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Research paper

Novel D-penicillamine carrying nanoparticles for metal chelation therapy in Alzheimer's and other CNS diseases

Zhengrong Cui[a], Paul R. Lockman[b], Craig S. Atwood[c,d], Cheng-Hsuan Hsu[a], Anshul Gupte[a], David D. Allen[b], Russell J. Mumper[a,*]

[a]Department of Pharmaceutical Sciences, Center for Pharmaceutical Science and Technology, College of Pharmacy, University of Kentucky, Lexington, KY, USA
[b]Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University HSC, Amarillo, TX, USA
[c]Department of Medicine, University of Wisconsin and the Geriatrics, Research, Education and Clinical Center, William S. Middleton Memorial Veterans Hospital, Madison, WI, USA
[d]Centre for Aging and Alzheimer's Disease, School of Biomedical and Sports Science, Edith Cowan University, Joondalup, WA, Australia

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Abstract

Metal ions accumulate in the brain with aging and in several neurodegenerative diseases. Aside from the copper storage disease, Wilson's disease, recent attention has focused on the accumulation of zinc, copper and iron in the Alzheimer's disease (AD) brain and the accumulation of iron in Parkinson's disease. In particular, the parenchymal deposition of β-amyloid (Aβ) and its interaction with metal ions has been postulated to play a role in the progression of AD. Thus, the strategy of lowering brain metal ions and targeting the interaction of Aβ peptide and metal ions through the administration of chelators has merit. Our recent finding that nanoparticle delivery systems can cross the blood–brain barrier has led us to investigate whether chelators delivered conjugated to nanoparticles could act to reverse metal ion induced protein precipitation. In the present studies, the Cu (I) chelator D-penicillamine was covalently conjugated to nanoparticles via a disulfide bond or a thioether bond. Nanoparticle-chelator conjugates were stable between pH 6–8 in aqueous suspension if stored at 4 °C, and did not aggregate when challenged with salts and serum. Release of D-penicillamine from the nanoparicles was achieved using reducing agents such as dithiothreitol (as a model for glutathione). Nanoparticles treated only under reducing conditions that released the conjugated D-penicillamine were able to effectively resolubilize copper–Aβ (1–42) aggregates. These results indicate that nanoparticles have potential to deliver D-penicillamine to the brain for the prevention of Aβ (1–42) accumulation, as well as to reduce metal ion accumulation in other CNS diseases.

Keywords: Wilson's disease; Alzheimer's disease; Metal chelation; β-Amyloid; Copper; Aggregates; Resolubilization

1. Introduction

The transition metals, copper and zinc, have both functional and toxic roles in the CNS. For example, copper is a cofactor for many enzymes catalyzing oxidation–reduction reactions such as superoxide dismutase, dopamine-β-monooxidase and peptidylglycine α-amidating monooxygenase. Yet excessive copper and zinc have deleterious effects in that they contribute to the oxidative stress and inflammation in the CNS associated with Wilson's disease, Alzheimer's disease (AD), and Parkinson's disease [1,2].

Wilson's disease is an autosomal recessive disease wherein copper metabolism is abnormal and accumulation is seen initially in the liver and eventually in other organs, including the brain. This accumulation results in neurologic symptoms of resting and intention tremors, spasticity, rigidity and chorea, dystonia, an unsteady gait,

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* Corresponding author. Department of Pharmaceutical Sciences, Center for Pharmaceutical Science and Technology, College of Pharmacy, University of Kentucky, 725 Rose Street, Lexington, KY 40536-0082, USA. Tel.: +1 859 257 2300x258; fax: +1 859 323 5985.
E-mail address: rjmump2@email.uky.edu (R.J. Mumper).

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and psychosis [3]. The current treatment for Wilson's disease is the administration of metal chelators D-penicillamine and trientine to facilitate the excretion of copper via the kidneys.

AD is a neurodegenerative disorder for which there is no cure or effective treatment [4]. Although, it is still controversial whether the deposits of β-amyloid (Aβ), a 39–43 amino acid peptide, are responsible for the neurodegeneration observed in AD, the parenchymal deposition of Aβ in the form of pre-amyloid lesions and neuritic plaques is one of the major neuropathological features of the AD [5]. Normal metal ion distribution is severely altered in AD patients [6]. Significant elevations in copper (~400 μM) and zinc (~1 mM) have been found in amyloid plaques and Alzheimer's neuropil regions compared to healthy brain (70 μM Cu; 350 μM Zn) [7,8]. Aβ has been found to avidly bind Cu(II) and Zn(II) [7,9–11]. The hypothesis that these biometals mediate the deposition of Aβ in AD was further confirmed by the finding that Aβ from post-mortem AD brain tissue can be resolubilized to the aqueous form by co-incubating with chelators such as trientine, D-penicillamine, and bathocuproine [12]. Although their hydrophilicity does not allow their passage across the blood–brain barrier (BBB), the hydrophobic metal chelator clioquinol has been shown to reverse amyloid deposition in APP-transgenic mice (APP2576) [13]. Oral administration of clioquinol (30 mg/kg/day) for 9 weeks in aged APP2576 transgenic mice with advanced Aβ deposition caused a 49% decrease in brain Aβ deposition (375 μg/g) as compared to sham-treated controls [14]. These changes were accompanied by no adverse effects and a significant improvement in behavior and general health parameters. The results were intriguing and suggested a potential approach to prevent and treat AD by targeting the interaction of Cu and Zn with Aβ. More recently, clioquinol was shown in a phase II clinical trial in AD patients to arrest cognitive deterioration and significantly lower plasma Aβ levels [15]. However, it is unclear whether this affect was a result of attenuated amyloid: metal ion interactions or some other mechanism. Although metal chelation therapy shows potential for disrupting amyloid:metal interactions, clioquinol was implicated in an epidemic of subacute myelo-optic neuropathy in the Japanese population during the 1970s, forcing its withdrawal from the market. In order for this novel and unique approach of treating AD via metal chelation to progress, a safer chelator in combination with alternative means of delivery is desired.

Previously, we reported on a novel nanoparticle-based drug delivery system. The nanoparticles were engineered from warm microemulsion precursors composed of emulsifying wax and surfactants such as Brij 78 and Tween 20 [16]. The nanoparticles have been successfully employed as carriers to deliver drugs ranging from lipophilic molecules such as gadolinium complexes for neutron capture therapy of tumors [17–19], Coenzyme Q_{10} [20], and hydrophilic molecules such as protein antigens [21] and plasmid DNA [22,23]. The nanoparticles have been shown to be long circulating in the blood [24] and to be taken up by the brain in situ and in vivo with no significant changes in BBB integrity or permeability [25,26]. Although, the exact mechanism of transport is not known, the absence of toxicity at the BBB both in vitro and in situ suggests that the nanoparticles may be transported through the barrier by endocytosis and/or transcytosis or by passive diffusion in the absence of barrier opening.

Using this novel nanoparticle-based drug delivery system, our long-term goals are to deliver D-penicillamine to the brain by systemic administration. Controlled delivery of D-penicillamine using nanoparticles may prove successful in lowering the effective concentration at the site of accumulated metal ions and also limit the removal of essential biometals. D-Penicillamine is currently approved by the FDA for the treatment of Wilson's disease and rheumatoid arthritis (CUPRIMINE®, Merck). However, D-penicillamine is too hydrophilic to cross the BBB, and is eliminated rapidly from the blood, e.g. biphasic kinetics with half life of 6–7 and 52–55 min, respectively [27]. Thus, nanoparticles may prolong the half life of D-penicillamine in the body as well as enhance brain uptake. However, even if the enhancement in brain uptake of D-penicillamine using circulating nanoparticles is only marginal, prolonging the residence time of D-penicillamine using long circulating nanoparticles in the blood may cause depletion of brain copper levels due to the formation of a peripheral sink.

The goal of the present work was to develop and characterize D-penicillamine carrying nanoparticles that could be targeted to the brain for potential metal chelation therapy of CNS diseases. The ability of the D-penicillamine carrying nanoparticles to resolubilize Aβ-copper aggregates in vitro was therefore investigated.

2. Materials and methods

2.1. Materials

Emulsifying wax was purchased from Spectrum Quality Products, Inc. (New Brunswick, NJ). Lactose, fetal bovine serum (FBS), NaCl, $CuCl_2$, ninhydrin reagent solution, DL-dithiothreitol (DTT), D-penicillamine, and Sephadex G-75 were from Sigma Chemical Co. (St Louis, MO). Brij 78 was from Uniqema (Wilmington, DE). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[4-(*p*-maleimidophenyl)butyramide] (sodium salt) (MPB-PE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[3-(2-pyridyldithio)-propionate] (sodium salt) (PDP-PE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

2.2. Methods

2.2.1. Engineering of nanoparticles

Nanoparticles containing either MPB-PE or PDP-PE were engineered as previously described with slight modification [16,17]. Briefly, exactly 2 mg of emulsifying wax was weighed and placed into 7-ml glass scintillation vials. After melting at 50–55 °C, de-ionized and filtered (0.22 μm) water (940 μl) was added to the vials and stirred at 50–55 °C until a homogenous milky slurry formed. Then, 60 μl of Brij 78 (100 mM in water) was added while stirring to obtain a final Brij 78 concentration of 6 mM. Stirring was maintained until microemulsions were formed (usually within about 3 min). The microemulsions were then cooled to room temperature while stirring to form solid nanoparticles. The sizes of the nanoparticles were measured at 25 °C by photon correlation spectroscopy using a Coulter N4 Plus Submicron Particle Sizer (Coulter Corporation, Miami, FL) at 90° angle for 120 s. Various amounts of MPB-PE or PDP-PE were incorporated in the nanoparticles by adding either 2, 10, 20, or 30 μl of MPB-PE or PDP-PE in chloroform (10 mg/ml) to the vials. The chloroform was evaporated prior to adding emulsifying wax to obtain a final MPB-PE or PDP-PE percentage of 1, 5, 10, and 15% w/w.

2.2.2. Conjugation of D-penicillamine to MPB- and PDP-nanoparticles

Coupling of D-penicillamine to MPB-PE containing nanoparticles (MPB-NPs) or PDP-PE containing nanoparticles (PDP-NPs) was completed using a method previously described with some modification [28,29]. To react D-penicillamine with MPB-NPs, freshly prepared MPB-NPs (containing about 200 μg/ml of MPB-PE, equivalent to 10% w/w) were mixed with 1 mg of D-penicillamine dissolved in de-ionized water. The pH was adjusted to 6.5 and the reaction mixture was stirred under N_2 gas for up to 14 h at room temperature. To react D-penicillamine with PDP-NPs, freshly prepared PDP-NPs (containing about 200 μg/ml of PDP-PE) were mixed with 1 mg of D-penicillamine dissolved in water. The pH was adjusted to 8.0 with NaOH and the reaction mixture was stirred under N_2 gas for up to 2 h at room temperature. The nanoparticles were separated from unconjugated D-penicillamine using gel permeation chromatography (GPC) using a Sephadex G75 column. For each fraction (0.5 ml), both the nanoparticle intensity measured by the counts per second (CPS) from the Coulter particle sizer and the D-penicillamine concentration were determined.

To study the reaction time course of D-penicillamine with PDP-NPs, D-penicillamine and PDP-NPs were mixed at pH 8.0 under N_2 gas as mentioned above. At 0, 0.5, 1, 2, and 4 h post-reaction, samples were withdrawn and applied to the GPC column. The D-penicillamine concentration was measured and the amount of D-penicillamine conjugated to the PDP-NPs at each time point was calculated.

To further confirm the conjugation of the D-penicillamine to the PDP-NPs, the GPC-purified D-penicillamine conjugated PDP-NPs were re-concentrated by ultrafiltration (MW cut-off, 50 kDa) and re-applied to the GPC column. In addition, the re-concentrated nanoparticles were incubated with 50 mM dithiothreitol (DTT, 50 mM) at 37 °C for 1 h and re-applied to the GPC column [28]. The GPC profiles of the DTT treated versus untreated samples were compared.

2.2.3. Determination of D-penicillamine

The D-penicillamine concentration was determined using the ninhydrin reaction with slight modification [30]. Briefly, samples were brought to 1 ml volume with de-ionized water in glass vials. After the addition of 0.5 ml ninhydrin reagent solution, the mixtures were gently vortexed and placed into boiling water bath for exactly 10 min. After cooling to room temperature, 2.5 ml of 95% ethanol was added into each vial. The OD570 of each sample was then measured using a U-2000 Hitachi Spectrophotometer (Danbury, CT). For each experiment, a standard curve was constructed with a known concentration of free D-penicillamine.

2.2.4. Stability of nanoparticles and D-penicillamine conjugated PDP-NPs

To study the short-term stability of PDP-NPs before and after conjugation with D-penicillamine, the nanoparticles were GPC-purified and stored at either 4 or 25 °C for 6 days in capped and sealed plastic tubes. The particle size of the nanoparticles was measured on day 0, 1, and 6 as described above.

To study the stability of the D-penicillamine conjugated PDP-NPs to salt and serum challenge, 100 μl of the GPC-purified nanoparticles were challenged (diluted) with 900 μl of 150 mM NaCl, 10 mM phosphate buffered saline (pH 7.4), 10% lactose solution, or 10% (v/v) FBS in normal saline. Particle size was measured continuously over 30 min at 37 °C.

To study the stability of the D-penicillamine PDP-NP conjugate to reducing agents, 100 μl GPC-purified and re-concentrated (using ultrafiltration, MW cut-off, 50 kDa) D-penicillamine conjugated PDP-NPs were mixed with 300 μl of phosphate buffered saline (pH 6, 7, and 8, and adjusted to 5 mM final concentration) or with dithiothreitol (DTT) at a final concentration of 50 mM and incubated at 25 °C for 18 h. The mixtures were then applied to the GPC column. The D-penicillamine concentration was measured and the amounts of D-penicillamine remaining conjugated to the PDP-NPs were calculated [23].

2.2.5. Resolubilization of copper–Aβ aggregates

Resolubilization of copper–Aβ aggregates was completed as described [11]. Briefly, Aβ 1–42 (5 μM) was induced to aggregate by incubation in 20 mM Tris/150 mM NaCl buffer at pH 7.4 with $CuCl_2$ (20 μM) for 1 h at 37 °C. Following precipitation, samples were incubated with, (1) no chelater (control), (2) EDTA (200 μM), (3) free

D-penicillamine (0.2, 2, and 10 mM), (4) D-penicillamine conjugated PDP-NPs, or (5) PDP-NPs for 1 h at 37 °C. Samples were then centrifuged (10,000×*g* for 20 min), and the supernatant was analyzed for protein concentration using Coomassie® Plus (Pierce) to determine percent total Aβ (1–42) in the soluble fraction. Experiments were carried out in triplicate. Bovine serum albumin was used to construct the standard curve. For D-penicillamine-conjugated PDP-NPs (final total D-penicillamine concentration = 1.5 mM), the nanoparticles were co-incubated under basic conditions (final NaOH = 0.1 N) at 45 °C for 1 h to allow for D-penicillamine release. Disulfide bonds are labile under basic conditions at increased temperature [31]. As such, the use of basic conditions and increased temperature, instead of reducing agents like DDT that can itself chelate copper, were used to cleave the disulfide bonds. The percentage peptide resolubilized by the D-penicillamine-conjugated nanoparticles was reported following correction with background from PDP-NPs alone. For unknown reasons, the supernatant of the nanoparticles alone developed weak blue color when mixed with the Coomassie reagent. Therefore, to calculate the percentage resolubilized peptide by the D-penicillamine-conjugated PDP-NPs prior to and after basic condition treatment, the value from the PDP-NPs alone was subtracted. Finally, D-penicillamine free PDP-NPs were also treated as above (NaOH of 0.1 N; 45 °C for 1 h) as control to ascertain whether the treatment conditions per se could resolubilize the copper–Aβ aggregates.

2.2.6. Statistical analyses

Except where mentioned, all statistical analyses were completed using a one-way analysis of variances followed by pair-wise comparisons with Fisher's protected least significant difference procedure. The type I error rate was set at 0.05 for all analyses. A *P*-value of <0.05 was considered to be statistically significant.

3. Results

Previously, we reported on a novel process to engineer nanoparticles from warm oil-in-water microemulsion precursors by simple cooling [16–20]. The microemulsion precursors were comprised of emulsifying wax as the warm oil phase (melting point, 50–55 °C) and Brij 78 (polyoxyethylene-20-stearyl ether) as the surfactant. The non-ionic emulsifying wax, comprised of cetylstearyl alcohol and polysorbate 60 in a molar ratio of about 20:1, was the core material of the nanoparticles. The wax is typically used in cosmetics and topical pharmaceutical formulations and is generally regarded as a non-toxic and non-irritant material. For example, cetyl alcohol is present in Exosurf Neonatal® for lung administration. Polysorbate 60 is used in many pharmaceutical products including parenterals. We further showed that the nanoparticles prepared with 2 mg/ml of emulsifying wax and 4–6 mM of Brij 78 were the most stable, whereas increasing final concentrations of Brij 78 resulted in less stable nanoparticles [32]. In the present studies, either MPB-PE or PDP-PE was incorporated into these nanoparticles to provide functional groups for conjugation with the –SH moiety of the D-penicillamine.

3.1. Engineering of MPB- and PDP-nanoparticles

To determine the maximum amount of MPB-PE that can be incorporated in the nanoparticles while keeping the size of the resulting nanoparticles small (<100 nm), different percentages (0, 1, 5, 10, and 15% w/w) of MPB-PE and PDP-PE were mixed with the emulsifying wax (2 mg/ml). Then, 6 mM Brij 78 was used to form microemulsions, which were then solidified to nanoparticles upon cooling. As shown in Fig. 1, without MPB-PE, small nanoparticles (68 ± 3 nm) were readily formed as expected. With increasing amounts of MPB-PE added, the size of the resulting nanoparticles increased although not significantly. However, when the MPB-PE percentage was increased to 15% w/w, microemulsions could no longer be formed and the resulting particle size was relatively large. Similar results were also obtained for PDP-PE (data not shown). Therefore, for the following studies, 10% w/w of MPB-PE and PDP-PE was used for all nanoparticle preparations. Based on the coating of several other hydrophobic ligands on the nanoparticles in our laboratories, it is likely that the lipophilic dioleoyl groups on both MPB-PE and PDP-PE molecules inserted into the lipophilic core of the nanoparticles, while the hydrophilic heads of MPB and PDP were exposed on the surface of the nanoparticles [16–20].

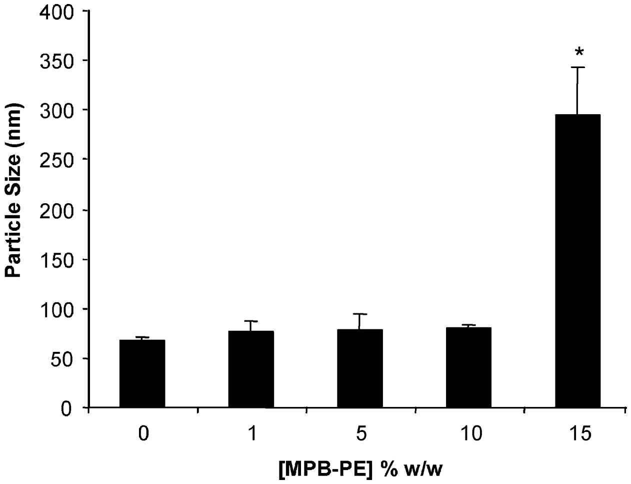

Fig. 1. The effect of MPB-PE concentration on the size of nanoparticles engineered from microemulsion precursors. Nanoparticles were cured from microemulsions prepared at 50–55 °C with emulsifying wax (2 mg/ml), Brij 78 (6 mM), and increasing concentrations of MPB-PE (0–15% w/w). * indicates that the size of particles with 15% of MPB-PE was significantly different than that of the others.

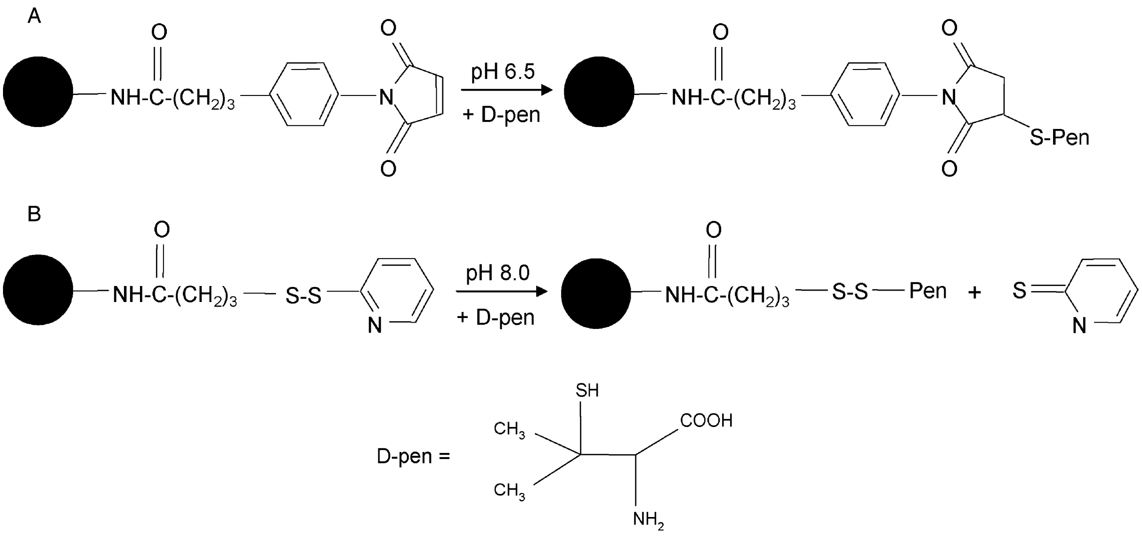

Fig. 2. Reaction schemes for the conjugation of D-penicillamine to MPB- and PDP-NPs.

3.2. Conjugation of D-penicillamine to MPB- and PDP-nanoparticles

Fig. 2 shows the covalent conjugation of D-penicillamine to either MPB-NPs or PDP-NPs. Papahadjopoulos and colleagues [28,29] have successfully applied these methods to conjugate antibodies on the surface of liposomes by taking advantage of the –SH groups on the antibodies. A comparison of PDP and MPB conjugates showed the MPB formed more stable complexes that survive in serum longer [29]. For the conjugation of MPB-NPs with D-penicillamine, the reaction was carried out at pH 6.5 under N_2 gas for 14 h. The unreacted D-penicillamine was then removed using GPC. Fig. 3A shows the GPC elution profile obtained when normal nanoparticles (free of MPB-PE) were subjected to the same reaction conditions. The nanoparticles eluted out mostly in the third fraction, whereas the free D-penicillamine eluted out in fractions 5–8. The low concentration of D-penicillamine (~0.4 μg/ml) detected in fraction 3 was most likely due to non-specific surface adsorption of D-penicillamine to the nanoparticles. These results demonstrated that the GPC conditions were sufficient to efficiently separate free D-penicillamine from nanoparticle-associated D-penicillamine. In contrast, when the D-penicillamine conjugated to MPB-NPs was applied to the same GPC column, the D-penicillamine concentration measured in fraction 3 was over 3.3 μg/ml strongly indicating D-penicillamine conjugation to the NPs. Since this sample was diluted about 10-fold by the GPC column, the final concentration of D-penicillamine conjugated to the nanoparticles corresponded to ~33 μg/ml. Nevertheless, since a large concentration of D-penicillamine (1 mg/ml) was added in the reaction, a large D-penicillamine peak (fractions 6–8) was observed in the elution profile.

Similar results were obtained when PDP-NPs were reacted with D-penicillamine. The reaction was carried out at pH 8.0 for 2 h under N_2 gas. Again, the elution profile of

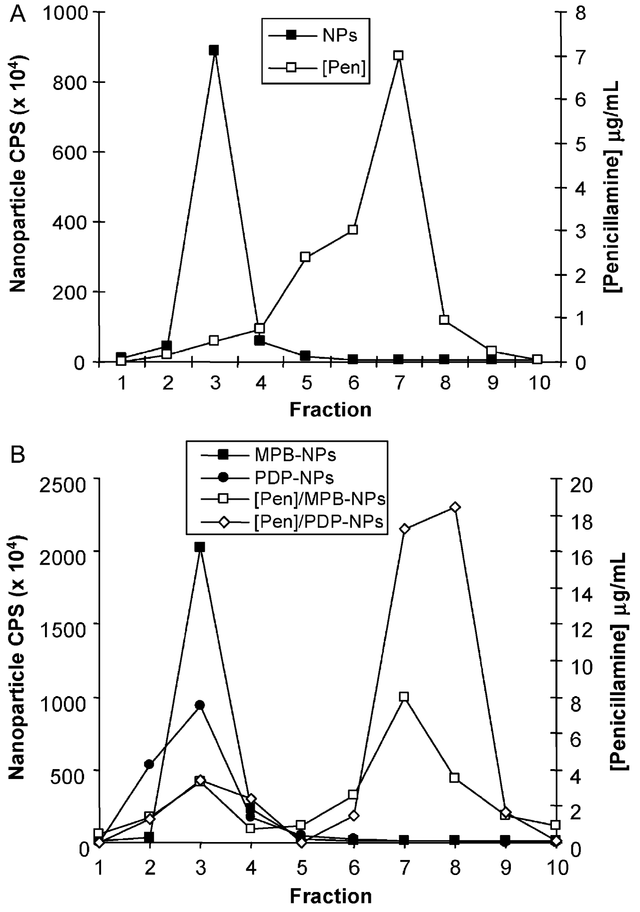

Fig. 3. Gel permeation chromatography profiles of: (A) normal nanoparticles (NPs) reacted with free D-penicillamine, and (B) MPB-NPs and PDP-NPs after reaction with D-penicillamine (1 mg/ml) at room temperature under N_2 gas. For MPB-NPs, the reaction was carried out at pH 6.5 for 14 h; for PDP-NPs, the reaction was carried out at pH 8.0 for 2 h. CPS indicates count per second from the particle sizer multiplied by 10^4. Normal NPs were reacted with D-penicillamine (1 mg/ml) for 14 h at pH 6.5 at room temperature and under N_2 gas. Similar results were obtained when the normal NPs were reacted with D-penicillamine for 2 h at pH 8.0 at room temperature and under N_2 gas (data not shown).

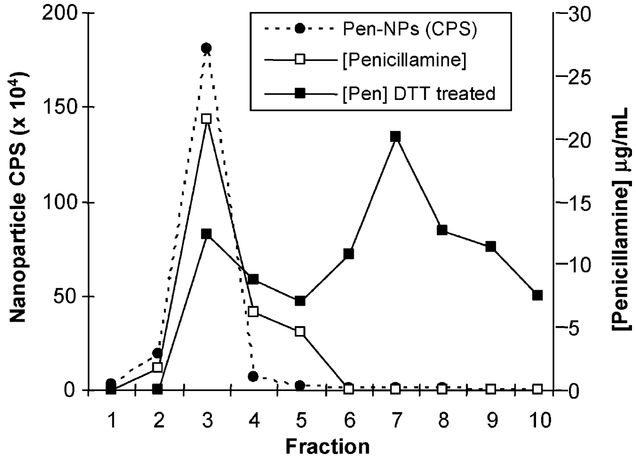

Fig. 4. Gel permeation chromatography profiles of purified and re-concentrated D-penicillamine conjugated PDP-NPs (Pen-NPs) before and after treatment with 50 mM dithiothreitol (DTT) at 37 °C for 1 h at pH 6.7. The NPs were detected by light scattering counts per second (CPS) from the particle sizer and the D-penicillamine concentration (μg/ml) was determined using the ninhydrin reaction as described in Section 2.2.3.

Table 1
The stability of PDP-nanoparticles (PDP-NPs) and D-penicillamine–NPs in aqueous suspension

	Particle size (nm)
PDP-NPs	105±36 (0.30)
PDP-NPs at 4 °C for 1 day	98±37 (0.36)
PDP-NPs at 4 °C for 6 days	94±33 (0.26)
PDP-NPs at 25 °C for 1 day	143±45 (0.17)
PDP-NPs at 25 °C for 6 days	220±76 (0.23)
PDP-NPs with D-penicillamine	119±42 (0.25)
PDP-NPs with D-penicillamine at 4 °C for 1 day	112±30 (0.13)
PDP-NPs with D-penicillamine at 4 °C for 6 days	115±47 (0.33)

PDP-NPs in aqueous suspension prior to or after conjugation with D-penicillamine were stored at 25 or 4 °C for 6 days. The particle sizes were measured on day 0, 1, and 6. Particle sizes are reported as the mean±SD (polydispersity index).

the PDP-NPs and D-penicillamine reaction mixture showed increased amount (~3.4 μg/ml) of D-penicillamine was co-eluted out at fraction 3 with the PDP-NPs, corresponding to ~34 μg/ml in the unpurified nanoparticle suspensions (Fig. 3B).

The GPC elution profiles in Fig. 3 provided evidence that D-penicillamine could be conjugated to both MPB-NPs and PDP-NPs using a thioether and disulfide bond, respectively. Since it is expected that the disulfide bond is less stable and could potentially be cleaved by reducing agents, additional studies were carried out to assess the stability of the D-penicillamine conjugated to the PDP-NPs. The GPC-purified nanoparticles (fraction 3 in Fig. 3B) were concentrated and re-applied to the GPC column. Fig. 4 shows that almost all the D-penicillamine co-eluted with the nanoparticles in fraction 3, indicting the stable association between the D-penicillamine and the PDP-NPs. Importantly, DTT was able to cleave a fraction of the D-penicillamine conjugated to the nanoparticles after 1 h incubation at 37 °C at pH 6.7. These results confirmed the conjugation of D-penicillamine to the nanoparticles via a disulfide bond that could be cleaved using a reducing agent such as DTT.

The time course of the reaction between D-penicillamine and PDP-NPs is shown in Fig. 5. The reaction reached saturation after 2 h, which agreed well with that reported by others [29]. Based on these results, all subsequent reactions were completed using a reaction time of 2 h.

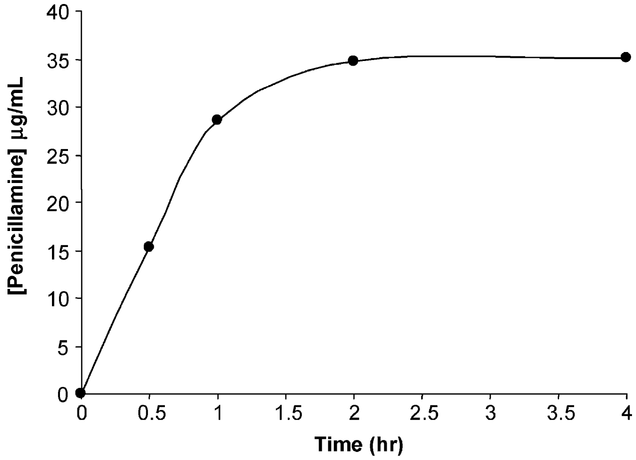

Fig. 5. Conjugation time course of D-penicillamine on the surface of PDP-NPs. PDP-NPs were co-incubated with D-penicillamine at room temperature and pH 8.0 under N_2 gas. Samples were withdrawn at time 0, 0.5, 1, 2, and 4 h. The D-penicillamine concentrations reported are those in the reaction mixture prior to GPC-purification.

3.3. Stability of the PDP-nanoparticles with and without D-penicillamine conjugation

Table 1 shows that the size of the GPC-purified PDP-NPs increased from 105±36 to 220±76 nm in 6 days when stored at ambient condition (25 °C). However, PDP-NPs and D-penicillamine conjugated PDP-NPs were stable when stored at 4 °C. Importantly, as shown in Table 2, D-penicillamine–NPs were stable when challenged at 37 °C for 30 min with several different biological vehicles including FBS. Additional studies were also performed to ascertain the stability of the disulfide bond. As shown in Table 3, the disulfide bond between D-penicillamine and PDP-NPs was quite stable over 18 h at 25 °C with about

Table 2
The stability of the D-penicillamine–NPs in simulated biological vehicles

	Size (nm) at 0 min	Size (nm) at 30 min
10 mM PBS	103.8±33.6 (0.18)	94.6±33.4 (0.25)
0.9% NaCl	106.4±35.6 (0.20)	87.0±33.5 (0.36)
10% FBS in saline	110.0±23.4 (1.38)	95.4±13.3 (1.39)
10% Lactose	117.9±44.2 (0.32)	111.2±32.5 (0.13)

D-Penicillamine conjugated nanoparticles were challenged with 10 mM phosphate buffered saline (PBS, pH 7.4), 0.9% NaCl (normal saline), 10% fetal bovine serum (FBS) in normal saline, or 10% lactose (w/w) at 37 °C for 30 min. The particle sizes were measured at 0 and 30 min. Particle sizes are reported as the mean±SD (polydispersity index).

Table 3
The stability of conjugated D-penicillamine on the nanoparticles

	Original D-penicillamine concentration (μg/ml)	Remaining D-penicillamine concentration (μg/ml)	% Remaining
pH 6 (5 mM PBS)	299	296	98
pH 7 (5 mM PBS)	299	280	94
pH 8 (5 mM PBS)	299	240	80
DTT (in H_2O)	299	206	68

D-Penicillamine conjugated PDP-NPs were incubated in phosphate buffered saline solution (PBS, 5 mM, pH 6, 7, and 8) or DTT solution (50 mM in water; pH 6.7) at 25 °C for 18 h. After incubation, the nanoparticles were purified using GPC and the D-penicillamine concentration was determined.

80% of the D-penicillamine still conjugated to the NPs even at pH 8. However, as expected, D-penicillamine could be partially cleaved from the nanoparticles using DTT.

3.4. Resolubilization of copper–Aβ aggregates by D-penicillamine conjugated PDP-NPs

Since metal ions are known to bind to Aβ and alter its solubility, we decided to test whether the D-penicillamine conjugated nanoparticles could resolubilize Aβ (1–42) precipitated by Cu(II). Copper–Aβ (1–42) aggregates were formed by mixing 5 μM Aβ (1–42) with 20 μM Cu(II) for 1 h at 37 °C. As previously reported, in the absence of chelator, Cu(II) induced the precipitation of most of the Aβ (1–42) (Fig. 6A). As expected, incubation with EDTA (200 μM) resolubilized over 80% of the copper–Aβ (1–42) aggregates. D-Penicillamine was less effective in resolubilizing the aggregates since with equal molar of D-penicillamine (to the EDTA) only around 40% of the aggregated Aβ (1–42) was resolubized. However, the effectiveness of the D-penicillamine in resolubilizing the copper–Aβ (1–42) aggregates could be enhanced by increasing the amount of D-penicillamine added. For example, as shown in Fig. 6B, when the molar ratio of the added D-penicillamine to the Cu used to induce the aggregates was increased from 10 to 100 (0.2–2 mM), the resolubilized Aβ (1–42) was increased from 39.3±3.8 to 66.6±9.6%. A further increase in the D-penicillamine to Cu ratio to 500 did not enhance the resolubilization of copper–Aβ (1–42) aggregates.

To determine the effectiveness of D-penicillamine conjugated to PDP-NPs in resolubilization of Aβ, we incubated D-penicillamine conjugated to PDP-NPs with Cu–Aβ (1–42) aggregates. D-Penicillamine conjugated to PDP-NPs was not effective in solubilizing copper–Aβ (1–42) aggregates (Fig. 7). However, after treating the D-penicillamine conjugated PDP-NPs under basic conditions at increased temperature (0.1 N NaOH at 45 °C for 1 h) to partially release the conjugated D-penicillamine, the nanoparticles together with the released D-penicillamine were then able to resolubilize ~40% of the copper–Aβ aggregates. The % released D-penicillamine was determined to be around 30%, which corresponds to ~135 μM of released free D-penicillamine. There was no statistical difference in the ability of free D-penicillamine (200 μM) and NP-released D-penicillamine (135 μM) to solubilize copper–Aβ (1–42) aggregates.

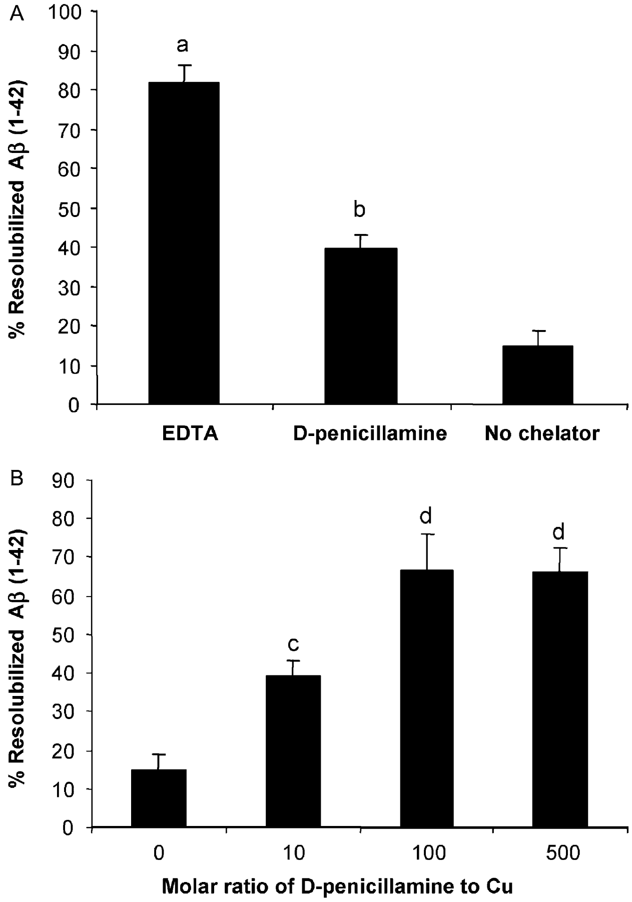

Fig. 6. Resolubilization of copper–Aβ (1–42) aggregates. Aβ (1–42) (5 μM) was precipitated by incubation in Tris-buffered saline with Cu(II) (20 μM) for 1 h at 37 °C. (A) Metal chelators (200 μM, EDTA or D-penicillamine) were then added to the reaction mixture for 1 h. The samples were centrifuged (10,000×*g*), and protein concentration in the supernatants was assayed with Coomassie Plus. (B) Dose response of the resolubilization by D-penicillamine. The molar ratio of D-penicillamine to Cu of 10, 100, and 500 corresponded to D-penicillamine concentration of 0.2, 2, and 10 mM, respectively. A and B indicate that the values for EDTA and D-penicillamine are different from each other and significantly higher than that for no chelator. C indicates that the value for the molar ratio of 10 was significantly higher than that for no chelator. D indicates that the values for the molar ratio of 100 and 500 were comparable but different from that of the others.

4. Discussion

There is growing interest in understanding the role that metals have in the CNS. For example, although copper has a positive role in facilitating reactions of cuproenzymes, it is also known that the reduction of Cu (II) to Cu (I) results in the formation of reactive oxygen species (i.e. superoxide

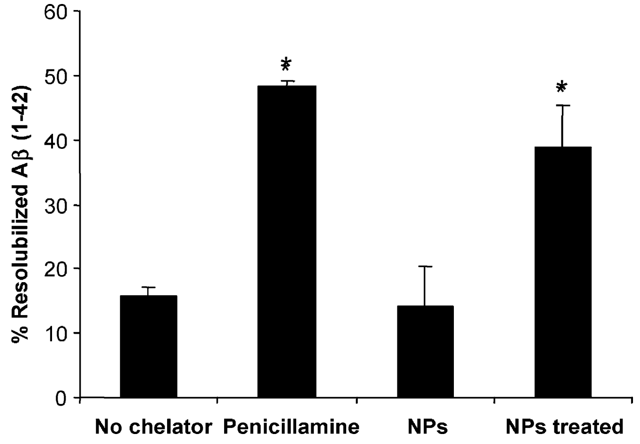

Fig. 7. Resolubilization of Cu–Aβ (1–42) aggregates by D-penicillamine conjugated PDP-NPs. The treatments included no chelator, D-penicillamine (200 μM), D-penicillamine conjugated PDP-NPs (NPs), D-penicillamine conjugated PDP-NPs after being treated in 0.1 N NaOH at 45 °C for 1 h (NPs treated). The values for the NPs (treated and untreated) were after subtraction of the values from unconjugated PDP-NPs (see Section 2.2). Experiment showed that the incubation conditions (0.1 N NaOH at 45 °C for 1 h) per se did not cause resolubilization. For the treated NPs, the released free penicillamine concentration was ~135 μM. * indicates that the values for D-penicillamine and NPs treated were not different from each other but were significantly higher than that for no chelator and NPs.

radical and hydroxyl radical) that can lead to intracellular damage [33]. Thus, maintaining the appropriate concentration of biometals such copper, iron, and zinc and preventing the formation of reactive oxygen species are of critical importance.

D-Penicillamine has established safe toxicity profiles and is currently used in the treatment of Wilson's disease, rheumatoid arthritis, and lead poisoning. However, D-penicillamine is highly hydrophilic, and there is no evidence in the literature indicating that it passes the BBB. Among the many ways to modify this chelator to increase its ability to pass the BBB, we chose to conjugate D-penicillamine to nanoparticles that have previously been shown to be long circulating in the blood [24,34] and to be taken up by the brain in situ and in vivo with no significant changes in BBB integrity or permeability [25,26]. In the present studies, it was shown that D-penicillamine could be conjugated to nanoparticles via either a thioether or disulfide bond. The D-penicillamine carrying nanoparticles were stable when 'challenged' with simulated serum suggesting their potential for delivery of chelating agents in vivo.

For both MPB-NPs and PDP-NPs, the final concentration of D-penicillamine conjugated to the nanoparticles was found to be 33–34 μg/ml or around 200 μM. This number agreed well with the theoretical maximum concentration (30 μg/ml) of D-penicillamine that may be conjugated based on the PDP-PE concentration in the PDP-NPs. The amount of PDP-PE and MPB-PE added into the nanoparticle preparation per ml was ~200 μM. Therefore, this suggested that all the PDP-PE and MPB-PE added was incorporated to the nanoparticles and that the PDP or MPB groups on the nanoparticles were saturated with D-penicillamine. If needed, the final concentration of the D-penicillamine can be increased by several methods, including increasing the amount of PDP-PE or MPB-PE incorporated in the nanoparticles with a modification of the composition of the nanoparticles, or by concentrating the nanoparticles by lyophilization/reconstitution or by ultracentrifugation. For example, a final concentration of 5 mM (750 μg/ml) of GPC-purified D-penicillamine-conjugated nanoparticles was obtained by ultracentrifugation for the copper–Aβ (1–42) aggregate resolubilization studies.

In addition to the disulfide linkage to nanoparticles, D-penicillamine was conjugated to the MPB-NPs via a thioether group. The thioether group is expected to be much more stable than the disulfide bond formed between D-penicillamine and the PDP-NPs [29]. Therefore, the PDP-NPs were mainly used in the present studies. Preliminary studies showed that the disulfide linkage was quite stable with pH ranging from 6.0 to 8.0, although as expected, the disulfide linkage was less stable at increased pH. The addition of DTT enhanced the release of D-penicillamine from the nanoparticles by disulfide exchange at pH 6.7. It is likely that under the conditions used in these experiments, DTT did not cleave all of the D-penicillamine due to oxidation and reformation of disulfide bonds [28]. However, the data did suggest that the release of D-penicillamine from the NPs could be expected to occur under normal physiological conditions by glutathione. Glutathione is the most concentrated non-protein thiol in cells and is present in concentrations the cell ranging from 1 to 6 mM [35].

The strategy of preventing or reversing the Aβ accumulation by targeting the interaction of Aβ peptide and metal ion through administering metal chelator(s) is intriguing and unique. Cherny et al. [12,14] proposed this potential treatment based on the findings by Bush and colleagues that Cu and Zn can prompt the precipitation of Aβ in vitro under simulated physiological condition. Also, treatment with chelators cannot only resolubilize the Aβ aggregates formed in vitro, but can also enhance the resolubilization of Aβ from post-mortem brain tissue [36]. In their initial proof-of-concept study, clioquinol was orally administered to transgenic AD mice. While clioquinol is quite lipophilic and has been shown to freely cross the BBB, it is known to cause subacute myelo-optic neuropathy [13]. Our studies demonstrate that both EDTA and D-penicillamine were effective in chelating copper and solubilizing Aβ (1–42). Fig. 6A demonstrates that at an equimolar concentration, EDTA appeared to be more effective than D-penicillamine in resolubilizing copper–Aβ (1–42) aggregates. However, D-penicillamine at higher concentrations was quantitatively as effective as EDTA in resolubilizing the aggregates. The ability of EDTA and D-penicillamine to solubilize Aβ (1–42) in vitro may be influenced by a number of different factors. EDTA and D-penicillamine chelate two different oxidation states of Copper. The stability constant (log Kapp) for EDTA and Cu (II) binding at pH 7.0 is 15.5 [11].

In contrast, D-penicillamine binds only Cu (I) with a stability constant of 18.8 [12]. Thus, it is possible that EDTA more effectively competes with Aβ (1–42) binding to Cu (II), whereas D-penicillamine complexed with Cu (II):Aβ (1–42) binds less efficiently due to the requirement of first reducing Cu (II) to Cu (I).

It has been shown in vitro that D-penicillamine:Cu (I) complexes have very poor aqueous solubility in the pH range of pH 1.9–7.6 due to the formation of neutral complexes [37]. However, in the body, D-penicillamine has been shown to form mixed (ternary) complexes with Cu (I) and glutathione or cysteine with very high stability constants resulting in complexes with high aqueous solubility due to overall negative charge [35,37,38]. It is this later phenomena that likely results in the ability of D-penicillamine to remove soluble Cu (I) from the body via kidney excretion. Another advantage of the use of D-penicillamine is that it preferentially binds Cu (I), and is less likely to extract Cu (II) involved in non-redox, structural capacity in proteins. Under the experimental conditions used in these studies, we cannot rule out the formation of insoluble ternary complexes of Cu (I), D-penicillamine, and Aβ (1–42) that were pelleted during centrifugation leading to lower free Aβ (1–42) detected in solution. It is also expected that performing the in vitro experiments in the presence of cysteine and glutathione may facilitate D-penicillamine-induced Aβ solubilization due to the formation of soluble ternary complexes [35,37,38].

It has been shown that Aβ forms insoluble fibrils in solution [12]. It is possible that under the conditions used to form copper–Aβ (1–42) aggregates in these studies, some Aβ fibrillized to form non-copper linked protein strands that could not be resolubilized by D-penicillamine. In contrast, the Aβ fibrils could be solubilized by EDTA. In fact, some metal chelators have the ability to both solubilize copper–Aβ (1–42) aggregates as well as intercalate Aβ fibrils [12].

Interestingly, D-penicillamine concentrations over 2mM did not result in increased resolubilization of the aggregates. This phenomenon was very similar to the results of Cherny et al. [12] wherein higher in vitro concentrations of D-penicillamine were equal or less effective than intermediate concentrations in releasing Aβ from homogenized AD brain samples. The reasons for this dose related effect of D-penicillamine remains unclear, but this effect has been observed for other chelators like NNNN-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN).

We have recently reported that our nanoparticle formulations crossed the BBB and that they had no effect on cerebral perfusion flow, BBB integrity and permeability in an in situ rat brain perfusion model [24–26]. In addition, these nanoparticles have been shown to be long circulating in the blood. In these present studies, we have shown that D-penicillamine, an approved metal chelator, can be attached to the nanoparticles via either disulfide or thioether conjugation. Moreover, D-penicillamine can be released from the nanoparticles under reducing conditions and released D-penicillamine can solubilize copper–Aβ (1–42) aggregates in vitro. These studies warrant the testing of the D-penicillamine nanoparticles in animal models of AD and Wilson's disease.

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