Neurochemical Research (2019) 44:1330–1345
https://doi.org/10.1007/s11064-018-2598-4

ORIGINAL PAPER

How to Study the Uptake and Toxicity of Nanoparticles in Cultured Brain Cells: The Dos and Don't Forgets

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Received: 15 March 2018 / Revised: 9 July 2018 / Accepted: 18 July 2018 / Published online: 7 August 2018

Abstract

Due to their exciting properties, engineered nanoparticles have obtained substantial attention over the last two decades. As many types of nanoparticles are already used for technical and biomedical applications, the chances that cells in the brain will encounter nanoparticles have strongly increased. To test for potential consequences of an exposure of brain cells to engineered nanoparticles, cell culture models for different types of neural cells are frequently used. In this review article we will discuss experimental strategies and important controls that should be used to investigate the physicochemical properties of nanoparticles for the cell incubation conditions applied as well as for studies on the biocompatibility and the cellular uptake of nanoparticles in neural cells. The main focus of this article will be the interaction of cultured neural cells with iron oxide nanoparticles, but similar considerations are important for studying the consequences of an exposure of other types of cultured cells with other types of nanoparticles. Our article aims to improve the understanding of the special technical challenges of working with nanoparticles on cultured neural cells, to identify potential artifacts and to prevent misinterpretation of data on the potential adverse or beneficial consequences of a treatment of cultured cells with nanoparticles.

Keywords Adsorption · Biocompatibility · Cell cultures · Endocytosis · Experimental strategies · Iron oxide nanoparticles

Introduction

Nanoparticles (NPs) have obtained increasing attention over the last two decades as the use of engineered NPs for various technical approaches, industrial and commercial purposes as well as for biomedical applications has dramatically increased [1, 2]. NPs are defined as particles that have a size < 100 nm in at least two dimensions [3]. Due to their small size and the resulting high surface-to-volume ratio, NPs differ in some of their chemical and physical properties from non-nanoscale particles or the bulk material of the same composition [3, 4]. NPs can be composed of inorganic and/or organic materials, including carbon, metals, metal oxides, silica, liposomes and polymers [5–7]. The basic NP material, the core, may define functional physical features of NPs such as plasmonic, superparamagnetic or fluorescent properties [8].

In biological environments, most NPs are not colloidally stable and tend to agglomerate or even precipitate [8, 9]. A common approach to prevent agglomeration of NPs and to increase the biocompatibility of NPs in physiological environments is the encapsulation of the NP core with a coat that consists of organic coating materials such as lipids, proteins, or synthetic polymers [1, 10–12]. Such a coating of NPs provides additional opportunities to further functionalize the NPs, for example with fluorescent dyes, drugs, antibodies or other compounds which enable better monitoring of the NPs and improve their use for specific applications [1, 9, 12, 13]. Due to the high variability in core and coat, NPs have become exciting tools for many biomedical applications including drug delivery and bioimaging [14, 15].

The increased usage of NPs and the intended or unintended release of NPs in the environment [2, 16, 17] makes it essential to study potential adverse and toxic consequences of NPs on mammalian cells. A large number of studies deals with the interaction between NPs and peripheral mammalian cells [14, 16], but less is known on the consequences of a

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NP exposure of brain cells [18, 19]. As frequently stated it is difficult to compare results described in different articles [2, 20–24], as experimental conditions differ strongly between reported studies. In addition, each component of the test system applied (media components, buffer, temperature, incubation time, inhibitors, coatings etc.) may severely affect the interaction between NPs and cultured cells by modulating either the physicochemical properties of the NPs (such as shape, size and surface charge), the properties of the cell cultures investigated or both NP properties and cell behavior. The list of potential modulators of NP–cell interaction is long and, accordingly, standardized protocols to enable a reliable comparison of different studies and to allow a valid risk assessment of NPs have been requested [17, 20, 25, 26].

Among the different types of engineered NPs, iron oxide nanoparticles (IONPs) have gained a huge interest due to their use for several biomedical and clinical applications. IONPs possess superparamagnetic properties at room temperature (therefore they are also abbreviated as SPIONs), meaning that IONPs consist of single magnetic domains, which can be magnetized by the application of an external magnetic field while this magnetization is lost after removal of the external field [27]. Furthermore, IONPs have a high surface-to-volume ratio and are non-toxic to most cell systems [1, 9, 12, 19]. These properties make IONPs useful for a number of biomedical applications [1], e.g. as contrast agent for magnetic resonance imaging [28, 29], for cancer treatment by induced hyperthermia [30, 31], and as therapeutic agents for targeted drug delivery [9, 32, 33] across the blood–brain barrier [34, 35]. The combination of magnetic and fluorescent properties in fluorescently labeled IONPs has even further increased the potential of IONPs for biomedical applications [31, 36].

The promising use of IONPs for diagnostic and therapeutic applications with the potential of such IONPs to enter the brain, makes it mandatory to study the consequences of an exposure of brain cells to IONPs [37]. In particular, the uptake, intracellular trafficking and the fate of intracellular IONPs should be investigated as a potential release of large amounts of redox active iron in brain cells may cause oxidative stress and should be considered in the context of the reported disturbances of brain iron homeostasis for neurodegenerative diseases [38–40].

In this review article we will describe and discuss basic experimental problems and challenges of an exposure of cultured neural cells with NPs. Our main focus will be on experiments to study the interactions of cultured neural cells with dimercaptosuccinate (DMSA)-coated IONPs (DMSA-IONPs). We have included some new experimental data for C6 glioma cells to give some examples how the interaction between such IONPs and cell cultures can be studied. We will draw attention to problems and limitations which we have encountered during our NP studies on neural cell cultures and which we recommend to consider for designing and performing experiments in order to gain reliable, conclusive and reproducible results and to prevent misinterpretation of the cell data obtained after exposure of neural cells to NPs. Such aspects are also important to consider for studies on the interactions of other types of NPs with other types of cultured cells. This review will not be able to give an overview on the current knowledge on the interactions of nanoparticles with the different types of brain cells, on the mechanisms of cellular uptake and intracellular trafficking of NPs, on the trafficking of NPs through the blood–brain barrier into the brain or on the potential use of NPs for therapeutic applications. For such important topics the reader is referred to recent review articles that cover these topics in great detail [19, 41–45].

Testing for Physicochemical Properties of NPs for Incubation Conditions

Cell binding and uptake of NPs takes place in the minute time range and depends on various factors, including the size, the charge and the shape of the NPs [1, 16, 17, 46]. The variety of NPs and the complexity of biological material makes it difficult to directly correlate cell uptake efficiency and NP size, but cellular internalization of NPs larger than 25 nm appears to be slower than that of NPs of a diameter below 25 nm, although this trend is not applicable to ultrasmall particles of a diameter of 2–3 nm [16, 47]. Also the colloidal stability of NPs depends on the size, charge and surface chemistry of the NPs [9, 12, 16] and is strongly affected by media composition and experimental conditions such as pH, salt concentrations and protein availability [8, 16, 17, 48]. Thus, physiochemical characterization of NPs for the conditions applied is crucial to understand and interpret the results that will be obtained in cell culture studies.

Many groups have the knowledge to synthesize and characterize the NPs of interest and many companies offer NPs with special features which make them interesting for cell based studies. Nevertheless, for each type of NPs, for each new compound added to the medium, for each cell culture type, and for each other alteration of incubation conditions it should be investigated whether the new condition may affect the physicochemical properties and the colloidal stability of the NPs. Several techniques are frequently used to characterize IONPs [12] and other types of NPs [49, 50]. Although the determination of each recommended parameter may be difficult due to unavailability of the required equipment, insufficient experience or high costs, a certain knowledge on physicochemical properties and confirmation of the colloidal stability of NPs for the incubation conditions used is essential to avoid artifacts and to prevent misinterpretations.

It cannot be excluded that minor amounts of contaminants may still be present from the synthesis procedure in the NP preparation provided by the supplier or that components of the NPs may have been released from the NPs during storage or handling (for example iron ions from IONPs). Therefore, it is strongly recommended to test for the potential presence and potential side effects of such contaminants. For example, this can be done by removing the NPs from the dispersion by filtration or high speed centrifugation [51, 52] and by subsequent testing of appropriate amounts of the NP-free filtrate or supernatant in the biological test system used for NP experiments.

Uncoated IONPs are frequently not stable as colloidal dispersion in physiological media due to strong magnetic attraction between the particles, van der Waals forces and their high energy surface leading to the agglomeration of the NPs, which strongly influence their interaction with cells [12, 53]. To avoid this, coatings are applied to IONPs which are intended for the use in physiological conditions. In addition, physiochemical properties and the stability of available IONPs can differ substantially due to variations in the synthesis procedure, the selection of an appropriate coat and the large number of additional functionalizations of the IONPs [1, 12]. As physicochemical properties and colloidal stability of NPs may strongly differ between dispersions in different solvents and incubation media, the determination of such parameters is essential for NPs dispersed in the physiological medium that will be used for cell incubations. Table 1 shows the summary of the physicochemical characterization of DMSA-IONPs in a physiological incubation buffer (IB) that we have frequently used to study uptake of IONPs into cultured neural cells.

Test for Size, Elemental Composition and Successful Coating of NPs

The nanoscale size of NPs can be best determined by electron microscopy. In electron microscopy a beam of highly accelerated electrons with short wavelengths is used and allows a visualization with a resolution down to atomic level, which can give direct structural and size information on the NPs investigated [57, 58]. Here the elemental composition of the NP core is important as the electron density of the core material determines the intensity of the signals obtained [59]. Especially transmission electron microscopy (TEM) is the method of choice for size and shape determination of intact NPs or for analysis of sections of NPs that contain metals or metal oxides [12, 53, 57, 60], while many non-metal coating materials will not be detectable by TEM. Thus, it should be considered that depending on the material of core and coat of a given type of NPs, the core and/or the coat of the NPs may not be visible by TEM. Furthermore, is has to be taken into account that the preparation of the sample such as drying or contrasting can influence physicochemical properties of the NPs such as size, morphology and dispersity [53].

Regarding the size distribution, NP preparations can be monodisperse (uniform; composed of NPs of the same size, shape and mass) or polydisperse (non-uniform) depending on the synthesis and the coating used [57, 60, 61]. The polydispersity index (PDI; dispersity) gives a number on the heterogeneity of the NP population in a given NP dispersion [49, 57, 62]. In addition, NPs can have a homogeneous structure and/or can be agglomerated or aggregated depending on the dispersant [16, 17, 63]. Information on the particle size in dispersion can be obtained by dynamic light scattering (DLS) which determines the hydrodynamic diameter of individual or aggregated NPs which does not give the absolute size but rather the larger diameter of the NPs with the solvation layer of associated water molecules [57]. An alternative method to analyze NP size is cryoTEM which allows visualization of NPs in sections of frozen NP dispersions [64]. These methods give also information about the size distribution and the colloidal stability of NPs or their agglomerates in a given dispersant [64–66]. An important parameter of dispersed NPs is also the surface charge that

Table 1 Characterization of DMSA-IONPs

Parameter	Method	Results/values
Shape	Transmission electron microscopy	Spherical
Size	Transmission electron microscopy	5–20 nm
Size distribution	Transmission electron microscopy Dynamic light scattering	Polydisperse
Hydrodynamic diameter	Dynamic light scattering	52–64 nm
Surface charge (ζ-potential)	Electrophoretic light scattering	− 15 to − 20 mV
Element content	Energy dispersive X-ray spectrometry	Iron, oxygen, sulfur
Magnetism	Vibrating sample magnetometer	Superparamagnetic
Coating present	Energy dispersive X-ray spectrometry	Presence of sulfur peak
	Electrophoretic light scattering	Negative surface charge in water

The results/values listed in this table have been taken and combined from the articles [19, 54–56]

is frequently analyzed by determination of the ζ-potential, which is defined as the potential at the hydrodynamic shear boundary measured by light scattering [65, 67]. Valuable information on the elemental composition of the NP core and coat can also be obtained from TEM analysis, if the equipment used allows elemental analysis by energy-dispersive X-ray spectroscopy (EDX), which uses a high energy beam to stimulate the emission of element characteristic X-rays from a sample [49, 66, 68].

Successful coating of NPs will modify physicochemical properties of NPs. For example the change of the surface charge [65, 67] or the colloidal stability in physiological medium are a good first indication for a successful coating. Further evidence for the presence of a coat around NPs can be obtained by identification of elements from the coat by EDX [54, 68, 69], by the demonstration of functional groups of the coat by infrared spectroscopy or by the presence of fluorophores as demonstrated by fluorescence spectroscopy [55, 69–72].

For DMSA-IONPs, TEM analysis revealed a polydispersed size distribution of 5–20 nm (Table 1; Fig. 1). Dispersed in physiological incubation buffer (IB) these IONPs had an average hydrodynamic diameter of around 60 nm (Fig. 1; Table 1), suggesting that some agglomeration of the primary particles had occurred. A polydispersity index of 0.257 demonstrated polydisperse size distribution in IB. The magnetic properties of the IONPs were used for magnetic separation during the synthesis process and were confirmed by recording a magnetization curve in a vibrating sample magnetometer [19]. The negative ζ-potential due to the carboxylate groups in the DMSA coat as well as the identification of sulfur by EDX [54–56] confirmed the successful coating of IONPs with DMSA (Table 1). For fluorescent DMSA-IONPs the presence of the introduced fluorophores in the obtained IONPs also confirmed successful coating of IONPs with fluorescent DMSA [55, 69].

Quantifying the NP Content of a NP Dispersion

For metal-containing NPs such as IONPs, CuONPs or AgNPs the metal content of the dispersions can be determined for example by atomic absorption spectroscopy (AAS) or inductively coupled plasma mass spectrometry (ICP-MS) [2, 53]. The iron content of IONP dispersions can also be determined reliably and sensitively by a ferrozine-based colorimetric assay [55, 73, 74]. However, for reliable quantification of metal contents of a NP dispersion the complete liberation of the metal from the NPs is required that will include the application of concentrated acids and may involve some ashing procedures [75–78]. Concentrations of NPs are frequently given as ng/mL or µg/mL which indicates the total amount of NP material that was dispersed in a given volume. These values include the metal components as well as all non-metal components of NP core and coat. Alternatively, concentrations of NPs can be given as molar concentration that refers only to the concentration of one component such as the iron content of IONPs, assuming complete dissolution of this component in the respective volume. For the DMSA-coated IONPs that we have frequently used for our studies a dispersion of 3.5 mg particles/mL corresponds to a total iron concentration of 40 mM [56].

If the NPs possess fluorescent properties these fluorescent properties can be used to quantify the NPs in dispersion and in cells by fluorescence spectroscopy, flow cytometry or fluorescence imaging techniques [58]. A drawback in the fluorescence quantification is the possible bleaching or quenching of the fluorescent signal, which could result in an incorrect quantification [27, 58, 79]. In addition, the type of fluorescence labeling can affect the fluorescent properties of the NPs. Fluorophores can either be encapsulated within the NPs or immobilized to the polymer coat of the NPs [36, 80, 81]. The distance between fluorophore and core can lead to quenching or alteration of the fluorescent signal [36]. Here

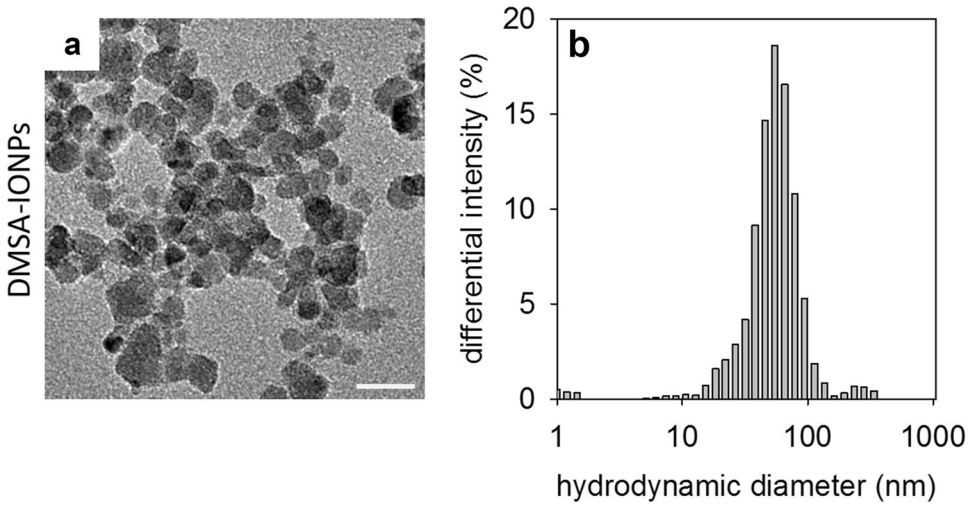

Fig. 1 DMSA-IONPs. The panels show a transmission electron microscopic (TEM) image (**a**) and the hydrodynamic diameter distribution curve (**b**) for 1 mM IONPs dispersed in physiological incubation buffer (IB) that were obtained as previously described [55, 56]. The size bar in the TEM image represents 20 nm. The TEM image (**a**) was kindly provided by Dr. Karsten Thiel (Fraunhofer IFAM, Bremen)

it is important to compare the fluorescence spectra of the fluorescent NPs with those of the free fluorescent dyes [55, 69, 71].

Modulation of NP Properties by Variation of the Coating Materials

The coating of the NPs provides opportunities to further functionalize the particles for example with fluorescence dyes [36, 54, 55, 80], with drugs, antibodies or other compounds [82–84] that may modify physicochemical properties and colloidal stability of the NPs and thereby the interaction with cultured cells. Thus, for each type of coating the NPs should be carefully characterized. For example, the use of different coating materials during the synthesis of IONPs caused strong differences in the hydrodynamic diameter of the NPs ranging from 20 to 200 nm [37, 85]. Such alterations in particle size but also size distribution, shape and surface charge are likely to affect binding of NPs to cells by electrostatic or hydrophobic interactions and subsequently also the uptake of NPs into cells.

Uncoated IONPs have a strong positive ζ-potential of around +40 mV and are colloidal stable in water, but they precipitate rapidly in salt-containing physiological media such as basal incubation buffer [56]. Such IONPs can be stabilized by coating with citrate [78] or DMSA [56]. DMSA appears to be bound to IONPs mainly by electrostatic interactions [19, 86, 87], although also covalent bonds of DMSA to the core have been discussed [86, 88, 89]. For 60 nm IONPs the DMSA-coating does not significantly increase the diameter of IONPs, but generates in water a negative ζ-potential (−60 mV) (Table 2), which maintains the coated IONPs in dispersion. Modification of the DMSA coat by low amounts of fluorescent dyes (around 1% of thiol groups present in DMSA used for coating) did not alter the physicochemical properties of the IONPs nor their binding or internalization into cultured neural cells [54, 55, 69], thereby defining such fluorescent IONPs as suitable tools to trace the internalization and the intracellular trafficking of DMSA-IONPs in cultured brain cells by fluorescence microscopy [54, 55, 69].

Modulation of NP Properties by the Components Added to the Incubation Media

In complex biological environments a variety of molecules are present including lipids, sugars and proteins that may adsorb onto the surface of the NPs [8, 16, 17], but even simple physiological buffers contain ions and other compounds that may affect the properties of dispersed NPs. In fact, as it is difficult to predict which compounds may similarly affect the stability and the properties of NPs, analysis of such parameters is recommended for each incubation medium used for cell exposure to NPs.

Table 2 shows the consequences of the presence of various compounds in a given incubation medium on the size (hydrodynamic diameter) and the surface charge (ζ-potential) of dispersed DMSA-IONPs. Compared to

Table 2 Modulation of physicochemical parameters of DMSA-IONPs by media components

Dispersant	Test compound	Hydrodynamic diameter (nm)	ζ-potential (mV)
H_2O		54 ± 11	−61 ± 13
IB		60 ± 8	−16 ± 4
IB	Phosphate (0.8 mM)	> 1000***	n.d.
IB	BSA (0.5 mg/mL)	75 ± 4*	−9 ± 2*
IB	FCS (10%)	95 ± 14*	−9 ± 2*
IB	Chlorpromazine (20 µM)	72 ± 11	−18 ± 7
IB	Dynasore (100 µM)	> 1000***	n.d.
IB	FCS (10%) + dynasore (100 µM)	111 ± 5**	−9 ± 1*
DMEM		> 1000	n.d.
DMEM	BSA (0.5 mg/mL)	> 1000	n.d.
DMEM	FCS (10%)	148 ± 22**	−10 ± 1

DMSA-IONPs were dispersed in a final concentration of 1 mM iron in the indicated dispersant water, or incubation buffer (IB; 20 mM HEPES, 145 mM NaCl, 5 mM D-glucose, 1.8 mM $CaCl_2$, 5.4 mM KCl, 1 mM $MgCl_2$, adjusted with NaOH to pH 7.4) or Dulbecco's modified Eagle's medium (DMEM; containing 20 U/mL penicillin G, 20 µg/mL streptomycin sulfate, 25 mM glucose and 1 mM sodium pyruvate) in the absence or the presence of the indicated test compounds before the average hydrodynamic diameter and the ζ-potential were determined. The data represent mean values ± SD of data obtained in three independent experiments performed on independently prepared batches of DMSA-IONPs

BSA bovine serum albumin, *FCS* fetal calf serum, *n.d.* not detectable

The significance of differences between the values obtained for the IONP-containing medium without or with the indicated test compounds is indicated by *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$

water as dispersant, the use of a physiological HEPES-buffered incubation buffer (Table 2) does not alter the average hydrodynamic diameter of the IONPs but increases the ζ-potential from − 60 to − 16 mV. The size of the NPs is moderately increased in the presence of proteins such as bovine serum albumin (BSA) or fetal calf serum (FCS), while the ζ-potential is further increased in the presence of proteins to around − 10 mV (Table 2). In contrast, already the presence of 0.8 mM phosphate, a component that is frequently present in physiological buffers used for metabolic studies on cultured brain cells [90], causes rapid agglomeration and precipitation of the IONPs as demonstrated by a diameter of the particle agglomerates in the micrometer range. Similarly, also dispersions of IONPs in DMEM culture medium that contains 0.9 mM phosphate were not stable and rapid agglomeration was observed which was prevented by the presence of 10% FCS, but not by BSA application (Table 2). Thus, the presence of proteins or protein mixtures can prevent agglomeration of IONPs, most likely by forming a protein coat (protein corona) around the DMSA-IONPs that affects NP properties and stability as well as their interactions with the cell surface [8, 91–93]. Formation of a protein corona will occur if NPs are dispersed in serum-containing media but also the many other compounds present in such complex media may affect physicochemical properties of the dispersed NPs and subsequently also the binding to cells and the cellular uptake of NPs.

To study the mechanisms of cellular uptake of NPs and to identify the endocytotic pathways involved in the internalization of NPs, a battery of more or less specific inhibitors for given endocytotic pathways are available and have been used [94]. This strategy has already been critically discussed due to the lack of specificity and severe side effects of some inhibitors [94–97]. In addition, a further disadvantage of some endocytosis inhibitors is their effect on the stability of NPs. Examples for such effects are shown in Table 2. Dynasore [98, 99] and chlorpromazine [97] are frequently applied to test for an involvement of clathrin-mediated endocytosis in the cellular uptake of NPs. However, the presence of dynasore leads to agglomeration and precipitation of IONPs, whereas the presence of chlorpromazine does not affect particle size and colloidal stability (Table 2). Furthermore, the agglomeration of IONPs by dynasore is almost completely prevented by the additional presence of 10% FCS (Table 2). These results underline the importance to investigate the potential effects of each compound that will be added to the given incubation medium to study the accumulation of NPs by cells, as modulation of the physiochemical properties and the stability of the IONPs may strongly affect the interaction between particles and cells as well as the biocompatibility or cytotoxicity of NPs [100–102].

Alteration of NP Properties by Cell-Derived Components

Last but not least, it has also to be considered that physicochemical properties and the colloidal stability of NPs could be changed by compounds that are released from the exposed cells during the incubation. For example, substances that are released from viable cultured astrocytes already within hours and are present in conditioned media lead to an agglomeration of IONPs and can therefore alter the binding to or the internalization of the IONPs into the cells [56, 73, 103, 104]. Such effects may be even more dramatic, if NPs have some toxic potential. As soon as the first cells have died and have released their content, the surface of the applied NPs can be covered by cellular biomolecules and cell debris that is likely to alter the interaction between NPs and cells. Therefore, it should be considered to characterize the extracellular NPs also during or after a given incubation of cells to learn whether cell-derived material has modulated the properties of the NPs during the incubation.

Using an Appropriate Cell Culture Test System for NP Studies

Cell Lines and Primary Cultures

In addition to the properties of NPs, also cellular parameters and the type of cell culture models chosen for NP interaction studies are important. Here, biomechanical properties of the cell membrane including membrane tension and bending modulus, composition and thickness of the phospholipid bilayer, cell size, proliferation rate, growth pattern, environmental factors as well as cell–cell-interaction play an important role in the NP adhesion and uptake [20, 46, 94, 105]. As the mechanisms of NP uptake depend on the cell type and the culture model investigated, it is important to select an appropriate test system for studying the consequences of an exposure of neural cells to NPs. Here, the characterization of the cell culture model system chosen is very important, as such models differ strongly in cell density, media requirements and sensitivity to given incubation conditions that are required for incubations with NPs. In addition, depending on the question addressed and the aim of the given study (biocompatibility or toxicity of NPs, mechanisms of uptake and trafficking of NPs, potential use of NPs for therapy of human tumors), a suitable cell culture model should be chosen, considering the type of neural cells to be addressed, the species origin of the cell cultures to be used as well as the investigation of primary brain cells or tumor cell lines of neural origin.

For first screening tests on the biocompatibility or toxicity of a given type of NP, immortalized cell lines of neural

origin are frequently used. Such cell lines are easy to handle, can be sub-cultured, batch-to-batch variability is considered to be low and the costs of such studies are reasonable [26]. However, as cell lines are mostly transformed, have a disturbed proliferation control mechanism, and possess when compared to the genuine brain cells genetic and chromosomal aberrations that may even increase with passage number [26], an interpretation of data obtained on cell lines for potential consequences of a respective NP incubation of genuine brain cells is problematic. In addition, due to the long handling of immortalized cell lines evolution of cells over the passaging procedure, potential contamination with mycoplasma as well as potential cross-contamination with other cell lines should be considered and/or excluded [106, 107]. Thus, cell lines used for NP studies should be characterized concerning their properties and authenticity, for example by testing for expression of the cell type specific markers and/or by genetic testing to confirm the cell line origin [107].

Primary cultures of brain cells, i.e., primary cultures of astrocytes, neurons, oligodendrocytes or microglial cells, have frequently been used to study properties and functions of the respective brain cell types in vitro [19, 90, 108–112]. Compared to cell lines, such cultures have a limited life span, show substantial variability between different culture preparations, require the access to living animals as cell donors and are more expensive. Nevertheless, primary cultures of brain cells are likely to reflect properties of the respective cell type in vivo more strongly than immortalized cell lines [26, 110, 113]. Thus, for studies to investigate the basic mechanisms of NP uptake, intracellular trafficking or the fate of internalized NPs in brain cells, primary cultures of brain cells, which are strongly enriched for one type of brain cells, appear to be a better model than cell lines of neural origin. If using primary cultures of brain cells, the cultures obtained should be characterized for the cell types present by immunocytochemical staining for cell type specific markers [90, 114] in order to learn about the enrichment of the desired cell type in these cultures but also about contaminations with other types of brain cells.

Primary cultures of brain cells are strongly enriched for one type of brain cells and lack therefore the complexity of the brain as the main architecture of the cells is lost and the interaction between different types of brain cells as well as potential support or potential competition is missing. Organotypic slice cultures and co-cultures of different types of brain cells can help to address and solve such problems [115]. Evidence that the presence of different types of brain cells can affect the accumulation of NPs has been presented for microglia-astrocyte cocultures. The data reported reveal that microglial cells in these culture accumulate IONPs more strongly than astrocytes, and thereby lower the uptake of IONPs into astrocytes [116, 117].

Potential Problems with Cell Density and Proliferation State

Depending on the cell cultures and the culturing conditions selected, the density of cells in cell cultures can substantially vary. Primary cultures of astrocytes as well as cell lines of neural origin can be investigated during the proliferation period and different cell densities can be established by variation of the number of cells seeded per dish. Primary astrocyte cultures will reach after a given culture time confluency, while cultures of neurons will not become confluent due to the post mitotic status of these cells [90].

Differences in cell density, cell–cell contacts, membrane tension and cell cycle phases have been described to strongly affect NP uptake [118–120]. An example for the effects of cell density on the cellular uptake of fluorescent Oregon Green (OG)–DMSA-IONPs is shown for C6 glioma cells (Fig. 2). With increasing seeding density, the protein content of the cultures increases almost linearly within a 24 h incubation period (Fig. 2a). Also the amount of iron and fluorescence accumulated by the cultured cells during a 1 h exposure to IONPs increased almost linearly, but only for low cell densities, while hardly any differences in accumulated iron or fluorescence was observed for cultures generated by seeding the cells at densities of 200,000 or 400,000 cells per well (Fig. 2b, c). As a consequence, at higher cell density a lower number of NPs may have been taken up per cell which could influence for example the toxic potential of NPs in the concentration applied. For particular NP experiments, it may be desirable to limit the potentially confounding effects of proliferation. In such cases, it is recommended to use neural cell lines or primary cells, which can be terminally differentiated prior to NP exposure by given treatments [26].

During the cell cycle the ratio of cholesterol and phospholipids, the expression of membrane proteins, surface antigens and receptors can vary [46, 122, 123], thereby influencing the interaction of cells with NPs and subsequently the uptake of NPs [123]. Accordingly, in synchronized cells the uptake of NPs can vary depending on the cell cycle phases [120, 124]. As most cultured cells used for NP uptake studies are not synchronized for their cell cycle, it should be considered that the data obtained on such cultures represent average values derived from cells in different phases of the cell cycle [120].

Potential Problems Due to Unspecific Binding to NPs to the Culture Plates

To improve binding of cells to cell culture plates, manufacturers frequently use chemical and/or physical surface modifications to alter the polystyrene surface of the culture plates [125, 126]. In addition, some cell types require special dish coatings to ensure cell attachment or to facilitate cell

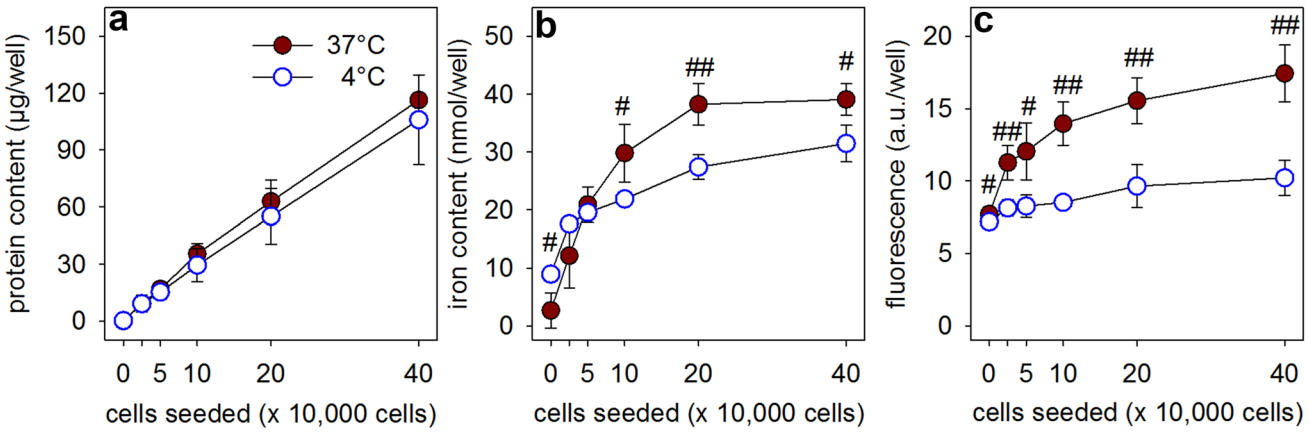

Fig. 2 Cell density-dependent accumulation of OG–DMSA-IONPs in C6 glioma cells. The cells were seeded in different densities and grown for 24 h before a 1 h incubation in IB with 1 mM fluorescent OG–DMSA-IONPs [55] was performed at 37 or 4 °C. The OG–DMSA-IONP incubation was terminated by washing the cells twice with ice-cold phosphate-buffered saline. The cells were lysed in 400 µL 50 mM NaOH and the lysates were used to determine the protein content (**a**), the cellular iron content (**b**) and the cellular OG fluorescence (**c**) using established assays as described before [55, 78, 121]. The data shown represent means ± SD of values obtained in four independent experiments. The significance of differences between the values obtained for cells that had been incubated at 37 and 4 °C is indicated by $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$

differentiation. For example, coating of cell culture dishes with collagen or poly-D-lysine is frequently used for primary neurons, hepatocytes or stem cells [90, 110, 127, 128]. As such coatings of plastic surfaces have the potential to bind NPs, a potential cell-independent adsorption of NPs to the surface of the culture dishes has to be considered. Data for such a cell-independent binding of NPs to coated dishes are shown in Table 3. While uncoated dishes hardly contain any detectable iron or fluorescence after incubation with fluorescent OG–DMSA-IONPs, substantial amounts of iron and OG fluorescence were determined for culture plates that had been preincubated (coated) with DMEM containing proteins or with a poly-D-lysine solution (Table 3). Such unspecific interactions between the NPs and the treated culture materials may strongly contribute to quantitative data obtained for the potential uptake of IONPs in cultures of low cell density.

Test for the Consequences of an Exposure of Cultured Neural Cells to NPs

Test for Biocompatibility or Toxic Potential of NPs

To study the cellular uptake mechanisms of NPs into neural cells, the cytotoxic potential of the chosen incubation conditions must be investigated in order to confirm that indeed

Table 3 Binding of fluorescence OG–DMSA-IONPs to cell culture plates

Preincubation medium	Iron content (nmol/well)	Fluorescence (a.u./well)
None	0.3 ± 0.1	1.6 ± 0.2
DMEM	2.3 ± 0.4**	3.3 ± 2.5
DMEM + BSA (0.5 mg/mL)	7.4 ± 1.8**	16.8 ± 7.1**
DMEM + FCS (10%)	6.0 ± 1.5**	13.6 ± 0.3***
Poly-D-lysine (15 µg/mL in H_2O)	22.0 ± 6.1**	23.0 ± 1.7***

24-well cell culture plates were preincubated for 24 h with 1 mL of the indicated solutions in a humidified atmosphere of an incubator at 37 °C for 24 h. Subsequently, the wells were washed twice with IB, incubated with 200 µL of 1 mM Oregon Green (OG)–DMSA-IONPs (200 nmol iron per well and 262 ± 26 a.u. fluorescence per well) in IB at 37 °C for 1 h, were washed twice with 1 mL phosphate-buffered saline and were treated with 400 µL 50 mM NaOH to solubilize bound IONPs that were subsequently quantified by determining the contents of iron and OG fluorescence [55, 56]. The data represent mean values ± SD of data obtained in three experiments performed on independently prepared batches of OG–DMSA-IONPs

DMEM Dulbecco's modified Eagle's medium, *BSA* bovine serum albumin, *FCS* fetal calf serum

The significance of differences between the data obtained for uncoated (none) and preincubated wells is indicated by $^{**}p < 0.01$ and $^{***}p < 0.001$

the uptake and internalization of NPs is monitored for viable cells. The size, shape, agglomeration state and surface coating play an important role in the cytotoxicity of NPs [100–102], because these factors highly influence the binding to the cells and their internalization. In this context it is important to considered that already partial cell toxicity will lead to substantial release of biomolecules from damaged cells which in turn can bind to the NPs, and thereby alters their physicochemical properties and also the interaction, uptake and toxic potential of cell debris-associated NPs for the viable cells present.

For IONPs, the cytotoxic potential is controversially discussed [2, 10, 18]. Several studies state that IONPs are not cell toxic [23, 129–131], whereas other studies observed a cytotoxic potential of IONPs depending on cell type and surface coating of the NPs [74, 132–134]. Smaller NPs have an increased reactive surface in relation to their volume and are usually faster internalized which may contribute to their higher cytotoxic potential [17]. Likely reasons for the controversial observations may be the use of different coating materials and different physicochemical properties of the IONPs applied that will affect binding to the cells and subsequent uptake [135, 136] as shown for differently coated IONPs on cultured primary neurons [137]. In addition, also cell-type differences strongly affect the cellular accumulation and the toxic potential of IONPs. For example, DMSA-IONPs were not toxic for cultured astrocytes or neurons even if millimolar concentrations of iron were applied as IONPs, while such concentrations showed strong toxic potential to cultured microglial cells [74].

To investigate the biocompatibility or the toxicity of a given treatment, a large number of assays is available and frequently used that address mostly either membrane integrity or metabolic functions [100, 138, 139]. For studies of potential toxicity of NPs it is recommended to apply at least two different biocompatibility assays that have independent underlying principles, in order to become aware of potential disturbances by NPs on the assay systems used.

Test for Potential Interferences of NPs with the Assay Systems Used to Determine Biocompatibility or Cell Toxicity

The determination of cellular integrity and function during or after an exposure of cells to NPs is important but not always easy as NPs may interfere with the assay applied. Similarly, also the quantification of cellular NPs or of cellular parameters and functions can be disturbed in the presence of NPs as their unique physicochemical properties, their high reactivity and their high adsorptive capacity may interfere with components of the test system applied [18, 92, 140, 141]. This can result in artifacts and a false-positive or false-negative interpretation of the data obtained. As a first qualitative approach to test for potential adverse consequences of an exposure of cultured cells to NPs we recommend microscopical inspection of treated and control cultures as severe effects of NPs on cell density or morphology can be easily visualized.

A large number of biocompatibility assays are based on colorimetric or fluorimetric dyes and depend on the light absorption and/or emission at a specific wavelength. It is obvious that the use of fluorescent IONPs limits the applicable number of these assays regarding interference of absorption and emission spectra. For example, measuring the generation of reactive oxygen species using Rhodamine 123 [142] would not be suitable after exposure of cells to OG–DMSA-IONPs due to the overlap of the excitation and emission spectra of these both dyes. But besides this obvious problem, nanomaterials have widely been shown to interfere with assays by altering the optical properties of dyes leading to under- or overestimation of the NP toxicity [2, 26]. For example, determination of the release of cellular LDH is considered as a good indicator for a loss in membrane integrity of cultured cells [90]. However, this assay should not be used for analysis of cellular consequences of CuONPs, as such NPs inactivate LDH [77]. Thereby, a quantification of released LDH is prevented which will lead to false-negative results suggesting absence of any cell toxicity of CuONPs.

To identify potential artifacts by NPs on the assay systems applied, several controls are recommended such as (1) exposing cells with NPs but without assay reagents [143], (2) applying a cell free control incubation [92], (3) removing or lowering the amount of NPs present in samples by centrifugation [140] and (4) intensive washing after the cell incubation before applying the assay system [143]. But even all these precautions cannot fully exclude interference in the results due to membrane adhesion and internalization of NPs by cells [18, 143]. Therefore, it is strongly recommended to run always additional controls including the addition of a standard concentration of NPs to cell samples that were obtained from viable control cells and a toxic control condition.

Test for Adsorption of NPs to the Cells and/or Uptake of NPs into Cells

The cellular uptake of NPs is a two-step process [144, 145]. First the NPs adsorb to the cell membrane and then the internalization by endocytosis takes place [104, 145, 146]. The surface properties are the crucial factor in cell–NP interaction as the engineered surface is first interacting with the cell membrane [46, 147]. This will mediate the binding of NPs to the cells before internalization of the NPs can take place. Uptake of NPs in cultured brain cells can be verified depending on the type of NPs investigated by electron, fluorescence or light microscopy.

The successful uptake of NPs into cells and the intracellular localization of NPs can be visualized by TEM. Concerning IONPs in cultured brain cells, a vesicular localization of electron dense material was reported for IONP-treated cultured primary astrocytes [56, 78, 103]. For such stainings even elemental analysis of the cellular NPs can be done and the material taken up into the cells can be confirmed. Fluorescence microscopy of neural cells that had been exposed to fluorescent IONPs allows also a qualitative view on the internalization of such NPs [54, 55, 69, 74, 148]. Finally cytochemical staining of the exposed cells for iron allows a qualitative view on the cellular presence of IONPs in cultured neural cells [54, 56, 69, 78, 149, 150]. For cytochemical or fluorescence analysis of the uptake of IONPs into cells it is important to consider that the signals obtained in light or fluorescence microscopic images do not represent the signals derived from individual fluorescent NPs but that rather a given amount of NPs has to be packed into vesicular structures in order to generate sufficient local density of NPs to allow detection.

Various methods can be applied to quantify cellular contents of NPs as long as the assay of choice is sufficiently sensitive for quantifying the low amounts of material that has been internalized into the cells by NP uptake. For NPs containing copper or silver, we have used highly sensitive AAS methods [75–78], and for IONPs a sensitive ferrozine-based colorimetric assay in microtiter plates [55, 56, 73, 74, 78]. However, for reliable quantification of metal contents of cellular NPs the complete liberation of the metal from the NPs is required which will include the application of concentrated acids and involves some ashing procedures. Concerning the NP quantification, it should be kept in mind, that the NP amount determined reflects the total amount of NPs in a well that consists of the amount of internalized NPs plus the amount of NPs bound extracellularly to cells plus the amount of NPs bound to the plastic of the dish in a cell-independent process.

In order to quantify the amount of internalized NPs it is important to discriminate between NPs that are adsorbed to the cells (and/or the culture plate) and the NPs that had been taken up by cellular endocytosis. This can be achieved by performing control adsorption experiments at 4 °C. This low temperature prevents the energy-dependent NP internalization, but does hardly affect adsorption and desorption processes [71, 104, 144, 145]. Thus, after a 4 °C incubation only the extracellularly adsorbed NPs are quantified as NP uptake is prevented, while the values obtained for a respective incubation at 37 °C represents the sum of adsorbed plus internalized NPs. The temperature dependence of IONP uptake in order to discriminate between adsorbed and internalized IONPs has been performed for several types of neural cell cultures and revealed that the amounts of adsorbed NPs after exposure of the cells to DMSA-IONPs represents between 50 and 70% of the total amount of IONPs [41, 54, 55, 114, 148]. Similar results were found for non-neural cells [144, 151].

Most of this extracellular adsorption of IONPs to neural cells may be caused by unspecific electrostatic interactions between the coat of the NPs and cell surface molecules which cannot be simply removed by additional washing steps. However, the binding of NPs to cells can also be made more specific by introducing cell receptor specific ligands, signal peptides or antibodies [84, 152–154]. In some cases, the presence of protein can reduce the adsorption of NPs to the cell membrane [55, 145, 146], and thereby also dramatically lower the cellular uptake.

After internalization into cells, NPs are encountering changes in their environment that can affect the stability and the cellular metabolism of the intracellular NPs. Uptake by endocytosis delivers NPs into the endosomal compartment that is characterized by a slightly acidic pH [155]. This may lead to degradation of the core and/or the coat of the NPs that may affect cellular parameters, cell viability but also the interpretation of the results obtained. Therefore, it is important to verify whether the internalized NPs are still intact and/or whether the coat is still colocalized with the core of the NPs [8, 16]. If the NPs are disintegrated or the coat is separated from the core of the NPs it has to be considered that components of core or coat are liberated from the NPs that can affect properties and functions of cells [155]. For example, IONP uptake causes severe toxicity of cultured microglia due to iron-mediated oxidative stress caused by lysosomal liberation of iron ions from the internalized IONPs [69, 74, 156]. The stability or lability of a given type of NPs in the acidic conditions found in lysosomes can be investigated by exposing NPs to lysosome-like acidic conditions in a cell-free system [157, 158].

An Example How to Analyse the Uptake of Fluorescent DMSA-IONPs in Cultured Neural Cells

Figure 3 shows experimental data that were obtained for an exposure of C6 glioma cells with OG–DMSA-IONPs. The fluorescent IONPs have been carefully characterized for their physicochemical properties and their colloidal stability in the incubation buffer (IB) used for the incubations [55]. Compared to the non-fluorescent DMSA-IONPs (Table 1), the presence of the low amounts of the fluorescent dye OG in the coat of the IONPs does not affect the physicochemical properties of the IONPs nor their interaction with cells [55]. For the cell experiments 100,000 C6 glioma cells were seeded per well of a 24-well plate in 1 mL of DMEM with 10% FCS. After 24 h incubation the cells were washed twice with IB and subsequently incubated for 1 h with given concentrations of OG–DMSA-IONPs in 200 µL IB at 4 °C or at 37 °C. The concentrations given for the IONPs represent

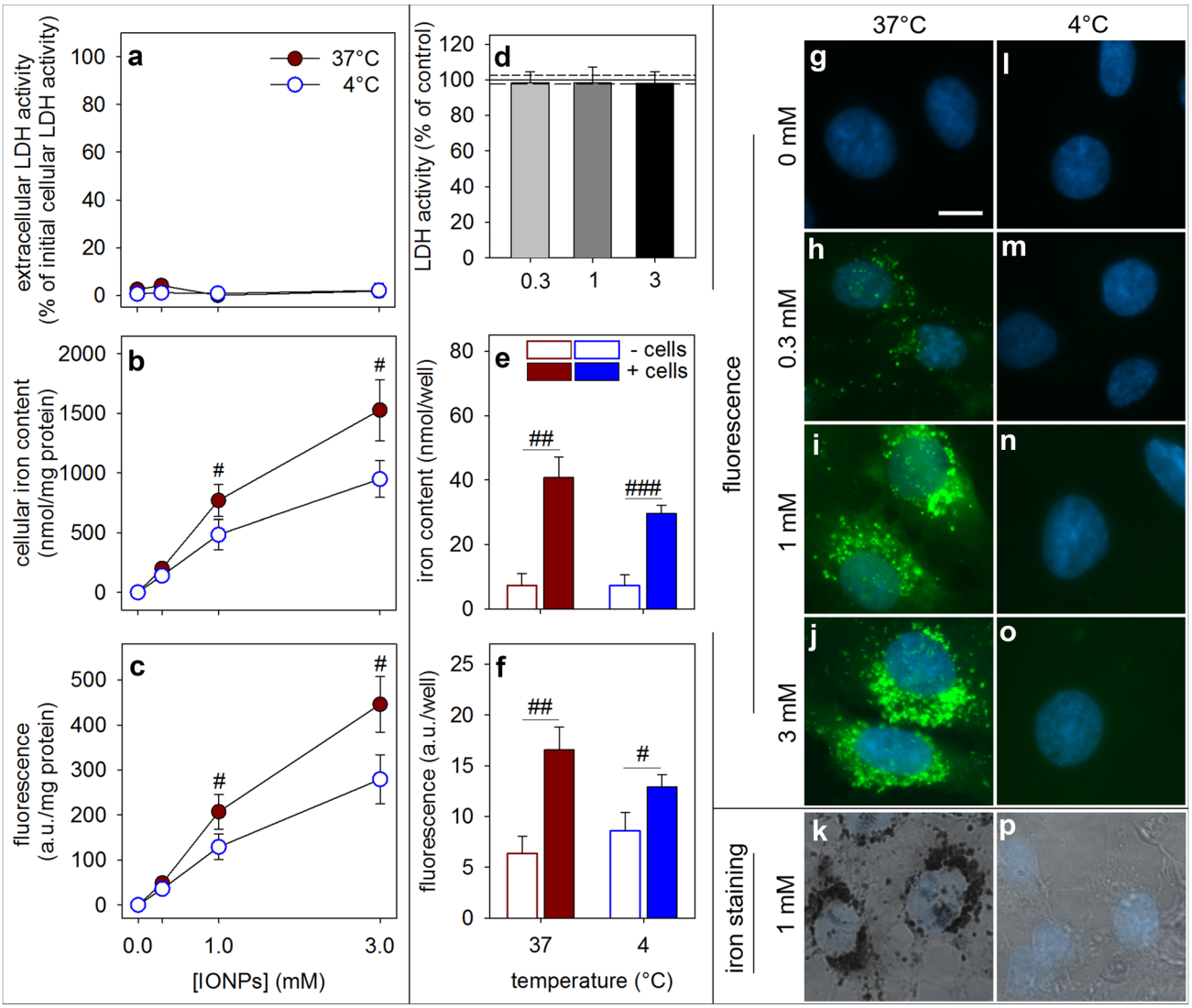

Fig. 3 Concentration-dependent accumulation of OG–DMSA-IONPs in C6 glioma cells. Per well, 100,000 cells were seeded and grown for 24 h before the cells were incubated for 1 h with the indicated concentrations of OG–DMSA-IONPs at 37 or 4 °C. The cell viability was investigated by testing for impaired membrane integrity by measuring the release of the cytosolic enzyme LDH (**a**) as previously described [90, 159]. The cellular iron content (**b**) and the cellular OG fluorescence (**c**) were determined from NaOH lysates (400 µL 50 mM NaOH) as recently described [55, 78]. The basal specific iron content of untreated C6 glioma cells is with 3.1 ± 4 nmol iron/mg protein in the range of the detection limit of the iron assay used [55]. Potential disturbances by IONPs on the LDH assay used were excluded by determining LDH activity in 1% Triton X-100 cell lysate in the presence of the given final concentrations of IONPs (**d**). The extent of cell-independent binding of OG–DMSA-IONPs to protein-coated wells (wells exposed to culture medium containing 10% FCS for 24 h) was determined in parallel to cell incubations (1 h at 37 °C or 4 °C with 1 mM IONPs) by measuring iron content (**e**) and fluorescence (**f**). The data shown represent means ± SD of values obtained in three independent experiments. The significance of differences between values obtained for incubations at 37 and 4 °C or for incubations in the presence or the absence of cells is indicated by $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$. Fluorescence microscopy was used to localize cellular OG fluorescence after 1 h incubation of cells with the indicated concentrations of OG–DMSA-IONPs at 37 °C (**g–i**) or 4 °C (**l–o**) and the cellular iron (**k, p**) was localized by cytochemical Perl's staining as previously described [55, 78]. The cell nuclei were stained with DAPI (blue) (**g–p**). The size bar in panel **g** represents 10 µm and applies to the panels **g–p**. (Color figure online)

the concentration of iron present in the IONPs applied and not the particle concentration.

Analysis of cell morphology did not reveal any obvious alteration after exposure to the IONPs (data not shown) and also the quantification of extracellular LDH activity after the 1 h exposure time did not show any increase in extracellular LDH (Fig. 3a). The lack of any influence of the NPs applied on the LDH assay performed was investigated by applying IONPs to lysates generated from cultured C6 cells before measuring LDH activity for the lysates. Even in final

concentrations of up to 3 mM iron as IONPs, the presence of IONPs did not affect the measurement of LDH activity in cell lysates (Fig. 3d), thereby excluding that a potential toxicity (LDH release) of the treatment could have been masked by inactivation of liberated LDH by IONPs. Analysis of the unspecific binding of IONPs to protein-coated cell culture plates (24 h preincubation with DMEM + 10% FCS) by quantification of iron and fluorescence revealed that the cell-independent binding of IONPs was low and even substantially lower than the adsorption of IONPs to the cells at 4 °C (Fig. 3e, f).

Analysis of the cellular fluorescence and cellular iron contents in cell lysates obtained from cells after exposure to OG–IONPs at 4 and 37 °C allows to discriminate between adsorbed IONPs and internalized IONPs (Fig. 3b, c). For incubations with 1 and 3 mM IONPs, the specific iron contents and the specific cellular fluorescence determined after incubations at 4 °C represented around 65% of the values observed for the respective incubations at 37 °C, demonstrating that after an incubation at 37 °C only around 35% of the cellular IONPs had been taken up into the cells (Fig. 3b, c). Despite of the substantial amounts of iron and fluorescence determined in lysates generated from cells after exposure to OG–IONPs at 4 °C, hardly any cellular fluorescence or iron signals were observed for this treatment (Fig. 3m–p), due to insufficient local density of the extracellularly adsorbed fluorescent IONPs to allow detection of iron or fluorescence [16, 58, 79]. In contrast, after the respective incubations at 37 °C, strong fluorescence signals and a strong cellular iron staining were detectable (Fig. 3h–k), indicating cellular uptake and intracellular packaging into vesicles of the fluorescent IONPs to local concentrations that are sufficiently high to allow detection of the internalization of OG–DMSA-IONPs by iron staining and florescence microscopy.

Conclusion

To test and screen for potential consequences of an exposure of brain cells to NPs, cultures of neural cells are good and easy to use model systems. However, due to their unique properties NPs will strongly interact with components of physiological media, with biomolecules and with cells. This can affect the physicochemical properties of the NPs but also the properties and the viability of cells. Therefore, it is important to analyze in detail the physicochemical properties of the NPs in the physiological media used for cell experiments, the potential effect of cell components on the NPs as well as the consequences of an NP exposure to the cells. A number of special control experiments are recommended to avoid artifacts and misinterpretations of data on the consequences of an exposure of brain cells to NPs. These dos and don't forgets will help to appropriately address the special challenges connected with studying the interactions between NPs and cultured cells and will help to avoid unnecessary artifacts in order to deliver robust and reproducible data and reliable and correct interpretations of the data obtained on the potential toxicity, uptake and intracellular fate of NPs in neural cells.

Acknowledgements The authors thank Dr. Karsten Thiel (Fraunhofer IFAM, Bremen) for kindly providing the TEM picture of the DMSA-IONPs.

Compliance with Ethical Standards

Conflict of interest The authors have no conflict of interest to declare.

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