VIP Gold Nanorods

DOI: 10.1002/anie.201107304

Quantitative Replacement of Cetyl Trimethylammonium Bromide by Cationic Thiol Ligands on the Surface of Gold Nanorods and Their Extremely Large Uptake by Cancer Cells**

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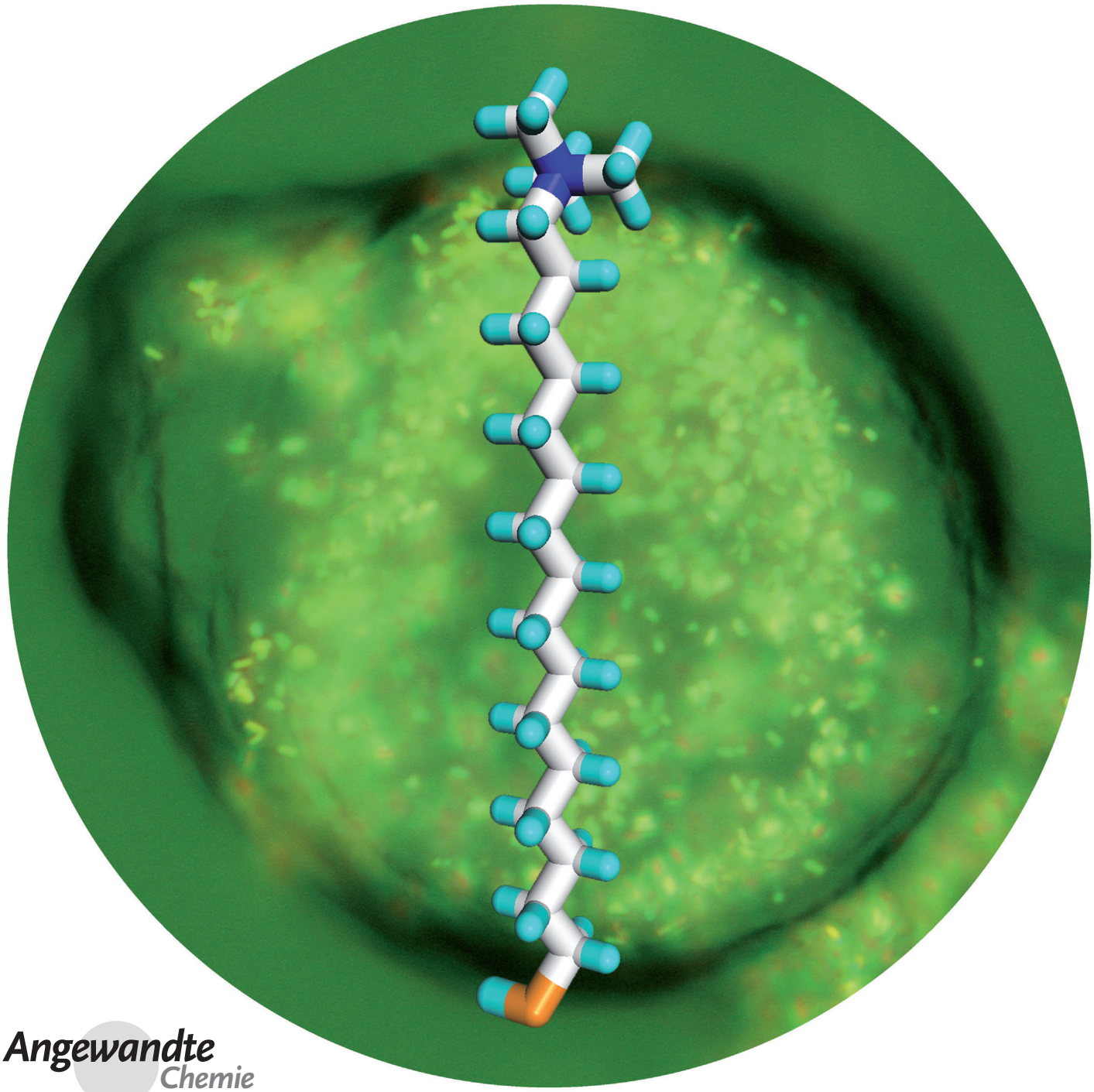


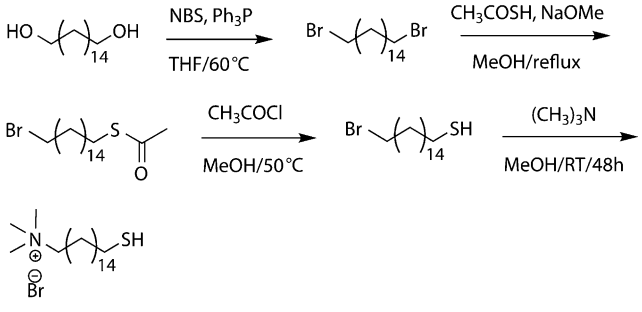
Over the past decade, gold nanorods (Au NRs) have received broad attention as possible therapeutic and diagnostic agents because of their anisotropic physical properties.[1–8] However, as-synthesized Au NRs based on the most common synthetic approach, the seed-mediated synthesis,[9,10] are not directly useable for these applications. They are synthesized in a concentrated cetyl trimethylammonium bromide (CTAB) solution and are noncovalently coated with a CTAB bilayer.[11] For CTAB-capped rods to remain soluble, the concentration of free CTAB in solution must remain above a certain value and there is a constant dynamic exchange of CTAB molecules between the solution and NR surfaces.[12] This feature limits the usefulness of these rods for many biological applications because free CTAB is known to be highly cytotoxic.[13,14] Various strategies have been developed to functionalize gold nanorods with a variety of different ligands to reduce their cytotoxicity.[12,15–20] Further reports have dealt with the problems of stability and biocompatibility of Au NRs by using various polymer shells,[21] polyelectrolytes,[22,23] peptides,[24] surfactants,[25] and lipids[14,26] to modify the Au NR surface. Partial replacement of CTAB was qualitatively confirmed in many of these cases, but the exact surface composition of nanorods and the amount of residual CTAB was either unknown or not possible to determine. For that reason, there is always some ambiguity in interpretation of in vitro and in vivo experiments involving such nanorods.[27]

Beyond just biocompatibility and low toxicity, there has been great interest in understanding the cellular uptake of Au NRs.[28–32] For example, if CTAB is replaced by nonionic polyethylene glycol, the cytotoxicity is substantially reduced, but the cellular uptake drops as much as 94%, and virtually no nanorods can enter the cells.[30] When polyelectrolytes are randomly wrapped around the CTAB bilayer, the uptake increases and the average number of rods that enter each cell ranges from hundreds[31] to one hundred thousand.[29] However, even polyelectrolyte-coated Au NRs are taken by cells in picogram quantities, which is only 0.1% of a typical cell mass. This small weight fraction may explain a somewhat limited success of photothermal oblation of tumor cells,[33] which is strongly dependent on the actual number of nanorods present inside the cells. Therefore, an obvious goal is to design stable and nontoxic (CTAB free) nanorods that could enter cancer cells in much larger quantities.

Here we report a strategy for complete exchange of CTAB for its thiolated analogue (16-mercaptohexadecyl)trimethylammonium bromide (MTAB) and we directly determine the chemical composition of the surface coating. Through ^{1}H NMR analysis, we are able to prove that CTAB is fully replaced by a covalent MTAB monolayer. By combining thermogravimetric analysis (TGA) with TEM size analysis of the nanorods, we are able to accurately determine the packing density of the self-assembled thiol monolayer on the surface of Au NRs. Cytotoxicity of these novel nanorods was studied by MTT assay on breast cancer cells (MCF-7), which were imaged by correlated optical and scanning electron microscopy (SEM). Extremely large number of nanorods (about 2×10^6 NRs per cell) were found inside the viable cells as confirmed by transmission electron microscopy (TEM) and quantified by inductively coupled plasma-optical emission spectrometry (ICP-OES).

Several reports have shown that even with systems that have successfully functionalized gold nanorods, CTAB is still observed to be present on the NRs.[28] We hypothesized that we could use a molecule with a structure very similar to CTAB to completely exchange the CTAB, but with the ability to bind more strongly to the NR surface. Thus, we chose to synthesize a thiolated CTAB analogue (MTAB) whose synthesis is shown in Scheme 1. Commercially available 1,16-hexadecanediol was converted to a corresponding dibromide under standard bromination conditions, which was followed by the synthesis of its monothioester. Cleavage of the acetyl group was carried out in anhydrous methanol using in situ generated hydrogen chloride. The resulting 16-bromo-1-hexadecanethiol was subjected to exhaustive methylation to yield the final product (see the Supporting Information). Importantly, the purified MTAB compound was found to be water-soluble, which offers an opportunity to perform ligand exchange in aqueous media. The MTAB ligand contains an entire CTAB moiety, but installs a pendant thiol group to be able to strongly anchor it to the gold surface. The cross-section of this molecule is small enough to form a compact monolayer on the surface of nanorods, thus providing their solution stability.

Stabilization of gold nanorods is generally difficult because of their small surface-to-volume ratio, and finding a compound that can directly exchange with CTAB is further complicated by the high-density positive charge and the amphiphilic nature of the CTAB bilayer (Figure 1). In the past, researchers have exchanged only the tips of gold nanorods because of relatively weak CTAB binding there and have noted that it is much stronger on the sides.[34–36] Furthermore, as the CTAB on the sides of the rods begins to exchange, it appears that the bilayer structure breaks down and rods tend to aggregate prematurely. However, choosing a



Scheme 1. Synthesis of (16-mercaptohexadecyl)trimethylammonium bromide (MTAB; NBS = *N*-bromosuccinimide, THF = tetrahydrofuran).

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[**] This work was supported by the NSF (grant numbers DMR-0547399 and DMR-1105878).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201107304.

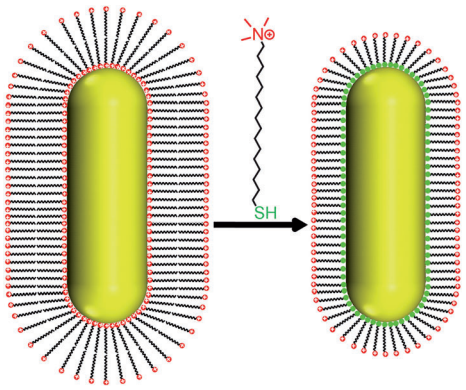

Figure 1. Exchange of the CTAB bilayer for the MTAB thiol monolayer.

cationic ligand with a chemical structure similar to native CTAB allows for the direct exchange in water to proceed smoothly as shown schematically in Figure 1. The noncovalent bilayer of CTAB is replaced with a compact thiolate monolayer of MTAB which is covalently anchored to the surface through gold–sulfur bonds. UV/Vis absorbance spectroscopy is often used to assess the stability of gold nanorods in solution as the intensity, the peak shape, and the peak position of the longitudinal plasmon resonance (LSPR) are sensitive to any aggregation that may occur. The UV/Vis spectrum (see Figure S1A in the Supporting Information) shows the typical absorbance spectrum for Au NRs and demonstrates what happens when excess CTAB is removed from solution through multiple rounds of centrifugation followed by dispersion in pure water. As expected, after just four rounds, the NRs have lost all solubility as evidenced by the lack of any absorbance. In stark contrast, when MTAB-modified NRs are purified by the same technique, there is no appreciable change in the absorbance spectrum even after five rounds of centrifugation and dispersion (see Figure S1B in the Supporting Information), indicating their much improved solution stability while also completely purifying the product.

Additional experiments show that these rods can be completely dried and kept in the solid state indefinitely without losing their water solubility. Surprisingly, a standard lyophilization technique can be applied to aqueous solution of MTAB NRs to produce a fluffy dark brown powder. Figure 2a shows a photograph of 15 mg of lyophilized Au NRs that occupy an area of several square centimeters. Most importantly, when a small amount of this powder is placed on the surface of pure water, rapid dissolution takes place as manifested by the concentration swirls and a continuous diffusion of the colored solute (Figure 2b). The nanorods dissolve in a matter of seconds without any heating or sonication. It is sufficient to flip the vial only once to form a homogeneous solution which does not contain any free organic molecules. The solution remains intact for at least several months as confirmed by UV/Vis analysis. This data clearly shows that a dense monolayer of small cationic thiol MTAB is capable of stabilizing large metallic particles in pure water and preventing their flocculation through entropically driven depletion interactions.[37] This data also showes for the

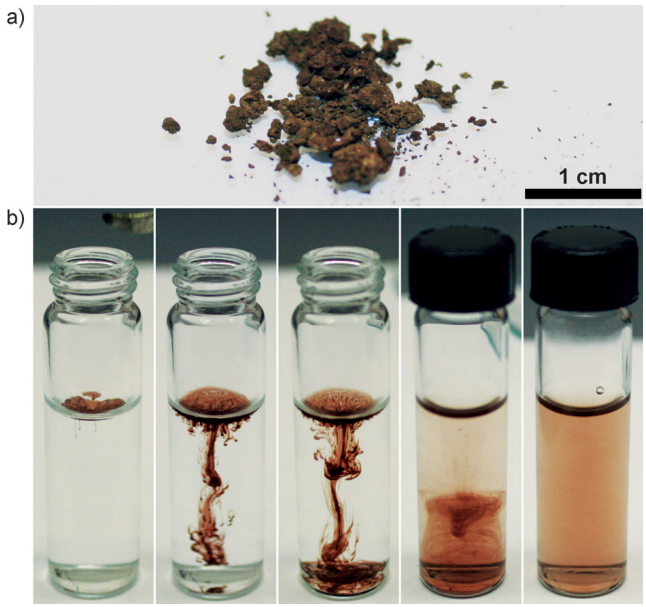

Figure 2. a) Photograph of lyophilized powder of MTAB-functionalized Au NRs and b) its spontaneous dissolution in pure water. The images were taken consecutively within intervals of five second.

first time that the presence of free surfactant in the solution of gold nanorods is not necessary as long as their surface is covalently functionalized by a dense organic shell.

Another indirect method that has often been used to characterize Au NR complexes is the zeta potential measurement, which can qualitatively describe the charge surrounding a nanoparticle. The zeta potential of the MTAB NRs was found to be +55 mV, demonstrating the cationic structures as expected from the high concentration of quaternary ammonium groups positioned around the NR surface. However, the zeta potential is unable to distinguish between CTAB and MTAB on the surface of the rods given that they both have the same cationic headgroup exposed to the solution and so is useful only as a secondary confirmation of structure. We chose ^{1}H NMR spectroscopy to analyze the organic component of the MTAB NRs which should be able to differentiate between the CTAB and MTAB molecules. We used KCN to dissolve the gold NR core and release any surface-bound organic material into solution[39] after having rigorously purified the functionalized NRs. Because of the low surface-to-volume ratio as well as high density of the gold core, we performed an oxidative dissolution of 105 mg of Au NRs in one milliliter of D_2O to get enough organic material for a sufficiently strong ^{1}H NMR signal. The dissolution proceeded very slowly, taking approximately two weeks to dissolve all of the gold, suggesting a high packing density for the MTAB surface functionality.[40] On the contrary, CTAB-coated NRs dissolved in about one hour under similar conditions. The dissolution procedure caused the organic component to precipitate from the D_2O solution, apparently because of the formation of a Au/CN/MTAB complex. The NMR spectrum of the D_2O supernatant showed no signals, confirming that the entire organic component had precipitated from the solution. The NMR spectrum of this precipitate

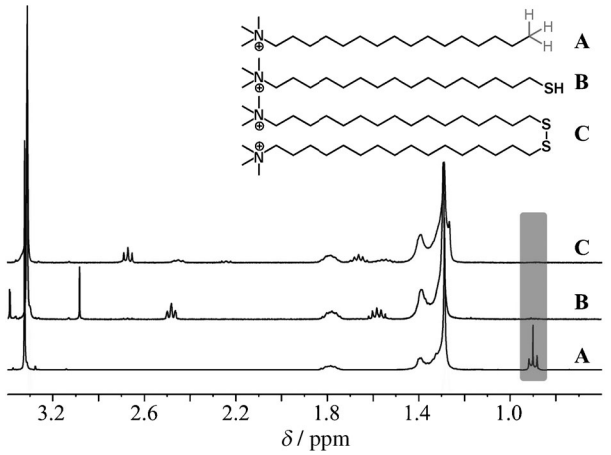

Figure 3. ^{1}H NMR spectra in deuterated methanol of CTAB (A), pure MTAB (B), and the organic product released upon oxidative dissolution of MTAB NRs (C).

dissolved in deuterated methanol, as well as that of pure CTAB and MTAB thiol, is shown in Figure 3. Comparing the spectra of CTAB and MTAB, it is clear that the isolated signal from the terminal methyl group of CTAB at 0.91 ppm is not present in the MTAB spectrum, making it a convenient marker for the presence of any CTAB in solution. Analysis of the NMR spectrum of the dissolved NRs (spectrum C in Figure 3) clearly shows that there is no signal corresponding to this methyl group, proving that no CTAB remained on the surface of the NRs after the exchange and the following purification. Further analysis of the NMR spectrum of the dissolved NRs shows the formation of the expected disulfide of MTAB as evidenced by the triplet at 2.75 ppm corresponding to the α-disulfide protons (instead of 2.5 ppm for α-thiol protons). Importantly, the NMR technique is capable of detecting small organic molecules at concentration close to 10^{-5} M, whereas the concentration of our solution was 10^{-2} M. This implies that even if there were some residual CTAB undetectable by NMR spectroscopy, it would not exceed 0.1 %. Therefore, for all practical purposes one can conclude that the method described hear offers an opportunity to quantitatively replace CTAB by its thiolated analogue.

Because the exact chemical composition of the NR surface agents was determined, it was possible to use TGA to further characterize the MTAB NRs. TGA analysis is a highly useful analytical technique for hybrid inorganic–organic nanostructures because it allows us to accurately quantify the weight percentage of a nanostructure that is organic compared to inorganic core.[39,41,42] For our study, more than 100 mg of MTAB NRs were synthesized and functionalized to be used for TGA and NMR measurements, which is quite a large amount when it comes to Au NR synthesis, requiring a synthesis on the four liter scale. However, even this amount of material is difficult to handle, forming an almost unusable thin film when dried normally. To solve this problem, the MTAB NRs were lyophilized from an aqueous solution, forming a low density powder which could be easily handled, as mentioned previously (Figure 2). TGA analysis of the lyophilized material (see Figure S2 in the Supporting Information) shows a weight loss of 5.3 % in the range between 200 and 450 °C corresponding to the percentage of organic material in the structure. Since the NMR analysis proved that no residual CTAB was left, this weight must be entirely because of MTAB ligands covalently attached to the surface of rods.

We then performed a careful TEM analysis by measuring over 300 nanorods, which determined that the average length and width of MTAB NRs were 41.88 and 9.87 nm, respectively. With this information, we were able to calculate a grafting density of about 3.7 molecules nm^{-2} for an MTAB monolayer and 5013 MTAB molecules residing on each Au NR (see the Supporting Information for detailed calculations). This is close to the grafting density of 4.5 molecules nm^{-2} for neutral alkanethiols on flat gold substrates[43] and may be slightly lower because of repulsive forces between the positively charged headgroups. We believe this is the first proof of a dense self-assembled thiol monolayer on gold nanorods with a calculated grafting density. Estimation of the extent of thiol functionalization has been carried out on DNA-functionalized Au NRs by measuring fluorescence intensity, but such structures contained only about 40 DNA molecules per nanorod[17] as compared to over 5000 MTAB molecules.

With the characterization complete, we performed cytotoxicity and cell uptake experiments to see if the MTAB NRs could be useful for biological applications. We first measured their in vitro cytotoxicity compared to regular CTAB-capped NRs using the standard MTT assay on MCF-7 breast cancer cells (see Figure S3 in the Supporting Information). These studies reaffirmed the cytotoxicity of regular CTAB-capped gold nanorods and showed the decreased cytotoxicity of the thiolated rods, which are not cytotoxic even up to concentrations of 0.1 g L^{-1}. This finding supports previous assertions that surface charge may not be a key factor for the cytotoxicity of nanoparticle systems.[31] Although previous reports have shown that it is mostly free CTAB that contributes to the cytotoxicity of CTAB NRs in the short term,[31] it is unknown what may happen to surface-bound CTAB in the longer term in in vivo experiments. Our system has the advantage of having quantitative CTAB replacement such that this is no longer an issue. Low cytotoxicity is particularly useful if the NRs can be taken up by cells efficiently. To study the uptake, MCF-7 cells were treated with a 20 μg mL^{-1} solution of MTAB NRs in Eagle's minimum essential medium for 24 h. The cells were subsequently rinsed multiple times with PBS solution to remove the excess of NRs that did not enter the cells. After that, the cells were trypsinized, redispersed into medium, and carefully plated onto a new culture slide. This approach allowed us to create a clear background without any free nanorods, which is critically important for differentiating between the internalized particles and those which are not associated with the cells. The dark field optical image (Figure 4 A) clearly shows a large uptake of MTAB NRs and demonstrates their high scattering efficiency. It also appears that NRs are clustered together inside the cells rather than being evenly distributed throughout their interior, suggesting that the MTAB NRs enter the cells through an endosomal pathway. To perform a

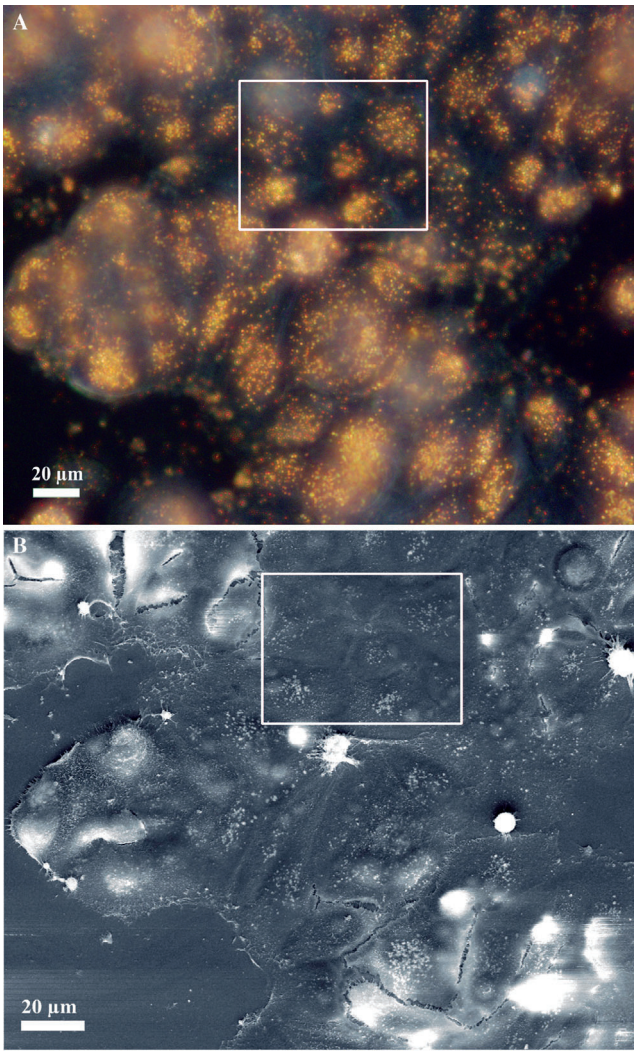

Figure 4. A) Dark field optical micrograph and B) SEM image of cells treated with MTAB NRs.

correlated SEM imaging of the exact same group of cells, we fixed the sample with glutaraldehyde and further cross-linked the cells with osmium tetroxide. This enabled us to prevent the collapse of cells under high vacuum conditions required for the SEM. The SEM imaging correlated with the optical experiments (Figure 4B) shows areas of increased brightness inside the cells because of clusters of internalized MTAB NRs. The locations of the NR clusters match up well with the bright spots seen in the optical micrograph, although there are some morphological changes that occurred during the fixation and dehydration processes. Most importantly, SEM imaging reveals that there are no MTAB NRs on the surface of the cells, which would be impossible to confirm by the optical microscopy alone. In contrast, the visualization of nanorods residing in thicker cell areas is more efficient by optical microscopy, displaying their strong potential as imaging contrast agents.

To further characterize the cellular uptake, we performed transmission electron microscopy (TEM) imaging of cells treated with the MTAB NRs as shown in Figure 5. Unlike SEM and optical microscopy, TEM images of the microtomed

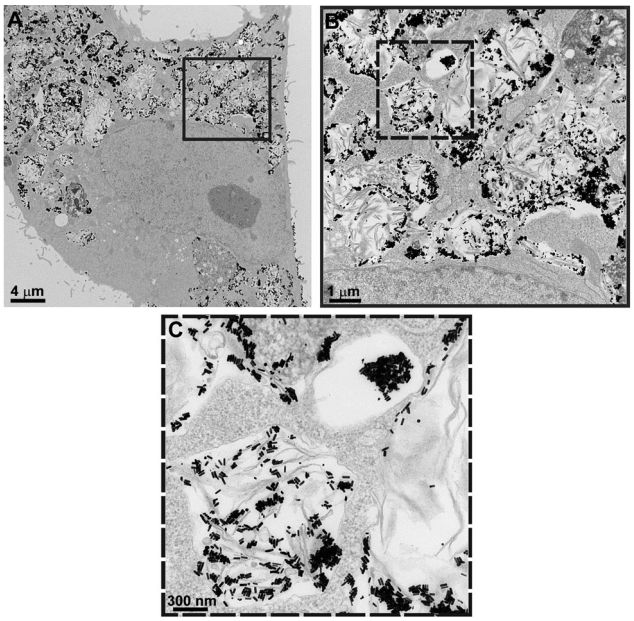

Figure 5. TEM images of microtomed MCF-7 cancer cells treated with the MTAB NRs (75 nm thick). A) View of an entire cell cross-section. B) Magnified image of the area outlined by the black box in panel A; C) Magnified image of the area outlined by the dashed box in panel B.

cross-sections reveal precise location and spatial distribution of individual nanorods. We prepared 75 nm thick sections that were cut through the center of cells. The TEM image of the entire cell cross-section (Figure 5A) shows an extremely large amount of NRs, which appear as dark particles. The volume of this cross-sectional sample is approximately 0.5% of the total cell volume (20 μm in diameter), which means that the total number of nanorods inside the cell is approximately 200 times greater than that present in this image (about 10000 NRs). Thus, even on the basis of TEM one can roughly estimate that there are at least two million nanorods per cell. If confirmed by quantitative methods, it would be significantly larger than the numbers reported in the literature for nanorods of similar size, which range from several hundred[31] to one-hundred-fifty thousand NRs per cell.[29] Even at low magnification one can see that NRs do not enter the nucleus and are clustered in endosomes. Higher magnification of regions outlined by the boxes (Figure 5B and C, respectively) clearly shows individual nanorods that do not appear to be free in the cytoplasm.[29,44–46] The amount of uptake was compared to another popular NR system, pegylated nanorods (PEG NRs), which are known to resist cellular uptake and thus form a good negative control for our system. To quantify the average number of NRs taken by cells, we performed ICP-OES analysis on dissolved cells which had been treated with both MTAB NRs and their pegylated analogues. Based on these results, while almost no PEG NRs were taken up (less than 1%), about 40% of MTAB NRs in solution were taken up by the cells. This corresponds to a level of about 2.17 million MTAB NRs per cell that have been treated with a solution of nanorods (see the Supporting Information for detailed calculations). Such a great amount of metallic gold increases the mass of cancer cell by 0.13 ng, which is approximately

13% of a typical cell mass (1 ng). It is particularly remarkable that the cells retain their viability and continue to proliferate with such a significant amount of gold nanostructures inside them. These results show that the MTAB NRs have a very low cytotoxicity and suggest that they may be promising for further biomedical applications such as drug/gene delivery and photothermal therapy.

In conclusion, we have synthesized highly stable, functionalized gold nanorods as an alternative to CTAB-capped NRs through a direct quantitative exchange with a thiolated CTAB analogue. The MTAB NRs showed a highly increased stability to multiple rounds of purification compared to CTAB NRs. We were able to quantitatively prove the complete removal of CTAB through the use of ^{1}H NMR spectroscopy, and thus determined the exact composition of the organic component of the hybrid nanostructures. We also successfully performed a TGA analysis to measure the exact organic versus inorganic composition of hybrid nanostructures and determined a grafting density for the MTAB thiol, proving the formation of a compact self-assembled monolayer on gold nanorods. The MTAB NRs were not only found to be nontoxic, but were also efficiently taken up by cancer cells in vitro in very large amounts as shown by a comprehensive combination of optical microscopy, SEM, TEM, and ICP-OES data.

Received: October 17, 2011
Published online: November 15, 2011

Keywords: cancer cells · cellular uptake · gold · nanorods · surface chemistry

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