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# Nanoparticle iron chelators: A new therapeutic approach in Alzheimer disease and other neurologic disorders associated with trace metal imbalance

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**Abstract**

Accumulating evidence suggests that oxidative stress may be a major etiologic factor in initiating and promoting neurodegeneration in Alzheimer disease. Contributing to this, there is a dyshomeostasis of metal ions in Alzheimer disease with abnormally high levels of redox-active metals, particularly iron, in affected areas of the brain. Although it is unclear whether metal excesses are the sole cause of oxidative stress and neurodegeneration or a by-product of neuronal loss, the finding that metal chelators can partially solubilize amyloid-β deposits in Alzheimer disease suggests a promising therapeutic role for chelating agents. However, the blood–brain barrier and toxicity of known chelators limit their utility. In this study, we suggest that covalent conjugation of iron chelators with nanoparticles may help overcome the limitations in blood–brain barrier permeability of existing chelation therapy. Using *in vitro* studies, we have shown that a chelator–nanoparticle system and the chelator–nanoparticle system complexed with iron, when incubated with human plasma, preferentially adsorb apolipoprotein E and apolipoprotein A-I, that would facilitate transport into and out of the brain via mechanisms used for transporting low-density lipoprotein. Our studies suggest a unique approach, utilizing nanoparticles, to transport chelators and chelator–metal complexes in both directions across the blood–brain barrier, thus providing safer and more effective chelation treatment in Alzheimer disease and other neurodegenerative diseases.
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*Keywords:* Blood–brain barrier crossing; Chelator nanoparticle conjugation; Lipoprotein absorption; Metal dysregulation; Neurodegeneration disease; Targeting chelation therapy

Although the etiology of Alzheimer disease (AD) is incompletely understood, accumulating evidence supports the concept that oxidative stress generated by various mechanisms may be a major factor that contributes to the initiation and promotion of neurodegeneration [9,18,21,29]. Transition metals, such as iron, copper, aluminum and zinc, have been found in high concentrations in the brains of patients with AD and studies suggest their involvement in the etiopathology of the brain changes seen in AD [13,16,25,26,28]. Overall, these studies indicate that the environmental conditions in AD, exacerbated by imbalances in several metals, have the potential to catalyze and stimulate free radical formation and enhance neuronal degeneration. This metal dysregulation may represent a therapeutic target for AD. Indeed, chelation therapy with desferrioxamine (DFO), ethylenediaminetetraacetic acid (EDTA) and iodochlorhydroxyquin (clioquinol) have been shown to induce clinical improvement in patients with AD [4,5,24]. Unfortunately, problems concerning chelator bioavailablity and toxic side-effects hindered further investigation, both to understand the pathologic role of metal dysregulation in AD as well as to evaluate the efficacy and safety of chelator therapy in AD.

Currently, drug delivery using nanoparticles to target the brain has shown promise in improved drug efficacy and reduced drug toxicity [14]. These particles have been shown to penetrate the blood–brain barrier (BBB) and preferentially adsorb lipoproteins [15]. It is possible that nanoparticles are able to cross the

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BBB by mimicking low density lipoprotein (LDL), thus permitting them to interact with the LDL receptor and resulting in their uptake by brain endothelial cells [14]. They may also be able to employ transferrin transcytosis for their transport [14]. More importantly, nanoparticles also have the potential to re-cross the BBB into the bloodstream. They may behave like lipoprotein particles by preferentially adsorbing apolipoprotein A-I (ApoA-I), known to facilitate removal of nanoparticles from the brain through the same transport systems [8]. These insights into nanoparticle transport have given an impetus to chelation therapy in AD. Indeed, since nanoparticles can be covalently conjugated to iron chelators without altering the chelator ability to bind metals, nanoparticles can be used not only to deliver chelators into the brain, but also to transport the chelator–metal complexes out of the brain. This novel approach to chelator therapy could significantly improve the efficacy and reduce the toxicity of chelation therapy.

In this study, we have tested iron removal from sections of fixed AD brain by various iron chelators in order to determine suitable chelators for conjugation with nanoparticles. In addition, to demonstrate the possibility of preferential adsorption of apolipoprotein E (ApoE) and/or ApoA-I by chelator–nanoparticle systems (CNPS) or chelator–nanoparticle systems chelated with iron (ICNPS), respectively, we have incubated these complexes with human plasma and, using 2D-gel electrophoresis, studied the pattern of proteins adsorbed. Our findings indicate that the CNPS is not only effective at chelating iron from human tissue but also binds to ApoE and ApoA-I that would facilitate brain entrance and exit, respectively.

The ability of chelators to remove iron from paraffin sections of brain tissue was examined histochemically as previously described to detect redox-active iron [28]. Brain sections from four AD patients (80–84 years) were either untreated or subjected to treatment with iron chelators and the degree of iron chelation in the treated sections compared, using differential interference microscopy, with the iron deposition in paired untreated sections. Briefly, hippocampal tissue from AD patients was fixed overnight in methacarn (methanol:chloroform:acetic acid in a ratio of 6:3:1). After dehydration in ascending concentrations of alcohol, the tissues were embedded in paraffin and 6 μm sections placed on silane-coated slides (Sigma). Sections were then deparaffinized with two changes of xylene for 10 min each and rehydrated through descending concentrations of ethanol to Tris-buffered saline (TBS) at pH 7.6 (50 mM Tris, 150 mM NaCl). Sections were incubated overnight at 37 °C in 40 μl of 0.01–0.1 M concentration of solutions of two chelators, 2-methyl-*N*-(2′-aminoethyl)-3-hydroxyl-4-pyridinone (MAEHP) and DFO in phosphate-buffered saline (PBS). Sections were thoroughly rinsed in TBS, incubated for 2 h at 37 °C in 7% potassium ferrocyanide in 3% aqueous hydrochloric acid, rinsed with Tris buffer and then incubated for 5–10 min in 3,3′-diaminobenzidine (Dako Corporation). Lastly, the sections were dehydrated through graded alcohol solutions, cover-slipped and submitted for microscopic examination.

To demonstrate the ability of iron chelators conjugated to nanoparticles, alone or complexed with iron, to selectively adsorb proteins, CNPS and ICNPS were incubated with human plasma and the pattern of adsorbed proteins studied using standard two-dimensional (2D) gel electrophoresis.

Iron chelators were conjugated to nanoparticles through the formation of an amido bond between a primary amino group in the chelator and a carboxyl group on the nanoparticle surface [1] to form CNPS (Fig. 1) which was coated at room temperature with a surfactant (polysorbate 80) to alter its surface properties. ICNPS was obtained by reaction of CNPS with ferric iron. Coated CNPS and ICNPS (100 μl of 2.5%, w/v solution of each in PBS) were incubated in 1 ml of citrated human plasma (Sigma) for 5 min at 37 °C [3]. After separation by centrifugation and washing four times with MilliQ water, the adsorbed proteins were eluted from the particle surface using a protein-solubilizing solution composed of 5% sodium dodecyl sulfate (SDS), 5% dithiothreitol (DTT), 10% glycerol and 60 mM Tris at pH6.8 [3] and analyzed by 2D gel electrophoresis. Separation in the first dimension using isoelectric focusing (IEF) was carried out in glass tubes with a 2.0 mm inner diameter using 2.0% pH 3.5–10 ampholines for 9600 V h. Separation in the second dimension, based on molecular weight (MW) of the protein, was carried out using SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Each tube from the first dimension electrophoresis was incubated for 10 min in pH 6.8 buffer (2.3% SDS, 50 mM DTT, 10% glycerol and 62.5 mM Tris) sealed on top of a 10% acrylamide slab gel (145 mm × 145 mm × 0.75 mm) and elec-

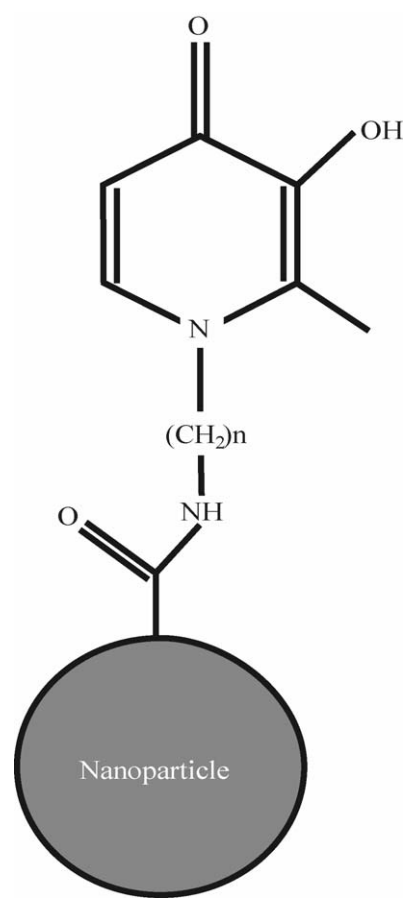


Fig. 1. Chemical structures of MAEHP ($n = 2$) and MAPHP ($n = 3$) that were covalently linked to nanoparticle through amido bond.

trophoresis carried out for 4 h at 12.5 mA/gel. The gels were dried between sheets of cellophane and silver-stained [3].

Differential interference microscopy demonstrated the ability of the iron chelators such as DFO and MAEHP to strip iron from methacarn-fixed, paraffin-embedded sections of AD brains. The efficacy of iron removal was dependent on the concentration and chemical structure of the chelator. Compared to DFO, MAEHP was a more effective chelator, even at a 0.01 M concentration. In contrast, DFO had a limited effect in removing iron at a concentration of 0.01 M although it depleted iron effectively at a 0.1 M concentration. Fig. 2 shows an AD brain section treated with 0.01 M MAEHP as a prototype of chelators (B) compared with untreated control (A). The treated section shows significantly less histochemical iron stain.

Fig. 3 shows the silver stain images of proteins adsorbed from human plasma by CNPS (MAPHP conjugated) and ICNPS and separated by 2D-gel electrophoresis. The protein patterns were entirely different from that of human plasma proteins (Fig. 3A). CNPS coated with the surfactant polysorbate 80, which altered its surface properties, preferentially adsorbed ApoE as shown in Fig. 3B. On the other hand, ICNPS showed preferential adsorption of ApoA-I (Fig. 3C).

In this study, we show that a CNPS not only retains ability to chelate metal ions from human tissue but also preferentially binds to ApoE in the free state and ApoA-I when loaded with iron. The results of our study suggest that nanoparticles may have the ability to carry chelators, across the BBB by preferential adsorption of ApoE. Studies by Kreuter et al. [14,15] have demonstrated that polysorbate 80-coated nanoparticles with a diameter of ∼300 nm can target the brain for drug delivery by mimicking LDL particles and using the LDL transport and/or transferrin transcytosis systems to cross the BBB. Studies have also shown that nanoparticle uptake by the brain does not cause significant change in BBB integrity or permeability [7], and that nanoparticles may deliver drugs to amyloid-β [10] and prevent amyloid-β-induced reactive oxygen species [27].

Additionally, for iron chelation to be effective and to prevent metal-associated oxidative damage and potential toxicity of chelator–metal complexes, an ideal chelator should, once complexed with the chelated ion, be capable of traversing the BBB in the reverse direction to leave the brain. This can be achieved by covalent conjugation of selected iron chelators to nanoparticles that are either non-degradable or can be degraded in a controlled manner to again serve as vehicles for transporting chelator–metal complexes out of the brain. Our study shows that ICNPS can preferentially adsorb ApoA-I which can facilitate removal of complexed nanoparticles from the brain via the LDL transport system. This result indicates that CNPSs possess the ability to leave the brain once they complex iron inside the brain after partial or total dissociation of ApoE from their surface. That said, dissociation may not be necessary since the chelators conjugated to nanoparticles can still expose themselves on the top of the absorbed ApoE layer in many cases (i.e., a long linkage between a chelator and a nanoparticle).

Conjugation to nanoparticles confers several additional advantages on chelators. Firstly, if bound to nanoparticles, a chelator need not be lipophilic to cross the BBB. Secondly, even being lipophilic, the chelator would be less toxic after conjugation. Thirdly, even hydrophilic iron chelators with large molecular weight such as hexadentate ones, which would not normally be able to cross the BBB, can be used. Various methods, depending on functional groups both in the chelator and on the nanoparticle surface, can be applied for conjugation with resulting good coupling yield [1], and with the metal-binding ability of the chelator being unaffected by conjugation to nanoparticle.

The iron chelators, MAEHP and 2-methyl-*N*-(3′-aminopropyl)-3-hydroxyl-4-pyridinone (MAPHP), used in this study, are derivatives of deferiprone or L1 that has high affinities for iron, aluminum, copper and zinc while lacking the ability to chelate calcium and manganese [11]. This favorable, selective chelation property makes them a suitable choice for the treatment of AD, where they can simultaneously deplete excess iron,

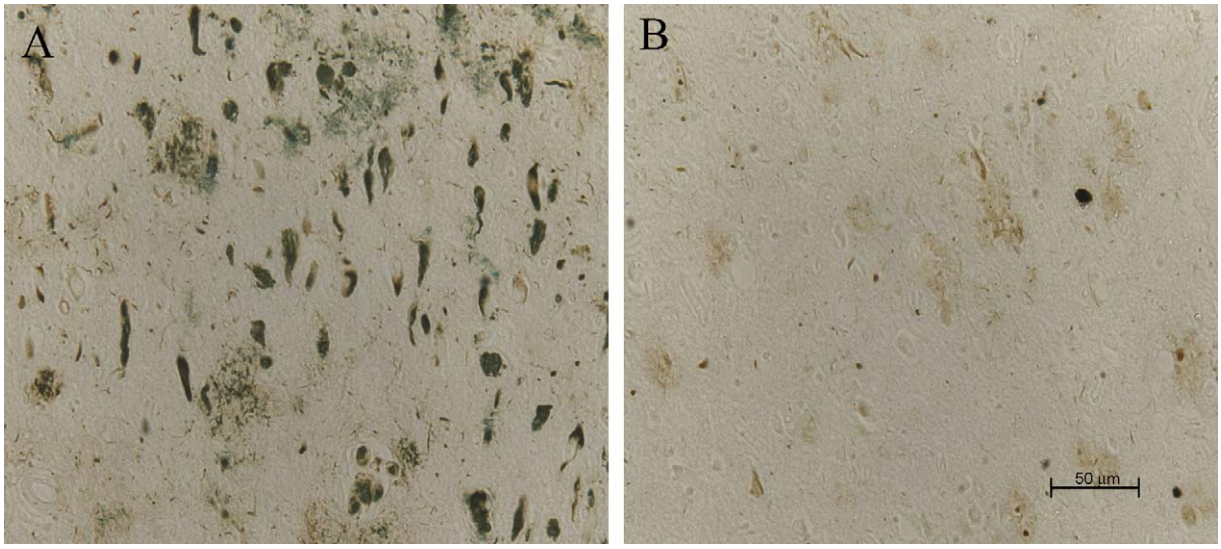


Fig. 2. Lesion-associated chelatable iron in AD brain sections was depleted with iron chelator (MAEHP as a prototypal chelator), which was detected histochemically with a modified Perl Stain. Saline (A) and MAEHP treated (B) sections.

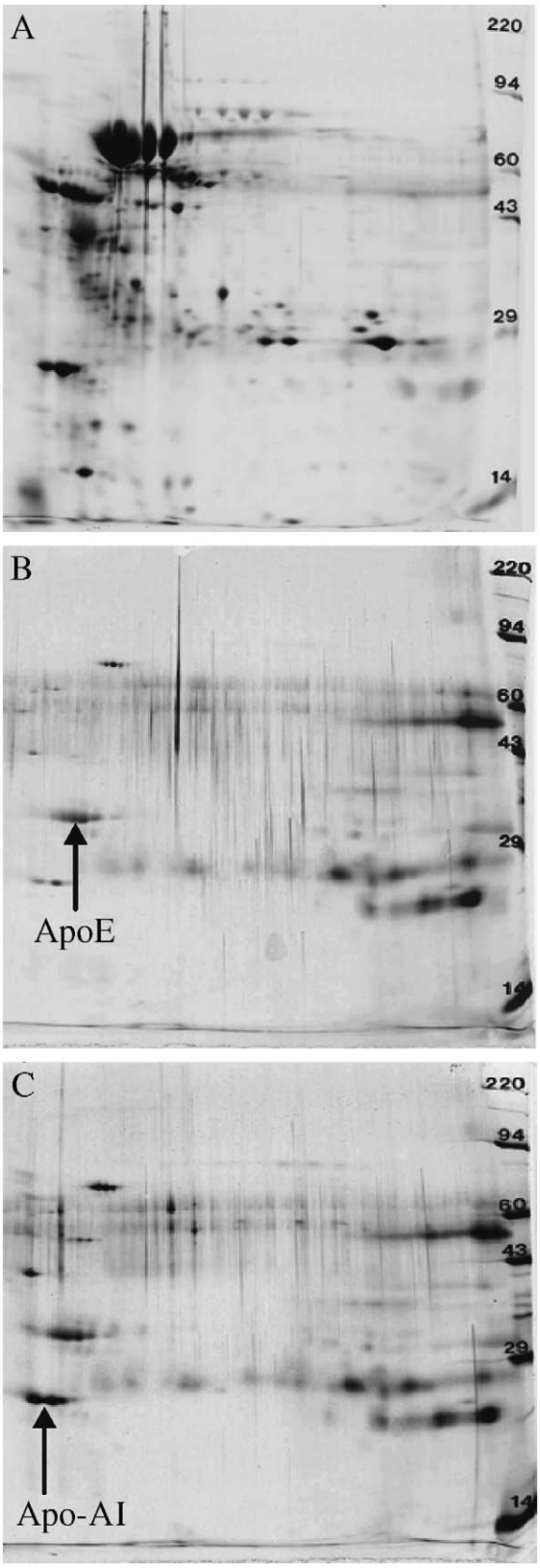


Fig. 3. Images of plasma protein patterns examined by 2D PAGE. (A) Plasma; (B) CNPS (MAPHP conjugated) coated with polysorbate 80; and (C) ICNPS.

aluminum, copper and zinc without affecting the essential elements, calcium and manganese.

Nanoparticles used in conjugation can be made of biocompatible synthetic or natural macromolecules [14] with functional amino and carboxyl groups on their surface for covalent bonding with chelators. Optimization of apolipoprotein adsorption by CNPS and ICNPS can be achieved using different chelators, linkages, coating materials as well as nanoparticles with different surfaces. The system may also be coated with ApoE, ApoB or ApoA-I to achieve predictable efficiency in crossing the BBB [15]. Further studies are warranted to demonstrate the efficacy of the CNPS, to determine their toxicity and to optimize their ability to cross the BBB.

DFO, an iron chelator approved by the FDA for the treatment of iron overload, has been shown, in a clinical trial, to significantly slow the rate of progression of AD [5]. This therapeutic effect has been ascribed to the affinity of DFO to bind iron, aluminum, copper and zinc [11], thus sequestering these metals and consequently preventing oxidative damage of brain tissue. Our study also shows chelatable iron in sections of AD brain. Chelatable iron is believed to catalyze free radical formation, causing oxidative damage to all classes of biomolecules [9]. Early reports by Smith et al. [28] have shown that chelation therapy can remove iron accumulation closely associated with senile plaques and neurofibrillary tangles in cases of AD. This iron, specifically localized to the characteristic lesions of AD, is capable of participating in *in situ* oxidation and of catalyzing $H_2O_2$-dependent oxidation [25]. Taken together, these studies indicate a potential role for chelation therapy in AD, to deplete excess metal in brain tissue and prevent metal-mediated oxidative damage. Our *in vitro* method of studying the effectiveness of chelators could serve as a useful tool for screening potential therapeutic agents capable of mobilizing iron from the AD brain.

The chelator DFO, in addition to its ability to simultaneously chelate several metals that are elevated in the AD brain, has the advantage of not binding to essential elements such as calcium and manganese. Thus, DFO may take precedence over other chelators such as clioquinol, D-penicillamine (low iron-binding affinity) and EDTA (high calcium and manganese binding) for therapy in AD. Unfortunately, DFO has serious neurotoxic side-effects [2]. It is also poorly absorbed via the gastrointestinal tract and is rapidly degraded after administration. To yield significant iron excretion, DFO requires prolonged subcutaneous administration [20]. Moreover, some studies show that DFO, due to its hydrophilic nature and large molecular weight, does not easily penetrate the BBB [17]. These disadvantages present serious hurdles for the use of DFO in AD treatment.

Another iron chelator, deferiprone or L1 (1,2-dimethyl-3-hydroxyl-4-pyridinone) is approved outside the US for clinical use in the treatment of iron or aluminum overload. Although L1 can be given orally and can cross the BBB due to its small molecular size and lipophilicity, it also has serious neurotoxicity [23]. In addition, studies have shown that L1, although it effectively binds iron, lacks the ability to remove iron from the brain [6], probably due to strong hydrophilicity of the iron-L1 complex. Derivatives of L1 with higher lipophilicity also have the ability to cross the BBB and bind brain iron, but they also possess con-

siderable neurotoxicity [23]. A newly developed iron chelator, deferasirox, is recently approved by FDA for treatment of transfusion iron overload. Although deferasirox is orally active, its long-term profiles are not yet available [19]. Also, its lipophilic nature like L1 may raise questions concerning potential toxicity in AD treatment.

The use of currently available iron chelators is, therefore, limited by their toxicity and/or poor transference across the BBB. Most bi-dentate iron chelators, such as L1 and its derivatives, with small molecular weight and high lipophilicity, have the ability to penetrate the BBB, but show toxicity [12]. On the other hand, hexadentate iron chelators are considered better candidates for chelation therapy than bi- and tridentate ones because of their lower toxicity before and after chelation [12], but they have difficulty penetrating the BBB [6,12] due to their hydrophilicity and relatively high molecular weight. Increasing their ability to cross the BBB by enhancing the lipophilicity and lowering the molecular weight of iron chelators is believed to come at the price of increased toxicity [22].

In conclusion, the nanoparticle chelator system developed here may provide a useful therapeutic option for the treatment of AD and the use of nanoparticles to transport iron chelators in and out of the brain could lead to safer and more effective therapy. This approach would help deepen our understanding of the pathogenesis of AD and pave the way for nanoparticle mediated delivery of other therapeutic agents in the management of AD as well as of other neurodegenerative diseases.

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