Cytotoxicity of Colloidal CdSe and CdSe/ZnS Nanoparticles

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ABSTRACT

Cytotoxicity of CdSe and CdSe/ZnS nanoparticles has been investigated for different surface modifications such as coating with mercaptopropionic acid, silanization, and polymer coating. For all cases, quantitative values for the onset of cytotoxic effects in serum-free culture media are given. These values are correlated with microscope images in which the uptake of the particles by the cells has been investigated. Our data suggest that in addition to the release of toxic Cd²⁺ ions from the particles also their surface chemistry, in particular their stability toward aggregation, plays an important role for cytotoxic effects. Additional patch clamp experiments investigate effects of the particles on currents through ion channels.

Introduction.

Recent progress in nanotechnology allows for the creation of new materials with properties tunable on the nanometer scale. Currently, already first attempts to use the functionality of such “smart” nanomaterials in life sciences are reported. Applications range from using fluorescent nanoparticles as dyes for fluorescence labeling of cells and for motility assays, using gold nanoparticles for immunostaining and gene delivery, to using magnetic nanoparticles as contrast agents for magnetic resonance imaging and for hyperthermia. In addition to working with cell cultures, nanoparticles have been successfully introduced in animal experiments as contrast agents, and future use in clinical applications is envisioned. It is evident that for any clinical application biocompatibility of the nanoparticles is crucial. Since nanoparticles are different from their respective bulk material due to their size, it is an obvious question to ask for potential dangers arising from their smallness. We can think of at least three different pathways by which nanoparticles introduced into an organism could interfere with its function and finally lead to impairment. (1) Most evident, introduced nanoparticles can be composed of toxic materials. This is true, for example, for fluorescent CdSe nanoparticles as well as for magnetic Co particles. Upon corrosion inside the organism, toxic ions could be released which finally poison the cells. Cytotoxic effects of Cd²⁺ (refs 14–18) and other metal ions are well-known. Compared to the respective bulk material, partial decomposition and release of ions is more likely for nanoparticles due to their enhanced surface-to-volume ratio. (2) There might be a negative effect of particles in general on cells, regardless of the material of the particles. It is known that particles can stick to the surface of cell membranes, and particles are also known to be ingested by cells. Mantling of the cell membrane and storage of particles inside cells might have impairing effects, even for absolutely inert particles that do not decompose or react. (3) There might be an effect caused by the shape of the (inert) particles. It has been reported for example, that carbon nanotubes can impale cells like needles. Particles of the same material but in a different modification can have a different toxic effect on cells in this way: Carbon nanotubes that have reached the lung are significantly more toxic than carbon-black and graphite. So far, most cytotoxicity studies on nanomaterials are focused on aerosols and involve particle uptake by the lungs. Such studies are important for many technical devices, for example, regarding the output of platinum nanoparticles from the catalysts of cars which might potentially endanger people who inhale these particles. In this study we focus on the toxic effects of colloidal nanoparticles dispersed in aqueous solution on cells. As already mentioned above, many different types of colloidal nanoparticles can be used for a broad variety of applications in life sciences and medicine. In this study we will focus on fluorescent CdSe and CdSe/ZnS nanoparticles.

To our knowledge, so far only two studies exist in which the cytotoxic effects of CdSe and CdSe/ZnS nanoparticles are investigated in detail. Derfus et al. have reported that
CdSe particles dissolved in aqueous solution release Cd$^{2+}$ ions and that the concentration of the Cd$^{2+}$ ions directly correlates with cytotoxic effects. The same authors have demonstrated that ion release is enhanced by oxidation, either through exposure to air or UV irradiation, but is repressed by encapsulating the particles with appropriate shells, as with ZnS and an additional organic shell. Shiohara et al. have investigated the cytotoxic effects of CdSe/ZnS particles of different size on three different cell types. The same group claims that for CdSe/ZnS particles the main source of cytotoxicity is not their cadmium content but rather the interaction of the particle surface with the cells. In our work described here we want to extend these studies to investigate the effect of different organic shells on cytotoxicity. Also, quantitative values for the onset of cytotoxic effects are determined.

Materials and Methods. Several cell lines (NRK fibroblasts, MDA-MB-435S breast cancer cells, CHO cells, RBL cells) were grown on standard cell culture substrates (All Materials and Methods can be found in detail in the Supporting Information). Nanocrystals of different materials and different surface coatings dissolved in water were added at different concentrations to adherent cells. In particular, CdSe, CdSe/ZnS, and Au nanocrystals coated with mercaptopropionic acid, embedded in a silica shell or embedded in an amphiphilic polymer shell, were investigated. In a first set of experiments, uptake of the nanocrystals by cells was investigated. For this purpose, green and red fluorescent CdSe/ZnS nanocrystals with different surface coatings were added to the cells, and after 18 h incubation the nuclei of the cells were stained with DAPI. Living cells were then imaged with differential interference contrast (DIC) and fluorescence microscopy. In this way the position of the nuclei and the ingested nanocrystals with two different surface coatings could be co-localized by their color of fluorescence (blue, green, red). In a second set of experiments, toxic effects of the ingested particles on the cells were investigated. From previous studies it is known that the adhesion behavior of NRK fibroblast is highly sensitive to toxic metal ions. In the study described here, the proliferation of the adherent cells was blocked by exchanging the culture medium to SATO medium. The number of adherent cells per labeled region on the cell culture substrate was counted and nanocrystals were then added at several concentrations. After 48 h of incubation, the nonadherent cells were removed by rinsing and the number of the adherent cells at the same region was counted. In this way the ratio $R$ of the number of adherent cells after and before incubation with nanocrystals, detected at the identical position, could be derived. The more toxic the effect of the nanocrystals on the cells, the lower this value is expected to be. As control, the constituents of the nanocrystals as Cd$^{2+}$ and Se$^{2-}$ ions and mercaptopropionic acid were added to the cells. In addition, the viability of the adherent cells before and after incubation with the nanocrystals was measured with a commercially available test (L3224, Molecular Probes). As a third set of experiments, nanocrystals were added to cells expressing hERG ion channels, and the effect of the nanocrystals on the ionic currents through these channels was investigated with an automated patch-clamp setup (Nanion). This idea is similar to a previous study. Uptake of nanocrystals by the cells was monitored by confocal microscopy.

Results and Discussion. Uptake of CdSe/ZnS Nanocrystals by MDA-MB-435S Breast Cancer Cells. In Figure 1 fluorescence and DIC images of MDA-MB-435S breast cancer cells that have been incubated for 18 h in serum-supplemented cell medium containing around $c$ (CdSe/ZnS particles) = 2–10 nM water-soluble CdSe/ZnS nanocrystals are shown. For each experiment red and green fluorescent nanocrystals with different surface coatings were used simultaneously, and experiments were performed for all different combinations. In particular, particles coated with mercaptopropionic acid (MPA), a silica shell bearing phos-phonate groups (phos-silane), a silica shell bearing polyethylene groups (PEG-silane), and an amphiphilic polymer (polymer) were used. From the images shown in Figure 1 it is evident that most of the different particles are ingested by the cells. By staining the nucleus with DAPI and manually changing the focus it could be shown that the ingested particles are stored in vesicular structures around the nucleus. For almost all combinations of particles with different surface coating, the ingested particles were stored in similar vesicular structures. Our data indicate that MPA, phos-silane, and polymer-coated particles are ingested by the cells in a very similar way, since these particles were found to co-localize inside the cells. However, different behavior was observed for PEG-silane coated particles. Whereas small green-fluorescent particles (hydrodynamic diameter of ~13 nm, unpublished results) were barely found inside the cells, bigger red-fluorescent particles (hydrodynamic radius of ~24 nm) could be detected inside the cells. These co-localization data demonstrate that water-soluble CdSe/ZnS particles are ingested by MDA-MB-435S cells in a nonspecific way, regardless of their size (in the range between 10 and 24 nm) and surface coating. Only for small PEG-silane coated particles uptake is significantly reduced. Nanocrystal uptake has also been demonstrated for other cell lines, such as NRK fibroblasts (data not shown).

Detachment of NRK Fibroblast from the Cell Culture Substrate upon Incubation with Nanocrystals. Since a previous study suggests that Cd$^{2+}$ ions released from the surface of Cd-containing nanocrystals are the main cause of toxic effects, we converted the concentration of CdSe and CdSe/ZnS particles to the concentration of Cd atoms on the surface of the CdSe core (see Supporting Information). This means that, in the case of a 1 nM concentration of CdSe particles with 100 surface Cd-atoms per particle, the concentration of Cd surface atoms is 100 nM. Analogously, the concentration of Au particles was converted to the concentration of Au atoms on the surface of the Au particles. The study of Shiohara et al. showed that for the same mass concentration (mg/mL) of particles cytotoxic effects are higher for smaller particles. Since for smaller particles the surface-to-volume ratio is higher, these findings support our idea to plot a “concentration parameter” proportional to the...
surface and not to the volume of the particles (i.e., to use the surface atom instead of the particle concentration). In Figure 2, the ratio $R$ between the number of adherent cells after and before incubation with nanoparticles is shown. As reference, this ratio is shown also for the case of incubation with Cd salts.

As is seen in Figure 2a, the same characteristic tendency can be observed in Figure 2b. For low Cd concentrations, $R$ adopts a constant value well below 1. With an increased number of Cd (surface) atoms, $R$ first decreases to a minimum value and then increases again to a value that can be close to 1. Thus, we can divide these curves in three different concentration regions. In the first region, the concentration of Cd atoms is very low and cell viability tests showed that all attached cells were alive (see Supporting Information). Exchanging the serum-containing cell culture medium to serum-free SATO medium resulted in the detachment of a significant fraction of cells, i.e., $R < 1$ even for $c(Cd) = 0$. Therefore, we assume that there are no toxic effects due to Cd in the first region of low Cd concentration and that the value of $R < 1$ can be simply explained by the effect of the SATO medium. In the second concentration region, $R$ drops. This means that more cells are detached due to the increased concentration of Cd (surface) atoms. Viability tests showed that attached cells in this region were still alive. For this reason we assume that in this region the viability of the cells is decreased due to poisoning by Cd$^{2+}$ ions in solution. Poisoned cells detach and therefore are not counted, which results in a decrease in $R$. We might refer to this effect as apoptosis. Upon further increment of the Cd concentration $R$ raises again, even sometimes to values larger than the value for very low Cd concentrations. Viability tests showed that the attached cells in this region were dead. We therefore assume that the concentration of Cd$^{2+}$ ions in solution was sufficiently high to immediately poison the cells before they could start to detach. We might refer to this effect as necrosis. This differentiation is suggested by the detailed study of López et al., in which apoptosis and necrosis are reported to be the pathway for cell death for low and high cadmium concentrations, respectively. The same authors also have shown that cytotoxic effects of Cd$^{2+}$ ions are more severe in serum-free medium compared to serum-containing medium. Therefore, our experimental conditions of serum-

Figure 1. Red and green fluorescent CdSe/ZnS nanocrystals with different surface coatings have been ingested by MDA-MB-453S cells. Images of the cells were recorded with differential interference contrast (DIC) microscopy, and the nanocrystals and the DAPI-stained nuclei were recorded with fluorescence microscopy. For each experiment, always red and green fluorescent nanocrystals with different surface coatings were used so that their position within the cells can be resolved by their color of fluorescence. The DAPI-stained nuclei appear in blue. The big images contain the merged DIC and fluorescence images, the smaller images show only the fluorescence. All scale bars represent 10 µm.
Figure 2. (a–e) Ratio $R(c)$ of adherent cells after/before incubation with Cd salts, CdSe and CdSe/ZnS particles, and Au particles. NRK fibroblasts have been incubated with (a) cadmium acetate hydrate (CAH) and cadmium perchlorate hydrate (CPH), (b) mercaptopropionic acid coated CdSe- and CdSe/ZnS nanocrystals, (c) silica-coated CdSe and CdSe/ZnS nanocrystals, (d) polymer-coated CdSe and CdSe/ZnS nanocrystals, and (e) polymer and mercaptoundecanoic acid (MUA) coated Au nanocrystals. The Cd salts fully dissociate in aqueous solution. Therefore, in (a) the $x$-axis represents the concentration $c$(Cd atoms) of Cd atoms in solution. In (b–d) the $x$-axis represents the concentration $c$(Cd surface atoms) of all the Cd atoms that are on the surface of the nanoparticles (see the Supporting Information; there also the scaling factor between $c$(Cd surface atoms) and $c$(CdSe particles) is given), and in (e) the $x$-axis represents the concentration $c$(Au surface atoms) of all the Au atoms that are on the surface of the nanoparticles. In each case results are shown for at least three separate series. For each data point within each series around 20000 cells were analyzed. For practical reasons in each series only a limited number of data points could be recorded (due to the limited number of cells that could be analyzed under the same conditions). For this reason not all series span the whole concentration range. Therefore, to compare the separate series for similar tendencies, the data points of each series were fitted with a double sigmoidal curve: $R(c) = a_1 - a_2/(1 + \exp((a_3 - c)/a_4) + a_5/(1 + \exp(a_6 - c)/a_7))$ with seven fit parameters $a_1$–$a_7$. The derived parameters for the respective series are enlisted in the table shown below. The value $R(c = 0)$ represents the control sample to which no Cd, or CdSe or CdSe/ZnS nanoparticles have been added, but in which cell growth has been affected by changing the serum-containing medium to SATO medium. Due to a limited amount of nanocrystals, the accessible concentration range was limited.
(a) and (b) clearly the double sigmoidal behavior of $R(c)$ can be seen. As indicator for the concentration limit of the onset of cytotoxic effects we use the $a_3$ parameter from the fits (a) $<a_3> = 0.35, 0.60, 0.48> \mu M = 0.48 \pm 0.13 \mu M$, (b) $<a_3> = 0.58, 0.58, 0.79> \mu M = 0.65 \pm 0.12 \mu M$ for CdSe and (c) $<a_3> = 4.9, 6.8> \mu M = 5.9 \pm 1.3 \mu M$ for CdSe/ZnS particles. In (c) no general tendency of $R(c)$ can be seen for PEG-silica coated nanoparticles and $R(c)$ seems to remain constant over the investigated concentration range. On the other hand, $R(c)$ decreases for increased concentrations of phosphate-silica coated nanoparticles. In (d) and (e) the shape of $R(c)$ can be approximated with a sigmoidal curve with the $a_3$ parameter from the fits as indicator for the concentration limit of the onset of cytotoxic effects: (d) $<a_3> = 0.83, 0.77> \pm 0.80 \pm 0.04 \mu M$ for CdSe and $<a_3> = 0.93, 1.0> \mu M = 0.98 \pm 0.07 \mu M$ for CdSe/ZnS particles, (e) $<a_3> = 3.2 \mu M$ for polymer and $<a_3> = 1.2, 2.7> \mu M = 2.0 \pm 1.1 \mu M$ for MUA-coated Au particles. In the case of CdSe and CdSe/ZnS particles $a_{core}$ denotes the wavelength of the first exciton peak of the CdSe core. In the case of Au and CdSe particles $d_{core}$ denotes the diameter of the particles, and in the case of CdSe/ZnS particles $d_{core}$ denotes the diameter of the CdSe core underneath the ZnS shell. The number of surface atoms per core in the case of CdSe, Au, and CdSe/ZnS particles is the number of Cd, Au, and Cd atoms on the surface of each CdSe particle. Au particle, and CdSe core underneath the ZnS shell, respectively. The terms $a_1, a_2, a_3, a_4, a_5, a_6, a_7$ are the resulting fit parameters for the function $R(c) = a_1 - a_2(1 + \exp((a_3 - c)/a_4)) + a_5(1 + \exp((a_6 - c)/a_7))$. $R(c)$ describes a double sigmoidal curve. Sigmoid curves are typically used in toxicology to describe dose–response functions. All values of one sigmoidal curve are between two asymptotes, determined by $a_1$ and $a_1+a_2$. The parameter $a_2/(a_1)$ controls the slope in terms of a rate at the inflection point $a_3$, which is the response halfway between the baseline and the maximum, also called $EC_{50}$. A double sigmoidal curve is the superposition of two single sigmoidal curves. (f) Overlay of phase-contrast and fluorescence image for NRK cells incubated for 48 h with CdSe/ZnS nanocrystals. The images of these cells correspond to the high concentration range shown in (b), (c), and (d). For MPA-coated CdSe/ZnS particles, cells incubated at high particle concentrations are dead, while the cell debris remains on the substrate. For silane coated particles no effect of the particles on the cells can be observed; even for high particle concentrations, the particles are ingested and stored around the nucleus and cells remain adherent. Polymer-coated particles at high concentrations tend to precipitate on the cell surface. Most cells detach from the surface, while the few still adherent cells are alive. Clearly different behavior dependent on the particle surface compared to the low concentration regime shown in Figure 1 can be seen.

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For CdSe and CdSe/ZnS particles, toxic effects start at concentrations of about $c$(Cd atoms) $= 0.48 \pm 0.13 \mu M$. The concentration of free Cd$^{2+}$ ions is released to solution as Cd$^{2+}$ ions, which are, according to this study, primarily responsible for cytotoxic effects. Our data suggest that, for our conditions (serum-free medium, 48 h incubation time, NRK cells), toxic effects of Cd$^{2+}$ ions start at concentrations of about $c$(Cd atoms) $= 0.48 \pm 0.13 \mu M$. Within the hypothesis that Cd$^{2+}$ ions released into solution are the main source for cytotoxic effects, a comparison of these values suggests that a significant amount of the Cd atoms on the surface of CdSe particles is released.
as Cd\textsuperscript{2+} ions into solution (≈0.48 μM/0.65 μM ≈ 75% as a very rough estimate), but that the mantling of CdSe with a ZnS shell drastically reduces this value to a rough estimate of 0.48 μM/5.9 μM ≈ 8%. Here we want to point out again that both the CdSe and the CdSe/ZnS particles had the same surface chemistry, a ligand shell of mercaptopropionic acid. Therefore, this direct comparison is another indicator that in this case indeed the release of Cd\textsuperscript{2+} and not eventual effects of the surface chemistry is responsible for the cytotoxic effects.

We also tested the other constituent of mercaptocarboxylic acid coated CdSe and CdSe/ZnS nanoparticles for cytotoxic effects. No cytotoxic effects of Se\textsuperscript{2−} (selenium acid) and Zn\textsuperscript{2+} (zinc chloride) ions could be observed up to concentrations of 40 μM (we were not able to investigate higher ion concentrations). The ligands used to stabilize the particles in water, mercaptopropionic acid (MPA), led to cell detachment at concentrations between 1 and 10 mM (the same value was found for an alternative ligand, mercaptoacetic acid). Compared to the onset of cytotoxic effect of Cd\textsuperscript{2+} ions at concentrations of about 0.48 μM, this suggests that cadmium is the main source of cytotoxic effects of mercaptopropionic acid stabilized CdSe and CdSe/ZnS nanocrystals (at most, one MPA molecule can be attached per Cd surface atom).

Instead of coating the nanocrystals with a monolayer ligand shell of mercaptopropionic acid, more stable coatings are also possible. First, we investigated the effect of embedding nanocrystals in a silica shell, which comprises many layers of cross-linked silane molecules. From Figure 2c it can be seen that a PEG-silica shell basically prevented toxic effects of particles on cells in the investigated concentration range. Even for PEG-silica coated CdSe nanoparticles, no clear toxic effects up to Cd surface atom concentrations of 30 μM were found. On the other hand, R\textsubscript{(c)} decreased upon increasing the concentration of phosphonate-silica coated CdSe nanoparticles. Microscopy images showed reduced particle uptake for PEG-silica coated particles, whereas phosphonate-silica coated particles were ingested by the cells and stored around the nucleus even for high Cd concentrations (Figures 1 and 2f). Viability tests showed that as well for PEG-silica as for phosphonate-silica coated particles within the whole concentration region, all adherent cells were living. For phosphonate-silica coated CdSe particle R\textsubscript{(c)} started to decrease only at higher Cd surface atom concentrations than MPA coated CdSe particles. Therefore, we conclude that the cross-linked silica shell around the particles in general is a good barrier against the release of Cd\textsuperscript{2+} ions from the particle surface. In addition, embedding PEG-groups in the silica shell reduced the uptake of the particles (see microscopy images Figure 1), which resulted in reduced cytotoxicity (see the R\textsubscript{(c)} curves shown in Figures 2b and 2c). Since silica shells embedding PEG and phosphonate groups should prevent the release of Cd\textsuperscript{2+} ions, in the same way we can further conclude that not only is the absolute concentration of Cd\textsuperscript{2+} ions responsible for cytotoxic effects but also the location where the Cd\textsuperscript{2+} ions are released is very important. If particles are ingested by cells, Cd\textsuperscript{2+} can be released directly inside cells (e.g., for phosph-silica coating), which seems to cause more severe cytotoxic effects than release of Cd\textsuperscript{2+} outside cells (e.g., for PEG-silica coating).

For polymer-coated CdSe and CdSe/ZnS nanoparticles, a gradual decrease of R upon increased Cd concentration was observed (Figure 2d). Viability tests showed that all adherent cells were living for all Cd surface atom concentrations, although there were basically no adherent cells left in the case of high Cd concentrations (right part of the curve in Figure 2d). Phase contrast and fluorescence microscopy analysis showed that, in the case of high concentrations, clouds of nanoparticles precipitated on the surface of the cells and on the cell culture substrate (Figure 2f). In the case of low concentrations no precipitation effects were observed, and it is important to point out that under these conditions polymer-coated particles were ingested by cells similar to silanized and mercaptopropionic acid coated ones (Figure 1). Reduction in the number of adherent cells already started at Cd surface atom concentrations of 0.80 ± 0.04 μM for CdSe and 0.98 ± 0.07 μM for CdSe/ZnS particles.

Most striking, for inert polymer-coated Au nanoparticles the same effects were observed as for polymer-coated CdSe and CdSe/ZnS nanoparticles (Figure 2e). This excludes the effect of Cd\textsuperscript{2+} ion release as the only source for poisoning of the cells in the case of polymer-coated particles. We therefore conclude that in the case of our polymer-coated particles the precipitation of the particles on the cells and not the release of Cd\textsuperscript{2+} atoms is the major factor for cell impairment. Again, we have to recall that for low particle concentration regular particle uptake by the cells has been observed and that therefore our polymer-coated particles are still useful for cellular labeling. Recently, other groups have reported a modified polymer coating procedure in which polyethylene groups are incorporated in the polymer shell,\textsuperscript{10} which should make these particles more stable against precipitation. Also, for other systems such as C\textsubscript{60} fullerenes, it has been observed that cytotoxicity correlates with water solubility and thus with the surface chemistry of the particles.\textsuperscript{43}

Impairment of Currents through Ion Channels upon Incubation of Cells with Nanocrystals. Two cell lines (RBL and CHO) were investigated regarding changes in their morphology and their electrophysiological properties upon incubation with nanocrystals. The CHO cell line was stably transfected to express the hERG channel, and the used RBL cell line endogenously expressed an inward rectifying potassium channel. For CdSe/ZnS particle concentrations in the nM range and incubation times of up to 2 days in serum-containing medium, no morphological changes of the cells were identified using high magnification light microscopy (see Supporting Information), although uptake of the nanocrystals by the cells was clearly verified by fluorescence microscopy (Figure 3a). Patch-clamp recordings under identical experimental circumstances of untreated and incubated cells revealed no changes of ion channel function and characteristic electrophysiological properties of the cells. In Figures 3b and 3c, currents through the ion channels of RBL and CHO cells before and after incubation with nanocrystals are displayed. As ion channels play an important role in the
cells and from one cell incubated with 5 nM MPA-coated CdSe/CdS nanocrystals (wavelength of the absorption peak of the CdSe core $\lambda = 508$ nm, corresponding to a CdSe core diameter of $d = 2.4$ nm; 61 Cd surface atoms per nanoparticle). The bright field and fluorescence image as well as the overlay of both of them are shown. (b) hERG currents as obtained from one nontreated CHO cell and from one cell incubated 3 h in 5 nM MPA-coated CdSe/ZnS nanocrystals (wavelength of the absorption peak of the CdSe core $\lambda = 508$ nm, corresponding to a CdSe core diameter of $d = 2.4$ nm; 61 Cd surface atoms per nanoparticle). (c) Endogenous K$^+$ inward rectifier currents of RBL cells without treatment and 3 h after incubation in 5 nM MPA-coated CdSe/ZnS nanocrystals (wavelength of the absorption peak of the CdSe core $\lambda = 508$ nm, corresponding to a CdSe core diameter of $d = 2.4$ nm; 61 Cd surface atoms per nanoparticle).

Conclusions. In addition to a previous study, we have determined the concentration limits of mercaptopropionic acid coated CdSe and CdSe/ZnS nanocrystals in a more detailed and absolute way. Poisoning of NRK cells due to the release of Cd$^{2+}$ ions starts at concentrations of 0.65 $\pm$ 0.12 $\mu$M and 5.9 $\pm$ 1.3 $\mu$M of surface Cd atoms for mercaptopropionic acid coated CdSe and CdSe/ZnS particles, respectively (Figure 2b). In agreement with previous studies, our data show that coating of CdSe particles with a ZnS shell increases the critical concentration up to which no toxic effects can be observed by almost a factor of 10. Since a ligand shell of mercaptopropionic acid around the particles is not very stable, such a shell cannot prevent the release of Cd$^{2+}$ ions from the particle surface. On the other hand, embedding the particles in a stable ligand shell of cross-linked silica molecules dramatically reduces the release of Cd$^{2+}$ ions. In the case of PEG-silica coated CdSe and CdSe/ZnS particles we could not observe any toxic effect up to concentrations of 30 $\mu$M Cd surface atoms (Figure 2c). We ascribe this to the fact of reduced uptake of these particles by cells. For polymer-coated nanocrystals, an additional pathway of poisoning was observed. For polymer-coated CdSe and CdSe/ZnS nanocrystals, cytotoxic effects started at concentrations of approximately 0.80 $\pm$ 0.04 $\mu$M and 0.98 $\pm$ 0.07 $\mu$M Cd surface atoms, respectively. These values seem to be independent of an optional ZnS shell around the CdSe particles. Cytotoxic effects for polymer-coated particles start at slightly higher concentrations than for MPA-coated CdSe, but already at significantly lower concentrations than for MPA-coated CdSe/ZnS particles. These data suggest that the polymer shell rather than an MPA shell is a better diffusion barrier against the release of Cd$^{2+}$ ions from CdSe surfaces. On the other hand, if Cd$^{2+}$ ions are drastically hindered by a ZnS shell around the CdSe particles, cytotoxic effects are more severe for polymer- than for MPA-coated particles. This effect could be attributed to a precipitation of the polymer-coated particles on the cell surface (Figure 2f).

We conclude that, in addition to the release of Cd$^{2+}$ ions from the surface of CdSe or CdSe/ZnS nanoparticles, cells can also be impaired if nanoparticles precipitate on the cell surface. Furthermore, cytotoxic effects are different in the case that particles are ingested by the cells compared to the case that particles are just present in the medium surrounding the cells. From this point of view it is important to correlate any study on the cytotoxicity of particles with a detailed microscopy analysis about the pathway of particle uptake. In accordance with another study, our data indicate a massive influence of the surface chemistry of the particles on their cytotoxic behavior in addition to the release of toxic molecules. Certainly many aspects of particle cytotoxicity still have to be investigated, since effects strongly depend on many parameters such as surface chemistry, type of the cell line, incubation time, serum-containing/free medium, etc. Clearly, Cd-containing nanoparticles cannot be considered as 100% biocompatible, but depending on the experimental situation conditions can be found under which no cytotoxic effects occur.

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Supporting Information Available: Details of Materials and Methods used in the study. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(I) Synthesis of the nanoparticles

The synthesis of hydrophobic CdSe, CdSe/ZnS, and Au nanoparticles is described in detail in the supporting information of a previous paper \(^1\). We have followed these protocols without change. The hydrophobic particles were transferred to aqueous solution using three different protocols.

1) The silanization of CdSe/ZnS particles with phosphonate silane has been described by Gerion et al. \(^2,3\) and the silanization of CdSe/ZnS particles with PEG silane has been performed as reported by Parak et al. \(^3\).

2) The polymer coating of Au and CdSe/ZnS particles has been described in detail by Pellegrino et al. \(^1\). We have followed these protocols without change. The same protocol was applied for the polymer coating of CdSe particles.

3) We have changed our previous protocol in which the transfer of particles to aqueous solution by ligand exchange with mercaptocarboxylic acids is described \(^2\).

The protocols for ligand exchange with mercaptocarboxylic acids as used in this study are reported below:
Coating of CdSe and CdSe/ZnS particles with mercaptopropionic acid

First, CdSe or CdSe/ZnS particles (as prepared in organic solvent as described above) were washed one time by precipitating them with methanol and redissolving them in chloroform. The particles were then precipitated by the addition of methanol and the sample was centrifuged and the supernatant discarded. The ligand exchange on the semiconductor particles was carried out by taking the wet precipitate (approximately 10 nmol of red fluorescent particles, or 40 nmol of green fluorescent particles) and adding 2 ml of anhydrous DMF (N,N-Dimethylformamide, purchased from Sigma, #22,705-6). A homogeneous distribution of the particles, which did not dissolve, was established by vortexing the sample. Then, equal molar amounts of mercaptopropionic acid (200 μl, purchased from Sigma, #M580-1) and 2-mercaptoethanol (160 μl, purchased from Sigma, #56,867-2) were added. After vortexing the solution turned transparent in the case of CdSe/ZnS particles. The CdSe cores did not produce a clear solution. This product was stored in the dark at room temperature until actual experiments with the particles were scheduled. Then, the particles were precipitated by adding 2 ml of deionized water and centrifugation. The particles were resuspended in deionized water. The addition of 50-100 μl Base (TMAH dissolved in methanol in a ratio of 1:3, tetramethylammonium hydroxide pentahydrate from Sigma, #T7505) yielded a clear solution with a pH of 10. The solution was then run through two NAP 25 gel columns (purchased from Amersham Biosciences, #17-0852-02) loaded with deionized water in order to neutralize the pH and to clean the product of remaining reactants.

Coating of Au particles with mercaptoundecanoic acid

Tetraoctylammonium bromide stabilized Au particles dissolved in toluene have been prepared according to standard protocols 4-7. We have followed the detailed description of the gold synthesis reported by Pellegrino et al. 1. In this report the tetraoctylammonium bromide ligands were then replaced by dodecanethiol ligands and the particles remained hydrophobic. Here we performed a ligand exchange to mercaptoundecanoic acid, which resulted in hydrophilic and thus water-soluble particles. For the ligand exchange with mercaptoundecanoic acid we followed the protocol of Mayya et al. 8.

100 ml of tetraoctylammonium bromide stabilized Au particles dissolved in toluene as prepared by Pellegrino et al. 1 were heated to 65°C. A hot solution (65°C) of 5.8 g mercaptoundecanoic acid (MUA, Sigma #45.056-1) dissolved in 10 ml toluene was added. This corresponds to a thirty-fold molar excess of MUA over the initial amount of gold. The solution was kept at 65°C for 1 hour. Then it was stirred overnight at room temperature. The day after a precipitate had formed. The solution was then centrifuged, and the translucent supernatant was discarded. The precipitate was dissolved in 0.1M TRIS buffer and sonicated to obtain a homogeneous, clear, violet solution of mercaptoundecanoic acid stabilized watersoluble Au-particles.
(II) Calculation of the number of surface atoms per nanoparticle and of the nanoparticle / surface atom concentrations

1) Calculation of the number of Cd surface atoms per CdSe particle

Given a certain radius of the particles, the number of surface atoms could be calculated by comparing the spherical particles with a small, faceted nanorod with the same surface. We assume, that we find the same number of atoms on a rod as we would find on a spherical particle. A rod has six lateral and two basal facets, see Figure S1a. On the lateral (non-polar) facets of the rods one finds one Cd-Atom per 30 Å². This can be understood by looking at a unit cell of a wurtzite structure, see Figure S1b. The unit cell has the same shape as the rod; especially it also has the six lateral facets. Each of these facets contains one Cd-Atom. The area of one of these facets is a • c, with a and c being the lattice constants of the CdSe-wurtzite structure. With a = 4.28 Å and c = 7.01 Å, one obtains 30.00 Å² for the area occupied by one Cd-atom on the lateral facets. A rod can be constructed by adding an adequate number of layers of atoms onto one core unit cell.

On the hexagonal basal facets of the unit cell there are three atoms. This facet can be split into six equivalent triangles, each of them with an area of 1/2 • 3\(^{1/2}/2\) • a\(^2\). So the total area per atom on this facet can be calculated to 15.86 Å². However, due to the intrinsic asymmetry of the wurtzite structure, and due to the growth mechanism, the (0001)-facet is only occupied by Cd atoms, whereas the opposing (000-1)-facet is composed exclusively of Se-atoms\(^{b}\), so that the overall density of atoms of one species on the basal facets, is one per 31.72 Å², which is roughly the same as on the lateral facets.

As the lateral facets mainly constitute the surface of the rod, a density of 1 atom per 30 Å² surface area is a good approximation. Overall the number of Cd-atoms on the surface of a CdSe particle can be calculated as \(N = 4 \cdot r^2/30\AA^2\), whereby \(r\) is the radius of the CdSe particle.

Figure S1: a) A schematic drawing of a rod. b) A unit cell of the wurtzite structure. Cd atoms are drawn in blue, Se atom in yellow. In each of the lateral facets there is one cadmium atom and one Selenium atom. Inner atoms are screened by atoms of the other species. From this picture one can also understand the difference between the two basal facets (0001) and (000-1). In this figure the Cd atoms have three bonds upwards, the selenium atoms only one. In the other direction the situation is contrary. From this derives the chemical difference of the two facets.
2) **Calculation of the number of Cd atoms on the surface of the CdSe core of CdSe/ZnS particles**

Since we identified Cd as the main source for cytotoxic effects, the ZnS shell around the CdSe cores rather has to be considered as a protection shell preventing the release of Cd atoms from the CdSe core than as an additional source of contamination. For this reason we consider the Cd atoms on the surface of the CdSe particle core as surface atoms, which are shielded by the ZnS shell. Thus, the number of Cd surface atoms for one CdSe/ZnS particle is $N = 4\pi r^2/30\AA^2$ (analogues to CdSe particles), whereby $r$ is the radius of the CdSe core and not of the whole CdSe/ZnS particle.

3) **Calculation of the number of Au atoms on the surface of Au particles**

To estimate the number of atoms on the surface of Au particles a method similar to that used for the estimation of surface atoms of the CdSe particles is employed. First, we calculate a mean surface area $A_{Au}$ per atom on a particle, then we calculate the number of atoms on the surface of a particle with given radius $r$ as $N = 4\pi r^2 / A_{Au}$. In the case of the gold particles we have to consider that gold grows in fcc-structure. We assume that the particles’ surface is mainly constituted by (111) and (100) facets. They have a close packing, and therefore are very stable. On the (100) facet of the unit cell there are two atoms, so with a lattice constant of $a = 4.08\ \text{Å}$ we find a density of one atom per $8.3\ \text{Å}^2$. On the (111) facet we find one atom per $7.21\ \text{Å}^2$. This can be understood by looking at a cross section of the fcc unit cell perpendicular to the [111] axis as marked in figure S2b. We find a triangle with three atoms on each side. The side length is $2^{1/2}\cdot a$. One of these triangles contains 2 atoms. So we find a density of one atom per $7.21\ \text{Å}^2$ on the (111) facet. Looking at the model systems Au$_{55}$ and Au$_{147}$ (see Figure S2a and b), which have closed shells, one can easily see that their surface is mainly constituted by (100) facets. Therefore, as an approximation, we used the value of $A_{Au} = 8\ \text{Å}^2$ surface area per atom. Overall the number of Au atoms on the surface of an Au particle can be then calculated as $N = 4\pi r^2 / 8\ \text{Å}^2$, whereby $r$ is the radius of the Au particle.

![Figure S2: a) A model of Au$_{55}$. This cluster is obtained by covering one single Au atom with two closed layers of atoms. b) A model of Au$_{147}$. Marked with arrows on the (100) plane is one face of the cubic unit cell. On the (111) plane the triangle marks six atoms that can be assigned to one cubic unit cell.](image-url)
4) Determination of the concentration of CdSe particles and Cd surface atoms

**Measurement of the CdSe particle concentration c(CdSe particles):**
The concentration of the CdSe nanocrystals is evaluated according to Yu et al. ⁹. Here, absorption spectra of the cores in the range of 200-800 nm were measured with an UV/vis spectra spectrophotometer (BIOCHROM Ultraspec 3100 pro). The absorption $A$ at first exciton peak at wavelength $\lambda_0$ is related to the particle concentration of the sample $c_p = c(\text{CdSe particles})$ by $A = \frac{c_p}{d} \frac{\mu_0}{A_{\text{CdSe}}} (P_0)$, where $\mu_0$ is size-dependent the extinction coefficient, $l$ the pathlength of the cuvette, and $d$ the dilution factor. Thus the particle concentration can be obtained from the absorption spectra.

**Calculation of the concentration of cadmium atoms $c(\text{Cd surface atoms})$ of the CdSe core surface in solution:**
For the estimation of the number $N_{\text{Cd}}$ of cadmium atoms on the surface of a CdSe core we calculate $N_{\text{Cd}} = \frac{A_{\text{core}}(\lambda_0)}{A_{\text{Cd}}}$, where $A_{\text{Cd}} = 30 \text{ Å}^2$ (see II.1) is the average area of one Cd atom and $A_{\text{core}}(\lambda_0)$ the core surface area. The latter value is calculated by $A_{\text{core}}(\lambda_0) = 4r_{\text{core}}^2$, where $r_{\text{core}}$ is taken from Yu et al. ⁹, in dependence of the wavelength $\lambda_0$ of the first exciton peak. Thus the particle concentration $c_p = c(\text{CdSe particles})$ can be converted to a cadmium surface atom concentration $c_N = c(\text{Cd surface atoms})$ according to $c_N = c_p \frac{\mu_0}{d} N_{\text{Cd}}$.

**Calculation of the mass concentration of CdSe nanocrystals in solution:**
The mass $m_{\text{NC}}$ of one CdSe particle was calculated according to $m_{\text{NC}} = m_{\text{core}} = d_{\text{CdSe}} \frac{V_{\text{core}}}{3}$, using the particle volume $V_{\text{core}} = \frac{4}{3} r_{\text{core}}^3$ and the mass density of CdSe $d_{\text{CdSe}} = 5.810 \frac{g}{cm^3}$ and ignoring the mass contribution of organic surface ligands. The mass concentration is $c_m = c_p m_{\text{NC}} N_A$, where $N_A = 6.022 \times 10^{23} \text{ mol}^{-1}$ is the Avogadro constant.

5) Determination of the concentration of CdSe/ZnS particles and Cd surface atoms

**Measurement of the CdSe particle concentration:**
In the case of CdSe cores with ZnS shell we assume the same extinction coefficient as for CdSe particles without shell to get the particle concentration. The extinction coefficient is taken from the data published by Yu et al. ⁹. We neglect the fact that upon growing a ZnS shell the first exciton peak of CdSe/ZnS particles is shifted to higher wavelength by 5 to 15 nm compared to CdSe cores.

**Calculation of the concentration of cadmium atoms $c(\text{Cd surface atoms})$ of the CdSe core surface of CdSe/ZnS particles in solution:**
For CdSe/ZnS particles we calculate the number of Cd surface atoms as the number of Cd atoms that lie on the surface of the CdSe core. For this purpose the first exciton peak of the CdSe cores has to be determined before growing the ZnS shell around them. From the wavelength of the first exciton peak the diameter of the CdSe core and thus the number of Cd atoms on the surface can be derived as described above for CdSe particles.
Calculation of the mass concentration of CdSe/ZnS nanocrystals in solution:
The mass \( m_{NC} = m_{core} \) of a CdSe particle without ZnS shell was calculated according to

\[
m_{NC} = m_{core} = d_{CdSe} \frac{4}{3} V_{core}, \text{ using } V_{core} = \frac{4}{3} r_{core}^3 \text{ and the mass density of CdSe}
\]

\( d_{CdSe} = 5.810 \frac{g}{cm^3} \) by ignoring the mass contribution of organic surface ligands. In the case of an additional ZnS shell around the CdSe particles the mass \( m_{ZnS} = d_{ZnS} \frac{4}{3} (r_{core} + r_{shell})^3 r_{core}^3 \) of the ZnS shell was added to the core mass \( m_{core} \) to get

\[ m_{NC} = m_{core} + m_{ZnS}, \quad d_{ZnS} = 4.090 \frac{g}{cm^3} \text{ is the mass density of ZnS and}
\]

\[ r_{shell} = 0.75 nm \text{ is an average empirical value for the thickness of a ZnS shell, derived from TEM measurements reported by Gerion et al.} \]

6) Determination of the concentration of Au particles and Au surface atoms

Measurement of the Au particle concentration:
As in the case of CdSe particles absorption spectra of the Au particles in the range of 200-800 nm were measured with an UV/visible spectrophotometer (BIOCHROM Ultraspec 3100 pro). The absorption \( A \) at the wavelength of the plasmon peak is

\[ A = c_p \frac{\bar{d} l \bar{P}_P}{d}, \text{ where } \bar{P}_P = 1.29 \times 10^7 \text{ M}^{-1} \text{cm}^{-1} \text{ is assumed as extinction coefficient for our gold particle with a radius of } r_P = 2.3 \text{ nm}, l \text{ the pathlength of the cuvette, d the dilution factor, and } c_p = c(Au \text{ particles}) \text{ the concentration of Au particles in the sample.}
\]

Calculation of the concentration of Au atoms located on the Au particle surface:
As in the case of CdSe particles, for the estimation of the number \( N_{Au} \) of Au atoms on the surface of an Au particle we use

\[ N_{Au} = \frac{A_p(r_P)}{A_{Au}}, \text{ where } A_{Au} = 8 \text{ Å}^2 \text{ (see II.3.) is the average area of an Au atom and } A_p(r_P) \text{ is the surface area of a particle with radius } r_P. \text{ The latter value is calculated by } A_p(r_P) = 4r_P^2 \bar{l}, \text{ where all the Au particles used in this study had a radius of } r_P = 2.3 \text{ nm as determined by TEM. Thus the particle concentration } c_p = c(Au \text{ particles}) \text{ was converted to an Au surface atom concentration } c_N = c(Au \text{ surface atoms}) \text{ according to } c_N = c_p \frac{N_{Au}}{A_p}.
\]

7) Table of the used CdSe, CdSe/ZnS, and Au samples

In order to compare our results with the observations of other groups we give here a table in which the conversion between different ways of quantifying nanocrystal concentrations is given.

<table>
<thead>
<tr>
<th>[\bar{l}] [nm]</th>
<th>c(CdSe particles) [nM]</th>
<th>c(Cd surface atoms) [(\bar{l})M]</th>
<th>c(CdSe particles) [(\bar{l})g/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>560</td>
<td>10</td>
<td>1.1</td>
<td>0.63</td>
</tr>
<tr>
<td>599</td>
<td>10</td>
<td>2.1</td>
<td>1.7</td>
</tr>
</tbody>
</table>
\[ \text{[nm]} = \text{wavelength of the first exciton peak in the absorption spectrum of the CdSe core particle} \]

\[ c(\text{CdSe particles}) [\text{nM}] = \text{concentration of CdSe particles} \]

\[ c(\text{Cd surface atoms}) [\text{M}] = \text{concentration of Cd atoms which are located on the surface of the CdSe particles} \]

\[ c(\text{CdSe particles}) [\text{lg/ml}] = \text{mass concentration of CdSe particles} \]

**CdSe/ZnS nanocrystals**

<table>
<thead>
<tr>
<th>[ \text{[nm]} ]</th>
<th>[ c(\text{CdSe/ZnS particles}) [\text{nM}] ]</th>
<th>[ c(\text{Cd surface atoms}) [\text{M}] ]</th>
<th>[ c(\text{CdSe/ZnS particles}) [\text{lg/ml}] ]</th>
</tr>
</thead>
<tbody>
<tr>
<td>508</td>
<td>10</td>
<td>0.61</td>
<td>0.86</td>
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<tr>
<td>599</td>
<td>10</td>
<td>2.1</td>
<td>3.3</td>
</tr>
</tbody>
</table>

\[ \text{[nm]} = \text{wavelength of the first exciton peak in the absorption spectrum of the CdSe core of the CdSe/ZnS particle (must have been determined before the growth of the ZnS shell)} \]

\[ c(\text{CdSe/ZnS particles}) [\text{nM}] = \text{concentration of CdSe/ZnS core/shell particles} \]

\[ c(\text{Cd surface atoms}) [\text{M}] = \text{concentration of Cd atoms which are located on the surface of the CdSe core of the CdSe/ZnS particles} \]

\[ c(\text{CdSe/ZnS particles}) [\text{lg/ml}] = \text{mass concentration of CdSe/ZnS particles} \]

**Au nanocrystals**

<table>
<thead>
<tr>
<th>[ c(\text{Au particles}) [\text{nM}] ]</th>
<th>[ c(\text{Au surface atoms}) [\text{M}] ]</th>
<th>[ c(\text{Au particles}) [\text{lg/ml}] ]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>8.31</td>
<td>5.92</td>
</tr>
</tbody>
</table>

\[ c(\text{Au particles}) [\text{nM}] = \text{concentration of Au particles} \]

\[ c(\text{Au surface atoms}) [\text{M}] = \text{concentration of Au atoms which are located on the surface of the Au particles with a diameter of 4.6 nm} \]

\[ c(\text{Au particles}) [\text{lg/ml}] = \text{mass concentration of Au particles with a diameter of 4.6 nm} \]
(III) Uptake of CdSe/ZnS nanocrystals by MDA-MB-435S breast cancer cells

MDA-MB-435S breast cancer cells were purchased from American Type Cell Culture (ATCC) and cultivated at 37°C and 5% CO₂ in the media proposed by ATCC. For uptake-experiments the adherent cells were rinsed twice with PBS without Ca²⁺ and Mg²⁺ (PBS, 9.55g/l, Biochrom AG, Germany) in order to reduce the concentration of adhesion-promoting Ca²⁺ and Mg²⁺ -ions inside the cells. The cells were then covered with Trypsin-EDTA-solution (Biochrom AG, Germany) and incubated at 37°C for 5 minutes. After incubation the now detached cells were diluted in PBS, filled into test tubes and centrifuged (5 min, 300g). The Trypsin-EDTA containing supernatant was removed and the cells were resuspended in media. The cells were diluted to a concentration of 7 * 10⁴ cells/ml. For all uptake-experiments 1 ml of cell-suspension was filled into a nunc-chamber® (Nalge, U.S.). To each chamber 10 µl of 2-10 µM solution of two types of different coated nanocrystals, fluorescing in red and green, respectively, were added. The final concentration of each type of nanocrystals in the serum-supplemented media was 2-10 nM. After 18 h of incubation at 37°C and 5% CO₂ the cells were rinsed twice with PBS and fresh medium was added. The cells were then incubated for additional 4 h to allow the cells to ingest the nanocrystals.

Before visualizing the cells their nucleus was stained with DAPI (Molecular Probes, U.S.) following the providers protocol for adherent cells. For visualizing the cells the nunc-chambers® were mounted onto a fluorescence microscope (Axiovert 200, Zeiss, Germany) using a 100x oil-immersion objective. For every image one channel in differential interference contrast mode (bw) and three individual channels in the fluorescence mode were recorded using the following filters: blue channel (DAPI): excitation: G 365, beamsplitter: FT 395, emission: BP 445/50; green channel (green nanocrystals): excitation: BP 450, beamsplitter: FT 480, emission: BP 535; red channel (red nanocrystals): excitation: BP 546, beamsplitter: FT 580, emission: LP 590. For excitation a 100 Watt Mercury lamp (HBO 100, Osram) was used at half power. The acquisition times varied between 100 and 500 ms for the different types of nanocrystals, as they have different quantum yields. Images were recorded with a Zeiss AxioCam  CCD camera. The recorded channels were overlaid using the microscope software (Axiovision 4.1).
(IV) Detachment of NRK fibroblast from the cell culture substrate upon incubation with nanocrystals

Chemicals
Ultrapure water (MilliQ-grade) was used in all experiments. Octadecyltrichlorosilane (OTS) was obtained from ABCR, Karlsruhe, Germany. Hexadecane, hexane, chloroform, mercaptopropionic acid (MPA), cadmium acetate hydrate (CAH), cadmium perchlorate hydrate (CPH), selenious acid (SA), zincchloride (ZC) PBS (phosphate buffer saline), Penstrep solution (containing 10,000 units/ml Penicillin and 10 mg/ml streptomycin-sulfate, and 0.9% NaCl), Liquid Media Supplement (ITS) as well as trypsin were purchased from Sigma-Aldrich. Dulbecco’s Modified Eagle Medium (DMEM, FG 0435), HAM’S F-12 Medium (FG 0815) and fetal bovine serum (FBS, S0115) were from Biochrom KG (Berlin, Germany). All chemicals were used as received.

Cell culture
Normal Rat Kidney (NRK)-fibroblasts (CRL-6509) were obtained from ATCC, Manassas, USA. As cell culture medium DMEM with 10% FBS (fetal bovine serum) and 1% Penstrep solution was used. During incubation with nanocrystals cells were cultivated in serum free SATO medium (HAMS F12 with 1% serum supplement ITS), which stopped cell proliferation. Measurements had to be performed in serum free medium since for our applied automated counting procedure (see below) it is important that cells do not proliferate and migrate too much. Otherwise it would be impossible to verify for each individual cell whether it is still adherent at the same place of whether it has detached from the surface.

NRK-fibroblasts were seeded in 6-wellplates (TPP, Switzerland; polyethylene, tissue culture treated) to a concentration of 3600 cells / cm². Special care was taken to achieve a cell population of separated single cells, in order to get optimal conditions for the optical counting procedure. Adhesion of the cells was observed after 5 to 24 hours. After cell adhesion, the cell layer was gently rinsed three times with PBS buffer, and the adherent cells were counted in PBS buffer within 20 min. The buffer then was replaced by the incubation medium (SATO and the toxin, i.e. Cd salts or nanocrystals, in a target concentration). SATO treatment always caused a certain loss of cells by detachment, even without toxin. After 48 hours of incubation, cells were rinsed three times with PBS buffer, removing detached cells, while the residual adherent cells were counted again in PBS buffer within 20 min.

Automated procedure to count adherent cells
A computer based optical counting method was used to sensibly detect the effects of very low toxin concentrations to the adhesion behavior of living cells. Cells were counted on images recorded with a phase contrast microscope before and after incubation with the toxin, and the ratio R(c) of cells adherent to the surface after / before incubation versus the toxin concentration c is plotted. For this purpose for each individual cell which was adherent before incubation with the toxin, it was tested if the same cell is still adherent after incubation with the toxin. Therefore, the position of each cell within the wellplate had to be registered before incubation with the toxin and had to be found again after 48 hours incubation with the toxin. While the motorized xy-stage of the microscope allowed for a very precise repositioning of the wellplate holder, placing the wellplates led to an overall repositioning error of approx. 100 μm. This contributes to the relative error of about 10% for the measured average ratios R. While a ratio R close to zero indicates the complete detachment of the cells from the surface, a ratio of R = 1 means that statistically every cell is found after the incubation time where it was seeded. The reference value R(c = 0) never is found to be = 1, because of the SATO medium. Changing the serum-containing medium to serum free SATO medium alone results in a detachment of some cells, even when no toxin is added (i.e. c = 0). Moreover, R(c) never
is found to be $= 0$ since always a certain amount of dirt, dust particles, or cell debris is counted wrongfully as adherent cells. In the worst case this background noise in $R(c)$ due to cell debris and other impurities could be as high as $R(c) = 0.34$. In the case of the polymer coated nanocrystals for high particle concentrations there are basically no adherent cells left and the constant $R(c) > 0$ background level is due to impurities wrongfully counted as adherent cells. Therefore, for the interpretation of our data it is important to correlate the $R(c)$ curve with microscopy images. Another important information additional to the $R(c)$ curves is result of the viability tests performed for different concentrations. These data indicate whether the counted adherent cells are alive or dead.

In practice, for each toxin concentration approximately 20000 cells were monitored in 94 pictures taken from two wellplate chambers. This was done with an inverted optical microscope (Axiovert 200, Carl Zeiss, Jena) in phasecontrast mode with a 4x Achromplan objective. Hydrophobic circular glass cover slides (preparation see below) were placed to float on the buffer surface to avoid light scattering through the meniscus of the liquid at the chamber walls, which would result in a brightness gradient in the pictures. The pictures, taken with a Zeiss AxioCam HRm CCD camera, showed a surface area of $2.6 \text{ mm} \times 3.5 \text{ mm}$ as a black background with the light spots of adherent cells (and dirt), see Figure S3. This high contrast and the low brightness variations of the background allowed for a computer based particle analysis with the data evaluation software Igor Pro 4.1, running on a Dual 2.0 GHz Macintosh PowerPC. Using the subroutine package of Igor Pro for image processing, it was possible to automate the cell counting. For particle analysis, a threshold in the grayscale was used for the particle detection based on the average brightness of a picture. The threshold and a minimum value for the spot size were the necessary parameters for a reliable counting of surface adherent particles.
Figure S3.1: a) Phase contrast image of NRK fibroblasts in SATO medium, cultured for 24 hours, before the addition of nanocrystals. b) Same image with the $N_A=198$ counted cells (marked in the image) that have been detected by the image analysis software. c) Phase contrast image of NRK fibroblasts after 48 hours of incubation in 50 nM MPA coated CdSe/ZnS nanocrystals wavelength of the absorption peak of the CdSe core $\lambda = 508$ nm, corresponding to a CdSe core diameter of $d = 2.4$ nm; 61 Cd surface atoms per nanoparticle) in SATO medium. This image shows the same area of the cell culture substrate as shown in a) and b). The area could be identified by the scratch in the surface. d) Same image with $N_B=108$ cells counted by the image analysis software (marked in the image). e) Overlay of image a) (black) and c) (red). This image compares the adherent cells before (a) and after (c) incubation with the nanocrystals. Note that a lot of cells detached from the surface upon incubation with the nanocrystals, while the still adherent cells moved during the 48 hours incubation time in SATO medium. They show a spindle-like shape and viability tests indicate that these cells are alive. The situation is typical for the intoxication of the NRK fibroblasts in serum free medium and for a low concentration of cadmium. The value $R = \frac{N_A}{N_B}$ is taken for the same concentration from in total 94 positions (pictures). The averaged value is plotted in the diagrams $R$ vs. concentration as one data point including the standard deviation of $R$ as error bar.
Figure S3.2: a) Phase contrast image of NRK fibroblasts in SATO medium, cultured for 24 hours, before the addition of nanocrystals. b) Same image with the $N_A=228$ counted cells (marked in the image) that have been detected by the image analysis software. c) Phase contrast image of NRK fibroblasts after 48 hours of incubation in 325 nM MPA coated CdSe/ZnS nanocrystals wavelength of the absorption peak of the CdSe core $\lambda = 508$ nm, corresponding to a CdSe core diameter of $d = 2.4$ nm; 61 Cd surface atoms per nanoparticle) in SATO medium. This image shows the same area of the cell culture substrate as shown in a) and b). d) Same image with $N_B=225$ cells counted by the image analysis software (marked in the image). e) Overlay of image a) (black) and c) (red). This image compares the adherent cells before (a) and after (c) incubation with the nanocrystals. Note that almost no cells detached from the surface upon incubation with nanocrystals. Adherent cells did not move on the substrate during the 48 hours incubation in the SATO medium. They show a sphere-like shape and viability tests indicate that the cells are dead. The situation is typical for intoxication of the NRK fibroblasts in serum free medium at high concentration of cadmium. In general, non-adherent cells and dirt were suppressed by rinsing with PBS buffer prior to image capturing. The value $R = \frac{N_B}{N_A}$ is taken for the same concentration from in total 94 positions (pictures). The averaged value is plotted in the diagrams $R$ vs. concentration as one data point including the standard deviation of $R$ as error bar.
Hydrophobic glass cover slides

A solution of 10 mM octadecyltrichlorosilane (OTS) in hexadecane was used to functionalize the surface of glass cover slides by dip coating for one hour. Finally the slides were rinsed with hexadecane, hexane and chloroform. The resulting surface showed advancing contact angles of approx. 92° for water.

Fluorescence microscopy to image the nanocrystals ingested by individual cells

Fluorescence microscopy was performed on an inverted microscope (Axiovert 200 from Carl Zeiss) with a Carl Zeiss FluoArc system, using a mercury lamp HBO 100. The filters for the fluorescence detection of the nanocrystals were the Q-dot TM 500 LP from AHF, Germany, as well as the filter sets 15 and 49 from Carl Zeiss.

Viability test

The Viability Kit L3224 from Molecular Probes was applied to distinguish between living and dead cells. Esterase activity of living cells converts non-fluorescent calcein-AM to intensely green fluorescent calcein. The second agent of this kit, Ethidium homodimer-1, enters defect cell membranes of dead cells and shows a 40-fold enhanced red fluorescence when it binds to nucleic acids of the cytoplasm. Following the manufacturers instructions for this test, cells were rinsed thoroughly with PBS buffer and a 4 µM solution of the agents was applied for 45 min. The filter set 00 from Carl Zeiss was used to watch the red fluorescence, while a 31001 filter from Chroma was used for the green fluorescence. In Figure S4 an example of this viability test is shown.

Similar viability tests have been used by other groups to determine cytotoxic effects of Cd ions, and CdSe and CdSe/ZnS particles\textsuperscript{11-13}. These tests are based on measuring metabolic activity of cells or on the staining of dead cells, and are from this point conceptually different from our experiments in which cell survival was measured in terms of cell adherence.

![Image](image.png)
Figure S4: NRK fibroblasts were cultivated for 24 hours in DMEM cell medium with serum. After this incubation they were rinsed with PBS buffer. Further culture was performed in serum free SATO medium containing cadmium perchlorate hydrate as cadmium source. The viability test was applied. Pictures show an overlay of the phase contrast image and the images of green and red fluorescence. a) NRK fibroblasts after incubation in 0.1 mM cadmium perchlorate hydrate. All adherent cells are marked as living (green color) and show a spindle like shape. b) NRK fibroblasts after incubation in 5 mM of cadmium perchlorate hydrate. Almost all adherent cells are marked as dead (red color) and show a spherical shape.
(V) Patch clamp experiments

To further evaluate the biocompatibility of nanocrystals we conducted electrophysiological studies of cells incubated with nanocrystals using the planar patch clamp technique\textsuperscript{14}. Two cell lines (RBL and CHO) were investigated in regard to changes in their morphology and their electrophysiological properties upon incubation with nanocrystals. The CHO cell line was stably transfected to express the hERG channel and the RBL cell line used endogenously expresses an inward rectifying potassium channel.

All cells were grown to 60-80% confluence in their standard serum-supplemented medium as described in the ATCC catalogue. The cells were incubated with nanocrystals at the desired concentration in their normal growth medium at 37 degrees Celsius and 5% CO\textsubscript{2} (several hours up to 2 days). After the incubation period, cells were isolated with a brief trypsin treatment. The resulting cell suspension was centrifuged twice at 1000 U/min for 2 minutes and the supernatant was pipetted off to remove cell fragments and debris. For resuspension the electrophysiological recording solutions were used. The cleaned cell suspension had a cell concentration of about one million cells per milliliter. Cells were examined with a confocal laser microscope to validate the quantum dot uptake by fluorescence measurements.

![Figure S5: The Port-a-Patch electrophysiology workstation. In a) the chip mounting station and the software steered suction control unit are shown. The full Port-a-Patch system including amplifier and PC is depicted in b) and c) displays the micro structured borosilicate glass chips, which are glued onto small twist caps. The caps contain an O-ring and are screwed onto the holder in the chip mounting station to obtain an airtight arrangement for suction application. Electrolyte solutions and cell suspension is pipetted onto the backside and top of the chip with standard pipettes as shown. The solutions are electrically contacted via Ag/AgCl electrodes, which are implemented in the system.](image-url)
Electrophysiological characterization and evaluation of cell function/viability of the cells ion channels were performed by whole cell recordings. All measurement have been performed with the chip-based patch clamp technique using the Port-a-Patch system by Nanion Technologies (www.nanion.de). The system uses micro structured borosilicate glass chips for whole cell patch clamp assays in an automated format. The Port-a-Patch was used in conjunction with an EPC-10 patch clamp amplifier (HEKA, Lambrecht, Germany) and is shown in Figure S5.

Cells were positioned and sealed on a micron-sized aperture in the glass chips by feedback controlled suction application. Brief suction pulses were applied to rupture the cells membrane across the aperture to obtain access to the cells interior, e.g. getting into the whole cell configuration. In the whole cell configuration, the current through the complete ensemble of all ion channels in the cell membrane is recorded, allowing the investigation of the ionic current conducted by the entire cell.

The chips for the recordings had an open aperture resistance of 2-3 MΩ. The recorded data were filtered at 3 kHz and sampled at 10 kHz. The electrophysiological recording solutions had the following ionic compositions (mM): extracellular (top of chip): NaCl (140), KCl (3), MgCl₂ (1), CaCl₂ (1), HEPES (10), pH = 7.4, 270 mOsm; intracellular (underside of chip): for RBL cells: K-Aspartate (120), EGTA (10), HEPES (10), NaCl (10), pH=7.2; for CHO cells: KCl (120), NaCl (10), EGTA (10), Heps (10), pH = 7.2. About 5 µl of cell suspension were pipetted onto the chip and standard suction protocols of the Port-a-Patch system were applied for cell positioning and achieving tight seal whole cell configurations. Standard voltage protocols were applied for the recordings of ionic currents.

After the electrophysiological characterization, the patch clamped cell on the aperture was examined with the confocal microscope to evaluate the fluorescence of nanocrystals inside the cell. In Figure S6, images of patch clamped cells on the chip are displayed, showing the fluorescence of the quantum dots inside the cell. Below the cell is the micron-sized aperture in the chip, which can be perceived as a small dot. The larger circle around aperture in the centre corresponds to the 3-D-microstructure in the chip. On the chip surface are more cells in the vicinity of the patch clamped cell and all show the characteristic fluorescence. By this procedure it was guaranteed, that all cells investigated had incorporated the nanocrystals.
Figure S6: Electrical properties of RBL cells after 4 h incubation with MPA-coated CdSe/ZnS nanocrystals (wavelength of the absorption peak of the CdSe core $\lambda = 508$ nm, corresponding to a CdSe core diameter of $d = 2.4$ nm; 61 Cd surface atoms per nanoparticle). Top: Currents as obtained after incubation in 10 nM, 50 nM, and 100 nM CdSe/ZnS particles. Measurements were performed in two different external K$^+$ concentrations showing the characteristic inward rectifying current of the fully functional potassium channels. The current at the higher potassium concentration is larger due to the abundance of permeating ions. Bottom: Each picture shows a cell positioned on the hole in the glass chip as seen under white light (WL, bright field) and as fluorescence while excitation with 488 nm laser light. The images at the bottom show an overlay of the bright field and fluorescence pictures.

In addition the cell morphology before and after incubation of the cells was investigated by optical microscopy (Figures S7, S8). No changes in cell morphology could be detected at the used concentrations and incubation time. Incubation with nanocrystals was performed for 4 hours in serum-supplemented medium.
Figure S7: RBL cells before and after 3-4 h incubation with MPA-coated CdSe/ZnS nanocrystals (wavelength of the absorption peak of the CdSe core $\lambda = 508$ nm, corresponding to a CdSe core diameter of $d = 2.4$ nm; 61 Cd surface atoms per nanoparticle). The images show RBL cells before (a) and 3 h after (b) incubation in 5 nM CdSe/ZnS particles; RBL cells before (c) and 4 h after (d) incubation in 10 nM; before (e) and 4 h after (f) incubation in 50 nM; before (g) and 4 h after (h) incubation in 100 nM CdSe/ZnS particles.

Figure S8: CHO cells expressing hERG before and after incubation with MPA-coated CdSe/ZnS nanocrystals. The images show cells before (a) and 3 h after (b) incubation in 5 nM nanocrystals (wavelength of the absorption peak of the CdSe core $\lambda = 508$ nm, corresponding to a CdSe core diameter of $d = 2.4$ nm; 61 Cd surface atoms per nanoparticle); before (c) and 3 h after (d) incubation in 5 nM nanocrystals (wavelength of the absorption peak of the CdSe core $\lambda = 547$ nm, corresponding to a CdSe core diameter of $d = 3.0$ nm; 92 Cd surface atoms per nanoparticle); before (e) and 3 h after (f) incubation in 200 nM nanocrystals (wavelength of the absorption peak of the CdSe core $\lambda = 547$ nm, corresponding to a CdSe core diameter of $d = 3.0$ nm; 92 Cd surface atoms per nanoparticle); before (g) and 10 h after (h) incubation in 200 nM (wavelength of the absorption peak of the CdSe core $\lambda = 547$ nm, corresponding to a CdSe core diameter of $d = 3.0$ nm; 92 Cd surface atoms per nanoparticle).
(VI) References