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Abstract

This critical review examines in vitro and in vivo evidence for the influence of engineered nanomaterial (ENM) physicochemical properties on their distribution into, and effects on, the nervous system. Nervous system applications of ENMs; exposure routes and potential for uptake; the nervous system and its barriers to ENM uptake; and the mechanisms of uptake into the nervous system and overcoming those barriers are summarized. The findings of English-language publications of studies that included at least two variations of an ENM physicochemical property and reported results of their pharmacokinetic and/or pharmacodynamic interaction with the nervous system that differed as a function of ENM physicochemical property(ies) are summarized in the Supporting Materials. A summary conclusion is drawn for each of the physicochemical properties on the strength of the evidence that it influences ENM-nervous system interaction.

Keywords

Chemical composition; shape; size; surface charge; surface coating

Abbreviations

BBB	blood-brain barrier
BMEC	brain microvascular (capillary) endothelial cell
CNS	central nervous system
ENM	engineered nanomaterial
NS	nervous system

I. Nervous system applications of engineered nanomaterials (ENMs)

There are reviews of the impact of the physicochemical nature of engineered nanomaterials (ENMs) on biological systems [1]; their circulation, biodistribution, cellular internalization, and trafficking [2]; the contribution of the biological corona to their effects [3]; and their impact on biological activity related to the brain and retinal diseases [4]. However, there has not been a critical review of the significance of the physicochemical properties of ENMs on the distribution into, and effect on, the nervous system (NS). This review addresses that information gap. It focuses on the influence of ENM physicochemical properties on their distribution/translocation to the NS and resultant effects. There is extensive interest in ENM use as drug and diagnostic agent delivery systems to the NS for pharmaco- and thermotherapy, as contrast agents for MRI visualization, as photosensitizers for diagnosis, and for cell labeling and cell replacement (e.g., for neurodegenerative disorders), including labeling mesenchymal stem cells to follow their fate. Much of the work has focused on cancer [5] and much research has investigated polymer-based ENMs. Most of the ENMs that have been studied are first generation, passive nanostructures, and second generation ENMs (active, such as targeted drugs). Third generation ENMs (nanosystems) such as neuro-electronic interfaces and fourth generation ENMs (molecular nanosystems), have not yet been studied in the NS.

II. ENM exposure routes and their potential to result in nervous system uptake

Due to the low bioavailability from inhalation, oral, and dermal exposure (below), ENM administration to achieve a medical goal usually requires systemic or local administration. Inhalation is the route of greatest concern for unintentional ENM exposure and uptake, most often from the lungs into systemic circulation and then to the NS from the blood. ENM translocation from the lungs to systemic circulation is < 5%, and to the NS very much less [6, 7]. Translocation from the lung to the brain after inhalation of 15 or 80 nm ^{192}iridium was 0.003 and 0.0003%, and for 12, 29, or 213 nm ceria was 0.01 to 0.4% of the dose [8, 9], whereas brain had 0.0001% of a 7 nm ceria after its intratracheal instillation [10]. Another route of uptake from inhalation exposure is via sensory nerve endings embedded in airway epithelia in the roof of the nasal cavity (the olfactory nerve and maxillary branch of the trigeminal nerve), followed by axonal translocation in unmyelinated neurons (fila olfactoria, which have a diameter of ~100 to 330 nm) to ganglionic and central nervous system (CNS) structures [11]. Uptake directly into the brain by this route bypasses systemic circulation and first pass intestinal and hepatic metabolism. Drug administration into the nasal cavity is quite easy to achieve. It is most amenable to potent agents. However, there are concerns about nasal cavity mucosal irritation, damage, and alteration of olfaction [12]. Numerous transporters are expressed by the olfactory and trigeminal cranial nerves that have terminations in the nasal epithelium, which might inhibit or facilitate ENM uptake [13, 14]. The olfactory nerve has been demonstrated to mediate uptake of viruses (30 nm polio [15]) and some ENMs (50 nm silver-coated gold colloid [16]; 36 nm ^{13}C [17]; 30 nm manganese oxide [18]; and 95 nm quantum dot loaded particles [19]). Other examples are in Tables S3 and S4. This

uptake appears to be mediated by endocytotic uptake into the neurons (rather than via transporters), retrograde axonal transport once they enter these sensory neurons, and interneuron translocation into the brain [20].

Non-inhalation routes of ENM uptake include the oral and dermal routes. Uptake into the brain after oral/gastric administration of 1 to 200 nm gold, 25 and 80 nm titania, and 7 and 30 nm ceria ENMs was ≤ 0.002% of the dose [10, 21-25]. Although ENMs have been shown to penetrate into skin, most studies have not shown transdermal penetration through intact skin. Disrupting this barrier with organics, abrasion, or flexing may enable ENM absorption into the hypodermis to reach blood and lymph vessels [26, 27]. It has been suggested that retrograde transport from nerve endings in the skin could take up ENMs into the dorsal root ganglia, although it does not appear that this has been demonstrated [28]. The only report suggesting translocation to the NS of ENMs applied topically was an increase of titanium in brain after application of Degussa P25, but not a 10, 25 or 60 nm titania, to the interscapular skin of hairless mice for 60 consecutive days [29]. Intradermal injection of quantum dots, bypassing the formidable barrier provided by the stratum corneum, resulted in translocation to the liver, lymph nodes, and kidney, but not the brain [30].

Intraperitoneal injection of scrapie virus (~25 nm) was thought to result in its uptake by sympathetic fibers into the NS by retrograde axonal transport. Prions (~10 nm) are thought to translocate in both directions between the periphery and the NS [31, 32]. These observations suggest ENMs might be similarly taken up. Daily intraperitoneal injection

of 5 nm anatase titania resulted in more titanium in the brain and greater effects than a comparable dose of bulk titania. Given the insolubility of titania ENMs, these results might indicate brain uptake, but verification of titania ENM in brain extravascular space was not reported [33]. Intraperitoneal injection of nanoscale aluminum, copper, gold, and silver increased levels of these metals in the brain. Changes in brain function were reported after intraperitoneal injection of these metal ENMs as well as after IL-13-coated liposomes (Tables S3 and S4), suggesting uptake from the peritoneal cavity. They may have been taken up directly to the brain via neuronal input or through the recently described lymphatic system of the brain [34], given the uptake of ENMs by the lymphatic system [35]. The presence of some 500 nm fluorescent latex particles in the brain after intramuscular injection to mice was attributed to their uptake and translocate by the lymphatic system [36].

Intravenous injection avoids the above barriers, providing 100% bioavailability. This route has been extensively investigated for ENM drug delivery and visualization. It is the best route to determine the potential for ENM entry into the brain's vasculature and parenchyma, and resultant effects.

III. The nervous system and its barriers to ENM uptake – The blood-brain barrier, blood-cerebrospinal fluid barrier, blood-spinal cord barrier, blood-retinal barrier, and blood-nerve barrier

The NS has two anatomical divisions, the CNS comprised of the brain and spinal cord, and the peripheral NS comprised of 12 pairs of cranial and 31 pairs of spinal nerves that

connect the CNS to organs, muscles and glands. The somatic NS includes afferent neurons that convey information from sensory organs to the brain, primarily to the cerebral cortex, and includes the olfactory nerve and maxillary branch of the trigeminal nerve mentioned above. Afferent neurons pass through the spinal nerve dorsal root ganglia, comprised of neuronal cell bodies that lie along the back of the vertebral column (spine). Dorsal root ganglia cells and rat PC12 cells are often used as models of neurons, as frequently cited in the Supporting Materials. The motor component of the somatic NS conveys efferent messages from the cerebral cortex via neurons to the skeletal muscles to enable voluntary movements. The autonomic nervous system afferent component conveys sensory impulses from the blood vessels and internal organs to brain regions, including the medulla, pons, and hypothalamus that elicit reflex responses through efferent autonomic nerves to the heart, blood vessels, and all the body's organs. The autonomic nervous system has two major components, the sympathetic and the parasympathetic systems, that often have opposite effects on end organs, such as the heart, thereby maintaining homeostasis. The healthy brain has neurons and glial cells (astrocytes, oligodendrocytes, and microglia). The nervous system has neurons and Schwann cells. The latter, like oligodendrocytes in the CNS, wrap neuronal axons in a myelin sheath.

Barriers for a material to reach an intracellular target in the NS include the blood-brain, blood-cerebrospinal fluid, blood-spinal, blood-retinal, and blood-nerve barriers, followed by the cell's plasma membrane, and then, depending on the target, perhaps an organelle membrane such as the nuclear envelope. To reach an intracellular target, a

multi-functional nanoconstruct, sequentially presenting different surface properties, may be required.

The anatomical basis of the blood-brain barrier (BBB) includes the brain microvascular (capillary) endothelial cells (BMECs) that line the ~5 to 10 μm diameter vessels that perfuse the brain. Adjoining cells have tight junctions, maintained by several proteins. The lack of 1 to 1.2 nm lanthanum flux through BBB endothelial cell tight junctions attests to this barrier's integrity [37]. ENMs are likely to pass through the endothelial cell membrane (transcellular) rather than between endothelial cells (pericellular) unless this space is enlarged. Serum proteins penetrated leaky cerebral vessels supplying blood to the subarachnoid space and pial surface as well as circumventricular organs (which lack a BBB so they can chemically communicate with blood) [38], suggesting the penetration of lipid ENMs into the brain through circumventricular organs is possible [39]. However, we did not see nanoceria in the median eminence or pituitary gland, which lack a BBB [40, 41].

The luminal surface of the BBB is coated with a carbohydrate rich glycocalyx layer bound to the endothelial cells by glycoproteins and proteoglycans, which contain sialic acid moieties. This provides a negative charge that is important to maintain BBB integrity and function. Cations that neutralize this charge can increase BBB permeability [42]. Heparan sulfate containing proteoglycans which constitute ~50 to 90 of the proteoglycans, such as the extracellular matrix proteoglycan perlecan and the transmembrane syndecan family, help to maintain and protect the BBB. These

proteoglycans can immobilize molecules, such as lipoproteins and chemokines, and HIV-1, and can mediate cellular uptake of apolipoprotein E (apoE)-containing lipoproteins and an apoE mimetic peptide Angiopep.

In addition to the barriers to ENM flux across the BBB presented by its physical components, the BBB expresses many components that protect it and the brain metabolically and enzymatically. The BMECs have numerous carrier-mediated influx and efflux transporters, including P-glycoprotein, multidrug resistance protein, and breast cancer resistance protein that transport lipophilic and other agents out of the BMECs into blood [43]. Most substrates of these transporters are small molecules. The BMECs also express enzymes, including monoamine oxidase, DOPA decarboxylase, cholinesterases, GABA transaminases, aminopeptidase, and endopeptidases, that metabolize neurotransmitters and many xenobiotics. A few cytochrome P 450 drug-metabolizing phase 1 enzymes, CYP1B1 (that metabolizes flavonoids and estradiol) and CYP2U1 (that metabolizes arachidonic acid, docosahexaenoic acid, and other long chain fatty acids), and some phase 2 enzymes, GSTP1, COMT, GSTM3, GSTO1 and GSTM2, are expressed [44]. Superoxide dismutase attenuates ROS-induced BBB disruption, protecting the brain from injury produced by ischemia, methamphetamine, and other insults [45, 46]. Further description can be found in [47-50].

The kinetics of ENM penetration of the blood-brain and blood-retinal barriers has been described in studies using methods that confirm distribution across the membranes, including imaging of ENMs in NS cells and use of the capillary depletion method that

separates brain parenchyma from brain endothelial cells. MWCNTs were seen in the parenchymal fraction 5 minutes after their intravenous administration [51]. One % polysorbate-coated PBCA and cationic-albumin PEG-poly(ε-caprolactone) ENMs were seen in the brain parenchymal fraction 30 minutes after their intravenous injection [52, 53]. Using in vivo multiphoton imaging of mice with a cranial window, stained nuclei were seen beginning 30 min after intravenous injection of nuclear stain-PS80 coated-PBCA ENM, amyloid plaque staining was seen beginning 15 minutes after intravenous injection PBCA-ENM coated with Alexa-488–conjugated anti-Aβ antibody, and PBCA-ENM loaded with a Trypan blue showed a time constant of brain entry of 18 minutes, corresponding to the BBB crossing time [54]. Imaging of rhodamine-labelled PBCA in retina showed blood-retinal barrier crossing in 20 to 25 minutes [55]. In the only found study of metal-based ENMs that showed short-term NS entry, transferrin-conjugated fluorescein-loaded Fe_3O_4 nanoparticles were seen 1 hour after their intravenous injection into rats, the only time studied [56].

IV. The mechanisms of substance uptake into the NS and overcoming barriers to ENM uptake

The mechanisms of substance uptake into cells include diffusion (adsorptive transcytosis), carrier-mediated transport, and receptor-mediated processes [57]. The receptor-mediated processes include facilitated diffusion, active transport, and endocytosis (the engulfing of particles and uptake in small vesicles into a cell) [58]. Diffusion across the BBB favors molecules < 500 D_a (~1 nm) and lipophilic substances [59, 60]. Endocytotic processes are believed to be the major mechanism of ENM cell

uptake [61]. Endocytotic processes involve phagocytosis and pinocytosis (macropinocytosis, caveolae, clathrin-coated pits, and clathrin- and caveolae-independent uptake). Phagocytosis can engulf spherical particles from ~200 to 3000 nm into a vacuole. Caveolar uptake occurs in non-fenestrated endothelial cells, involving an invagination of the cell membrane surrounded by the protein caveolin on the cytoplasmic surface, receptor proteins, and invagination into the cell. The caveolae-mediated uptake pit diameter is ~50 to 80 nm. Although endothelial cells in the mammalian brain have fewer pinocytotic vesicles than most other tissues [62], this route was shown to mediate uptake of neutral and cationic ENMs across a co-culture of bovine brain microvascular endothelial cells and mixed glial cells [63]. The clathrin coated- and clathrin/caveoli-independent pit diameters are ~120 and ~90 nm, respectively. However, one should not think that these diameters limit the size of ENMs that can be taken up by these processes [64].

Several approaches to enhance brain ENM uptake have been investigated; molecular Trojan horse approaches to enable hitchhiking through the BBB. These include surface functionalization/conjugation to transferrin (to be recognized by the transferrin receptor subtype-1 for receptor-mediated endocytosis), transferrin receptor antibodies, lactoferrin (to be recognized by the lactoferrin receptor for receptor-mediated endocytosis), apolipoprotein E (apoE) and the peptide Angiopep (an apoE-mimetic peptide ligand) that are recognized by the low density lipoprotein receptor, insulin-like growth factor binding protein (for recognition by the insulin-like growth factor receptor), and a rabies virus-derived peptide [65-67]. The BBB can be intentionally compromised to enhance

distribution into the CNS. Focused ultrasound that creates microbubbles has been shown to open targeted BBB regions for a few hours to enhance local brain uptake [68], and has been used to transiently increase BBB permeability to enhance brain gold ENM delivery as well as doxorubicin and gadolinium in polymers [69-72]. The BBB tight junctions can be temporarily opened by intra-carotid infusion of hyper-osmotic (~25%) mannitol, which has been used for brain cancer chemotherapy [73]. No reports were found that investigated the interaction of physicochemically-different ENMs with the brain when these methods were used to open the BBB.

V. Addressing the knowledge base of this review

This review is based on English-language publications of ENM studies which had at least 2 variations of a physicochemical property that resulted in different ENM interaction (pharmacokinetic and/or pharmacodynamic) with the NS or its components. The physicochemical properties of both the synthetic identity (the ENM as made) and the bioidentity (biological identity, transformed from the synthetic identity by protein coating, aging, etc.) were considered. It is assumed that the response to a transformed ENM will not be the same as to its synthetic identity [74]. For example, aging (oxidation) of zero valent iron decreased its toxicity [75] and MWCNT oxidation altered cell response and ENM distribution and degradation [76-78]. Publications were reviewed for results related to five ENM physicochemical properties (chemical composition, size, shape, surface charge, and surface coating). For in vitro studies, reports were reviewed for comparative results of the five physicochemical properties on eleven NS cell types (or mixtures thereof); stem, blood-brain barrier, blood-peripheral nerve barrier, blood-retinal

barrier, microglia, astrocytes, oligodendroglia, neural, peripheral NS cells, mixtures of NS cells, and tumor cells. For in vivo studies, reports were reviewed for comparative results of the five physicochemical properties studied in healthy vs. disease model animals.

The strategy to identify the literature examined for this review included PubMed, Web of Science, and SciFinder searches, followed by searches and examination of references cited by the identified reports and reviews. Five PubMed database searches were conducted between November 2012 and January 2016. The cumulative yield of 1344 English-language citations produced ~ 550 unique citations. The PubMed search strategy used a combination of relevant controlled vocabulary terms from Medical Subject Headings [Mesh] and Text Words (words or phrases found in either an article title or abstract). Core anatomic and disease MeSH terms included Nervous System OR Nervous System Diseases, which yields more specific terms indexed below the main terms in the PubMed tree structure. To increase initial yield, text words were also searched, including Neuro* OR Nerv* OR Brain OR Astrocyte* OR Retinal OR Microglia* OR Apoptosis OR Cerebrospin* OR Mening* OR Encephal* OR Alzheimer* OR Parkinson* OR Dementia. Asterisks (*) were used to force truncation and find variable endings to root terms. Nanotoxicology search criteria primarily relied on "Nanostructures"[Mesh], plus text words. Core terms included Nanotox* OR Nanotech* OR Namomolec* OR Nanomaterial* OR Nanotech* OR Nanoparticl* OR Nanodot* OR Nanotub* OR Biotransform* OR Ultra fine OR Quantum Dot. Additionally, Title Word searches for terms such as Genotox* OR Cytotox* OR Neurotox* OR Toxic* were used,

then combined with the neurotoxicology search terms. Additionally, the PubMed “Similar Articles” algorithm was used for articles that appeared to be of high relevance.

The PubMed keywords were used to devise the Web of Science search strategy. Three separate searches were conducted during the same timeframe as the PubMed searches. The strategy was filtered, focusing on title words and research design. This returned 325 citations.

Ten targeted SciFinder searches were conducted in October, 2015 to look for publications to fill in ENM physicochemical property pharmacokinetic and/or pharmacodynamic interaction cells lacking entries. Search terms were: stem cells nano nervous system, blood-peripheral nerve barrier nano, peripheral nerve barrier nano, peripheral nerve nanomaterial, peripheral nerve nano, blood-retinal barrier nano, blood-nerve barrier nano, oligodendroglia nano, astrocyte nano, and astrocyte nanoparticle nanomaterial.

The author read the abstract of all returned citations to select the reports that appeared to report studies that included at least two variations of an ENM physicochemical property. Those reports were read to extract the relevant details, resulting in the ~ 235 reports summarized in the Supporting Materials and > 230 reports that did not include at least two variations of an ENM physicochemical property that resulted in ENM physicochemical property-dependent different responses.

Introduction to Sections VI to IX

Summaries of the influence of the physicochemical properties of ENMs on their interaction with the NS, organized according to the five physicochemical properties and study material (in vitro by cell type or in vivo) have been summarized in 4 tables in the Supporting Materials. Tables S1 and S2 report in vitro results, Tables S3 and S4 report in vivo results. Tables S1 and S3 contain summaries of reports of studies that determined pharmacokinetic endpoints, and Tables S2 and S4 contain summaries of reports of studies that determined pharmacodynamic (effect) results. Tables S1 and S2 include the eleven cell types searched. Tables S3 and S4 distinguish between studies conducted in healthy vs. disease model animals, noting the NS region or cell type studied, animal species, and route of ENM administration. Entries under a physicochemical property and study material are chronological; the oldest listed first. The absence of an entry under a physicochemical property for a cell type (Tables S1 and S2) or animal status (Tables S3 and S4) indicates no information was found.

The level of evidence that a differentiating ENM physicochemical property influences NS interaction, based on the reports summarized in Tables S1 to S4, is presented in Tables 1 to 4. An entry of No indicates no evidence. N/S indicates the evidence is not strong, often because only one report addressed this condition. An entry of S indicates strong evidence, based on more than one well-conducted and interpreted study and/or multiple supporting studies in the absence of multiple studies with conflicting results. For many studies, it is difficult to attribute a different response to two or more ENMs to a single physicochemical property because the structural/chemical difference(s) among

the ENMs represent more than one physicochemical property, the entanglement of their physicochemical properties [79]. This is particularly relevant when trying to attribute a difference to surface charge which is often confounded by the functional groups that provide the different charges. For N/S and S entries, reports that provide the strongest evidence are cited.

VI. In vitro studies reporting the influence of ENM physicochemical properties on their pharmacokinetic responses (uptake, distribution, and persistence)

Table 1 indicates the level of evidence (based on studies summarized in Table S1) that each of the five physicochemical properties has on the pharmacokinetics of ENM cell type/cell mixture interaction. Only 4 reports with stem cells, 1 with oligodendrocytes, and 4 with normal astrocytes studied alone were found, preventing very many conclusions that physicochemical properties influence ENM pharmacokinetic interaction with these cells. Although ENMs have been studied as scaffolds for regeneration of peripheral nerve cells, no reports were found of ENM pharmacokinetic interaction with peripheral NS cells (other than dorsal route ganglia cells that are included with neurons) or the blood-nerve barrier, accounting for the absence of entries for these targets in Table 1. Some conclusions can be drawn from the studies cited in Table 1. More than half of the studies summarized in Table S1 were of BBB models. Of these, nine used hCMEC/D3 cells. Reports using these human-derived cells were given more credence than reports using other cells when summarizing the strength of evidence in Table 1. The literature consistently shows an inverse relationship between ENM size and extent of distribution across in vitro models of the BBB. Results with tumor-derived cells suggest greater cell

association or uptake of 40 to 50 nm ENMs than larger or smaller ones, consistent with the conclusion that ~ 50 nm in the optimum size for uptake by non-phagocytic eukaryotic cells [80]. There is insufficient information to know if this is true for non-tumor NS cells. Permeation through the BBB appears to be favored for ENMs with closer to, or with, neutral surface charge. Cell membrane surfaces, including brain microvasculature endothelial cells, are negatively charged, so ENMs with a net negative surface potential would be expected to have difficulty approaching the cell membrane. However, this is not consistent with the conclusion that increasing surface charge, either positive or negative, favors particle uptake by non-phagocytic eukaryotic cells [80]. The evidence that surface coating influences the pharmacokinetics of ENMs on NS cells comes from the many studies that investigated methods to deliver ENMs across the BBB to the brain, and some studies that assessed the risk of brain parenchyma ENM entry. Two of the four studies that compared non-tumor- and tumor-derived cells show different response, suggesting more work is warranted to selectively target ENMs to NS tumor cells.

VII. In vitro studies describing the influence of ENM physicochemical properties on their pharmacodynamic responses (effects/responses of the cell type)

Table 2 indicates the level of evidence (based on studies summarized in Table S2) that each of the five physicochemical properties has on the effects produced by ENMs on NS cell types or cell mixtures. As with pharmacokinetic endpoints, the lack of sufficient studies (none were found for blood-peripheral nerve barrier or peripheral cells, only one

was found for the blood-retinal barrier and for oligodendrocytes, and three with normal astrocytes studied alone) prevents conclusions of the influence of physicochemical properties on the effects of ENMs on these barriers and cells. Generally, from a few to a few hundred nm, effects on cells decreased as size increased. This trend was seen with stem, blood-brain barrier (which represented < 20% of the entries in Table S2), neuronal (which represented 35% of the entries in Table S2), and tumor cells. Only 1 study compared surface coating in non-tumor and tumor cells [81], providing insufficient information to conclude if they respond similarly.

VIII. In vivo studies reporting effects of the influence of ENM physicochemical properties on their pharmacokinetic responses (uptake, distribution, and persistence)

Table S3 contains summaries of reports of studies that determined pharmacokinetic endpoints in the NS of the mouse, rat, and rabbit (1 study) of more than one ENM. There are many reports concluding that ENMs enter the brain. For ENMs from < 2 to 500 nm, there was generally an inverse relationship between size and brain association after intravenous administration; supported by studies cited in Table 3. For most studies concluding that ENMs enter the brain, the methods employed were not able to determine ENM distribution into brain parenchyma. Most studies used methods that do not account for the ENM in the blood within the vasculature of the brain. Blood occupies ~2% of brain volume in the cortex and a greater space in some other brain regions [82, 83]. Rats perfused to remove blood 4 h after intravenous injection of gold glyconanoparticles had only ~4% as much ENM in their brain as rats that had not been perfused [84].

Similarly, perfusion reduced gold in three brain regions to 7 to 18% of that seen on non-perfused rats after intra-abdominal nanogold injection [85]. These results, and the rapid ENM decline over time in the whole brain or brain regions, e.g., [85-89], which are interpreted as not reflecting parenchymal entry, and the decrease in ENM in brain capillaries but not parenchyma over 24 hours [90], suggest many studies that reported brain ENM in the absence of removal of blood in the brain significantly over-estimated the amount of ENM that entered brain parenchyma. Some studies accounted for the contribution of blood to brain ENM [23, 83, 91], however this does not fully remove the contribution of ENM in sites other than brain parenchyma, such as adsorption to the luminal wall of brain vasculature and ENM presence in cellular and membrane components of the BBB [41, 92]. In several studies differences seen in short-term time points did not persist to later times. None of these studies verified ENM distribution into brain parenchyma. These results suggest that not all of the ENM penetrated into brain parenchyma, but that the temporal difference might be due to ENM in blood within the brain or adherent to the luminal wall of brain vasculature that subsequently distributed away from these sites [93-98]. A few reports verified ENM brain parenchyma entry [99, 100], but one cannot conclude from one of these [99] that size influenced brain levels of gold because this ENM was given by intraperitoneal injection. The difference in brain gold ENM could be due to differences in uptake from the peritoneal cavity. Because the distinction between ENM in the brain vs. brain parenchyma has seldom been made, reports that claimed brain ENM entry were assessed for evidence that the ENM entered brain parenchyma. The findings are noted in Table S3.

As noted above, it is difficult to isolate surface charge without confounding factors from other variables. Several studies, although all from the same group, found less distribution through brain for negative than near neutral ENMs when introduced into ex vivo brain [72, 101]. In vivo results addressing the relationship between surface charge and brain association are not consistent, preventing a conclusion [102-106]. A large number of studies showed evidence that surface coating affected brain association, reflecting the extensive efforts to overcome the restrictions to brain entry presented by the BBB. No attempt was made to relate results from in vitro studies of brain-derived cells (Table S1) to the in vivo situation (Table S3) due to the great restriction of the BBB to brain entry.

IX. In vivo studies describing how ENM physicochemical properties affect their pharmacodynamic responses (effects/organism responses)

Table S4 contains summaries of reports of studies that determined response/effect endpoints in the mouse, rat, guinea pig, and rabbit NS of more than one ENM. One would expect greater response when a greater amount of ENM associates with the brain. This was seen in a study that determined both endpoints [107]. Smaller ENMs produced greater responses than larger ENMs [99, 108, 109] but a firm conclusion that size correlates with NS response is prevented by the entanglement of their physicochemical properties. Although ENM size affects its NS response, the relationship is not as simple as its influence on brain association. No studies investigating the influence of size were identified using ENM intravenous administration to the studied animal where more than one ENM was investigated. Uptake from the exposure site (oral, intraperitoneal, intranasal) may influence the NS response, preventing attribution of NS response to

size when these routes were employed. Only one study employing the intravenous route suggests cationic surface charge was associated with greater response, as might be predicted by neutralization of the negative charge on the BBB [102]. A firm conclusion that surface charge correlates with NS response is again prevented by the entanglement of their physicochemical properties.

X. The ENMs that have been studied for their physicochemical properties that influence pharmacokinetic and/or pharmacodynamic interaction with the nervous system

A minority of the studies cited in the Supporting Materials investigated polymer-based ENMs, primarily focused on targeting or permeating the blood-brain barrier, entering the brain, or targeting cancer or cancel cells. The polymer-based studies were generally published sooner (median 2007, range 1990 to 2015) than the metal- and carbon-based ENM studies (median 2012, range 2001 to 2016). A contributor to the difference may be the concern about adverse and persistent effects of the generally insoluble carbon-, silica-, metal-, and metal oxide-based ENMs.

XI. Conclusions

It is well established that ENM physicochemical properties can affect their pharmacokinetics (uptake, distribution, and persistence) and resulting responses. This has been demonstrated in organ systems other than the NS, evidenced by the extensive clearance of ENMs into the liver and spleen, and ENM modifications that reduce this to target other sites. It has been less well demonstrated for the NS and not

previously reviewed. Of the ENM physicochemical properties that have been investigated for their influence on NS distribution and effects (chemical composition, size, shape, surface charge, and surface coating) the greatest emphasis has been on surface coating, particularly studies attempting to preferentially target ENM delivery to, and effects on, the brain. Studies with stem cells, blood-brain barrier cells, neurons and neuron-like cells, and tumor cells, as well as whole animals, have shown the influence of ENM surface coating on distribution and effects. Size has been shown to influence ENM distribution, as an inverse relationship for distribution across in vitro BBB models, into the brain of whole animals, and effect on neurons and neuron-like cells; and greater tumor cell association or uptake of 40 to 50 nm ENMs than larger or smaller ones. Strong evidence for the influence of chemical composition, shape, and surface charge on NS pharmacokinetics and effects is generally lacking.

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