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Laser-induced delivery of gold nanoparticles into living cells

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Introduction

The uptake of metal nanoparticles (NPs) by mammalian cells and subsequent irradiation with light in the plasmon resonance band has been suggested for various diagnostic and therapeutic applications.[1-4] In particular, laser irradiation of intracellular NPs may present an interesting approach to cancer therapy.[3,4] Several properties make NPs highly suitable for this purpose: (i) functionalisation with antibodies or proteins allows for targeting of specific cells; (ii) only low light intensities are required due to the high absorption cross section; and (iii) the absence of photobleaching means that long exposure times are possible.

While there are examples in the literature of successfully inducing cell death in the presence of NPs,[3,4] the mechanisms involved remain hypothetical. Photothermal effects are most frequently suggested, but irradiation of NPs could have other effects, such as release of toxic material from the particle surface or localised destruction of the cellular structure, which may induce cell death without a major increase in temperature.

Here, we studied the mechanism of cell death at the subcellular level. More importantly, we also evaluated the scope of laser irradiation for the controlled release of NPs carrying a payload into the cell cytosol without compromising cell viability, which would have many potential applications in the field of gene and drug delivery or intracellular labelling.

Materials and methods

HeLa cells were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum and nonessential amino acids at 37°C in a humidified atmosphere of 5% CO_2. Citrate-stabilised gold NPs with a diameter of (16±1) nm were synthesised by adding trisodium citrate solution to a boiling $HAuCl_4$ solution (Turkevich-Frens procedure) and characterised using UV-vis spectroscopy and transmission electron microscopy (TEM).

Except where stated otherwise, HeLa cells were incubated with 2 nM gold NPs for 24 hours and then rinsed with phosphate buffer solution (PBS) prior to laser exposure in order to remove non-internalised NPs. Laser irradiation was performed with a cw argon laser (Coherent Innova 300C) at 514 nm (plasmon resonance). The beam (max. 2 W, 1.55 mm dia.) was directed onto the cell culture from above through a thin layer of PBS.

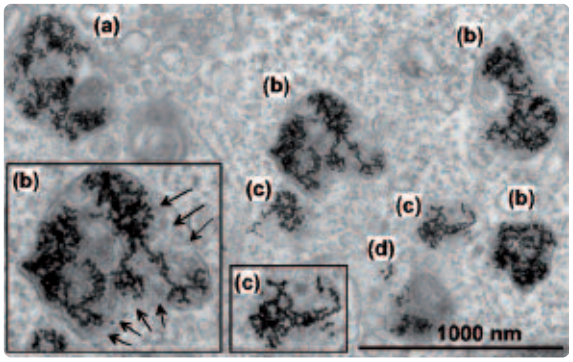

Figure 1. TEM image of NP-containing endosomes after 90 s of irradiation at 37 W/cm², showing an intact endosome (a), partially disrupted endosomes (b), endosomes without membrane (c) and particles released into the cytosol (d). Insets are magnified images of some of the features, with arrows indicating regions of partially disrupted membrane.

Cell viability was tested with the trypan blue dye exclusion assay: cells were incubated with 0.4% trypan blue for 5 min, then rinsed with PBS and observed under a phase contrast microscope to count the number of stained (dead) cells. For TEM, cells were fixed in 1% osmium tetroxide for 1 hour, rinsed with water, stained with 0.5% uranyl acetate for 1 hour, dehydrated in ethanol and embedded in expoxy resin, which was polymerised at 60°C for 48 hours. Ultrathin sections (50-75 nm) were stained with 5% uranyl acetate and 2% lead citrate and imaged in a 120 kV FEI Tecnai Spirit TEM. For confocal microscopy, cells were incubated for 20 min with rhodamine-labelled dextran (0.2 mg/ml). After laser irradiation, the cells were fixed with 4% paraformaldehyde and imaged on a Zeiss LSM510 confocal microscope (exc. 543 nm, emission >560 nm).

Cellular uptake of gold nanoparticles

In spite of their inherent instability at high ionic strength, citrate-stabilised gold NPs do not significantly aggregate immediately after addition to the cell culture, probably due to proteins replacing citrate. A slight red-

shift of the plasmon band was observed upon addition to the cell medium, which is indicative of protein adsorption.[5] TEM analysis shows that gold NPs are readily taken up by cells via endocytosis and end up trapped in endosomes, see Fig. 1 (a). Before laser irradiation, individual particles freely dispersed in the cytosol are not even observed as minority species. Viability upon nanoparticle incubation was higher than 98%, indicating no toxicity of NPs.

Cell death and morphology changes upon irradiation

Irradiation of cell cultures without added NPs at intensities of up to 106 W/cm^2 for up to 5 minutes did not result in any cell death, as assessed by the trypan blue dye exclusion assay, or morphological changes, as assessed by TEM.

For cell cultures incubated with NPs, the number of dead cells was found to increase with exposure time and power density. At 106 W/cm^2, a significant portion of cells die and become detached from the culture already after 2 min. On the other hand, upon irradiation at 37 W/cm^2, the fraction of dead cells remains small (< 1%) even after 5 min. TEM images show severely damaged cells after irradiation at 58 W/cm^2 or more for a few minutes. Irradiation at 37 W/cm^2, on the other hand, leaves cells with normal morphology, although cytosol protein density starts to decrease after 2 min, indicating cellular stress.

Irradiation-induced endosome rupture

TEM images at higher magnification revealed that even after irradiation at low intensities which did not cause cell death, morphology changes or changes in protein density not all NP-containing endosomes were intact.

Different scenarios could be observed depending on the stage in the endosomal pathway. Release of NPs into the cytosol was observed mostly from early endosomes, whose outer membrane was found partially or totally disrupted. By contrast, in the case of late endosomes and multivesicular bodies (formed at later stages during endocytosis), often no release was observed even in the occasional case of damage to the outer membrane.

The various stages of the disruptive process caused by longer exposure to the laser beam (37 W/cm^2, 90 s) are collectively captured by the TEM image in Figure 1, which shows an intact late endosome (a), partially disrupted late endosomes (b), early endosomes without membrane (c) and particles released into the cytosol (d). Under these irradiation conditions, not only early endosomes were affected, but also the outer membrane of late endosomes and multivesicular bodies. At the same laser intensity, but a reduced irradiation time (30 s), on the other hand, we observed the disruption only of early endosomes.

We also studied the effect of even lower laser intensities (11 W/cm^2) at a range of irradiation times (3-7 min). In all cases, TEM images confirmed that the cells remained healthy with no morphological disruption. Already after 3 min irradiation, many early endosomes were disrupted, but even late endosomes and multivesicular bodies were found which had released NPs into the cytosol, Fig. 2. Increased

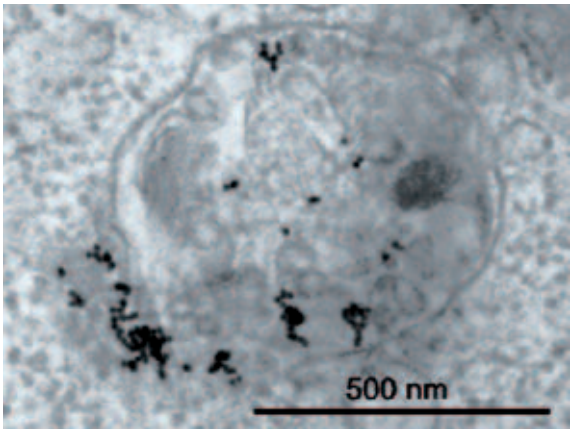

Figure 2. TEM image of NP-containing endosome after 3 min of irradiation at 11 W/cm^2, showing early stage of disruptive process of a multivesicular body.

irradiation times resulted in more effective release of NPs, i.e. a larger fraction of late endosomes and multivesicular bodies were found to have been affected.

We note that in all cells analysed endosomes containing relatively few NPs were affected more significantly than similar endosomes with a relatively large number of NPs, which remained mostly intact.

Confocal microscopy was used to confirm endosome disruption. Cells were incubated with rhodamine-labelled dextran, a fluorescent marker for endosomes. Irradiation was performed at 37 W/cm^2 for 60 s. The control experiment (no NPs present) showed that the marker is strictly confined to the endosomes, Fig. 3 (left). In contrast, the marker had diffused throughout the cell after irradiation in the presence of NPs, Fig. 3 (right).

Discussion

Our results show that cellular uptake of gold NPs by endocytosis and subsequent irradiation at the plasmon resonance wavelength can have various effects on the cells. Irradiation conditions can be adjusted to produce a very diverse range of outcomes, from cell death to selective rupture of particle-loaded organelles while preserving high cell viability.

In previous publications which observed cell death under similar conditions the effect was ascribed to photothermal heating of the cell.[3,4] However, a simple estimate of the steady-state temperature increase expected under our irradiation conditions using

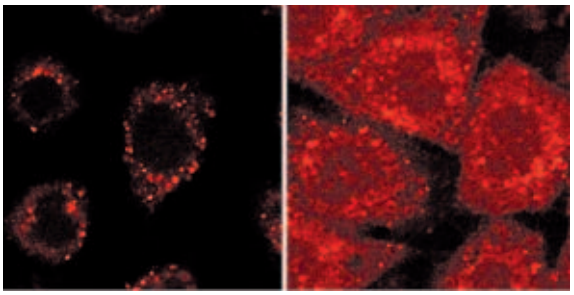

Figure 3. Confocal fluorescence images of cells without (left) and with (right) NPs, incubated with rhodamine-labelled dextran, after 60 s of irradiation at 37 W/cm^2.

Fourier's law yields only small values. Even for our highest irradiation intensity (106 W/cm^2), a temperature increase of only 0.16°C is expected at the surface of an endosome with a diameter of 300 nm, loaded with 1000 NPs. This clearly is not sufficient to provoke disruption of the organelles or cell death. Thus, the effects of irradiation on NP-loaded endosomes and whole cells can not be due to a photothermal mechanism. This is also supported by our observation that the effect of irradiation is more pronounced for endosomes with less NPs, whereas a positive correlation between the number of internalised NPs and irradiation effects would be expected for a photothermal mechanism.

At present, the mechanism leading to endosome rupture and cell death is not known. It may involve photochemical processes, e.g. the creation of photoelectrons at the NP surface. At higher NP concentrations, such photoelectrons would be expected to be quenched more efficiently, which potentially could explain the experimentally observed anti-correlation between the number of NPs in an endosome and the effect of irradiation.

The results presented here are of great relevance for the fields of drug delivery and intracellular labelling. It may be possible to develop methods for highly controlled intracellular delivery using NPs carrying the active compound and functional groups for targeting specific cells or cell organelles. Endocytosis of such NPs would place them into the cell, but still away from the target site; their activation could then be locally and temporally controlled by irradiation-induced release from the endosome.

Conclusions

Endocytosed gold NPs can be released into the cell cytoplasm by mild laser irradiation which does not affect cell viability or morphology. Early endosomes are most susceptible to laser irradiation, but also late endosomes and multivesicular bodies can be ruptured with sufficient irradiation time. Although the mechanism for membrane rupture (and cell death upon intense irradiation) is not known, photothermal effects can be ruled out.

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References

1. A. P. Alivisatos, *Nature Biotechnology*, **22**, 47 (2004).
2. X. Wu, *et al*., *Nature Biotechnology*, **21**, 41 (2003).
3. I. H. El Sayed, *et al*., *Cancer Letters*, **239**, 129 (2006).
4. X. Huang, *et al*., *Journal of the American Chemical Society*, **128**, 2115 (2006).
5. H. Joshi, P. S. Shirude, V. Bansal, K. N. Ganesh and M. Sastry, *Journal of Physical Chemistry B*, **108**, 11535 (2004).