Uptake of silica nanoparticles in the brain and effects on neuronal differentiation
using different *in vitro* models

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Abstract

Nanomedicine offers a promising tool for therapies of brain diseases, but they may be associated with potential adverse effects. The aim of this study was to investigate the uptake of silica-nanoparticles engineered for laser-tissue soldering in the brain using SH-SY5Y cells, dissociated and organotypic slice cultures from rat hippocampus. Nanoparticles were predominantly taken up by microglial cells in the hippocampal cultures but nanoparticles were also found in differentiated SH-SY5Y cells. The uptake was time- and concentration-dependent in primary hippocampal cells. Transmission electron microscopy experiments demonstrated nanoparticle aggregates and single particles in the cytoplasm. Nanoparticles were found in the endoplasmic reticulum, but not in other cellular compartments. Nanoparticle exposure did not impair cell viability and neuroinflammation in primary hippocampal cultures at all times investigated. Neurite outgrowth was not significantly altered in SH-SY5Y cells, but the neuronal differentiation markers indicated a reduction in neuronal differentiation induction after nanoparticle exposure.

Keywords: nanomedicine; neuroinflammation; nanoparticle uptake; neurodifferentiation.

Background

The use of nanoparticles (NPs) in food production, agriculture, cosmetics, and medicine increased tremendously in recent years due to the enormous potential of NPs in these fields [1]. Although nanomedicine seems promising and offers innovative opportunities also as therapy of brain diseases, their potential to cause adverse effects, e.g. inflammatory reactions, possible neurotoxicity and neurodegeneration, needs to be evaluated.

The severity of cytotoxicity has been shown to depend on the chemical composition, size, shape, aggregation, surface chemistry and surface energy of the NPs used [2]. Thus, understanding effects of NP exposure including neurotoxicity is important to address the safety of newly synthesized NPs, especially when the particles are used in biomedical applications.

Translocation and accumulation of TiO₂ NPs have been demonstrated after nasal administration in mice leading to oxidative stress, proliferation of glial cells, tissue necrosis, and hippocampal cell apoptosis. Moreover, this NP exposure was associated with altered regulation of genes related to immune response, memory, learning, brain development or neurodegeneration [3]. Intranasal instilled TiO₂ NPs were shown to cause histopathological changes in the hippocampus and high inflammatory responses by elevated levels of tumor necrosis factor α (TNF α) and interleukin β (IL-1 β) [4]. Activation of microglia is observed rapidly in case of brain injury and it contributes to neuronal vulnerability due to synthesis and release of immunomodulators such as cytokines and chemokines [5, 6]. Polystyrene-NPs of different sizes were demonstrated to exert no effect on cell viability in different neural cells *in vitro*. The particles changed the metabolic activity in a cell-dependent

manner, but cellular damage was only found at very high NP concentrations [7]. In the models used, neurons did not internalize particles, while microglial cells ingested a large amount of carboxylated but almost no PEGylated NPs [7].

Our study aimed at investigating possible effects of NPs designed for laser tissue soldering in the brain [8], e.g. cerebral bypass surgery as described previously [9, 10]. In a previous study, NP exposure did not result in increased cytotoxicity and apoptosis in microglial and neuron-like cell lines even though a transient depletion of glutathione indicating reactive oxygen species (ROS) formation was measured [9]. Moreover, NPs were demonstrated to be taken up by microglial cells in a time- and particle-dependent manner [10], but no modulation of cytokine secretion and autophagy was found [10].

Besides microglia, a possible uptake of the NPs in neurons and other cells of the brain needs to be investigated as well as the effects the NPs may have on these cells.

Ex vivo models including organotypic brain slices and dissociated primary hippocampal cells offer certain advantages because they mimic the *in vivo* situation in more detail. Human SH-SY5Y cells have been used as an *in vitro* model for neuroscience and neurotoxicity experiments and these cells have been extensively used to study *in vitro* neurodegenerative diseases [11-16]. Therefore, in the present study, the effects of two Si-NPs types, (ICG)/polycaprolactone (PCL)-rhodamine-doped NPs (PCL-NPs) and Si-ICG/polycaprolactone-poly(lactic acid) (PLLA)-rhodamine-doped NPs (PLLA-NPs) on neurotoxicity and neuronal differentiation were investigated in distinct brain cell culture models including differentiated SH-SY5Y cells, organotypic hippocampal slice cultures and primary dissociated hippocampal cultures. The uptake of the two silica NP types, their intracellular targets and their

potential pro-inflammatory effects were analysed. Besides the qualitative analysis of NP uptake in cell organelles, quantitative analyses offer improved tools to explore cellular uptake.

Methods (see supplementary S1)

Cell culture models

Organotypic hippocampal slice cultures - Hippocampi were prepared as described previously [17]. Briefly, 16 Wistar rat pups (Janvier Elevage, France) were sacrificed on postnatal day 7 by a lethal dose of pentobarbital (150 mg/kg i.p., G. Streuli & Cie. SA, Uznach, Switzerland). Animal experiments were performed in accordance with institutional guidelines and national regulations. All experimental protocols were approved by the Animal Research Ethics Committee of the Canton Bern, Switzerland.

Dissociated primary hippocampal cultures - Primary cultures were used for cell viability assessment and detailed uptake analysis. Hippocampal cell suspensions were obtained using a slightly modified protocol compared to the one described previously [18, 19].

All cultures were randomly assigned to the NP groups and to untreated controls (without NP exposure).

Neuronal cells - SH-SY5Y cells were obtained from ATCC (Manassas, VA, USA), and culturing was done as previously described [9]. At day *in vitro* (DIV) 1, the cells were exposed to NPs in the same medium supplemented except only containing 1% FBS for 24 h at the end of the exposure (DIV2). The medium containing NPs was removed and cells were cultured with the same medium used for NP treatment, but supplemented with [10 μ M] retinoic acid (RA) to induce differentiation.

Nanoparticle exposure

PCL-NPs and PLLA-NPs were obtained from the Department of Chemistry and Bioanalytics, Academy for Life Science, Muttentz, Switzerland. NP synthesis and characterization as well as the, chemical and physical properties of PCL-NPs have been described previously [8-10]. The average NP size was 90 nm for PCL-NPs and 95 nm for PLLA-NPs and the Zeta potential was -25.4 mV for PCL-NPs and -15.9 mV for PLLA-NPs. An amount of 5 μ l of the respective NP suspension was deposited on top of the organotypic slices at DIV8 for one day or five days. For both primary cell cultures and SH-SY5Y cells, final NP concentrations of $[2.6 \times 10^9]$, $[5.2 \times 10^9]$, $[2.6 \times 10^{10}]$ PCL-NPs/ml and $[2.9 \times 10^9]$, $[5.8 \times 10^9]$, $[2.9 \times 10^{10}]$ PLLA-NPs/ml were used.

Nanoparticle uptake (Immunofluorescence staining, transmission electron microscopy)

At the end of the culture period (see supplementary S1), cultures were fixed with 4% paraformaldehyde. Briefly, after one hour blocking with 10% normal horse serum in 0.4% Triton-X PBS, primary antibodies were applied overnight at 4°C (mouse anti-GFAP (1:1000), rabbit anti-Iba-1 (1:500), mouse anti- β -3-tubulin (1:500), chicken anti-MAP2 (1:1000), mouse anti-calnexin (1:500), rabbit anti-giantin (1:1000) and mouse anti-ATPB (1:500) in 0.4% Triton-X PBS for both cryosections and cultures. The obtained pictures were analyzed using a Zeiss Axio Imager Z1 coupled with an Apotome 1 (Carl Zeiss Vision Swiss AG, Feldbach, Switzerland).

For cellular uptake analysis of primary cells, the cell cytoskeleton was stained using acti-stain 488 phalloidin and nuclei using Hoechst.

Transmission electron microscopy (TEM) experiments in primary cell cultures were performed as described previously [9]. NP uptake was measured in a quantitative manner using high-content analysis [10].

Assessement of cell viability and neuroinflammation

Cell viability of primary cells exposed to NPs and the respective controls was evaluated by Presto Blue reagent (Life Technologies, Switzerland).

IL-1 β (Invitrogen, Switzerland) and TNF α (Life Technologies, Switzerland) were analyzed by ELISA according to manufacturer's protocol.

In organotypic slice cultures the number of Iba-1 positive cells (was detected by high-content analysis and reported as percentage of the total number of cells. Iba-1 positive cells in each field were analyzed per slice and expressed as number of Iba-1 cells per area unit. Three serial sections for 3 slices per group were analyzed and 6-12 fields were counted for each section.

Neuronal differentiation

Differentiated SH-SY5Y cells were stained with β -3-tubulin and Hoechst 33342 and acquired with the IN Cell Analyzer 2000 (General Electric Healthcare, Glattbrugg, Switzerland). A number of 4 to 30 regions (= fields) per well was selected and images were obtained using a wide field epifluorescence microscope. Images were analyzed using the IN Cell Investigator 1.6.2 software (GE Healthcare, Glattbrugg, Switzerland).

Measurement of PI3-k/Akt and MAP-K/ERK in differentiating SH-SY5Y cells

SH-SY5Y cells were incubated with NPs for 24 h before differentiation was induced by [10 μ M] all-trans retinoic acid (RA, Sigma). On day *in vitro* (DIV) 6, protein extraction was performed and the samples were analyzed by Western Blot. Briefly,

NP-treated SH-SY5Y cells were lysed in protein lysis buffer containing phosphatase and proteinase inhibitors. The lysed cell suspension was incubated on ice for 15 min, then sonicated for 10 sec at 40% amplitude and centrifuged at high speed for 10 min at 4°C. The protein content of the supernatant was quantified using the Pierce protein assay reagent (Bio- Rad Laboratories, CA, USA). Equal amounts of protein from each test sample were loaded and subsequently separated on 12% SDS-PAGE gels before the protein was transferred onto PVDF membranes. Subsequently, the primary antibodies, rabbit anti-phospho-Akt (1:1000), rabbit anti-Akt (1:1000), mouse anti-phospho-pMAP-K (ERK1/2) (1:2000), mouse anti-pMAP-K (ERK1/2) (1:2000), mouse anti-MAP-2 (1:500) and the monoclonal mouse antibody anti-β-actin (1:10,000). Blot quantification was performed using ImageJ analysis measuring the ratio between the intensity of the obtained band of a specific marker versus the corresponding band of actin using arbitrary units.

Statistical analysis

A one-way ANOVA was conducted, followed by the Dunett's test or Tukey's multiple comparison tests to compare the mean of all treatments with the respective controls (GraphPad Software Inc., La Jolla, USA). P-values ≤ 0.05 were considered significant. Three independent experiments were performed in triplicates for all analyses. Data are presented as mean + standard error of the mean (S.E.M.).

Results

Analysis of NP uptake in distinct *in vitro* models

The uptake of both NP types in SH-SY5Y cells was analyzed after 24 h exposure followed by 6 days of differentiation using RA. At the end of the differentiation period, 31.6 +/- 2% and 43.8 +/- 2.8% neurons had taken up PCL- or PLLA-NPs, respectively (Figure 1A). NP co-localization is shown in differentiated SH-SY5Y cells (Figure 1B).

In rat primary hippocampal culture, the quantitative uptake analysis showed a time- and concentration-dependent NP uptake as illustrated in Figure 2. Longer incubation times resulted in an increased NP uptake when the analysis was performed for each concentration separately (Figures 2A and 2B). A comparison of same incubation times revealed a concentration-dependent NP uptake (Figures 2C and 2D). The number of cells containing PCL-NPs was found to be significantly higher after an exposure time of 6 h and 24 h at a concentration of $[2.6 \times 10^9]$ and $[5.2 \times 10^9]$ PCL-NPs/ml and after a 24 h exposure at $[2.6 \times 10^{10}]$ PCL-NPs/ml when compared to an exposure time of 1 h. For PLLA-NPs, the differences were statistically significant and more pronounced for all times and for each concentration tested. The maximal uptake of both particle types was reached after 24 h for the lowest concentration investigated, 77.4 +/- 2.1% and 76.8 +/- 1.4% for PCL- and PLLA-NPs, respectively. An equivalent uptake rate was already achieved after 1 h for the highest concentration $[2.6 \times 10^{10}]$ PCL-NPs/ml and $[2.9 \times 10^{10}]$ PLLA-NPs/ml (69.8 +/- 1.3% and 74.2 +/- 0.7% for PCL- and PLLA-NPs, respectively). The number of NP-positive cells significantly increased with increasing concentrations of PCL-NPs for the same exposure times. Significant differences between PCL-NP concentrations for each exposure time are depicted in Figure 2C. After PLLA exposure, the number of NP-positive cells significantly increased in a concentration-dependent manner within

1 same exposure time. Significant differences between PLLA-NP concentrations for
2 each exposure time are depicted in Figure 2D.
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5 A comparison of the cytoskeleton staining of rat primary hippocampal cells incubated
6 with both NP types revealed no difference in morphology or organisation of the cell
7 skeleton when compared to control cells (data not shown). In accordance with these
8 observations, no significant difference in cell viability was found for rat primary
9 hippocampal cells exposed to NPs for 6 h and 24 h in comparison to non-exposed
10 control cells. Cell viability remained above 80% for all concentrations and exposure
11 times tested (Table 1A-1B). A cell specific analysis of the percentage of neurons (β -
12 3-tubulin-positive) versus total cells was performed in NP-exposed cells and control
13 cells indicating no neuron-specific neurotoxicity.
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16 NP uptake was also investigated using organotypic hippocampal slice cultures
17 exposed for 24 h and 5 days to both NP types. No time-dependent difference was
18 observed between the 2 exposure times and for PCL- and PLLA-NPs. PLLA-NPs
19 were found in microglial cells as shown in Figure 3A but not in astrocytes (GFAP-
20 positive cells) and neurons (MAP-2- positive cells) after exposure to $[5.2 \times 10^9]$ PLLA-
21 NPs/ml (Figures 3B and 3C). Similar results were obtained with PCL-NPs at a
22 concentration of $[5.8 \times 10^9]$ NPs/ml (data not shown).
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25 For technical reasons, the high-content analysis could not be performed in the
26 organotypic slice cultures because of the thickness of the slices. Therefore, we used
27 a dissociated primary culture model consisting of rat hippocampal cells. In this model,
28 both NP types were found in microglial cells but not in neurons after an exposure
29 time of 24 h (Figure 4A). NP co-localization was observed with the endoplasmic
30 reticulum (ER) marker (Figure 4B) indicating that both particle types entered this
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compartment (only PLLA-NPs are shown). In contrast, no particles were found in neurons (Figure 4C), mitochondria (Figure 4D) and the Golgi apparatus (Figure 4E).

TEM analysis demonstrated that both NP types were observed in the cytoplasm in either vacuole-like structures (Figure 5B) or free (Figure 5C).

Effect of NP exposure on neuro-inflammation

In primary hippocampal cells, the production of IL-1 β and TNF α measured 24 h after NP exposure (Table 2) revealed no significant change in the release of both cytokines. ELISA measurements of cytokine secretion in the culture medium performed after NP exposure for 24 h incubations did not change for both NP types in dissociated primary hippocampal cells when compared to untreated control cells.

In the *ex vivo* slice culture model, a semi-quantitative analysis of microglial cell numbers was performed to evaluate potential inflammatory responses. No significant differences in the number of microglial cells were found when rat brain slices exposed to NPs and untreated control slices were compared for an exposure time of 1 day and 5 days indicating that both silica NP types used in our study do not induce inflammatory response. These results are depicted in Figure 6.

Effect of NP exposure on neuronal differentiation

Possible effects of NPs on neuritogenesis were investigated by analyzing the morphology of SH-SY5Y cells, namely the length and the number of neurites after exposure to the highest NPs concentration tested in primary cultures, i.e. [2.6 x 10¹⁰] PCL-NPs/ml or [2.9 x 10¹⁰] PLLA-NPs/ml. In the model used, a significant reduction

of cellular processes smaller than 11 μm was observed after exposure to both NP types in differentiated SH-SY5Y cells. An increase in neurite length in all neuritis larger than 21 μm was found in NP-exposed compared to control cells. While this effect was seen for both NP types, it was not statistically significant (Figure 7). Measures using high-content analysis of NP-treated SH-SY5Y cells exhibited no significant alteration of neurite outgrowth in comparison to untreated differentiated control cells as illustrated in Figure 7. Quantitative analysis of the total number of neurons revealed a tendency for a higher number of neurons compared in NP-treated cells compared to untreated differentiated cells, but this effect was not statistically significant (data not shown).

Kinases have been demonstrated to be involved in neurite elongation (PI3-K/Akt) and in synaptic plasticity (MAP-K/ERK) of neurons. Therefore, we investigated the effect of NP exposure on the expression of these kinases. Four groups of SH-SY5Y cells were analyzed: undifferentiated SH-SY5Y cells (not RA treated), differentiated SH-SY5Y cells (RA treated for 6 days) and cells exposed for 24 h to both NPs types followed by 6 days of differentiation using RA. At the end of the culture period, an upregulation of phosphorylation of both Akt (P-Akt) and MAP-K (P-p42/44-MAP-K) and was found indicating an enhanced differentiation process between undifferentiated control and differentiated control SH-SY5Y cells (Figure 8). Exposure with both NPs for 24 h before differentiation resulted in a significant downregulation of phosphorylated-Akt (P-Akt) (Figure 8D) when compared to differentiated control SH-SY5Y cells at DIV6. Likewise, a significant decrease in phosphorylated-p44-MAP-K (P-p44-MAP-K) was seen in NP-exposed SH-SY5Y cells when compared to differentiated control SH-SY5Y cells ($p \leq 0.0001$) (Figure 8E). The P-p42-MAP-K was slightly upregulated in differentiated SH-SY5Y cells exposed to PCL-NPs when

1 compared to differentiated untreated control SH-SY5Y cells (Figure 8F). The
2 exposure with both NPs for 24 h before differentiation resulted in a significant
3 downregulation of the differentiation marker MAP-2 (Figure 8G).
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Discussion

Uptake

The uptake of Si-PCL- and -PLLA-NPs was investigated using *in vitro* and *ex vivo* models, namely neuron-like cells (SH-SY5Y), organotypic brain slices and primary hippocampal cells. Both NP types were taken up by SH-SY5Y cells. In contrast, in rat organotypic hippocampal slice cultures and dissociated primary hippocampal cell cultures, PCL- and PLLA-NPs were found in microglial cells but not in astrocytes or neurons. Several groups reported that physicochemical properties such as surface charge and size of particles changes the incorporation of NPs into specific cell types [20-23]. In our study, the size of both NP-types used did not differ, and despite the different charge, both NP-types were taken up by both microglia and neurons. Interestingly, the uptake differed between models used. While the SH-SY5Y cells consist of one cell type, the dissociated hippocampal cells and the rat organotypic slice cultures represent a mixed cell population. Therefore, in the latter two models, microglia cells may have taken up the NPs preferably, hence the neurons were found devoid of NPs. NPs can interact with various cellular components as already demonstrated [24], but the uptake depends on the type and physiological status of a particular cells. Uptake of silica particles was shown for neuronal cells and microglial cells [9], whereas, we only found in microglial cells in primary hippocampal culture. However, about 80% of the cells took up NPs, while the culture consisted of about 20% microglial cells only. NP-uptake was quantified using a cytoskeleton marker, whereas β -3-tubulin used for analyzing NPs in neurons only stains parts of the cytoplasm. Hence, we cannot exclude NP uptake in neurons.

Our results are in accordance with another study where internalization of diverse Si-NPs was reported in NE-4C neuroectodermal stem cells and primary forebrain

1 cultures, while neurons did not take up NPs in the 2 culture model used [25]. A
2 specific targeting of neurons, however, is possible using specifically engineered NPs
3 as shown in the hippocampal slice culture model by Walters et al. [20, 21]. Taken into
4 account that the NPs used in this study are designed for laser-assisted tissue
5 soldering in the brain, the organotypic slice cultures were chosen to compare the
6 results from the *in vitro* models (cell lines) with a model that mimics the *in vivo*
7 conditions more closely. Immunohistochemical observations obtained from this model
8 indicated that mature neurons did not take up NPs in the experimental settings used.
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11 Both NP types formed aggregates of different sizes in dissociated primary
12 hippocampal cell cultures, and they were found freely or in vacuole-like structure in
13 the cytoplasm close to the nucleus. Similar findings were reported previously for
14 differentiated SH-SY5Y cells with the exact same NP types used in this study [9]. The
15 behavior of Si-NPs forming agglomerates in vacuole-like structures in the cytoplasm
16 has been demonstrated for brain-derived primary cells (forebrain origin), NE-4C
17 neuroectodermal stem cells and mice microglial cells. The NP exposure had been
18 associated with active cellular processes, which drive particles into endocytotic
19 vesicles, lysosomes or autophagosomes by energy-dependent endocytotic or
20 intracellular sorting mechanisms [7].
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22 In this study, both NP types were observed in the ER of primary cells of the
23 hippocampus. The uptake of the particles in the ER indicates a transport from the
24 lysosomes to the ER [26] or an uptake of the nanomaterial into caveosomes, which
25 are structures that are known to migrate with the ER [27-29]. It has been reported
26 that a possible consequence of the NP uptake into the ER includes protein misfolding
27 [30, 31]. The observation that both NP types appeared as single particles or as
28 aggregates either located in vacuoles or free in the cytoplasm suggest different
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mechanisms of Si-NP uptake which might also be related to the Si-NP concentration used as previously shown in mice primary microglia [32].

We demonstrated a time-dependent uptake of both NP types in rat primary hippocampal cells. A similar finding had been demonstrated recently for the uptake of Si-NPs in mouse bone marrow dendritic cells [33]. Other studies have also shown a time- and concentration-dependent uptake of PEG-PCL-NPs by U87 glioma cells and Raw246.7 macrophage cells [34].

We performed the time and concentration-dependent analysis in primary hippocampal cultures only. In the other models, the highest concentration was used for the analysis and no effects on viability and neuroinflammation were found. In contrast, Izak-Nau et al. reported that SiO₂-NPs induce cytotoxic response in NE-4C neuroectodermal stem cells and primary forebrain culture and an increase in cell death at high particle concentrations. This effect was also dependent on the coating of the NPs [25]. Again, the characteristics of each NP types used are likely to change the NP behavior and may explain the differences observed.

Neuroinflammation

NP exposure did not affect cell viability and neuroinflammation under the experimental conditions used, in accordance with a recent report where silica particles were shown to have no significant effects on *in vitro* viability and mortality on different neural cells [7]. No recruitment of microglial cells was observed in our *ex vivo* model after NP exposure for 24 h and 5 days. The release of inflammatory cytokines was not influenced by NP exposure for 24 h in dissociated cultures. The combined results obtained from the *ex vivo* and the *in vitro* models did not show an activation of microglia.

1 If NP exposure results in NP uptake in microglia and the synthesis and release of
2 neuroinflammatory molecules, neuronal degeneration may occur. Several studies
3 showed inflammatory responses *in vivo* after exposure to TiO₂-NPs associated with a
4 significant increase in TNF- α and interleukin IL-1 β levels [4]. In contrary, our findings
5 showed no significant increase in microglial cell numbers after exposure for 24 h and
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Neurodifferentiation

A transient exposure to NPs (24 h) did not significantly impair neuronal differentiation in SH-SY5Y cells. However, these cells subjected to PCL- and PLLA-NPs for the same time resulted in a significant downregulation of activated kinases such as AKT and ERK, whereas no significant modulation of the neurite growth was found in comparison to control cells. A recent study demonstrated that Ag-NP exposure for one week promoted neuronal differentiation of SH-SY5Y cells after increasing intracellular ROS generation. In agreement with our results, the kinases AKT and ERK were also shown to be increased in SH-SY5Y cells [35]. Several research groups have linked ROS-mediated kinase activation to the neuronal differentiation process [36, 37]. We previously demonstrated a transient depletion of glutathione (GSH) in SH-SY5Y cells indicating an increase of ROS generation after exposure to the same NPs as the one used in this study [9]. Here, we demonstrated that this effect is likely to be related to a modulation in phosphorylation of AKT and ERK kinases as already demonstrated [36-38]. Hence, our results suggest that the NP exposure modulates the intracellular signaling pathways leading to a modification of

neuronal differentiation, namely neurite outgrowth, which possibly could not have been detected at the time the analysis was performed. It was reported that ERK could activate the expression of neuronal specific genes in the cell nucleus [39], and it may therefore be essential for synaptic plasticity, learning, and memory [40, 41]. The PI3K/AKT signaling pathway has been also demonstrated to improve neurite elongation resulting in an increase in the dendritic length of hippocampal neurons in primary neuronal cells [42, 43]. In contrast, Guo et al. reported that silica nanoparticles induced oxidative stress caused by activation of MAPK/Nrf2 pathway and NFkB signaling [44]. In our study, MAPK downregulation was accompanied by a significant downregulation of the mature neuron marker, MAP-2 indicating an effect on differentiation. Baranes and coworkers have shown axonal elongation rather than forming complex branching trees in primary neurons and an immature neuronal cell line (PC12) when cultivated on gold NP scaffolds [45]. In contrast, a downregulation of both kinases ERK and AKT in NP-exposed SH-SY5Y cells was found in our study indicating a potential loss of function and possibly on neurite outgrowth of SH-SY5Y cells after NP exposure. Further investigations on different and specific neuronal phenotypes, namely dopaminergic and cholinergic neurons, will be performed to further evaluate the potential risk of the NPs when used for laser-tissue soldering in the brain.

Acknowledgements

The authors thank Véronique Gaschen (Division of Veterinary Anatomy, University of Bern, Switzerland) for her excellent technical assistance and for providing the electron micrographs.

The authors thank Dr. phil. nat. Denis Grandgirard (Institute for Infectious Diseases, University of Bern, Switzerland), Microscopy and High-content analysis was performed on equipment supported by the Microscopy Imaging Center, University of Bern, Switzerland.

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Figure legends

Figure 1 SH-SY5Y cells were exposed to PCL and PLLA-NPs at a concentration of $[2.6 \times 10^{10}$ PCL-NPs/ml] and $[2.9 \times 10^{10}$ PLLA-NPs/ml] for 24 h before differentiation with RA. Quantitative high-content analysis of NP uptake was performed in differentiated SH-SY5Y cells positively-stained with the neuronal marker β -3-tubulin (A). The data represent the mean percentage of cells positive for β -3-tubulin containing NPs versus total number of cells. Error bars correspond to S.E.M. from $n = 120$ fields randomly chosen per group from 4 independent experiments. No significant differences in NP uptake were found between the two NP types. A representative photomicrograph of NP uptake (red) in SH-SY5Y cells stained with the neuronal marker β -3-tubulin (green). Cell nuclei were counterstained with Hoechst (blue). Scale bar 10 μ m (B).

Figure 2 PCL- (A, C) and PLLA- (B, D) nanoparticle (NP) uptake in rat primary hippocampal cells using cytoskeleton staining and quantitative high-content analysis. NP uptake is shown as percentage of primary cells with NPs versus the total number of cells counted after 1, 6 and 24 h incubation at NP concentrations of $[2.6 \times 10^9]$ PCL-NPs/ml and $[2.9 \times 10^9]$ PLLA-NPs/ml grey bars, $[5.2 \times 10^9]$ PCL-NPs/ml and $[5.8 \times 10^9]$ PLLA-NPs/ml dark grey bars and $[2.6 \times 10^{10}]$ PCL-NPs/ml and $[2.9 \times 10^{10}]$ PLLA-NPs/ml black bars. Error bars correspond to S.E.M.; significant differences between the different incubation times for the same NP concentration (A, B) are labeled with asterisks (* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$).

Concentration-dependent differences at the same incubation time are depicted as circles (C, D) ($^{\circ} = p \leq 0.05$; $^{\circ\circ} = p \leq 0.01$; $^{\circ\circ\circ} = p \leq 0.001$; $^{\circ\circ\circ\circ} = p \leq 0.0001$).

Figure 3 Microscopic images showing PLLA-nanoparticle (NP) uptake in rat organotypic hippocampal slice cultures. After NP exposure $[2.9 \times 10^{10}]$ PLLA-NPs/ml for 5 days, NPs (red) were mainly found in microglial cells (A) stained with the marker Iba-1 (green), but were absent in astrocytes (B) (GFAP staining, green) and neurons (C) (MAP-2 staining, green). Cell nuclei were counterstained with Hoechst (blue). The arrows depict NP co-localization with microglia, the arrowheads show NPs observed without co-localization. Magnification 400x, scale bars 10 μ m.

Figure 4 Representative microscopic images from rat hippocampal primary cultures on DIV 8 showing PLLA-nanoparticles (NPs) taken up after 24 h exposure into microglial cells (Iba-1-positive cells) (A), and in the ER compartment (calnexin-positive organelles) (B), whereas no NPs were found in neurons (β -3-tubulin-positive cells) (C), Golgi apparatus (giantin-positive compartments) (D) or mitochondria (ATPB- positive organelles) (E). Cell nuclei were counterstained with Hoechst (blue). $[2.9 \times 10^{10}]$ PLLA-NPs/ml. Magnification 400x, scale bars 10 μ m.

Figure 5 Representative transmission electron microscopy (TEM) photomicrographs of PCL-nanoparticles ($[2.6 \times 10^{10}]$ PCL-NPs/ml) (NPs) taken up by hippocampal primary cultures (DIV 8) after 24 h of exposure. Images provide subcellular details of microglial cells identified by dense heterochromatin lining the

nuclear membrane. The nucleus is visible (NC) and the localization of NPs is shown in the cytoplasm (arrowhead) and in vacuole-like structures (arrows). Magnification 5600x, scale bar 5 μm (A), scale bars 1 μm (B, C).

Figure 6 Semi-quantitative high-content analysis (In Cell Analyzer) of microglial cells stained with the microglial marker Iba-1 in hippocampal slice culture. Analysis was performed on slice culture exposed to each nanoparticle (NP) type (PCL and PLLA) for 1 and 5 days (1d and 5d). Matching controls were done in parallel (Co). The number of Iba-1 positive cells counted were reported to the total number of cells (i.e. total number of nucleus) for each field analyzed per slice (3 slices/group, 3 sections/per slice, 6 to 12 fields /section). The ratio Iba-1 cells versus total cell numbers is expressed per area unit. NP exposure did not significantly change the number of microglial cells when compared to the respective control.

Figure 7 Quantitative high-content analysis of neurite outgrowth in undifferentiated control SH-SY5Y cells, differentiated control SH-SY5Y cells and SH-SY5Y cells exposed to both NP types ($[2.6 \times 10^{10}]$ PCL-NPs/ml; $[2.9 \times 10^{10}]$ PLLA-NPs/ml) for 24 h before differentiation with RA. The data are represented as mean + S.E.M. from $n = 120$ fields per group of 4 independent experiments. Significant differences to undifferentiated controls are labelled with asterisks (**** = $p < 0.0001$). No significant difference in morphological differentiation was seen between differentiated control SH-SY5Y cells and differentiated SH-SY5Y cells first exposed to NPs.

Figure 8 Markers of the MAP kinase, PI3 kinase pathways and of mature neurons (MAP-2) were used to evaluate neuronal differentiation in SH-SY5Y cells exposed to PCL- or PLLA- nanoparticles (NPs) ($[2.6 \times 10^{10}]$ PCL-NPs/ml; $[2.9 \times 10^{10}]$ PLLA-NPs/ml) for 24 h before differentiation with RA until DIV6. Untreated controls, undifferentiated SH-SY5Y cells (Co undiff) and differentiated SH-SY5Y cells (Co diff) were performed in parallel. Representative Western blots are shown in (A, B, C). The histograms represent the variation of the ratio phosphorylated Akt (P-Akt) (D), phosphorylated p42/44-MAP-K (P-p42/44-MAP-K) (E, F) to total Akt and p42/44-MAP-K. The histogram of the ratio MAP-2/actin is shown in (G). The actin signal was used as loading control. Respective protein levels were assessed using digital quantification of immunoblots, and are presented as relative intensity compared to total protein. The analysis was done using Image J. Values are expressed with arbitrary units. Error bars represent S.E.M. Significant differences to undifferentiated controls are labeled with asterisks (*= $p \leq 0.05$; ****= $p \leq 0.0001$); significant differences to differentiated controls are labeled with plus (+ = $p \leq 0.05$; +++ = $p \leq 0.001$).

Table1A: Viability of hippocampal cultures after PCL nanoparticle exposure

Exposure time (h)	Control	[2.6 x 10 ⁹ NPs/ml]	[5.2 x 10 ⁹ NPs/ml]	[2.6 x 10 ¹⁰ NPs/ml]
6	100.0 ± 1.6	84.8 ± 7.0	92.9 ± 3.5	93.5 ± 5.3
24	100.0 ± 1.2	88.9 ± 3.3	86.4 ± 1.9	89.8 ± 4.1

Table1B: Viability of hippocampal cultures after PLLA nanoparticle exposure

Exposure time (h)	Control	[2.9 x 10 ⁹ NPs/ml]	[5.8 x 10 ⁹ NPs/ml]	[2.9 x 10 ¹⁰ NPs/ml]
6	100.0 ± 1.6	83.1 ± 1.5	98.2 ± 1.9	89.1 ± 3.8
24	100.0 ± 1.3	91.7 ± 3.5	87.8 ± 5.3	92.1 ± 2.6

Table 2: Cytokine levels in hippocampal cultures after nanoparticle exposure

Exposure	TNFα (pg/ml)	IL-1β (pg/ml)
Control	5.8 ± 0.7	54.7 ± 1.6
PCL	7.7 ± 0.3	52.5 ± 2.2
PLLA	7.8 ± 0.3	49.1 ± 1.3

Figure 1 R1

Figure 2 R1

Figure 3 R1

Figure 4 R1

Figure 5 R1

Figure 6 R1

Figure 7 R1

Figure 8 R1

Rat hippocampal primary cultures

Si-NP uptake

Microglia/NPs

ER/NPs

Organotypic rat slice cultures

Microglia/NPs

Astrocytes/NPs

Neurons/NPs

Neurons/NPs

Differentiated SH-SY5Y cells