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Amyloid β -Protein Assembly: The Effect of Molecular Tweezers CLR01 and CLR03

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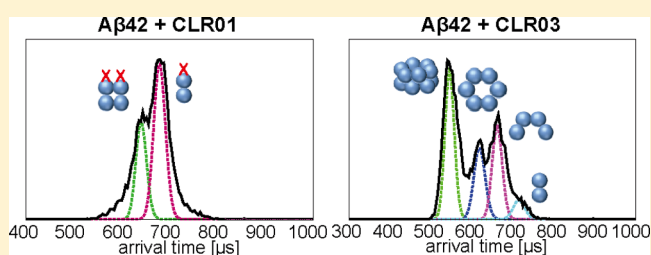
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S Supporting Information

ABSTRACT: The early oligomerization of amyloid β -protein ($A\beta$) has been shown to be an important event in the pathology of Alzheimer's disease (AD). Designing small molecule inhibitors targeting $A\beta$ oligomerization is one attractive and promising strategy for AD treatment. Here we used ion mobility spectrometry coupled to mass spectrometry (IMS-MS) to study the different effects of the molecular tweezers CLR01 and CLR03 on $A\beta$ self-assembly. CLR01 was found to bind to $A\beta$ directly and disrupt its early oligomerization. Moreover, CLR01 remodeled the early oligomerization of $A\beta_{42}$ by compacting the structures of dimers and tetramers and as a consequence eliminated higher-order oligomers. Unexpectedly, the negative-control derivative, CLR03, which lacks the hydrophobic arms of the tweezer structure, was found to facilitate early $A\beta$ oligomerization. Our study provides an example of IMS as a powerful tool to study and better understand the interaction between small molecule modulators and $A\beta$ oligomerization, which is not attainable by other methods, and provides important insights into therapeutic development of molecular tweezers for AD treatment.



INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia, affecting over 44 million people worldwide.¹ AD is a progressive brain disorder that damages synapses and brain cells and causes decline of memory, loss of cognitive and executive functions, and eventually death.² There is no known cure for AD, and the etiology of the disease is not well understood. Previous studies have shown that amyloid β -protein ($A\beta$) plays an important role in AD pathogenesis.³ $A\beta$ is, in reality, not one but a group of peptides derived from the type-1 transmembrane protein, amyloid β -protein precursor (APP), through cleavage by β - and γ -secretases.

$A\beta$ exists *in vivo* primarily as 40 or 42 amino acid long peptides with $A\beta_{40}$ constituting 90% and $A\beta_{42}$ ~9% of all $A\beta$ species.⁴ Even though $A\beta_{42}$ is a relatively minor constituent, it has been found to be the primary component of amyloid plaques, which are an important pathologic hallmark of AD.⁵ In the plaques, $A\beta$ is found as fibrillar, β -sheet-rich aggregates. Formation of $A\beta$ fibrils *in vitro* and *in vivo* is a complex process involving multiple intermediate oligomeric species, which are highly neurotoxic and are believed to be the proximal neurotoxins acting in AD.^{6–9} Immediately upon dissolution *in vitro*, $A\beta_{42}$ forms small oligomers, including dimers and tetramers, as well as paranuclei (pentamers and hexamers) that self-associate to form decamers and dodecamers.^{10–12}

Among these species, the 56 kDa dodecamer has been identified as a plausible cause of memory deficits in the AD brain¹³ and in transgenic mice.¹⁴ Thus, targeting and remodeling $A\beta_{42}$ oligomers is a primary therapeutic strategy for AD.¹⁵

One strategy for AD treatment and prevention is preventing $A\beta$ formation, which could be achieved by inhibiting or modulating the β - and/or γ -secretase enzymes.¹⁶ However, this approach has been problematic because both secretases cleave substrates other than APP, which are important in other functional biological processes.^{17–19} Another potential strategy is enhancing clearance of $A\beta$ oligomers and aggregates from the brain.²⁰ This can be accomplished either by facilitating degradation of $A\beta$ by proteases²¹ or other clearance mechanisms, or by directly remodeling the aggregation of $A\beta$ into clearance-prone structures using suitable peptides or small molecules.

Many natural proteins, peptides, and small molecules have been discovered to interact with $A\beta$ and modulate $A\beta$ self-assembly.²² Among them, small molecules are particularly attractive as a direct therapeutic strategy for the treatment of

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AD.^{23,24} For instance, biologically active molecules from green tea ((-)-epigallocatechin-3-gallate, EGCG) or the Indian spice turmeric (curcumin) have been found to prevent A β aggregation and inhibit A β -induced toxicity.^{25,26} Inositol stereoisomers have been found to interact with A β and attenuate its neurotoxic effects.^{27,28} Z-Phe-Ala-diazomethylketone (PADK), which acts as a lysosomal modulator up-regulating the expression of cathepsin B in lysosomes, has been shown to interact with A β 42 directly and modulate A β 42 oligomerization.²⁹ C-terminal fragments of A β 42 and many polyphenol molecules have been shown to inhibit A β oligomerization, aggregation, and toxicity,^{30–34} and molecules have been specially designed to inhibit not only aggregation of A β but also its metal binding and oxidation.³⁵

Molecular tweezers (MTs), which possess a torus-shaped cavity with a surrounding belt of alternating aromatic and aliphatic rings, were designed to serve as host molecules binding specifically to lysine and to a lesser extent to arginine residues.^{36–38} MTs were shown to be modulators of the aggregation of A β and other amyloidogenic proteins and effective inhibitors of the toxicity of these proteins.³⁹ A lead MT derivative, CLR01 (Figure 1a), was shown to inhibit the toxicity of multiple amyloidogenic proteins in cell viability assays using cell lines and primary cell cultures^{39–42} and protected synaptic integrity and function of hippocampal and cortical neurons against the synaptotoxicity of A β 42.⁴¹ In addition, peripheral administration of CLR01 in transgenic mice led to a decrease in amyloid plaques, neurofibrillary tangles, and brain inflammation, suggesting that it is a promising candidate for therapeutic development.⁴¹

Mechanistic investigation showed that disruption of A β self-assembly is mediated by CLR01 binding to the two lysine and the single arginine residues in A β .³⁹ Dynamic light scattering (DLS) and electron microscopy (EM) experiments suggested that CLR01 does not prevent oligomer formation but rather modulates A β self-assembly into formation of structures that are neither amyloidogenic nor toxic.³⁹ Interestingly, by the relatively low resolution of DLS and EM, these structures were similar in size to the toxic and amyloidogenic oligomers of A β alone, suggesting that subtle conformational changes in A β might account for the apparent loss of amyloidogenic potential and toxic activity. However, the low-resolution methods could not provide information about what these changes might be. In addition, whether CLR01 binds A β monomers, oligomers, and/or larger aggregates has not been demonstrated directly.

In several previous studies, a derivative called CLR03 (Figure 1b) was used as a negative control.^{39,41} This compound shares the polar bridgehead structure with CLR01 but lacks the hydrophobic arms, and therefore is not expected to bind specifically to lysine or arginine. Consequently, CLR03 indeed acted as a negative control and was not found to inhibit the aggregation and/or toxicity of amyloidogenic proteins. Nonetheless, how CLR03 interacts with A β and whether it has any effect on early A β oligomerization of A β remains to be uncovered.

To address all these questions, here we used ion mobility spectrometry coupled mass spectrometry (IMS-MS)^{43,44} to investigate the effect of CLR01 and CLR03 on A β assembly. IMS-MS has been utilized successfully in the past to study A β and amyloid assembly^{12,45–49} and the effects of various small molecules on the assembly process.^{29,32,35,50}

EXPERIMENTAL METHODS

Peptide and Sample Preparation. Full-length A β 40 and A β 42 were synthesized by *N*-9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The peptides were purified by reverse-phase HPLC and their integrity validated by mass spectrometry and amino acid analysis as described previously.⁵¹

The samples were prepared in 10 mM ammonium acetate, and the pH was adjusted to 7.4. Samples contained 10 μ M A β 42 and molecular tweezers at different concentration ratios. An A β 42 sample without MTs was prepared under the same procedure as a positive control.

Transmission Electron Microscopy (TEM). Microscopic analysis was performed using a FEI T-20 transmission electron microscope operating at 200 kV. The A β samples with and without molecular tweezers were prepared using the same procedure as that for the mass spectrometry analysis. The samples were kept at 4 °C for 2 weeks. For TEM measurements, 10 μ L aliquots of samples were spotted on glow-discharged, carbon-coated copper grids (Ted Pella, Inc.). The samples were stained with 10 mM sodium metatungstate for 10 min and gently rinsed twice with deionized water. The sample grids were then dried at room temperature before TEM analysis.

Mass Spectrometry and Ion Mobility Spectrometry Analysis. Samples were analyzed on a home-built ion mobility spectrometry-mass spectrometer⁴³ which is composed of a nanoelectrospray ionization (nano-ESI) source, an ion funnel, a temperature-controlled drift cell, and a quadrupole mass filter followed by an electron multiplier for ion detection.

Briefly, for ion-mobility measurements, ions are generated continuously by a nano-ESI source, focused and stored in the ion funnel. A pulse of ions is injected into a temperature-controlled drift cell filled with 3–5 Torr helium gas, where they gently pass through under the influence of a weak electric field. The injection energy can be varied from \sim 20 to \sim 150 eV, but it is usually kept as low as possible to minimize thermal heating of the ions during the injection process. The ions exiting the drift cell are mass analyzed with a quadrupole mass filter, detected by the conversion dynode and channel electron multiplier, and recorded as a function of their arrival time distribution (ATD).

The ions in the drift cell experience a constant force from the electric field, E . This force is balanced by a retarding frictional force due to collisions with the buffer gas, resulting in a constant drift velocity, v_d . The drift velocity is proportional to the electric field:

$$v_d = KE \quad (1)$$

Here, the proportionality constant K is termed the ion mobility. The absolute ion mobility is dependent on the temperature (T) and the pressure (P) of the buffer gas, so it is typically converted to the reduced mobility K_0 :

$$K_0 = K \frac{P}{760} \frac{273.16}{T} \quad (2)$$

The ions exiting the drift cell are mass analyzed and detected as a function of the arrival time, t_A . The reduced mobility K_0 can be determined from the instrument parameters by using eq 3 and plotting t_A versus P/V ⁵²

$$t_A = \frac{l^2}{K_0} \frac{273.16}{760T} \frac{P}{V} + t_0 \quad (3)$$

In eq 3, l is the length of the drift cell (4.503 cm), V is the voltage across the drift cell, and t_0 is the time the ions spend outside the drift cell before hitting the detector. All of these quantities are either known constants or are measured for each experiment.

The reduced ion mobility K_0 can be related to the collision cross section Ω using kinetic theory⁵³

$$K_0 = \frac{3q}{16N} \left(\frac{2\pi}{\mu k_B T} \right)^{1/2} \frac{1}{\Omega} \quad (4)$$

Here, q is the ion charge, N is the buffer gas number density at STP, μ is the reduced mass of the ion–He collision, and k_B is the Boltzmann constant. The measured reduced mobility (K_0) and the collision cross section (Ω) provide information about the three-dimensional configurations of the ions. For peptide and protein ions, the secondary and tertiary structural information and the oligomeric states can be identified by comparison with modeling.

Experimental arrival time distributions can be fitted by calculating the flux of ions exiting the drift tube using ion transport theory.⁵³ The ion packet is taken as a periodic delta function, and the flux is given by eq 5:

$$\Phi(0, z, t) = \frac{sae^{-\alpha t}}{4(\pi D_L t)^{1/2}} \left(v_d + \frac{z}{t} \right) [1 - e^{-r_0^2/4D_T t}] \times e^{-r_0^2/4D_T t} \quad (5)$$

Here z is the ion charge, r_0 is the radius of the initial ion packet, a is the area of the exit aperture, D_L and D_T are the longitudinal and transverse diffusion coefficients, s is the initial ion density, and α is the loss of ions due to reactions in the drift cell. The fitted feature represents the theoretical ATD for one species with a given cross section. If a feature in the experimental ATD is broader than the fitted one, then the feature possibly represents a family of structures, rather than a single structure.

RESULTS

For the IMS-MS experiments performed here, $A\beta$ and the molecular tweezers were prepared in ammonium acetate buffer, in contrast to previous experiments in which they were studied in sodium phosphate buffer. This change in condition was not expected to cause major changes in $A\beta$ assembly or its inhibition. To verify this expectation experimentally, we assessed samples of $A\beta$ 42 in the absence or presence of CLR01 or CLR03 by TEM. $A\beta$ 42 was incubated with CLR01 at 1:1 or 1:10 concentration ratio, respectively, and with CLR03 at 1:10 concentration ratio.

As shown in Figure S1 (Supporting Information), $A\beta$ 42 shows long fibrils in the absence of tweezers or in the presence of CLR03, as observed previously in sodium phosphate buffer.³⁹ In the presence of an equimolar concentration of CLR01, the $A\beta$ 42 sample shows a few protofibril-like structures and amorphous structures. At 1:10 $A\beta$ 42:CLR01 concentration, only small amorphous structures are observed. These TEM results show that CLR01 inhibits the fibril formation by $A\beta$ 42, whereas CLR03 does not, which is consistent with previous studies in sodium phosphate buffer. These results indicate that the change of buffer has a minimal effect on $A\beta$ fibrillogenesis and the way it is impacted by CLR01 or CLR03, supporting the comparison between the data shown below and previous biophysical investigations of these systems.

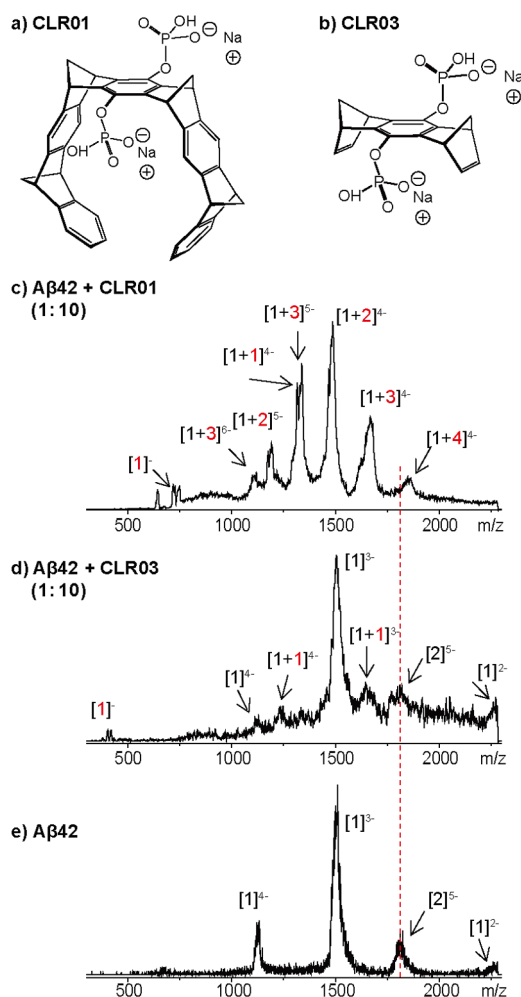


Figure 1. Different binding effects of CLR01 and CLR03 on $A\beta$ 42. (a, b) Molecular structures of CLR01 and CLR03 compounds; (c–e) mass spectra of $A\beta$ 42 samples: (c) 1:10 mixture of $A\beta$ 42 and CLR01; (d) 1:10 mixture of $A\beta$ 42 and CLR03; (e) $A\beta$ 42 alone. Each species is noted in brackets where the first number is the number of $A\beta$ 42 molecules and the second number represents the number of bound small molecules. The charge is noted as a superscript.

Mass Spectrometry Reveals Different Binding Effects of CLR01 and CLR03 on $A\beta$ 42.

Mass spectra of $A\beta$ 42 samples in the absence or presence of MTs are shown in Figure 1. In the mass spectrum of $A\beta$ 42 alone (Figure 1e), there are four peaks, which correspond to $A\beta$ 42 species with charge states $z/n = -4, -3, -5/2$, and -2 (where z represents charge and n represents oligomer order), respectively, as described previously.^{12,45} In the mass spectrum of a 1:10 mixture of $A\beta$ 42 and CLR01 (Figure 1c), there are three sets of peaks which correspond to $-4, -5$, and -6 charge states of the complexes of $A\beta$ 42 with one, two, three, or four CLR01 molecules bound. As the mass spectrometry study was conducted in negative ion mode, the binding form of CLR01 is with loss of sodium ions, which results in producing $A\beta$ 42 and CLR01 complexes with higher charge states. Note that no $-5/2$ $A\beta$ 42 peak ($m/z = 1805$), which represents dimer or higher order oligomers, is observed. This indicates that CLR01 disrupts the formation of $A\beta$ 42 dimers and higher order oligomers. No peaks of uncomplexed $A\beta$ 42 are observed in the mass spectrum, suggesting that CLR01 binds to $A\beta$ 42 directly with high

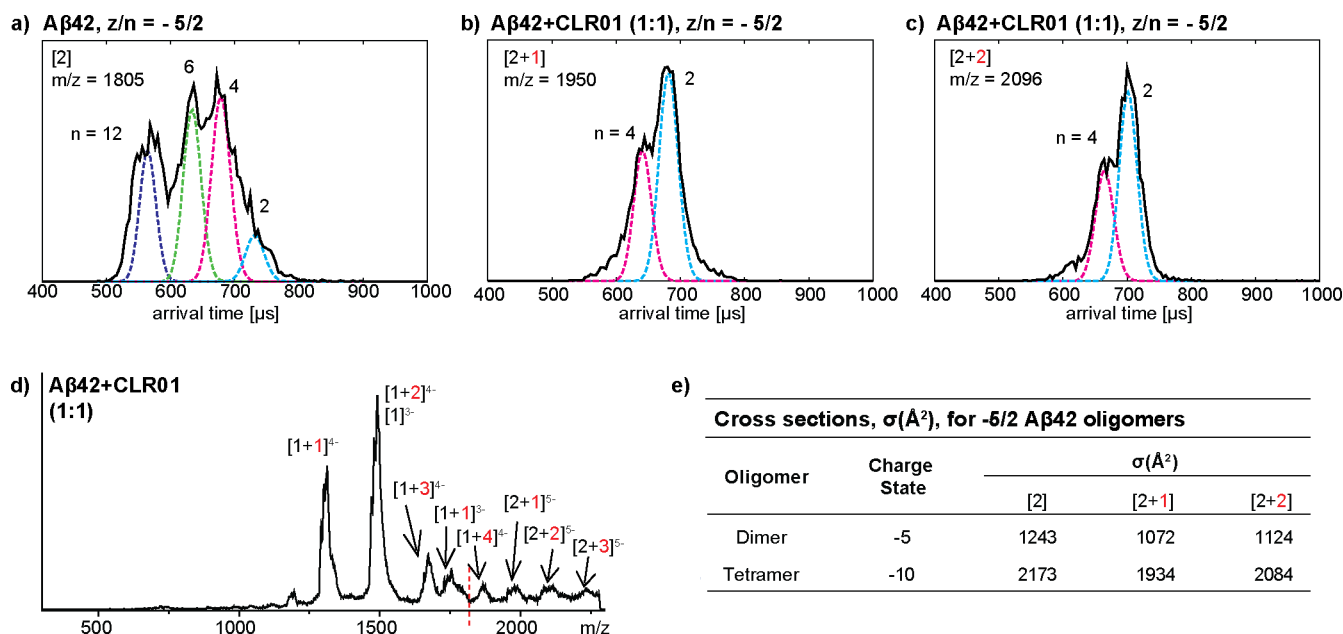


Figure 2. Effects of low concentration CLR01 on $A\beta 42$ early oligomerization. ATDs of (a) $z/n = -5/2$ $A\beta 42$ ($m/z = 1805$) in an $A\beta 42$ sample without CLR01; (b) $z/n = -5/2$ $A\beta 42$ and CLR01 complex ($m/z = 1950$); and (c) $z/n = -5/2$ $A\beta 42$ and CLR01 complex ($m/z = 2096$) in a 1:1 mixture of $A\beta 42$ and CLR01. Each ATD is fit with multiple features using the procedure described in the Experimental Methods section, and the oligomer order (n) is noted for each feature. (d) A mass spectrum of the 1:1 mixture of $A\beta 42$ and CLR01 is shown as an example. Each species is noted in brackets where the first number is the number of $A\beta 42$ molecules and the second number represents the number of bound CLR01 molecules. The charge is noted as a superscript. The dashed line represents the theoretical position for the uncomplexed $-5/2$ peak. Mass spectra of mixtures of $A\beta 42$ and CLR01 with different ratios are shown in Figure S2 (Supporting Information). (e) Cross sections of dimer and tetramer in the uncomplexed or CLR01-complexed $-5/2$ $A\beta 42$. The error for the cross sections reported here is between 0 and 1%.

affinity and there are no CLR01-free $A\beta 42$ species present in solution.

In contrast, the spectrum of a 1:10 mixture of $A\beta 42$ and CLR03 (Figure 1d) shows four peaks corresponding to the -4 , -3 , $-5/2$, and -2 $A\beta 42$ species, similarly to $A\beta 42$ alone (Figure 1e). There are two additional small peaks tailing the -3 and -4 $A\beta 42$ peaks corresponding to the -3 and -4 complex species of $A\beta 42$ with one CLR03 bound, respectively. The intensities of these two complex peaks are much lower than those with CLR01 bound, suggesting that the affinity of CLR03 binding to $A\beta 42$ is much lower than that of CLR01.

To better understand the effects of CLR01 on $A\beta 42$, the mass spectra of $A\beta 42$ with different ratios of CLR01 (1:1, 1:2, 1:5, and 1:10) were recorded. The mass spectrum of a 1:1 mixture is shown in Figure 2d as an example, and others are provided in Figure S2 (Supporting Information). The mass spectrum of the 1:5 mixture of $A\beta 42$ and CLR01 is similar to that of the 1:10 mixture (Figure 1c) with peaks corresponding to complex species $A\beta 42$ and CLR01 with charge states -4 , -5 , and -6 . As the concentration of CLR01 decreases (1:2 and 1:1 ratios), the complex species of $A\beta 42$ and CLR01 with lower charge states (-4 , -3 , $-5/2$) are observed in the mass spectra. One possible explanation is that CLR01 itself is slightly basic in aqueous solution and the observed binding form of CLR01 is CLR01 with loss of sodium ions; therefore, the complexes tend to carry more charges in the presence of high concentration CLR01. Note that no $z/n = -5/2$ CLR01-free $A\beta 42$ dimer peak is observed in any of the mixtures. However, in the low-ratio mixtures (1:1 and 1:2, see Figure 2d and Figure S2, Supporting Information), the $-5/2$ complex peaks of $A\beta 42$ oligomers with CLR01 molecules bound are observed ($m/z = 1950$, 2096, and 2241 representing $[2+1]$, $[2+2]$, and $[2+3]$ $A\beta 42$ -CLR01 complexes, respectively). These results suggest

that CLR01 not only binds to $A\beta 42$ monomers but also to small $A\beta 42$ oligomers with relatively high affinity, thereby disrupting the formation of larger $A\beta 42$ oligomers even at 1:1 ratio. As the concentration of CLR01 increases, the $A\beta 42$ oligomers decrease in abundance or altogether disappear.

Ion Mobility Spectrometry Reveals CLR01 Inhibiting Early $A\beta 42$ Oligomerization. To better understand the effects of CLR01 on $A\beta 42$ oligomerization, an ion mobility study was conducted. No ATDs for the $z/n = -5/2$ $A\beta 42$ peak ($m/z = 1805$) could be recorded, as it was not observed in any of the mixtures of $A\beta 42$ and CLR01 (Figures 1c and 2d and Figure S2, Supporting Information). The ATDs of the $[2+1]$ and $[2+2]$ complex peaks were recorded and are shown in Figure 2b and c (the signal of $[2+3]$ complex species was too weak to obtain a reliable ATD). The ATD of the $-5/2$ $A\beta 42$ peak of pure $A\beta 42$ (Figure 2a) shows four features with arrival times of ~ 712 , 680, 620, and 540 μs , which were previously assigned as $A\beta 42$ dimer, tetramer, hexamer, and dodecamer, respectively, based on their cross section values (see refs 12 and 45 for a detailed discussion of the $-5/2$ peak assignment). In contrast, in the 1:1 mixture of $A\beta 42$ and CLR01, the ATD of the $m/z = 1950$ peak (Figure 2b, labeled as $[2+1]$ species) shows only two features with arrival times of 690 and 640 μs , which are assigned as dimer and tetramer, respectively. This indicates there is one CLR01 molecule bound to the $A\beta 42$ dimer and two CLR01 molecules bound to the $A\beta 42$ tetramer, respectively. No features at lower arrival times are observed, suggesting there are no $A\beta 42$ hexamers, dodecamers, or other larger oligomers formed in the presence of CLR01. The ATD of the $m/z = 2096$ peak (Figure 2c, labeled as $[2+2]$ species) also shows two dominant features with arrival times of ~ 700 and 660 μs , which correspond to $A\beta 42$ dimer and tetramer, respectively. This indicates that there are two CLR01 molecules

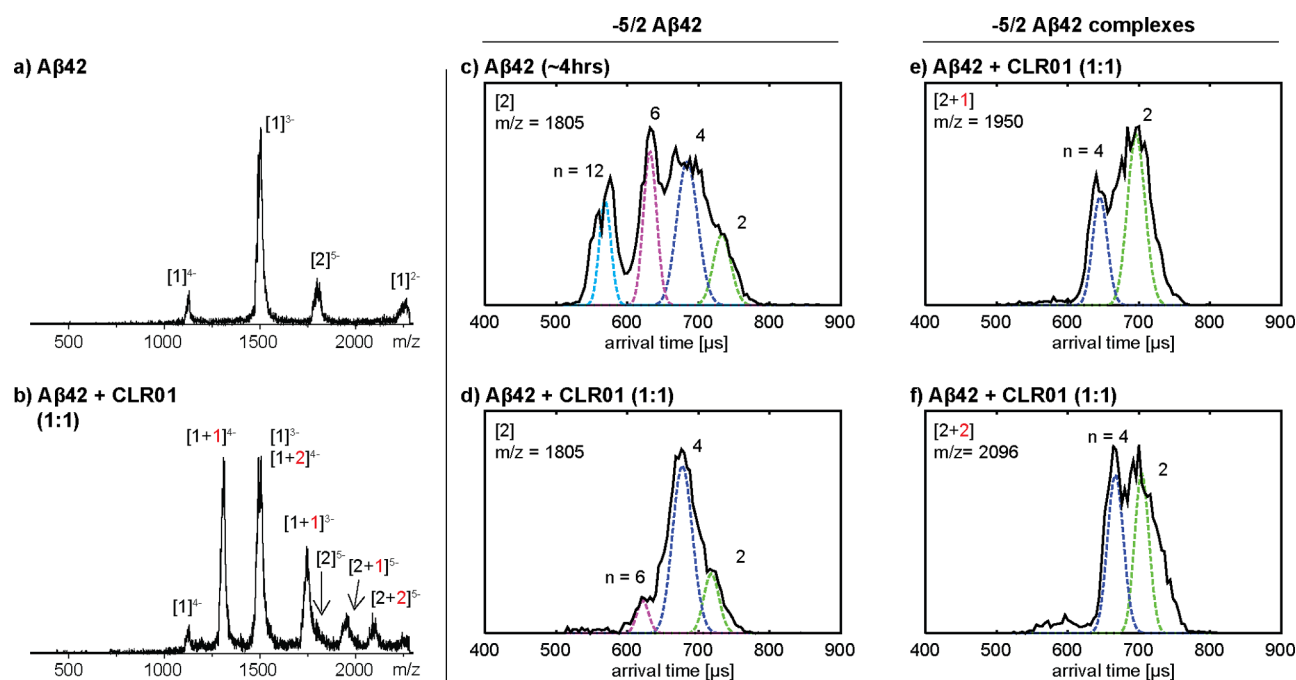


Figure 3. CLR01 remodels the early oligomerization of $A\beta_{42}$. (a) Mass spectrum of $A\beta_{42}$ alone with ~ 4 h of incubation on ice; (b) mass spectrum of the $A\beta_{42}$ sample immediately after the addition of 1:1 CLR01. Each species is noted in brackets where the first number is the number of $A\beta_{42}$ and the second number represents the number of bound CLR01 molecules. The charge is noted as a superscript. (c) ATD of the $z/n = -5/2$ $A\beta_{42}$ peak for the $A\beta_{42}$ in the absence of CLR01 after ~ 4 h of incubation on ice. (d) ATD of the $z/n = -5/2$ $A\beta_{42}$ peak after addition of 1:1 CLR01. (e and f) ATDs of $-5/2$ $A\beta_{42}$ oligomer complexes after addition of 1:1 CLR01 to the preaggregated $A\beta_{42}$ sample. Each ATD is fit with multiple features using the procedure described in the Experimental Methods section, and the oligomer order (n) is noted for each feature.

bound to the $A\beta_{42}$ dimer and four CLR01 molecules bound to the $A\beta_{42}$ tetramer. Again, the highest oligomers with CLR01 bound observed are tetramers and absence of features at lower arrival times indicates no hexamer, dodecamer, or higher order oligomer formation. These results indicate that CLR01 not only binds to $A\beta_{42}$ monomers but also to small oligomers and inhibits the formation of hexamer and higher order oligomers.

The cross sections of oligomer complexes are given in Figure 2e. Interestingly, the cross sections of dimers with one or two CLR01 molecules bound are significantly smaller than those of the dimer with no ligands attached, even though their mass has increased. This result suggests CLR01 induces more interaction between the two monomers, leading to a compact conformation and overall size reduction. Similarly, the cross sections of tetramers with two or four CLR01 molecules bound are smaller than those of the tetramer with no CLR01 bound. In addition, the tetramer ATD peaks with CLR01 bound (Figure 2b, c) are much narrower than in wild type $A\beta_{42}$. This indicates there is little structure variation in the CLR01 bound tetramer while in wt $A\beta_{42}$ the tetramer family of structures is both larger and more varied. The unbound $A\beta_{42}$ tetramer normally adopts a family of structures that have a bent arrangement ($\sim 120^\circ$ angle).¹² It is likely that the tetramers with CLR01 bound adopt either a more closed square ring structure or a pyramidal structure accounting for the fact they are smaller than CLR01-free $A\beta_{42}$ tetramers and that dimer cannot be added to form hexamer. A similar effect prevents $A\beta_{40}$ from growing beyond tetramer and explains its greatly reduced toxicity relative to $A\beta_{42}$.¹²

CLR01 Remodels Preformed $A\beta_{42}$ Oligomers. To explore whether CLR01 can remodel the early oligomerization of $A\beta_{42}$ not only immediately upon dissolution but also after the oligomers have already formed, $A\beta_{42}$ was incubated for 4 h

on ice, following which CLR01 was added to the samples. The samples were incubated at a low temperature to allow quasi-equilibrium of small oligomers to be reached but avoid extensive aggregation, which happens at higher temperatures and leads to clogging of the nano-ESI capillaries, preventing further analysis. The results are shown in Figure 3.

Following incubation, the mass spectrum of $A\beta_{42}$ (Figure 3a) shows four peaks with charge states of $z/n = -4, -3, -5/2$, and -2 . The ATD of the $-5/2$ peak shows four features corresponding to dimer, tetramer, hexamer, and dodecamer, similar to Figure 2a. Upon addition of CLR01 at 1:1 concentration ratio, new peaks appeared corresponding to $z/n = -4, -3$ monomer complexes and $z/n = -5/2$ oligomer complexes (Figure 3b). Overall, the spectrum was similar to the one obtained in the inhibition study (Figure 2d), with the exception that no peaks were observed with 3 or 4 CLR01 molecules bound. The data indicate that CLR01 binds to $A\beta_{42}$ monomers and preformed $A\beta_{42}$ oligomers directly, mostly with 1:1 or 1:2 stoichiometry, whereas binding of additional CLR01 molecules is less likely after incubation. This suggests the existence of two main binding sites for CLR01 on $A\beta$, Lys16, and Lys28.³⁹

Interestingly, the ATD of the $z/n = -5/2$ unbound $A\beta_{42}$ peak (Figure 3c) showed only three features representing dimer, tetramer, and a small amount of hexamer after the addition of equimolar CLR01. The feature representing $A\beta_{42}$ dodecamer was eliminated after the addition of CLR01, suggesting that the binding of CLR01 dissociated the preformed $A\beta_{42}$ dodecamer. Moreover, the relative intensity of the hexamer decreased significantly compared to that before CLR01 addition (Figure 3c), suggesting CLR01 began to also dissociate hexamers. The ATDs of the $z/n = -5/2$ $A\beta_{42}$:CLR01 oligomer complex peaks (Figure 3e, f) showed

only the two features corresponding to dimer and tetramer with CLR01 molecules bound, as observed immediately upon mixing of $A\beta 42$ and CLR01 (Figure 2b and c), suggesting that binding of CLR01 to $A\beta 42$ dimers or tetramers changes their structure so that additional dimers cannot be added to form hexamers. The ATDs of the $z/n = -5/2$ peaks were monitored again after 1 day of incubation at 4 °C, and the results (see Figure S3, Supporting Information) were similar to those obtained following 4 h of incubation, suggesting that CLR01 maintained the distribution of $A\beta 42$ oligomers, in which dodecamers were excluded and hexamers were a minor species.

To test the effect of the $A\beta 42$:CLR01 concentration ratio on the remodeling of $A\beta 42$ oligomerization, higher ratios of CLR01 (1:5 and 1:10, respectively) were added to 4 h incubated $A\beta 42$. The mass spectrum of the 1:5 mixture is shown in Figure S4c (Supporting Information) (the result of the 1:10 ratio was similar to that of the 1:5 mixture and therefore is not shown). The mass spectrum of the 1:5 mixture showed mostly complexes of $A\beta 42$ monomer with CLR01 molecules. No $z/n = -5/2$ peak of CLR01-free or CLR01-complexed $A\beta 42$ was observed. These results suggest that high concentrations of CLR01 dissociate preformed $A\beta 42$ oligomers. Taken together, these results indicate that CLR01 remodels $A\beta 42$ oligomerization both at low and high concentration ratios.

Ion Mobility Spectrometry of $A\beta 42$ Monomer Complexes. The ATDs of $z/n = -3$ of $A\beta 42$ monomer in the absence or presence of CLR01 (1:1) are shown in Figure S5 (Supporting Information). In the ATD of the -3 monomer peak of $A\beta 42$ alone, there are two dominant peaks with arrival times of ~ 640 and ~ 680 μs , previously identified as a solvent-free conformer and a solution-like conformer, respectively.⁴⁹ The ATD for the -3 peak of $A\beta 42$ complexed with one CLR01 molecule shows two similar features with arrival times of ~ 668 and ~ 712 μs . By analogy, these are assigned as the solvent-free and solution-like conformers of $A\beta 42$ monomer with one CLR01 bound, respectively.

The ATDs of $z/n = -4$ and -5 complexes of $A\beta 42$ with one, two, three, or four CLR01 molecules bound in a 1:5 mixture, respectively, are shown in Figure S6 (Supporting Information). Those for $z/n = -4$ have two features in their ATDs, and as the number of bound CLR01 molecules increases, the intensity of the compact, shorter-time feature increases, relative to the more extended, longer-time feature. Overall, there are no features with shorter arrival times detected which indicates that only monomer with CLR01 complexed is present in solution. The cross sections of the $A\beta 42$:CLR01 monomer complexes are shown in Figure S6c (Supporting Information). Addition of each CLR01 ligand increases the size of the complex by an amount comparable to the size of CLR01, suggesting that no major structural transitions occur in the monomers upon CLR01 binding.

Effects of CLR01 on $A\beta 40$ Assembly. $A\beta 40$ has an identical sequence to that of $A\beta 42$ except for absence of Ile41 and Ala42 residues at the C-terminus but has very different assembly and pathological properties. Thus, it is interesting to examine how CLR01 affects its early oligomerization in comparison to $A\beta 42$. The mass spectra of $A\beta 40$ alone and $A\beta 40$ mixed with CLR01 at different ratios are provided in Figure S7 (Supporting Information). The mass spectrum of $A\beta 40$ alone shows three peaks which correspond to $z/n = -4$, -3 , and $-5/2$, similar to the spectrum of the $A\beta 42$. The mass

spectra of mixtures of $A\beta 40$ and CLR01 at different ratios show sets of peaks at charge states -3 , -4 , -5 , and -6 corresponding to $A\beta 40$:CLR01 complexes. Up to four CLR01 molecules are observed bound to $A\beta 40$. At lower CLR01 concentration (1:1 ratio, Figure S7b, Supporting Information), there are three $z/n = -5/2$ peaks at $m/z = 1876$, 2021, and 2167, corresponding to $[2+1]$, $[2+2]$, and $[2+3]$ oligomer complexes of $A\beta 40$ and CLR01. At higher concentrations of CLR01 (1:5 and 1:10 ratios), no $-5/2$ oligomer complexes were detected, suggesting that no dimer or higher-order oligomers formed.

The ATDs of these $-5/2$ oligomer peaks are shown in Figure 4 (the signal of the $[2+3]$ complex was too weak to

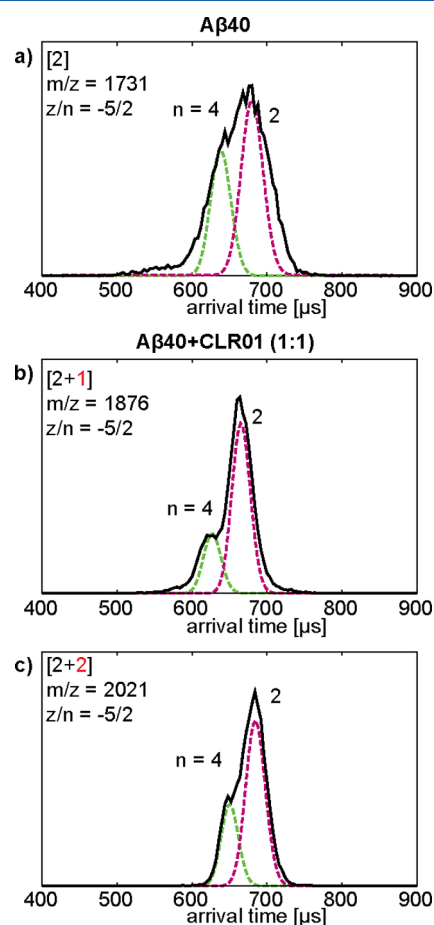


Figure 4. Effects of low concentration CLR01 on $A\beta 40$ oligomerization. (a) ATD of $z/n = -5/2$ $A\beta 40$ ($m/z = 1731$) for $A\beta 40$ alone; (b and c) ATDs of $z/n = -5/2$ $A\beta 40$ and CLR01 complexes ($m/z = 1876$ and 2021) in the 1:1 mixture of $A\beta 40$ and CLR01. Each ATD is fit with multiple features using the procedure described in the Experimental Methods section, and the oligomer order (n) is noted for each feature. Note the ATDs with CLR01 bound, panels b and c, are significantly narrower than wild type, panel a.

obtain a reliable ATD and therefore is not shown). The $-5/2$ peak of $A\beta 40$ ($m/z = 1731$, Figure 4a) shows two features with arrival times of ~ 690 and ~ 620 μs which previously were assigned as $A\beta 40$ dimer and tetramer (see ref 12 for a detailed discussion of the $-5/2$ peak assignment). For the 1:1 mixture of $A\beta 40$ and CLR01, the ATDs of $-5/2$ $[2+1]$ and $[2+2]$ oligomer complexes (Figure 4b and c) show two primary features, which can be assigned as dimer and tetramer based on their cross sections. These results indicate that there are one or

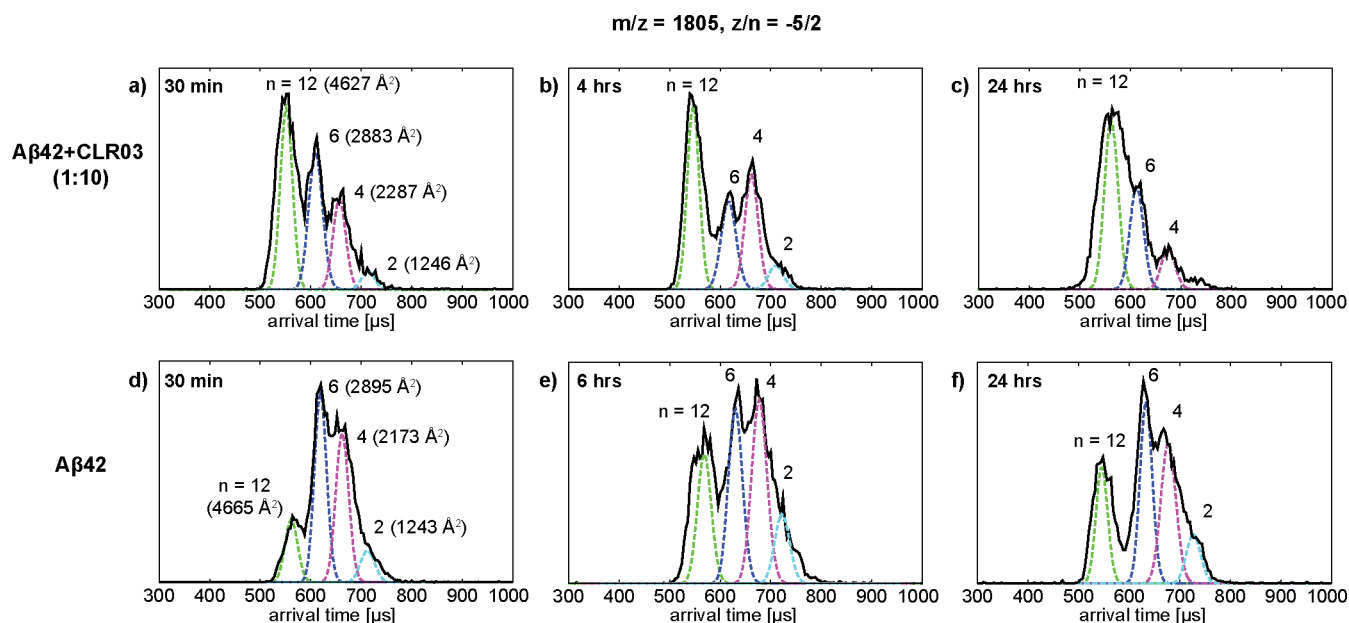


Figure 5. Time-dependent ion mobility study of the effects of CLR03 on $A\beta 42$ early oligomerization. (a–c) ATDs of the $-5/2$ $A\beta 42$ peak ($m/z = 1805$) for the 1:10 mixture of $A\beta 42$ and CLR03 at different time points; (d–f) ATDs of the $-5/2$ $A\beta 42$ peak ($m/z = 1805$) for $A\beta 42$ alone at different time points. Each ATD is fit with multiple features using the procedure described in the Experimental Methods section. The oligomer order (n) and cross section are noted for each feature.

two CLR01 molecules bound to $A\beta 40$ dimers and two or four CLR01 molecules bound to $A\beta 40$ tetramers. No features at shorter arrival times were observed, indicating that there are no hexamer or larger oligomers formed. Interestingly, the intensities of the tetramer feature for $[2+1]$ and $[2+2]$ complex species (Figure 4b and c) are lower than that of the $-5/2$, CLR01-free $A\beta 40$ tetramer feature, which indicates that the formation of tetramer is slower in the presence of CLR01 in the $A\beta 40$ sample than in its absence.

The ATDs of monomer complexes with charge states $z/n = -3, -4, -5$, or -6 are shown in Figures S8 and S9 (Supporting Information). The $z/n = -4$ species (Figure S9a, Supporting Information) show two features in their ATDs corresponding to the solvent-free conformer and the solution-like conformer of $A\beta 40$ complexed with CLR01. Taken together, these results indicate that CLR01 binds to $A\beta 40$ with relatively high affinity and inhibits its early oligomerization. The cross sections of $A\beta 40$ monomer complexes are shown in Figure S9b (Supporting Information). Similarly to the $A\beta 42$ case, addition of each CLR01 ligand increases the size of the monomer complex by an amount comparable to the size of CLR01, suggesting that no major structural transitions occur in the monomers upon CLR01 binding.

IMS Reveals That CLR03 Facilitates Early $A\beta 42$ Oligomerization. As noted above, CLR03 has been used as a negative-control compound, which was not expected to inhibit $A\beta$ oligomerization or aggregation. Hence, we felt it was important to do similar experiments that are reported here.

A time-dependent study of the ATDs of the $-5/2$ $A\beta 42$ peak ($m/z = 1805$) of $A\beta 42$ alone and the 1:10 mixture of $A\beta 42$ and CLR03 is shown in Figure 5. The ATD of the $-5/2$ $A\beta 42$ peak for the 1:10 mixture of $A\beta 42$ and CLR03 at 30 min (Figure 5a) shows four features that can be assigned as $A\beta 42$ dimer, tetramer, hexamer, and dodecamer based on their cross sections, which is similar to the results of $A\beta 42$ alone at 30 min (Figure 5d). Interestingly, the intensity of the dodecamer

feature of the $-5/2$ peak for the mixture of $A\beta 42$ and CLR03 is relatively higher than other features, whereas the intensity of the dodecamer feature of $A\beta 42$ alone at 30 min is relatively lower than other features, suggesting that the growth of dodecamer in the presence of CLR03 is faster than in its absence. After up to 24 h of incubation, the dodecamer in the $A\beta 42$ and CLR03 mixture becomes an even more dominant feature in the ATDs (Figure 5b and c), whereas the CLR01-free $A\beta 42$ sample does not change substantially and appears to be in a state of quasi-equilibrium (Figure 5e and f). The observation of dodecamer and the significant rapid growth of dodecamer in the $A\beta 42$ sample in the presence of CLR03 suggest that CLR03 not only does not inhibit the formation of $A\beta 42$ dodecamer but actually facilitates the dodecamer formation.

ATDs of $z/n = -3$ and -4 peaks for $A\beta 42$ alone and a 1:1 mixture of $A\beta 42$ and CLR03 are shown in Figure 6. The ATD of the $z/n = -3$ $A\beta 42$ peak for the mixture of $A\beta 42$ and CLR03 (Figure 6b) shows two features at ~ 640 and $\sim 680 \mu s$ corresponding to the compact and extended conformers, respectively, which is similar to those of $A\beta 42$ alone (Figure 6a). Remarkably, there is another feature at a substantially shorter arrival time ($\sim 500 \mu s$) as noted by the arrow in addition to the two monomer features, which is not observed for $A\beta 42$ alone. This indicates the presence of relatively large oligomers ($n \geq 2$) formed in the presence of CLR03. A similar feature is observed in the ATD of the $z/n = -3$ peak of $A\beta 42$ with one CLR03 bound, which corresponds to large oligomers ($n \geq 2$). In Figure 6e and f, dominant features with shorter arrival times are observed in the ATDs for $z/n = -4$ peaks of $A\beta 42$ with and without CLR03 bound, in addition to the monomer feature, indicating the presence of large oligomers ($n \geq 2$). These results are consistent with the results of the ATDs of the $z/n = -5/2$ $A\beta 42$ peak ($m/z = 1805$, Figure 5) and further support the fact that CLR03 facilitates self-assembly of $A\beta 42$.

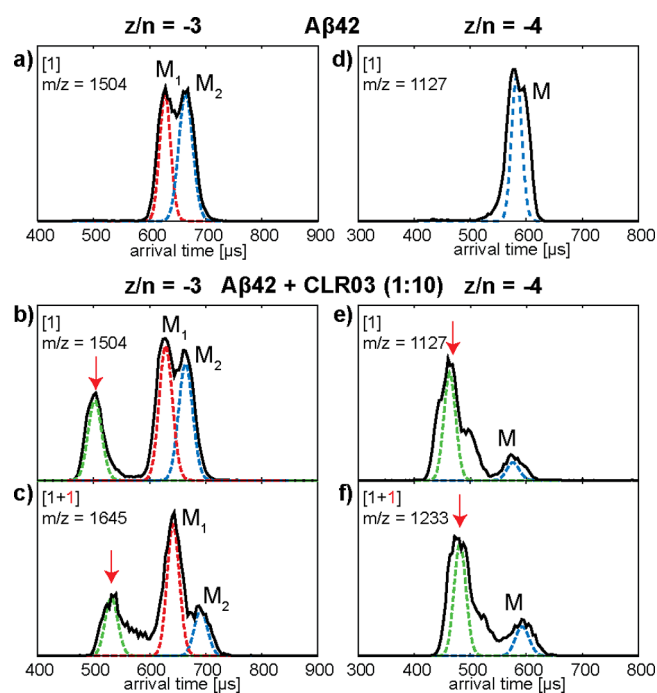


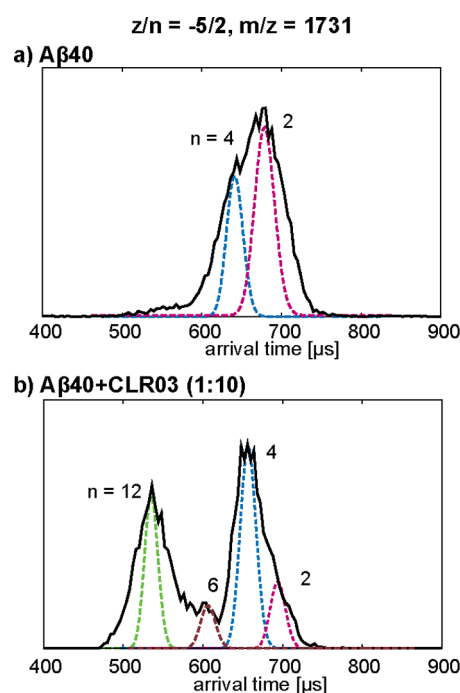
Figure 6. CLR03 facilitates $A\beta_{42}$ oligomer formation: (a) ATD of the $z/n = -3$ peak for $A\beta_{42}$ alone, (b and c) ATDs of $z/n = -3$ $A\beta_{42}$ without and with CLR03 bound for a 1:10 mixture of $A\beta_{42}$ and CLR03, (d) ATD of the $z/n = -4$ peak for $A\beta_{42}$ alone, (e and f) $z/n = -4$ $A\beta_{42}$ without and with CLR03 bound for a 1:10 mixture of $A\beta_{42}$ and CLR03. Each ATD is fit with multiple features using the procedure described in the Experimental Methods section. M_1 and M_2 represent two conformations of the $A\beta_{42}$ monomer. The arrows indicate the formation of oligomers ($n \geq 2$) in the mixture of $A\beta_{42}$ and CLR03.

CLR03 Facilitates $A\beta_{40}$ Assembly. The effect of CLR03 on $A\beta_{40}$ oligomerization is shown in Figure 7. The mass spectrum of a 1:10 mixture of $A\beta_{40}$ and CLR03 shows peaks corresponding to $z/n = -3$ and $z/n = -4$ monomer with one CLR03 bound but no CLR03 attachment to the $z/n = -5/2$ peak (Figure S10b, Supporting Information). However, the ATD of the $-5/2$ $A\beta_{40}$ peak ($m/z = 1731$) in the presence of CLR03 (Figure 7b) shows formation of both $A\beta_{40}$ hexamer and dodecamer based on their cross sections. In Figure 7c, the cross sections for the dimers and tetramers of $A\beta_{40}$ are given for $A\beta_{40}$ alone and for $A\beta_{40}$ mixed with CLR01 and CLR03. Note that CLR03 significantly increases both cross sections, whereas CLR01 significantly decreases both cross sections. Further aggregation is enhanced by CLR03 and inhibited by CLR01.

The ATDs of $z/n = -3$ and -4 peaks of the 1:10 $A\beta_{40}$:CLR03 mixture (Figure S11, Supporting Information) show features at arrival times shorter than those of monomers, suggesting formation of large oligomers ($n \geq 2$) in the presence of CLR03. Overall, these results reveal that CLR03 facilitates early oligomerization of $A\beta_{40}$.

DISCUSSION AND CONCLUSIONS

Our mass spectrometry study of the lead molecular tweezer, CLR01, and the related derivative, CLR03, provides novel observations that could not have been obtained previously due to the low resolution of the methods used. Our investigation reveals that CLR01 and CLR03 bind to $A\beta$ with very different affinities. Three CLR01 molecules bind with relatively higher



c) Cross sections for $-5/2$ $A\beta_{40}$ oligomers

Charge State Oligomer	$\sigma(\text{\AA}^2)$	
	-5 Dimer	-10 Tetramer
$A\beta_{40}$	1161	2084
$A\beta_{40} + \text{CLR01}$	1084*	1936*
$A\beta_{40} + \text{CLR03}$	1223	2215

* Cross sections of dimer with one CLR01 bound and tetramer with two CLR01 bound.

Figure 7. CLR03 facilitates $A\beta_{40}$ assembly. (a, b) ATDs of the $-5/2$ $A\beta_{40}$ peak ($m/z = 1731$) for the $A\beta_{40}$ samples in the absence or presence of CLR03. Each ATD is fit with multiple features using the procedure described in the Experimental Methods section, and the oligomer order (n) is noted for each feature. (c) Cross sections of $-5/2$ $A\beta_{40}$ oligomers for samples of $A\beta_{40}$ in the absence or presence of CLR01 or CLR03. The error for the cross sections reported here is between 0 and 1%.

affinity and a fourth weakly, but only one CLR03 molecule binds weakly to $A\beta$.

The $A\beta$:CLR01 stoichiometry found in our study is consistent with previous data suggesting that there are three possible binding sites for molecular tweezers on $A\beta$ at Arg5, Lys16, and Lys 28.³⁹ Possible explanations for our observation of a fourth CLR01 molecule weakly binding to $A\beta_{40}$ and $A\beta_{42}$ could simply stem from differences in instrumentation, or might reflect nondiscriminating electrostatic and/or aromatic interactions between $A\beta$ and CLR01 molecules, which might have been broken under harsher ionization conditions in the study by Sinha et al.³⁹ These nonspecific dispersive interactions could also explain the weak binding of one CLR03 molecule to $A\beta$, which was not observed within the limits of NMR detection in the previous study.³⁹

The observation of three $z/n = -5/2$ oligomer complexes in the 1:1 and 1:2 mixtures of $A\beta_{42}$ and CLR01, respectively, suggests that CLR01 not only binds to $A\beta_{42}$ monomers but also to small $A\beta_{42}$ oligomers (dimers and tetramers). Moreover, IMS reveals that CLR01 inhibits the formation of

hexamers and dodecamers. This is important, as dodecamers have been identified as probable toxic agents in AD.^{12–14} Understanding the mechanism of how CLR01 blocks dodecamer formation is crucial for developing a therapeutic strategy for AD. Remarkably, the cross sections of the dimer and tetramer decreased substantially upon binding of CLR01 (see Figure 2e). These results suggest that CLR01 interacts with A β 42 to change the folding of the monomer, which in turn changes the binding interface in dimer and tetramer formation, resulting in compact structures that resist further aggregation. Once multiple CLR01 ligands bind to the monomer, even dimer formation is prevented.

The ability of CLR01 to remodel early A β 42 oligomerization after the oligomers had an opportunity to form and reach a quasi-equilibrium state for 4 h was assessed by IMS as well. Interestingly, even at low concentration (1:1 ratio), CLR01 was capable of removing preformed A β 42 dodecamers and hexamers. At high concentrations (1:5 and 1:10 ratios), CLR01 removes essentially all preformed A β 42 oligomers. These data are consistent with the inhibition results immediately upon mixing A β 42 and CLR01. It is possible that CLR01 binds to A β 42 monomers and oligomers and redirects them into either a slower aggregation process or an off-pathway set of structures. In either case, the resulting structures are nonamyloidogenic and nontoxic.^{39–41} Our data reveal for the first time that the loss of amyloid-formation propensity and toxicity correlate with disruption of the oligomerization process and compaction of oligomers formed in the presence of CLR01.

Surprisingly, the related derivative, CLR03, was found to facilitate the early aggregation of A β 42, especially promoting the formation of hexamers and dodecamers. Perhaps even more surprisingly, CLR03 also facilitated the formation of hexamers and dodecamers in A β 40, which does not form these oligomers on its own. Previous studies showed that CLR03 did not inhibit A β 42- or A β 40-induced neurotoxicity.³⁹ However, how CLR03 interacts with A β has been unclear. Given the fact that CLR03 is an organic phosphate, we wondered whether it is possible the addition of CLR03 has a simple “salting out”-like effect, which would thereby promote A β aggregation. To address this possibility, an organic phosphate, *p*-nitrophenylphosphate (PNPP), was added to A β samples and was found not to induce significant changes in A β oligomerization (see Figure S12, Supporting Information). This result implies a simple “salting out”-like effect is not occurring for CLR03 and that CLR03 interacts with A β in a specific manner.

A major difference between the structures of CLR01 and CLR03 is that CLR01 has a torus-shaped cavity whereas CLR03 does not have a cavity structure. CLR03 carries a bridge-like structure with negatively charged phosphate groups on each side. It is possible that one of the phosphate groups on CLR03 interacts with positively charged residues (Lys, Arg, N-terminus) of A β through Coulombic interactions. The other phosphate group on the other side of CLR03 could then attract a positively charged residue of another A β or A β oligomer. Thus, by attracting positively charged residues in A β , CLR03 could facilitate A β oligomerization and aggregation.

Alternatively, CLR03 may interact weakly with two positively charged groups in a single A β , resulting in no observation of peaks for single A β with multiple CLR03 in the mass spectra (Figure 1d). As a consequence, conformation change may occur that promotes A β assembly. This is a form of salting out, and it may be more effective than PNPP. However, the fact that

assembly is promoted to structures similar to wt A β 42 speaks against salting out as a dominant assembly mechanism.

CLR01, which also carries two phosphate groups, however, does not catalyze A β oligomerization. This suggests that inclusion of lysine or arginine inside the cavity of CLR01 is of paramount importance for CLR01's mode of action. The central hydrophobic region of A β is regarded to be important for the hydrophobic clustering of A β . Recently, a macrocyclic inhibitor cucurbit[7]uril has been shown to inhibit amyloid fibrillation by hydrophobic interactions with nonpolar phenylalanine residues of A β .⁵⁴ CLR01, which has hydrophobic arms, is likely to have additional hydrophobic interactions with lysine residues. The binding of CLR01 to lysine residues, especially Lys16 which is close to the central hydrophobic region of A β , may result in conformation change of A β and compaction of A β oligomers. Our data suggest that binding of CLR01 causes A β monomers to either resist oligomer formation altogether or to redirect them to nontoxic oligomer assembly.

Previous detailed analysis showed that A β 40 formed a nearly closed planar tetramer that resisted further dimer addition.¹² Here, we found that adding CLR01 to A β 40 significantly reduced the cross sections of both the dimer and tetramer, leading to nearly isotropic assembly and reducing the likelihood of even forming the tetramer, much less higher-order oligomers. On the other hand, the presence of CLR03 in the solution significantly extended both the dimer and tetramer, yielding cross sections similar to those of the corresponding A β 42 oligomers and leading to hexamer and dodecamer formation.

The essential features of these results are given in cartoon style in Figure 8. A β 42 wt rapidly forms dodecamer, but addition of the molecular tweezer CLR01 eliminates dodecamer formation by inducing the dimer and tetramer to form compact species that cannot add additional A β 42. The opposite

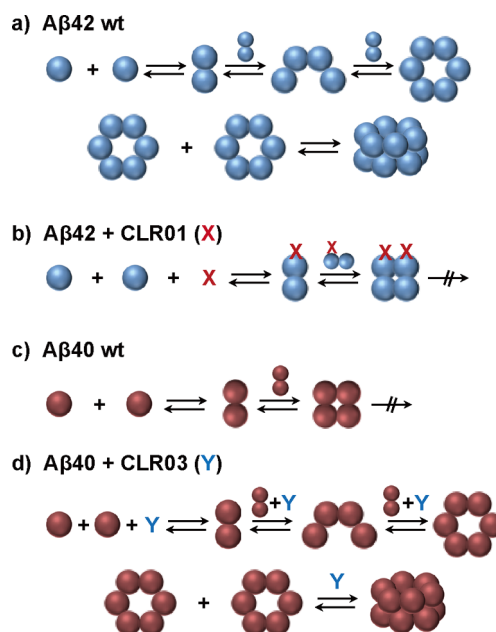


Figure 8. Different effects of CLR01 and CLR03 on A β early oligomerization. Oligomerization of (a) A β 42 wild type, (b) A β 42 with the presence of CLR01, (c) A β 40 wild type, and (d) A β 40 with the presence of CLR03. A β 42 and A β 40 are represented with blue and red balls, respectively. CLR01 and CLR03 molecules are noted as X and Y.

effect is obtained by CLR03. A β 40 wt forms terminal compact tetramers, but addition of CLR03 leads to open tetramer formation and eventual dodecamer formation. These contrary effects are potentially of great importance in A β assembly and require further study to reveal the details involved. These studies, which will include both high level molecular dynamics modeling and additional direct sampling of structures of A β oligomers, are underway.

■ ASSOCIATED CONTENT

● Supporting Information

TEM images, mass spectra of A β -MT mixtures, ATDs and cross sections of A β -MT complexes, and effects of PNP on A β . This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare the following competing financial interest(s): F.-G.K., T.S., and G.B. are co-authors and co-inventors of International Patent No. PCT/US2010/026419, USA Patent No. 8,791,092, and European Patent Application 10 708 075.6. G.B. is a Director and a Co-Founder of Clear Therapeutics, Inc..

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