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Metal chelators coupled with nanoparticles as potential therapeutic agents for Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is a devastating neuro-degenerative disorder characterized by the progressive and irreversible loss of memory followed by complete dementia. Despite the disease's high prevalence and great economic and social burden, an explicative etiology or viable cure is not available. Great effort has been made to better understand the disease's pathogenesis, and to develop more effective therapeutic agents. However, success is greatly hampered by the presence of the blood-brain barrier that limits a large number of potential therapeutics from entering the brain. Nanoparticle-mediated drug delivery is one of the few valuable tools for overcoming this impediment and its application as a potential AD treatment shows promise. In this review, the current studies on nanoparticle delivery of chelation agents as possible therapeutics for AD are discussed because several metals are found excessive in the AD brain and may play a role in the disease development. Specifically, a novel approach involving transport of iron chelation agents into and out of the brain by nanoparticles is highlighted. This approach may provide a safer and more effective means of simultaneously reducing several toxic metals in the AD brain. It may also provide insights into the mechanisms of AD pathophysiology, and prove useful in treating other iron-associated neurodegenerative diseases such as Friedreich's ataxia, Parkinson's disease, Huntington's disease and Hallervorden-Spatz Syndrome. It is important to note that the use of nanoparticle-mediated transport to facilitate toxicant excretion from diseased sites in the body may advance nanoparticle technology, which is currently focused on targeted drug delivery for disease prevention and treatment. The application of nanoparticle-mediated drug transport in the treatment of AD is at its very early stages of development and, therefore, more studies are warranted.

Introduction

Alzheimer disease (AD) is the most common form of dementia and the fourth leading cause of death among people aged 65 and older [1,2]. AD is a devastating neurodegenerative disease with progressive and irreversible damage to thought, memory, and language [3]. At present, over 24 million people suffer from dementia worldwide and the affected population will exponentially increase to 42 million by year 2020 and to 81 million by year 2040 [4] if no effective prevention and treatment become available [5,6]. In addition, AD places an incalculable emotional and physical drain on families and caretakers. As such, the disease poses a heavy economic and societal burden with associated annual cost of care over \$100 billion and about \$500 billion by year 2020 in the United States alone [3,7]. The ravaging effects of

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the disease call for efforts to prevent, forestall, and reverse the disease. Unfortunately, despite much interest, currently available FDA-approved drugs, targeted towards managing cognitive symptoms such as changes in memory and perception, provide only partial benefit to select patients and fall tremendously short as adequate means of therapeutic management [8,9]. Obviously, there is a great need for a better understanding of AD development and more effective therapeutic agents.

Although the etiology of 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 [10–16]. Compared with other tissues, the central nervous system may be particularly susceptible to oxidative damage [17, 18]. Oxidation reactions are catalyzed by transition metals such as iron and copper [19] and, as such, the likelihood that an oxidation reaction will take place is probably increased by the regional concentrations of the metals [10,19]. In fact, some 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 [20–24]. 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, and this metal dysregulation may represent a therapeutic target for AD. Indeed, chelation therapy with desferrioxamine (DFO), ethylenediaminetetraacetic acid (EDTA) and iodochlorhydroxyquin (clioquinol) has been shown to induce clinical improvement in patients with AD [25–27]. However, problems concerning chelator bioavailability to cross blood-brain barrier (BBB) and toxic side-effects hindered further investigation, both to understand the pathologic role of metal dysregulation as well as to evaluate the efficacy and safety of chelator therapy in AD.

Currently, drug delivery using nanotechnology has shown promise in targeting the central nervous system, which other approaches may not achieve [28,29]. Nanoparticles made of natural or artificial polymers ranging in size from about 10–1000 nm [28,30] present a possible tool to transport drugs across the BBB [28] and substantial studies have demonstrated the possibility [31–33]. The advantages of nanoparticles include reduced drug toxicity, improved biodistribution and therapeutic efficacy [34]. In this review, we discuss the current development of chelators coupled with nanoparticles as potential therapeutics for AD.

1. Copper chelator conjugated to nanoparticles for preventing and reversing A β deposition

Although copper and zinc are essential in the body, their excess is toxic. Accumulation of these metals in the central nervous system is believed to contribute to the oxidative stress and inflammation associated with AD [19,35,36]. Studies reveal that A β has the ability to bind copper and zinc with high affinity and that these metals may, in turn, mediate the A β deposition in AD [37–44]. The evidence showing the association of these metals with A β deposition is that simple co-incubation of A β from post-mortem AD brain tissue with copper and/or zinc chelators results in re-soluble aqueous A β [45]. This association is also demonstrated by other findings from both animal experimental studies and human clinical trials [27,46–49]. Severe changes in some metal distributions are found in the brain of AD patients compared with healthy controls [36]. Concentrations of copper and zinc are significantly elevated in amyloid plaques and neuropil regions in the brains of AD patients (about 400 mM for copper and 1 mM for zinc) compared to the healthy brains (70 mM for copper and 350 mM for zinc) [21,37]. Treatment of APP transgenic mice (an Alzheimer's disease mouse model) with the copper and zinc chelator, clioquinol, was shown to reverse A β deposition [50], leading to a near halving of A β deposition in the animal brain, compared with sham treated controls [47]. The reduction of A β deposition was accompanied by a significant improvement in behavior and general health

parameters. Clinical studies also demonstrate that AD patients treated with clioquinol show significantly slowed cognitive deterioration and lowered plasma A β levels [27,46]. These results suggest a potential approach that can prevent and treat AD by targeting the interaction of copper and zinc with A β using chelation agents. However, an implication of clioquinol toxicity in an epidemic of subacute myelo-optic neuropathy in Japan has forced withdrawal of the drug from the market. For neuro-chelation therapy, the BBB represents a major hurdle for most available chelation therapeutics [8,51]. To gain a deeper understanding of the roles of metals in AD development and to make progress in AD chelation therapy, there is a great need to develop more effective and safer chelation agents. Nanoparticle-mediated drug transport as an alternative means of drug delivery to the brain may provide an opportunity for such development [8,52].

A recent study showed that D-penicillamine, a specific copper chelator for Wilson's disease (copper overload), can be successfully conjugated to nanoparticles through covalent bonding [53]. The nanoparticles are designed to have a long circulating ability in the blood, and have been shown to be taken up by the brain *in situ* and *in vivo* without significant changes in BBB integrity or permeability [53–56]. The particles contain 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide] (MPB-PE) or 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine-N-[3-(2-pyridyldithio)propionate] (PDP-PE) to provide functional sites for the covalent conjugation with a sulfhydryl group in D-penicillamine. The sulfhydryl group can either react with MPB-PE or PDP-PE to form thio-ether or disulfide bonds, respectively, which link the chelator and nanoparticles [57–59] (Scheme 1). Both D-penicillamine conjugated PDP-PE nanoparticles and MPB-PE nanoparticles are stable when treated with simulated serum, thus being suitable for chelator delivery *in vivo*. D-penicillamine conjugated MPB-PE nanoparticles are more stable than D-penicillamine-PDP-PE conjugated ones because of the formation of a more stable thioether linkage of D-penicillamine-MPB compared to the less stable disulfide link between D-penicillamine and PDP [59].

It should be mentioned that the linkages using sulfhydryl group of D-penicillamine totally deprive its copper binding activity. This, however, may provide a targeting chelation approach by which D-penicillamine can chelate copper within the brain only if it is released from nanoparticles, thus minimizing chelator toxicity in other places. To restore the chelation activity of D-penicillamine, it was proposed that the release could take place when the D-penicillamine conjugated nanoparticles react with reducing agents such as glutathione under normal physiological conditions. In cells, glutathione is the most concentrated non-protein thiol and its concentration is about 1–6 mM [53,60]. In order to test this hypothesis, a reducing agent, dithiothreitol (DTT) was used to incubate with the D-penicillamine-conjugated nanoparticles. After 1-hour incubation at 37 °C in water at pH 6.7, D-penicillamine was partially released from PDP-PE containing particles by DTT, indicating cleavage of the disulfide bond between D-penicillamine and PDP. Although this result indicates the possibility of D-penicillamine release from nanoparticles by reducing agents, further studies are warranted such as increasing the releasing efficacy in both *in-vitro* and *in-vivo* investigations. Because A β is known to bind metals with high affinity and its aggregates are associated with copper, the ability of D-penicillamine, D-penicillamine-nanoparticles and D-penicillamine released from nanoparticles to reverse copper mediated A β aggregates were evaluated in this study. The A β deposition was obtained from incubation of A β 1–42 with cupric chloride in Tris buffer (pH 7.4) for 1 hour at 37 °C and then incubated with those chelation agents for a further 1 hour at 37 °C. To determine the re-dissolved copper A β aggregates in the soluble fraction, the samples were centrifuged and the supernatants were analyzed for protein concentrations using the Pierce method [53]. In order to release D-penicillamine from nanoparticles, the chelator-conjugated PDP-PE containing particles were incubated in basic aqueous solution (0.1 M, sodium hydroxide) at 45 °C for 1 hour. Under these conditions, the disulfide bond of D-penicillamine-particle linkage was more labile [57,61]. Results show that the D-penicillamine

conjugated to nanoparticles (NPs) can no longer re-solubilize the insoluble A β aggregates mediated by copper while the free D-penicillamine does. D-penicillamine released from nanoparticles (NPs treated) restores its ability to effectively re-solubilize the copper-A β aggregates (Fig. 1).

This study provides a method to prepare D-penicillamine-conjugated nanoparticles that may have the ability to cross the BBB without adverse effects on cerebral perfusion, BBB integrity and permeability [53,55,56]. This study also shows that the nanoparticle-delivered chelator may be released after targeting and re-solubilize metal-mediated A β deposition, thus being able to prevent and treat AD. D-penicillamine is used to treat Wilson's disease, wherein copper is accumulated initially in the liver and eventually in other organs including the brain [62]. D-penicillamine can conveniently be administered orally and its effect on urinary copper excretion is dramatic [63]. However, development of D-penicillamine intolerance is frequently seen among patients and the ability of D-penicillamine to cross the BBB is not clear [53,64, 65]. Thus, nanoparticle D-penicillamine delivery may increase the brain targeting of D-penicillamine and reduce its intolerance. Although more *in vivo* studies are needed and this chelation may be imperfect due to the possible accumulation of the chelator-metal complexes in the brain, this study provides evidence to support nanoparticle drug delivery as a potential approach for AD management.

2. Nanoparticles as a vehicle to transport iron chelators and metal chelator complexes into and out of the brain, respectively

a. Rationales of iron chelators conjugated with nanoparticles for AD treatments

It is already known from the above that copper and zinc play an important role in the accumulation of A β deposits, which in the form of pre-amyloid lesions and neuritic plaques are one of the key neuropathological features of AD [53,66]. Other metal ions are also known to be toxic and may be potential risk factors for AD. For example, substantial studies show that the metabolism of iron is involved in AD and that the concentration of iron, like copper, in the brain of AD patients is elevated [35,67–71]. The iron levels in neuropil of human brain are 39 mg/g for AD patients and 19 mg/g for controls determined by microparticle-induced X-ray emission [21,72–74]. Studies also demonstrate that iron can induce A β aggregation at acidic pH [43], a pathological value found in inflammatory disorders. The toxic effects of excess iron or copper result largely from their ability to catalyze free radical generation through the Fenton reaction or Haber-Weiss reaction [10]. Iron also plays a key role in the regulation of cellular functions such as respiration, oxygen transport, DNA synthesis, nitric oxide formation and other redox reactions [10]. Therefore, iron levels in particular biological compartments have a close association with various disease processes. Interestingly, another metal ion, aluminum has also received attention in AD [75] although its role in AD development has not yet been convincingly demonstrated. Nonetheless, aluminum is found in high concentrations in both senile plaques and intra-neuronal fibrillar tangles in the brains of subjects with AD, which suggests that this metal may be involved in the etiopathology of AD [20,35,70,76]. Aluminum, unlike iron and copper, is unable to participate in redox cycles of electron transfer reactions due to a fixed oxidation state of 3+ in biological systems, but growing evidence suggests that it can act synergistically with iron to increase free radical damage [17,77]. A recent study shows that accumulated aluminum in the central nervous system modulates A β formation and deposition [78]. As mentioned early, evidence already shows that copper [14,21,23,79–82] and zinc [21,40,70,83,84] are implicated in the development of AD. In the AD brain, the concentration of copper is elevated in the rim of senile plaques and the concentration of zinc is significantly elevated in senile plaques. Taken together, these studies suggest that the conditions of imbalances in several metals, have the potential of catalyzing and stimulating

free radical formation [8], and of facilitating A β aggregation and deposition [85], thus enhancing neuro-degeneration.

Simultaneously elevated levels of multiple metals promoting oxidative damage to the brain present a complex system of pathophysiology that is not yet fully understood. Despite this complexity, metal dysregulation may in fact be the Achilles' heel of AD, opening a door for chelation therapy. A chelator, regardless of synthetic or natural origin, may have high affinity for one metal ion, but it can also undesirably chelate other metals in the body leading to serious side effects. The affinity for multiple metals such as aluminum, copper, iron and zinc may pose useful rather than detrimental effects since various metals are implicated as oxidative instigators. Perhaps this is why desferrioxamine (DFO), a specific iron chelator also with high affinities for aluminum, copper, and zinc, has demonstrated some therapeutic benefits for patients with AD. Thus, simultaneous chelation of several excess metals in the AD brain may provide a novel approach for safe and effective treatment of AD. However, this promising approach is hindered by chelator toxicity and/or poor bioavailability, especially BBB impenetrability. A few clinically available iron chelators also negatively affect the development of iron chelation therapy for this disease.

DFO and deferasirox (ICL670, Exjade®) are the only iron chelation drugs approved by the FDA for iron overload disease (Fig. 2). Their therapy can promote iron excretion and have led to great improvements in the quality and duration of life of patients who suffer from β -thalassemia and other refractory anemias [86,87]. Although there are no reports yet on deferasirox for potential treatment of AD, DFO is found to significantly slow the progression of AD in one clinical trial [26]. In this study, only the chelation of aluminum was examined, but it is possible that the therapeutic effect may also have been due to removal of iron since DFO preferably chelates iron forming 1:1 complex (DFO, a hexadentate chelator) [88,89]. DFO also has an appreciable affinity for copper and zinc [88,90]. The affinity constants of DFO for Fe(III), Al(III), Cu(II), and Zn(II) are 30.6, 22.0, 14.1, and 11.1 (logK), respectively [91]. In this particular clinical study, copper and zinc were not monitored. So, it is unclear if the DFO treatment simultaneously removes these metals and/or whether such a removal plays a role in slowing the clinical progression of AD. However, 2 years after the initial publication, a verbal report at the International Conference on Alzheimer's Disease (Padua, Italy, 1992) provides evidence that iron and zinc concentrations are decreased in a postmortem analysis for DFO-treated patients [72,88]. This clinical study in addition to the follow-up report provides strong evidence that multiple metal accumulations in the brain may be key contributors to AD development and simultaneous removal of them mitigates disease progression. Aside from effective chelation of several metals, DFO also inhibits nigrostriatal degeneration induced by 6-hydroxydopamine [92]. Unfortunately, DFO has serious side effects including neurotoxicity and neurological changes [19,75,93–98]. Furthermore, DFO is poorly absorbed by the gastrointestinal tract and rapidly degrades after administration [99]. Therefore, it requires long subcutaneous administration to yield significant iron excretion [86,89,100]. Moreover, some studies show DFO does not easily penetrate the BBB due to its hydrophilic nature [101], although this point remains open to debate [102]. Indeed, some penetration may occur due to a compromised BBB via lesion sites [101]. Nonetheless, the neurotoxicity and difficulty of administration and delivery present serious hindrances to the use of DFO for AD treatment.

Deferiprone, another iron chelator (L1 or 1,2-dimethyl-3-hydroxyl-4-pyridinone) is a bidentate chelator and approved in Europe, but not in the United States, for iron or aluminum overload diseases [103–105] (Fig. 2). It chelates iron and aluminum with very high stability constants of $\log\beta=37$ and 32, respectively. Since it can also chelate copper and zinc with appreciable efficiencies ($\log\beta=19.6$ for copper and 13.5 for zinc), L1 is capable of simultaneously chelating these metal ions. L1 virtually lacks affinity for calcium or magnesium, therefore, unlike many other chelators, L1 has no toxic effects due to chelation of these essential metals [105]. L1 also

has high oral activity and BBB penetration because of its lipophilicity and relatively small molecular size. However, its use is limited because of serious side effects [106,107]. Moreover, studies show that L1 lacks the ability to remove iron from the brain [102] probably due to strong hydrophilicity of the iron-L1 complex. Additionally, there is no carrier-mediated transport system available to remove the complex from the brain. Other L1 derivatives with higher lipophilicity also have the ability to cross the BBB and complex iron in the brain, but they also possess considerable neurotoxicity [93,96,106]. Deferasirox is a tridentate iron chelator with oral bioavailability, which is currently approved by FDA for treatment of transfusional iron overload in Thalassemia. However, its long-term profiles are not yet available [87]. Furthermore, its lipophilic nature like L1 may raise questions concerning potential toxicity in AD treatment.

Thus, the use of the currently available iron chelators to simultaneously remove several excess metals in the brain of AD is limited because of their toxicity and/or poor transference across the BBB. Most bi- or tridentate iron chelators with small molecular weight and high lipophilicity have the ability to penetrate the BBB, but show toxicity [108]. 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 [108], but they have difficulty penetrating the BBB [102,108,109] due to their hydrophilicity and relative high molecular weight. One strategy to increase the BBB penetration is by enhancing the lipophilicity and lowering the molecular weight of the iron chelators, but this is believed to increase toxicity [110]. In addition, the increase in lipophilicity of iron chelators will decrease the solubility in aqueous solution with probable a decrease in bioavailability [107]. Also, it is possible that some lipophilic chelators, which normally should cross the brain endothelial cells, are rapidly pumped back into the blood stream by extremely effective efflux pumps [111].

Some existing USP drugs such as clioquinol (Iodochlorohydroxyquin) that possess chelation properties and BBB penetration ability have shown therapeutic benefits in AD [27,46]. Clioquinol (Fig. 2) is an antibiotic agent and chelator with high affinity for zinc and copper and less for calcium and manganese [47]. As mentioned before, oral administration of clioquinol in APP transgenic mice with advanced A β deposition leads to a significant reduction of A β deposition in the mouse brain compared to non-treated animals. This reduction of A β deposition is reported to be associated with copper and zinc chelation by clioquinol [47]. In clinical studies with clioquinol, the clinical rate of cognitive decline is slowed in a subset of AD patients compared with controls [27,46]. Interestingly, the zinc concentration in plasma of AD patients treated with clioquinol was significantly higher than that of controls although no parallel change in plasma copper concentration has been indicated [27]. However, further investigation with this new approach is hindered by clioquinol toxicity. It should also be mentioned that this type of chelator generally may have a low affinity for iron and would be toxic at doses needed for sufficient iron removal [91]. EDTA has also shown some clinical improvements in the patients with dementia, including AD, in a small clinical trial and a large retrospective study [25,112].

In some current experimental studies, chelators with a hydroxyquinoline backbone or with high lipophilicity show BBB permeability and neuroprotective potential [48,113,114]. However, the toxicity of these chelators related to their lipophilicity has not been studied. Other therapeutic approaches are being explored to overcome the impediment of the BBB. For example, a prochelator has been designed for the purpose of easily entering the BBB. The functional groups of the prochelator are then activated by enzymatic or non-enzymatic reactions after they have entered the target organ [115]. Another example is to use simple inorganic silicate that can form very stable complexes with many metals and probably has the ability to enter the BBB [116]. A newly developed copper chelator (tetrathiomolybdate) for Wilson's disease is also suggested as a potential treatment for AD [117,118]. Iron chelators

designed with near-optimal lipophilic/hydrophilic balance of the free chelator and iron complex for the purpose of passage into and out of the cell have been synthesized and studied [107]. However, no clear-cut clinical evidence for a beneficial effect of these chelators in AD has been demonstrated, and new approaches are necessary.

The use of nanoparticles to transfer chelation agents represents an exciting therapeutic option that may provide safer and more effective chelation therapeutics. One of the possible mechanisms by which the nanoparticles deliver drugs into the brain may be involved in the preferential absorption of apolipoprotein E (ApoE) and/or B. The particles also appear to mimic LDL and interact with the LDL receptor, resulting in their uptake by brain endothelial cells [28,31,119,120]. The transferrin transcytosis systems may be also employed by the particulated drug delivery systems to deliver drugs into the brain [28,119,121]. There are five advantages to this particle chelator delivery approach. First, the chelators need not be lipophilic to cross the BBB. Second, the lipophilic character of the chelators no longer contributes to potential toxicity. Third, improved iron binding capability. And fourth, hydrophilic hexadentate iron chelators with large molecular weights may be used. Finally, the most important advantage is that the chelator conjugated to nanoparticles has the potential to leave the brain after complexing metals, thus further improving chelation safety and efficacy.

For metal chelation to be effective, the chelators must be capable of leaving the brain with the corresponding complexed metal ions. Many chelators are unable to prevent iron from catalyzing free radical formation even though the chelators can complex iron tightly [122]. Thus, the metal-chelator complexes in the brain may still catalyze free radical formation, with possible new adverse effects on the brain. In fact, there is no systematic solution for this problem and finding the solution remains a big challenge in chelation therapy for AD as well as other neurodegenerative diseases associated with metal toxicity. If the nanoparticles are not biodegradable within the brain and can mimic lipoprotein particles by preferentially absorbing Apo A-I, known to facilitate the removal of particles from the brain [28,123], the same carrier mediated transport systems of LDL will be able to carry the metal-complex nanoparticles out of the brain. This is in contrast to lipophilic chelators that can enter the brain, but when complexed with metals, are unable to cross the BBB due to a change in their lipophilicity. For example, the distribution coefficient (DC) of free L1 determined in n-octanol/Tris-HCl buffer system is 0.24, but when complexed with iron is down to about 0.001 [124]. Therefore, although L1 can reportedly penetrate the BBB, it fails to remove iron from the brain [102]. Obviously, the use of nanoparticles may provide a novel approach to transport of metal-chelator complexes out of the brain. The use of nanoparticles conjugated with chelators to mimic lipoprotein particles and transfer iron chelators into and out of the brain through selected apolipoprotein absorption not only provides a useful means of improving the efficacy and minimizing the toxicity of chelation therapy, but also provides insights into the mechanisms of AD development. This novel approach can also open a new area for nanoparticle technology to clean unwanted substances from diseased sites.

b. Experimental results

For covalent conjugation of nanoparticles, iron chelators should have a functional group to react with an active moiety on the nanoparticle surface. The functional group introduced into the iron chelators should not possess adverse effects on the chelator-metal binding. Synthetic methods to produce a series of iron chelators with such functional side chains have been developed [125–130]. The preparation approaches are simple and production yields are good. The synthetic chelators have been characterized using standard methods such as ¹H-NMR, MS, UV-vis and elemental analysis. Figure 3 shows chemical structures of some prototypic chelators. They include 2-methyl (or Ethyl)-N-(2'-hydroxyethoxy)methyl-3-hydroxyl-4-

pyridinone (MHEHP or EHEHP) and 2-methyl-N-(2'-aminoethyl or 3'-aminopropyl)-3-hydroxyl-4-pyridinone (MAEHP or MAPHP).

The metal binding properties of these chelators and some biological properties such as the *in vitro* ability to remove iron from tissue sections of AD brains, and from ferritin (an important protein for iron storage) have been examined [125,127]. The reaction of chelators with iron was indicated by a color change of buffer solutions. The chelators and iron formed purple complexes with the chelator-iron stoichiometry of 3:1 [125,127], which was determined spectrophotometrically by titration of chelators in Tris buffer with freshly prepared iron solution. Typical titration curves using MHEHP and EHEHP are presented in Fig. 4. To further examine the ability of the chelators to complex metals, the reaction of chelators with ferritin was evaluated by incubation of horse spleen ferritin with chelators in physiological condition. This protein is important for iron storage, and in brain tissue most of non-heme iron is in the form of ferritin [67,131]. Evidence also shows that iron metabolism is altered in the brain of AD patients, with alterations including chelatable iron, ferritin and transferrin [68, 69,76,132]. The *in vitro* extraction of iron from the ferritin core by chelators is a convenient measurement of the potential efficacy of *in vivo* removal of iron from iron-overloaded organs [133]. The chelators can mobilize iron from ferritin in a slow reaction, which is monitored by the increase in absorbance of the iron-chelator complexes using a spectrophotometric method [125,127,133]. The formation of iron-chelator complexes in all the reactions indicated a common kinetic mobilization pattern that is characterized by initial rapid iron removal, followed by a slower iron removal rate. The end of the reaction as judged from stable absorbance. The chelators were more effective than DFO in mobilizing iron from ferritin. The initial rate of removal was faster with the chelators than with DFO, while the rate after 24 hours was about the same, which can be seen in Fig. 5.

Furthermore, *in vitro* tests of iron removal from AD brain sections with various iron chelators were also performed using a histochemical method [24], and examinations of the sections using differential interference microscopy demonstrated that iron chelators have the ability to strip iron from the brain tissues. Results showed that the iron removal efficacy depends on the chelator chemical structures and concentrations used. For example, both MAEHP and MHEHP had better ability to remove iron from AD brain sections, compared with DFO, at concentrations of 0.01 M (Table 1). The histochemical evaluation of iron in an AD brain section treated with MAEHP is presented in Fig. 6, showing remarkably less staining for iron compared with an untreated control. Importantly, this finding provides further support for the possible mechanism of metal-associated oxidative damage in AD development, by showing the existence of chelatable iron in the AD brain. The chelatable iron is believed to be one of the culprits that catalyze free radical formation, causing oxidative damage to all types of biomolecules [10]. Moreover, this result indicates the potential of chelation therapy of AD by depletion of excess metals, hence preventing metal-mediated neuro-oxidative damage. These results are consistent with early reports that chelation treatment can affect iron accumulations that are closely associated with the places of senile plaques and neurofibrillary tangles in cases of AD [24]. This iron specifically localized to the lesions of AD could participate in *in-situ* oxidation and catalyze an H_2O_2 -dependent oxidation [24]. This study also identifies a useful tool to screen potential chelators, which have suitable ability to mobilize iron from the AD brain *in vitro*.

Methods for conjugation of various iron chelators to nanoparticles have also been developed. The particles can be made of biocompatible synthetic or natural macromolecules [28,30,111] with functional groups such as amino or carboxyl groups on their surface for covalent bonding with chelators [57,134]. A variety of covalent bonds such as amido, amino, ether and thioether can easily be formed for conjugation, depending on the existing functional groups located on chelator side chains and on the surface of particles [57,128,134,135]. Here, a simple method

of particle-chelator conjugation by forming an amido bond is presented as a prototype. Monodispersed polystyrene particles with carboxyl groups on the surface were used to conjugate MAPHP or DFO, each of which contained a free primary amino group available for the conjugation. The preparation of the chelator-particle conjugates is presented in Scheme 2. Additionally, other kinds of chelators are also conjugated to nanoparticles using the similar procedures. As mentioned before, it also is possible to synthesize chelators containing different functional groups that readily react to the nanoparticle surface active sites without any adverse effects on the chelator metal binding.

Prior to conjugation, the carboxylic acid groups were activated using carbodiimide in a buffer solution at an acidic pH value to form an active intermediate ester. To remove excess carbodiimide, the particles were rapidly washed with cool Mill-Q water since the ester is unstable and undergoes hydrolysis. An alternative method, such as adding a water-soluble N-hydroxyl compound like sulfo-N-hydroxysuccinimide (NHS), could increase the coupling yield since NHS is known to form a more stable intermediate ester by replacing the oacylisourea intermediate formed by carbodiimide. The NHS-formed ester is less susceptible to hydrolysis but still highly reactive toward amino groups [136,137]. The primary amino group in the chelators reacts with active ester intermediates derived from carboxyl groups on the particle surface, consequently forming covalent amido bond linkages in good quality yields (over 70%). Coupling yields were determined by (1) measuring the free chelator concentration in solution before and after conjugation, using HPLC or UV-visible spectrophotometer readings, and (2) calculating the difference between the two concentrations to indicate the amounts of the chelators conjugated to particles. The particle size distribution was measured after the conjugation reaction and compared with the distribution prior to conjugation. A distribution change was not observed, which indicated that there was no particle clumping.

After conjugation, the ability of the chelator-particle systems to bind iron was determined. To test whether chelators retain their ability to complex metals after conjugation with nanoparticles, the chelator-nanoparticle systems were incubated in a $\text{Fe}(\text{NO}_3)_3$ solution. After the reaction was complete, the solution was removed and non-complexed iron was determined by adding MAPHP and measuring the iron-MAPHP complexes using UV-visible spectroscopy. Results indicated that about two MAPHP molecules conjugated to particles complexed with every iron atom. Because each MAPHP can provide only two oxygen donors to chelate iron, two more iron-binding sites are needed to form a highly stable iron complex with an octahedral coordination sphere. According to our earlier studies [138], the oxygen atoms in amido groups may be involved in chelating iron; thus, two oxygen donors from two amido groups and four oxygen donors from two MAPHPs may assemble into a hexadentate chelator, using particle surface linkages as the backbone (Fig. 7). This is significant, as hexadentate iron chelators possess many advantages including kinetic stability, concentration independence of iron affinity, and low toxicity [108]. In addition, attempts to convert bi- or tridentate iron chelators into hexadentate chelators with various backbones suffer mainly from decreased bioavailability and the risk of potential toxicity. By contrast, the use of nanoparticles to convert bi- or tridentate chelators to hexadentate chelators may circumvent these problems due to the particle targeting ability. In this manner, many bidentate or tridentate iron chelators, even with intolerable toxicity, can be modified by conjugation with nanoparticles, since the toxic character of the free chelators may be diminished after particle conjugation. Our results also revealed that DFO still retains the 1:1 complex ratio with iron, an indication that conjugation with particles does not affect its iron binding ability. It is worth mentioning that the bioavailability of some hexadentate iron chelators with high molecular weights and hydrophilicity can be improved after forming the chelator-particle delivery systems [139].

In order to demonstrate the possibility that chelator-nanoparticle systems enter the brain, and that the chelator-nanoparticle systems leave the brain after chelation with metals, the protein

absorption patterns of MAPHP-particle system and Iron-MAPHP-particle system were evaluated [28,140,141]. 2-D PAGE analyses were used to conduct this evaluation after incubation of the systems with human plasma. The 2-D PAGE images are presented in Fig. 8, and show that the protein patterns on the systems are totally different from that of the human plasma protein (Fig. 8. a). Through changing the surface properties of MAPHP-particle system by coating surfactants such as polysorbate 80, the system preferentially absorbed ApoE (Fig. 8. b). On the other hand, after complexing of MAPHP-particle system with iron, the Iron-MAPHP-particle system possessed the absorption preference for Apo A-I (Fig. 8. c). Such preferential protein absorptions may allow the MAPHP-particle system and iron-MAPHP-particle system to mimic the ApoE and ApoA-I nanoparticle, respectively, and therefore to enter brain without metal chelation and leave the brain with complexed metals via LDL transport mechanisms [33,123]. Uniform coating of the systems with ApoE, B, or A-I can also be achieved by overcoating of these apolipoproteins, which may enable the systems to cross the BBB with high efficiency [31]. These studies indicate that iron chelator-nanoparticle systems have the potential to enter the brain and bring excess metals out of the brain, thus effectively preventing metal-associated oxidative damage. The results also show the potential to obtain chelator-nanoparticle systems with optimal surface properties via changing chelators, linkages, coating materials, and nanoparticles with different surfaces. Further studies are warranted to demonstrate the chelation efficacy of the chelator-nanoparticle systems, to evaluate the system toxicity and to optimize their capability to cross the BBB.

Summary

The approach to simultaneously chelate excess iron and other metals such as Al, Cu and Zn in the brain may provide a useful therapeutic option for AD treatment. The use of nanoparticles to transport iron chelators into and out of the brain could lead to a safer and more effective chelation therapy for AD by means of decreasing the multiple metal load in neuro-tissue and thereby staving off the harmful effects of oxidative damage and its sequelae. Iron chelators with functional groups can be synthesized and the functional groups can be used for conjugation with nanoparticles. The conjugation of such chelators to nanoparticles does not alter the chelator metal binding ability, and in some cases, the conjugation even improves the chelation coordination property. The nanoparticles can be made of biocompatible synthetic or natural macromolecules with functional groups on their surface for conjugation with chelators. The macromolecules can be non- or controlled biodegradable for specific purposes. Changing nanoparticle surface properties can optimize the preferential protein absorption, thus improving the ability of the particle to cross the BBB. Many approaches to adjust particle surface properties can be used, such as coating with different surfactants or proteins, conjugating with different chelators or with various linkers, and using antibodies to increase lipoprotein preferential absorption. Further studies with the chelator-nanoparticle system may provide more insights into the mechanisms of AD pathophysiology, and may show the possibility of prevention and treatment for AD and other iron-mediated neurodegenerative diseases such as Friedrich ataxia, Huntington's disease, Parkinson's disease and Hallervorden-Spatz Syndrome. These studies may also open the door for the use of a nanoparticle-mediated transport to facilitate extraction of toxins from defective places in the body for disease prevention and treatment.

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Fig. 1. Studies of Cu-A β (1–42) aggregate resolubilization. The treatments are control (no chelator), D-penicillamine, D-penicillamine conjugated to PDP-NPs (NPs) and D-penicillamine conjugated to PDP-NPs (NPs) after treated in 0.1 N NaOH at 45 °C for 1 h (NPs treated). An asterisk indicates that the values for D-penicillamine and NPs treated are not different from each other, but are significantly higher than that of no chelator and NPs. (with permission after ref. 53).

Fig. 2.
Chemical structures of A. DFO; B. ICL670; C. L1; and D. Clioquinol.

Fig. 3. Chemical structures of chelators with functional chains. A. MAEHP ($n = 2$) and MAPHP ($n = 3$); and B. MHEMHP ($R = \text{Me}$) and EHEMHP ($R = \text{Et}$).

Fig. 4. Titration of MHEMHP (IIa) and EHEMHP (IIb) with iron. (with permission after ref. 127).

Fig. 5. Removal of iron from ferritin by the chelators of MHEMHP (IIa), EHEMHP (IIb) and DFO. (with permission after ref. 127).

A.

B.

Fig. 6.

Lesion-associated chelatable iron in AD brain sections was depleted with iron chelator, which was evaluated by histochemistry. A. Control and B. MAEHP treated section (with permission after ref. 141)

Fig. 7. Four oxygen donors from two MAPHPs and two oxygen donors from amido groups constructing a hexadentate chelator through particle surface as the backbones.

Fig. 8. Images of plasma protein patterns examined by 2-D PAGE. A. Plasma; B. Nanoparticle-chelator systems coated with polysorbate 80; and C. Nanoparticle-chelator systems complexed with iron (with permission after ref. 141).

Scheme 1.

Conjugation reactions of D-penicillamine to (A) MPB- and (B) PDP-nanoparticles (minor modification from ref.53).

Scheme 2.

Reactions of MAPHP and DFO with nanoparticles, forming chelator-particle conjugates (with permission after ref. 128).

Table 1

Iron evaluation by histochemical staining in AD brain sections after treated with various iron chelators

	0.1 M	0.01 M
Untreated	Positive	Positive
DFO	Negative	Very positive
MHEMHP	Negative	Weakly positive
MAEHP	Negative	Weakly positive