Ecotoxicity and uptake of polymer coated gold nanoparticles


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Abstract
Bioconjugated gold nanoparticles (Au NPs) are a promising tool for pharmaceutical applications. However, the ecotoxicity of these types of NPs has hardly been studied. We investigated the ecotoxicity and uptake of 4–5 nm Au NPs to which two types of polymer coatings were attached. One coating was an amphiphilic polymer only and the other an amphiphilic coating to which 10 kDa polyethylene glycol chains were attached. In both 72 h algal growth inhibition tests with the alga Pseudokirchneriella subcapitata and in 24 h resazurin cytotoxicity tests with the rainbow trout gill cell line RTGill-W1, the pegylated Au NPs were found less toxic compared to the amphiphilic coated particles. No uptake or direct interaction between particles and algal cells was observed. However, uptake/adsorption in fish gill cells reached up to $>10^6$ particles/cell after 1 h and particles were eliminated for $>96\%$ after 24 h depuration. Both particle types were found within membrane enclosed vesicles in the cytoplasm of RTGill-W1 cells.

Keywords: Environmental toxicology, nanoparticles, surface chemistry, algae, RTGill-W1

Introduction
Colloidal gold nanoparticles (NPs) have been used since ancient times, among others as a staining component in glass. Faraday was the first to scientifically report the synthesis and optical characteristics of gold colloids (Faraday 1857). However, only in the last few years have gold NPs gained interest regarding their potential catalytic and bio-nanotechnological applications.

In addition to their flexible use as a catalyst (Han et al. 2009; Li et al. 2009; Tsunoyama et al. 2004), the optical properties of gold NPs make them an interesting labelling tool in various fields of biological research. Because of their strong visible light absorbing and scattering ability, a variety of detection techniques can be used, like dark field microscopy, photothermal imaging, photo-acoustic imaging and fluorescence microscopy (Sperling et al. 2008). Gold NPs also exhibit high contrast in transmission electron microscopy (TEM) and X-ray imaging. Besides biological labelling, gold NPs can be used as a carrier of DNA and drugs for specific and non-specific delivery into cells. The molecules of interest are adsorbed to the NP surface and once taken up in the cells, the molecules detach from the surface. A similar strategy is of particular interest for cancer therapy, where gold NPs can be covered with ligands specifically binding to receptors predominantly present in the membrane of cancer cells (Jain et al. 2007).

Besides their intrinsic characteristics useful in the above-mentioned and many other applications, the most important reason for their use arises from the possibility to synthesise, stabilise and modify gold NPs with application-specific tailored properties. First, gold NPs can be synthesised in aqueous and organic media through reduction of gold salts, like AuCl₃ (Sperling et al. 2007). Often, the particles are stabilised by a surfactant. The surface of gold NPs can then be modified in subsequent reactions aimed at producing a NP with the ligand of choice. Typically, molecules containing a thiol functional group are anchored to the gold surface through formation of Au-S bonds (Brust et al. 1994). If desired, these molecules can serve as a template for the attachment of other molecules (Woehrle et al. 2005).

Despite the high potential gold NPs offer for biomedical applications, concern has been raised regarding their biocompatibility and the potential risks to human health and the environment (Murashov 2009; Ray et al. 2009). Although gold is an inert material, size reduction towards the nanoscale can cause increased reactivity and toxicity. Due to their expected increased use, especially in pharmaceutical formulations, humans and the environment may be exposed to a wide variety of functionalised Au NPs. Therefore, their (eco-)toxic effects need to be addressed.
In this study, the ecotoxicity of two types of polymer coated gold NPs used as a precursor in biological applications was assessed using an algal species (*Pseudokirchneriella subcapitata*) and a rainbow trout gill cell line. Toxicity data were corrected for the interference caused by the optical properties of the particles. Furthermore, uptake of the particles in both cell types was investigated using TEM and confocal laser scanning microscopy.

**Materials and methods**

**Polymer coated gold NPs**

Gold NPs were synthesised according to previously published protocols (Lin et al. 2008; Zhang et al. 2010). The inner gold core of the NPs was around 4.5 nm in diameter and the surface was capped with dodecanethiol. One batch (amphiphilic coated gold NPs; AP) was coated with an amphiphilic polymer, consisting of a hydrophobic part (dodecylamine) and a hydrophilic part, poly-(isobutylene-alt-maleic anhydride). Following the coating procedure with the amphiphilic polymer (Lin et al. 2008; Zhang et al. 2010), another batch (pegylated amphiphilic coated particles; APma10kPEG) was prepared through post modification of the amphiphilic polymer with polyethylene glycol (PEG) molecules with a molecular weight of 10,000 g.mol\(^{-1}\) (Sperling et al. 2006), each containing a methyl and an amino group at the outer ends, whereby the amine functional group served as an anchor point for attachment to the functionalised NP surface. Both particle types are presented in Figure 1. Size determination of the gold core, the amphiphilic coated gold NPs and the pegylated (i.e., post-modified with 10 kDa PEG) APs was performed immediately after synthesis. The TEM derived size of the gold core was 4.6 nm. The particles were stored in 10 mM NaHCO\(_3\) and 100 mM NaCl solution at pH 10 and 4°C. Gold particle stock concentrations were 6.4 and 3.8 \(\mu\)M of AP and APma10kPEG particles, respectively. This corresponded to an estimated mass concentration of 2.5 and 1.5 g.l\(^{-1}\), respectively. More details on the synthesis and purification procedure and on the calculation of mass concentration are given in the online supplementary material. After purification, reaction by-products such as excess precursor molecules or empty polymer micelles were removed and the solution only contained the NPs (Sperling et al. 2007). In addition, any residual solvent was removed after synthesis during phase transfer (Lin et al. 2008). More details on the characterisation studies performed after synthesis are given in the online supplementary material. In the following paragraphs, amphiphilic coated gold NPs are referred to as ‘AP particles’ and the pegylated amphiphilic coated particles as ‘APma10kPEG’.

The polymer coating of the NPs was optionally modified with a fluorophore, cresyl violet, for confocal microscopy experiments (Zhang et al. 2010). The fluorophore was embedded rather inside than outside the polymer shell and did not increase the hydrodynamic diameter of the particles (Lin et al. 2008; Fernández-Argüelles et al. 2007).

**Characterisation in test media used for in vitro experiments**

Twenty-four hours after suspension preparation, the actual hydrodynamic size of both gold NP types was determined at 0.13 \(\mu\)M Au NP concentration (~50 mg Au.l\(^{-1}\)) and 25°C in the two ecotoxicity test media used in this study, i.e., the OECD algal medium (OECD 2006) supplemented with 3.6 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer at pH 7.4 and the Leibovitz’s L15 medium (Leibovitz 1963) without serum, using a PCS 4700 SM (Malvern Instruments, Worcestershire, UK) dynamic light scattering device. Incident light was produced by a 5 mW HeNe laser and scattered light was detected at an angle of 150°. A 7032 CN correlator (Malvern Instruments) was used for data processing. Monomodal analysis was performed to determine particle size distributions, whereas the harmonic intensity weighted average hydrodynamic diameter was obtained by the cumulant analysis option of the automeasure software (Malvern Instruments). All samples were analysed in triplicate. After 96 h at 25°C under illumination, the particle size distributions of AP and APma10kPEG in OECD algal medium were analysed again.

The light absorbance spectrum of 0.05 \(\mu\)M Au NPs (~20 mg Au.l\(^{-1}\)) particle suspensions in Leibovitz’s L15 test medium was recorded using an Aquamate spectrophotometer (Thermo Electron Corporation, Waltham, MA, US) between wavelengths of 315 and 900 nm.

![Figure 1](image-url) 4-5 nm Au nanoparticles (NPs) capped with dodecanethiol, coated with an amphiphilic polymer, consisting of dodecylamine and poly-(isobutylene-alt-maleic anhydride) (PMA) (A), to which 10 kDa polyethylene glycol (PEG) molecules are attached (B).
Ecotoxicity testing

Algal assays
The alga *Pseudokirchneriella subcapitata* was obtained from the Culture Collection of Algae and Protozoa (CCAP 278/4, 121 Oban, Scotland) and was cultured at Ghent University in ES-medium at 1/2 strength (Provasoli 1968) which was added to carbon filtered aerated tap water, supplemented with 1.4 mg l⁻¹ FeSO₄.7H₂O, 15 mg l⁻¹ NaH₂PO₄.2H₂O, 150 mg l⁻¹ NaNO₃ and 2.35 mg l⁻¹ MnCl₂.4H₂O. Four days prior to the start of 72 h algal growth inhibition experiments, a new algal culture was prepared and allowed to grow on a shaking table at 20 ± 1°C in continuous light (70 μE·m⁻²·s⁻¹). This new culture was subsequently used to inoculate each replicate of a growth inhibition experiment with 10,000 cells ml⁻¹.

Test concentrations, which were prepared in OECD algal test medium supplemented with 3.6 mM MOPS buffer at pH 7.4 one day in advance of the test and equilibrated at 25°C, varied between 0.0012 and 0.12 μM Au NPs (0.046–46 mg Au l⁻¹). Both NP types were tested twice according to the standard OECD guideline No. 201 (OECD 2006). For each test concentration, three replicates and one blank correction (no inoculation with algae) were included. During the 72 h test, all flasks were incubated at a temperature of 25°C under continuous illumination (70 μE·m⁻²·s⁻¹) and were shaken manually three times a day. Every 24 h, the fluorescence of extracted chlorophyll in a sample from each replicate was measured. To this end, 3 ml extraction mixture (dimethylsulfoxide:acetone (1:1): was added to a 0.75 ml sample, vortexed and allowed to stand in the dark for at least 20 min. Chlorophyll fluorescence was recorded with a spectrophotometer (LS50B, Perkin Elmer, Zaventem, Belgium) in a 1 cm quartz cuvette at a wavelength of 671 nm, using an excitation wavelength of 431 nm (Mayer et al. 1997). The fluorescent signal of each sample was subsequently converted to an algal cell density using chlorophyll fluorescence measurements of algal concentration series prepared in the control medium and in each Au NP test concentration (M(10) and 100 mg Au l⁻¹) AP Au NPs, the detected chlorophyll fluorescence. The slope of the linear regression decreased from 3.29 in the control to 2.79 in 0.057 μM Au NPs (22 mg l⁻¹) suspensions. By preparing a concentration series in each concentration 20.0057 μM, we could correct for this interference. A possible shading effect caused by the gold NPs was investigated using the approach outlined by Hund-Rinke & Simon (2006). Briefly, the chlorophyll contents upon spatially separating Au NPs and algal cells in two different 96-well plates, one transparent plate positioned on top of a white plate, was compared to the chlorophyll contents when algal cells and NPs were added to the same well and allowed to stand for 3 days at 25°C under continuous illumination. The initial cell density in the wells was 10,000 cells ml⁻¹ and the plates were shaken manually three times a day. Again, algal cell densities were determined relative to a concentration series prepared in the control medium or in the corresponding Au NP concentration.

Assays with the fish gill cell line RTGill-W1

The RTGill-W1 cell line was first developed and described by Bols et al. in 1994.

The cell line originated from a primary culture of gill epithelial cells of the rainbow trout (*Oncorhynchus mykiss* (Walbaum)). The cells exhibit epithelial morphology and are believed to have derived from undifferentiated precursor gill stem cells. The cells were a gift from Dr. Kristin Schirmer (Eawag, Dübendorf, Switzerland) and were routinely cultured at 20°C in 75 cm² tissue culture flasks with 10 ml of L15 supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS).

*In vitro* cytotoxicity tests were performed with 25,000 cells per well attached overnight to the surface of an opaque 96-well plate. For attachment, each well contained 100 μl L15 medium supplemented with 10% FBS. Before exposure, the cell layers were washed with 100 μl L15 medium. The cells were subsequently exposed for 24 h to a 100 μl gold NP suspension in L15 medium, following colorimetric measurement of cell metabolic activity using the resazurine dye (O’Brien et al. 2000). The tests involved five replicates of each test concentration (including control) containing cells and one no-cell treatment for each test concentration (including control). Following the 24 h exposure period, 10 μl of a 500 μM resazurin solution in phosphate buffered saline (PBS) was spiked to each well and allowed to react for 3.5 h at 20°C. Ninety microlitre reaction product from each well was subsequently transferred to a white 96-well plate. Finally, immediately before measurement, 20 μl AP or APm10kPEG suspension in L15 medium was added in order to set the final Au NP concentration in each well to the highest test concentration used, i.e., 0.12 μM Au NPs (~46 mg Au l⁻¹). In this way, the decrease in fluorescent signal of resorufin was equal for each replicate. Resorufin fluorescence was recorded using the LS50B device, adopting excitation and emission wavelengths of 560 and 590 nm, respectively.

The need to adjust the final Au NP concentrations in each well to the maximum test concentration arose from the interference of the Au NPs with the fluorescent detection of resorufin (Sigma Aldrich, No. R3257), which is shown in online supplementary Figure S6. In presence of 0.026 and 0.26 μM (~10 and 100 mg Au l⁻¹) AP Au NPs, the detected...
fluorescence of resorufin strongly decreased, resulting in a decrease of the slope of the linear regression from 40.8 to 31.4 and 7.2, respectively.

Statistical data treatment
In algal assays, concentration–response curves of AP and APma10kPEG particles were fitted to the log-logistic and modified log-logistic model, respectively. Because of the appearance of a horizontal plateau in the concentration–response curves of AP particles, the modified log-logistic equation better described the experimental data of AP than the log-logistic equation (Van Hoecke et al. 2008). In fish gill cell assays, effect concentrations were determined using the log-logistic model. Effect concentrations of AP and APma10kPEG established in the algal growth inhibition assays and in the cytotoxicity tests were compared using t-tests for dependent samples (α = 0.05). To assess the importance of shading, Jonckheere-Terpstra step down trend tests were used to identify treatments that showed a significant difference compared to the control (Jonckheere 1954; Terpstra 1952).

Cellular uptake studies
Fluorescently labelled gold NPs
Uptake of gold NPs by RTGill-W1 cells, both qualitatively and quantitatively, was studied using the same two types of gold NPs to which a cresyl violet perchlorate fluorescent label was attached (Zhang et al. 2010). Uptake and/or strong adsorption to the cell membrane was qualitatively detected using a confocal laser scanning microscope (LSM 510 META, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with excitation at 543 nm with a helium-neon laser and selection of emission wavelength with a 560 nm long pass filter. The images were taken with a 100× immersion oil objective (N.A. 0.55). Samples were prepared by exposing ~80% confluent cell layers in 75 cm² culture flasks for 24 h to 10 ml L15, L15 + 0.013 μM (~5 mg Au.l⁻¹) AP and APma10kPEG particles. No serum was supplemented. After exposure, the medium was poured off and the cell layer was treated with 5 ml trypsin working solution (Invitrogen, Merelbeke, Belgium, No. 25300). Cells were then transferred to 50 ml centrifugation tubes containing 10 ml L15 medium, followed by centrifugation for 5 min at 150 × g. The pellet was resuspended in 1.5 ml L15 medium and transferred to an Eppendorf tube, after which a second 5 min centrifugation at 150 × g was performed. The pellet was resuspended in formalin (Sigma Aldrich No. HT501128), followed by centrifugation (150 × g, 5 min). Again, the pellet was resuspended in formalin. The fixed samples were used for confocal laser scanning microscopy.

Uptake of AP particles was quantified using a short exposure time (1 h), after which the cells were still intact and apoptosis did not yet result in a substantial loss of cells. Exposure concentrations used were 0.0026, 0.0057, 0.012, 0.026, 0.057, 0.12 and 0.26 μM Au NPs (~1.0, 2.2, 4.6, 10, 22, 46 and 100 mg Au.l⁻¹). In 24-well plates, 200,000 cells/well were allowed to attach overnight and were rinsed with L15 medium. The cell layer was subsequently exposed to 1 ml medium with or without fluorescently labelled gold particles. For each treatment, six replicates containing cells and six replicates without cells were included. After 1 h exposure, the medium was removed and all wells were rinsed three times with PBS at 4°C. Then, 250 μl L15 medium and 60 μl 1% Triton X-100 were added to each well in order to lyse the cells. The cell homogenate was transferred to a 0.5 ml Eppendorf tube and centrifuged for 2 min at 1000 × g. Finally, 200 μl of the supernatant was used for fluorescent detection in a white 96-well plate, using an excitation wavelength of 600 nm and an emission wavelength of 630 nm. The concentration of Au NPs in both the cell homogenates and identically treated no cell controls was calculated relative to a standard series in the same matrix (L15 + 0.2% Triton X-100) and finally the concentration in cell homogenates was corrected for the Au NPs still present in the no cell controls.

In a separate experiment, the elimination of AP particles by the RTGill-W1 cells over a 24 h period post exposure to 0.12 μM Au NPs (46 mg.l⁻¹) was investigated. An identical procedure was used, except that after the 1 h exposure to AP particles rinsing of the cell layer was performed with PBS at 20°C instead of at 4°C, in order not to induce any changes due to a temperature decrease. Elimination was allowed in 1 ml L15 medium per well. After 4 and 20 h, the L15 medium was renewed in each well.

Transmission electron microscopy
TEM was used to investigate uptake of the two gold NP types in both the algae and the fish cells. The exposure conditions were identical to the cytotoxicity experiments, except for the fact that fish cells were exposed in a 75 cm² culture flask. Algal cells and RTGill-W1 cells were exposed to AP particles at concentrations of 0.0026 μM (~1.0 mg Au.l⁻¹) and 0.0057 μM (~2.2 mg Au.l⁻¹), respectively, and to APma10kPEG particles at concentrations of 0.012 μM (~4.6 mg Au.l⁻¹) and 0.0057 μM, respectively. Sample pre-treatment was performed as described in Van Hoecke et al. (2009). Ultrathin sections (100 nm) were cut using an ultramicrotome (RMC, PowerTomeXL, Tucson, Arizona, USA) with a diamond knife (Diatome, Biel, Switzerland). These samples were imaged with an FEI Tecnai G2 Spirit Biotwin TEM (Fei, Hillsboro, OR, USA) at an operating voltage of 120 kV.

Results
Characterisation in test media
Gold NP suspensions were stable under experimental conditions in both test media. The intensity-weighted particle size distributions of both AP and APma10kPEG indicated small aggregates up to 150 nm; however, less than 2% of the signal arose from aggregates >100 nm. Average diameters (standard deviation) and polydispersity indexes are given in Table I. At the end of an algal growth inhibition test, the suspensions were still stable. Particle size distributions are presented in online supplementary Figure S7. Data correspond reasonably with previous studies which had been performed in different media (Sperling et al. 2007).

The absorbance spectra of both Au NP types in L15 medium are given in online supplementary Figure S8.
These particles absorb over the entire visible light spectrum with a peak maximum around 515 nm, which corresponds to the plasmon peak (Sperling et al. 2007). The absorbance spectrum between 315 and 900 nm in L15 was similar to that obtained immediately after synthesis (online supplementary Figure S3). This indicates that the NPs were well dispersed.

**Effects on the alga Pseudokirchneriella subcapitata**

In the algal growth inhibition tests, 10, 20 and 50% effect concentrations were established in the low milligram per litre range. Mean values and standard deviations obtained in the two experiments are given in Table II. Based on 72 h 10 and 20% effect concentrations on growth rate (i.e. the concentration that gives rise to a 10 and 20% decrease in algal growth rate relatively to the control, respectively) (72 h-E-C10 and E-C20), t-tests for dependent samples (α = 0.05) indicated that the amphiphilic coated Au NPs were more toxic than the APma10kPEG particles. For E-C10 and E-C20 p values of the t-tests were 0.037 and 0.045, respectively. However, no significant difference between 72 h-E-C10 values was identified by the t-test for dependent samples (p = 0.071). Figure 2 presents the concentration–response curves obtained in the two experiments.

In addition, it was investigated if light limitation might have contributed to the observed reduction in algal growth rate. The 96-well plate experiment showed that algal cells have contributed to the observed reduction in algal growth two experiments.

**Table I. Mean Au particle sizes and polydispersity indexes (PI) determined by DLS in OECD algae medium and in Leibovitz’s L15 medium without serum.**

<table>
<thead>
<tr>
<th>Medium</th>
<th>AP Diameter (standard deviation) (nm)</th>
<th>PI (standard deviation)</th>
<th>APma10kPEG Diameter (standard deviation) (nm)</th>
<th>PI (standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OECD (after 24 h)</td>
<td>17.6 (3.8) 0.293 (0.155)</td>
<td></td>
<td>35.6 (0.7) 0.354 (0.048)</td>
<td></td>
</tr>
<tr>
<td>OECD (after 96 h)</td>
<td>19.3 (2.0) 0.213 (0.112)</td>
<td></td>
<td>39.3 (2.9) 0.281 (0.080)</td>
<td></td>
</tr>
<tr>
<td>L15</td>
<td>21.5 (0.7) 0.415 (0.025)</td>
<td></td>
<td>37.3 (1.3) 0.292 (0.064)</td>
<td></td>
</tr>
</tbody>
</table>

In vitro cytotoxicity assays with the gill cell line RTGill-W1

In four separate 24 h resazurin assays with both particle types, mean EC10 values (standard deviation) of 0.012 (0.009) and 0.039 (0.025) μM Au NPs (or ~4.7 (3.3) and 15 (10) mg.l⁻¹) were calculated for amphiphilic and pegylated amphiphilic coated Au NPs, respectively. Mean EC20 values (standard deviation) were 0.039 (0.015) and 0.14 (0.05) μM Au NPs (~15 (6) and 54 (18) mg.l⁻¹), respectively. The 24 h-EC10 values obtained for both NP types were not significantly different (p = 0.135). However, based on the 24 h-EC20 values the AP particles were more toxic than the pegylated APma10kPEG particles (p = 0.010). Concentration–response curves for both NP types are given in Figure 4. The latter figure presents data points as overall mean values (standard deviation) of individual replicates assessed in four separate experiments. The four individual concentration–response curves of each experiment are shown in the online supplementary material. In Figure 5, morphology of the cell layer exposed to both types of Au NPs (0.12 μM) and in the control is shown. Clearly, cell lysis in cells exposed to Au NPs affected the cell density on the well surface. Furthermore, the shape of cells exposed to Au NPs changed from elliptical typically for epithelial cells to more spherically shaped due to swelling of the cells. The purple colour originated from the interaction between the light microscope and the opaque colour of NP suspensions. The pictures were taken before addition of the resazurin and hence the opaque colour did not originate from resorufin.

**Cellular uptake**

Qualitative and quantitative uptake using fluorescently labelled particles

The images in Figure 6 represent the RTGill-W1 control and gill cells exposed to both fluorescently labelled AP and APma10kPEG Au NPs. Fluorescence images are shown in online supplementary Figure S11. Uptake of AP was detected using confocal laser scanning microscopy (Figure 6B and C). Using the overlay images, the particles were found distributed all over the cytoplasm, but no internalisation into the cells’ nuclei was observed. The APma10kPEG were taken up to a much lesser extent (Figure 6D). However, the labelling efficiencies of the NPs with fluorophores varied from batch to batch. For this reason, the difference in fluorescence observed in the cells does not directly relate to the difference in toxicity.

**Table II. Effect concentrations (standard deviation) (n = 2) of AP and APma10kPEG particles obtained in 72 h algal growth inhibition tests.**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>AP (μM)</th>
<th>APma10kPEG (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-C10</td>
<td>0.0031</td>
<td>0.013</td>
</tr>
<tr>
<td>E-C20</td>
<td>0.0049</td>
<td>0.028</td>
</tr>
<tr>
<td>E-C50</td>
<td>0.019</td>
<td>0.10</td>
</tr>
<tr>
<td>E-C10 (mg.l⁻¹)</td>
<td>1.2</td>
<td>5.1</td>
</tr>
<tr>
<td>E-C20 (mg.l⁻¹)</td>
<td>1.9</td>
<td>11</td>
</tr>
<tr>
<td>E-C50 (mg.l⁻¹)</td>
<td>7.5</td>
<td>39</td>
</tr>
</tbody>
</table>
in amount of particles taken up. In the images shown, the fluorescent signal of the cresyl violet label was weaker for the pegylated particles (factor 8) compared to the non-pegylated particles. In Panel D of Figure 6, it was difficult to distinguish the fluorescence with the naked eye. However, observation of the fluorescence image in the dark confirmed the uptake of the pegylated particles in the cells.

In the quantitative study, the concentration of AP particles in the cell homogenate of cells exposed to 0.0026, 0.0057 and 0.012 μM NPs (~1.0, 2.2 and 4.6 mg Au.l⁻¹) was lower than the method detection level (<1.2 × 10⁻⁴ μM Au NPs or <0.048 mg Au.l⁻¹). However, cells exposed to 0.026, 0.057, 0.12 and 0.26 μM NPs (~10, 22, 46 and 100 mg Au.l⁻¹) suspensions increasingly sequestered the particles up to 0.0078 μM Au NPs (~3.1 mg Au.l⁻¹) in the cell homogenate (concentration corrected for no cell controls) of cells exposed to 0.26 μM (~100 mg Au.l⁻¹) AP particles. Table III lists the results of the uptake study in RTGill-W1 cells, with quantities expressed as concentration, absolute mass and an estimation of the amount of particles per cell. The amount of particles per cell was estimated in the order of 10⁶.

In a separate experiment, the elimination of AP particles was studied: cells were exposed for 1 h to 0.12 μM (~46 mg Au.l⁻¹) suspensions followed by a 24 h depuration period. The concentration of AP particles in the cell homogenate was lower than the method detection level. Hence, it can be concluded that at least 96% of the particles initially taken up were eliminated within the 24 h depuration period.

**Transmission electron microscopy**

Exposure to AP and APma10kPEG particles did not affect the algal cell morphology, as can be observed from the TEM images in Figure 7. Moreover, no particles were observed in the intracellular environment, attached to the cell membrane or closely surrounding the cells. Algal cell walls were intact. Possibly, only weak interactions between algal cells and polymer coated Au NPs took place, which has led to the complete elimination of particles during TEM sample pretreatment.

No effect on fish gill cell membrane could be observed. Online supplementary Figure S10 shows a cross section of both a cell in the control and a cell exposed to APma10kPEG particles.

All particles taken up in the cells could be visualised in the dark shaded cellular vesicles to which the OsO₄ bound. No large aggregates could be observed in the vesicle and the particles appeared as single spheres or as clusters of a few particles. The size of the gold core of individual particles inside the vesicle shown in panel C of Figure 8 ranged between

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**Figure 2.** Concentration–response curves of AP and APma10kPEG particles obtained in two independent 72 h algal growth inhibition tests.

**Figure 3.** Results of 96-well plate experiment to assess the shading effect induced by AP (part A) and APma10kPEG (part B) particles. The white bars indicate algal cells directly exposed to Au nanoparticles (NPs) in the same well, while the black bars indicate algal cells spatially separated from the particles, in which light first had to cross an Au NP suspension in OECD medium. Treatments significantly different from the control are indicated by an asterisk (*).
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the luminescent Photobacterium phosphoreum cytocyte culture exposed to 5 \textit{in vitro} maximum test concentration of 17.4 mg.l\(^{-1}\). However, a significant increase in cellular ROS levels was observed relative to the control after a 15 day exposure in soil, indicating that the Au NPs indirectly affected growth of lettuce plants. In contrast, Barrena et al. (2009) did not observe any significant toxic effect of 10 nm citrate coated gold NPs on lettuce and cucumber germination at 62 mg.l\(^{-1}\) concentration.

Two \textit{Daphnia magna} studies using citrate coated Au NPs with diameters around 20 nm reported a 48 h acute lowest observed effect concentration of 0.5 mg.l\(^{-1}\) \(\text{mg Au.l}^{-1}\) and a 48 h EC50 of 70 mg.l\(^{-1}\) (Lovern et al. 2008; Li et al. 2010). Gold NPs distributed along the gut and eventually were eliminated from the digestive tract within 24 h timeframes each (Lovern et al. 2008). External appendages were covered with Au NPs, causing changes in \textit{Daphnia's} swimming pattern (Li et al. 2010).

In contrast with above referenced studies in which only mild effects were described, Renault et al. (2008) reported mortality of the green alga \textit{Scenedesmus subspicatus} during exposure to 10 nm Au NPs saturated with a 1.7 kDa polymer coating containing a positively charged amine group at the outer end. At the highest test concentration of \(1.6 \times 10^5\) Au NPs per algal cell, 50% algal mortality was observed after 24 h. Furthermore, the Au NPs were found to strongly adsorb to the cell wall, including the cellulosic layer after penetration into the trilaminar layer of the cell wall. However, no uptake was observed into the intracellular environment. The high affinity of the amine coated Au NPs for the \textit{Scenedesmus subspicatus} algal cell wall could be explained by the electrostatic attraction between the negatively charged cell surface and the positively charged amine groups (Renault et al. 2008). This situation was clearly different from our experiments with \textit{Pseudokircheriella subcapitata}, in which the Au NPs had a coating with uncharged organic functional groups at the outer ends (APma10kPEG) or with negatively charged carboxyl groups (AP). Therefore, the particles were not electrostatically attracted to the algal cells and perhaps the bulky organic PEG coating might even have caused steric repulsion in our experiments. Furthermore, the experimental approaches in our study and in the study by Renault et al.

Figure 4. Concentration–response curves of AP and APma10kPEG Au nanoparticles (NPs) obtained in 24 h resazurin cytotoxicity assays with RTGill-W1 cells in L15 medium without serum. Data points present mean values (standard deviation) assessed in four separate experiments.

3.19 and 7.97 nm (see online supplementary Figure S12). Furthermore, no change on cellular arrangement could be detected at the low NP concentrations used in the TEM study.

**Discussion**

Amphiphilic and PEG + amphiphilic coated Au NPs were stable under experimental conditions. The DLS derived average hydrodynamic sizes were larger compared to the TEM observed particle sizes. This is due to the fact that the TEM shows contrast only for the inorganic particle core but not for the surrounding polymer coating. In addition, a solvent layer is expected to be associated with the coated Au NPs, which can also explain the larger hydrodynamic size. A similar observation was made by Uboldi et al. (2009).

So far, several ecotoxicity studies used gold NPs. Because of the variety in Au NP sizes, coatings and test organisms used ecotoxicity studies are difficult to compare. However, with few exceptions, observed toxic effects were not severe and only occurred at high test concentrations of at least tens of mg.l\(^{-1}\). One study reported the absence of effects on \textit{in vitro} metabolic activity in a rainbow trout primary hepatocyte culture exposed to 5–10 nm citrate coated Au NPs at a maximum test concentration of 17.4 mg.l\(^{-1}\). However, a threefold increase in cellular ROS levels was observed (Farkas et al. 2010). Barrena et al. (2009) described no significant effects on anaerobic microbial communities exposed to 10 mg.l\(^{-1}\) citrate coated 10 nm Au NPs. Also, the luminescent \textit{Photobacterium phosphoreum} was not affected at 28 mg.l\(^{-1}\). On the other hand, the metabolic activity of luminescent bacteria decreased upon exposure to 5 nm Au NPs coated with bovine serum albumin, with 50% effect concentration of 26 \(\mu\)M. However, no apparent cell death was observed (Zheng et al. 2010). Shah & Belozerova (2009) were unable to detect any significant effect on a soil microbial community exposed for 15 days to dodecanethiol functionalised gold NPs of undefined size spiked to the soil at 0.013 and 0.066\% (w/w). On the other hand, increase in the shoot-root ratio of germinated lettuce seeds was observed relative to the control after a 15 day exposure in soil, indicating that the Au NPs indirectly affected growth of lettuce plants. In contrast, Barrena et al. (2009) did not observe any significant toxic effect of 10 nm citrate coated gold NPs on lettuce and cucumber germination at 62 mg.l\(^{-1}\) concentration.

Figure 5. RTGill-W1 cell morphology in the control (A) and exposed to 0.12 \(\mu\)M (–46 mg Au.l\(^{-1}\)) AP (B) and APma10kPEG (C) particles in L15 without serum after 24 h.
are completely different. Renault et al. (2008) studied algal cell mortality in a dense algal suspension (30 x 10^6 algal cells.mL^-1), while in our study algal growth inhibition of a starter culture of 1.0 x 10^4 cells.mL^-1 was the endpoint. However, when expressing exposure concentrations of both studies as number of NPs.l^-1, it becomes clear that the amine coated Au NPs used by Renault et al. (2008) caused the 50% decrease in algal cell survival at a concentration of 4.8 x 10^15 NPs.l^-1, while only 10% decrease in algal growth rate was observed in our study at AP and APma10kPEG particle concentrations of 1.9 x 10^15 and 7.9 x 10^15 NPs.l^-1, respectively. Hence, for a similar Au NP concentration, the observed effect on algal cell mortality was much more severe for the amine coated Au NPs compared to the amphiphilic and APma10kPEG.

In the study by Renault et al. (2008), the contaminated algae were subsequently fed to the benthic mollusc Corbicula fluminea. Despite the detectable uptake of Au NPs in various tissues of the digestive tract, the uptake was limited to the epithelial layer and did not cause any morphological damage. A TEM analysis revealed the presence of the particles in lysosomal vesicles inside the cells, but not freely in the cytoplasm or the cells' nucleus. Tedesco et al. (2010) studied accumulation of 5 nm mercaptopropionic acid

Table III. Quantitative uptake of AP particles in RTGill-W1 cells exposed to various Au nanoparticle (NP) concentrations in L15 medium without serum during 1 h.

<table>
<thead>
<tr>
<th>Exposure concentration</th>
<th>Concentration in cell homogenate (standard deviation)</th>
<th>Mass taken up by cell layer^a (standard deviation)</th>
<th>Number of particles per cell^b (x10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.l^-1</td>
<td>μM Au NPs</td>
<td>mg.l^-1</td>
<td>μM Au NPs</td>
</tr>
<tr>
<td>10</td>
<td>0.026</td>
<td>0.05 (0.10)</td>
<td>0.0001 (0.0003)</td>
</tr>
<tr>
<td>22</td>
<td>0.057</td>
<td>0.29 (0.12)</td>
<td>0.0007 (0.0003)</td>
</tr>
<tr>
<td>46</td>
<td>0.120</td>
<td>1.2 (0.1)</td>
<td>0.0031 (0.0003)</td>
</tr>
<tr>
<td>100</td>
<td>0.260</td>
<td>3.1 (0.1)</td>
<td>0.0078 (0.0003)</td>
</tr>
</tbody>
</table>

^aCalculated as c_h V_h, where c_h is the concentration of Au NPs in the cell homogenate (mg.l^-1) (= outcome of the measurement) and V_h is the volume of the cell homogenate (= 310 μl); ^bCalculated as: N_AuNP = \frac{c_h N_A V_h}{389.10^6 N_c} where c_h is the concentration Au NPs in the cell homogenate (mg/l), N_A is Avogadro’s number (6.02.10^23 mol^-1), V_h is the volume of the cell homogenate (310 μl) and N_c is the number of cells per well (200,000). The factor 389 X 10^6 arises from the conversion between Au NP mass concentration (mg Au.l^-1) and Au NP molar concentration (M Au NPs) as explained in section 5 in the supplementary material.

Figure 6. Overlap laser scanning microscopy images of RTGill-W1 cells in the control (A) and exposed to 0.013 μM (~5 mg Au.l^-1) AP (B, C) and APma10kPEG (D) particles. The height of the Z plane where the fluorescence was registered is given in parts B, C and D.
coated Au NPs in the bivalve *Mytilus edulis*, which predominantly occurred in the digestive gland. Indications of reactions with thiol containing proteins and oxidative stress were reported at 0.5 mg.l⁻¹ concentrations. No such effects were observed with similar 13 nm Au NPs (Tedesco et al. 2008). So far, it is unclear whether the toxicity observed in our experiments is due to oxidative stress as well.

From the discussion above, it is clear that coating characteristics and NP size affect Au NP toxicity. However, in a previous study it has been demonstrated that in our case cytotoxicity arises from the Au core rather than from the polymer shell. The polymer coated gold particles induced pro-inflammatory response in *in vitro* studies, while the plain polymer shell did not (Lehmann et al. 2010). To date, however, no experimental data are available on any possible post-modification of the polymer under intracellular conditions.

Likewise, *in vitro* toxicity studies with Au NPs in the context of human toxicology have used diverse NP sizes, coatings and cell types. Those *in vitro* toxicity studies point out that predominantly only minor effects were observed following exposure of various cell lines to Au NPs. Differences in toxicity can be due to differences in NP size, whereby smaller NPs were found to be more toxic (Pan et al. 2007). Importantly, toxicity can specifically be due to the stabilising coating used and in general, positively charged coatings induced a more severe effect compared to negatively charged coatings (Wang et al. 2008; Goodman et al. 2004).

Concerning Au NP cellular uptake, every study published so far confirmed that Au NPs with different coatings were readily taken up by a large variety of cell types. For example, Chithrani et al. (2006) established uptake half-lives of about 2 h of 14, 50 and 74 nm particles. Furthermore, several studies concluded that endocytic pathways were responsible for Au NP uptake in cells, since particles were mostly observed in the cytoplasm encapsulated by membrane surrounded vesicles (Shukla et al. 2005; Uboldi et al. 2009). Shukla et al. (2005) reported intracellular movement of lysosomes containing Au NPs towards the nucleus and subsequent perinuclear arrangement without internalisation into the cell’s nucleus. In contrast, Gu et al. (2009) convincingly demonstrated the affinity of di-amine PEG coated Au NPs towards the HeLa cells’ nuclei. Both NP types used in our study were not freely dispersed into the cytoplasm, but were encapsulated by closed membrane vesicles, in agreement with Shukla et al. (2005) and with Uboldi et al. (2009). In the quantitative uptake study, more than 10⁶ amphiphilic coated Au NPs were found strongly adsorbed or taken up by each cell after 1 h. The same order of magnitude was reported by Gu et al. (2009).
for pegylated Au NPs with a 3.7 nm Au core after 6 h treatment. After 24 h, the amount of cells taken up reached a plateau. Nevertheless, the amounts taken up in our study seem to exceed the ones reported by Gu et al. (2009). Possible explanations include the fact that our experimental approach reflects the sum of particles strongly attached to the cell wall and particles taken up into the intracellular environment. Possibly, the amount of particles adsorbed to the cell wall represents a substantial fraction of the final result. The increase of fluorescent signal because of the presence of free fluorescent molecules or the leakage of fluorophores from the particles cannot have played a major role in the present study because of the very high colloidal stability of the polymer coated NPs. As the particles are highly stable they could be subjected to vigorous purification by size exclusion chromatography or gel electrophoresis. Furthermore, the fluorophore is covalently attached to the inner polymer shell (Fernández-Argüelles et al. 2007). On the other hand, pegylation was found to decrease cellular uptake in the present study, which points to the importance of surface coating for particle uptake. Size is clearly not the reason why pegylated Au NPs are internalised to a lesser extent. In a previous study, the hydrodynamic diameter of Au NPs with amphiphilic polymer shell and with amphiphilic polymer shell + 10 kDa PEG has been determined to be around 12 and 28 nm, respectively (Sperling et al. 2007). An increase in size from 12 to 28 nm is not likely to be the predominant effect. On the other hand, it has been reported that PEG, due to reduced nonspecific interaction with the environment, reduced uptake by cells due to surface chemistry (Wang et al. 2010). Therefore, clear differences in in vitro and in vivo uptake in the dependence of the surface coating can be observed (Brandenberger et al. 2010; Lipka et al. 2010).

In total, about a dozen of in vitro studies have shown the absence or only moderate toxicity of surface modified Au NPs, which increases their value as a potential candidate in advanced pharmaceutical applications. Likewise, in the present study, only moderate toxicity was observed. Indeed, no more than 60% decrease in algal growth rate and 40% decrease in RTGill-W1 cell survival and proliferation was detected at 0.12 μM Au NPs (~46 mg Au.1⁻³) concentrations.

In comparison, organic polymer NPs like polyethylene imine (PEI), which have similar medical applications, have been shown to be less efficient (factor 8) than functionalised Au NPs in, for example, mediating DNA translocation across the cellular membrane (Goodman et al. 2004). In addition, PEI as free polymer and as polyplex with DNA was found to be highly toxic towards Pseudokirchneriella subcapitata, with 72 h EC₅₀ values of 41 and 67 μg PEI.1⁻¹ from 220 μg PEI.1⁻¹ on, algal cells were deteriorated (Robbens et al. 2010).

Acknowledgement

This work was supported by the European Union Sixth Framework Programme NanoInteract (NMP4-CT-2006-033231) and the Agency for Innovation through Science and Technology (IWT, Belgium). Parts of this work were supported by BMBF (WP, project UMSICHT). Furthermore, we thank Dr. Kristin Schirmer and Myriam Claey's.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supporting information including details of studies on the synthesis, characterization, ecotoxicity and uptake of gold nanoparticles are available online.
Ecotoxicity and uptake of polymer coated gold nanoparticles:
supporting information


1 Synthesis

Gold nanoparticles (NPs) arose through reduction of a gold solution in an organic phase. Basically, the synthesis of the gold NPs involved 4 steps: (1) phase transfer of gold solution to an organic phase, (2) reduction reaction, (3) surface modification with dodecanethiol and (4) purification. Finally, (5) Au NPs were coated. First, synthesis took place in toluene, followed by a surface modification with dodecanethiol. The thiol group bound to the gold NP surface, while the outer end served as an anchor point for subsequent attachment of polymers. Subsequently, particles were precipitated in methanol and resuspended in toluene in three washing steps in order to remove any excess dodecanethiol. Purified particles were dried in a N₂ stream. The detailed procedure was based on Lin et al. (2008) and on Zhang et al. (2010).

1.1 Phase transfer of gold solution to organic phase

In a beaker, 6.51 g of tetraoctylammonium bromide was dissolved in 240 ml of toluene and transferred into a 500 ml separation funnel. Tetrachloroauric acid (900 mg) was weighted into a 20 ml vial to which 75 ml Millipore water was added in three washing steps to yield a yellow translucent solution which was transferred to the separation funnel. The funnel was shaken for about 5 minutes in order to transfer the AuCl₄⁻ ions into the organic
phase. The transfer could be visually observed from a color change from transparent to dark red, while the initially yellow aqueous phase turned colorless. The toluene phase now contained tetraoctylammonium-gold ion pairs (see Equation 1). The toluene phase was transferred to a 500 ml round flask.

\[
\text{AuCl}_4^- (aq) + N(C_8H_{17})_4^+ (tol) \rightarrow N(C_8H_{17})_4^+ \text{AuCl}_4^- (tol)
\]  
(Equation 1)

1.2 Reduction reaction

In a beaker, 1002 mg of sodium borohydride was dissolved in 75 ml Millipore water under vigorous stirring, which caused hydrogen escaping from the solution (Equation 2).

\[
\text{BH}_4^- (aq) + 3\text{H}_2\text{O} \rightarrow \text{B(OH)}_3(aq) + 3\text{H}_2(g) + \text{H}^-
\]  
(Equation 2)

This clear solution was then pipetted dropwise within one minute into the red solution of tetraoctylammonium-gold in toluene. Upon stirring for a few seconds, the color changed from red to red-violet. This color change indicated the nucleation of gold clusters mediated by sodium borohydride. The residual sodium borohydride in solution reduced the remaining gold ions, providing additional monomers for the growth of the nuclei.

\[
n\text{AuCl}_4^- (tol) + 3n\text{e}^- \rightarrow 4n\text{Cl}^- (aq) + \text{Au}_n
\]  
(Equation 3)

The Br⁻ ions are supposed to be attached to the surface of the gold clusters, attracting again the N(C₈H₁₇)₄⁺ counter ions. The solution was stirred for 1 hour, transferred to the cleaned separation funnel and 75 ml of 10 mM HCl was added in order to remove the excess sodium borohydride. The funnel was shaken for 1 minute and the aqueous phase on the bottom was discarded. Thirty ml of 10 mM NaOH was added to the funnel to remove the excess acid and after shaking for 1 minute the aqueous phase was again discarded. Finally, 75 ml of Millipore water was added to remove excess ions, the funnel was shaken
for 1 minute and the aqueous phase was discarded. This last washing step was repeated 4
times. The aqueous phase and the eventually remaining emulsion were discarded. The
organic phase was then transferred to a 500 ml round flask and stirred overnight to allow
the particles to Ostwald ripen to a thermodynamically stable average size and size
distribution.

### 1.3 Surfactants change

Thirty ml of dodecanethiol was added to the Au nanocrystals dissolved in toluene.
The solution was heated to 65 °C and stirred for 2 – 3 hours. During this process the
mercapto groups of the dodecanethiol molecules displace the \( \text{Br}^- \) ions and yield
dodecanethiol coated Au nanocrystals. The solution was then cooled to room temperature
and split into several half–filled 20 ml vials.

### 1.4 Purification

The Au nanocrystals were precipitated by the addition of about the same amount of
methanol, followed by centrifugation for 5 min. After discarding the clear supernatant, the
precipitate of each vial was dissolved in as little toluene as possible and pooled again. This
and the following precipitation steps removed the excess dodecanethiol molecules. The
nanocrystals were first centrifuged for two minutes to precipitate larger aggregates, the
supernatant was then taken out and precipitated again by the addition of methanol, followed
by centrifugation. The slightly colored supernatant was discarded and the precipitate was
redissolved toluene.

### 1.5 Polymer Coating
1.5.1 Polymer synthesis

Poly-(isobutylene-alt-maleic anhydride) was designated to form the hydrophilic backbone on the amphiphilic polymer. Hydrophobic side chains were attached by reacting the amino-groups of dodecylamine (C\textsubscript{12} carbon chain) with the anhydride rings of the hydrophilic backbone in order to generate an amphiphilic polymer. The mixture was chosen in such a way that 75 % of the available anhydride rings were going to react with the amino-group of dodecylamine. Afterwards, the polymer still had a 25 % of anhydride rings that could react with cross-linker. For the synthesis of the amphiphilic polymer, 3.084 g of poly-(isobutylene-alt-maleic anhydride) (average MW ~6000 g.mol\textsuperscript{-1}) was placed in a round flask. All the solvents employed for the synthesis of this polymer were anhydrous, in order to avoid the presence of water in the medium, which would have caused subsequent aperture of the anhydride rings. In another flask, 2.7 g (~15 mmol) of dodecylamine was dissolved in 100 ml of tetrahydrofuran anhydrous, (THF, ≥ 99.9 %). This solution was added as quickly as possible under stirring to the polymer powder. This mixture was sonicated for ~20 seconds and heated to 55-60 °C (just below boiling point of THF) for 1 hour. Afterwards, the solution was pre-concentrated to about one third of the original volume in a rotavapor system under reduced pressure (p = 200-100 mbar) in order to enhance the reaction between the polymer and the amine. Once the sample had been concentrated, it was left stirring overnight. Then, the solvent was slowly evaporated until the polymer was completely dried (pale yellow solid). Finally, the amphiphilic polymer was re-dissolved in 25 ml anhydrous CHCl\textsubscript{3} with a resultant concentration of 0.8 M monomers (= 20 mmol/25 ml).

1.5.2 Coating procedure
The mixture of nanocrystals and polymer according to the best ratio (100 monomers/1 nm² NP surface, cfr. Lin et al., 2008) were added to the round flask and mixed well. After that, the flask was mounted on an evaporation device, the pressure was gradually lowered to 0 mbar in order to evaporate the chloroform slowly. In the end, the apparatus was ventilated two more times in order to remove the residual chloroform. Finally, the polymer coated particles were resuspended into an aqueous phase, consisting of 50 mM sodium borate buffer (SBB) pH 12.

2 HPLC purification

Polymer coated gold nanoparticles (AP) were purified by HPLC. The HPLC system used was an Agilent 1100, consisting of the solvent degasser G1322A, the quaternary pump G1311A, the multiwavelength detector G1365B and the fraction collector G1364A. The column was an Omnipol ID x L = 15 x 400 mm, filled with Sephacryl S-300 HR gel (Amersham Biosciences, # 17-0599-10). The running buffer was 50 mM SBB pH 9, with 100 mM NaCl, as suggested for the column media. The pH was adjusted to 9 to keep the particles better in suspension.

Each time 0.9 ml sample obtained after the procedure described in § 1.5.2 was run with a constant flow of 1 ml.min⁻¹ of running buffer. The detector was typically set to the wavelength of the plasmon peak of the Au NPs at 515 nm and to some other UV wavelengths, e.g. 220 nm and 280 nm to be able to observe the elution of free polymer molecules or aggregations of these and PEG molecules in case of PEGylated particles (Figure S1). The fraction collector was programmed to collect samples from t = 24 min to t = 29 min, filling vials 5-10. The pooled fractions were filled into a Viva cell 70 100K MWCO (Sartorius Stedim biotech VS6041) and concentrated up to 6 µM.
For fluorescent nanocrystals and pegylated particles, the procedure was the same. The
detector was set to the wavelength of the exciton peak and the UV wavelengths mentioned
above, respectively. The fraction collector was set to different time slices for collection.

3 Post Modification by ma10kPEG

EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) chemistry has
been used for post modification of purified polymer coated gold nanoparticles (AP) by
mA10kPEG (10 kDa PEG with methoxy and amine on consecutive ends). The EDC
amount used was 64000/NP and PEG molecules were 500/NP. A schematic overview of the post modification is given in Figure S2. The carboxyl groups of the hydrophilic PMA coating on the gold NPs was first activated by EDC (step 1 in Figure S2). EDC modified particles then reacted with the amines' outer end of the 10 kDa PEG molecules (step 2 in Figure S2). Excess EDC and PEG molecules were again purified by HPLC as explained earlier (§ 2). This time running buffer was 10 mM sodium bicarbonate buffer pH 10.1 with 100 mM NaCl instead of SBB pH 9. The trace in Figure S1-C shows two peaks at 220 nm, of which the peak in time interval 50-60 corresponds to excess PEG. NPs were finally dispersed in sodium bicarbonate buffer pH 10 containing 100 mM NaCl.

![Figure S2 - Activation of carboxyl groups with EDC and subsequent attachment of aminecontaining Molecules (Sperling et al., 2006).]

4 Characterization

In the following images results from the characterization studies after the synthesis in chloroform are given (Sperling et al., 2007). Figure S3 shows the light absorption spectrum of the particles over the wavelength interval 300-800 nm.

![Figure S3 - Absorption spectrum of gold NPs after synthesis in chloroform (Sperling et al., 2007).]
The particles absorbed light over the entire wavelength interval, but a clear distinct plasmon peak could be observed around 515 nm. The particle concentration was determined from the absorption at the plasmon peak (at 515 nm) by using a molar extinction coefficient of $8.63 \times 10^6 \text{ M}^{-1}\text{cm}^{-1}$. **Figure S4** panel A shows a TEM picture of the freshly synthesized particles, from which a size distribution was derived using computational analysis (panel B).

**Figure S4** – Panel A: TEM image of uncoated gold NPs in chloroform. The scale bar presents 10 nm; Panel B: particle size distribution derived from TEM picture. (Sperling et al., 2007.)

5 **Calculation of mass concentration of Au NP suspensions**

The concentration of Au NP stocks was converted from micromolar NP concentration ($\mu$M Au NPs) to mass concentration (mg Au.l$^{-1}$) based on particle volume and density of Au. Hence, a 4 nm Au NP has a volume of $3.35 \times 10^{-26} \text{ m}^3$. A 6.4 $\mu$M AP particle stock contains $3.8 \times 10^{18} \text{ NPs.dm}^{-3}$. As a consequence, the total Au volume in suspension is $1.3 \times 10^{-7} \text{ m}^3 \text{ Au.dm}^{-3}$ stock suspension. When converting the Au volume to mass based on the density of gold ($1.93 \times 10^7 \text{ g.dm}^{-3}$), it can be concluded that a 6.4 $\mu$M Au NP suspension corresponds to a 2.5 g Au.l$^{-1}$ suspension. Similarly, a 3.8 $\mu$M APma10kPEG NP stock
corresponds to a mass concentration of 1.5 g Au.l⁻¹ and a 1 µM Au suspension to 0.389 g Au.l⁻¹. Intermediate calculations for AP stock are summarized in Table S1. In this estimation the contribution of the organic coating is neglected. In a separate study, the theoretical calculation was checked using ICP-MS analysis of dissolved Au NP cores. Both results corresponded reasonably well, showing a relative difference of 5 % (Ali et al., 2011).

Table S1 - Conversion of molar Au NP concentrations to Au mass concentrations illustrated for AP stock.

<table>
<thead>
<tr>
<th>Au NP radius (m)</th>
<th>2x10⁻⁹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au NP volume (m³)</td>
<td>2.5</td>
</tr>
<tr>
<td>6.4 µM AP NP concentration¹ (NPs.dm⁻³)</td>
<td>3.8x10⁻¹⁸</td>
</tr>
<tr>
<td>AP volume concentration (m³ Au.dm⁻³ stock)</td>
<td>1.3x10⁻⁷</td>
</tr>
<tr>
<td>Density of gold (g.m⁻³)</td>
<td>1.93x10⁷</td>
</tr>
<tr>
<td>AP mass concentration (g Au.dm⁻³ stock)</td>
<td>2.5</td>
</tr>
</tbody>
</table>

6 Interference of Au NPs with chlorophyll and resorufin detection

Figure S5 - Chlorophyll fluorescence of algal concentration series in standard OECD medium supplemented with amphiphilic coated Au NPs (AP) in various concentrations. Straight lines are linear least squares regressions.

1 l = 1 dm³
7 Characterization of Au NPs in toxicity test media

Figure S7 - Particle size distributions of AP and APma10kPEG particles in L15 without serum and in OECD medium.

Figure S8 - Light absorbance spectrum of 20 mg.l\(^{-1}\) AP and APma10kPEG particles in L15 medium without serum.
8 Cytotoxicity and uptake of Au NPs in RTGill-W1 cell line

Figure S9 shows the concentration-response curves of all four 24 h resazurine cytotoxicity experiments. In each of those experiments, AP and APma10kPEG particles were tested simultaneously. The effect concentrations established in each experiment are given in Table S2.

Table S2 – 10 and 20 % effect concentrations (95 % CI), expressed as molar concentration of particles and as Au mass concentrations for AP and APma10kPEG Au NPs established in 4 separate 24 h resazurin cytotoxicity assays with RTGill-W1 cells.

<table>
<thead>
<tr>
<th></th>
<th>EC10 (µM Au NPs)</th>
<th>EC20 (µM Au NPs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AP</td>
<td>APma10kPEG</td>
</tr>
<tr>
<td>Exp. 1</td>
<td>0.010 (0.005-0.022)</td>
<td>0.0072 (0.0008-0.0596)</td>
</tr>
<tr>
<td>Exp. 2</td>
<td>0.0085 (0.0026-0.0280)</td>
<td>0.068 (0.042-0.111)</td>
</tr>
<tr>
<td>Exp. 3</td>
<td>0.024</td>
<td>0.044</td>
</tr>
</tbody>
</table>
Cross-sections of RTGill-W1 cells in the control and exposed to 0.0057 µM Au NPs were visualized using TEM. Images are shown in Figure S10. Figure S11 shows confocal laser scanning microscopy images, where the overlay with light microscopy images is omitted.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>EC&lt;sub&gt;10&lt;/sub&gt; (mg Au.l&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>EC&lt;sub&gt;20&lt;/sub&gt; (mg Au.l&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(0.012-0.051)</td>
<td>(0.017-0.112)</td>
</tr>
<tr>
<td></td>
<td>(0.039-0.087)</td>
<td>(0.08-0.41)</td>
</tr>
<tr>
<td>Exp. 4</td>
<td>0.0051 (0.0023-0.0113)</td>
<td>0.036 (0.018-0.069)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>AP</th>
<th>APma10kPEG</th>
<th>AP</th>
<th>APma10kPEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td>4.0</td>
<td>2.8</td>
<td>8.7</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>(1.9-8.4)</td>
<td>(0.3-23.2)</td>
<td>(4.8-15.6)</td>
<td>(9-97)</td>
</tr>
<tr>
<td>Exp. 2</td>
<td>3.3</td>
<td>26.5</td>
<td>14.4</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>(1.0-10.9)</td>
<td>(16.4-43.0)</td>
<td>(7.2-28.7)</td>
<td>(36-68)</td>
</tr>
<tr>
<td>Exp. 3</td>
<td>9.5</td>
<td>17</td>
<td>22.6</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>(4.5-20.0)</td>
<td>(6-43)</td>
<td>(15.1-33.8)</td>
<td>(31-159)</td>
</tr>
<tr>
<td>Exp. 4</td>
<td>2.0</td>
<td>14</td>
<td>14.9</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>(0.9-4.4)</td>
<td>(7-27)</td>
<td>(7.1-31.5)</td>
<td>(35-124)</td>
</tr>
</tbody>
</table>
Figure S11 – Laser scanning microscopy images of RTGill-W1 cells in the control (A) and exposed to AP (B, C) and APma10kPEG (D) particles. The height of the Z plane where the fluorescence was registered is given in parts B, C and D.
Figure S12 - Magnification of Figure 8 Panel C which shows the Au NP cores taken up inside a cellular vesicle. Size of individual particles range between 3.19 and 7.97 nm. A few small aggregates are present. M = vesicle membrane, Agg = aggregate of Au NPs and Au= individual Au NP.

References


