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***In vivo* nanoneurotoxicity screening using oxidative stress and neuroinflammation paradigms**

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Abstract

Iron oxide nanoparticles (IONPs) are promising neuroimaging agents and molecular cargo across neurovascular barriers. Development of intrinsically safe IONP chemistries requires a robust *in vivo* nanoneurotoxicity screening model. Herein, we engineered four IONPs of different surface and core chemistries: DMSA-Fe₂O₃, DMSA-Fe₃O₄, PEG-Fe₃O₄ and PEG-Au-Fe₃O₄. Capitalizing on the ability of the peripheral nervous system to recruit potent immune cells from circulation, we characterized a spatiotemporally controlled platform for the study of *in vivo* nanobiointerfaces with hematogenous immune cells, neuroglial and neurovascular units after intraneural IONP delivery into rat sciatic nerve. SQUID magnetometry and histological iron stain were used for IONP tracking. Among the IONPs, DMSA-Fe₂O₃ NPs were potent pro-apoptotic agents in nerve, with differential ability to regulate oxidative stress, inflammation and apoptotic signaling in neuroglia, macrophages, lymphocytes and endothelial cells. This platform aims to facilitate the development of predictive paradigms of nanoneurotoxicity based on mechanistic investigation of relevant *in vivo* bio-nanointerfaces.

Keywords

magnetic nanoparticles; nanotoxicity; blood-brain barrier; iron oxide; theranostic

INTRODUCTION

Neurovascular barriers, such as the blood-brain barrier (BBB) and the blood-nerve barrier (BNB), are important physiological barriers that limit delivery of drugs and bioactive molecules into the central and peripheral nervous systems (CNS/PNS). Engineered nanoscale (< 100 nanometers, nm) particles (NPs) have been increasingly used for drug and gene delivery across the intact neurovascular barriers [1]. Magnetic iron oxide nanoparticles

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(IONPs) are promising magnetic resonance imaging contrast agents and nanocarriers crossing neurovascular barriers, based on their controllable movements in response to magnetic fields and ability to be readily functionalized with various molecules [2–7]. Although the majority of NPs are BBB-impermeable, engineered IONPs reach the brain after intravenous, intraperitoneal or inhalation administration [8–11]. This unprecedented ability to cross neurovascular barriers, combined with the high surface area and reactivity of NPs [12], makes the nervous system vulnerable to potential nanotoxicity [6, 13]. The lack of robust screening approaches for nontoxic NP chemistries, and poor grasp of predictive paradigms of nanoneurotoxicity are the major obstacles in translating the advancing NP designs into viable biomedical platforms.

Current *in vitro*, *in vivo* and *in silico* approaches of nanoneurotoxicity assessment have major limitations. *In vitro* tests are poorly interpretable *in vivo* as over 90% of the NPs exerting genotoxicity in cultures are not genotoxic in animal tests [12]. This discrepancy may be caused by limitations of NP biodistribution within a cell compared with a whole organism, resulting in rapid NP overload. The use of traditional *in vivo* drug toxicity testing approaches are impractical (requiring over a billion dollars and 30 years to complete even for nanomaterials already developed [14]) and are not designed to account for the unique, often unknown features of NPs (e.g., new portals of entry, ability to activate innate immune responses, cross neurovascular barriers and undergo axonal transport), expected to influence *in vivo* biodistribution, permeability, biodegradation, clearance and toxicity patterns, and to covary with the physicochemical parameters of NPs (e.g., chemical composition, size, shape, aspect ratio, surface coating and charge, redox activity, state of agglomeration) [14–18].

Generally, NPs produce cytotoxicity via generation of reactive oxygen species (ROS) and activation of oxidative stress (OS), inflammation, and apoptosis signaling, causing cell death and various forms of mutagenesis and genotoxicity [17, 19]. In order to minimize exhaustive toxicity testing and streamline the development of safe-by-design NPs crossing the intact neurovascular barriers, there exists an acute need for an intermediate *in vivo* screening platform, which would provide relevant, spatiotemporally controlled *in vivo* nanobiointerfaces and utilize the mechanistic knowledge acquired *in vitro* [14–18, 20].

IONPs are employed in biomedicine as the least hazardous among metal NPs [21, 22]. However, CNS accumulation of iron, a potent redox-active metal and ROS generator, has been linked to the pathogenesis of many neurodegenerative disorders [23] and used in detection of biogenic IONPs (a complex of ferritin with iron) to diagnose Alzheimer's disease [24]. Modification of IONPs surfaces (e.g., with biopolymers, lipids, inorganic molecules), necessary to improve their functionalization property, solubility and permeability across tissue barriers [2, 6, 7], determines their stability and toxicity parameters in neural cells [25].

The objective of the present study was to develop a robust intermediate *in vivo* nanoneurotoxicity screening model. Capitalizing on the ability of PNS to recruit immune cells from the circulation [26] after intraneural IONP delivery into the sciatic nerve in rat, we studied *in vivo* interaction and toxicity of IONPs with immune cells, neuroglial and neurovascular units in a spatiotemporally environment.

METHODS

IONP Synthesis

All reagents were analytical grade and purchased from Sigma Aldrich (St. Louis, MO) unless otherwise indicated. *Fe₂O₃ NPs, ~10 nm diameter (general method)* were prepared

using Massart's method [27, 28]. FeCl_3 was added to DI water and, separately, FeCl_2 to a 2 N solution of HCl. The solutions were mixed in DI water under vigorous stirring followed by addition of 2 M ammonia solution, forming a black precipitate of Fe_3O_4 . The solution was washed 5 times by centrifugation at 900g for 5 min, and redispersed each time in DI water. The IONP solution was heated to 80°C, and oxidized to Fe_2O_3 by bubbling oxygen for 2 h. *Fe_3O_4 NPs, ~10 nm diameter (general method)*: A mixture of 24 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 9.82 g $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ was reacted with 50 ml of ammonium hydroxide under nitrogen gas at 80°C. The solution was allowed to react for 1.5 h after the addition of 3.76 g oleic acid. The fabricated magnetite NPs were washed with deionized water until a neutral pH was reached and then were transferred *in situ* into phenyl ether.

DMSA- Fe_2O_3 NPs—Dimercaptosuccinic acid (DMSA) and the maghemite solution [40] were prepared separately in deoxygenated DI water (via 1 h of N_2 bubbling). Both solutions were deoxygenated for 2 h, pH adjusted to 3.0 with HNO_3 , mixed with constant nitrogen bubbling for 30 min, spun down at 800g for 5 min and resuspended in 200 ml of DI water. The pH was adjusted to 9.5 with NaOH, maintained for 30 min, and reduced to pH 7.5 with HCl. The solution was centrifuged at 1000g for 10 min, and the precipitate removed; the brown supernatant was diluted with deoxygenated DI water and stored under nitrogen at 4°C.

DMSA- Fe_3O_4 NPs—800 mg of magnetite nanoparticles in 35 ml toluene was sonicated with 90 mg DMSA in 5 ml dimethyl sulfoxide for 5 min and then allowed to react at room temperature (RT) for 24 h. After the reaction, toluene was added and then nanoparticles were centrifuged, washed with ethanol and acetone.

PEG- Fe_3O_4 NPs—A mixture of 560 mg polyethylene glycol (PEG), 280 mg *N*-hydroxysuccinimide, 420 mg *N,N*-dicyclohexylcarbodiimide, 178 mg dopamine hydrochloride, and 1.4 g anhydrous Na_2CO_3 in 280 ml CHCl_3 and 140 ml dimethylformamide was reacted at RT for 3 h. After the addition of 0.7 g of magnetite nanoparticles in 5 ml CHCl_3 into the above mixture, the reaction was allowed to occur at RT for 22 h. After the reaction, hexane was added and nanoparticles were collected using a magnet, then washed with acetone and water.

PEG-Au- Fe_3O_4 NPs—A mixture of 170 mg Fe_3O_4 and 1.6 g $\text{Au}(\text{OOCCH}_3)_3$ in 40 ml phenyl ether was reacted with 3.1 g 1,2-hexadecanediol, 0.5 ml oleic acid, and 3 ml oleylamine under nitrogen gas at 180°C for 1.5 h. The fabricated Au- Fe_3O_4 NPs were washed with deionized water and ethanol. The mixture of 100 mg of Au- Fe_3O_4 NPs in 2 ml hexane and 1 g of PEG-SH (M.W.=5000 Da) in 500 ml DI water was sonicated for 1 h and then allowed to react at RT for 65 h. After the reaction, 100 ml hexane was added and nanoparticles were separated and washed.

Transmission Electron Microscopy

The IONPs were analyzed using transmission electron microscopy (TEM). Carbon-coated copper TEM grids were immersed for 15 min in the ferrofluid solutions of the IONPs described above, removed, drained, air-dried, and imaged with FEI Sphera Tecnai T2 TEM at 180–200 KV.

Energy Dispersive X-ray Analysis

PEG-Au- Fe_3O_4 NP dispersed solution was placed on silicon substrate. The sample was dried and analyzed by Energy Dispersive X-ray (EDX) analysis (Oxford EDX with Inca software).

Animals and IONP delivery

Adult female Sprague-Dawley rats (200–250 g, Harlan Labs) were housed in plastic cages in a temperature-controlled environment with 12:12 light:dark cycle and free access to food and water. Anesthesia was achieved using 4% isoflurane (IsoSol; Vedco, St. Joseph, MO) in oxygen. Sciatic nerves were exposed at mid-thigh level. Intraneural injections of IONPs (10–35 mg/ml; diluted in DI water to the total of 0.5, 15 or 150 mM of Fe and sterilized using 0.45 µm-pore-size filter), DMSA (4.4 mg/ml), PEG (4.4 mg/ml) or recombinant rat tumor necrosis factor- α (TNF- α , R&D Systems, Minneapolis, MN, 25 ng/ml) were performed using a 33-gauge needle in 10 µl volume. Sham injection consisted of nerve exposure, followed by a poke with a 33-gauge needle. At 2 h to 7 days post-injection, the rats were sacrificed by intraperitoneal injection of an overdose of a Nembutal (Abbott Labs, North Chicago, IL) and Diazepam (Steris Labs, Phoenix, AZ) cocktail in saline, followed by Euthasol (Virbac, Fort Worth, TX). The procedures were carried out according to the NIH Guidelines for Animal Use and protocol approved by the Institutional Animal Care and Use Committee.

SQUID Magnetometry

The nerves were isolated, freeze-dried in liquid nitrogen and stored at -80°C . The nerves were placed inside a gelatin capsule for M-H magnetization measurement, performed on a Quantum Design MPMS 2 superconducting quantum interference device (SQUID) magnetometer with the applied field up to 1 T. Magnetization behavior of samples were obtained at both RT and 10 K.

Neuropathology

The nerves were isolated, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% aqueous osmium tetroxide, dehydrated in a graded alcohol series and propylene oxide and embedded in araldite. Transverse 1 µm sections were cut with a glass knife on an automated Leica RM2065 microtome and stained with Methylene blue Azure II for light microscopic examination [29]. The images were acquired using a Leica DMR microscope and Openlab 4.04 imaging software (Improvision, Waltham, MA).

Antibodies for Immunodetection

Rabbit anti-extracellular signal-regulated kinases (ERK, Cell Signaling, Danvers, MA), rabbit anti-phospho-ERK (Cell Signaling), mouse anti-heme oxygenase (HO-1, GTS-1clone Abcam, Cambridge, MA), rabbit anti-HO-1 (Abcam), rabbit anti-caspase-3 (Cell Signaling), rabbit anti-cleaved caspase-3 (Cell Signaling), mouse anti-human matrix metalloproteinase-9 (MMP-9, Calbiochem, San Diego, CA), rabbit anti-interleukin 1 (IL-1 Abcam), mouse anti-CD68 (ED1 subclone, Serotec, Raleigh, NC), mouse anti-T-cell receptor (TCR, Serotec), mouse anti-CD3 (Abcam), mouse anti-S100 (Abcam), and mouse anti- α -actin (Sigma).

Iron Staining, Immunofluorescence and Imaging

Animals were perfused transcardially with 4% paraformaldehyde in 0.2 M phosphate buffer. The sciatic nerves were isolated, post-fixed, and embedded in paraffin or OCT compound. *For iron staining*, 10 µm-thick transverse sections were incubated with Perl's solution (2% HCl, 2% potassium ferrocyanide) for 30 min at RT, rinsed in DI water and incubated in 0.5% diaminobenzidine in Tris buffer containing 0.15% hydrogen peroxide [5, 30]. *For immunofluorescence*, the sections were deparaffinized in xylenes and rehydrated in Tris-buffered saline (TBS), as required. Non-specific binding was blocked in TBS containing 5% normal goat serum and 0.25% Triton X-100. The slides were incubated for 16–18 h at 4°C with a primary antibody followed by 1 h incubation at RT with a species-specific secondary

antibody conjugated with Alexa 488 (green) or Alexa 594 (red). The nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI, Molecular Probes, Grand Island, NY, 5 min). The absence of non-specific staining was confirmed using the non-immune serum. The sections were mounted in a Slowfade Gold antifade reagent (Molecular Probes). The images were acquired using a Leica DMR microscope and Openlab 4.04 imaging software (Improvision). The staining intensity and individual cell counts were calculated in 4 randomly selected areas per rat from 3 rats per group.

Immunoblotting and Zymography

Nerves were isolated, freeze-dried in liquid nitrogen and stored at -80°C . Nerve extracts were prepared using 50 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, 150 mM NaCl, 10% glycerol, 0.1% SDS, and protease inhibitors (5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, aprotinin and leupeptin, 1 $\mu\text{g}/\text{ml}$ each) for immunoblotting, or in protease inhibitors-free for zymography. Extract aliquots (20–60 μg protein, determined by BCA Protein Assay, Pierce) were analyzed using 15% acrylamide gels (Bio-Rad, Hercules, CA), or 10% acrylamide gels co-polymerized with 0.1% gelatin, respectively. *For immunoblotting*, the gels were transferred to a nitrocellulose membrane using an iBlot dry blotting system (Invitrogen, Carlsbad, CA). The membranes were blocked using 5% non-fat milk (Bio-Rad) in TBS, incubated overnight at 4°C with the primary antibodies specified above (diluted in 5% bovine serum albumin), washed in TBS containing 0.1% Tween and then incubated with a horseradish peroxidase conjugated goat anti-rabbit or anti-mouse secondary antibody (Cell Signaling). The blots were developed using enhanced chemiluminescence (GE Healthcare). The membranes were re-probed using a β -actin antibody to control equal protein loading. *For zymography*, the gels were washed in 2% Triton X-100 for 30–60 min at RT, incubated 18 h at 37°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM CaCl_2 and 1 μM ZnCl_2 and 0.2 mM sodium azide, and stained with Coomassie Blue R250 to visualize the gelatinolytic activity, as a clear band on a dark background of undegraded gelatin. The band density was measured in 3–4 rats/group using Image J software.

Data Analyses

Statistical analyses were performed using KaleidaGraph 4.03 and SPSS 16.0 software by a two-tailed, unpaired Student's t-test or analysis of variance (ANOVA), followed by the Tukey's Kramer post-hoc test. $p < 0.05$ values were considered significant.

RESULTS

Physicochemical Characterization of IONPs

We synthesized roughly spherical, polydisperse ($3 \text{ nm} < D_{\text{TEM}} < 15 \text{ nm}$), maghemite (Fe_2O_3) or magnetite (Fe_3O_4) IONPs of high purity. Hydrophilicity was achieved using DMSA or PEG coating. Coating with DMSA, a negatively charged, sulfur-containing chelating agent used as a therapy for metal poisoning, enhances the colloidal stability of IONPs, and by interacting with the cationic pockets of the plasma membrane facilitates their high-efficiency intracellular uptake via adsorptive endocytosis [31–33]. Hydrophilic PEGylated IONPs are internalized by fluid-phase endocytosis and amphiphilic affinity to lipid bilayers on plasma membranes [2]. DMSA- Fe_2O_3 and DMSA- Fe_3O_4 NPs were used to assess the effects of iron oxide core chemistry. DMSA- Fe_3O_4 and PEG- Fe_3O_4 NPs were used to assess the effects of surface chemistry. PEG-Au- Fe_3O_4 NPs were synthesized and compared to PEG- Fe_3O_4 NPs to test the effect of an intermediate gold layer, believed to provide a barrier against iron oxide core oxidation [2, 34].

The resulting IONPs (DMSA-Fe₂O₃, DMSA-Fe₃O₄, PEG-Fe₃O₄ and PEG-Au-Fe₃O₄) formed dark-brown concentrated aqueous ferrofluids, stable over a wide range of pH (3 to 11) and salt concentrations. All IONPs were confirmed to be roughly spherical at 8–10 nm using TEM (Figure 1A–B shown a representative micrograph). The presence of Au was confirmed using EDX analysis (Figure 1C and Table 1).

Immune cell infiltrate the IONP-injected nerve

The IONPs were injected into an intact sciatic nerve by intraneural injection (Figure 2A–B) at 0.5 and 15 mM Fe for the studies of iron detection sensitivity and toxicity, respectively. Due to comparable results on immune cell infiltration among IONPs, data obtained with DMSA-Fe₂O₃ NPs, known to cause dose-dependent toxicity of PC12 cells [28], was selected as representative. Accumulation of DMSA-Fe₂O₃ NPs in the nerves was confirmed at 48 h post-injection using SQUID magnetometry of the nerves (Figure 2C). An increase in M-H magnetization almost saturated at an applied field of ~2000 Oe. The coercivity (less than 5 Oe) increased as the temperature was lowered. The IONPs were detectable at all Fe concentrations, including the lowest tested 0.5 mM Fe (Figure 2C). DMSA-Fe₂O₃ NP accumulation was uniform across the nerves section (a), as confirmed using Perl's stain for iron (Figure 2D). Control injection of DMSA or PEG alone produced no magnetization values in nerve (data not shown), or significant accumulation of endogenous iron (Figure 2D(b)).

The ultrastructural nerve changes were analyzed after intraneural IONP injection (Figure 2E). After control DMSA injection (a–b), the nerve bundles maintained normal morphology, displaying intact axons and myelin sheath, no visible change in endoneurial or epineurial vessel endothelia, and the perineurium consistently presented as a thin, pink collagen layer (b). In contrast, nerves exposed to DMSA-Fe₂O₃ (15 mM Fe) NPs displayed clusters of infiltrating phagocytes in perivascular (c–d) and subperineurial (e) spaces. A remarkably organized array of IONP-laden phagocytes in subperineurial spaces was accompanied by subperineurial edema (e). Certain perivascular areas displayed severe edema and cell accumulation suggestive of disrupted BNB (c–d, f–g). Spindle-like cells and fiber loss were occasionally noted in perivascular areas (g).

A significant increase in the numbers of macrophages/monocytes (identified with CD68) and lymphocytes (identified CD3) was observed across the nerve fascicle and the perivascular areas after DMSA-Fe₂O₃ NP injection (Figure 2F). The CD68 and CD3 reactive cell numbers remained exceedingly low after DMSA injection and comparable to sham injection. The immune cell infiltration corresponded to the 44% increase in nuclear profiles after DMSA-Fe₂O₃ NP compared to DMSA injection (Figure 2F, graph).

IONP surface chemistry influences neural OS signaling *in vivo*

A working paradigm of NP-induced toxicity includes persistent generation of ROS leading to inflammation, mediated by mitogen-activated protein kinases (MAPKs) and apoptosis (cleaved (cl.) caspase 3) [15, 16, 35, 36]. Thus, nerve levels of MAPK/ERK and caspase 3 were analyzed at 48 h after intraneural injection of DMSA-Fe₂O₃, DMSA-Fe₃O₄, PEG-Fe₃O₄ and PEG-Au-Fe₃O₄ NPs (15 mM each). The ratios of phospho (p)- to total ERK were significantly increased in all IONP-injected relative to intact nerves (Figure 3). Compared with DMSA-Fe₃O₄, DMSA-Fe₂O₃ NPs stimulated lower levels of ERK activation, but higher levels of caspase 3 activation. Cl. caspase 3 (19 and 17 kDa) levels also increased after PEG-Au-Fe₃O₄ compared with PEG-Fe₃O₄ NP injection. High reproducibility of the data is evident in three rats per group.

Phenotypic characterization of cells susceptible to IONP toxicity in nerve

Next, phenotypic markers for macrophages/monocytes (CD68); T lymphocytes (TCR); and Schwann cells (S100) were used to identify the neural cell types susceptible to DMSA-Fe₂O₃ NP toxicity, as the most potent OS3 inducer (Figure 3). Another advantage of using DMSA-Fe₂O₃ NPs for this experiment is their universal mechanisms of internalization by various cell types [31–33]. The endpoints of nanotoxicity used were the levels and distribution of HO-1, a phase II anti-oxidant enzyme and a marker of tier 1 OS [37]; MMP-9, an extracellular protease and a biomarker of NP-mediated macrophage activation [35], which promotes migration of IONP-loaded cells [38]; IL-1 and p38, the inflammatory mediators stimulated by metal NPs, and cl. caspase 3 for apoptosis [15, 16, 35, 36]. TNF- (25 µg/ml) was used as a positive control for neuroinflammation [39].

HO-1 (32.8 kDa) was undetectable in nerve after sham or DMSA injection. Its levels significantly increased at 2 and 24 h after DMSA-Fe₂O₃ NP injection (Figure 4A). The early time-points were used for the tier 1 OS. HO-1 increased mildly at 24 h after TNF- injection. DMSA-Fe₂O₃ NP injection produced accumulation of the HO-1-reactive cells in perivascular spaces (Figure 4B), identified as CD68+ macrophages, TCR+ lymphocytes and endothelial cells. S100+ Schwann cells or axons were not reactive for HO-1.

IL-1 (31 kDa, pro-form) levels increased after DMSA-Fe₂O₃ NP compared to sham, DMSA or TNF- injection (Figure 5A). Comparable albeit less dramatic changes in phospho-p38 were observed (Figure 5A). Gelatinolytic MMP-9 activity (92 kDa) was poorly detectable in normal, sham- or DMSA-injected nerves and significantly elevated after DMSA-Fe₂O₃ NP and TNF- injections (Figure 5B). These inflammatory factors were detected in crescent structures of Schwann cells, CD68+ macrophages and vessel endothelia (MMP-9 is shown, Figure 5C).

Both, inactive (35 kDa) and activated, cl. caspase 3 (19 and 17 kDa) levels were elevated after DMSA-Fe₂O₃ NP compared to sham, DMSA or TNF- injections (Figure 6). Cl. caspase 3 was reactive in axons, S100+ Schwann cells, TCR+ lymphocytes, but not CD68+ macrophages.

The nerve one week after IONP exposure

By day 7 after DMSA-Fe₂O₃ NP injection, the MAPK, MMP-9, IL-1 and cl. caspase 3 levels normalized (data not shown). Only at a higher (150 mM Fe) dose of DMSA-Fe₂O₃ NPs mild pro-IL-1 (31 kDa) and inactive caspase 3 (35 kDa) elevation remained (Suppl. Figure 1A). The nerves no longer showed evidence of phagocytes. Nonetheless, certain abnormalities in perivascular morphology persisted (Suppl. Figure 1B).

DISCUSSION

Pharmaceutical advancement of nanodesigns into clinical neuroscience has been hindered by the lack of robust toxicity screening models. *In vitro* nanotoxicity data are poorly interpretable *in vivo* [12], whereas preclinical drug toxicity testing approaches are both highly impractical [14] and fail to account for the unique, often unknown biodistribution, biodegradation and clearance parameters of NPs, as well as their properties to activate immune response and cross tissue (e.g., neurovascular) barriers. The development of ‘intermediate’ *in vivo* toxicity models minimizing these variables is key to screening and identifying intrinsically nontoxic NP chemistries, sizes, shapes, and other key physiochemical parameters [14–18, 20, 40–43].

We have engineered four spherical, 8–10 nm IONPs with different core and surface chemistry (DMSA-Fe₂O₃, DMSA-Fe₃O₄, PEG-Fe₃O₄ and PEG-Au-Fe₃O₄) and

demonstrated that intraneural NP injection into the sciatic nerve in rat is a unique nanoneurotoxicity platform, which allows for (1) the comparative screening for a relatively neuro-biocompatible NP chemistry (Figure 3); (2) the mechanistic investigation of *in vivo* neural OS and inflammation markers (Figures 4–6); (3) the concurrent analyses of *in vivo* nanointerfaces with hematogenous immune cells (macrophages and lymphocytes), neuroglia (Schwann cells) and neurons, and endothelial cells of the neurovascular unit (Figures 2E–F, 4–6); (4) high spatiotemporal control of the NP distribution and dose, resulting in high within-group reproducibility of the sensitive mechanistic endpoints (Figure 3); and (5) IONP tracing along the nerve tract, using SQUID magnetometry (Figure 2C), Perl's iron stain (Figure 2D) or a detectable tag, making it potentially suitable for the studies of axonal transport [44] of NPs.

In contrast to intraneural injection, intravenous administration results in high (up to 80%) clearance of NPs by the reticuloendothelial system of liver and spleen, providing poor spatiotemporal and dose control of NPs interfacing with the CNS/PNS [13]. Physiochemical parameters (e.g., surface/core chemistry) that influence the mechanisms and the efficiency of NP clearance and transcytosis across the BBB/BNB, introduce variables at multiple stages between NP administration and the CNS/PNS exposure. Direct NP injection into the nervous system limit these variables. Compared with the CNS, the PNS is superior in its ability to recruit potent macrophages from circulation [45], offering spatiotemporal control of this critical nanobiointerface to the studies of nanotoxicity. Unlike other nerves of the PNS, the largest and most accessible mixed (i.e., sensory and motor) sciatic nerve is suitable for the studies of both sensory and motor alterations after NP exposure. Relative to less sentient types of nervous system, intraneural injection in rodents allow for complex neurotoxic behavioral testing (e.g., pain) followed by high-throughput *in silico* mechanistic investigation in the same animal group [46, 47].

This platform, however, cannot predict the ability of NPs to cross the BBB/BNB and should be used where indications of a given NP reaching the CNS/PNS exist [1, 6]. It is important to note that, in a biological system, the 'corona' of protein binding to the primary NP surface, the 'secondary coating', may facilitate transcytosis of NPs not intended to cross the BBB/BNB. The secondary coating may reduce or increase neuro-biocompatibility of NPs. For example, DMSA-Fe₂O₃ NPs, the most potent cell death inducer in nerve among the tested IONPs, are thought to produce toxicity due to their high efficiency of intracellular uptake, leading to an iron overload and excessive ROS generation [6]. The albumin coating reduces the IONP uptake [2]. Relative to DMSA, PEG coating on Fe₃O₄ NPs reduced ERK activation, consistent with the PEG property to reduce protein binding and macrophage uptake, resulting in immune evasion, employed to increase IONP circulation after intravenous delivery [2]. An effect of intermediate Au coating in PEG-Au-Fe₃O₄ to precipitate neural apoptosis compared with PEG-Fe₃O₄ NPs may relate to the reduced speed of their biodegradation [2, 34] or DNA damage in neural cells [48]. The specific influence of the iron core oxidation state on neurotoxicity remains unclear as of yet; DMSA-coating of the fully oxidized maghemite (Fe₂O₃) core associated with elevated cl. caspase 3 levels compared to those of the magnetite (Fe₃O₄) core. Dextran-coated Fe₃O₄ NPs, commonly used for MRI enhancement agents and known to readily cross the intact BBB [8–10], are generally neuro-biocompatible [5], although long-term mechanistic data is lacking. Poor functionalization capacity of dextran limits utility of such IONPs for the layered nanodesigns necessary for targeted delivery of drugs and molecules.

Based on light microscopy, macrophages were the main cells to internalize DMSA-Fe₂O₃ NPs in nerve. Whereas these anionic particles are believed to enter various cell types by a universal mechanism [33], involving binding to pockets of positive charge on the plasma membrane, followed by adsorptive endocytosis [31–33], differential accumulation of IONPs

in macrophages suggests endoneurial phagocytosis of the IONPs as a means of clearance. Macrophages depart the nerve by the lymphatic system; however, perivascular accumulation of IONP-laden macrophages after intraneural delivery may imply entry into systemic circulation and transport to other organs. Dextran-coated IONPs also accumulate in endoneurial macrophages after intracardiac injection; this property is being successfully utilized for diagnostic imaging of neuroinflammation in nerve [49]. Despite the relatively high Fe dose, DMSA-Fe₂O₃ NP injection produced no apoptosis in macrophages, whereas endothelial and Schwann cells, neurons, and T cells were vulnerable to cell death. It is conceivable that the anti-oxidative response in macrophages is superior in quenching redox signaling to other cells.

Thus, the nanotoxicity-related three-tier OS, i.e., a continuum in HO-1/ROS, inflammation and apoptosis signaling [16, 50], manifested only in T cells in nerve at the analyzed time-points. IONP-induced neural OS was evident in all but Schwann cells, which primarily activated inflammation (MMP-9, IL-1) signaling. MMP-9 promotes migration of IONP-loaded cells through the extracellular matrix [35, 38] and macrophage recruitment into the injured nerve [29]. Immunocompetent Schwann cells [45] readily stimulate MMP-9 and cytokine production in response to nerve damage independent of OS [29, 39, 46]. In agreement, TNF- injection stimulated neural MMP-9 but not HO-1. Thus, NP-induced inflammation signaling may represent an innate immune rather than toxic response employing toll-like receptor and other systems [15].

Collectively, these data suggest that intraneural NP injection is a powerful tool in characterizing the mechanistic interactions at the nanobiointerface with cells of the immune, neuroglial and neurovascular units *in vivo* and the development of a predictive paradigm of nanoneurotoxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of IONPs

A–B, High-resolution TEM using Sphera Tecnai T2 TEM demonstrates roughly spherical, polydisperse particles with a mean diameter of ~8–10 nm: DMSA-Fe₂O₃ (A) and PEG-Au-Fe₃O₄ (B). **C,** Energy Dispersive X-ray (EDX) analysis of the PEG-Au-Fe₃O₄ NPs.

Figure 2. IONP tracking and immune cell accumulation in nerve

A, An illustration of sciatic nerve in rat, and **B**, of intraneural IONP injection into the nerve fascicle. **C**, SQUID magnetometry of nerves using Quantum Design model MPMS2 at 77°K; a field sweep between +1 to -1 tesla at RT and 10 K at 48 h after DMSA-Fe₂O₃ (0.5 mM Fe) NP injection. Representative data of n=4–5/group. **D**, Perl's stain for iron (brown) in a nerve section after DMSA-Fe₂O₃ NP (1.5 mM Fe, a) or DMSA (b) injection. Methylene blue counterstain. Representative data of n=3/group. Scale bars = 50 μm. **E**, Nerve ultrastructure after DMSA (a–b) or DMSA-Fe₂O₃ NP (15 mM Fe, c–g) injection. Methylene blue Azure II stain. Intact axons, myelin sheath (a–b), endoneurial and epineurial vessel endothelia and perineurium (b) after DMSA injection. Perivascular (c–d) or subperineurial (e) clustering of phagocytes and other cells and edema after DMSA-Fe₂O₃ NP injection (c–d, f–g). V, vessel; ax, axon; p, perineurium. Representative data of n=4/group. Scale bars = 25 μm (b, e) and 10 μm (a, c–d, f–g). **F**, CD68 (macrophage, green) and CD3 (lymphocyte, red) immunofluorescence across the nerve fascicle and around the vessels (v) 48 h after DMSA-Fe₂O₃ (15 mM Fe) NP but not DMSA injection. Scale bars = 50 μm. The mean DAPI+ cell numbers ± SEM in n=3/group and 4 areas per n (graph, *, p<0.05).

Figure 3. IONP core/surface chemistry influence neural inflammation and apoptosis

A, ERK and Caspase 3 immunoblotting in nerve (β-actin, equal loading control, 40 μg/lane) 48 h after intraneural IONP (15 mM Fe) injection. N, normal nerve. **B**, The mean optical density for the pERK to ERK, and Caspase 3 to β-actin ratios from n=3 rats/group (graph, *, p<0.05).

Figure 4. DMSA-Fe₂O₃ NPs induced neural antioxidant response

A, HO-1 immunoblotting (β-actin, equal loading control, 40 μg/lane) in nerve 2 and 24 h after intraneural sham (Sh), DMSA, DMSA-Fe₂O₃ (15 mM Fe) NP or TNF- (25 ng/ml) injection. The mean optical density ± SEM from n=3/group (graph, *, p<0.05). **B**, Immunofluorescence for HO-1 (red), Schwann cells (S100, green), macrophages (CD68, green) and T lymphocytes (TCR, green) in nerve 24 h after DMSA-Fe₂O₃ (15 mM Fe) NP injection; DAPI, blue; V, vessel. Representative data of n=3/group. Scale bars = 50 μm.

Figure 5. DMSA-Fe₂O₃ NPs induce neural inflammation

A, IL-1 and p38 immunoblotting (β-actin, equal loading control, 40 μg/lane) in nerve 48 h after intraneural sham (Sh), DMSA, DMSA-Fe₂O₃ (15 mM Fe) NP or TNF- (25 ng/ml) injection. The mean optical density ± SEM from n=3/group (graph, *, p<0.05). **C**, Gelatin zymography for MMP-9 activity in nerve. The mean optical density ± SEM from n=3/group (graph, *, p<0.05). **D**, Immunofluorescence for MMP-9 (red) and macrophages (CD68, green) in nerve 48 h after intraneural DMSA-Fe₂O₃ (15 mM Fe) NP injection. DAPI, blue. MMP-9 localizes in CD68+ macrophages (yellow), vessel (V) endothelia (red) and Schwann cells (crescent structures, arrows). Representative data of n=3/group. Scale bars = 35 μm (A) and 50 μm (D).

Figure 6. DMSA-Fe₂O₃ NPs induce neural apoptosis

Caspase 3 immunoblotting (β-actin, equal loading control, 40 μg/lane) in nerve 48 h after intraneural sham (Sh), DMSA, DMSA-Fe₂O₃ (15 mM Fe) NP or TNF- (25 ng/ml) injection. The mean optical density ± SEM from n=3/group (graph, *, p<0.05). **B**, Immunofluorescence for cl. caspase 3 (red or green, as indicated), Schwann cells (S100, green), macrophages (CD68, red) and T lymphocytes (TCR, green) in nerve 48 h after DMSA-Fe₂O₃ (15 mM Fe) NP injection. DAPI, blue. Representative data of n=3/group. Scale bars = 50 μm.

Table 1

Physicochemical characteristics of PEG-Au-Fe₃O₄ NPs.

Element	App	Intensity	Weight%	Weight%	Atomic%
	Conc.	Corrn.		Sigma	
C _K	0.25	1.0929	28.67	0.89	57.71
O _K	0.18	1.5298	15.14	0.60	22.88
Si _K	0.01	1.1281	0.98	0.15	0.85
Fe _L	0.18	0.5949	37.99	1.45	16.45
Al _M	0.10	0.7020	17.21	0.91	2.11
Totals			100.00		