Original Article

Cytotoxicity of nanoparticles independent from oxidative stress

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ABSTRACT — The use of nano-sized materials offers exciting new options in technical and medical applications. On the other hand, adverse effects on cells have been reported and may limit their use. In addition to physico-chemical parameters such as contamination with toxic elements, fibrous structure and high surface charge, the generation of radical species was identified as key mechanism for cytotoxic action of nanoparticles. The cytotoxic potential of nanoparticles in the absence of radical generation is less well investigated. This study aims to investigate the size-dependent effect of carboxyl polystyrene particles on cells to identify potential adverse effects of these particles. Particles were characterized in different solutions to assess the influence of the medium on size and surface charge. Viability, membrane integrity, apoptosis, proliferation and generation of oxidative stress were investigated. In addition the intracellular localization of the particles was recorded. 20 nm polystyrene particles induced cellular damage by induction of apoptosis and necrosis. These particles generated radicals to the same degree as larger polystyrene particles. Particles were taken up into endosomes and lysosomes in a size-dependent manner. Protein containing solutions led to increases in particle size, decreased cytotoxicity and reduced cellular uptake. It can be concluded that even in the absence of high surface reactivity and not linked to the generation of radicals nano-sized particles may cause cell damage. The mechanism of this damage includes apoptosis, necrosis and inhibition of proliferation.

Key words: Nanotoxicology, Cytotoxicity, Nanoparticles, Subcellular localization, Apoptosis

INTRODUCTION

Nano-sized materials are increasingly used in technical and medical applications and it is expected that in the future the use of nanotechnology will further increase. On the other hand there is concern that this technology may also present a risk for health of users and producers of these particles. This concern is based on the finding that nano-sized particles differ from bulk materials regarding many physicochemical and biological properties and that several nanoparticles, which could be used in medical applications, act cytotoxic (Lewinski *et al.*, 2008). Plain nano-sized gold particles for instance show size-dependent organ distribution and cytotoxic effects (Pan *et al.*, 2007; Semmler-Behnke *et al.*, 2008). In addition to

size the coating has a pronounced influence on biocompatibility: This effect has been studied systematically for poly(amidoamine) polymers by Boas *et al.* (2006). Systematic studies, however, are difficult because changes in surface charge are often accompanied by changes in size and/or hydrophobicity and therefore a combined effect is evaluated. In addition, often the effect of surface charges can be evaluated in single particles because aggregation occurs.

Polystyrene particles are widely used as model and reference particles to study cellular uptake (Panyam *et al.*, 2002; Rejman *et al.*, 2004), as well as for *in-vitro* (Geiser *et al.*, 2005; Olivier *et al.*, 2003; Papageorgiou *et al.*, 2007) and for *in-vivo* studies (Brown *et al.*, 2001; Demoy *et al.*, 1997; Fernandez-Urrusuno *et al.*, 1995; Gibaud *et*

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al., 1996; Kanai *et al.*, 1996; Kato *et al.*, 2003; Nemmar *et al.*, 2002; Silva *et al.*, 2005; Yacobi *et al.*, 2008). Polystyrene is not cytotoxic and the spheres are commercially available in reproducible sizes and surface charges. For the assessment of size it is fortunate that all particles independent of their sizes possess the same surface charge. When working with nanoparticles the interaction with proteins especially from blood or from epithelial mucous is important. The change of particle characteristics from suspension in distilled water or in buffer to suspension in cell culture medium with serum albumin is only recently been taken into account. The binding of proteins to nanoparticles called 'protein corona' determines the biological effects of the particles to a great extent. Surprisingly, even particles of different sizes made from the same material and having the same surface charge display a corona with different composition (Lundqvist *et al.*, 2008).

The mechanism of cell damage of nanoparticles generally is explained by the generation of radicals through a large catalytic surface (Nel *et al.*, 2006). Several types of nanoparticles like for instance metal oxides generate oxidative stress in cells (Limbach *et al.*, 2007; Park *et al.*, 2008). In many cases the generation of oxidative stress can be demonstrated also in the absence of cells (Xia *et al.*, 2006). Polystyrene particles, with the exception of strongly cationic ones, allow the evaluation of cell damage in the absence of oxidative stress because these particles appear to induce radicals only in professional phagocytes such as macrophages but not in the absence of cells (abiotic condition, Xia *et al.*, 2006) and in non-phagocytotic cells (Swain *et al.*, 2004).

This study was conducted to find out if polystyrene particles can induce cell damage independent from generation of oxidative stress. Further, we examined i) at which size cytotoxicity is observed, ii) how the presence of serum proteins changed the cytotoxic effect, iii) which mechanisms of the cell damage were involved, iv) if cytotoxicity is correlated to the ability to produce radicals and v) if cytotoxicity was correlated to uptake into specific organelles.

MATERIALS AND METHODS

Particles

Carboxyl polystyrene latex beads in sizes of 20-40-60-140-200-500 nm, 20 nm carboxylate-modified polystyrene latex beads and 200 nm amidine polystyrene latex beads were obtained from Invitrogen. For cellular uptake studies yellow-green (505/515) fluorescently labelled carboxyl PS particles in sizes of 20, 40 and 200 nm and red (580/605) fluorescent-

ly labeled carboxyl PS particles in nominal sizes of 20, 40 and 200 nm (FluoSpheres, Invitrogen, Lofer, Austria) were used. 200 μ M H₂O₂ and 500 μ g/ml quartz particles (BCR66, size standard particles, 1 μ m, Duke Scientific, Fremont, CA, USA) served as positive controls.

Physico-chemical characterization of particles was performed by dynamic light scattering using a Malvern Zetasizer 3000 HS. For the measurements particles were diluted with the respective medium to concentrations less than 1 mg/ml and sonicated for 20 min. After equilibration of the sample solution to 25°C size and zeta potential were measured at 633 nm and a detection angle of 90°. For sample analysis nonnegative least-squares (NNLS) software was used.

Cell culture

The human endothelial cell line EAhy926 (kind gift from Dr. C.J. Edgell) was cultured in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin. Cells were cultured at 37°C in a humid 95% air/5%CO₂ atmosphere. Cells were pre-cultured in this medium 24 hr before treatment.

Cells were exposed to the particles in concentrations of 500-250-125-62.5 and 31.25 μ g/ml. The particles were suspended in DMEM with 0%, 1%, 5% and 10% FBS to evaluate the influence of protein on the biological effect. All particle suspensions were sonicated for 20 min before addition to the cells. Exposures were performed at 37°C in a 95% air/5%CO₂ atmosphere and two time points (4 hr and 24 hr) were evaluated.

Screening assays

For the determination of ATP-content the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Mannheim, Germany) was used according to the manufacturer's instruction and luminescence was read on a Lumistar (BMG Labtech, Offenburg, Germany).

For Neutral Red uptake the assays kit Neutral red for 1,200 tests without microplates (Aniara Corporation, Mason, OH, USA), for formazan bio-reduction CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega) and for protein content (sulforhodamine B staining) the *Invitro* Toxicology assay kit Sulforhodamine B Based (TOX-6 assay, Sigma-Aldrich, Wien, Austria) was used. Absorbance was read at 690 nm for Neutral Red uptake, at 490 nm for formazan bio-reduction and at 565 nm for the sulforhodamine B staining on a SPECTRA MAX plus 384 (Molecular Devices).

Protein content (leucin uptake)

1 μ Ci methyl-³H Leucine (GE Healthcare, Wien, Austria) was added 4 hr prior to cell harvesting with a 96 filter plate FilterMate Cell Harvester (Perkin Elmer) on filter plates (Unifilter-96 GF/C white 96 well plates + foil from, Perkin Elmer). Filter plates were air-dried overnight and covered with sealing foil at the backside of the plate. 20 μ l Scintillation Cocktail (Betaplate Scint, Perkin Elmer) was added to each well and the plates covered with a clear sealing foil to prevent drying up. Scintillation was read at a Wallac Trilux Beta counter (Perkin Elmer).

Calcein AM/ Ethidium homodimer (EthD-1) staining

Cells were seeded in chamber slides (BD-Falcon) and cultured 24 hr prior to the actual experiment. After medium change PS particles were added and cells cultured for additional 24 hr. Medium was discarded and walls removed from the chamber slide. LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Invitrogen) at 5 μ M concentration was used and cells were incubated for 30 min at RT in the dark. After washing with PBS, pictures were taken with a confocal laser scanning microscope LSM510 Meta (Zeiss, Wien, Austria). Images were acquired at 488 nm excitation wavelength using a BP 505-530 nm band-pass detection filter for the green channel (Calcein AM) and 543 nm excitation wavelength in conjunction with LP 560 for the red channel (EthD-1). For quantification on a fluorometer cells were seeded in plates and fluorescence was read at Ex/Em wavelength of 485/520 nm for Calcein AM and 544/612 nm for EthD-1 staining at a fluorometer (FLUOstar Optima, BMG Labtech).

Proliferation*Thymidine uptake*

1 μ Ci methyl-³H Thymidine (GE Healthcare, Wien, Austria) was added 4 hr prior to cell harvesting with a 96 filter plate FilterMate Cell Harvester (Perkin Elmer) on filter plates (Unifilter-96 GF/C white 96 well plates + foil from, Perkin Elmer). Filter plates were air-dried overnight and covered with sealing foil at the backside of the plate. 20 μ l Scintillation Cocktail (Betaplate Scint, Perkin Elmer) was added to each well and the plates covered with a clear sealing foil to prevent drying up. Scintillation was read at a Wallac Trilux Beta counter (Perkin Elmer).

BrdU-uptake

Cells were seeded in chamber slides (BD-Falcon) and cultured 24 hr prior to the actual experiment. Cells were treated for 24 hr with PS particles in total; for the last 4

hr 10 μ M BrdU (Roche Diagnostics, Wien, Austria) was added. Medium was discarded and walls removed from the chamber slide. Cells were fixed for 10 min in absolute methanol at -20°C and for 3 min in acetone. Slides were washed three times with PBS at RT and subsequently incubated with Anti-BrdU AlexaFluor488-labelled antibody (mouse, 1:40, Invitrogen) for 30 min. Slides were rinsed again 3 x 5 min in PBS and nuclei counterstained with 0.5 μ g/ml propidium iodide (Invitrogen) for 30 min at RT. Slides were viewed at the confocal laser scanning microscope LSM510 Meta (Zeiss). Images were acquired at 488 nm excitation wavelength using a BP 505-530 nm band-pass detection filter for the green channel (BrdU) and 543 nm excitation wavelength in conjunction with LP 560 for the red channel (propidium iodide).

Apoptosis*Caspase 3/7 activation*

Activation was determined using Caspase-Glo 3/7 Assay (Promega) according to the instruction manual and luminescence read on a Lumistar (BMG Labtech).

TUNEL

For the detection of double-strand breaks the APO-BrdUTM TUNEL Assay Kit (Invitrogen) was used according to the instructions in the manual. Cells were treated in chamber slides. Slides were viewed at the confocal laser scanning microscope LSM510 Meta (Zeiss). Settings were the same as for BrdU-uptake.

YoPro-1/propidium iodide staining

The Vybrant Apoptosis Assay Kit #4 (Invitrogen) composed of 100 μ l YO-PRO-1 and 1.5 mM propidium iodide was used according to the instructions in the manual. Cells were treated in chamber slides and viewed at the confocal laser scanning microscope LSM510 Meta (Zeiss). Settings were the same as for BrdU-uptake.

Detection of cleaved cytokeratin 18

Cells were treated in chamber slides for microscopy. After treatment, cells were washed with PBS. After fixation with methanol at -20°C for 30 min and three rinses in PBS, cells were incubated with M30 Cyto Death ALX-804-590 antibody (Eubio, Wien, Austria, 1:100) for 30 min at RT. For negative controls mouse IgG (Linaris Biologische Produkte, Wertheim-Bettingen, Germany, 1:1000) was used. Subsequently, slides were rinsed in PBS (3 x 10 min at RT) and counterstained with propidium iodide (5 μ g/ml, 10 min at 37°C in AB-Diluent from Dako) and rinsed again in PBS (3 x 10 min at RT). Slides

were viewed after mounting in Mounting Medium (Dako Diagnostica, Wien, Austria) and viewed at the confocal laser scanning microscope LSM510 Meta (Zeiss). Settings were the same as for BrdU-uptake.

Membrane integrity

Lactate dehydrogenase release

The CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Mannheim, Germany) was used according to the instructions given by the producer and fluorescence recorded with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. After subtraction of the blank value the average fluorescence from the samples was normalized to the maximum LDH release (lysis control).

Adenylate kinase release

The detection was carried out using ToxiLight BioAssay Kit (Lonza, Köln, Germany) according to the instructions given by the producer. Luminescence was read after 5 min on a luminometer (Lumistar, BMG Labtech).

Propidium iodide uptake

Cells were treated in chamber slides. After treatment, 10 µl 1 mg/ml propidium iodide (Invitrogen) (final concentration 5 µg/ml) was added to the culture medium for 10 min. Walls from the chamber slides were removed and the slides rinsed in PBS (3 x 10 min at RT), mounted in Mounting Medium (Dako Diagnostica) and viewed at the confocal laser scanning microscope LSM510 Meta (Zeiss). Images were acquired with excitation at 543 nm and detection with a LP 560 filter.

Oxidative stress

Cells were treated with subtoxic concentrations of particles and positive control (hydrogen peroxide, H₂O₂) to exclude an influence of the signal by cell loss.

Oxidation of dehydroethidium

Generation of radicals was evaluated after 4 hr and 24 hr in the presence of 10 µM dihydroethidium (DHE) (Invitrogen) and PS particles or 50-200 µM H₂O₂ in cell culture medium. Cells were either treated in chamber slides for microscopy or in cell culture plates for evaluation in the fluorometer. After the treatment cells were washed with PBS. Walls from the chamber slides were removed, mounted in Mounting Medium (Dako Diagnostica) and viewed by a fluorescence microscope. Fluorescence in the plates was read with 544 nm excitation and 612 nm emission at a fluorometer.

Oxidation of dichlorodihydrofluorescein

Cells were grown for 24 hr in cell culture plates and loaded with 10 µM 2,7-dichlorodihydrofluorescein diacetate (Invitrogen) in medium for 30 min at 37°C. Subsequently, cells were rinsed and cultured for 4 hr and 24 hr with PS particles or 50-200 µM H₂O₂ as positive control. Fluorescence was read with 485 nm excitation and 520 nm emission at a fluorometer.

Co-localization studies with organelles

For co-localization studies cells were seeded in chamber slides and incubated with 25 µg/ml carboxyl Fluospheres in medium for 30 min and 24 hr. After this incubation, cells were labeled with the organelle trackers, which were all obtained from Invitrogen.

For labeling of the endoplasmic reticulum cells were washed twice first with medium + 10% FBS and secondly with HBSS and incubated with 1 µM ER-Tracker Red (Bodipy TR glibenclamide) in HBSS for 20 min at 37°C. Subsequently, cells were washed with HBSS and viewed under the microscope. For labeling of mitochondria, cells were rinsed twice in medium + 10% FBS and incubated with 200 nM MitoTracker DeepRed 633 in complete medium for 20 min at 37°C. After washing with complete medium cells were viewed.

For labeling of lysosomes cells were incubated with 50 nM Lyso Tracker Green DND 26 in complete medium. After 5 min of incubation at 37°C the cells were viewed under the microscope.

As marker for endosomes the receptor-mediated uptake of labeled transferrin as marker for early and recycling endosomes (Peters *et al.*, 2001) was used. 25 µg/ml Transferrin Conjugates Alexa Fluor 488 was added together with the particles and incubated for 30 min at 37°C. Subsequently, the cells were rinsed three times in PBS, fixed in 4% paraformaldehyde, rinsed again in PBS and viewed.

Labeling of microtubules was performed by incubation of cells in 100 nM TubulinTracker Green Reagent for Live-Cell Tubulin Labeling (OregonGreen 488Taxol, bis-acetate) for 30 min at 37°C. After three rinses in HBSS cells were viewed under the microscope.

Slides were viewed at the confocal laser scanning microscope LSM510 Meta (Zeiss). Images for co-localization of Fluospheres with Lyso Tracker Green DND and ER-Tracker Red were acquired at 488 nm excitation wavelength using a BP 505-530 nm band-pass detection filter for the green channel (Lyso Tracker, YG Fluospheres) and 543 nm excitation wavelength in conjunction with a LP 560 nm long pass filter for the red channel (ER-Tracker Red, red Fluospheres). Co-localization of YG

Fluospheres with MitoTracker DeepRed 633 was viewed with 488 nm excitation and BP 505-530 nm detection for the Fluospheres and excitation at 633 nm in conjunction with detection at BP 679-754 nm for the MitoTracker.

For co-localization of red Fluospheres with Transferin Alexa Fluor 488 and with Tubulintracker Green Reagent images were acquired at 488 nm and 543 nm excitation in conjunction with BP 505-530 nm and LP 560 nm filter, respectively.

RESULTS

Physico-chemical properties

Size and surface charge of the particles in the dynamic light scattering differed according to the medium in which they were suspended. In distilled water dispersion was best and smallest sizes were recorded. In medium + 5% FBS size for most particles was highest and surface charge was reduced. No difference was seen between particles in medium + 5% FBS and in medium + 10% FBS. The negative surface charge of carboxyl PS particles decreased with increasing concentrations of FBS in the medium. Amidine PS particles lost positive surface potential already in medium without FBS. For overview see Table 1.

Cytotoxicity screening

For screening and mode of action studies the EAhy926 cell line was used. This cell line is derived from human umbilical vein endothelial cells and retains endothelial features to a high degree (Edgell *et al.*, 1983; Unger *et al.*, 2002). 20 nm carboxyl and carboxylate-modified PS particles, which are more hydrophilic according to the

producer, showed similar cytotoxic action in the screening assays and the fluorescently labeled carboxyl PS particles showed the same cytotoxicity profile as the not labeled ones. All other particles, carboxyl PS particles ≥ 40 nm and amidine PS particles, did not show indication for cytotoxicity.

In the EAhy926 endothelial cell line the sensitivity of the different screening assays was compared. For comparison the half maximal inhibitory concentration (IC_{50}) as measure for the effectiveness of a compound in inhibiting biological or biochemical process was chosen. The IC_{50} of the ATP-content was at a concentration of 125 $\mu\text{g/ml}$ 20 nm carboxyl PS (Fig. 1a). IC_{50} values in Neutral Red uptake, formazan bioreduction, sulforhodamine-B staining and leucine uptake assays ranged between 120 and 150 $\mu\text{g/ml}$ upon this treatment. Results from these spectroscopic and radiometric screening assays were consistent with microscopical evaluation of calcein AM/ethidium homodimer staining (Fig. 1c). Table 2 shows that all viability screening assays used in this study produced very similar results.

The presence of 1% FBS in the medium reduced the cytotoxic effect of 20 nm carboxyl PS particles only slightly: in the formazan bioreduction assay for instance IC_{50} values increased from 150 $\mu\text{g/ml}$ in medium without FBS to 250 $\mu\text{g/ml}$ in medium with 1% FBS. At 5% and 10% FBS in the medium size of the particles increased (see Table 1) and cytotoxicity decreased markedly. 20 nm carboxyl PS particles at 500 $\mu\text{g/ml}$ decreased viability to 70% and 80%, in medium with 5% and 10% FBS, respectively.

Table 1. Hydrodynamic diameter and sizes in different media of PS particles used in this study

Functionalization, size	Size in distilled water (nm)	Size (DMEM, 0% FBS, nm)	Size (DMEM, 1% FBS, nm)	Size (DMEM, 5% FBS, nm)	zeta-Pot. (DMEM, 0% FBS, mV)	zeta-Pot. (DMEM, 1% FBS, mV)	zeta-Pot. (DMEM, 5% FBS, mV)
Carboxyl modified, 20 nm	24	35/110	40/180	45/120	-30.0	-15.9	-10.3
Carboxyl, 20 nm	26	42/300*	35/250*	77	-41.2	-13.6	-11.6
Carboxyl, 40 nm	34	45/105*	40/176*	132	-36.6	-13.6	-11.1
Carboxyl, 60 nm	62	56/142*	81/210*	123	-35.1	-12.7	-11.8
Carboxyl, 80 nm	82	81	144	114	-33.0	-13.6	-10.7
Carboxyl, 100 nm	93	158	223	184	-36.8	-10.7	-9.5
Carboxyl, 200 nm	160	230	227	263	-36.6	-10.1	-10
Amidine, 200 nm	220	200/1922*	320/1050*	350/1150*	-6.2	-11.3	-10.1

*: two peaks in the size distribution recorded.

Abbreviations: FBS: fetal bovine serum; DMEM: Dulbecco's modified Eagle's medium.

Fig. 1. Effect of 20 nm carboxyl PS particles after 24 hr on EAh926 cells regarding viability with ATP content (a) as parameter and in calcein AM/ethidium staining (c) and regarding apoptosis in terms of caspase 3/7 activation (b) and in YO-PRO-1/propidium iodide co-staining (d). ATP content starts to decline at 100 µg/ml (a); at this concentration still more vital cells (green) than dead cells (red) are seen (c). Apoptosis in the caspase 3/7 activation is significantly increased at 100 µg/ml (b). Similar amounts of apoptotic cells (uptake of YO-PRO-1, green), of secondary apoptotic cells (uptake of both dyes, yellow) and of necrotic cells (incorporation of propidium iodide, red) are seen (d).

Table 2. Sensitivity of screening assays for toxicity of 20 nm carboxyl PS spheres in the cell line EAhy926 after 24 hr of exposure. For comparison IC₅₀ values are given.

Name of the assay	Principle	IC ₅₀ (μg/ml)
Formazan bioreduction	Metabolic activity	150
ATP content	Metabolic activity	125
Neutral Red uptake	Metabolic activity	120
Sulforhodamine B staining	Protein content	120
Leucin uptake	Protein content	160

Mode of action

Apoptosis

Caspase 3/7 activation as indication for apoptosis was significantly increased at 100 μg/ml of 20 nm carboxyl PS particles (Fig. 1b). At this concentration similar amounts of apoptotic cells (uptake of YO-PRO-1, of secondary apoptotic cells (uptake of both dyes) and of necrotic cells (incorporation of propidium iodide) are seen (Fig. 1d). In the TUNEL staining, in contrast, only a minority of cells were labeled at 100 μg/ml and the fraction of TUNEL positive cells was higher at 200 μg/ml. The fraction of cells showing fragmented cytokeratin 18 was highest at 100 μg/ml, but the signal in general was low; at the maximum 50% of the cells were stained.

Membrane damage

LDH-release was significantly increased at 100 μg/ml of 20 nm carboxyl PS particles (Fig. 2a). The release of adenylate kinase was not suited for the detection of membrane damage by carboxyl PS particles because no increase was recorded in the presence of these particles. Cells with disintegrated plasma membrane integrated propidium iodide into the DNA: intensely stained nuclei were seen upon exposure to 100 μg/ml 20 nm carboxyl PS particles (Fig. 2d). Propidium iodide positive cells contained a higher amount of fluorescently labeled (green) 20 nm carboxyl PS particles.

By the use of fluorescently labeled particles the correlation of uptake and membrane damage was studied: cells with a higher particle load in general showed propidium iodide uptake.

Proliferation

³H-thymidine uptake was significantly reduced already at ≥ 50 μg/ml 20 nm carboxyl PS particles (Fig. 2b). The reduction was confirmed by detection of BrdU-uptake in

the presence and absence of carboxyl PS. At a concentration of 100 μg/ml only few cells with BrdU-uptake (d, green) were identified (Fig. 2d).

Oxidative stress

Cells were incubated with 20 nm and 200 nm carboxyl PS particles at sub-toxic concentrations of 0-100 μg/ml. Absolute signals in cells were markedly higher after 24 hr than after 4 hr exposure but relative changes were similar. Incubation with dihydroethidium showed that few nuclei were stained in the controls (Fig. 3a) and particles of both sizes produced slightly higher but roughly similar increase in the incorporation of ethidium in the nuclei (Figs. 3b and c). In the positive control (100 μM H₂O₂) all nuclei were stained (Fig. 3d). Detection of oxidative stress by oxidation of dichlorodihydrofluorescein produced similar results: both particles generated more fluorescence than untreated controls and much less than the positive control (200 μM H₂O₂, Fig. 3e).

Cell uptake and localization

Uptake of PS particles was highest for 20 nm and lowest for 200 nm; 500 nm particles were not taken up. Uptake rates were higher after 24 hr than after 30 min. To identify intracellular distribution of the particles and to find out if the smallest PS particles can penetrate into nucleus, mitochondria and endoplasmic reticulum a panel of organelle markers was used. Co-labelling (Figs. 4a and b) of 20, 40 and 200 nm PS spheres with transferrin-Alexa 488 as marker for early and recycling endosomes (Peters *et al.*, 2001) and with LysoTracker as marker for late endosomes and lysosomes was seen. No colocalization, however, of these particles with markers for endoplasmic reticulum and for mitochondria was recorded (Figs. 4c and d). 20 nm and 40 nm particles were associated to TubulinTracker positive structures (Figs. 4e and f). In time series it appeared that the particles were transported along these microtubules. Occasionally, 20 nm PS particles were seen in the nucleus.

DISCUSSION

This study on the cytotoxic mechanism of polystyrene particles revealed no difference in the generation of oxidative stress between 200 nm and 20 nm carboxyl PS particles 20 nm: carboxyl PS spheres induce cell damage while 200 nm particles did not. Carboxyl PS particles of ≥ 40 nm were not cytotoxic. 20 nm refers to the size of the particles in distilled water. In culture medium the PS particles were considerably larger. Earlier conclusions on the toxicity of nanoparticles were based on data originating

Fig. 2. Effect of 20 nm carboxyl PS particles after 24 hr on EAhy926 cells regarding membrane damage demonstrated by lactate dehydrogenase release (a) and by propidium iodide uptake (c) and regarding proliferation assessed by ^3H -thymidine uptake (b) and by BrdU-uptake (d).

At 100 $\mu\text{g/ml}$ 20 nm Carboxyl PS particles LDH-release is significantly increased. Cells with disintegrated plasma membrane integrated propidium iodide (red) into the DNA. Propidium iodide positive cells contained a higher amount of fluorescently labeled (green) 20 nm carboxyl PS particles.

^3H -thymidine uptake (b) was significantly reduced already at ≥ 50 $\mu\text{g/ml}$. At a concentration of 100 $\mu\text{g/ml}$ only few cells with BrdU-uptake (d, green) are seen.

Cytotoxicity of nanoparticles

Fig. 3. Generation of oxidative species in EAhy926 cells after exposure to 20 nm and 200 nm carboxyl PS particles assessed by oxidation of dihydroethidium (DHE) and by dichlorodihydrofluorescein (DCF). Cells treated with 50 μg/ml of 20 nm and 200 nm PS particles for 24 hr (b and c) showed slightly more positive (red) nuclei than the untreated controls (a) and much less than in the positive (H₂O₂-treated) control (d). e: Production of DCF is slightly enhanced in cells treated with PS particles but markedly less than in the positive (H₂O₂-treated) control.

from dry particles or from suspensions in distilled water. More recently, changes induced by physiological media such as loss of surface charge and changes in size/aggregation were identified: particles such as carbon black,

metal oxides and carbon nanotubes take advantage from a protein containing dispersion solution because their average size decreases (Bihari *et al.*, 2008; Sager *et al.*, 2007). These particles are more toxic in protein containing solu-

Fig. 4. Co-localization of organelle markers with 20 nm and 40 nm fluorescently labeled carboxyl PS particles in EAhy926 cells. Carboxyl PS particles (red) of both sizes (40 nm particles shown) in part co-localize to transferrin-Alexa 488 positive structures (green) (a) and to lysosomes identified with LysoTracker (b). Co-localization is shown in yellow by merging of the red and green channel. Even the smaller 20 nm particles, however, were not seen in mitochondria or in the endoplasmic reticulum (c, d). Particles of both sizes (40 nm particles are shown) are seen in the vicinity of microtubules identified by Tubulin tracker green in the confocal image (e) and in the 3D reconstruction (f).

tions because the size of the aggregates is reduced. For PS particles an increase in toxicity is not seen because the average size increased in physiological solutions.

Increase in the size of dispersed PS particles most probably was due to aggregation and not caused by coating of the particles with protein. Coating with protein called corona formation and concomitant increase in the hydrodynamic diameter is seen for PS particles due to

their hydrophobicity (Lundqvist *et al.*, 2008). Bihari *et al.* (2008) reported that increases in size caused by corona formation with serum albumin typically causes increases in dynamic light scattering of 14.5 nm corresponding to 2 molecules of serum albumin. On the other hand, neutralization of the surface charge by salts or proteins favors the formation of aggregates. This effect caused larger increases than coating: sizes of 60 nm amine and amidine

PS particles are 5-6 times larger in medium with FBS than in distilled water (Xia *et al.*, 2006). 20 nm particles in our study were 2-4 times larger in physiological solutions suggesting the presence of aggregates.

For the assessment of the mode of cytotoxic action various assays were used. This was done firstly because some assays could have a lower sensitivity and therefore the effect would be obvious only at higher concentrations of particles. Secondly, interactions of nanoparticles with assay systems have been reported (Wörle-Knirsch *et al.*, 2006) and should be identified. In general, no marked differences in the sensitivities of the assays were seen and interaction with the assay was rare and seen only for the luminescent detection of adenylate kinase release. This indicates that interferences can also occur in the absence of color and reductive or oxidative activity of the tested particles.

Not cationic PS microparticles or nanoparticles generally are regarded as non-toxic. Down to sizes of 60 nm PS particles did not induce cell damage in not phagocytotic cells and *in-vivo* (Olivier *et al.*, 2003; Schöler *et al.*, 2000; Silva *et al.*, 2005). In our study we also found that 60 nm PS particles did not cause cell damage. The 20 nm PS particles, however, acted cytotoxic. Consistent with earlier studies (Swain *et al.*, 2004) we did not detect prominent generation of oxidative stress. Although the level of oxidative species in cells treated with PS particles was higher than in un-treated cells, there was no significant difference between the generation by the cytotoxic 20 nm PS particles and the non-cytotoxic 200 nm PS particles.

Caspase activation and lactate dehydrogenase release were noted at concentrations lower than decrease in viability. We, therefore, conclude that disturbance of membrane integrity may be one mechanism for the cytotoxic action in PS nanoparticles.

Functionalized gold particles and nanotubes are able to cause holes in the plasma membrane by lipid peroxidation (Panessa-Warren *et al.*, 2008). Polyamine-terminated dendrimers cause holes or enlarge holes in plasma membranes; the formation of such holes appears to be linked to cellular uptake of particles (Leroueil *et al.*, 2007). For the uptake of polystyrene particles into erythrocytes adhesive and diffusive mechanism were proposed (Geiser *et al.*, 2005). PS particles can bind a variety of proteins and, therefore, an interaction of the PS particles and binding of membrane proteins is likely. As plasma membrane contains attached and integrated proteins (Nelson and Cox, 2004) accounting for about 50% of the total plasma membrane this binding may disrupt the plasma membrane integrity. We observed that cells with membrane damage,

identified by propidium iodide staining, contained more particles than cells with integer membrane.

It has been suspected that cellular uptake correlates with cell damage (Hong *et al.*, 2006), which is consistent with our observation that 20 nm PS particles, which were cytotoxic, were taken up to a higher degree than 200 nm ones, which were not cytotoxic. PS particles of sizes ≥ 200 nm were taken up to a low degree as shown in other studies. Kato *et al.* (2003) reported that un-coated 240 nm PS particles are taken up by macrophages but not by endothelial cells. 80 nm and 200 nm PS particles are also taken up by erythrocytes whereas 1 μ m large particles are not (Geiser *et al.*, 2005). For fibroblasts PS particle uptake up to 500 nm has been reported (Olivier *et al.*, 2003). Despite a lower uptake rate of 200 nm PS particles compared to 20 nm particles in our study the subcellular distribution of both particles was essentially similar and the majority of the particles was seen in the endosomal compartment, which is consistent with the localization of other PS particles reported by other groups (Panyam *et al.*, 2002). We did not see any co-localization with markers for ER and mitochondria for particles of both sizes.

The access to other organelles is size-dependent: Not targeted 9 nm in contrast to 30 nm gold nanoparticles have been detected in the nucleus (Berry *et al.*, 2007). Targeted gold nanoparticles, however, transverse the nuclear core complex up to 39 nm of size (Panté and Kann, 2002) and polyimide-coated core-shell silica nanoparticles in non-cytotoxic concentrations were seen in the nucleus even at a size of 66 nm (Fuller *et al.*, 2008). We observed 20 nm sized PS particles in the nucleus only rarely. For the endoplasmic reticulum the size exclusion is 13 nm (Chang *et al.*, 2008) and for mitochondria 3 nm (Salnikov *et al.*, 2007) but particles causing cell damage by generation of oxidative stress are seen in mitochondria until larger sizes like fine ($< 2.5 \mu$ m) and ultrafine ($< 0.15 \mu$ m) ambient particles, which are seen inside structurally damaged mitochondria (Li *et al.*, 2003). In view of these dimensions it is not surprising that the PS particles, which were tested at sub-toxic concentrations, were seen neither in mitochondria nor in the endoplasmic reticulum. Due to technical limitation of confocal microscopy the presence of single PS particles in these organelles cannot be excluded.

It can be concluded that nanoparticles can also damage cells by mechanisms not correlated to the generation of oxidative stress. These mechanisms may include induction of apoptotic and necrotic cell death and decreased proliferation. The pattern is not unique for PS particles but resembles for instance the one described for un-loaded chitosan particles (Qi *et al.*, 2005). The signalling cas-

cares, which are involved in these processes, have to be studied in more detail.

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