The Cellular Interactions of PEGylated Gold Nanoparticles: Effect of PEGylation on Cellular Uptake and Cytotoxicity

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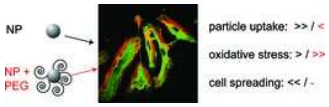
The Cellular Interactions of PEGylated Gold Nanoparticles: Effect of PEGylation on Cellular Uptake and Cytotoxicity



Nanoparticle PEGylation influences cell interaction. PEGylation of gold nanoparticles is studied under both identical nanoparticle doses and similar intracellular nanoparticle concentrations. PEGylation is found to impede cell uptake in a cell-specific manner and impedes nanoparticle effects on cell morphology. However, PEGylation increases oxidative stress and does not always appear to be optimally suited for in vitro cell labeling.

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The Cellular Interactions of PEGylated Gold Nanoparticles: Effect of PEGylation on Cellular Uptake and Cytotoxicity

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Keywords: nanotoxicology, gold nanoparticles, poly(ethylene glycol), cytotoxicity, cell labeling

ABSTRACT: Poly(ethylene glycol) (PEG) is frequently used to coat various medical nanoparticles (NPs). As PEG is known to minimize NP interactions with biological specimens, the question remains whether PEGylated NPs are intrinsically less toxic or whether this is caused by reduced NP uptake. In the present work, the effect of gold NP PEGylation on uptake by three cell types is compared and evaluated the effect on cell viability, oxidative stress, cell morphology, and functionality using a multiparametric methodology. The data reveal that PEGylation affects cellular NP uptake in a cell-type-dependent manner and influences toxicity by different mechanisms. At similar intracellular NP numbers, PEGylated NPs are found to yield higher levels of cell death, mostly by induction of oxidative stress. These findings reveal that PEGylation significantly reduces NP uptake, but that at similar functional (= cell-associated) NP levels, non-PEGylated NPs are better tolerated by the cells.

1. Introduction

The interest in the use of nanoparticles (NPs) for biomedical applications is vastly increasing owing to the wide plethora of enticing features that NPs possess.[1] This makes them frequently studied as tools for improved noninvasive in vivo and in vitro cell imaging, cancer therapy, cell transplantation, and as carriers for drug and/or gene delivery.[1] There are no "naked" NPs in biological environments as their surface is always covered with organic matter.[2] One frequently used intentional organic surface coating of NPs is based on poly(ethylene glycol) (PEG).[3] This flexible molecule, available in various chain lengths and terminal functional groups, effectively shields the surface from the surrounding environment. Thereby, it provides colloidal stability by sterically hindering NP agglomeration.[2] Furthermore, PEGylation of NPs under in vitro conditions has shown reduced cellular uptake and improved biocompatibility.[4] Under in vivo conditions, PEGylation promotes the in vivo blood circulation time of NPs by reducing their opsonization and thereby impeding clearance of the NPs by the reticuloendothelial system.[4e,5] Recently, however, the use of PEG has been described to induce inflammation and cause hypersensitivity.[5a] In vitro, the effect of PEG density and PEG chain length has been well studied with respect to their effect on cellular NP uptake,[4d] where PEG has been shown to reduce NP binding and associated uptake levels of NPs where the uptake kinetics of bound NPs were not affected.[6] Indirectly, some studies have also pointed to a higher biocompatibility of PEGylated NPs, which is likely to be a direct

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result of the lower amount of cell-associated nanomaterials.[4d] For cell labeling applications in which a minimal number of cell-internalized NPs is essential, it is as of yet unclear whether higher levels of PEGylated NPs would be a better choice than lower levels of non-PEGylated NPs (resulting in similar cellular NP levels) as little data are available on the effect of PEG on cell homeostasis at similar cellular NP levels.

Nanotoxicology is a complex field where minor changes in NP surface chemistry can drastically alter the interaction of NPs with cells.[7] Therefore, it is of interest to investigate the effect of applying a PEG coating to Au NPs in terms of their cytotoxicity. PEG can be attached directly via thiol groups to the surface of Au NPs.[4c,8] Alternatively, PEG can also be covalently attached to more complex NP surfaces, such as NPs stabilized with an amphiphilic polymer,[9] for example, poly(isobutylene-*alt*-maleic anhydride)-dodecylamine (PMA). PMA-coated Au NPs were prepared, analogously as previously described.[9b] Presence of PEG can be conveniently verified by retarded mobilities in gel electrophoresis experiments (see Supporting Information).[2,9b] The number of PEG molecules per NP can also be quantified by NMR.[10]

2. Results and Discussion

2.1. Nanoparticle Characterization

It has to be noted that PEGylation does not only change the surface chemistry of NPs but also influences other physicochemical parameters.[2] Obviously, addition of PEG increases the hydrodynamic diameter of the NPs.[9b] However, it also changes the surface charge of the NP by the complexation of cations.[11] In addition, PEGylation, in general, improves the colloidal stability of NPs by introducing sterical repulsion between the NPs.[2,12] In the present study, polymer (PMA)-coated Au NPs with a saturated methoxy-PEG (mPEG) shell were investigated and compared with the same Au NPs without PEG, which were analyzed in a previous study.[13] Au NPs with a core diameter of 4.6 ± 1.1 nm were used (**Figure 1**). Whereas the polymer-coated Au NPs without saturated PEG shell had a hydrodynamic diameter of 12.6 ± 1.1 nm and a ζ-potential of – 31.9 ± 5.2 mV.[13] The addition of a saturated shell of methoxyl-terminated PEG (M_w = 2 kDa) resulted in a hydrodynamic diameter of 21.7 ± 2.9 nm and a ζ-potential of −7.6 ± 0.8 mV, as measured by dynamic light scattering (DLS) using a zetasizer. The hydrodynamic diameter determined here for the PEGylated NPs is significantly higher than the ones determined with different methods in previous studies. Data indicate an increase in hydrodynamic diameter and a less negative ζ-potential due to the complexation of cations upon PEGylation.

To evaluate the effect of PEGylation on cellular uptake of NPs and its consequent cytotoxicity, a recently described multiparametric methodology was used.[14] This methodology allows to define the noncytotoxic concentration of the NP tested for a variety of cell types and to identify the mechanisms underlying the NP's cytotoxic profile. Furthermore, this methodology enables to evaluate and compare the intrinsic toxicity levels and the mechanisms underlying these events. Although the

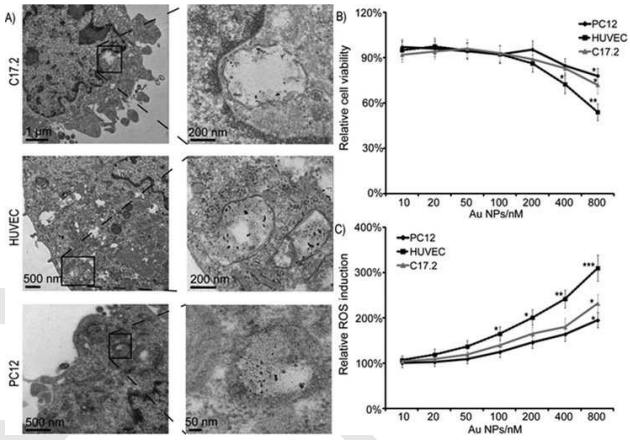

Figure 1. A) Transmission electron microscopy (TEM) images of C17.2, HUVEC, and PC12 cells exposed to 50 × 10^{-9} M of PEGylated Au NPs for 24 h revealing clear cellular uptake. B,C) Effects of PEGylated Au NPs on B) cell viability and C) reactive oxygen species (ROS) induction at different Au NP incubation concentrations. Data are expressed relative to untreated control cells as mean ± SEM (n = 5). When appropriate, the degree of significance is indicated (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

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Au NPs evaluated in this study are very interesting for biomedical research, their toxicological profile still has not been fully unraveled.[15] Previously, the same Au NPs as used in this study, but without the PEG shell, were evaluated through this methodology, revealing NP-concentration-dependent cytotoxicity (see **Table 1**) and defining the nontoxic concentration of the NPs at 10 × 10^{-9} M.[13] In the present work, identical Au NPs saturated with methoxy-terminated PEG (M_w = 2 kDa) are used to evaluate the effect of PEGylation on NP uptake and interactions with cultured cells.

2.2. Cellular Nanoparticle Uptake

In the present study, the PEGylated Au NPs were used to label three different cell types, being human umbilical vein endothelial cells (HUVECs), murine C17.2 neural progenitor cells, and rat PC12 pheochromocytoma cells. These cells have previously shown to be efficient model systems for cytotoxicity studies,[16] where both HUVEC and C17.2 cells are extensively explored in cell transplantation studies and are thus often used for dosing with nanomaterials in order to facilitate in vivo cellular tracking.[17] These cells additionally differ quite a bit, including human and rodent cells as well as endothelial and neural cell types, which are common target cell types. Therefore, these cells can provide a nice overview of how cells, in general, interact with a certain type of NP. Additionally, these cells have been used in various previous studies, allowing a direct comparison of any toxicity data obtained for different types of materials.

In terms of uptake, PEGylated Au NPs were found to be taken up by all three cell types used. As evidenced by transmission electron microscopy (TEM) (Figure 1A), the Au NPs are typically located in endosomal structures, though it is known

Table 1. Overview of the total NP numbers of non-PEGylated and PEGylated Au NPs at which significant levels of bio-effects were occurring.

	Non-PEGylated NPs		PEGylated NPs	
Bio-effect	Exposure conc. [$\times 10^{-9}$ M]	Intracellular NP amount [$\times 10^5$ NPs/cell]	Exposure conc. [$\times 10^{-9}$ M]	Intracellular NP amount [$\times 10^5$ NPs/cell]
Cell death	200	*62*	400	*27.4*
ROS	50	*21*	100	*9.3*
Cell morphology	50	**21**	400	**27.4**
PC12 functionality	20	*9*	100	*4.5*
No toxicity observed	10	*6*	50	*3.5*

*The total NP numbers of non-PEGylated and PEGylated Au NPs at which significant levels of bio-effects were occurring are given for both the concentration of NPs in the media upon incubation (expressed as M) as well as the intracellular number of NPs (expressed as 10^5 particles per cell). For all effects, the level of NPs in HUVEC cells was selected, apart from PC12 functionality, where the level is chosen in PC12 cells and the level where no toxicity is observed is the average value for all three cell types. Bio-effects, which occurred at lower intracellular NP numbers for PEGylated NPs, are indicated in italic, those which occurred at lower levels of non-PEGylated NPs are highlighted in bold.

that they also can reach other intracellular compartments.[4c] The images themselves reveal the presence of the NPs in structures consisting out of a unilamellar-enclosed cytoplasmic compartment, which is typical for endosomal compartments. Recently, the role of NPs in inducing autophagy as a key nanotoxicity mechanism has gained a lot of importance.[18] The TEM structure revealed in these images, however, differs from those observed for autophagosomes,[19] in that the latter compartments consist of a double lamellar membrane.[18a] As such, no clear signs of autophagy have been observed, suggesting that autophagy likely does not play a major role in the cellular processing of these nanomaterials, but more research on this interesting phenomenon should be performed before any firm conclusions can be drawn.

An important finding in this regard is the localization of the NPs with endosomal compartments, which is similar to the localization observed for the non-PEGylated Au NPs.[13] This finding reveals that PEGylation of the Au NPs did not affect the intracellular distribution of the NPs and suggests that a similar uptake mechanism is involved. Although, in neither study, any NPs were observed in the cell nuclei, the transfer of a limited number of NPs toward the nuclear region cannot be ruled out completely and may also follow a dose-dependent trend.[20] The similar intracellular distribution of the NPs is of great importance for a direct comparison of any toxicological effects as the nature and degree of any effects observed depends on the exact intracellular location of the NPs.[20]

The TEM images qualitatively show large differences in the levels of NP uptake between the different cell types, where HUVECs have the highest uptake levels, followed by C17.2 and PC12 cells. This qualitative measurement was further confirmed by quantitative determination of cellular NP levels by inductively coupled plasma–mass spectrometry (ICP–MS) after 24 h (Table S2, Supporting Information). A similar trend was observed and the uptake efficacy of HUVECs was approximately twofold higher than that of the C17.2 and PC12 cells. These data are in sharp contrast with the data for non-PEGylated Au NPs, as shown in Table 1. For non-PEGylated NPs, C17.2 and HUVEC cells had similar uptake levels, where the addition of PEG has significantly reduced the uptake of the NPs in all cell types,[13] in accordance with previous studies.[4c] However, the extent of the effects of PEGylation on NP uptake is clearly cell type-dependent, where for HUVECs, maximally a fourfold reduction is observed, compared with an eightfold reduction for PC12 cells and a tenfold reduction in C17.2 cells. These data clearly indicate that the effect of PEGylation on NP uptake can vary significantly between different cell types and that specific targeting will be significantly mediated by PEGylation of the NPs.

2.3. Effect of PEGylated Nanoparticles on Cell Viability and Oxidative Stress

After 24 h of incubation, cell viability was found to be affected at high Au NPs doses (starting from 400×10^{-9} M of exposure concentrations) and was found to be cell type-dependent. These data were in correlation with the cell uptake levels (Figure 1B), resulting in highest toxicity levels for HUVEC cells compared with PC12 or C17.2 cells. Similarly, a concentration-dependent induction of reactive oxygen species (ROS) was observed (Figure 1C) in all three cell lines. Interestingly, the addition of 5×10^{-3} M *N*-acetylcysteine (NAC), an FDA-approved free-radical scavenger, completely inhibited ROS induction and restored cell viability (Figure S9, Supporting Information). Furthermore, high NP levels were found to affect mitochondrial membrane potential, cytoplasmic Ca^{2+} levels, and DNA damage (**Figure 2**) that are important pathways involved in ROS-dependent cell death.[21] Compared with non-PEGylated Au NPs, the induction of ROS at identical incubation doses was approximately 1.7-fold lower,[13] which is in line with other studies where PEGylation of NPs resulted in a lower induction of oxidative stress.[22] However, more interestingly, when comparing similar intracellular NP levels (as determined by ICP–MS) the induction of ROS was approximately 1.6-fold higher for PEGylated NPs in all three cell types. These findings are in contrast with the general belief that PEGylation reduces ROS levels and can be explained by the fact that, here, ROS levels were compared at similar intracellular levels of PEGylated and non-PEGylated NPs whereas in nearly all previous studies, NPs have been compared with respect

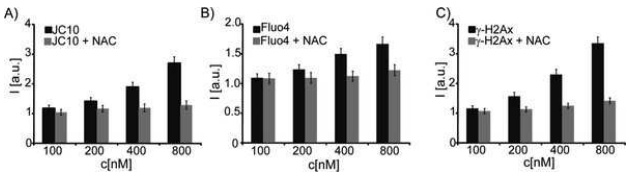

Figure 2. Effects of PEGylated Au NPs (incubation concentration c [$\times 10^{-9}$ M]) on HUVEC cell as given as relative signal intensities I [a.u.]. A) mitochondrial membrane potential, B) cytoplasmic calcium levels, and C) DNA double-strand breaks in the absence (dark gray) and presence of 5×10^{-3} M NAC (light gray), when cells were exposed to PEGylated Au NPs for 24 h at 100, 200, 400 or 800 $\times 10^{-9}$ M. Data are expressed relative to the values obtained for untreated control cells as mean ± SEM ($n = 4$).

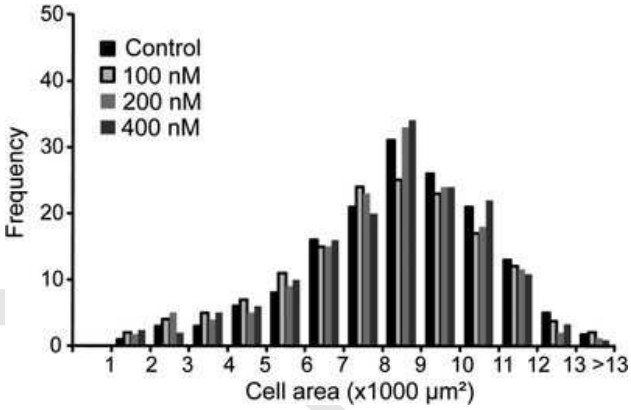

Figure 3. Histogram representing the distribution of cell areas of control HUVEC cells or cells exposed to PEGylated Au NPs at 100, 200, or 400 $\times 10^{-9}$ M incubation concentration for 24 h and analyzed at 24 h post-cell labeling, as described in the Supporting Information.

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to their exposure concentrations.[4d,22] However, in plant cells, PEG chains have been found to induce ROS indirectly as a result of altering water stress levels.[23] The precise mechanism for the enhanced ROS production here remains unclear but can probably be attributed to the altered physicochemical properties of the NPs upon PEGylation. One important contributor to the level of oxidative stress may be p53, a key transcription regulator for many intracellular pathways including inflammation and oxidative stress sensing.[24] It would be interesting to see whether PEG chains in themselves are ROS inducing and whether this involved p53 activity. Taken together, these data point to the importance of ROS in the toxicological profile of the PEGylated Au NPs, with a cell type-dependent correlation between ROS levels, stemming from the intracellular localization of the NPs and a reduction of cell viability.[25] Interestingly, ROS-associated secondary effects, such as DNA damage, are significantly higher for PEGylated NPs than for their corresponding non-PEGylated counterparts for similar intracellular NP levels (= the number of NPs per cell).

2.4. Effect of PEGylated Nanoparticles on Cell Morphology

Apart from ROS, which is an important mediator in the toxic potential of NPs,[26] several other mechanisms have been suggested to play an important role, such as cellular deformations and effects on the cell cytoskeleton architecture.[27] To further define the mechanisms underlying the toxicity of the PEGylated Au NPs, the effect of the NPs on HUVEC cell morphology and cytoskeleton architecture was analyzed as described previously (**Figure 3**).[16] For this analysis, only the HUVEC cells were considered as the rounded PC12 and C17.2 cells are intrinsically far less spread and are therefore not well suited for this type of analysis. The functionality of these cell types is investigated using specific assays later on in this manuscript. No effect on cell morphology or cytoskeleton architecture could be observed at nontoxic concentrations of the PEGylated Au NPs, i.e., up to 400 $\times 10^{-9}$ M (added exposure concentration). These observations were different to the results obtained for various types of non-PEGylated Au NPs,[28] including PMA-coated Au NPs, where cellular deformations were already observed at 50 $\times 10^{-9}$ M.[13] Interestingly, at 400 $\times 10^{-9}$ M (added exposure concentration) of PEGylated Au NPs, higher intracellular NP levels (1.3-fold) and higher ROS levels (1.6-fold) were found than for non-PEGylated Au NPs at 50 $\times 10^{-9}$ M (external concentration). However, even at higher intracellular NP levels, PEGylated NPs were not found to induce any morphological or cytoskeletal alterations (Table 1). These data suggest that the cellular morphological changes observed previously for the Au NPs are ROS independent and furthermore, the addition of PEG appears to be able to overcome these effects.

To test this more thoroughly, the effect of the PEGylated Au NPs on the expression and activation of two key signaling pathways [NFκB and focal adhesion kinase (FAK)] was analyzed in the HUVEC cells. Whereas the NFκB pathway is known to be an important mediator in ROS-induced signaling,[29] the activation status of FAK is linked to the cytoskeletal architecture[30] and was previously shown not to be prone to direct ROS-induced signaling.[16a,16b] **Figure 4**A,B shows a clear concentration-dependent activation of the NFκB pathway, which could be overcome by co-treatment with NAC, indicating a ROS dependence. No effect was seen on the expression or activation of FAK (Figure 4C,D), supporting our earlier findings that PEGylation of Au NPs protects the cells from cytoskeletal deformations, even at conditions where similar intracellular NP concentrations have been obtained (Table 1). In the latter experiment, PC12 cells were not included as these cells are only semi-attached and therefore only form minimal focal adhesions. The level of FAK activation is closely linked to the formation of fully mature focal adhesions, which results in minimal levels of active FAK in the PC12 cells.

2.5. Effect of PEGylated Nanoparticles on Cell Functionality

As a final test, the effect of the PEGylated Au NPs on PC12 functionality was assessed, where the ability of the cells to induce the outgrowth of neurites was evaluated as described elsewhere.[31]

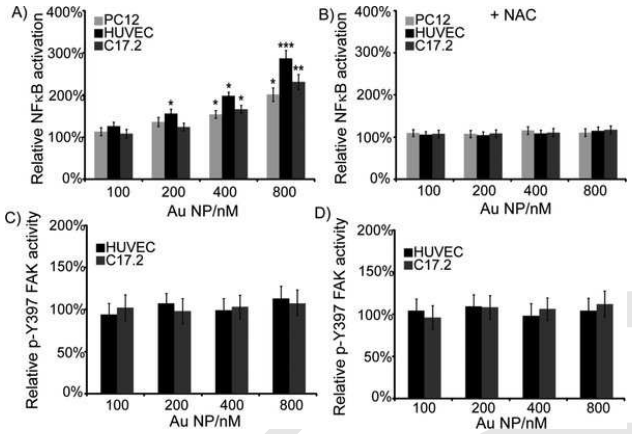

Figure 4. The effects of PEGylated Au NPs in dependence of incubation concentration on activation of A,B) NFκB and C,D) FAK in the A,C) absence or B,D) presence of NAC, a ROS scavenger.

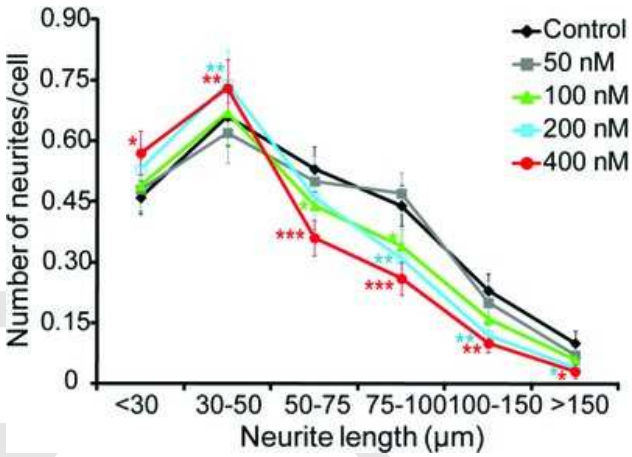

Figure 5. Effect of PEGylated Au NPs on neurite induction from PC12 cells exposed to incubation concentrations of Au NPs of 0, 50, 100, 200, and 400 $\times$ 10^{-9} M for 24 h followed by 48 h exposure to nerve growth factor. Data are expressed as the total number of neurites of a certain length per cell as mean $\pm$ SEM ($n = 4$).

Figure 5 shows a clear concentration-dependent inhibition of PC12 functionality, where effects were noticeable from 100 $\times$ 10^{-9} M, which is in line with the onset of ROS induction. These data show that the induced ROS effects can result in reduced cell functionality, which has important consequences for the use of such labeled cells for any biomedical purposes. Based on these results, we define the nontoxic concentration of PEG Au NPs as 50 $\times$ 10^{-9} M (exposure concentration), which is higher than the nontoxic concentration of 10 $\times$ 10^{-9} M (exposure concentration) for non-PEGylated Au NPs, which was obtained using exactly the same methodology.[13] However, given the lower uptake efficiency of cells for PEGylated Au NPs, the higher level of PEGylated Au NPs in the incubation medium does not correspond to a higher level of intracellular Au NPs, as is also shown in Tables S2 and S3 (Supporting Information).

Overall, the current study highlights the importance of a thorough investigation into the effect of altering the surface chemistry of the NP of interest with regard to its effect on cultured cells. PEGylation in itself will drastically alter the chemistry of the NP surface. As shown in Section 2.1., addition of the PEG chains increases the hydrodynamic diameter and alters the surface charge of the NPs. Additionally, the colloidal stability of NPs is generally improved by PEGylation due to steric repulsion between NPs. The effect of PEGylation therefore influences quite a large number of different parameters, all known to affect cellular interaction.[11,12] However, the precise effect of any of these individual parameters is hard to predict as all of them are closely linked. For instance, with regard to cellular uptake, the enhanced colloidal stability likely results in higher uptake values for smaller NPs, where extensive agglomeration is known to impede NP uptake.[32] Alternatively, the increase in hydrodynamic diameter, the decrease in surface charge, and the presence of a long, flexible hydrocarbon chain that hinders the actual NPs of coming into close contact with the cell membrane will all impede cellular uptake. One key aspect of the addition of PEG chains is their influence on the protein corona, which forms around any type of nanomaterial in serum-containing media.[33] Given the loss in charges and the flexible nature of the PEG chains, PEGylation is known to impede protein binding onto the particle surface.[34] Therefore, the protein corona formed around non-PEGylated and PEGylated particles will be quite different. This protein corona is however extremely important in determining "cell vision," i.e., the way in which cells will see the foreign NPs and how they will interact with them.[35] This effect of PEGylation is therefore quite important and cannot be ignored when trying to understand the precise mechanisms behind the observed effects in our study.

3. Conclusion

The data summarized in Table 1 indicate that on average, for the different cell types, higher intracellular levels of non-PEGylated Au NPs can be achieved without inducing any toxic effects than is the case for PEGylated NPs. In general, these data therefore show that the reduced toxic effects of NPs upon PEGylation is primarily driven by a significant drop in cellular NP uptake levels, as at least for PMA-coated Au NPs, the intrinsic toxicity for similar intracellular numbers of NPs appears to be higher upon PEGylation. Interestingly, PEGylation of the Au NPs had a clear cell type-dependent effect on NP uptake and toxicity. In addition, in terms of toxicity mechanisms, PEGylation of NPs was found to affect the underlying causes, resulting in higher ROS levels and higher secondary ROS mechanisms but no effect on cytoskeletal aberrations for similar intracellular levels of PEGylated NPs compared with non-PEGylated NPs (Table 1). The exact reason for this currently remains unclear, but the addition of a flexible shielding layer appears to drastically alter cell–NP interactions and requires further studies to shed more light on this complex issue. The question therefore

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remains whether PEGylation of NPs is actually beneficial or potentially hazardous for in vitro labeling of cultured cells.

4. Experimental Section

A full experimental methodology including NP synthesis and characterization, and setup of cell–NP interaction studies can be found in the Supporting Information that accompanies this manuscript.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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