Chapter 28
Application of Mass Spectrometry to Characterize Localization and Efficacy of Nanoceria In Vivo

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Abstract In vivo study of nanomaterials is complicated by the physical and chemical changes induced in the nanomaterial by exposure to biological compartments. A diverse array of proteins can bind to the nanomaterial, forming a protein corona which may alter the dispersion, surface charge, distribution, and biological activity of the material. Evidence suggests that unique synthesis and stabilization strategies can greatly affect the composition of the corona, and thus, the in vivo properties of the nanomaterial. Protein and elemental analyses techniques are critical to characterizing the nature of the protein corona in order to best predict the in vivo behavior of the nanomaterial. Further, as described here, inductively coupled mass spectroscopy (ICP-MS) can also be used to quantify nanomaterial deposition in tissues harvested from exposed animals. Elemental analysis of ceria content demonstrated deposition of cerium oxide nanoparticles (CeNPs) in various tissues of healthy mice and in the brains of mice with a model of multiple sclerosis. Thus, ICP-MS analysis of nanomaterial tissue distribution can complement data illustrating the biological, and in this case, therapeutic efficacy of nanoparticles delivered in vivo.

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Abbreviations

AC	Analytical centrifugation
AUC	Area under the curve
BBB	Blood–brain barrier
CA/EDTA	Citric acid/EDTA
CeNPs	Cerium oxide nanoparticles
DLS	Dynamic light scattering
EAE	Experimental autoimmune encephalomyelitis
ICP-MS	Inductively coupled mass spectrometry
LC-MS/MS	Liquid chromatography-mass spectrometry
MS	Multiple sclerosis
PEGylation	Polyethylene glycol addition
ROS	Reactive oxygen species
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography

28.1   Introduction

The extravasation of drugs and nanomedicines through the blood–brain barrier (BBB) represents a significant obstacle for the development of effective therapeutics for neurological diseases. Of the over 40,000 current medicinal formulations, less than 1 % of the drugs gain entry and are active in the central nervous system [1]. Consequently, for many therapeutics, the BBB is a principle obstruction in the development of pipeline drugs to treat central nervous system disorders. Brain microvascular endothelial cells and glial end-feet, which constitute the anatomical basis of the BBB, form tight junctions due to a lack of fenestration, thereby reducing the diffusion of molecules across the vascular bed. However, cell and molecular biology studies have provided insights into the function of the BBB and the transport systems, enzymes, and receptors that regulate the penetration of molecules into the brain. Further, the field of nanotechnology has opened the door to the delivery of drugs and medicines through the BBB; several nanoparticle-bound drugs including doxorubicin [2–4], loperamide [5, 6], a novel anticonvulsive agent [7], and others [8–10] can be successfully delivered across the BBB, often times utilizing the brain's endogenous transport mechanisms.

Nanomaterials are systems (1–100 nm) that are potentially useful tools in medicine; by virtue of their small size, they readily interact with endogenous biomacromolecules (DNA, protein, lipids) and, thus, are able to act at the cellular, molecular, and genetic level. Both targeting nanoparticles to specific organs and cell types and understanding the interactions of nanomaterials with endogenous biomolecules remain significant challenges in nanomedicine development. Regardless of the route of administration, all nanomaterials encounter various cell types, complement system

enzymes, lipids, and a myriad of proteins both in the blood and the extravascular space. The complexity of the mammalian serum proteome is immense and estimated to contain over 5,000 different proteins at concentrations spanning over 10 orders of magnitude [11, 12]. While changes in the physical and chemical properties of nanomaterials can alter their affinity for biomolecules, predicting these interactions, a priori, is difficult at best. In vitro test beds have not shown good translational efficacy compared to the effects observed in intact animals, except for the most toxic of materials. This discordance in findings may occur, in part, as a result of the dynamic change in the protein corona in vivo which can evolve with changes in protein concentration and affinity to the nanomaterial as well as the ionic strength of the biological compartment the particle is resident in. For example, it is not unusual for a nanoparticle delivered to the lung to cross ~15 different physiological barriers (and hence a number of unique biological compartments) to reach a target in the brain.

Characterizing the interactions between nanomaterials and the proteins with which they associate is critical for the prediction and understanding of biological outcomes [13–18]. The protein corona can change the intrinsic physical–chemical properties of the nanoparticle (size, degree of subsequent particle aggregation, and surface properties) which in turn affect the biological activity of the nanomaterial. Understanding the biomolecular relationship between the protein corona and tissues may permit more selective targeting to cell receptors, cellular compartments or organelles, and specific biomolecules. In particular, the corona has been shown to affect nanoparticle uptake [19, 20], blood coagulation [21], targeting [15, 16], protein aggregation [22], and particle distribution [23].

In biosystems, the corona may evolve and take on an evolving identity during its lifetime as the nanomaterial traverses through biological systems [24]. Once a nanomaterial enters a biological system, the most abundant proteins are adsorbed on the surface. However, over time they are replaced by higher affinity proteins in a process termed the *Vroman Effect* [25], which may continually alter the properties and behavior of nanomaterials in biological systems. Thus, understanding the dynamic nature of the corona of nanomedicines would greatly aid in predicting the potential biological interactions and the evolution of nanomaterial efficacy and distribution within intact organisms over time (Fig. 28.1).

28.2 Structure and Composition of the Corona

The majority of adsorbed biomacromolecules on the surface of nanomaterials in blood plasma are proteins, and recently some minor traces of lipids have also been reported [26]. The protein corona is thought to be divided into two parts distinguished by binding affinities. Proteins with high affinity for the nanomaterial are known to comprise the "hard" corona, consisting primarily of proteins that bind tightly to the nanoparticle and have very slow exchange rates. Proteins of lower affinity comprise the "soft" corona and consist of proteins that interact loosely with the proteins that make up the "hard" corona and have more rapid exchange rates [27].
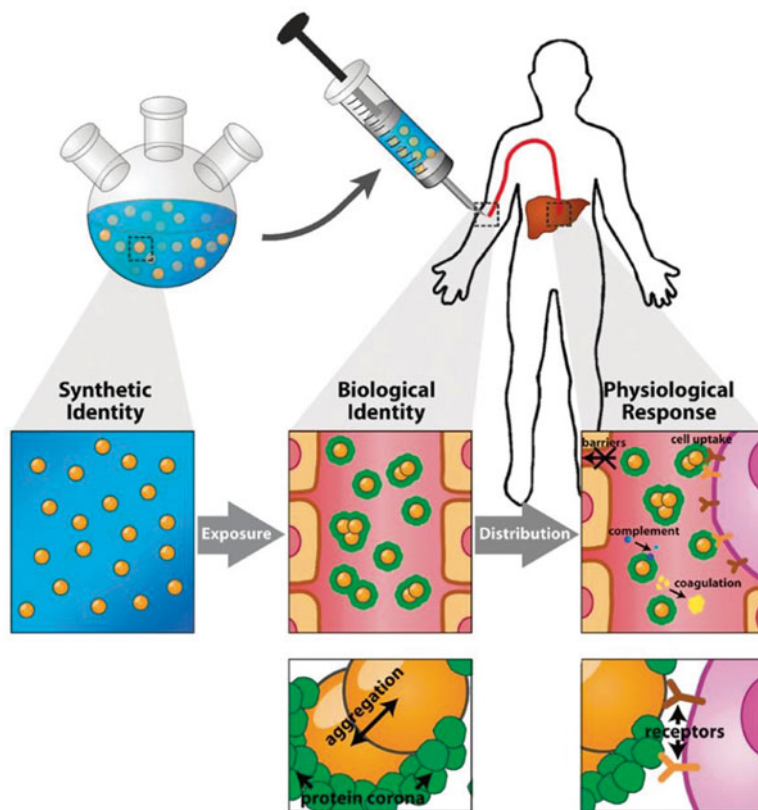
Fig. 28.1 The relationship between the identity of a nanoparticle and its physiological response. The synthetic identity of the nanoparticle includes the many physical and chemical properties of the synthetic nanoparticle. Upon exposure to biosystems, the nanoparticle becomes encapsulated by a corona of adsorbed proteins and begins to take on its biological identity. The biological identity determines the physiological response, including the distribution and activity of the nanoparticle. Reproduced from [27] with permission of The Royal Society of Chemistry

In their recent review of 63 nanomaterials from 26 studies, Walkey and Chan [27] noted 125 unique plasma proteins, termed the "adsorbome," in the protein corona of one standardized nanomaterial. This list is relatively new and will certainly be expanded as more studies are performed on the corona of nanomaterials. In this study, the most common plasma protein corona consists of approximately 2–6 proteins that are adsorbed in high abundance with many more proteins adsorbed in lower abundance. Interestingly, the adsorbed proteins in high abundance were not conserved across materials, and only a small subset of the adsorbome proteins bind to most nanoparticles [27]. However, some trends in protein binding have been observed. Dextran coated nanoparticles are typically bound by antibodies, while cationic and hydrophobic nanoparticle surfaces show strong affinity for albumin [28]. Hydrophobic surfaces tend to attract apolipoproteins, while surfaces presenting hydroxyl groups and positive charges promote binding of proteins from the complement system (C3) [28].

28.3 Hard vs. Soft Corona

The influence of differing nanoparticle surface modification on the protein corona has been reviewed [23], and it was observed that albumin, IgG, fibrinogen, and apolipoproteins are present in the corona of all of the studied nanomaterials. This is not surprising, given the relative abundance of these molecules in the blood, but it is also likely that in time these molecules are desorbed and replaced by higher affinity proteins as they traverse different biological compartments. Such association of proteins with the surface of the nanomaterial is primarily entropy driven [17]. Many water molecules associated with the nanomaterial are liberated when one protein, such as fibrinogen, apolipoprotein, or albumin, binds to the surface. The increase in entropy of the released water molecules is larger than the decrease in entropy of the corona-bound proteins. Proteins bound to the surface in this manner generally do not lose their active conformation [17].

Because of this type of interaction, the hard corona can evolve over time [29]. In the study by Lundqvist (2008), nanoparticles were first incubated in plasma followed by a secondary incubation in salt solutions that mimicked cytosol (i.e., intracellular fluid). Following this second incubation, the composition of the corona was characterized and compared to control samples that were incubated in each fluid separately. The results suggested that there is a distinct evolution of the corona, and that the final corona displays a roadmap or blueprint of its history. The authors of this work suggest that the identity of the corona may be used to potentially predict nanoparticle behavior in biosystems [29].

Though it is apparent that the protein corona has significant biological implications, it remains difficult to predict the interactions between biomacromolecules and nanomaterials. The coating and surface characteristics of nanoparticles appear to provide some indication of the nature of their corona interactions but predicting these interactions has proven difficult and will require intensive study if these materials are to be developed for biological applications.

28.4 Minimizing the Extent of Protein Corona Formation

Several polymers, such as polysaccharides, have been used to coat nanoparticles to efficiently increase circulation time [30]. Among the various approaches, addition of polyethylene glycol (PEG) (abbreviated here PEGylation), is the most widely used strategy to create a steric barrier on the surface of nanoparticles and has been a mainstay in functionalizing a wide variety of nanoparticles [31, 32]. PEGylation can change the physical and chemical properties of the nanomaterial, including its conformation, charge, and hydrophobicity. For many nano-formulations, PEGylation increases drug stability, decreases protein adsorption, improves solubility, and retention time of the conjugates in blood, and reduces proteolysis, renal excretion, and uptake by reticuloendothelial organs (i.e., liver and spleen). These factors result in a prolonged blood residence time, thereby allowing a reduced dosing frequency

[33]. For example, Walkey et al. [34] demonstrated that the surface density of PEG influences serum protein adsorption and the subsequent biological fate of gold nanoparticles, including their uptake by macrophages.

Unfortunately, PEGylation does have drawbacks. PEGylation strongly inhibits cellular uptake and endosomal escape, which results in significant loss of activity of the delivery system [35]. If the biological impact of the particle involves surface chemistry, high density PEGylation can dampen catalytic activity, and the desorption of PEG can lead to immune responses directed against the PEG molecule [36]. Despite its limitations, however, PEGylation continues to be an important strategy in improving the biocompatibility and performance of many nanomaterials.

Recently, we have shown that a stabilizer package of citric acid/EDTA (CA/EDTA) confers many of the benefits of PEGylation without the drawbacks. Citric acid stabilization by itself is easily washed off the particle surface, however the addition of EDTA generates a shell layer that is very durable even when exposed to high ionic strength solutions. Interestingly, the unique CA/EDTA stabilizer decreases nanoceria aggregation, lengthens circulation time, and limits reticuloendothelial organ deposition, without impairing the catalytic properties of this nanomaterial as it participates in redox reactions [37]. Although this approach to stabilization may not be amenable to all nanomaterials, it is readily applied to metal oxides. The addition of CA/EDTA also allows the particle to retain a smaller hydrodynamic radius compared to a similar particle that has been PEGylated. Importantly, intracellular localization and translocation of the particles across the BBB are improved by the addition of CA/EDTA; removal of the stabilizer prevents extravasation of the particle into the brain parenchyma (unpublished data).

28.5 Techniques to Study Protein Corona Composition

Thus, native nanoparticles can certainly be changed by decoration with proteins. The corona thickness and density determine the overall size of the nanoparticle as well as the underlying nanoparticle surface area exposure. The identity and quantity of adsorbed proteins must also be considered, as these factors regulate biological distribution and route of metabolism. Observation of such characteristics can be accomplished by a few widely employed techniques, including dynamic light scattering (DLS), analytical centrifugation (AC), and size exclusion chromatography (SEC) [38, 39].

Identification of the proteins decorating the corona is generally performed after isolation of the adsorbed protein from the nanomaterial surface. Subsequently, the protein corona is removed from the nanomaterial with high temperatures, high salt concentrations, detergents, enzymes, thiols, or a combination of the aforementioned treatments. Depending on the strength of the nanomaterial–protein interaction, the isolation may result in only partial removal of proteins, which could certainly bias the results. Upon isolation of proteins from the corona, the proteins are generally

separated by size using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by band extraction from the gel, digestion with trypsin [40], and identification via tandem liquid chromatography-mass spectrometry (LC-MS/MS) [13, 41].

Since it is not possible to recover nanoparticles once they are injected into an animal, protein–nanomaterial interactions are often modeled by incubating the nanomaterials in a physiological medium containing plasma or serum to mimic blood protein adsorption. With this technique, the corona forms, the nanomaterial is washed, and the proteins comprising the "hard corona" are identified. However, a drawback is that neither serum nor plasma fully replicates the in vivo environment; serum lacks coagulation factors and plasma lacks blood enzymes. In addition, this strategy does not address the changes in protein composition as the particle traverses through tissues, cells, and organelles.

28.6   In Vivo Properties of Nanomaterials

The goal of nanomedicine is to administer nanomaterials to whole organisms for diagnostic or therapeutic purposes [42]. Since the physical and functional characteristics of nanomaterials are so heavily impacted by formation of the corona, the in vivo behavior of these materials may not directly correlate with observed in vitro effects. Aside from altered biological activity, the nanomaterials may have unexpected or undesired distribution to organ systems. For example, the reticuloendothelial organs, liver, and spleen are common sites of nanoparticle deposition when materials are delivered intravenously or subcutaneously [43]. This distribution pattern is likely due in part to the uptake of nanoparticles by circulating or tissue-resident immune cells (i.e., Kupfer cells) [43], a process that is enhanced by the binding of blood complement proteins to the surface. Though strategies have been implemented in attempt to specifically target nanomaterials to desired locations by the addition of moieties during synthesis [44], more general nanoparticle stabilization (or certainly no stabilization) allows organismal distribution to be driven more by the biochemistry of serum protein interactions.

Deposition of nanomaterials to the liver and spleen can result in tissue toxicity and organ dysfunction [45, 46], in particular if levels of the nanoparticles in these tissues remain elevated over time. Unless the nanomaterial is injected directly into a target tissue or organ, there are relatively low levels detected in other organs following nanomaterial administration [47–49], which can limit the range of applications of such substances. As previously noted, delivery of materials into the brain through the BBB is a challenge regardless of the size and composition. The exception to this may be nanomaterials that are small enough to pass through the tight junctions between endothelial cells making up the BBB. However, to reach the brain in the first place, the nanoparticles must avoid uptake by phagocytic immune cells [43, 50], aggregation of particles themselves [51], and binding to other large proteins that would significantly increase their size [50].

Recently, we developed a method to synthesize very small, monodispersed cerium dioxide nanoparticles (2 nm) that are stabilized with CA/EDTA [37]. Cerium oxide nanoparticles (CeNPs) that are unstabilized or minimally stabilized with sodium citrate have illustrated limited deposition in brain tissues [46, 52, 53], though high levels were detected in liver and spleen tissues. A predominant functional property of CeNPs is their ability to fluctuate between +3 and +4 states, which provides unparalleled antioxidant activity that has been demonstrated in both acellular [54–57] and cellular [58–62] in vitro systems. As noted, however, these in vitro models have limitations in their translation to outcomes in intact biological systems, since the nanomaterials are applied directly to the cells, instead of having to traverse biological compartments in order to reach potential target sites. Thus, though the in vitro capabilities of CeNPs are attractive as a therapeutic mechanism, there is little guarantee that this antoxidant activity would be preserved and observed in vivo regardless of the organ target.

28.7 Use of Murine Multiple Sclerosis Model to Demonstrate Biological Efficacy of Cerium Oxide Nanoparticles

A number of neurodegenerative and autoimmune diseases involve high levels of reactive oxygen species (ROS) and other free radicals as at least part of their pathogenesis. The mechanism of such molecules is to steal electrons from other macromolecules such as DNA and lipids, which results in stabilization of the free radical, but causes damage to cells and cellular structures as a result [63]. Thus, antioxidants that can donate or accept electrons from other molecules, generally free radicals, limit the reactivity of these molecules and theoretically block disease development or progression [64]. Previously we have shown that CeNPs are capable of reducing the accumulation of many biologically relevant free radicals in the brain including superoxide anion, nitric oxide, and peroxynitrite [58, 65]. The disease multiple sclerosis (MS) is a neurodegenerative, autoimmune disease involving the targeting of antigens in the myelin sheath surrounding neurons [66]. Immune cells mediate damage to the myelin, including macrophages that are recruited by activated autoreactive T cells. Macrophages play a critical role in protection from pathogens by producing high amounts of ROS (among other killing mechanisms) [67], but in the context of an autoimmune disease, the ROS are inappropriately targeting a "self" tissue instead of providing protection against infection. Destruction of the myelin sheaths of neurons in MS patients causes poor nerve impulse conduction that can result in cognitive and motor function deficits [66].

The murine model of MS, experimental autoimmune encephalomyelitis (EAE) [68], provides an excellent opportunity to test the efficacy of nanomaterials for several reasons. First, neutralization of free radicals can theoretically provide alleviation of disease pathogenesis, so the efficacy of antioxidant CeNPs could be measured by tracking disease symptom severity. Second, the site of disease pathogenesis in the brain and spinal cord allows assessment of how well nanoceria can be delivered

to these organs without direct administration into these sites. Since EAE development involves disruption of the BBB, additional healthy animals must be similarly treated with nanoparticles to confirm similar brain deposition of nanoceria in organisms with intact BBBs.

Given limited brain deposition of other nanoceria formulations and the understanding that physical and chemical characteristics of nanoparticles affect protein adsorption and delivery, development of a novel nanoceria formulation was undertaken as previously noted. Synthesis of these CeNPs involved stabilization with a unique combination of citrate and EDTA [37]. The zeta potential of these custom-synthesized CeNPs is considerably less negative than other formulations (−23.5 mV), and the particles are smaller (hydrodynamic radius = 2.9 nm). Further, the particles remain highly dispersed in physiological salt solutions and resist pelleting even at very high centrifugation rates (100,000 × *g*) [37]. The protein–nanomaterial interactions of our CeNPs were modeled by incubating the nanomaterial in a physiological medium containing mouse serum to mimic blood protein adsorption. After incubation in the serum, the protein-decorated CeNPs were collected by centrifugation and analyzed. Isolated hard corona proteins were separated via SDS-PAGE and the gel bands were excised from the gel and digested with trypsin, and the resulting peptide mixtures were then extracted from the gel pieces and concentrated on a SpeedVac concentrator and then cleaned with a C18 Micro ZipTip (Millipore). The clean peptide mixtures were then analyzed by nanoliquid chromatography tandem mass spectrometry (nanoLC-MS/MS) using a NanoAcquity UPLC coupled with a QTOF Micro mass spectrometer (both from Waters Corporation). The ions were analyzed in data-dependent mode. The resulting raw data were converted into pkl files using ProteinLynx Global Server (version 2.4. from Waters Corporation) and the pkl files were used to perform database searches using the web-based Mascot database search engine. The Mascot search parameters included: Swissprot database, peptide tolerance of ±0.9 Da, MS/MS tolerance of ±0.5 Da, enzyme used: trypsin, one missed cleavage, and cysteines modified to carbamydomethyl as fixed modification.

The full procedure for protein digestion and peptide extraction, as well as the parameters used for nanoLC-MS/MS and data processing are described in detail elsewhere [69–73]. An example of an outcome of such a proteomics experiment is shown in Table 28.1 (note: the numbers on the right are the Mascot scores; the higher the score, the higher the probability that a protein is identified with high confidence). Far fewer proteins were observed in the corona than predicted [74], likely due to the presence and characteristics of the CA/EDTA stabilizer.

The relatively minimal protein corona generated as a result of the addition of highly durable stabilizers also appears to affect nanoceria biodistribution, as we observed high quantities of CeNPs in the brain [37], relative to those reported by other studies with differing nanoceria formulations [46, 52, 53]. Though ceria was detected in the spleens and livers of healthy mice injected with CeNPs, the levels were nearly 100-fold lower than those measured in animals that received citrate stabilized or unstabilized nanoceria [46, 52, 53]. This unique distribution pattern may be due to high concentrations of ApoA1 and serum albumin (relative to immunoglobulins) bound to the CeNPs. The near impossibility of isolating untagged

Table 28.1 The protein
composition of the hard
corona of monodispersed
cerium dioxide nanoparticles
(2 nm) stabilized with
CA/EDTA[a]

Proteins found in corona	Score
Alpha-2-macroglobulin	331
Serum albumin	288
Ig alpha chain C region	271
Ig mu chain C region secreted form	249
Hemoglobin subunit beta-1	207
Alpha-1-antitrypsin 1–2	180
Complement C3	172
Apolipoprotein A-I	171
Ig kappa chain V–III region PC 2880/PC 1229	110
Ig gamma-2B chain C region	96
Haptoglobin	82
Ig gamma-2A chain C region, A allele	76
Hemoglobin subunit alpha	61
Alpha-2-macroglobulin	57
Hemopexin	46
Ig heavy chain V region 441	44
Ig gamma-3 chain C region	38
Ig gamma-1 chain C region, membrane-bound form	35
Kininogen-1	33
Serine protease inhibitor A3K	30

[a]Previously unpublished results

CeNPs from such biological tissues necessitates the use of ICP-MS to most efficiently quantify the ceria content of these organs as a proxy for better, direct assessment of nanoparticle content. Transmission electron microscopy (TEM) could be used, but it is time consuming and only qualitative. STEM-EELS could add elemental analysis capability to the TEM technique, but often the deposition of the element of interest is so low that it cannot be detected in thin sections by this method.

Administration of the CeNPs to animals induced with a chronic progressive form of EAE illustrated similar brain deposition to that observed in healthy animals (Fig. 28.2) [37]. TEM imaging of cerebellum tissue harvested from a CeNP-treated animal revealed the presence of the nanoparticles within cells including the mitochondria, neuronal myelin sheaths, and cell membranes (Fig. 28.2). However, simple target tissue deposition was insufficient to demonstrate the retention of the CeNPs' potential biological activity. EAE-induced mice develop progressive loss of motor function, first in the hind limbs and later in the front limbs [75]. A disease assessment paradigm exists to assign a clinical score of disease severity to each animal twice daily, based upon its limb mobility. The scale for these scores is 0–5, with higher scores reflecting more severe symptoms. Intravenous CeNP administration at varying doses (10, 20, or 30 mg/kg) followed a preventative or therapeutic regimen relative to disease induction: preventative: days −1, 0, 1, 3, 7, 14, 21, 28, 35; 3-day therapeutic: days 3, 7, 14, 21, 28, 35; or 7-day therapeutic: days 7, 14, 21, 28, 35 [37]. Using clinical scores as an indication of disease severity, the
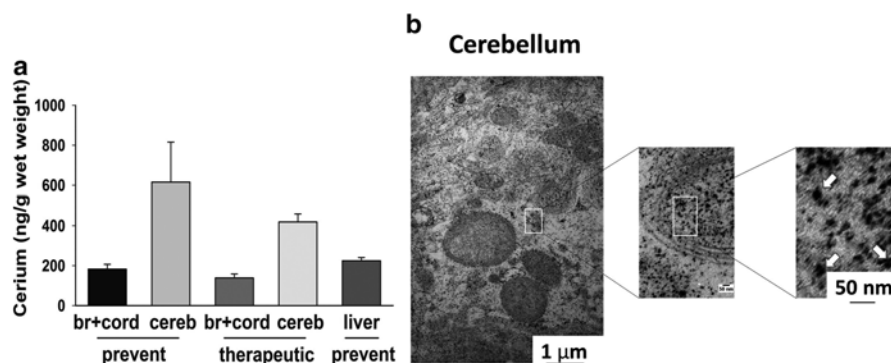
Fig. 28.2 ICP-MS analysis demonstrated the presence of CeNPs in the brains of mice with EAE. Ceria was detected in the cerebellum of CeNP-treated EAE animals (**a**) with intracellular distribution shown by transmission electron microscopy (**b**). *Arrows* indicate nanoparticle location. Reprinted with permission from Heckman, K. L., W. Decoteau, A. Estevez, K. J. Reed, W. Costanzo, D. Sanford, J. C. Leiter, J. Clauss, K. Knapp, C. Gomez, P. Mullen, E. Rathbun, K. Prime, J. Marini, J. Patchefsky, A. S. Patchefsky, R. K. Hailstone, and J. S. Erlichman, 2013, Custom Cerium Oxide Nanoparticles Protect against a Free Radical Mediated Autoimmune Degenerative Disease in the Brain: ACS Nano. Copyright 2013 American Chemical Society

preventative and therapeutic treatment regimens were effective at alleviating EAE symptoms, in particular at higher doses (Fig. 28.3) [37]. Area under the curve (AUC) analysis allows for assessment of the cumulative disease severity throughout the entire disease course (Fig. 28.4).) In fact, the efficacy of the CeNPs was similar to that of the currently used MS drug fingolimod (Figs. 28.3 and 28.4) [37].

The cumulative amount of CeNPs delivered to the EAE mice correlated with the improvements in disease severity (measured by clinical scores) and also with the amount of ceria detected in the brains of treated animals (detected by ICP-MS) (Fig. 28.4) [37]. Given these correlations, it seems logical to conclude that the CeNPs reach the brain and protect against this ROS-mediated disease. However, these metrics do not specifically demonstrate the mechanism utilized by the CeNPs to interfere with disease pathogenesis and thus protect against symptom progression. To test whether levels of ROS in the brain may have been neutralized by the antioxidant properties of the CeNPs, brains were harvested from EAE animals late in the disease course, and sections were stained with CM-DCFDA, a total ROS indicator dye (Fig. 28.5) [37]. Levels of ROS were reduced approximately 30 % in brain sections of CeNP-treated animals, relative to those of control or fingolimod-treated animals, indicating that antioxidant activity was indeed observed in those animals treated with CeNPs and is likely a mechanism of CeNP-mediated disease protection.

Complete analysis of the biological properties and efficacy of nanoceria is not only dependent upon the standard biological metrics of disease severity, but also upon the biochemical techniques that enable both analysis of in vivo ceria deposition and more fundamental in vitro CeNP characterization. However, in context of the in vivo
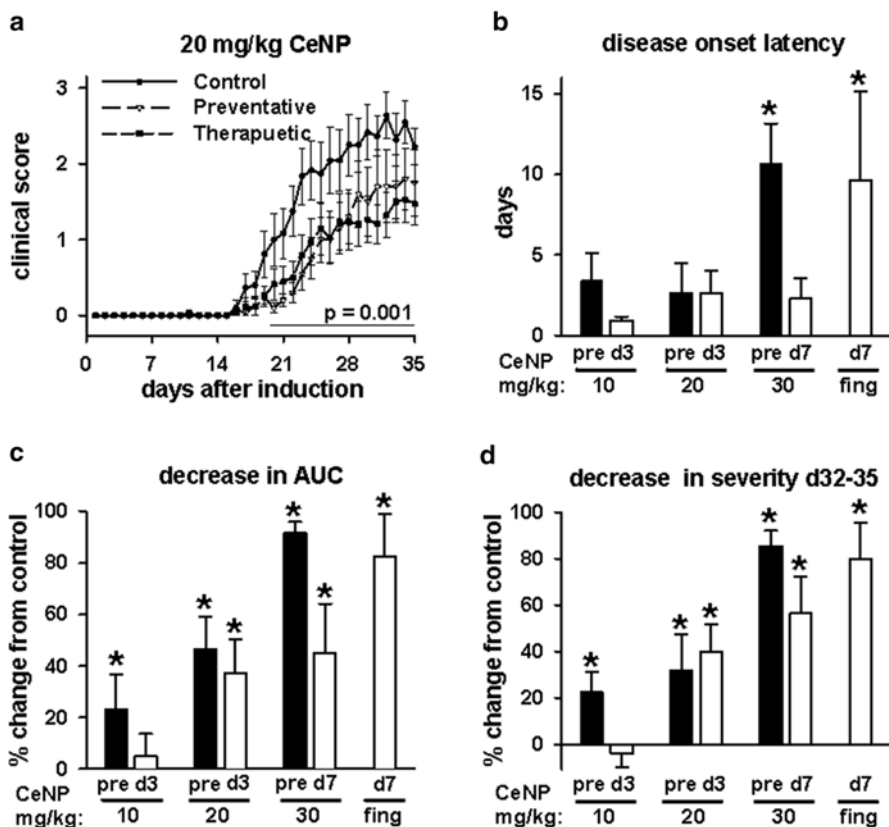
Fig. 28.3 EAE disease severity was alleviated by CeNP treatment in preventative and therapeutic treatment regimens. Higher clinical scores are indicative of worse disease symptoms; (**a**) depicts the kinetics of disease progression in mice treated with 20 mg/kg CeNPs compared to control. Disease onset (**b**) and area under the curve (AUC) analysis (**c**) illustrate the improved efficacy of CeNPs delivered at higher doses, in particular compared to the currently used drug, fingolimod. The longevity of the CeNP treatment is illustrated by decreased clinical scores at late stages of disease (**d**). Reprinted with permission from Heckman, K. L., W. Decoteau, A. Estevez, K. J. Reed, W. Costanzo, D. Sanford, J. C. Leiter, J. Clauss, K. Knapp, C. Gomez, P. Mullen, E. Rathbun, K. Prime, J. Marini, J. Patchefsky, A. S. Patchefsky, R. K. Hailstone, and J. S. Erlichman, 2013, Custom Cerium Oxide Nanoparticles Protect against a Free Radical Mediated Autoimmune Degenerative Disease in the Brain: ACS Nano. Copyright 2013 American Chemical Society

EAE studies, there is still an incomplete understanding of what unique protein corona enables the delivery of the CeNPs into the brain in a configuration that preserves their antioxidant activity. The ideal experiment would be to somehow retrieve the CeNPs from the brain tissue for characterization of bound proteins. Given the small size of the nanoparticles, this is a formidable task, especially given the intracellular compartmentalization that has likely occurred. In the absence of an efficient retrieval
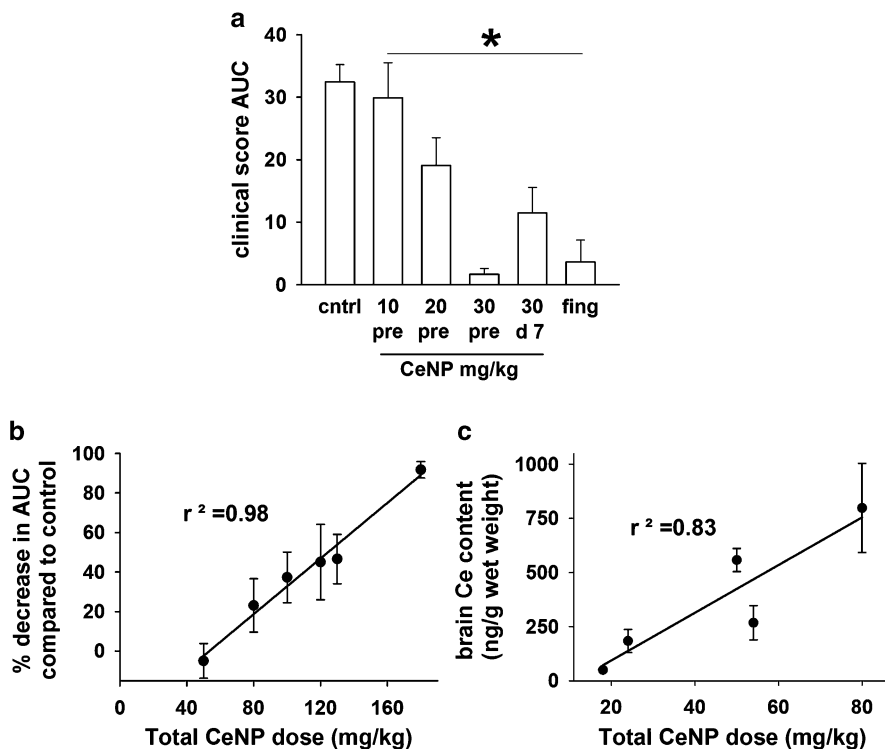
Fig. 28.4 Cumulative disease severity was lessened by CeNP treatment, as dosage correlated with biological activity and tissue deposition. AUC analysis indicates that overall EAE disease severity was worse in control animals and those treated with lower doses of CeNPs (**a**). However, as CeNP dose increased, so did the reduction in clinical disease severity (**b**). Further, the increased deposition of ceria in the brains of CeNP-treated animals (**c**) suggests that the presence of the nanoparticles provided this protection against disease pathogenesis. Reprinted with permission from Heckman, K. L., W. Decoteau, A. Estevez, K. J. Reed, W. Costanzo, D. Sanford, J. C. Leiter, J. Clauss, K. Knapp, C. Gomez, P. Mullen, E. Rathbun, K. Prime, J. Marini, J. Patchefsky, A. S. Patchefsky, R. K. Hailstone, and J. S. Erlichman, 2013, Custom Cerium Oxide Nanoparticles Protect against a Free Radical Mediated Autoimmune Degenerative Disease in the Brain: ACS Nano. Copyright 2013 American Chemical Society

technique, immunohistochemistry analysis of brain sections of CeNP-treated animals could be useful. If the CeNPs could be molecularly tagged in a manner that would not drastically increase size or disrupt protein corona interactions, the nanoparticles could be detected along with staining to detect the co-localization of the nanomaterial with a bound, probable serum protein. Further description of the protein corona that allowed for the successful deposition of these custom CeNPs in the brain (with simultaneous limited deposition in reticuloendothelial organs) will allow for the synthesis of new nanomaterials specifically targeted for brain delivery.
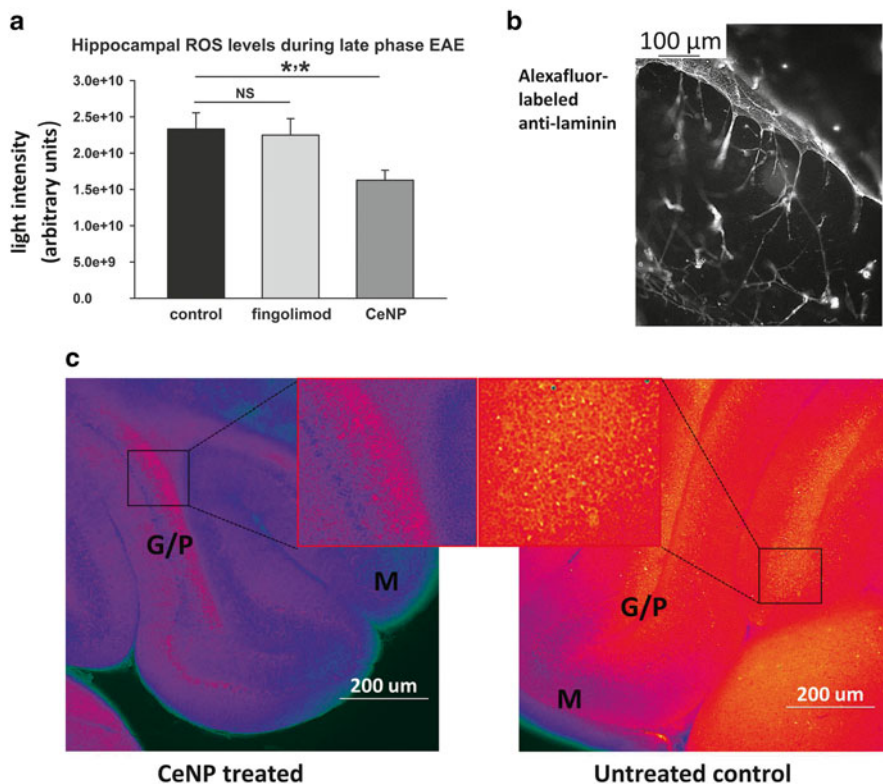
Fig. 28.5 Lower levels of ROS were detected in the hippocampus of CeNP-treated EAE animals. Late in the disease course, hippocampus brain slices were stained with a total ROS indicator dye (**c**); quantitative analysis (**a**) demonstrated that the CeNPs retained their antioxidant activity upon reaching the brain. Microvasculature staining (**b**) and lower magnification analysis of the ROS indicator dye staining in the brain (**c**) demonstrated that reduced ROS levels are found outside the brain vasculature. In particular, ROS levels in the granular cells (typically a site of neurodegeneration in MS) were lower in the CeNP-treated mice compared to controls. Reprinted with permission from Heckman, K. L., W. Decoteau, A. Estevez, K. J. Reed, W. Costanzo, D. Sanford, J. C. Leiter, J. Clauss, K. Knapp, C. Gomez, P. Mullen, E. Rathbun, K. Prime, J. Marini, J. Patchefsky, A. S. Patchefsky, R. K. Hailstone, and J. S. Erlichman, 2013, Custom Cerium Oxide Nanoparticles Protect against a Free Radical Mediated Autoimmune Degenerative Disease in the Brain: ACS Nano. Copyright 2013 American Chemical Society

28.8 Summary

Nanotechnology is a "toolbox" that provides nanosized building blocks for new materials, devices, and systems for broad scientific applications. Engineered nano-materials have unique physical–chemical properties that make them promising candidates for many biomedical applications, including tissue regeneration, drug,

and gene delivery, and in vivo monitoring of disease processes. The burgeoning possibilities of nano-scale structure manipulations will likely increase. Furthermore, because cellular molecules and man-made nanoparticles have similar dimensions, the latter may directly interact with vital cellular processes. Understanding the nano-biological interface requires a thorough understanding of factors that influence protein adsorption which are central in dictating biodistribution, fate and, to varying degrees, biological action. Consequently, new biological testing paradigms must be developed to examine the physiological effects of these man-made materials to assess both their biomedical potential as well as toxicology. Mass spectroscopy in its various forms (ICP-MS, LCMS) is a critical tool in the development of this technology in biological settings.

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