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Site-specific chirality-conferred structural compaction differentially mediates the cytotoxicity of A β 42 \ddagger

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Growing evidence supports the confident association between distinct amyloid beta (A β) isoforms and Alzheimer's Disease (AD) pathogenesis. As such, critical investigations seeking to uncover the translational factors contributing to A β toxicity represent a venture of significant value. Herein, we comprehensively assess full-length A β 42 stereochemistry, with a specific focus on models that consider naturally-occurring isomerization of Asp and Ser residues. We customize various forms of D-isomerized A β as natural mimics, ranging from fragments containing a single D residue to full length A β 42 that includes multiple isomerized residues, systematically evaluating their cytotoxicity against a neuronal cell line. Combining multidimensional ion mobility-mass spectrometry experimental data with replica exchange molecular dynamics simulations, we confirm that co-D-epimerization at Asp and Ser residues within A β 42 in both N-terminal and core regions effectively reduces its cytotoxicity. We provide evidence that this rescuing effect is associated with the differential and domain-specific compaction and remodeling of A β 42 secondary structure.

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Introduction

Naturally occurring D-amino acid substitution, also known as amino acid D-epimerization, has been observed in many disease-associated peptides and proteins, including amyloid beta (A β), one of the putative biomarkers and drug targets for Alzheimer's disease (AD).^{1–6} A β is of significant interest due to the prevalence of post-translational D-epimerization in AD brain samples.⁷ In brain samples extracted from AD patients, the two most common residues isomerized are aspartic acid and serine.^{5,8,9} Notably, previous studies have experimentally demonstrated that A β D-epimerization is age-dependent and

that such isomerization is long lived.^{2,5,8} Although most prior reports aimed at AD biomarker and drug target discovery lack the ability to detect and evaluate the impact of D-isomerized A β due to technical limitations,^{5,10–12} an increasing number of recent reports have focused on the role of A β D-epimerization in AD through novel assays capable of D-isomerized A β -targeted identification and purification.^{7,13,14} Recently, directed D-epimerization has also been intensively explored in terms of its impact on the cytotoxicity and oligomerization of A β .^{3,4,15–17} Notably, point D-amino acid substitution can effectively reduce A β cytotoxicity, highlighting the critical role of Ser26 residue.^{16,17} Meanwhile, Makarov and coworkers have found that, isomerization at Asp7 residue alone increases the amyloid cytotoxicity in both mouse models and human neuronal cells.^{18,19} Although a systematic chirality survey of Asp and Ser residues within A β 42 is still lacking, emerging data supports the prospects of a chiral regulation mechanism capable of rescuing A β in cytotoxicity,^{3,15–17} which may be further probed in the context of enzymatic regulation and phage display.^{20,21} Remaining challenges confronting a systematic survey concerning the chiral effects of Asp and Ser residues of A β 42 consist not only of those associated with the analytical discrimination and preparation of A β 42 stereoisomers, but also of the inadequate molecular understanding of the combinatorial structural consequences of D-epimerization at all Asp and Ser residues within different domains of A β 42.

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While many prior reports have described technical advancements associated with the chiral discrimination and separation of D-amino acid containing peptides,^{22–30} there remains a dearth of tools capable of targeting A β 42 stereochemistry. Traditional biophysical techniques frequently suffer from an inability to reconcile the mixture states created from the relatively low structural stability and high aggregation propensity of A β 42. These challenges are amplified when one maps the subtle structural impacts induced by D-epimerization at few sites onto the mixture of oligomeric states created within most A β samples. Ion mobility-mass spectrometry (IM-MS) has increasingly become an important alternative for the chiral separation of A β stereoisomers.^{13,31–37} IM-MS offers high analytical speed, low sample consumption and the ability to resolve small structural differences in peptide analytes, driven by recent technological advancements in IM-MS.^{34–39} While molecular dynamics (MD) simulations have been combined with IM-MS to reveal the structural consequences of D-epimerization within small neuropeptides,⁴⁰ Li *et al.* has recently reported a multi-dimensional IM-MS (md-IM-MS)-based structural analysis strategy, based on the metal-bound chiral amplification and oligomer-resolved data integration method, to facilitate the study of the chiral effects on monomer structure, oligomeric propensity and receptor binding for A β fragments.^{34,41} However, results from truncated A β (*e.g.* A β N-terminus and core region fragments) cannot be reliably extrapolated to predict those for the full length bioactive A β forms (*e.g.* A β 42).

To fully probe the chiral effects in full length, bioactive A β 42 including the differential roles of the N terminal and core regions, we herein provide an improved md-IM-MS^{34,41} approach for the systematic study of the structural consequences of domain-specific Asp/Ser D-epimerization within A β 42 (Table 1 & Scheme S1†). Cytotoxicity of A β isoforms in the context of neuronal cell lines were examined using a series of custom-synthesized peptides, which were also subjected to structural analysis using an improved md-IM-MS platform based on composite IM measurements across multiple instrument platforms. Analytical challenges facing the MS characterization of full length A β 42 have been successfully tackled through an improved sample preparation workflow. Moreover, IM-MS-guided replica exchange MD (REMD) simulations were employed to construct and refine the 3D structures of different A β 42 stereoisomers. Our analysis reveals that co-D-epimerization at Asp and Ser residues of A β 42 in both the N-terminal and

core region effectively and synergistically reduces its cytotoxicity, seemingly related to the differential and domain-specific changes in both overall and local 3D shapes, among which structural compaction exhibits the most significant effect.

Results and discussion

In vitro characterization of A β stereoisomer-receptor binding affinity

First, we customized a variety of A β stereoisomers with D-Asp and/or D-Ser residues to systematically construct site-specific, domain-constrained isomer models. As shown in Table 1, dD A β refers to A β isoforms with all Asp D-isomerized, dS A β refers to A β isoforms with all Ser D-isomerized, while dDdS A β refers to A β with all Asp and Ser co-D-isomerized. Scheme S1† shows the sequence and chemical structure for A β 42, where N-terminal and core regions are highlighted in different colors. To be more specific, A β 10/A β 16 and A β (17–36) are designated as N-terminal and core peptides, respectively, all of which are synthesized in four isoforms, WT, dD, dS and dDdS (Table 1). In total, we studied 12 A β stereoisomers in three groups to inspect domain-constrained chiral effects.

Immunoaffinity-based isolation is one of the most popular strategies in AD biomarker purification, which heavily relies on the binding specificity and affinity between the chosen antibody and the target (*e.g.* A β).^{7,10} Not surprisingly, we found that standard A β antibodies exhibit significantly varied performance in their ability to purify A β stereoisomers, as revealed by surface plasmon resonance (SPR) binding curves (Fig. 1). While in Fig. 1A the binding affinity for WT A β 42 to its specific antibody, 6E10, is calculated to be around 5.55 μ M, isomerization at both Asp and Ser residues significantly weakens A β 42 binding. This series of experimental observations starkly illustrate the challenges involved in quantitatively evaluating the contribution from A β 42 stereoisomers in AD pathology.

Metal ions, particularly copper (Cu²⁺), have been considered as an important regulator for A β structural flexibility and self-assembly.^{34,42,43} As such, we performed a series of native IM-MS experiments on A β 42-Cu²⁺ complexes in order to compare the copper binding affinities over differentially D-isomerized A β 42 isoforms. Representative mass spectra in Fig. S1† support the differential copper binding capabilities (including affinity and stoichiometry) of A β 42 stereoisomers. Further, triplicate binding affinity measurements enable quantitative comparison of their copper-binding affinities. As shown in Table 2, native

Table 1 Domain-constrained structural flips on A β 42

D-Residues	N-terminus A β 10/A β 16 ^a	Core region A β (17–36) ^b	Full length A β 42 ^c
WT	—	—	—
dD	Asp _{1/7}	Asp ₂₃	Asp _{1/7/23}
dS	Ser ₈	Ser ₂₆	Ser _{8/26}
dDdS	Asp _{1/7} Ser ₈	Asp ₂₃ Ser ₂₆	Asp _{1/7/23} Ser _{8/26}

^a A β 10/A β 16: DAEFRHDSGY/DAEFRHDSGYEVHHQK. ^b A β (17–36): LVFFAEDVGSNKGAIIGLMV. ^c A β 42: DAEFRHDSGY EVHHQKLVFF AEDVGSNKGAIIGLMVGGVV IA.



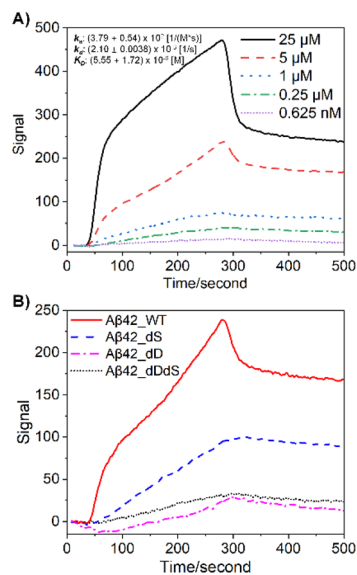


Fig. 1 Differential antibody binding affinity mediated by site-specific chiral inversion at full-length A β 42. (A) SPR response curves for various levels of WT A β 42 with A β antibody, 6E10, immobilized on a COOH sensor chip. Calculated binding kinetics information: k_a , $(3.79 \pm 0.54) \times 10^3$ 1/(M*s); k_d , $(2.10 \pm 0.0038) \times 10^{-3}$ 1/s; K_D , $(5.55 \pm 1.72) \times 10^{-6}$ M. (B) Response curves for WT and differentially D-isomerized A β 42 at 5 μ M each, showing the significantly decreased antibody binding affinity upon D-epimerization.

Table 2 Copper-binding affinity^a for A β 42 stereoisomers

A β 42	K_{D1} [μ M] (+1 Cu ²⁺)	K_{D2} [μ M] (+2 Cu ²⁺)	Fold change	
			K_{D1}	K_{D2}
WT	0.04 \pm 0.01	27.04 \pm 1.88	1.00	1.00
dD	1.50 \pm 0.02	33.31 \pm 3.26	34.12	1.23
dS	0.70 \pm 0.14	46.35 \pm 3.85	15.84	1.71
dDdS	0.31 \pm 0.06	19.77 \pm 2.63	7.15	0.73

^a All data in this table represents triplicate measurements with SD values for error ranges. The calculation of K_D fold changes is based on the normalization of WT group to be 1.0.

MS reports strong copper binding to WT A β 42 to form 1 : 1 complex, with K_D values of \sim 40 nM, with significantly lower binding affinity measured for subsequent copper binding events (\sim 27 μ M). While the formation of 1 : 2 Cu : A β complexes are not significantly affected upon site-specific isomerization (fold changes <2), 1 : 1 binding affinity diminishes significantly, resulting in a 7–34 fold change in Cu binding K_D . Notably, the largest fold change was observed for dD A β 42, which likely alters the known copper-binding domain located at N-terminus of A β 42. Interestingly, the Cu affinity recorded for dDdS A β 42 exhibits stronger affinity than the peptides carrying individual isomerized residues, which seemingly indicates the ability of A β 42 to structurally compensate to create an altered binding pocket for Cu binding. In addition, these Cu-binding data may have implications for the biological activities of A β 42

stereoisomers, as discussed above, Cu-regulated self-assembly and oligomerization is one of the putative molecular mechanisms for regulating A β neurotoxicity.^{42,44}

Cytotoxicity characterization of A β stereoisomers

Next, we aimed to interrogate and compare the biological impact of chiral inversion on A β 42, targeting multiple sites of different functional domains. Cell viability experiments were then performed by using N2a cell lines. As shown in Fig. 2A, incubation of N2a cells with 20 μ M WT A β 42 reduced the cell viability by \sim 82% and the half maximal inhibitory concentration (IC_{50}) as calculated from the S curve was estimated to be \sim 13 μ M. While previous studies have reported effective reduction in cytotoxicity through chiral inversion of all amino acids within A β 42,³ it was reported that isomerization at Asp7 residue alone increased the cytotoxicity burden.^{18,19} Our results demonstrate that D-epimerization of a few specific Asp and Ser residues may be sufficient to reduce its cytotoxicity. As WT A β 42 showed high cytotoxicity at 20 μ M and the trend lines for D-isomerized A β 42 are well separated, we thus conducted experiments with concentrations no higher than 100 μ M. The IC_{50} value for dD A β 42 is \sim 2.6 folds higher than WT A β 42 while the cell viability only reduced by \sim 18% with 20 μ M incubation, suggesting a significantly reduced cytotoxicity within N2a cell line. However, the IC_{50} values for dS A β 42 and dDdS A β 42 cannot be derived from the concentration range studied herein. Noting their reduced viabilities are \sim 0% and \sim 12%, respectively, further expansion of our methodology may establish the possible cytotoxicity reduction. These dose–response experiments using N2a cell lines clearly reveal site-specific, domain-constrained structural inversion differentially reduces amyloid cytotoxicity of A β 42. This agrees with previous studies on different cell lines that used similar A β 42 stereoisomer samples.^{2,3}

Notably, A β 42 does not function alone. Instead, as previously reported, its biological activities rely on several cofactors including metal ions and cellular transporters (*e.g.* human serum albumin, HSA).^{42,43} Knowing this, it is of topical interest to inspect the cytotoxicity of A β 42 in the presence of representative cofactors. Fig. 2B shows the viability results for four A β 42 stereoisomers in the presence of Cu²⁺ and HSA. For WT A β 42, the cell viability is approximately 60% at 10 μ M in the absence of cofactors (dark bar), while the viability was reduced further by \sim 10% with copper co-incubation (grey bar). Interestingly, the copper-induced cytotoxicity was successfully reduced with the addition of HSA (white bar), which is in accordance with previous reports.⁴³ Agreeing with our previous dosing experiments (Fig. 2A), we also observed systematically reduced cytotoxicity of A β 42 in the absence of cofactors. However, no significant viability changes were observed for dD A β 42 with both copper and HSA co-incubation when compared to WT A β 42. Contrasting this observation, we did detect significantly reduced cytotoxicity of dS A β 42 and dDdS A β 42 in the presence of both copper and HSA. To further interrogate the domain-specific cytotoxicity, we then measured cell viability in the presence of A β 42 N-terminus (Fig. 2C) and core region



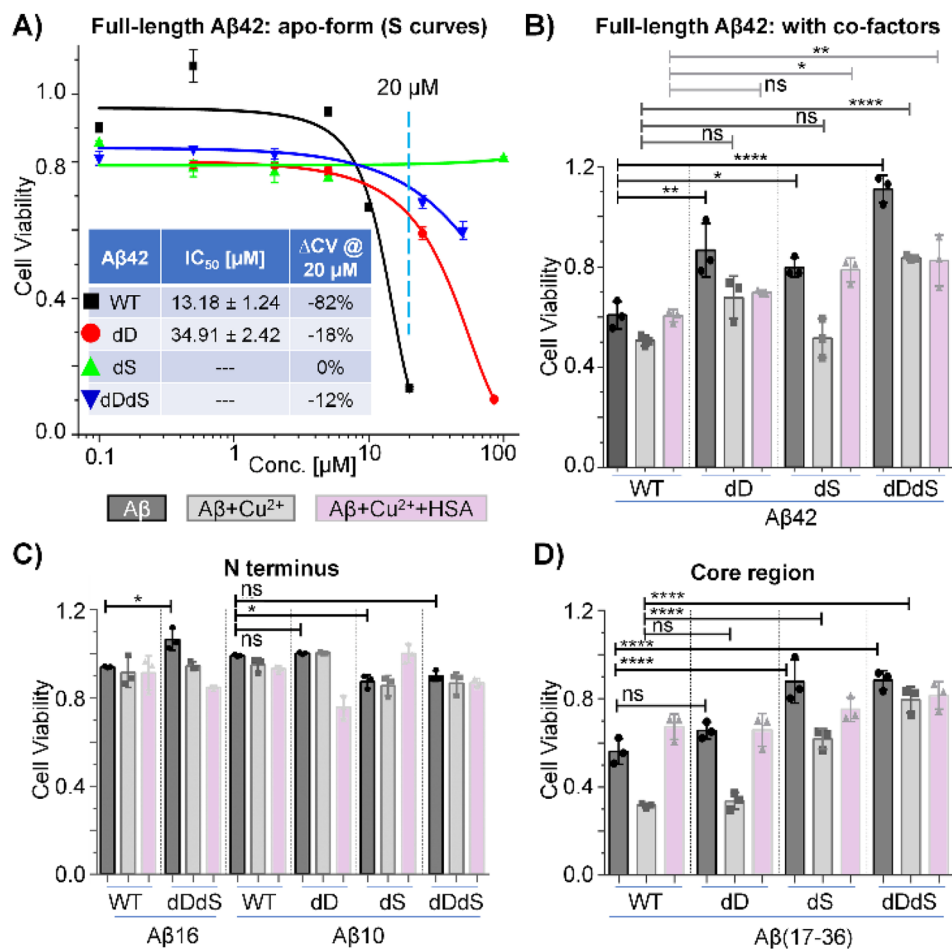


Fig. 2 Amyloid cytotoxicity mediated by site-specific, domain-constrained structural flips. (A) Cellular viability of N2a cell line in response to varied dosing of four A β 42 stereoisomers in the absence of co-factors. (B)–(D) Cell viability of N2a cells treated with A β 42 (B), A β N-terminus (C), and A β core region fragment (D) samples generated upon 24 h preincubation of cofactors including Cu²⁺ and HSA. Error bars represent standard deviations from three independent experiments. Statistical comparisons are based on Student's *t*-tests: ns, no significance; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. Conditions: [A β] = 10 μ M; [Cu²⁺] = 10 μ M; [HSA] = 10 μ M.

fragments (Fig. 2D). While we observe the copper-enhanced and HSA-related cytotoxicity rescuing phenomenon in most groups, all N-terminus fragments of A β 42 displayed only slight cytotoxicity whereas core regions present comparable cytotoxicity to that of full-length A β 42. It should be noted that the observed reduction in cytotoxicity when screening the chiral A β 42 core region correlates directly with our previous report that demonstrated reduced receptor binding affinity by \sim 7–13-fold upon chiral inversion of this same truncated peptide.³⁴ Taken together, we conclude that the site-specific, domain-constrained structural flip is beneficial for cytotoxicity reduction both in the presence and absence of cofactors. Our data demonstrate that A β 42 cytotoxicity is largely mediated by the core region, with observed contribution from the N-terminus. Co-D-epimerization of Asp and Ser residues within A β 42 likely imparts significant steric alteration of these two structural domains, resulting in cooperative reduction in cytotoxicity. The hypothesized synergistic relationship between N-terminus composition and A β toxicity is further reinforced by previous reports that D-isomerized Asp residues within this region.² The

results based on N2a cell line should represent a general trend for the cytotoxicity of A β stereoisomers from the perspective of a proof-of-concept demonstration, although it is also useful to extend future tests to more neuronal cell lines, including PC12 and SH-SY5Y.

Molecular mechanism for structure–activity relationship of A β 42 stereoisomers

To understand the underlying molecular mechanisms behind the altered cytotoxicity induced by D-epimerization, we aimed to firstly characterize the structural impacts of chiral inversion of A β 42. Our previously reported md-IM-MS strategy^{34,41} was the clear method-of-choice, given its ability to rapidly discriminating the subtle structural differences of A β 42 stereoisomers. Expansion of this method for the analysis of full-length A β 42 requires overcoming two pressing obstacles: (1) the propensity of A β 42 to form aggregates in solution, crippling electrospray signal stability during IM-MS analysis; (2) the inadequate collision cross-section (CCS) resolution of IM-MS for A β 42 stereoisomers when using a single IM regime. The first obstacle



cytotoxicity and CCS in response to chiral inversion between A β 42 dDdS group and A β 42 D23S26 group suggests that the N-terminus plays an important regulatory role in conferring A β 42 structure and function.

A further survey of A β 42 domain-linked fragments enables more detailed observations of domain-constrained chiral effects. For N-terminus fragments, no significant A β cytotoxicity (Fig. 2) was observed alongside chiral inversion-induced structural expansion (Fig. S6–9, discussions in ESI †). For core region fragments, decreased A β cytotoxicity was observed in tandem chiral inversion-induced structural compaction (Fig. S6–9, discussions in ESI †).

In summary, co-D-epimerization at Asp and Ser residues of A β 42 at both the N-terminus and within the core region effectively and synergistically reduces its cytotoxicity. This reduced toxicity coincides with the significant alteration of A β 42 conformation, which are shown to display domain-specific structural features, including structural compaction.

Having overcome the two obstacles preventing reproducible, high-resolution analyses of full-length A β 42, the data obtained reveal two additional questions of significant interest. First, is it possible to illuminate the relationship between specific chiral inversion and local conformation changes in A β 42? Second, how do reciprocal CCS changes in domain specific fragments (+1.3% & -1.1%, Fig. S6–9, discussion in ESI †) contribute to the integrative CCS changes observed in full length A β 42 (-3.4%, Fig. 3)?

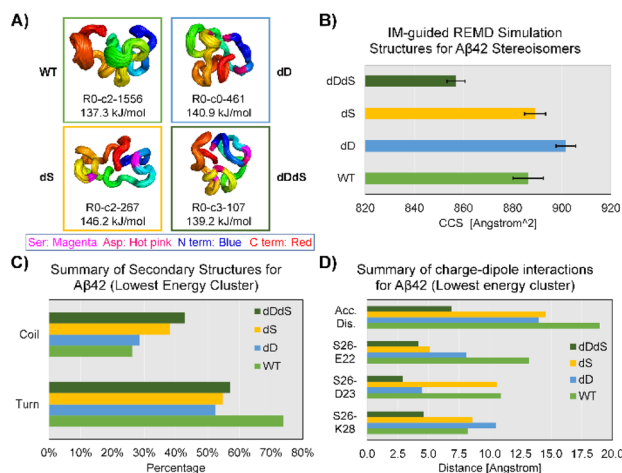


Fig. 4 IM-guided REMD simulation results for full-length A β 42 stereoisomers (WT/dD/dS/dDdS, [A β 42 + 4H] $^{4+}$) and corresponding structural analysis. (A) Lowest energy and most dominant/major REMD structural clusters for A β 42 stereoisomers. Conformer name, R0-c3-107: R0 (rep #0), c3 (cluster #3), 107 (total structure numbers). N-terminus, blue; C-terminus, red; Ser residues, magenta; Asp residues, hot pink. Energy for each conformer is also listed right below the cartoon illustration. (B)–(D) Calculated CCS distributions, secondary structures and charge-dipole interactions for REMD-simulated A β 42 stereoisomers. Charge-dipole interactions are characterized with the spatial distances between certain residues (e.g. S26–E22 (d_1), S26–D23 (d_2) and S26–K28 (d_3)), which is termed Accumulated Distance (Acc. Dis.). Accumulated Distance is calculated from the Euclidian distance between all interacting residues: $d_{acc} = (d_1^2 + d_2^2 + d_3^2)^{0.5}$.

To this end, we performed IM-MS-guided molecular dynamics (MD) simulations of A β 42 stereoisomers. CCS values based on TIMS measurements were used to guide replica exchange MD (REMD), which means only structural models within experimental CCS error ranges were adopted to create final cluster models. REMD results in distinct cluster models that are energetically favorable for A β 42 stereoisomers (Fig. S10–13 †). Fig. 4 illustrates the lowest energy clusters for WT, dD, dS and dDdS A β 42, ranging from 137.3 kJ mol $^{-1}$ to 146.2 kJ mol $^{-1}$. These chosen clusters of lowest energy are considered as representative conformers, as they possess significant percentages of total structure numbers, ranging from 107 (29% of all cluster models) to 1556 (61% of all cluster models). A β 42 cluster models generated from 300 K REMD simulations displayed mean CCS values of $886.3 \pm 3.2 \text{ \AA}^2$ (4+), $901.7 \pm 2.0 \text{ \AA}^2$ (4+), $889.2 \pm 2.1 \text{ \AA}^2$ (4+) and $857.0 \pm 1.9 \text{ \AA}^2$ (4+), for WT, dD, dS and dDdS, respectively. The generated WT, dD and dS A β 42 models match well with our experimental results (Fig. 3A), especially conf. #2, with CCS deviations spanning 1.3–3.0%. As well, the *in silico* dDdS A β 42 model is in accordance with our experimentally observed conf. #1, deviating by only 1.5%. From these results, we can conclude not only a strong agreement between our experimental results and theoretical models, but also that our hypothesis regarding structural compaction post D-epimerization can be rigorously tested.

In order to distill more structural detail, we further evaluated the REMD-generated A β 42 stereoisomer models. Secondary structure analysis, as shown in Fig. 4C, suggests an increased coil fraction but decreased turn content in A β 42 after chiral inversion. The D-isomerized A β 42 clusters exhibit a coil fraction range of 29–43% while the turn fraction ranges between 52–57%. These observed changes of secondary structural elements appear to be linked to the structural compaction observed in A β 42 isomers. Taking Ser26 as an example, we found that charge-dipole interactions 17 are effectively altered at both long (WT: S26–E22 distance up to 13 Å, d_1 , Fig. 4D) and short ranges (dDdS: S26–E22 distance of 4.2 Å). This type of alteration in charge-dipole attraction is also prevalent across two other pairs of residues, S26–D23 (d_2) and S26–K28 (d_3). To characterize these overall charge-dipole interactions, we cumulatively evaluated the distances of these three pairs (d_{acc} , Fig. 4D). In total, the d_{acc} values for Ser26 were diminished from ~ 19 Å (WT A β 42) to 6.9 Å (dDdS A β 42), indicative of the elevated charge-dipole attractions upon D-epimerization. Therefore, REMD simulations suggest that the major consequences of D-epimerization are the enhanced S26-related dipole-charge attractions and increased coil fraction at the C-terminus, which together result in the compaction of full-length A β 42.

Conclusions

Collectively, we present integrated, comprehensive IM-MS datasets on the analyses of A β 42 stereochemistry, with focus on two most frequently observed D-epimerization residues in nature, Asp and Ser. Site-specific, domain-constrained chirality inversion weakens both antibody and metal ion binding capacity of full-length A β 42. Simultaneously, D-epimerization at



all Asp and Ser residues of A β 42 inhibits its cytotoxicity, likely by inhibiting the membrane disruption pathway that shown to be directed by the core or C-terminal regions.^{34,45} This is inferred from the structural compaction of full-length A β 42 and core region fragments, observed both experimentally and *in silico*. The findings might be of substantial interest to the field and potentially generalizable to other intrinsically disordered proteins and peptides.

Our further efforts in both IM-MS and REMD give rise to refined structural models of all stereoisomers in the gas phase, providing unprecedented information regarding local structural features that confirm our hypothesis surrounding A β 42 structural compaction-based toxicity modulation. As shown, domain-constrained amino acid isomerization alters and generally compacts A β 42 while promoting differential, cooperative and domain-specific structural changes. Our results suggest that enhanced interactions between Ser26 and neighbouring residues, as well as the increased amount of coil structure at the C-terminus are accommodated in A β 42 chiral variants. This study provides a comprehensive insight into the molecular mechanisms behind the naturally occurring D-epimerization of Ser/Asp in A β 42 and serves to inform on the molecular foundation that enables amino acid-level chiral control that can reduce A β 42 cytotoxicity^{3,15–17} through enzymatic regulation and phage display.^{20,21} The analytical approaches described here may lay the groundwork for future AD drug development and clinical trials targeting the control of D-epimerization-promoting enzymes with specific residues in amyloid protein as substrates, such as racemase.^{20,46} It should be noted that, not all D-substitutions will lead to effective attenuation of cytotoxicity. In contrast, only specific site mutations, mostly Ser26-based, are beneficial for decreasing cytotoxicity. This may rationalize the fact that aged AD patients will accumulate all kinds of A β stereoisomers but they still suffer from the disease, and are not effectively reduced by the beneficial isoforms when combating with other harmful forms. Future efforts, from a clinical application perspective, may be directed to elevate the level of D-substitution towards Ser26 residue rather than other sites.

Data availability

Data can be available from the corresponding authors (G. L., L. L. and B. T. R.) upon reasonable request.

Author contributions

G. L., L. L. and B. T. R. designed the project. G. L. collected MS data, C. K. J. performed REMD, M. M. carried out cell experiments. G. L. drafted the manuscript. Y. J., Z. Z., D. G. D., Ga. L. and E. R. helped the collection of MS data and involved in figure preparation. All authors involved in the data analysis, wrote the paper and approved the final version of this manuscript.

Conflicts of interest

There are no conflicts to declare.

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