Unraveling In Vivo Brain Transport of Protein-Coated Fluorescent Nanodiamonds

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1. Introduction

Reaching the brain with an effective therapy or imaging reagent is still considered one of the most challenging tasks in treating neurological diseases. The complexity of neurological causes and syndromes as well as the limited understanding of the exact disease pathophysiology have impeded the development of efficient therapies without long-term side effects.[1] In addition, the blood–brain barrier (BBB) still represents a major challenge for effective central nervous system (CNS) delivery of therapeutics and imaging probes. The BBB consists of brain capillary endothelial cells, pericytes, astrocytes, and neurons, all referred to as the neurovascular unit (NVU).[2] The role of the NVU is to maintain a constant homeostatic environment and to prevent xenobiotics (including many potential drugs) from entering the brain.[3] The BBB is not only a physical barrier but also a dynamic interface influenced by the individual physiological and pathophysiological conditions.[4] Thus, there is a need for a more personalized therapy for patients with CNS diseases. However, translating science into solutions for personalized therapies is still a major challenge in research and development. Personalized medicine is based on the knowledge of the individual genetics, disease status, and pharmacokinetics. Theranostics, a combination of diagnosis and therapy in one system, is becoming an attractive research field to address the transition from conventional to personalized therapeutic plans.[5,6] Many nanomaterials, such as different types of radionuclide-labeled particles like antibodies, liposomes, polymers, and micelles have already been used for theranostic applications, both in animal models and in patients.[7] Most optical tools such as fluorescent dye labels,[8] expression of fluorescent[9,10] or nonfluorescent proteins like luciferase[11] are limited by photobleaching, strong interference with autofluorescence from background tissue, and low transfection efficiencies. Poly(lactic-co-glycolic acid; PLGA) NPs are the most studied nanosystems for successful BBB crossing.[12] Nonetheless, they do not possess intrinsic optical properties useful for nanotheranostics. Additionally, in a recent study Medina et al. demonstrated that PLGA NPs were not transported to the brain in healthy animals but only in diseased animals with impaired BBB.[13,14] Inorganic nanomaterials such as quantum dots have been considered as alternatives for
brain targeting, but their cytotoxicity, for example due to release of toxic metal cations, represents a major limitation for in vivo studies. Carbon dots (CDs) also exhibit desirable optical properties and a few studies report their spontaneous BBB crossing ability. Given their recent discovery, the potential of CDs as a platform in the diagnosis and therapy of CNS diseases still needs further investigation addressing new functionalization strategies to avoid rapid clearance and heart accumulation that might induce toxicity.

Here, fluorescent nanodiamonds (NDs) were investigated as an emerging class of carbon nanomaterials providing essential features of nanotheranostics. NDs are characterized by peculiar optical and magnetic properties, biocompatibility, high thermal conductivity and electrical resistivity as well as chemical stability and nondegradability even in harsh environments. The implementation of elemental defects (e.g., nitrogen vacancy; NV) in the carbon lattice leads to an outstanding photostable far-red fluorescence with an emission maximum at 700 nm, which is higher than most cellular autofluorescence. It was shown that NV-NDs improve the sensitivity of magnetic resonance imaging (MRI) due to their dynamic nuclear spin polarization from the NV center. However, the high potential of NDs as in vivo theranostics is still limited by their tendency to aggregate in biological surroundings. Typical commercial suspensions of NDs usually contain larger aggregates hindering their biological applicability. Haziza et al. have previously shown in vitro tracking of fluorescent NDs in mouse hippocampal neurons for monitoring the biological effects of nocodazole and genetic factors. Another recent study demonstrated the neuronal action potential magnetic sensing in marine worms using fluorescent NDs. The great potential of NDs for therapeutic and bioimaging applications in neuronal diseases has been highlighted. However, in vivo studies are lacking that demonstrate NDs delivery after systemic administration across an intact BBB in vivo. The study of Xi and colleagues, for example, showed delivery of the anticancer drug doxorubicin by NDs, which adsorbed doxorubicin at the surface, after direct location into brain tumor tissue via invasive convection-enhanced delivery. Therefore, to our knowledge, it has not yet been demonstrated whether NDs are able to cross an intact BBB without invasive strategies, which would be of fundamental importance to evaluate their application in neuroscience.

Consequently, there is an urgent need for new studies, which assess the trafficking and transport of NDs across the most restrictive biological barrier, i.e., the blood–brain barrier.

In this study, NDs stabilized by a protein-derived biopolymer coating (cationic, PEGylated denatured human serum albumin; dcHSA-PEG) were evaluated for their ability to cross an intact BBB in vitro and in vivo, which is a prerequisite for their application as nanotheranostic platform for neurological disease treatment. dcHSA-NDs reveal high colloidal stability over a broad pH range (pH 2–8). The high number of reactive functional groups of the dcHSA-PEG coating provides an easy to functionalize platform for the attachment of drugs or targeting sequences, thus circumventing the disadvantage of challenging surface chemistry of uncoated NDs.

We believe that none of the reported nanoplatforms for brain targeting exhibit, simultaneously, all of the dcHSA-NDs features: 1) stable fluorescence, 2) potential for hyperpolarization of nuclear spin for magnetic resonance imaging, 3) temperature sensing, 4) colloidal stability, 5) multitude of functionalization groups on dcHSA-PEG, 6) biocompatibility. Transport of dcHSA-NDs across the BBB after intravenous injection would allow brain delivery of multiple drugs, possibility of treatment tracking via magnetic resonance and sensing of brain temperature changes occurring in a number of common human diseases in one platform. Thus, dcHSA-NDs provide not only a potential biomedical strategy but also an efficient platform to study biological processes in vivo.

To evaluate dcHSA-NDs for brain targeting in vivo via BBB crossing, we demonstrated that dcHSA-NDs 1) were able to cross the BBB in an in vitro model and 2) were endocytosed by brain endothelial cells partly incorporated in endosomal compartments and lysosomes and released on the abluminal side. Here the particles were taken up by neurons and astrocytes, and 3) were transported via cell-to-cell connections of tunneling nanotubes. 4) Cell viability or BBB integrity in vitro was not affected. 5) Most importantly, investigated fluorescent dcHSA-NDs crossed BBB in healthy mice reaching the brain parenchyma and could be tracked at the single cell level. Our results clearly demonstrate the great potential of fluorescent dcHSA-NDs as a delivery platform for BBB transport and provide promising evidence for future personalized nanotheranostic applications in neurological diseases and the study of disease mechanisms in the brain.

2. Results and Discussion

2.1. Characterization of dcHSA-NDs

To design a NDs system, which is able to cross an intact blood–brain barrier, their transport needs to be controlled addressing endocytosis and transcytosis. Therefore, we selected denatured and cationized albumin as NDs coating, which facilitates cell penetration and intracellular trafficking and final release on the brain side after transcytosis. Cationization of HSA is accomplished by reacting about 100 carboxylic acid groups of the aspartic acid and glutamic acid side chains with ethylenediamine in excess yielding cHSA with about 159 primary amino groups as reported previously. cHSA provides the positive charges necessary for NDs coating by electrostatic interactions with the negatively surface charges of NDs (Figure 1A) and facilitates to enhanced cellular uptake due to interactions with the negatively charged cellular membrane. Furthermore, about 20 polyethylene glycol (PEG) polymer chains with an average molecular weight of 2000 g mol⁻¹ were covalently attached addressing about 20 of the about 159 amino groups on the denatured HSA (Figure S1, Supporting Information) to form the final dcHSA-PEG depicted in Figure 1. PEG(2000) chains, which provide an extended hydration shell, impart high colloidal stability in biological solutions, and reduce protein binding in plasma. For coating, the NDs were first dispersed in boric acid buffer (20 × 10⁻⁴ M, pH 8.4) by sonication. Then, the dispersion was added to dcHSA-PEG-BDP or dcHSA-PEG-Rho (12 mg) and dissolved in a larger volume of boric acid buffer (pH 8.4) by a dropping funnel. The mixture was stirred at room temperature overnight. After coating overnight, dcHSA-NDs were separated from the unbound dcHSA-PEG by centrifugation.

Figure 1
All synthesis steps and all characterizations are given in the Supporting Information.

The zeta-potential measurement clearly showed a change from the negatively-charged, uncoated NDs to positively-charged, protein-coated dcHSA-NDs (zeta potentials: NDs (−28.5 ± 1.0 mV), dcHSA-NDs (26.3 ± 0.7 mV), and dcHSA-PEG (11.9 ± 3.7 mV); Figure S3, Supporting Information). To address the size-dependent impact of dcHSA-NDs on in vitro BBB transport, dcHSA-NDs of two different sizes (about 40 nm for dcHSA-NDs 1 and 55 nm for dcHSA-NDs 2) were separated from the same batch. The hydrodynamic diameter after coating was assessed by DLS (dynamic light scattering) in MilliQ water. The average diameters changed from 28.47 ± 0.22 nm before coating to 41.55 ± 0.20 nm for dcHSA-NDs 1 (40 nm) and 36.68 ± 0.18 nm to 55.87 ± 0.92 nm for dcHSA-NDs 2 (55 nm), respectively (Figure 1G). The morphology of the NDs before and after coating was characterized by transmission electron microscopy (TEM). From the TEM images, histograms were created displaying the sizes of NDs 1 (18.7 ± 3.4 nm) and NDs 2 (25.5 ± 5.5 nm) that support the results obtained from the DLS measurements (Figure S2, Supporting Information). Furthermore, uncoated NDs formed clusters visible in TEM images (Figure 1C,D). In contrast, discrete nanoparticles of dcHSA-NDs were observed (Figure 1E,F) indicating the successful coating and stabilization of the NDs by the dcHSA-PEG shell. In addition, we recorded high-resolution TEM (HRTEM); it was accomplished to visualize the typical diamond lattice structure (Figure 1B).

The stability of the coated polymer shell on the surface of NDs (dcHSA-NDs 1 and 2), was further assessed in agarose gel (Figure S4, Supporting Information). The overlap of the three signals for NDs, the protein coating, and its rhodamine label proved that the coating of dcHSA-NDs even remained stable in harsh condition during gel electrophoresis (1x TAE (Tris base-Acetic acid-EDTA) buffer and electric field).

2.2. dcHSA-NDs Are Transported Across BBB in an In Vitro Model

To investigate the ability of dcHSA-NDs to cross the BBB, an in vitro model composed of the endothelial cell line bEnd.3 cells
seeded on the luminal side of a transwell insert was applied (Figure 2A). In this in vitro model, the luminal compartment represents the bloodstream and the abluminal compartment represents the brain. The in vitro BBB model has been validated after bEnd.3 cell seeding by continuously monitoring the transendothelial electrical resistance (TEER) using the CellZscope system, which indicates whether these cells have formed a tight cell barrier. TEER values higher than 40 Ω × cm² were considered adequate to exclude relevant paracellular transport (Figure 2B). Additionally, the apparent permeability coefficient (P_app) as a validation parameter of a hydrophilic tracer, FITC-dextran 4 kDa, was measured to ensure the formation of tight cell junctions restricting paracellular diffusion. The mean P_app was 2.98 ± 1.23 × 10⁻⁶ cm s⁻¹, which is in the range of optimal P_app for BBB model.

The two different sizes of tested nanodiamonds, dcHSA-NDs 1 (40 nm) and dcHSA-NDs 2 (55 nm), were applied with a concentration of 30 µg mL⁻¹ in the luminal compartment of the transwell BBB model to assess their ability to traffic from luminal across bEnd.3 cells to abluminal and to assess the impact of NDs size changes on BBB transport. After 24 h treatment, dcHSA-NDs fluorescence was quantified in collected abluminal medium to determine the transport rate. A transport of 100% indicated passive diffusion of dcHSA-NDs in transwell inserts without cells. dcHSA-NDs were capable to efficiently cross the BBB in vitro. Transport of 45.17 ± 7% (one-way analysis of variance (ANOVA); p < 0.05; n = 6 wells from 3 cultures) for dcHSA-NDs 1 (40 nm) and 38.6 ± 7.6% (one-way ANOVA; p < 0.05; n = 6 wells from 3 cultures) for dcHSA-NDs 2 (55 nm) was measured compared to the 100% control (100% dcHSA-NDs 1: 100 ± 5.4%; 100% dcHSA-NDs 2: 100 ± 3%; n = 6 wells from 3 cultures) (Figure 2C). The transport rate did not show a significant size dependent difference (dcHSA-NDs 1 vs dcHSA-NDs 2: one-way ANOVA, p > 0.05).

2.3. dcHSA-NDs Undergo Internalization and Intracellular Trafficking in Brain Endothelial Cells

In order to understand the mechanisms underlying the dcHSA-NDs endothelial transport, their uptake and intracellular localization were studied in more details. Using fluorescence microscopy, dcHSA-NDs-containing vesicles were detected in bEnd.3 cells (Figure 3A) indicating that the transport of dcHSA-NDs is an active endocytosis and transcytosis process. Fluorescent NDs were tracked by reflection as well as NV signals and the dcHSA-PEG-shell was detected independently by following the signal of bodipy-488-labelling on dcHSA-PEG to evaluate, whether the NDs coating remains stable in the cellular environment. Colocalization of the signals was observed (Figure S5, Supporting Information) demonstrating that dcHSA-NDs reached intracellular compartments and that the biopolymer shell remained attached to the NDs during uptake and intracellular trafficking. Furthermore, comparing dcHSA-NDs with and without NV-centers, we found no difference in cell uptake, hence dcHSA-NDs with and without NV-centers were used.

Next, we studied some of the mechanisms involved in cellular uptake and intracellular trafficking. bEnd.3 cells were treated with dcHSA-NDs in combination with FITC-labeled transferrin (TF, via clathrin-mediated uptake) or the B subunit of cholera toxin-alexa-488 (CTX, via caveolae-mediated uptake) for 24 h to identify their endocytotic pathway. Cellular coupate was
analyzed by quantification of colocalizing vesicles in confocal z-stacks. dcHSA-NDs vesicles colocalized 27.7 ± 3.07% with TF and 70.55 ± 3.9% with CTX positive vesicles showing a significantly higher preference of dcHSA-NDs for caveolae-mediated endocytosis (TF vs CTX: n = 12 regions of interest from 3 cultures, Mann–Whitney U test, p < 0.0001) (Figure 3B,C). Caveolae-mediated uptake has been shown to be fundamental, specifically for cationized-albumin, more than native albumin in brain endothelial cells[36,37] and our data clearly showed that dcHSA-NDs also followed predominantly this pathway.

After cellular uptake via both clathrin-mediated or caveolae-mediated endocytosis, early endosomes (EE) typically mature to late endosomes (LE) and lysosomes.[38] To assess whether dcHSA-NDs localize in EE or LE/lysosomes, costainings were performed with early endosome antigen (EEA) marker or lysosomal-associated membrane protein 1 (LAMP-1) marker, respectively. Confocal microscopy revealed that dcHSA-NDs appear within both EE and LE/lysosomes (Figure 3D). Quantitative analysis showed a minor colocalization of 2.4 ± 0.5% with EE and about 15.14 ± 4.15% with LE/lysosomes (n = 12 regions of interest from 3 cultures, Mann–Whitney U test, p < 0.0001) (Figure 3E). These results can be expected, considering that the retention time in EE usually is only a few minutes.[38] After 24 h most of the dcHSA-NDs-containing EE already undergo maturation in LE and LE/lysosome fusion, which explains why LE/lysosomes represent the endosomal compartments with most pronounced colocalization. These data correspond to previous data of dcHSA-NDs in LE/lysosomes of cancer cells. Cells treated with doxorubicin-loaded-dcHSA-NDs released the drug doxorubicin into the cytosol by an endosome/lysosome escape mechanisms.[30] The transport data confirm these results, although part of dcHSA-NDs localize in LE/lysosomes, a consistent percentage of dcHSA-NDs is able to follow a transcytotic pathway thus avoiding intracellular entrapment. We further assessed whether dcHSA-NDs induced autophagy and were subsequently internalized in autophagosomes. Co-staining with the autophagosome marker microtubule-associated protein 1A/1B-light chain 3 (LC3) was performed to study possible dcHSA-NDs autophagosome incorporation. Representative orthogonal z-stacks showed LC3-positive vesicles in both CTR and dcHSA-NDs treated cultures (Figure S6A, Supporting Information). Only 0.98 ± 0.52% (n = 10 regions of interest from 3 cultures) of dcHSA-NDs colocalized with LC3-positive-vesicles (Figure S6B, Supporting Information), which is a negligible value considering the high rate of basal autophagy in endothelial cells.[39] Thus, no induction of autophagy and no dcHSA-NDs in autophagosomes were observed. Since an increase in autophagy is also a marker for cellular stress, dcHSA-NDs-related cellular stress could be excluded by this test.

2.4. dcHSA-NDs Are Taken up by Neurons and Astrocytes

High intracellular trafficking into endothelial cells underlines the potential of dcHSA-NDs to cross the BBB after transcytosis or LAMP-1 as late endosomes marker; scale bar = 7 µm. E) Quantification of colocalization for dcHSA-NDs-containing vesicles with EE or LE; n = 12 ROIs from 3 cultures, Mann–Whitney U test, ***p < 0.001.
to reach the abluminal compartment in vitro. In physiological conditions dcHSA-NDs may also interact with other cell types such as neurons and astrocytes after trafficking from blood to the brain parenchyma. Therefore, we studied whether dcHSA-NDs were also taken up by murine primary neuronal cells. Astrocytes were stained against glial fibrillary acidic protein (GFAP) while neurons were stained against the neuronal nuclear protein NeuN to visualize the cell body or β-III-tubulin for imaging of axons and dendrites. Orthogonal view from z-stacks of dcHSA-NDs-treated astrocytes showed high uptake predominantly in the perinuclear region (Figure 4A). However, in neurons, confocal microscopy showed higher localization of dcHSA-NDs in axons and dendrites than in the perinuclear region, which is clearly visible in the β-III-tubulin and in the NeuN staining (Figure 4B). In summary, our data demonstrate that dcHSA-NDs are able to cross the BBB model system under in vitro conditions and provide high potential for passing the BBB to reach different target cells in the brain, which is an essential feature to track delivery and modulate pathophysiology of neurological disorders at different target sites.

2.5. dcHSA-NDs Tracking Reveals Direct Cell–Cell Transport

Most dcHSA-NDs-containing vesicles were localized in the perinuclear region in bEnd.3 cells and astrocytes. However, dcHSA-NDs-containing vesicles were also detected in distal subcellular areas, and this peripheric localization was mostly in neuronal cells, which prompted us to investigate distal

Figure 4. dcHSA-NDs are taken up by neurons and astrocytes in vitro. A) Representative confocal orthogonal views of dcHSA-NDs (red) uptake in astrocytes labeled by GFAP marker (green). B) Neuronal uptake of dcHSA-NDs. Neuronal cell body stained by NeuN (magenta) and dendrites and axons stained by β-III-tubulin (green); scale bar = 5 µm.
localization of dcHSA-NDs. Intriguingly, after staining the cellular cytoskeleton with rhodamine–phalloidin, fluorescent dcHSA-NDs vesicles were detected in a cell structure connecting two adjacent cells (Figure 5A). Actin bridges have been identified as tunneling nanotubes (TNT) that have been observed in vitro for many cell types as well as in vivo for developing embryos of different species.[40,41] TNTs are thin membranous bridges connecting cells over long distances and transferring various cellular components from cell to cell.[41]

In order to study cell–cell migration of dcHSA-NDs, live cell imaging was carried out to track dcHSA-NDs-vesicles moving along TNTs (Video S1, Supporting information). Detection of dcHSA-NDs fluorescence at 0 and 45 min merged with brightfield pictures at 0 min allowed visualization of different vesicle movements at varying positions along the same TNT (Figure 5B). Time-lapse tracking clearly showed a unidirectional migration of dcHSA-NDs-vesicles from an initiating cell to a target cell and accelerated movement when...
dcHSA-NDs-vesicles approached the target cell (Figure 5B; white arrows). We speculate that the differences in movement could be due to restricted available space, a “bottle-neck,” during the entry phase, in which the vesicle diffuses randomly in the cytosol. This could explain the observed slower migration of dcHSA-NDs vesicles close to the beginning of the TNTs (Figure 5B), which then changes to a directional motion in the TNT, induced by molecular motors in actin- and tubulin-mediated transport.\(^{[42]}\)

TNT formation was also demonstrated in neurons and astrocytes.\(^{[41,43,44]}\) In an immature neuronal/glial coculture, fluorescent dcHSA-NDs-vesicles could also be tracked by live cell imaging in membrane cell–cell connections as observed for bEnd.3 cells (Figure 5C; Video S2, Supporting Information). Co-stainings with GFAP, β-III-tubulin, and rhodamine–phalloidin showed TNTs connecting astrocytes and neurons (Figure S7, Supporting Information). Our data complements previous studies on TNT transport of fluorescent NDs in HEK293T and SH-SY5Y neuroblastoma cells in vitro for intercellular cargo delivery.\(^{[42]}\) However, we demonstrate for the first time intercellular transport of fluorescent dcHSA-NDs in brain endothelial cell line and primary neuronal/glial cells. Our data show migration of dcHSA-NDs from cell to cell for NVU cells along TNTs without the need of being released to the extracellular environment. Intriguingly, these data strongly suggests direct cell–cell transfer of dcHSA-NDs across a biological barrier for brain delivery. The direct cell–cell migration across the BBB would be of high value due to a possible faster and more specific delivery pathway avoiding extracellular interactions of dcHSA-NDs.

To summarize, these in vitro results suggest intracellular trafficking leading to transcytosis and direct cell–cell transport of dcHSA-NDs from the luminal to abluminal compartment with no involvement and induction of autophagy.

2.6. dcHSA-NDs Have No Impact on BBB Integrity and Cell Viability

The biocompatibility of dcHSA-NDs is crucial for bioapplications. Thus, the impact of dcHSA-NDs on bEnd.3 cell monolayer integrity and astrocyte and neuron viability was investigated. TEER was constantly measured using a CellZscope during the dcHSA-NDs treatment in transwell inserts to monitor bEnd.3 monolayer integrity. Comparing TEER measurement of control (CTR) and dcHSA-NDs-treated inserts, showed a slight tendency of the CTR to decrease compared to dcHSA-NDs samples (\(n = 6\) wells from 3 cultures, two-way ANOVA, \(p > 0.05\)) (Figure 6A). These results indicate preservation of BBB integrity after dcHSA-NDs treatment. The general decrease of TEER values at the beginning of the treatment is due to perturbations in the assay related to medium exchange. The treatment at time \(t = 0\) produced a fluctuation-dependent instability in the TEER measurement that became stable again within the following hours.

Figure 6. dcHSA-NDs do not affect BBB integrity and cell viability. A) BBB in vitro integrity investigated for 24 h of dcHSA-NDs treatment on bEnd.3 cell monolayer. TEER was measured by CellZscope and the values at \(t = 0\) were set to 100% and each following measurement was expressed in relative percentage; \(n = 6\) wells from 3 cultures. B,C) Cell viability after dcHSA-NDs treatment in astrocytes B) or in neurons C) for 24 h with concentrations from 0.5–30 \(\mu g\) mL\(^{-1}\) quantified by Alamar Blue assay. Dead cell control: cell toxin staurosporine (Stauro). \(n > 7\) from 3 cultures; one-way ANOVA; ***\(p < 0.001\).
Fluorescent dcHSA-NDs Cross the BBB and Can be Tracked at the Single Cell Level in Astrocytes and Neurons In Vivo

To assess the potential of fluorescent dcHSA-NDs for clinical applications, we investigated whether fluorescent dcHSA-NDs injected intravenously in mice were able to reach the brain. 24 h after dcHSA-NDs application (500 µg mL⁻¹ of blood), brain and liver were screened for dcHSA-NDs signals. Liver was used as positive control since biodistributional studies have clearly demonstrated that many cationic nanoparticles are highly taken up in the liver.[45] Thus, high uptake in liver slices allowed comparison of dcHSA-NDs signals (reflection, bodipy488-labeled-dcHSA-shell, NV) intensity in tissue (Figure S8, Supporting Information). Colocalization of signals originating from NDs and Bodipy-488 labeling on dcHSA-PEG-shell suggest that at least the dcHSA-NDs, which show colocalization, maintain their integrity in vivo. Detection by reflection represents the most reliable detection system since it images all NDs in the sample, independent of the presence or absence of NV centers (not all NDs have NV centers). Therefore, brain slices were carefully analyzed for dcHSA-NDs reflection signals. The orthogonal view of confocal z-stacks showed dcHSA-NDs in brain slices (Figure 7A). In order to identify dcHSA-NDs-positive cells, astrocytes and neuronal cell bodies were stained for GFAP and NeuN, respectively. dcHSA-NDs were clearly detectable at the single cell level close to blood vessels and some NPs-containing vesicles colocalized with GFAP-positive astrocytes surrounding vessels or NeuN-positive neurons as observable from 3D reconstructions (Figure 7B,C). Biodistribution studies were accomplished to identify organs showing dcHSA-NDs uptake. Liver and spleen showed the highest uptake after 24 h (Figure S9, Supporting Information). However, the observed brain uptake indicates an adequate circulation time of dcHSA-NDs in the bloodstream required for transport into the brain. Additionally, the organs with the highest uptake are also the ones involved in the clearance of drugs and exogenous molecules. Observed kidney uptake in particular in an area identified as the glomeruli, the region of ultrafiltration, suggests clearance of the particle (Figure S9, Supporting Information). It is important to underline that after dcHSA-NDs ultrafiltration from blood to primary urine, they might still be reabsorbed to the bloodstream. Comparing the biodistribution of dcHSA-NDs to published data for 50 nm uncoated NDs, both systems were found in liver, spleen, kidney, and lung, while our dcHSA-NDs clearly revealed uptake in the brain, more uptake in spleen and less in lung. Both particle systems exhibit low uptake rates for the heart.[46] Also, for other brain-targeted particles such as liposomes, bispecific antibodies or gold nanoparticles targeting the transferrin receptor, high uptake in liver, kidney, and spleen was observed, while uptake into the brain of healthy WT mice was in most cases about 20 times less pronounced.[47–49]

Thus, these data clearly confirm the ability of dcHSA-NDs to cross the BBB and reach the brain parenchyma in vivo. Combined with the in vitro data, we assume a transcytotic process involving also direct cell–cell migration from brain endothelial cells to neurons via astrocytes incorporation occurring in vivo as also observed in vitro.

3. Conclusions

Previous studies on NDs were exclusively based on invasive in situ brain delivery approaches that were not able to solve the challenge of systemic NDs delivery into the brain after intravenous injection.[20,27] Herein, for the first time, we evaluated fluorescent NDs with a dcHSA-PEG protein coating in mice after systemic application and revealed efficient transport across an intact BBB in vivo. The NDs reached the abluminal side via intracellular trafficking and were taken up by astrocytes and neurons. We also provided new insights in dcHSA-NDs trafficking via direct cell–cell interactions mediated by TNTs. We demonstrate successful BBB crossing and tracking of fluorescent dcHSA-NDs at the single cell level within a complex neuronal system, thus achieving for the first time systemic delivery of fluorescent dcHSA-NDs into the brain.

Fluorescent NDs offer great potential as nanotheranostics due to their optical properties such as stable fluorescence, the option for nuclear spin polarization allowing hyperpolarization and MRI detection,[50,51] and biocompatibility.[24] Therefore, we propose dcHSA-NDs as nanotheranostic for neurological diseases such as glioma, as they combine within one platform 1) the fluorescent properties of fluorescent NDs and detection by refraction due to the diamond lattice, 2) the natural albumin-mediated “recruitment” at the tumor site as well as 3) multiple available groups to attach anticancer drugs. It is conceivable that HSA conjugates enrich at tumor sites in vivo. Matsumura and Maeda have shown that the accumulation of intravenously injected Evans blue-albumin complex accumulates in tumor tissue.[52] Since then others have demonstrated accumulation of albumin in many other carcinomas as well[53] and these attractive features were also reported for cationized albumin.[54,55] dcHSA-NDs conjugated with multiple copies of the anticancer drug doxorubicin were applied previously to target cancer cells in a human breast cancer xenograft model and pronounced antitumor efficacy was demonstrated, as well as high particle stability, which is a critical concern for further clinical application.[56]

In summary, we show for the first time successful brain delivery and tracking of fluorescent dcHSA-NDs at the single cell level in vivo. Our data in combination with the observed antitumor efficiency of doxorubicin-coated dcHSA-NDs in the peripheral breast cancer model suggest that dcHSA-NDs could...
emerge as nanotheranostics for systemic applications in personalized treatment of neurological diseases such as glioma.

4. Experimental Section

**Ethical Approval:** All experimental procedures were approved by the ethical committee of the “Landesuntersuchungsamt Rheinland-Pfalz” and the authority “Landesuntersuchungsamt Rheinland-Pfalz” protocol number: “Aktenzeichen: 23 177-07/16-1-1-024.” Principles of laboratory animal care (European, national and international laws) were followed.

**Materials:** If not stated otherwise, chemicals were obtained from Sigma-Aldrich, Seelze or Hamburg, Germany.

**In Vitro Transport Assay:** With mean TEER values of at least $40 \, \Omega \times \text{cm}^2$, dcHSA-NDs 1 (40 nm) and 2 (55 nm) were applied in the luminal compartment of the in vitro BBB model with a concentration

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**Figure 7.** In vivo fluorescent dcHSA-NDs cross the BBB and are tracked on single cell level in neurons and astrocytes. A) Representative confocal orthogonal views of dcHSA-NDs (white) signals in brain slices. B,C) Representative confocal orthogonal view of dcHSA-NDs (white) localization in GFAP-positive astrocytes (green) B) and NeuN-positive neurons (green) C); scale bar = 40 \(\mu\)m.
of 30 μg mL⁻¹. Crossing rate was quantified by rhodamine labeling of dcHSA-NDs. Fluorescence was measured with Infinite F1000 TECAN plate reader. Fluorescence intensity in abluminal compartment of transwell inserts without cells was set to 100% and the percentage of crossing was calculated as relative values.

_Uptake and Coupake of dcHSA-NDs in bEnd.3_ Cells: dcHSA-NDs or fluorescent dcHSA-NDs were applied in a final concentration of 30 μg mL⁻¹ to bEnd.3 cells, primary murine astrocytes or neurons seeded on coverslips for 24 h. For coupake studies, dcHSA-NDs were applied in combination with 120 μg mL⁻¹ transferrin-Alexa Fluor 488 (T13342, Invitrogen) or 15 μg mL⁻¹ cholera toxin B subunit-FITC (C1655). Cells were finally fixed with paraformaldehyde (PFA) 4%.

**Live Cell Imaging:** bEnd.3 cells or neurons/glia cocultures (DIV 2) were treated with fluorescent dcHSA-NDs (30 μg mL⁻¹) for 24 h. Cells were washed with PBS and fresh medium was added to start live cell imaging. 45-minutes live cell imaging was carried out with IX81 microscope and a monochrome fluorescence CCD camera X10 using the cellF Software (Olympus). Movies were edited with Windows Live Movie Maker. 45 min recording time are presented in 2 s videos for bEnd.3 or 6 s videos for neurons.

**Quantification of Colocalization:** Images were taken by TCS SP5 confocal (Leica). For quantification of intracellular colocalization, z-stacks were deconvolved by Huygens Essential software. Using Image J software, the percentage of vesicles containing dcHSA-NDs colocalizing with a certain intracellular compartment was analyzed setting arbitrary a threshold of 40% of colocalization for each vesicle to avoid false positive values. In addition, deconvoluted z-stacks were analyzed by JACoP plugin in ImageJ to calculate Van Steensel’s cross-correlation functions (CCFs) as proof of colocalization data.[38]

**Cell Viability Assay:** Cytotoxicity on astrocytes and neurons was investigated using the cell viability Alamar Blue assay. Cells were treated with dcHSA-NDs for 24 h after reaching confluance for astrocytes and at a cell density of 200 000 cells/well for neurons. Cells were washed with HBSS−/−, and resazurin (R7017) was applied in each well with a final concentration of 16.66 μg mL⁻¹ in HBSS++ (100 μL). After 1 h incubation, fluorescence was measured spectrophotometrically using Tecan Infinite F1000 plate reader (Tecan, Salzburg, Austria). Fluorescence values were corrected to fluorescence of media without cells (blank). Cell viability (%) was related to untreated control cells (100%).

**In Vivo Fluorescent dcHSA-NDs Uptake:** Fluorescent dcHSA-NDs with bodipy488-labeled-dcHSA-PEG in PBS were intravenously injected (500 μg mL⁻¹) in C57BL/6 P21-29 mice for 24 h. After treatment mice were perfused with PBS/heparin-natrium (Ratiopharm, 5000 E.I.) and subsequently with PFA 4%. Liver and brain were collected and incubated overnight in PFA 4%. The day after, organs were washed with PBS followed by equilibration in 30% of sucrose. 20 μm slices were obtained using a freezing microtome (Leica CM 1255).

**Data Analysis:** Data were analyzed with GraphPad Prism 5 software (Graph Pad, La Jolla, CA, USA) and presented as mean ± SEM. Statistical analyses were performed with Mann-Whitney U test, ANOVA one way, p < 0.05 was considered as statistically significant*, p < 0.01**, p < 0.001***.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

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blood–brain barrier, drug delivery, nanodiamonds, nanotheranostics, tunneling nanotubes

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