Imaging and Organelle Distribution of Fluorescent InGaP/ZnS Nanoparticles in Glial Cells

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Abstract:

Aim: To assess the effects of oleic acid treatment on subcellular distribution of indium gallium phosphide–zinc sulfide (InGaP/ZnS) nanoparticles in microglia and astrocytes. **Materials & methods:** The extent of colocalization between the nanoparticles and organelles was assessed by confocal microscopy, spectrofluorometry and cell sorting. **Results:** Cell treatment with a common fatty acid (oleic acid) within the range of physiological concentrations markedly enhanced the InGaP/ZnS uptake by microglia and afforded their colocalization within lipid droplets/lysosomes but not with mitochondria. **Conclusion:** These results suggest that the availability of mono-unsaturated fatty acids, such as oleic acid, in different cells could significantly alter nanoparticle uptake and localization, which can in turn affect the functions of cells and tissues coexposed to nanoparticles.

Keywords: cell organelles | imaging | lipid droplets | lysosomes | microglia | mitochondria | quantum dots

Article:

One of the challenges in nanomedicine is the determination of the distribution of nanoparticulate carriers and nanoparticles in the body, as well as in individual cells, in real time [1] . Most of the single cell studies performed to date were done in different cell lines, but studies in primary neural cultures are still relatively sparse [2-6] . The main objective of the present study was to examine the intra-organelle distribution of indium gallium phosphide-zinc sulfide (InGaP/ZnS)-coated nanoparticles in primary neural glial cells obtained from the cortical brain region, and to assess the role of exogenous oleic acid as a putative agent that can influence nanoparticle subcellular distribution. The rationale for selecting InGaP/ZnS nanoparticles is that they have excellent photophysical properties and they are less toxic within a low nanomolar concentration range (1-10 nM) than several other nanoparticles [7,8] . Primary cortical-dispersed mixed culture

systems and microglia N9 cells were used to examine uptake and subcellular distribution of InGaP/ZnS nanoparticles in various cell types of the CNS and to find out how cell treatments with oleic acid can alter their distribution.

Oleic acid is a monounsaturated fatty acid (C18:1), one of the most common fatty acids present in mammalian tissues and a common component of various foods (e.g., olive oil). Oleic acid is produced by astrocytes in the brain [9-11] , and is utilized by neurons, particularly during nervous system development [12] . Interestingly, it has also been used as a component of different pharmaceutical preparations such as gels [13] , nanoparticles [13-16] and transdermal drug penetration enhancers [17-19] . It was suggested that oleic acid can be employed as an ion-pairing agent to facilitate transfer across lipoidal membranes [20] but others attribute this enhancement to the *cis* -structure of oleic acid, which disrupts lipid bilayers when applied to the skin's surface [21] . In addition, in cultured human hepatocytes, oleic acid was found to induce lipid droplet formation [22] . Lipid droplets are organelles that have been extensively studied in adipose tissues, but not in the CNS. Lipid droplets were detected in brain glioma cells but their function still remains unclear [23] . Lipid droplets are intracellular neutral lipid-storage sites that can also interact with other organelles [24,25] . The functional significance of lipid droplet interactions with different organelles is not clear; it appears that they provide fuel to mitochondria and could alter the transfer of phospholipids, cations and signaling proteins. Functional roles of lipid droplets in cells exposed to nanoparticles were not previously explored and are currently unknown.

In this study, we examined the uptake and colocalization of (negatively charged mercaptohexadecanoic acid [MHDA]) InGaP/ZnS nanoparticles in primary cortical cultures and microglia N9 cells. We further investigated whether the induction of lipid droplets in the presence of oleic acid will alter the InGaP/ZnS nanoparticle subcellular distribution. So far, there are no reports examining lipid droplet-nanoparticle colocalization in glial cells. The rationale for focusing on glia in this study is that astroglia and microglia are most likely the major players in retaining and delivering nanoparticles in the CNS [26,27] . The delivery of therapeutic agents and diagnostic compounds to specific brain regions is restricted by several barriers including the blood-brain barrier, the blood-cerebrospinal fluid barrier and other specialized barriers. Strategies to advance translational research into brain barriers was recently reviewed by Neuwelt *et al.* and recommendations to advance the research in this area have been proposed [28] . Several imaging and drug-delivery strategies to brain tumors have been developed [29,30]and new nanomedicines are in clinical trials for glioblastomas.

Several groups, including our laboratory, have demonstrated that metallic and other nanoparticles are taken up by microglia, macrophages of the nervous system [31,32] . Glial cells, astrocytes and especially microglia, avidly respond to various insults to the nervous system [33] and play a critical role in the functioning of the CNS at all stages of life, under both normal and pathological conditions [34,35] . In addition, microglia constitute a critically important 'surveillance cell network' in the nervous system. They are highly dynamic and upon focal activation they switch from a patrolling to a shielding mode. Time-lapse experiments showed

microglia processes as remarkably motile, with processes contacting neurons and astrocytes [36] . Brain astrocytes are activated by some, but not all, nanoparticles, as shown by real-time imaging in mice [31] . In the current study we show that microglia are the major glial cell type that internalize InGaP/ZnS nanoparticles in mixed primary cortical cultures. In addition, we show that the distribution and uptake of InGaP/ZnS nanoparticles within individual organelles (lysosomes, mitochondria and lipid droplets) are markedly affected in neural cells exposed to oleic acid.

Materials & methods

* Nanomaterials & vital dyes

InGaP/ZnS (Evident Technologies Troy, NY) nanoparticles are functionalized with hydrophobic ligands such as trioctyl-phosphine oxide, therefore soluble only in organic solvent. To render them soluble in aqueous solution for application in biological media, the trioctyl-phosphine oxide capping groups were exchanged with MHDA; these InGaP/ZnS nanoparticles were characterized as previously described [8] . The InGaP nanoparticles are coated with ZnS, have an overall negative surface charge, have a core size of 5 nm and emit at 680 nm. Stock solutions of InGaP/ZnS nanoparticles were diluted in Neurobasal A cell culture medium to yield 1-10 nM working concentrations.

Some of the critical physical and photophysical properties of these and other employed nanoparticles in this study are summarized in Table 1. The cadmium telluride (CdTe) nanoparticles are not coated with ZnS; they have mercaptopropionic acid and an overall negative charge (-9.6 mV) with an emission wavelength at 535 nm and a core size of 2.8 nm. Similar nanoparticles were described previously [3] . Two types of cadmium selenide (CdSe) nanoparticles were used: those coated with ZnS and capped with mercaptopropionic acid (core size 4.3 nm) and those with polyethylene glycol (PEG) with a hydrodynamic radius of 18-20 nm. These PEGylated CdSe nanoparticles have neutral surfaces and an emission wavelength maximum at 705 nm (Invitrogen, Ontario, Canada). Additional details of cores and surfaces are discussed in the section 'Results & discussion'.

Vital dyes used for labeling cellular organelles were purchased from Invitrogen. Lysosomes were visualized with LysoTracker green DND-26 (0.5 µM, 3 min; λ$_{ex}$ 504 nm, λ$_{em}$ 511 nm). Mitochondria were stained in cortical primary cultures with MitoTracker Green FM (0.5 µM; 3 min; λ$_{ex}$ 490, λ$_{em}$ 516 nm). Lipid droplets were visualized with Bodipy 493/503 (0.01 mg/ml, 3 min; λ$_{ex}$ 493 nm, λ$_{em}$ 503 nm).

* Cell cultures & treatments

Dissected cortices of mice (129T2/SV, postnatal day 5, from at least six animals), were freed from meninges, pooled and stored into ice-cold sterile Ca^{2+} /Mg^{2+} free phosphate-buffered saline (PBS; Invitrogen). Small pieces were dissociated mechanically by gentle trituration using sterile

1 ml pipette tips (Fisher, Montreal, Quebec, Canada) and trypsinized with 0.25% trypsin/ethylenediaminetetraacetic acid (15050-15065, Invitrogen) at 37°C for 10 min. Dissociated cells were resuspended in Dulbecco's Modified Eagle Medium (11995-11073, Invitrogen) containing 10% fetal bovine serum (26140-26079, Invitrogen) and penicillin/streptomycin/neomycin cocktail (15640-15055, Invitrogen). Cells in suspension were seeded onto poly-L-ornithine (P-3655, Sigma, Ontario, Canada) and laminine (L-2020, Sigma)-coated 12-mm glass cover slips (12-545-580, Fisher) and incubated in a 95% air/5% CO_2 atmosphere at 37°C. Attached cells were placed in DMEM medium plus supplements for 24 h in 24-well plates (Corning plates from Fisher). The next day, cells were washed twice with prewarmed Neurobasal A media (12349-12015, Invitrogen) and, finally, serum-free Neurobasal A medium containing B27 (17504-17044, Invitrogen), l-glutamine (G-7513, Sigma) and penicillin/streptomycin/neomycin supplements. The cultures were maintained in 95% air/5% CO_2 atmosphere at 37°C. The cells were used for experiments at *in vitro* day 8, treated with oleic acid (400 µM complexed with bovine serum albumin, Sigma; 24 h) and then treated with InGaP/ZnS nanoparticles (6 × 1012 nanoparticles/ml) in the presence of oleic acid for 4-72 h. Control cells received only the equivalent volume of the cell culture medium used for cell treatment with nanoparticles. All measurements were performed in triplicate in at least two independent experiments.

*** Uptake of nanoparticles by flow cytometric analysis (fluorescence-activated cell sorting)**

Microglia cells grown in Iscove's Modified Dulbecco's Medium and PC12 cells cultured in Roswell Park Memorial Institute (RPMI) medium 1640 phenol-red free medium containing 10% fetal bovine serum in 24-well plates were used 24-48 h after plating. 24 h after incubation with nanoparticles (Table 1, CdTe, CdSe, InGaP), all cells were exposed to 1-10 nM nanoparticle concentrations for 4 and 24 h, cells were washed twice in PBS and detached by vigorous shaking. Cells were centrifuged at 2500 rpm for 6 min, resuspended in cold PBS with 1% bovine serum albumin and finally placed on ice before the analysis. Flow cytometric analysis was done with a triple-laser flow cytometer (BD FACS[trademark] Area, Becton-Dickinson, CA, USA) and data analysis was performed using the Flowjob Treestar Software (Ashland, Oregon, USA). For detection of nanoparticle emission at 535, excitation was done at 488 nm (blue laser) and the emission was determined by a dichroic 530/30/FITC bandpass filter. For CdSe/ZnS PEG nanoparticles and InGaP nanoparticles, optimal excitation was achieved with 633 nm laser and a 660/40 nm bandpass filter. The median of measured fluorescence was used to calculate the relative uptake of nanoparticles relative to controls that were not exposed to any nanoparticles but received an equivalent volume of the corresponding cell culture media. All experiments were performed in triplicate. Fluorescent signal from 5000 or 10,000 cells was collected and expressed as mean fluorescence intensity (MFI). MFI is the arithmetic mean of the data; the linear-scaled fluorescence values are averaged and the result is weighted by outliers at the high end of the distribution. Measurements were done at least twice and values are averages of at least six measurements (n = 6). Results were analyzed by analysis of variance (ANOVA).

*** Confocal microscopy**

Cells were grown on 12 mm^2 glass cover slips (2×10^5 cells/well) in 24-well chambers (Sarstedt, Montreal, Quebec, Canada) and InGaP/ZnS nanoparticles were added to designated wells. The cells were incubated for different time periods with individual vital dyes to visualize organelles. Live imaging was carried out on a Leica SP2 confocal microscope. Double- and triple-labeled samples were imaged for colocalization. Images were obtained by sequential imaging to prevent bleed-through. All images were taken using a HCXPLAPO 63X lens.

Before imaging, cells were washed with PBS or with serum-free medium. No background fluorescence was detected in cells under the settings used. Images were acquired at a resolution of 512 × 512 and 1024 × 1024. The scan size was 146.2 × 146.2 µm. The images were averaged four times to improve the signal:noise ratio with a Z-section of 0.25 µm. Images were transferred to Imaris software (Bitplane Ag, Switzerland[101]) for 3D visualization and colocalization quantification studies. As a negative control we performed colocalization studies on glial fibrillary acidic protein and Hoechst 33342 (for labeling cell nuclei), which do not colocalize. Lysosomes were visualized with LysoTracker green DND-26, (0.5 µM, 3 min); λ$_{ex}$ 504 nm, λ$_{em}$ 511 nm). Mitochondria were stained with MitoTracker Green FM, (0.5 µM; 3 min; λ$_{ex}$ 490, λ$_{em}$ 516 nm) and lipid droplets were visualized with Bodipy 493/503 (0.01mg/ml, 3 min, λ$_{ex}$ 493 nm, λ$_{em}$ 503 nm). Figures were created using Adobe® Photoshop and Microsoft Office® PowerPoint 2003.

*** Colocalization measurements**

The extent of colocalization of the two labels (i.e., InGaP/ZnS nanoparticle and organelle-specific fluorescent dye) was measured using the 'colocalization' module of Imaris, 64-bit version (Bitplane AG) [37] . This program allows the analysis of stacks of confocal sections obtained in two channels. Each of the confocal sections consists of an array of square elements called pixels. A voxel is defined from a pixel as a prism in which the base is the pixel and the height is the thickness of the confocal section.

The Imaris Colocalization module is used to determine the intensity threshold of the dynamic range (in the 0-255 scale of pixel intensity) for each of the two labels [38] . Any voxels with intensities above this threshold are considered to be above the background noise. In the software, a voxel is defined as having colocalization when the intensities of both labels are above their background thresholds. The program has an automatic threshold feature to avoid investigators' bias in setting the threshold [38] .

The data from this produces a colocalization measure for each of the labels. This method may lead to an overestimate in the amount of colocalization, as the disadvantage is that it does not reflect whether the intensities of the two labels increase and decrease together in the same voxels, whereas when there is a high degree of colocalization the intensities of the two labels would be expected to vary together. We also used the Pearson coefficient in voxels with

colocalization. The Pearson coefficient is a number between +1 and -1, with positive values indicating a direct correlation, negative values indicating an inverse correlation and values near 0 indicating no correlation. Negative values of the Pearson coefficient are seldom encountered in colocalization studies. In this case, the Pearson coefficient measures the correlation between the intensities of the two labels only in the voxels with colocalization. This measure is more precise than 'the percentage of material colocalized,' as it has an additional requirement that the intensities of the two labels vary together, but the Pearce coefficient may underestimate the amount of colocalization [37] .

The following procedure was used to measure colocalization. A computer folder containing the stack of confocal sections for the two labels was generated by the Leica SP2 confocal microscope. A region of interest was defined as all the voxels in which the intensity of one of the labels was above a pixel intensity of 10 (in the dynamic range 0-255 scale). A pixel intensity of 10 was well within the background for all images. We then used the automatic threshold to calculate the thresholds for each label. Threshold values varied between pixel intensities of 10 and 95, and generally agreed with background levels estimated visually. Once the thresholds were set, the program was able to output the measures of colocalization as described previously.

*** Statistical analysis**

Data were analyzed using SYSTAT 10 (SPSS, Chicago, IL, USA). Statistical significance was determined by Student's t-test, one-way ANOVA followed by multiparametric Dunett's *post hoc* test, or two-way ANOVA. Differences were considered significant where p was less than 0.05.

Results & discussion

The purpose of this study was to explore subcellular distribution of InGaP/ZnS nanoparticles in glial cells and to examine whether the cell treatment with a common fatty acid (oleic acid) can alter nanoparticle uptake and distribution in organelles of glial cells.

Results from these studies show that nanoparticles made of elements from group III/V, such as InGaP/ZnS, can be readily detected in primary living glial cells; InGaP/ZnS nanoparticles are distributed in several organelles, including lipid droplets, induced by oleic acid and in its presence; uptake of InGaP/ZnS nanoparticles and their subcellular distribution is markedly enhanced by oleic acid treatment. This organelle was not previously studied in the context of nanoparticle distribution in living cells of the CNS.

We first tested several fluorescent nanoparticles (Table 1) to select the ones with best-suited photophysical properties for subcellular distribution studies by confocal microscopy. These nanoparticles can be excited within a wide range of wavelengths and have relatively narrow fluorescence maxima. Their core components (CdTe, CdSe, InGaP) and sizes are different.

InGaP/ZnS nanoparticles (type III-V core elements), CdTe and CdSe nanoparticles (type II-VI) had negative surfaces. Commercially available CdSe/ZnS nanoparticles coated with PEG were recently presented as promising tools for multicolor bioimaging [2,31] . In particular, CdSe/ZnS/PEG nanoparticles emit near-infrared light (core size: 2.6 nm; hydrodynamic radius: 18-20 nm; emission at 705 nm) with minimal or no tissue background autofluorescence and have excellent photostability. They have neutral surfaces, due to the methoxy terminal on the PEG polymer. As such, they were designed to decrease nonspecific adhesion properties and to reduce their heavy metal-induced cell death. Nevertheless, II-VI nanoparticles still contain heavy metal core elements (cadmium, selenium) and thus possess a potential risk for inducing cytotoxicity in a concentration-time-dependent manner, due to the initiation of unspecific cellular degradation processes [39] . Similarly, CdTe nanoparticles without a ZnS coating with overall negative surface charge, due to the carboxylate terminal group from mercaptopropionic acid, are poorly fluorescent. These nanoparticles were prepared and characterized as described in the previous study [40] and discussed in [39] . They are prone to degradation and cadmium release in biological media [41,42] . To overcome this limitation we employed InGaP/ZnS nanoparticles (core size: 5.0 nm; emission at 680 nm), which appeared to be good candidates for live cell and *ex vivo* imaging with desirable photophysical properties [8] . InGaP/ZnS nanoparticles capped with MHDA have an overall negative surface charge, owing to the carboxylate terminal group. PEGylated InGaP/ZnS nanoparticles with a negative surface charge are now commercially available (eFluoro 700 [Carboxyl]; emission: 703.5 nm; quantum yield: 30%, using reference dye Rhodamine 6G) and they compare favorably to those described previously [8] . The initial internalization studies were performed with all three negatively charged nanoparticle types using fluorescence-activated cell sorting analysis and two different cell lines (Figure 1). Pheochromocytoma, PC12 cells are well characterized and frequently used *in vitro* as a model system in basic neuroscience research. The mouse microglia are phagocytic cells in the CNS.

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Figure 1. Uptake of nanoparticles in living PC12 cells and microglia.

(A) Uptake of nanoparticles in PC12 cells and **(B)** microglia N9 determined by fluorescence-activated cell sorting analysis. **(C)** Live cell imaging of InGaP/ZnS nanoparticles compared with CdSe/ZnS-PEG and CdTe nanoparticles in PC12 cells. All cells were treated with nanoparticles under comparable conditions (10 nM, for 4 h). **(1-3)** are control cells not exposed to any quantum dots. **(4-6)** are cells treated with CdTe, CdSe/ZnS-PEG and InGaP/ZnS quantum dots. All measurements were done in triplicate in at least two independent experiments. Scale bar = 5 μm.

Results from these studies showed that the MFI in PC12 cells treated with cadmium telluride nanoparticles was very low and indistinguishable from the autofluorescence of the cells. CdSe/ZnS nanoparticles with the same surface (mercaptopropionic acid, net negative charge) and the PEGylated ones with the same cores could be barely detected in the low nanomolar concentration range (0.1-1 nM), but MFI was significantly higher with treatments in the 10 nM concentration (Figure 1). By contrast, InGaP/ZnS nanoparticles were detectable in cells even in 1

nM concentration and the MFI was significantly higher compared with other nanoparticles in the 10 nM concentration ($p < 0.05$). The MFI value for 10 nM InGaP/ZnS nanoparticles was 875.05 ± 41.69. Similar trends in relative fluorescence intensity values were observed in living N9 microglia cells treated with the equivalent (10 nM) InGaP/ZnS nanoparticles for 4 h (Figure 1B). Spectrofluorometric determinations of internalized nanoparticles yielded similar results confirming that InGaP/ZnS nanoparticles are the most promising tools for imaging and subcellular distribution studies (not shown). Preliminary measurements of internalized concentrations of InGaP/ZnS nanoparticles at different times after the initial exposure showed InGaP/ZnS in approximately 2 nM cytoplasmic concentration after 4 h of exposure. The concentration increased (4.3-5.0 nM) after 10-12 h and did not significantly increase after 24 h (5.3-5.9 nM). These data are consistent with internalization mechanisms for CdSe/ZnS quantum dots recently suggested by Zhang [41] .

Fluorescent microscopy (Figure 1C) analyses further confirmed that the strongest fluorescent signal was observed with InGaP/ZnS nanoparticles (Figure 1C, panel 6; the top panels 1, 2 and 3 correspond to control cells without nanoparticle treatment but with only 30 µl of cell culture media added. Panels 4, 5 and 6 show cells treated with nanoparticles dispersed in 30 µl). On average 150-200 cells were analyzed per condition. Typical appearances of single cells containing fluorescent nanoparticles (bottom panels) and their corresponding controls (without nanoparticle treatment, top panels) are shown. Some PC12 and microglia N9 cells did not internalize nanoparticles, regardless of the time of exposure (1-3 days) and within 1-10 nM concentrations. A strong signal (panel 6) from the internalized InGaP/ZnS nanoparticles suggested the existence of aggregated nanoparticles (notice a punctate distribution of InGaP/ZnS nanoparticles in the cytoplasm but not in the nucleus). Carboxylic groups found at the terminal end of the capping ligand add coulombic repulsion to sterically protect the nanoparticles. Therefore, the loss of the hydrophilic MHDA capping ligand can induce aggregation of bare InGaP/ZnS nanoparticles [43] . In addition, the smallest change in pH may cause aggregation in the cytoplasm due to the high affinity between nanoparticles [44] . Neither InGaP/ZnS nanoparticles nor CdSe/ZnS nanoparticles were blinking, suggesting intracellular nanoparticle aggregates.

In order to determine more precisely the intracellular localization sites of InGaP/ZnS nanoparticles in different organelles, confocal microscopy was performed and z-stacks from individual cells from the primary cortical cultures were analyzed. These cortical cells in primary cultures consist of several different cell types, including different glial cells and neurons. Initially, for *in vitro* cortex imaging, primary neural cerebral cultures from 5-day-old mice were characterized by immunostaining using specific antibodies to identify glial cells and neurons (Figure 2). Most of the cells were astrocytes (>70%) as revealed by antibodies recognizing glial fibrillary acidic protein; relatively smaller populations were neurons (10-15% were [beta]-III tubulin positive cells) and microglia (<10% cells were Iba-1 and Mac-2 positive).

Primary cultures were maintained for 7 days *in vitro* prior to being treated with InGaP/ZnS nanoparticles and analyzed by confocal fluorescent microscopy. This approach allowed for the generation of z-stacks and a closer look at nanoparticle localization in association with the plasma membrane and intracellular localizations. The localization of the InGaP/ZnS nanoparticles within glial cells and intracellular organelles was performed in live cells using selected vital dyes for mitochondria and acidic organelles.

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Figure 2. Immunostained neural cells in primary cortical mouse dispersed cultures.

(A) GFAP-positive cells (astrocytes); **(B)** [beta]-tubulin (neurons); **(C)** Iba-1- and Mac-2-positive microglia. All pictures were taken with 20×.

GFAP: Glial fibrillary acidic protein.

Since microglia are the major nanoparticle 'consumers', we immunolabelled astrocytes and microglia (green) and assessed whether InGaP/ZnS nanoparticles were taken up by both astrocytes and microglia, or preferentially one glial cell type (Figure 3). Immunostaining with antibodies specific for astrocytes (Figure 3A-C) and microglia (Figures 3D-F) showed that InGaP/ZnS nanoparticles were largely taken up by microglia (Figures 3E & F). In addition, cell-sorting analysis of nanoparticle-treated microglia cells not mixed with any other cell type confirmed that microglia avidly took up InGaP/ZnS nanoparticles (Figure 3G).

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Figure 3. Uptake and subcellular distribution of InGaP/ZnS nanoparticles in primary cortical cultures after 4 h of treatment.

(A-C) Astrocytes were labeled with Abs against GFAP and **(D-F)** microglia were labeled using Mac-2 Abs. Scale bar = 20 μm. **(G)** The uptake of InGaP NPs (10 nM) in microglia N9 after 4 h of treatment as assessed by fluorescence-activated cell sorting. All measurements were done in triplicates from three independent experiments and the values are expressed as the mean ± standard error of the mean. Significant difference when $p < 0.01$. Nuclei are shown in blue.

*$p < 0.01$.

Ab: Antibody; a.u.: Arbitrary units; GFAP: Glial fibrillary acidic protein; InGaP/ZnS: Indium gallium phosphide-zinc sulfide;

NP: Nanoparticle; RFI: Relative fluorescence intensity.

Since the high uptake of nanoparticles can cause untoward effects, with the final outcome being cell death, we used two different methods to assess if the cell membranes of cells exposed to InGaP/ZnS nanoparticles become leaky or cause cells to die. An assessment of plasma membrane leakage, using propidium iodide, and cell viability in these primary cortical cultures

and microglia did not reveal significant cell loss or plasma membrane leakage with up to 10 nM concentrations of InGaP/ZnS nanoparticles 1-4 h or 24 h after exposure, but there was approximately 5% cell loss measured after 48 h (data not shown). Studies showing a significant reduction in cell viability after exposure to CdTe nanoparticles were reported [3,40,45] and compared with other nanoparticles [39] .

To assess the subcellular distribution of InGaP/ZnS nanoparticles in glial cells, vital dyes were used for labeling acidic organelles (LysoTracker), mitochondria (MitoTracker) and lipid droplets (Bodipy 493/503). A 3D reconstruction of cells from z-stacks and Imaris software were used to determine colocalization of nanoparticles with organelles by determining the volume overlaps between the nanoparticles and organelles. Colocalization of two labels means that the two signals are close enough that they cannot be resolved optically. The Imaris colocalization module permits the analysis of the entire confocal stack by measuring the intensity of each label within each voxel. The program uses an iterative procedure to determine an intensity threshold of the dynamic range (0-255 scale of pixel intensity) for each of the two labels [38] . The extent of colocalization is expressed by 'percentage of material colocalized' or 'relative volume overlap' (RVO), given by the equation, where SAi is the intensity of the ith voxel for label A, *i* ε*coloc* are the voxels of label A with colocalization with label B, and *i* ε*objectA*are the voxels above threshold for label A.

[Formula omitted]

The data gathered from this equation produce a colocalization measure for each of the labels. The advantage of this approach is that it takes into account the number of voxels with colocalization as well as the intensities ('immuno-data') of the two labels in each voxel. A representative picture of multicolor-labeled organelles in primary cortical cultures exposed to InGaP/ZnS nanoparticles and quantification of volumes occupied by the nanoparticles is shown (Figure 4). A total of six fields (20-30 cells) were analyzed for each paradigm. An assessment for the RVO indicated that the colocalization of InGaP/ZnS nanoparticles with mitochondria was very low (less than 2%), Pearce coefficient was 0.003, whereas the colocalization with acidic compartments was significantly higher (five- to sixfold higher than in mitochondria; Figure 4J, middle bar). There was a significant increase in the number of InGaP/ZnS nanoparticles in lysosomes after oleic acid treatment from 17% to 58%, Pearce coefficient 0.41. Also, there was an increase in colocalization of InGaP/ZnS nanoparticles to lipid droplets after oleic acid treatment from 13% to 46%, Pearce coefficient 0.3. Lipid droplets, organelles not previously explored as a subcellular interaction site with nanoparticles, showed small but significantly higher colocalization than mitochondria in astrocytes and microglia (Figure 4G-I and lipid droplets bar, Figure 4J).

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Figure 4. Multiple organelle labeling and subcellular location of InGaP/ZnS NPs in live primary cortical cells.

Confocal images showing organelles stained with vital dyes for MT (MitoTracker Green FM, 0.5 µM; 3 min; **A & C**); lysosomes (LysoTracker green-DND 26, 0.5 µM, **D & F**); LDs (Bodipy 493/503, 0.01 mg/ml, 3 min, **G & I**) and InGaP/ZnS NPs (10 nM, **B,E & H**). An overlay of individual organelles with InGaP/ZnS NPs are shown in (**C,F & I**). Panel **(J)** at the bottom shows mean values and STDs for the RVO between the organelles (MT, Lyso, LDs) and InGaP NPs from triplicate measurements in three independent experiments. RVO of InGaP/ZnS NPs with lysosomes was significantly larger ($p < 0.05$) than in MT or LDs **(J)** .

InGaP/ZnS: Intraorganelle distribution of indium gallium phosphide-zinc sulfide; LD: Lipid droplet; Lyso: Acidic organelles; MT: Mitochondria; NP: Nanoparticle; RVO: Relative volume overlap; STD: Standard deviation.

We next studied whether cotreatment with oleic acid, a fatty acid well known for inducing lipid droplets in many cell types, would affect the uptake and distribution of InGaP/ZnS nanoparticles [22,46] .

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Figure 5. OA treatment of primary cortical mixed-cell cultures and microglia enhances InGaP/ZnS nanoparticle uptake and promotes their colocalization with lipid droplets and lysosomes.

(A) Uptake of InGaP/ZnS nanoparticles in microglia. Ordinate: RFI. A minimum of 5000 cells were analyzed (in triplicate) for each condition (two independent experiments) for uptake experiments using fluorescence-activated cell sorting analysis. **(B & C)** Confocal images of mitochondria, **(D & E)** lysosomes and **(F & G)** lipid droplets before and after OA treatment. **(H-J)** show quantitative data from confocal microscopy. The determination of RVO used using Imaris software. Changes were considered significant when $p < 0.05$.

*$p < 0.05$.

a.u.: Arbitrary units; Ctrl: Control; InGaP/ZnS: Intraorganelle distribution of indium gallium phosphide-zinc sulfide; OA: Oleic acid; RFI: Relative fluorescence intensity; RVO: Relative volume overlaps; STD: Standard deviation.

Oleic acid treatment (400 µM, 24 h) induced lipid droplet formation in primary cortical cells. Cell sorting analysis showed that oleic acid-treated cells internalize significantly larger quantities of InGaP/ZnS nanoparticles than the untreated cells (Figure 5A). To assess the intracellular location of nanoparticles in cells exposed or unexposed for 24 h to oleic acid, confocal microscopy was performed. Confocal images of mitochondria (Figures 5B & C), lysosomes (Figures 5D & E) and lipid droplets (Figurse 5F & G) show that the most marked effects of oleic acid are seen within cells labeled with Bodipy 493/503, indicating the presence of lipid droplets.

Determination of organelle volumes and their overlaps with volumes of InGaP/ZnS nanoparticles in cells without and with oleic acid treatment show significant differences (Figures 5H & J). The bar graphs show the mean values and standard deviations for the RVO from triplicate measurements in three independent experiments for mitochondria, lysosomes and lipid droplets. Oleic acid did not change the RVO significantly for InGaP/ZnS in mitochondria, (Figure 5H) but there was a significant increase in the RVO between nanoparticles and lysosomes (Figure 5I) after oleic acid treatment (400 µm, 24 h). Also, oleic acid significantly enhanced the RVO between lipid droplets (Figure 5J) and InGaP/ZnS nanoparticles.

Lavado and coworkers reported that the addition of oleic acid modified the uptake of glucose in cultured rat astrocytes [47] , and others demonstrated significant acidic drug penetration through the skin by long-chain unsaturated fatty acids (i.e., oleic acid). It is possible that the enhanced uptake of InGaP/ZnS nanoparticles by microglia treated with oleic acid reported here could be attributed to their amphiphylic character-modifying quantum-dot hydrophobicity. Alternatively, oleic acid may potentially disrupt and increase the fluidity of the lipid packing in the cell membrane, due to its kinked structure arising from the *cis* double bond, and hence decrease the diffusional resistance to permeants[48] . Recent studies by Khatchadourian *et al.* showed that oleic acid treatment can temporarily increase PC12 cell viability under starvation conditions by providing fuel and reducing the extent of oxidative stress caused by cadmium telluride nanoparticles [42] . Further internalization and subcellular distribution studies are warranted to better understand the mechanism behind the oleic acid enhancement of internalization effects and modulation of cellular functions in cells from the CNS.

InGaP/ZnS nanoparticles were previously studied in proximal renal porcine tubule cell line LLC-PK-1 and in rat PC12 cells but not in glial cells[7,8] . In these previous studies InGaP/ZnS nanoparticles were found to be less toxic than CdSe nanoparticles in nanomolar concentrations[7,8] . Several recent reports show that bionanoparticles and artificial nanoparticles can enter brain tissue [26,49-54] , therefore excessive uptake of nanoparticles and distribution in cell organelles as in glia may have adverse effects on the functions of these and neighboring cells.

Microglia and astrocytes respond to nanoparticles even if they are exposed to minute quantities of nanoparticles, barely or not even detectable by common biochemical or biophysical means. We have previously demonstrated that the degree of activation of astrocytes was directly related to the physicochemical properties of tested nanoparticles and it was clear that luminescent nanoparticles with reactive and unprotected surfaces markedly activated astrocytes at the site of administration [28] . However, PEGylated CdSe nanoparticles induced only a small and transient activity of astrocytes surrounding the injection site [31] . It was difficult to obtain a more detailed subcellular distribution pattern from fixed tissue slices and, therefore, in the present study we explored InGaP/ZnS-nanoparticle distribution in living cell organelles using vital dyes. Data from confocal micrographs demonstrate that nanoparticles made of type III/V elements (indium and gallium) were distributed within several organelles in primary cortical cultures.

Among the best-known distribution studies with nanoparticles are those with gold nanoparticles, because their shapes, sizes and high density allow for detailed analysis using an electron microscope (EM) [55] . Nanoparticles can be seen using an EM in fixed cells and are readily detected in living cells by fluorescence microscopy. However, optically degenerate nanoparticles require silver staining for direct visualization in biological samples under bright-field microscopy [56] . Fluorescence studies of nanoparticles in living cells are complementary to EM studies. An advantage of the approach illustrated here is that 3D reconstructions from z-stacks (from living cells) avoid potential artifacts owing to the inevitable cell fixation necessary for preparation of EM samples.

The common fatty acid, oleic acid, is widely explored as one of the omega-9 fatty acids playing a role in development, differentiation and death of neural and other cells [57,58] . The present study demonstrates that oleic acid induces lipid droplet formation and enhances the distribution of nanoparticles within glial cells (Figure 6). This finding is relevant to studies aimed at the application of nanoparticles for medical purposes.

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Figure 6. Changes in InGaP/ZnS nanoparticle uptake and distribution in glial cells without and with oleic acid treatment.

InGaP/ZnS: Indium gallium phosphide-zinc sulfide; QD: Quantum dot.

Although levels of free fatty acids are strictly controlled in the body, there are circumstances when they are elevated or very high [59] . Under normal physiological conditions, they will be high during periods of fasting (overnight fast); otherwise, persistently elevated levels of free fatty acids are a sign of metabolic problems or disease such as obesity or diabetes [59] . Thus, the metabolic status of the organism has to be taken into consideration when testing various nanoparticles for their distribution, and their potential hazard or benefit. Further studies are needed in single cells and whole animals to better understand effects of fatty acids on uptake and distribution of nanoparticles.

Lipid droplets induced by oleic acid treatment may facilitate subcellular redistribution of nanoparticles and change interorganelle communication. Examples of functional interaction between lipid droplets and other organelles have been previously reported [60] . It was shown that lipid droplets are frequently seen in close proximity (6-10 nm) to mitochondria in porcine oocytes, as revealed by fluorescence resonance energy-transfer analyses. The authors have postulated that lipid droplets are an important source of energy substrate for the developing oocytes [61] . We have also observed a change in interorganelle communication between lipid droplets and lysosomes in PC12 cells treated with or without oleic acid. This change in interorganelle communication resulted in changes in cell viability [42] . The interaction between lipid droplets and other cellular organelles is a new area of research relevant to biological applications of nanoparticles.

The oleic acid augmentation of nanoparticle colocalization with lipid droplets could be interesting in many aspects of cell biology and animal studies, because disturbed lipid distribution and metabolism is associated with and/or casually related to numerous pathologies [62] . Thus, distribution of nanoparticles could be affected in normal and pathological states where lipid composition is altered. In addition, abnormalities in endogenous lipid distribution and intake of high-fat diets could considerably change nanoparticle cellular and tissue biodistribution, resulting in altered cellular functions.

Conclusion

InGaP/ZnS nanoparticles are taken up by glial cells of the CNS. Their subcellular distribution is altered within the organelles in the presence of oleic acid, which induces lipid droplet formation. Oleic acid treatment markedly enhances InGaP/ZnS-nanoparticle internalization and promotes colocalization with lipid droplets in glial cells. Since oleic acid and other fatty acids are common constituents of food, they could also alter internalization and organelle distribution of other nanoparticles, ultimately affecting cellular functions.

Future perspective

Fluorescent nanoparticles, particularly those emitting in the far red and infra-red spectral regions, are increasingly being used to investigate the interactions between cells and nanoparticles, in addition to the fate of nanoparticles in different tissues. This study demonstrates that oleic acid, an omega-9 fatty acid, induces lipid droplet formation in glial cells, resulting in the recruitment of nanoparticles and a change in the subcellular distribution of this organelle. Treatment of neural cells with oleic acid enhances the internalization of nanoparticles by glial cells, especially by microglia. Further studies should be carried out to understand the mechanisms involved in fatty acid promotion of nanoparticle entry into cells and how they alter cellular functions. Since lipid droplets interact with other organelles, these interactions could be disturbed under pathological conditions, ultimately affecting nanoparticle fate. Future *in vivo* studies will demonstrate if and how nanoparticle fate is altered in animal models of different diseases (e.g., metabolic syndrome and diabetes) so that better imaging systems can be designed as diagnostics and drug carriers for therapeutic interventions in states of disturbed metabolism.

Table 1. Properties of nanoparticles.

Core material	**ZnS-coated**	**Surface polymer**	**Size (nm)**	**Surface charge**	**λ$_{ex}$(nm)**	**λ$_{em}$(nm)**
CdTe	No	None	2.8	Negative	<400	535
CdSe	Yes	None	4.3	Negative	<400	595
CdSe	Yes	PEG	18-20	Neutral	<400	705

InGaP	Yes	None	5.0	Negative	<400	680
InGaP (Evident Tech)	Yes	PEG-COOH	18-20	Negative	<400	703

λ$_{ex}$: Wavelength excitation; λ$_{em}$: Emission wavelength; CdSe: Cadmium selenide; CdTe: Cadium teluride; InGaP: Indium gallium phosphide; PEG: Polyethylene glycol; ZnS: Zinc sulfide.

Executive summary

Indium gallium phosphide-zinc sulfide nanoparticles for real-time imaging

* Indium gallium phosphide-zinc sulfide (InGaP/ZnS)-coated nanoparticles are higly luminescent (E_{max} = 680 nm) and compare favorably to CdSe/ZnS nanoparticles.

* They are suitable for both single-cell and whole-animal imaging in real-time.

Subcellular distribution of InGaP/ZnS nanoparticles in organelles

* Multicolor imaging of organelles in living neural cells treated with InGaP/ZnS nanoparticles reveal their distribution in several organelles, including lipid droplets and acidic organelles.

Effect of oleic acid treatment on uptake & subcellular distribution of InGaP/ZnS nanoparticles

* Uptake of InGaP/ZnS nanoparticles is significantly enhanced in cells treated with an unsaturated omega-9 fatty acid, that is, oleic acid.

* Oleic acid treatment induces lipid droplet formation in cortical cultures.

* Lipid droplet induction by oleic acid treatment causes subcellular redistribution of nanoparticles and changes i nterorganelle communication.

* Enhancement of InGaP/ZnS uptake could be attributed to the ability of oleic acid to modify the hydrophobicity of nanoparticles and the formation of lipid droplets in cells.

* The current study suggests that nanoparticle subcellular distribution will be affected by different intra- and extra-cellular contents of unsaturated fatty acids.

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The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.