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The Toxicity of Silver Nanoparticles Depends on Their Uptake by Cells and Thus on Their Surface Chemistry

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A set of three types of silver nanoparticles (Ag NPs) are prepared, which have the same Ag cores, but different surface chemistry. Ag cores are stabilized with mercaptoundecanoic acid (MUA) or with a polymer shell [poly(isobutylene-alt-maleic anhydride) (PMA)]. In order to reduce cellular uptake, the polymer-coated Ag NPs are additionally modified with polyethylene glycol (PEG). Corrosion (oxidation) of the NPs is quantified and their colloidal stability is investigated. MUA-coated NPs have a much lower colloidal stability than PMA-coated NPs and are largely agglomerated. All Ag NPs corrode faster in an acidic environment and thus more Ag(I) ions are released inside endosomal/lysosomal compartments. PMA coating does not reduce leaching of Ag(I) ions compared with MUA coating. PEGylation reduces NP cellular uptake and also the toxicity. PMA-coated NPs have reduced toxicity compared with MUA-coated NPs. All studied Ag NPs were less toxic than free Ag(I) ions. All in all, the cytotoxicity of Ag NPs is correlated on their uptake by cells and agglomeration behavior.

1. Introduction

Silver nanoparticles (Ag NPs) are frequently used in industry, mainly because of their antimicrobial properties,[1] with applications in an increasing number of medical and consumer products. However, their antibacterial features and extremely small size, which makes them to have a high surface area and to be more reactive, also suggest a toxicological risk when these NPs come into contact with biological systems.[2] Countless toxicological studies involving Ag NPs have already been carried out in different organisms ranging from bacteria to humans.[3–5] Although it is generally known that corrosion (oxidation) and subsequent release of Ag(I) ions is a major source of toxicity,[6,7] there is still a lack of detailed general knowledge concerning the origin of the Ag NPs' toxicity. Most important shortcomings are that individual studies are often based on different NPs, and also on different types of cells, which complicate the comparison and extrapolation of results. In some studies, the final toxicity resulted to be an unclear combination of effects among the Ag NPs and their released Ag(I) ions, whereas in other works the Ag(I) ions showed a greater (or even the only) contribution to the toxicity than the Ag NPs per se.[3,7–9] Several studies describe the release of Ag(I) ions upon NP oxidation and subsequent partial dissolution of the Ag NPs over time as amplification of the toxicity of the (undissolved) Ag NPs, which can cause intracellular reactions, as, for example, in the mitochondria.[5] Other studies even claim that the only effect originates from Ag ions, which acts on the cell membrane, whereas under the same conditions Ag NPs had no effect.[9] Studies indicate that toxicity depends on size,[10] shape,[11] charge,[12] and colloidal stability[13] of the Ag NPs.

Although correlation of the physicochemical properties of NPs to their interaction with cells is attempted by a large-body research studies, a comprehensive picture is still missing (not only for Ag NPs but in general) albeit many effects are well established. This is partly due to the fact that not all physicochemical properties are easy to be determined experimentally, and most of them are entangled (e.g., loss in colloidal stability/reduced dispersion also increases the effective hydrodynamic diameter of the NPs).[14] The interaction of NPs with cells is not only governed directly but also it is strongly influenced by interplay with the medium. Salt can reduce colloidal stability, and the NP surface will be covered with a corona of proteins, which provides signature to the NP surface.[15] This interaction is not static, but rather dynamic and may change with time.[16] Although this already gives a complex scenario for the NP–medium interaction, modern techniques such as fluorescence correlation spectroscopy (FCS) allow for detailed

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investigation of the physicochemical properties of NPs in many protein-containing media[17] (but not in all, for instance, not in blood). Characterization is, however, complicated as soon as NPs enter cells. Going back to the dynamic picture of the protein corona,[15] its composition certainly will change inside cells. Enzymes in endo/lysosomal compartments can, for example, digest part of the protein corona. [18] Also the pH in these compartments, in which NPs are typically residing, is highly acidic. In particular in the case of Ag NPs, this may enhance oxidation of the inorganic NP core by release of Ag(I) ions, which in turn would affect toxicity. In order to single out such effects, highly defined NPs, in which parameters are varied in a controlled way, and appropriate test media, are warranted.

In the present study, we wanted to investigate two hypotheses. The first one is that toxicity of Ag NPs is correlated with their uptake by cells, i.e., the more internalized Ag NPs, the greater their toxicity. This was motivated by previous studies based on different types of NPs.[19] In order to test this hypothesis, cells need to be exposed to the same amounts of Ag NPs, but different amounts are internalized. One possibility to enhance uptake of Ag NPs is coupling them to magnetic NPs. Enhanced internalization of Ag NPs via magnetofection has been demonstrated to increase cytotoxicity.[20] In our study, we wanted to attempt an alternative strategy. Ag NPs are internalized via endocytotic pathways,[21] whereby uptake can be controlled by the surface chemistry of the NPs. Polyethylene glycol (PEG) of 10 kDa molecular weight is commonly used to reduce NP uptake by cells in vitro [22] and also to increase in vivo retention times.[23] Thus, in this study, we modulate uptake of Ag NPs by PEGylation and compare toxic effects obtained for Ag NPs with PEG to those obtained for the same Ag NPs but without PEG. The second hypothesis is that toxicity of Ag NPs is correlated to how fast they corrode, i.e., how fast they dissolve under physiological conditions. Also this hypothesis has been motivated by previous reports.[7] As pointed out above, one hereby needs to consider that NPs internalized in endo/lysosomal structures are exposed to highly acidic pH, which enhanced NP dissolution, in contrast to the neutral pH in extracellular media. In our study, we attempt to modulate oxidation of Ag NPs by different protective surface coatings. Often Ag NPs are capped by a monolayer of surfactant, and thus have been also termed previously as monolayer-protected clusters.[24] Additionally, we protected and stabilized the NP surface by wrapping a polymer around the original surfactant shell.[25] Altogether the key point of our study is that all experiments are based on the same Ag cores. By modification of the surface chemistry, both uptake by cells and oxidation are controlled. As the actual Ag cores of the different Ag NPs are identical, and also the final Ag NPs are well defined and characterized, comparative studies can be performed.

2. Materials and Methods

2.1. Synthesis of Hydrophobic Ag NPs [26]

Ag NPs capped with dodecylthiol were synthesized following the protocol of Mari et al.[27] These hydrophobic NPs were dispersed in chloroform and had a core diameter (i.e., the diameter of the inorganic Ag NP, excluding the organic capping [14] of $d_c = 4.2 \pm 0.4$ nm and an experimentally determined molecular extinction coefficient of $\varepsilon = 16.9 \times 10^6\ M^{-1}\ cm^{-1}$ at the surface plasmon peak at 430 nm. Based on this, we calculated that each Ag NP in average contains $N_{Ag} \approx 2200$ Ag atoms in total, whereby ≈30% ($N_{Ag,surf} \approx 650$) Ag atoms are located on the surface of each NP. For a detailed description of the synthesis protocol and the calculations, we refer to the Supporting Information. In the following section, different strategies on how these NPs were transferred to aqueous phase will be described.

2.2. Synthesis of Hydrophilic Ag NPs

The hydrophobic Ag NPs were transferred via two strategies to aqueous phase. First, the hydrophobic dodecylthiol ligands were replaced with hydrophilic mercaptoundecanoic acid (MUA) ligands by a ligand-exchange procedure, resulting in water-soluble Ag–MUA NPs. Second, an amphiphilic polymer (dodecylamine-modified poly(isobutylene-alt-maleic anhydride) PMA) was wrapped around the original ligand shell, leading to water-soluble Ag–PMA NPs. Advantages and disadvantages of both procedures have been compared in previous works.[28] The resulting Ag NPs were stringently purified by gel electrophoresis and size exclusion chromatography, which allows for sufficient removal of excess of reactants and polymeric micelles[29] (for the case of polymer-coating). Using EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) chemistry, 10 kDa amino-modified polyethylene glycol NH_2–PEG–CH_3 (Rapp Polymere) was linked via the amino group to the carboxyl groups on the surface of Ag–PMA NPs. NPs saturated with PEG (Ag–PMA–satPEG NPs) and with exactly one PEG per NP (Ag–PMA–1PEG NPs) were obtained and fractionated via gel electrophoresis. Thus, four different surface modifications based on the same original hydrophobic NPs were obtained: Ag–MUA, Ag–PMA, Ag–PMA–1PEG, Ag–PMA–satPEG (**Figure 1**). Details of the surface modification protocols are reported in detail in the Supporting Information.

2.3. Characterization of Colloidal Properties

The colloidal properties of the Ag NPs were characterized according to our standard protocols,[30] including determination of the hydrodynamic diameter (d_h) by dynamic light scattering (DLS), the zeta-potential (ζ) [31] by laser Doppler anemometry (LDA), and stability in salt-containing solutions (**Table 1**). For the stability assays, changes in the d_h upon the presence of NaCl were recorded (**Figure 2**).

2.4. Characterization of NP Oxidation

Despite stringent purification after synthesis, the Ag cores of the Ag NPs corrode over time, leading to the release of Ag(I) ions.[7] Release of Ag(I) was quantified both, at neutral (pH = 7) and acidic (pH = 3) conditions using ultrafiltration and inductively coupled plasma mass spectrometry (ICP-MS).

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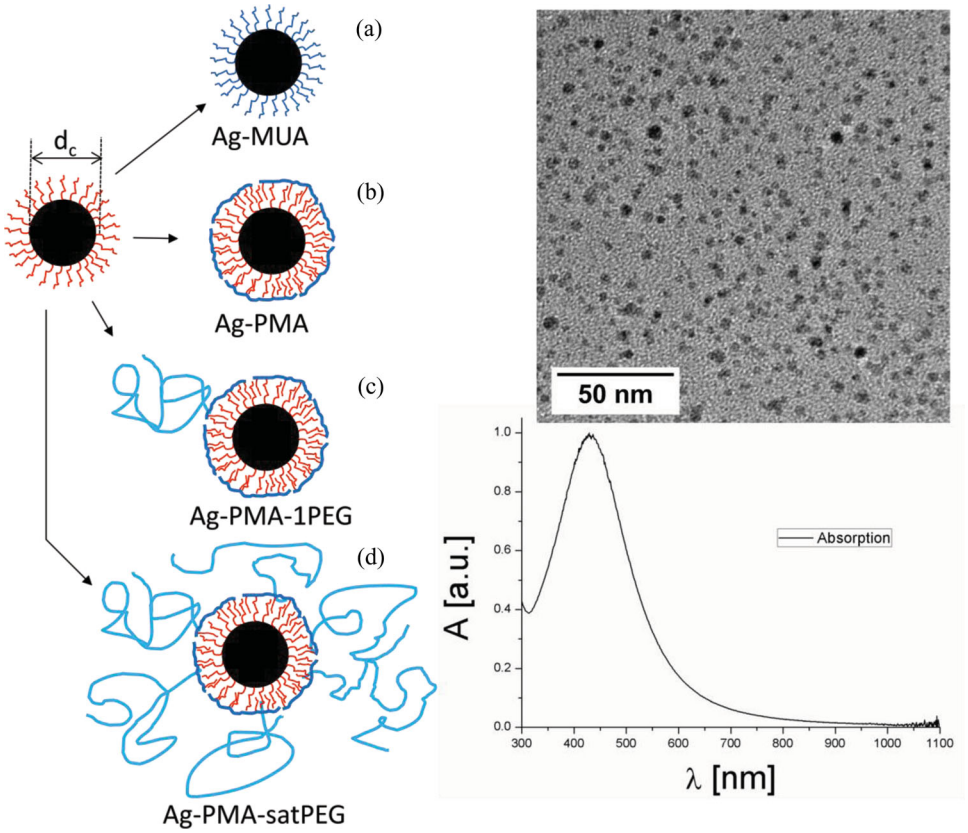

Figure 1. Sketch of the four types of surface modifications, which were applied to the same hydrophobically capped Ag NPs. The Ag cores (with core diameter d_c) are depicted in black, and the original hydrophobic ligand shell is depicted in red. The following surface modifications have been applied to obtain water-soluble NPs. a) Ligand exchange to mercaptoundecanoic acid (Ag–MUA NPs; the hydrophilic ligands are depicted in blue). b) Overcoating with an amphiphilic polymer (Ag–PMA NPs). c) Overcoating with an amphiphilic polymer and conjugation with one PEG molecule per NP (Ag–PMA–1PEG NPs). d) Overcoating with an amphiphilic polymer and subsequent saturation of the surface with polyethyleneglycol (Ag–PMA–satPEG NPs).

2.5. Analysis of NP uptake by Cells

The cellular uptake of Ag NPs was analyzed by confocal laser scanning microscopy (CLSM). NIH/3T3 embryonic fibroblasts were seeded on Ibidi-Plates at cell densities of $2 \times 10^{[4]}$ cells/well in 300 µL growth medium (DMEM-F12 Ham's basal medium supplemented with 10% calf serum, 1% L-glutamine, and 1% penicillin/streptomycin). The next day, the cells were rinsed with phosphate-buffered saline (PBS) and incubated with Dy636-modified fluorescent Ag NPs (Supporting Information) at a concentration of c(Ag NP) = 10×10^{-9} M ($\lambda_{excitation}$ 645 nm and $\lambda_{emission}$ 671 nm) for 15 h at 37 °C and 5% CO_2. The cells were then analyzed with a CLSM (Zeiss LSM Meta; **Figure 3**).

Table 1. Colloidal (hydrodynamic diameter d_h and zeta-potential ζ) and toxic properties (concentration of half viability, LD_{50}) of Ag NPs with different surface coating, with a core diameter of d_c = 4.2 nm. The concentrations refer to the number of Ag atoms in solution (c(Ag)). The LD_{50} value for $AgNO_3$ was determined as $0.022 \pm 1.24 \times 10^{-5} \times 10^{-3}$ M.

Sample	d_h [nm]	ζ [mV]	LD_{50} [$\times 10^{-3}$ M]
Ag–MUA	11 ± 4	–24.9 ± 1.7	$0.04 \pm 2.83 \times 10^{-4}$
Ag–PMA	12 ± 3	–31.0 ± 1.3	$0.65 \pm 8.66 \times 10^{-3}$
Ag–PMA–1PEG	13 ± 4	–41.1 ± 1.5	$0.73 \pm 6.48 \times 10^{-3}$
Ag–PMA–satPEG	12 ± 3	–10.9 ± 0.4	$1.34 \pm 2.49 \times 10^{-2}$

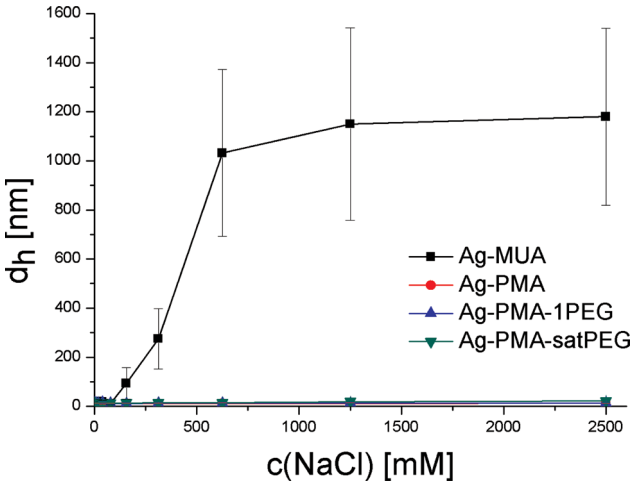

Figure 2. Change of the hydrodynamic diameter (d_h) of the Ag NPs upon the presence of NaCl (d_h was detected directly after exposure of the Ag NPs to AgCl). The error bars belong to the width of the peak.

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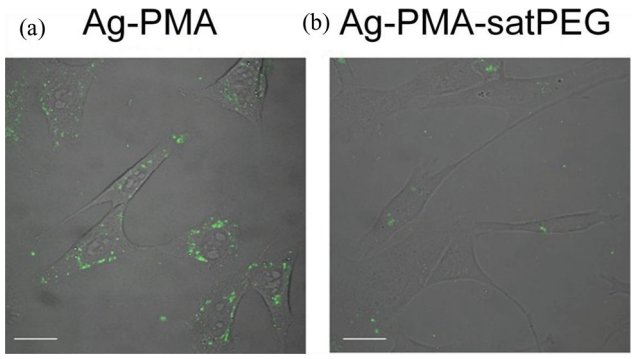

Figure 3. Fluorescence images of 3T3 fibroblasts, which had been exposed for 15 h to fluorescence (DY636) labeled a) polymer-coated Ag NPs or b) polymer-coated Ag NPs whose surface has been saturated with 10 kDa PEG molecules. The images correspond to the overly images of the transmission and fluorescence channel. Scale bars correspond to 20 µm.

2.6. Analysis of Cell Viability

Viability of NIH/3T3 embryonic fibroblasts upon incubation with Ag NPs was probed with a standard resazurin assay.[32] Silver nitrate was used as positive control. Resazurin is a blue, non-fluorescent sodium salt, which is converted to resorufin by metabolically active cells. Resorufin is a pink, fluorescent sodium salt that accumulates outside the cells. This reduction process requires functional mitochondrial activity, which is inactivated immediately after cell death. Thus, cell viability was assessed by the increase in the fluorescence signal and is given as mean of normalized intensities against the concentration of Ag. The response curves were fitted with a sigmoidally shaped curve, of which the point of inflection was taken as LD_{50} value, i.e., the concentration when cell viability was reduced by half (**Figure 4**).

3. Results and Discussion

3.1. Colloidal Properties

Four different surface modifications applied to the same Ag cores were compared. All NP samples have comparable hydrodynamic diameters (Table 1) in PBS. PEGylation increases the diameter, which is indicated in gel electrophoresis and size exclusion chromatography experiments (Supporting Information). Surprisingly, we were reproducibly not able to observe this also with DLS, which is however within the error bars. In the DLS data, MUA and PMA provide negative charge to the NPs and thus colloidal stability via electrostatic repulsions

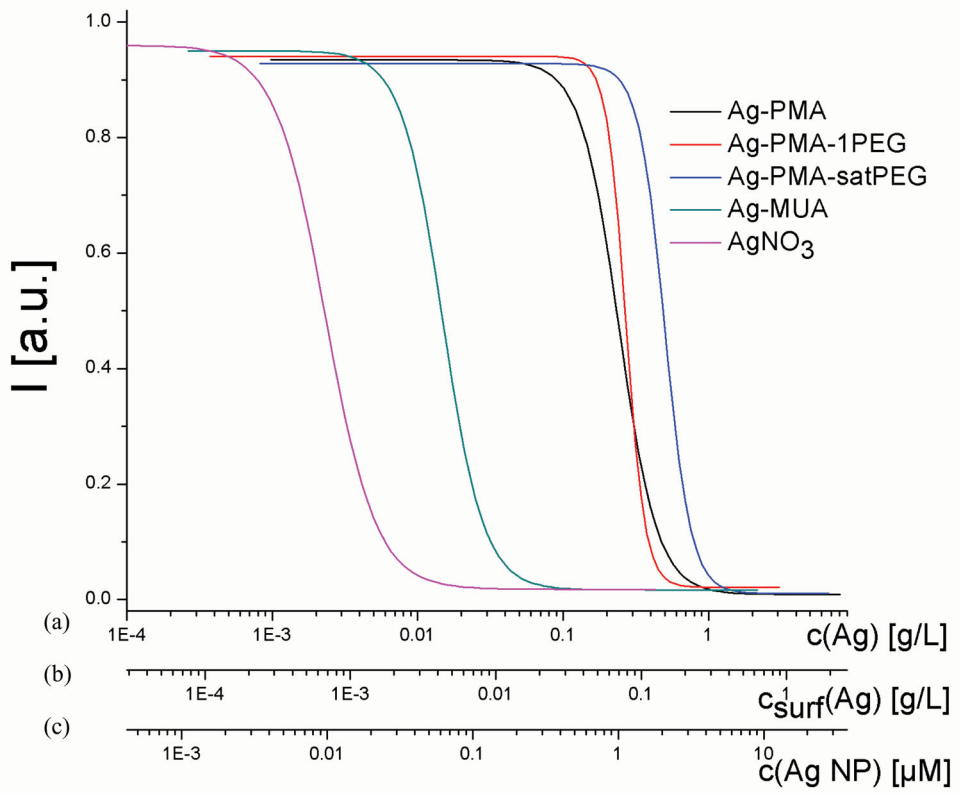

Figure 4. Resazurin-based viability test of 3T3 fibroblasts, which had been incubated for 24 h with Ag NPs. Onset of fluorescence (as quantified by the measured intensity I) is an indicator for viability of cells. The amount of Ag is quantified in a) the total amount of Ag (c(Ag)), b) the amount of Ag atoms, which are present on the NP surface (c_{surf}(Ag)), and c) the amount of Ag NPs (c(Ag NP)). The following scaling factors were used (Supporting Information): $c_{surf}(Ag) = 0.29\ c(Ag)$ and $c(Ag\ NP) = 4.2 \times 10^{-9}$ mol mg^{-1} c(Ag). In case of $AgNO_3$ as silver source, only the c(Ag) concentration scale is valid; in case of all the NPs, all three concentration scales are valid .

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(the zeta-potential data in Table 1). Saturation of the NPs surface with PEG (Ag–PMA–satPEG NPs) reduces the negative charge, but the colloidal stability is conducted by steric repulsions. However, as soon as NaCl is added to PBS, dramatic differences in colloidal stability of the NPs are visible. While for Ag–PMA NPs, the hydrodynamic diameter upon the presence of NaCl (up to 2.5 M) remains relatively unaffected, Ag–MUA NPs completely agglomerate, which is visible by their huge hydrodynamic diameters (Figure 2). Please note that colloidal characterization was performed in neutral PBS buffer containing different amounts of NaCl, not in the actual cell media. The presence of NaCl can provoke electrostatic screening and thus induces agglomeration. The presence of proteins (in serum-containing cell media) also could act on colloidal stability. However, as proteins and the NPs in this study have hydrodynamic radii on the same order of magnitude DLS is hard to perform.[33] Data with similar NPs (the same polymeric surface coating but Au instead of Ag cores) demonstrate that the presence of proteins can under our experimental conditions further reduce colloidal stability.[33] Thus, we can conclude that Ag–MUA NPs are highly agglomerated even at neutral pH and without the presence of proteins, whereas Ag–PMA NPs (with/without PEGylation) are much better dispersed. This is due to the more negative zeta-potential of PMA versus MUA coatings, which leads to stronger electrostatic repulsion for Ag–PMA NPs than for Ag–MUA NPs. For PEGylated NPs, the loss of colloidal stability due to electrostatic screening by NaCl is compensated by steric repulsions.

3.2. NP Oxidation

The Ag NPs were stringently cleaned after synthesis, leading to the removal of excess Ag(I) ions in solution. Kittler et al.[7] demonstrated release of Ag(I) from the Ag NPs due to oxidation. In order to study the release of Ag(I), NP samples incubated in water were analyzed by ultrafiltration followed by ICP-MS. At pH 7, even after 7 days of incubation, the amount of Ag(I) released from the Ag NPs was below the detection limit (<0.0015%; Supporting Information for details). On the other hand, after 7 days of incubation at pH 3 solution, around 1% of the Ag from the Ag NPs was detected as Ag(I) in solution. This suggests that Ag NPs inside acidic endo/lysosomal compartments release more Ag(I) than NPs in the extracellular neutral medium. No significant differences among the four different NP samples were observed. This can be explained by the fact that all are capped by thiolated hydrocarbon chains of comparable length (mercaptoundecanoic acid for Ag–MUA NPs and dodecylthiol for Ag–PMA NPs, respectively). Thus, changes in the outer surface cappings as PEGylation did not have significant effects on NPs oxidation. We also found in agreement with Kittler et al.[7] that Ag NPs are not completely dissolved and only a certain amount of Ag(I) is released. Analyses were performed in water at different pH, and not in the cell medium. To the best of our knowledge, proteins as particular enzymes in the cell medium can partly disintegrate the organic shell around,[18] but not extensively dissolve inorganic (e.g., Ag) cores.

3.3. NP Internalization

As can be clearly seen in Figure 3, the uptake of Ag–PMA NPs was strongly reduced when the surface of the NPs was saturated with PEG molecules of 10 kDa size (Ag–PMA–satPEG NPs vs Ag–PMA NPs). We did not quantify uptake as this has been already previously done with similar NPs,[22,33] and the qualitative results are sufficiently indicative. Data also clearly demonstrate that Ag–PMA NPs are internalized to a much higher extent than Ag–PMA–satPEG NPs. Unfortunately, we were not able to quantitatively compare the uptake of Ag–MUA NPs and Ag–PMA NPs. This is due to the fact that on the basis of different surface chemistry, the Dy636 fluorescence labels were attached differently to the NPs. Besides different amount of fluorophore per NP, they also will have different distance to the Ag cores and thus different quenching effects. The main statement of the uptake experiments thus is that PEGylation drastically reduces the uptake of NPs by cells, in accordance with previous works.

3.4. Cell Viability

As expected, free Ag(I) ions ($AgNO_3$ solution as positive control) significantly reduce the viability of NIH/3T3 cells, as quantitatively demonstrated in dose-response curves (one time point, one type of assay, and one cell type; Figure 4). Also in case Ag(I) is introduced in the cells via Ag NPs, the cell viability is reduced. In comparison to the MUA coating, the PMA coating was effective in reducing the toxicity of the Ag NPs. The surface modification of PMA-coated Ag NPs with one single PEG molecule (10 kDa) per NP (Ag–PMA–1PEG NPs) exhibited a similar dose-response curve (with similar LD_{50} values) to Ag–PMA NPs. In contrast, dose-response curve for Ag–PMA–satPEG NPs was strongly shifted to higher concentrations values. This indicates that much lower concentrations of nonPEGylated Ag NPs than of PEGylated NPs are necessary to induce cell death.

4. Conclusions

Correlation of the interaction of the NPs with cells and their physicochemical properties allows for a set of conclusions. i) Toxicity of Ag NPs was lower in comparison to Ag(I) ions, in case of normalization of the absolute amount of Ag. In Figure 4 NP concentration is given in terms of c(Ag) gram per liter, which allows for direct comparison. Even if one assumed that in the case of Ag–MUA NPs all Ag atoms on the NP surface went into solution, there is still a lower toxic effect in comparison to the same amount of free Ag(I) ions (from $AgNO_3$). Even in the worst case (Ag–MUA NPs), the toxicity from all Ag atoms on the surface still is lower than the one for free Ag(I), in case the c_{surf}(Ag) scale for Ag NPs is compared with the c(Ag) scale for $AgNO_3$. In addition, while ≈30% of the Ag within Ag NPs is bound on surface, our ICP-MS data indicated that even at pH 3, only ≈1% of Ag is released from the Ag NPs into solution. Thus, under our experimental conditions, Ag NPs are less toxic compared to Ag(I) ions in terms of absolute amounts of Ag, as most of the Ag in NPs is bound to the NPs and not available as Ag(I).

Please note that this is the opposite as reported by Cronholm et al.,[9] who, however, worked under different experimental conditions (other NPs and cells). In our case, even if one assumed the outer capping of Ag NPs to be oxidized there is still lower toxicity compared to $AgNO_3$. Additionally, PMA coating did not reduced the release of Ag(I) versus MUA coating, but reduced cytotoxicity. ii) Toxicity of Ag NPs depends on their uptake by cells. One may argue that the 1% of released Ag(I) ions from internalized Ag NPs has a more cytotoxic effect than assuming a scenario in which all Ag atoms situated at the Ag NP surface (≈30%) would be released extracellularly. Ag(I) as free ion is not membrane permeable (although it can be complexed by serum proteins) and thus Ag NPs are an efficient carrier to transport Ag(I) ions inside cells. The difference between intracellular and extracellular Ag is demonstrated upon comparison of Ag–PMA and Ag–PMA–satPEG NPs. PEGylation reduces cellular uptake, which goes hand in hand with a lower cytotoxicity. iii) Agglomeration is a key parameter in Ag NPs toxicity. Comparing the cytotoxic effects between Ag–MUA NPs and Ag–PMA NPs, different agglomeration behavior is the most likely reason. Ag–MUA NPs showed a greater tendency to agglomerate what finally resulted in an increased cytotoxicity. Toxic effects may be explained by clouding the cell membrane, as has been speculated previously.[34] Although our data clearly demonstrate that toxicity due to intracellular Ag is higher than that due to extracellular Ag (note that in contrast in the report by Cronholm et al.,[9] toxicity was generated by the extracellular interaction of Ag(I) to the cell membrane, unfortunately we could not quantify this in terms of the contribution of Ag from Ag(I) or Ag NPs. Characterization of physicochemical properties of internalized NPs still remains a future challenge, which, however, is strongly needed for further unraveling pathways of cytotoxic effects and their attribution to distinct physicochemical properties.

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