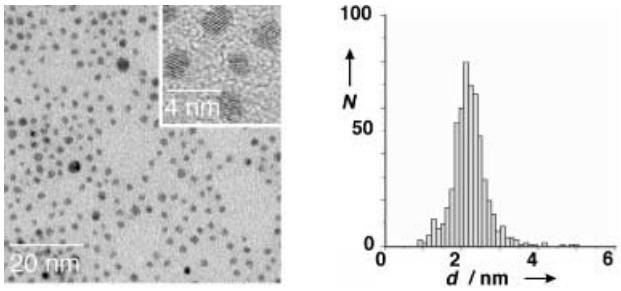


Figure 2. TEM image of the silica-coated amino-functionalized gold nanocrystals and the respective histogram.

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amine ligands from binding to the surface of a second particle thus causing coagulation.[17] This assumption is confirmed by the absorption spectrum (Figure 3) which would display an additional band at $\lambda > 600$ nm if interparticular binding was occurring. The IR spectrum of the cleaned particles shows all vibrations of the coupled ligand shell expected; detailed assignments are given in the Supporting Information.

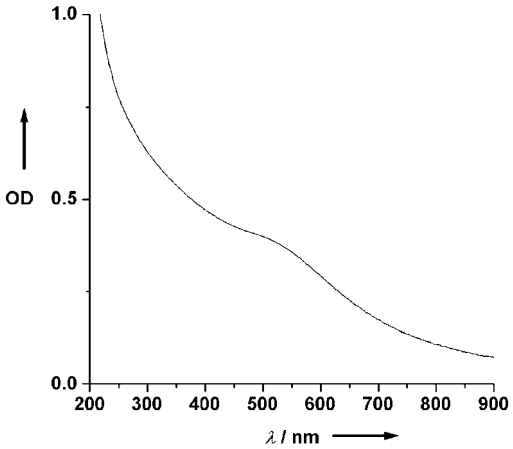

Figure 3. UV/Vis spectrum of a colloidal solution of the silica-coated and amino-functionalized gold nanoparticles in water. OD = optical density.

We determined the number of the freely accessible amino groups at the surface of the particles by using the fluorescence indicator fluorescamine.[18–19] Figure 4a shows the number of amino groups translated into number per gold particle as a function of the quantity of the ligand **3** added during polycondensation. It can be seen that already relatively small concentrations of **3** are sufficient to verify three to four amino ligands per particle and that the number of detectable amino ligands increases only slightly after further increasing the concentration of **3**. Owing to the relatively small required surface area of the ligands chosen, the small number of amino groups per particle is hard to understand, one may, moreover, suspect that during polycondensation the aminopropyl group is not only incorporated in a way that the amino groups point outwards, but also that backbonding to the particle surface takes place. This is readily explained as the voluminous trimethoxysilyl end groups of the originally used ligands **1** prevent the gold particles from being completely covered by thiolate groups. Back-bonded amino groups which are fixed to the particle surface cannot be detected with fluorescamine as this molecule only reacts with free primary amines and hardly reaches the particle surface.

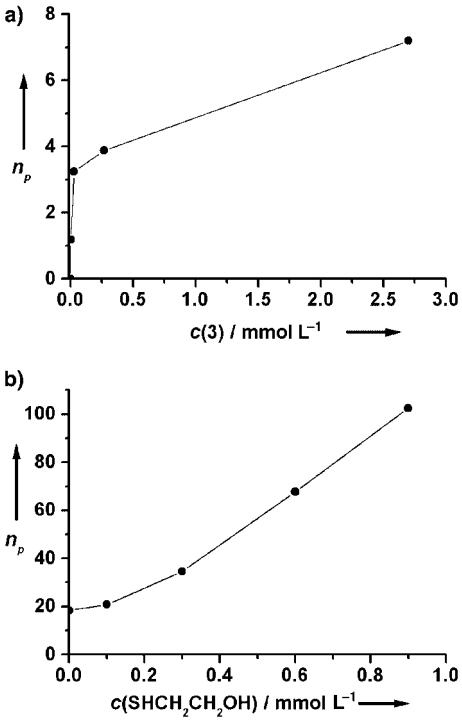

Figure 4. Number of amino groups per particle (n_p) determined with fluorescamine a) as a function of the concentration of compound **3** presented during condensation, and b) as a function of the 2-sulfanylethanol concentration. The latter compound was added as a supplementary ligand before the condensation with compound **3**.

To support the theory of amino backbonding and to possibly prevent the latter, we added the short-chained ligand 2-sulfanylethanol during polycondensation. We intended to block the free coordinating sites at the particle surface thus impeding amino backbonding. Figure 4b shows the number of amino groups detectable by fluorescamine, this time as a function of the 2-sulfanylethanol concentration. One can clearly recognize that the 2-sulfanylethanol molecules quasi force the amino groups outwards.

With the synthesis described one obtains water-soluble nanoparticles with a tightly bound ligand shell and amino groups pointing outwards which are particularly suitable for coupling reactions with biological molecules. The amino group can be brought to react with the activated ester of biotin,[20–22] a molecule that conjugates with outstanding selectivity and specificity with avidin proteins. Biotin–avidin coupling represents an extraordinary example for the molecular key-and-lock system in biological processes. With increasing biotinylation the formerly hydrophilic nanoparticle surface becomes more and more hydrophobic and the biotinylated particles precipitate gradually in aqueous media. We solved this problem by adding during polycondensation besides the ligand **3**, the ligand **4** functionalized with quaternary ammonium. Quaternary ammonium groups cannot bind to biotin, they only cause a positive surface charge of the particles over the entire pH range and give rise to the desired water solubility of the particles during the biotinylation of the amino ligands. It is possible to adjust the number of coupling groups at the surface by varying the ratio of ligands **3** and **4**.

To achieve a possible coupling of negatively charged biomolecules such as DNA, ligand **4** can be exchanged for ligand **5** which results in a negative surface charge of the particles preventing coagulation due to Coulomb attraction. Preliminary experiments with DNA were successful, and details will appear in a future article.To prove the conjugation of biotinylated particles, we mixed fluorescently labeled avidin with varying quantities of biotinylated particles. The resulting conjugates have a molar mass of such a magnitude that they can easily be separated from the solution by centrifugation. The fluorescence intensity F of the super-

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natant solution is shown in Figure 5 as a function of the added quantity of biotinylated particles. The ratio of biotinylated particles to the avidin substrate is indicated on the *x* axis (*r*). It can be seen that already from about two gold particles per

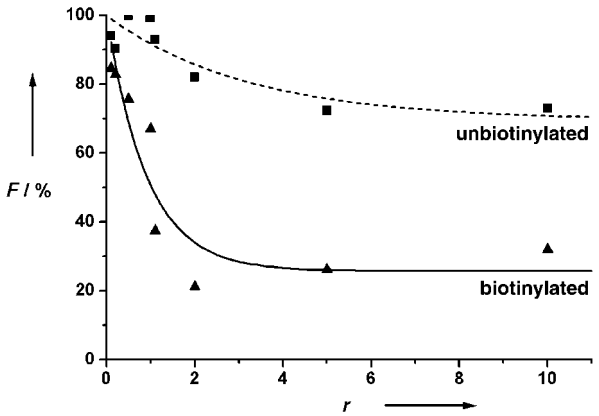

Figure 5. Decrease of fluorescence by FITC–avidin in the supernatant solution after the centrifugation of the mixtures of FITC–avidin with biotinylated and unbiotinylated particles as a function of the molar particle/avidin ratio.

avidin molecule the fluorescence intensity of the supernatant solution has decreased to about 20% of the original value, which suggests a specific coupling. From the fact that the fluorescence intensity does not decrease completely we conclude that either some of the avidin is not able to link, a certain percentage of the conjugates could not be separated by centrifugation, or that a small quantity of the fluorescence probe molecules are available as monomers and are not coupled to avidin. To further elucidate this phenomenon, we added to an identical avidin substrate the respective quantity of unbiotinylated (but in other respects identical) gold particles. We observed a slight decrease of fluorescence after centrifugation which may be explained by the small degree of nonspecific adsorption.

In summary, we present for the first time a synthesis for functionalized nanoparticles that allows one to covalently link the ligand shell at minimum thickness, thus binding it statically to the particle surface. Furthermore, the total amine ligand shell is provided in such a way that it points outward into the solution with an adjustable number of functional coupling groups and at the same time the solubility and surface charge can be adjusted by additional covalently bound auxiliary ligands. The particles surface-modified in such a way remained stable for months and did not show any signs of coagulation before and after biotinylation. The outstanding suitability for specific bioconjugation is confirmed by the biotin–avidin coupling.

Experimental Section

All chemicals used were of highest available purity, and were supplied by Merck, Sigma, Aldrich, Fluka, and ABCR. The Millipore water had a specific resistance of 18.2 MΩ cm.

UV/Vis absorption spectra were recorded with a Varian Cary 50 single beam spectrometer. Fluorescence spectra were measured at room temperature with a FluoroMax-2 ISA Instruments Inc. fluorescence spectrometer.

High-resolution transmission electron microscopic images were recorded with a Philips CM 300 UT microscope, which operates with an LaB_6 cathode at 300 KV and is equipped with an EDX detector (EDAX) and a CCD camera type Gatan 694.

Particle synthesis: For the synthesis of the gold nanocrystals stabilized with ligand **1**, $KAuCl_4$ was reduced with $NaBH_4$ in the presence of **1**. Accordingly, $KAuCl_4$ (1.5 mg) was dissolved in H_2O (80 μL) and then 2-propanol (20 mL) was added, thereafter ligand **1** (0.58 μL) and $NaBH_4$ (0.2 mg, dissolved in H_2O (240 μL)) were added under stirring. The surface of the resulting 2.2 nm gold colloids was condensed in the presence of various alkoxysilanes.

For the amino functionalization four freshly prepared 20 mL aliquots of gold colloids were mixed with compound **3** (*x* μL) each replenished to 10 μL by addition of dry DMF (*x* μL (equivalent to *c*(**3**) in the reaction mixture); 0.01 μL (2.7 μM); 0.01 μL (27 μM); 1 μL (270 μM); 10 μL (2.7 mM)), with a 50% solution of compound **4** (40 μL) in methanol, and 25% aqueous NH_3 (1.5 mL) and then stirred for 16 h. The resulting colloidal precipitate was washed three times in propanol and redispersed in H_2O (10 mL). The number of NH_2 groups was determined with sols cleaned in this way.

In a further series of experiments the surface of the gold particles was protected with 2-sulfanylethanol before the condensation step. Directly after reduction 2-sulfanylethanol (*x* μL (equivalent to *c* in the reaction mixture); 0.14 μL (100 μM); 0.42 μL (300 μM); 0.84 μL (600 μM); 1.26 μL (900 μM)) were added to 20 mL aliquots of freshly prepared colloidal solution followed by condensation in the presence of compound **3** (10 μL) and 25% aqueous NH_3 (1.5 mL). The colloids were cleaned as described above and redissolved in H_2O (10 mL).

In a blank experiment we added the respective amounts of 2-sulfanylethanol to the readily formed colloidal solutions after shell preparation and determined the number of NH_2 groups. No increase was found.

Determination of the NH_2 groups with fluorescamine: To each cleaned colloidal solution (600 μL), H_2O (400 μL), 50 mM borate buffer pH 9 (1 mL), and a fluorescamine solution (100 μL, 2.8 mg in 1 mL of acetone) were added and stirred for 30 min. Absorption spectra and emission spectra ($\lambda_{ex} = 390$ nm) of the solutions diluted to 1/5 were recorded.

The negatively charged, amino-functionalized gold nanocrystals were synthesized in methanol. The reduction took place using the same quantities as described above. Successively, a 50% methanolic solution of ligand **5** (45 μL), ligand **3** (10 μL), H_2O (500 μL), and a 50% methanolic solution of tetramethylammonium hydroxide (500 μL) were added under stirring and refluxed for 25 min. After the mixture had been allowed to cool, trimethylchlorosilane (26 μL) was added and the mixture was further refluxed for 10 min. A 5-mL sample of the resulting clear solution was mixed with H_2O (1.5 mL), concentrated to 1 mL using a rotary evaporator, and then purified by using a Sephadex G-25 column.

Biotinylation: The colloids from a 20-mL preparation, which had been condensed with a mixture of ligand **3** (10 μL) and ligand **4** (40 μL), were redissolved in H_2O (3 mL) and intensively dialyzed against a 5 mM K_2HPO_4 buffer. Succinimidyl 6-(biotinamido)hexanoate (0.7 mg) was dissolved under dry N_2 in dry DMF (140 μL) and then added to the gold colloids under stirring. After 14 h the resulting clear solution was purified by dialysis.

The quantitative determination of the NH_2 groups before and after the biotinylation served as an indicator for the success of biotinylation and resulted in an average covering of two biotin groups per particle.

FITC–avidin–particle conjugates: For a series of experiments with various particle/avidin ratios we replenished *x* μL of the purified solution of biotinylated colloids (*x* μL (particle/avidin ratio); 7.5 μL (0.25); 15 μL (0.5); 30 μL (1); 75 μL (2.5); 150 μL (5); 300 μL (10)) to 300 μL with H_2O, added an FITC–avidin solution (15 μL, 0.15 mg protein mL^{-1}) and shook the mixture for 20 min.

The mixtures were filled up with H_2O to 3 mL and centrifuged at 13000 rpm for 5 min. Emission spectra of the supernatant solutions were recorded at an excitation wavelength of 495 nm.

An identical series of experiments was carried out with the same, but unbiotinylated particles.

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