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An amphiphilic material arginine–arginine–bile acid promotes α -synuclein amyloid formation†

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Amyloid generation plays essential roles in various human diseases, biological functions, and nanotechnology. However, developing efficient chemical and biological candidates for regulating amyloid fibrillation remains difficult because information on the molecular actions of modulators is insufficient. Thus, studies are needed to understand how the intermolecular physicochemical properties of the synthesised molecules and amyloid precursors influence amyloidogenesis. In this study, we synthesised a novel amphiphilic sub-nanosized material, arginine–arginine (RR)–bile acid (BA), by conjugating positively charged RR to hydrophobic BA. The effects of RR–BA on amyloid formation were investigated on α -synuclein (α SN) in Parkinson's disease and on K18 and amyloid- β (1–42) (A β 42) in Alzheimer's disease. RR–BA showed no appreciable effect on the kinetics of K18 and A β 42 amyloid fibrillation because of their weak and non-specific interactions. However, RR–BA specifically bound to α SN with moderate binding affinity through electrostatic interactions between the positively charged RR and the negatively charged cluster in the C-terminus of α SN. In addition, hydrophobic BA in the α SN–RR–BA complex transiently condensed α SN for primary nucleation, thereby accelerating α SN amyloid fibrillation. We propose an electrostatic binding and hydrophobic condensation model of RR–BA-driven amyloid formation of α SN, which will contribute to the rational design and development of molecules for controlling amyloid aggregation in diverse fields.

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Introduction

Amyloid fibrils are highly insoluble, ordered protein aggregates consisting of cross- β structures.¹ The formation and deposition of amyloids in cells and tissues are thought to cause more than 50 serious human diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD).^{2,3} However, the beneficial roles of amyloid fibrils, *i.e.* functional amyloids, in melanin biosynthesis and biofilm formation have also been reported.⁴ The discovery of functional amyloids has motivated the utilisation of non-toxic amyloid fibrils in biotechnology.^{5–7} Although the molecular mechanisms governing amyloid formation, the structural and morphological properties of amyloid fibrils, and amyloid cytotoxicity have been widely studied, protein self-assembly into amyloid fibrils is still not well understood.

In general, the self-assembly of soluble monomeric proteins into insoluble amyloids involves two steps: nucleation and elongation. Nucleation is a slow process that is kinetically limited by a high-energy barrier, whereas elongation, *i.e.*, amyloid growth, occurs rapidly by incorporating monomers to the active end of amyloid fibrils. Notably, the overall process of amyloid formation is highly sensitive to physicochemical and

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biological factors,^{8–10} preventing a complete understanding of amyloid formation. Depending on the ambient conditions, amyloid proteins, such as amyloid- β (A β) and tau in AD and α -synuclein (α SN) in PD, show complicated aggregation pathways and various amyloid structures such as the polymorph of the amyloid fibril.^{8,11–14} The aggregation pathway of A β is altered when the solvent conditions are changed, resulting in the formation of different types of aggregates.⁸ Tau and α SN formed amyloid fibrils *via* the liquid-to-solid phase transition and underwent liquid–liquid phase separation depending on the physicochemical properties of the binding partners and solution conditions.^{9,15} Transthyretin in cardiac amyloidosis impeded the aggregation kinetics of A β by slowing down nucleation without affecting the elongation step because of the physicochemical properties of the interacting residues of both proteins.¹⁶ Interestingly, recent studies demonstrated that lipid bilayers can either promote or inhibit the amyloid formation of A β and α SN in a dose-dependent manner.^{17–20} Furthermore, alterations in the lipid components changed the physicochemical features, including the charges, hydrophobicity, and fluidity of lipid bilayers, leading to distinct dual effects.¹⁹

Nanosized materials have been applied in various fields including biomedicine, therapeutics, and diagnosis because of their versatile physicochemical properties such as size, reactivity, charges, hydrophilicity, and hydrophobicity.^{21,22} Importantly, various nanomaterials have been developed that are physicochemically tailored for regulating amyloid formation through molecular interactions.²³ For example, a self-destructive nanosweeper, generated through conjugation of peptides to chitosan nanoparticles, selectively bound to A β peptides *via* polar interactions such as hydrogen bonding, and led to the inhibition of A β aggregation and the promotion of A β degradation.²⁴ Graphene quantum dots (GQD), a promising carbon-based nanomaterial, interfere with α SN amyloidogenesis through an attractive charge–charge interaction between the negatively charged carboxyl groups of GQD and the positively charged region of α SN.²⁵ In addition, carbon nanotube-based biomimetic nanostructures effectively captured amylin monomers through hydrophobic interactions and hydrogen bonding and blocked amyloid formation by changing the disordered structure of amylin to an α -helical conformation.²⁶

In this study, we synthesised sub-nanosized non-cytotoxic arginine–arginine (RR)–bile acid (BA) as a positively charged amphiphilic conjugate and evaluated how the intermolecular physicochemical properties between the materials and amyloid precursors affect amyloidogenesis. The amphiphilic sub-nanosized material RR–BA is composed of a positively charged RR peptide and hydrophobic BA (Fig. 1A). BA has been widely used for medicinal purposes and previous studies suggested that BA can be used in therapy for transthyretin cardiac amyloidosis.²⁷ We selected three types of amyloidogenic proteins, α SN, K18, and A β 42, as target molecules for RR–BA by considering their physicochemical properties and relationships with the neurodegenerative diseases of PD and AD.

α SN, a major pathological protein in PD, is composed of three distinct regions: a highly alkaline (positive charge) N-terminal region (residues 1–60), a hydrophobic non-amyloid β component (NAC) domain (residues ~60–100), and a highly acidic (negative charge) C-terminal region (residues 100–140) (Fig. 1C). K18, a key tau fragment responsible for forming amyloids and neurofibrillary tangles in AD, is positively charged and contains four microtubule-binding repeats (R1–R4) (Fig. 1D). A β (1–42) (A β 42), whose aggregation is critical for amyloid plaque deposition in AD, shows high hydrophobicity in its central hydrophobic region and C-terminus (Fig. 1E). By combining several biophysical methods, we determined the mechanistic details of the distinct impact of the amphiphilic sub-nanosized material RR–BA on the three types of amyloidogenic proteins based on their aggregation behaviours and intermolecular interactions. We also newly proposed an electrostatic binding-hydrophobic condensation model to rationalize how an amphiphilic modulator acts on protein aggregation on the basis of the physicochemical intermolecular interactions. Our results provide insight for designing candidate molecules against protein misfolding diseases.

Experimental section

Materials

Lyophilised A β 42 (purity $\geq 95.0\%$) was obtained from Peptide Institute, Inc. (Osaka, Japan) and used without further purification. NaOH and HCl solutions (1 M) were purchased from Merck Millipore (Darmstadt, Germany). The C-terminal-modified arginine–arginine peptide (RR–NH₂ ethylamide; 357.4 Da) was synthesised by Anigen (Gwangju, Korea). *N,N*-Diisopropylethylamine (DIPEA), anhydrous *N,N*-dimethylformamide (DMF), ursodeoxycholic acid, diethyl ether, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxy succinimide (NHS), heparin (average molecular weight of 15 000), and other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). Ursodeoxycholic acid was used to synthesise RR–BA. Unless otherwise stated, BA represents ursodeoxycholic acid.

Synthesis

RR–NH₂ (50 mg) was dissolved in anhydrous DMF. BA (~27.5 mg) was added to the peptide-containing DMF. After stirring for 5 min, ~8.1 mg of NHS and ~40.2 mg of EDC were added to the solution. Next, ~4.9 μ L of DIPEA was added and mixed to avoid the effects of salt. The solution was continuously stirred at 600 rpm at room temperature for 24 h, and the product was slowly precipitated twice in 14 mL of cold ether/chloroform (6 : 1) co-solvent. The precipitate was collected by centrifugation at 2000 rpm for 4 min. After discarding the supernatant, the solvent remaining in the precipitant was removed by evaporation for 40 min. The product was rehydrated in 7 mL of distilled water, and the solution was freeze-dried for 48 h. The samples were further purified using Sep-

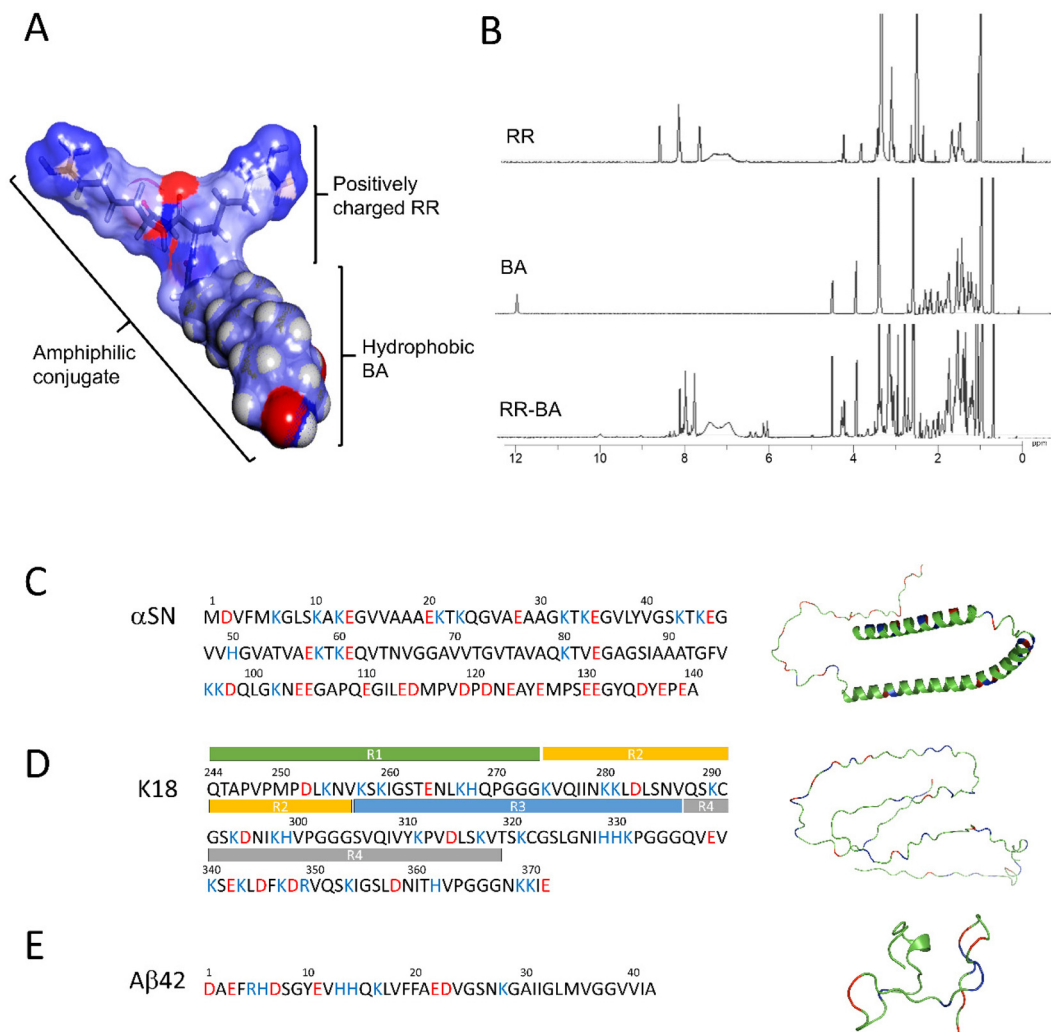


Fig. 1 Physicochemical and structural properties of RR-BA and three types of amyloidogenic proteins. (A) Molecular structure and physicochemical properties of RR-BA. (B) 1D proton NMR spectra of positively charged RR (top), hydrophobic BA (middle), and the synthesised amphiphilic conjugate (RR-BA) (bottom). Amino acid sequences of α SN (C, left), K18 (D, left), and A β 42 (E, left) and their conformations (C–E, right). Negatively charged residues (Asp and Glu) and positively charged residues (His, Lys, and Arg) are coloured in red and blue, respectively. Green colour in the structures indicates non-charged residues. Four microtubule-binding repeats, R1–R4, in K18 are indicated in different colours.

Pak C18 Plus cartridges (Waters, Milford, MA, USA) and freeze-dried to obtain a white powder.

ThT fluorescence assay

The kinetics of amyloid formation in α SN, K18 and A β 42 were traced in a thioflavin T (ThT) fluorescence assay at 37 °C. We prepared 70 μ M α SN and 30 μ M K18 in 20 mM sodium phosphate buffer (pH 7.5), 100 mM NaCl, and 5 μ M ThT; this buffer containing 5 μ M ThT was used for 5 μ M A β 42. The concentrations of RR-BA and RR used for α SN, K18, and A β 42 were 70 and 350, 30 and 150, and 5 and 25 μ M, respectively. Heparin (5 μ M) was used to induce K18 amyloid formation. Amyloid formation of α SN and A β 42 was monitored using a SpectraMax iD3 microplate reader (Molecular Devices, Sunnyvale, CA, USA). A Synergy H1 plate reader (BioTek Instruments, Winooski, VT, USA) was used for K18 experi-

ments. Each sample (100 L per well) was loaded in triplicate into a 96-well plate with a nonbinding surface (Corning, Inc., Corning, NY, USA). To prevent sample evaporation, a sealing film (PowerSeal Cristal View, Greiner-Bio-One, Kremsmünster, Austria) was affixed to the plates. α SN and K18 samples were continuously shaken with 3 mm stainless steel beads; however, A β 42 amyloid formation without beads was observed under quiescent conditions. The fluorescence intensity of ThT was recorded from the top of the plate at excitation and emission wavelengths of 445 and 490 nm, respectively.

The kinetic parameters of amyloid formation were determined by fitting the ThT emission curves using the following equation:¹⁰

$$Y = y_i + m_i t + \frac{y_f + m_f t}{1 + \exp[-k(t - t_0)]}$$

where $y_i + m_i t$ and $y_f + m_f t$ are the initial and final baseline values, respectively. t_0 indicates the half-life at which the ThT fluorescence intensity reaches 50% of the maximum amplitude. k represents the elongation rate constant. The lag time was obtained based on the following relationship: lag time = $t_0 - 2(1/k)$. The average and error values of the lag time and elongation rate constant were calculated for three separate samples in a single set.

CD spectroscopy

Circular dichroism (CD) measurements were performed on a JASCO J720 spectrophotometer (Tokyo, Japan) using a quartz cuvette with a 0.1 mm path length at room temperature. α SN and K18 (10 M) containing 100 mM NaCl and 5 μ M A β 42 without NaCl were prepared in 20 mM sodium phosphate buffer (pH 7.5). The concentrations of RR-BA and RR in the α SN and K18 samples were 10 and 50 μ M, respectively, and half concentrations (5 and 25 μ M) were used for the A β 42 samples. The K18 samples contained $\sim$ 1.7 μ M heparin. Far-UV CD spectra of monomeric α SN, K18, and A β 42 with and without RR-BA were immediately measured after sample preparation. To obtain the CD spectrum of amyloid fibrils, the spectrum of the amyloid solution was subtracted from that of the supernatant obtained by centrifuging the amyloid solution at 15 000 rpm for 30 min. The bandwidth and scan rate were 1 nm and 100 nm min⁻¹, respectively. CD signals are presented as the mean residue ellipticity (deg cm² dmol⁻¹). The content of secondary structures was predicted using the method reported previously.²⁸

NMR spectroscopy

Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker Avance II 700 MHz spectrometer equipped with a cryogenic probe (Bruker BioSpin, Billerica, MA, USA) at 15 °C for ¹⁵N-labelled α SN and 10 °C for ¹⁵N-labelled K18 and A β 42. For two-dimensional (2D) ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) measurements of α SN, 50 μ M ¹⁵N-labelled α SN solutions in the absence and presence of 250 μ M RR-BA or RR were prepared in 20 mM sodium phosphate buffer (pH 7.5) containing 100 mM NaCl and 10% D₂O. For 2D ¹H-¹⁵N band-selective excitation short-transient transverse relaxation optimised spectroscopy (BEST-TROSY) measurements of K18, a stock solution of $\sim$ 500 μ M ¹⁵N-labelled K18 was prepared, as described in the ESI.† Next, 50 μ M ¹⁵N-labelled K18 solutions were prepared with and without 50 μ M heparin and 250 μ M RR-BA in 20 mM sodium phosphate buffer (pH 7.5) containing 10% D₂O and 100 mM NaCl. For 2D ¹H-¹⁵N selective optimised flip angle short transient heteronuclear multiple quantum coherence (SOFAS-HMQC) measurements of A β 42, the stock solution of $\sim$ 200 μ M ¹⁵N-labelled A β 42 was diluted to 30 μ M in 20 mM sodium phosphate buffer (pH 7.5) containing 10% D₂O and treated with 150 μ M RR-BA. The data were processed and analysed using NMRPipe²⁹ and Sparky.³⁰ The backbone resonances of α SN and A β 42 were assigned based on previously assigned information.^{18,31,32} For backbone resonance assignment of

K18, TROSY-based triple resonance NMR experiments were conducted as described in the ESI.† The equation used to calculate the chemical shift perturbations (CSPs) ($\Delta\delta_{\text{NH}}$) is as follows:³³

$$\Delta\delta_{\text{NH}} = \sqrt{\Delta\delta_{\text{H}}^2 + \left(\frac{\Delta\delta_{\text{N}}}{6.5}\right)^2}$$

where $\Delta\delta_{\text{H}}$ and $\Delta\delta_{\text{N}}$ represent alterations in the chemical shifts in the ¹H and ¹⁵N dimensions, respectively.

MD simulation

The solution NMR structure of micelle-bound α SN (PDB ID:1XQ8) was used for molecular dynamics (MD) simulation.³⁴ First, molecular docking simulation between an RR-BA molecule and the C-terminal part of α SN was conducted using the AutoDock program. The residues in the C-terminal region of α SN that displayed meaningful CSPs were set as binding sites. After confirming intermolecular binding, MD simulations were performed using BIOVIA Discovery Studio 2022 software (San Diego, CA, USA). Briefly, molecular binding between one α SN molecule and two conjugates was performed using the Chemistry at HARvard Molecular Mechanics force field. Next, the standard dynamics cascade was sequentially performed with the complex for 500 ps. The generalised Born with a simple switching solvent model was used for MD simulation because of the effects of water molecules. All molecular interactions and binding energies between RR-BA and α SN were investigated using BIOVIA Discovery Studio 2022 software.

Results

Synthesis and characterization of RR-BA

To develop a modulator and investigate its effect on amyloid formation, we designed an amphiphilic molecule by considering that all amyloid proteins accommodate charged, polar, and hydrophobic regions with different portions. The positively charged amphiphilic conjugate RR-BA was synthesised in a one-step reaction between the positively charged RR and the hydrophobic BA (Fig. 1A and S1A†). The C-terminus of the arginine dipeptide RR was blocked by amidation to remove the negative charge and avoid other undesired reactions, which endowed RR-BA with only positive charges. Arginine is safe for human consumption. BA, which has been approved by the U. S. Food and Drug Administration as a drug, was used in this study due to its less toxicity compared to other family members, including deoxycholic acid and chenodeoxycholic acid.³⁵

The conjugation of hydrophilic RR to hydrophobic BA was confirmed using high-performance liquid chromatography (HPLC) (Fig. S1B†). RR-BA was eluted at $\sim$ 12 min with a single peak, indicating its high purity. RR-BA was further characterised using one-dimensional (1D) ¹H and ¹³C NMR spectroscopy, mass spectrometry, and dynamic light scattering

(DLS) (Fig. 1B and S2–S4†). The characteristic peaks of BA (~1 to ~3.5 ppm) and RR (~8 ppm) were observed in the 1D proton NMR spectrum of RR–BA, verifying the complete synthesis and purification of RR–BA without forming aggregates. The successful synthesis of RR–BA was further confirmed by the ^{13}C NMR spectrum that displayed sharp NMR signals of RR and BA (Fig. S2†). In the mass measurement, two types of protonation signals corresponding to RR–BA, $([\text{RR–BA} + \text{H}]^+)$ and $([\text{RR–BA} + 2\text{H}]^{2+})$ were observed at 732.6 and 366.9 m/z , respectively (Fig. S3†). DLS analysis revealed that the average hydrodynamic radius (R_{H}) of RR–BA was ~0.3 nm, indicating that RR–BA remained soluble because of the repulsive electrostatic interactions between the positive charges in RR (Fig. S4†). To assess the *in vivo* availability of RR–BA and its two components, we tested the cytotoxicity of RR, BA, and RR–BA towards fibroblasts. As presented in Fig. S5,† no significant cell death was observed with compounds in the range of 0–100 μM , indicating that all molecules were non-toxic.

Impacts of RR–BA on amyloid formation of the three types of amyloid proteins

To determine whether the amphipathic RR–BA conjugate affects the aggregation of several amyloidogenic proteins, we selected αSN , K18, and A β 42, whose physicochemical properties differ, and performed biophysical analyses. We first evaluated the aggregation kinetics using the ThT fluorescence assay to quantify the formation of amyloid fibrils. In the absence of RR–BA, the ThT fluorescence intensity of αSN increased after a lag time of ~30 h and reached a plateau at ~35 h (Fig. 2A). The sigmoidal kinetic profiles of αSN suggested the occurrence of nucleation-dependent amyloid fibrillation, which is commonly observed in amyloidogenesis such as in that of amylin and β 2-microglobulin.^{36–39} The end-state samples were characterised using CD spectroscopy (Fig. 2J) and atomic force microscopy (AFM) (Fig. S6A†). The far-UV CD spectrum of αSN in the initial state showed a largely unfolded conformation with a negative CD signal at 200 nm, suggesting that αSN maintained an intrinsically disordered structure. In contrast, the far-UV CD spectrum of αSN after incubation exhibited a characteristic profile with a minimum at ~218 nm, indicating a β -strand-rich structure of amyloid fibrils. The AFM image revealed the formation of aggregates with a fibrillar morphology (Fig. S6A†). Overall, αSN amyloid fibrils formed in the absence of RR–BA.

The influences of RR–BA on the structure and aggregation of αSN were then investigated. As shown in Fig. S7A,† the far-UV CD spectra of αSN monomers in the presence of RR–BA were mostly overlapped with the spectrum in the absence of RR–BA. The secondary structure analysis further demonstrated that monomeric αSN with and without RR–BA had almost identical secondary structures (Table S1†). Thus, these results suggest that RR–BA would not change the energy level of αSN monomers without affecting the monomeric αSN structure (Fig. S8A†). Notably, the presence of RR–BA led to a significant acceleration in the amyloid fibrillation of αSN . Sigmoidal ThT profiles were also detected for αSN amyloid fibrillation at 1

(70 μM) and 5 (350 μM) molar equivalents of RR–BA with respect to the concentration of αSN (70 μM) (Fig. 2A), indicating that αSN still aggregated into amyloid fibrils in a nucleation-dependent manner, even in the presence of RR–BA. Kinetic analysis showed that the average lag time was shortened from ~30 h without RR–BA to ~14 and ~4 h in the presence of 70 and 350 μM RR–BA, respectively (Fig. 2D). The elongation rate constant also exhibited significant changes. At low RR–BA concentrations (70 μM), the elongation rate constant increased from ~1 h^{-1} (without RR–BA) to ~1.7 h^{-1} , and further increasing the concentration of RR–BA to 350 μM increased the elongation rate constant to ~3.2 h^{-1} (Fig. 2G). These kinetic results suggest that RR–BA might significantly decrease the energy barrier, which determines the kinetics of αSN amyloid formation (Fig. S8A†). In accordance with the enhanced ThT intensities, the far-UV CD results revealed the formation of a β -strand-rich structure of αSN with RR–BA (Fig. 2J). The AFM images revealed that fibrillar aggregates formed after incubation (Fig. S6B†).

To further examine whether the promoting effect of RR–BA was solely related to RR, the same experiments were conducted using 1 (70 μM) and 5 (350 μM) molar equivalents of RR with respect to the concentration of αSN (70 μM). In contrast to RR–BA, RR did not cause noticeable changes in αSN amyloid fibrillation (Fig. 2D and G, and S9A†). The ThT fluorescence assay reported that the lag times and elongation rate constants with 70 and 350 μM RR were similar to those without RR. The far-UV CD spectra of αSN at both RR concentrations almost overlapped with those of αSN with and without RR–BA, demonstrating that the same secondary structure was formed in all amyloid fibrils (Fig. 2J). The AFM images also revealed that fibrillar aggregates generated with RR had a similar morphology to that of amyloid fibrils prepared with or without RR–BA (Fig. S6C†). Thus, the promoting effect of RR–BA on αSN amyloidogenesis is not solely related to RR.

Next, we examined the effect of RR–BA on K18 amyloid formation. The ThT fluorescence of the K18–RR–BA mixture was traced without heparin in real time (Fig. S10†). Several studies reported that heparin induces the amyloid formation of full-length tau and its fragments.^{14,40–43} In agreement with these studies, the ThT fluorescence intensities of K18 without heparin showed minimal changes throughout incubation at all concentrations of RR–BA (Fig. S10A†). The results of CD and AFM analyses confirmed that K18 remained as a disordered monomer with and without RR–BA (Fig. S10B and C†). These results showed that RR–BA did not induce the amyloid generation of K18. However, as expected, the addition of negatively charged heparin at 5 μM led to a remarkable increase in the ThT intensity of K18 after the lag phase of ~1 h (Fig. 2B). The far-UV CD spectra and AFM images revealed the formation of β -structure-rich amyloid fibrils (Fig. 2K and S6D†).

Next, we examined how RR–BA influenced the structure and amyloid formation of K18 in the presence of heparin. As shown in Fig. S7B,† the far-UV CD spectra of K18 monomers with and without RR–BA were mostly similar, suggesting no effect of RR–BA on the secondary structure of K18. The ThT

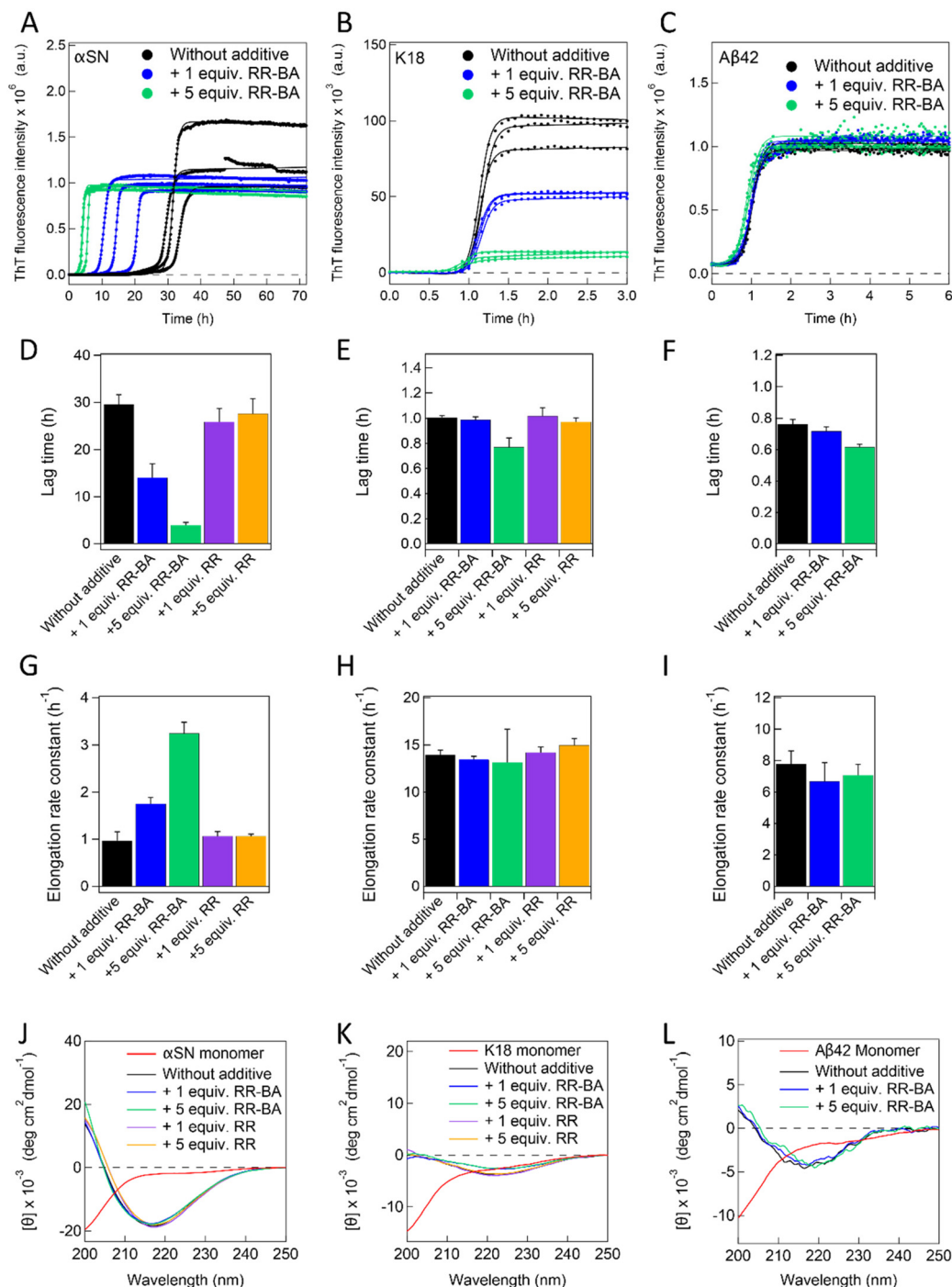


Fig. 2 Kinetic and structural investigation of the amyloid formation of α SN, K18, and A β 42 with compounds. Amyloid fibrillation of α SN (A), K18 (B), and A β 42 (C) in the absence and presence of 1 or 5 molar equivalents of RR-BA monitored using ThT fluorescence. Solid lines represent the fit curves. Lag times and elongation rate constants of the amyloidogenesis of α SN (D and G), K18 (E and H), and A β 42 (F and I) in the absence and presence of RR-BA or RR. Far-UV CD spectra of α SN (J), K18 (K), and A β 42 (L) after incubation. CD spectra (red lines) of monomeric α SN (J), K18 (K), and A β 42 (L) were recorded for comparison. Experimental and analysed data obtained at different concentrations of RR-BA and RR are shown in different colours: black (without RR-BA and RR), blue (1 molar equivalent of RR-BA), green (5 molar equivalents of RR-BA), purple (1 molar equivalent of RR), and orange (5 molar equivalents of RR).

fluorescence profile showed that the lag time and elongation rate constant of K18 with and without RR-BA were not distinguishable (Fig. 2B, E and H). However, the maximum inten-

sity of the ThT fluorescence of the K18 sample solution without RR-BA was ~5-fold higher than that with 5 molar equivalents of RR-BA. This large difference in the ThT fluo-

rescence may be attributable to the polymorphism of amyloid generation depending on the environment. Previous studies demonstrated that the polymorphic aspect of amyloid fibrils showing varying amyloid structures in spite of the same precursor proteins leads to distinct fluorescence intensities due to the different binding sites of ThT on amyloid fibrils.^{20,44} Indeed, the presence of RR-BA in the K18 solution after incubation altered the CD spectrum (Fig. 2K), suggesting the formation of amyloid fibrils with distinct secondary structures. Moreover, K18 amyloid fibrils grown with and without RR-BA showed different spectra in the fluorescence dye-binding assays. The Nile red fluorescence spectrum of K18 amyloid fibrils grown without RR-BA presented a blue shift of the maximum wavelength with an enhanced fluorescence intensity compared to that formed with RR-BA (Fig. S11A†). The curcumin fluorescence spectrum of K18 amyloid fibrils grown with RR-BA resulted in a shift of the maximum wavelength of K18 amyloid fibrils generated without RR-BA from ~517 to ~513 nm (Fig. S11B†). Consistent with the results of the CD and fluorescence dye-binding experiments, TEM micrographs exhibited that K18 amyloid fibrils formed without RR-BA were thicker and longer than those formed with RR-BA (Fig. S11C and D†). Formation of a different structure with almost the same kinetics suggests that RR-BA alters the thermodynamic energy level of K18 amyloid aggregates without affecting the kinetic energy barrier of amyloid formation (Fig. S8B†). In contrast, RR did not affect the heparin-induced amyloid fibrillation of K18 (Fig. S8B†), as observed in α SN amyloid formation (Fig. S8A†). Kinetic analysis of amyloid aggregation revealed that the lag time and elongation rate constant remained unchanged at all RR concentrations examined (Fig. 2E and H). The same CD spectrum revealed the formation of a β -strand-rich structure of K18 at a 5 : 1 molar ratio of RR : K18 without conformational differences (Fig. 2K).

Finally, we investigated the effects of RR-BA on the structure and amyloid aggregation of A β 42. Analogous to α SN and K18, nucleation-dependent amyloid fibrillation was detected in A β 42 (Fig. 2C). In the absence of RR-BA, the ThT fluorescence emission of A β 42 increased after an average lag time of ~0.7 h with an elongation rate constant of ~8 h⁻¹ (Fig. 2C, F and I). CD experiments revealed the conformational conversion from an initial random-coil structure to a β -strand-rich structure after incubation (Fig. 2L). Together with the AFM image (Fig. S6E†), these results showed that A β 42 amyloid fibrils were formed. The addition of 1 or 5 molar equivalents of RR-BA did not affect the far-UV CD spectra of A β 42 monomers and the kinetics of A β 42 amyloid formation (Fig. 2C, F and I, and S7C†). Furthermore, the CD spectra of A β 42 with and without RR-BA after incubation were similar (Fig. 2L). Together, these results support that RR-BA does not affect A β 42 amyloid generation with no change in the energy landscape of A β 42 amyloidogenesis based on the thermodynamics and kinetics (Fig. S8C†).

Complexation between RR-BA and the three types of amyloidogenic proteins

To better understand how the amphiphilic RR-BA conjugate modulates the amyloidogenicity of α SN, we first performed 2D

¹H-¹⁵N HSQC NMR experiments of the uniformly ¹⁵N-labelled α SN with and without RR-BA. As shown in Fig. 3A, the ¹H-¹⁵N spectrum of α SN without RR-BA displayed a narrow distribution of NMR peaks between ~7.8 and ~8.4 ppm, indicating the highly disordered nature of α SN, which is consistent with the results in the far-UV CD spectrum (Fig. 2J). Upon treatment with 5 molar equivalents of RR-BA, significant CSPs were detected for amino acid residues F94, E105, M116, D119, D121, N122, A124, E126, M127, S129, E130, G132, Y133, D135, Y136, and E137 (Fig. 3D). This observation indicated that RR-BA specifically binds to the negatively charged C-terminal region of α SN (Fig. 1C). There was no change in the NMR peak intensity (Fig. 3G), indicating that the binding of RR-BA did not induce rapid aggregation of α SN. In addition, 2D NMR experiments were performed for ¹⁵N-labelled α SN with RR (Fig. S12†) to investigate their interactions. Similar to RR-BA (Fig. 3D and G), the addition of RR led to remarkable CSPs in the C-terminal region of α SN without significantly changing the NMR peak intensity (Fig. S12B and C†), indicating the selective binding of RR to the C-terminus of α SN.

To evaluate the binding affinity of RR-BA for α SN, we performed biolayer interferometry (BLI) experiment. The BLI sensorgram showed a dose-dependent change in the association and dissociation curves with an increase in the concentration of RR-BA (Fig. S13A†). Of note, both the association and dissociation phases consisted of fast and slow phases, suggesting that RR-BA binds to α SN through multiple binding models. Although sensorgrams are commonly used for fitting analyses of intermolecular binding properties, the too fast association and dissociation in the current case are not applicable for accurate fitting. Thus, to obtain an apparent dissociation constant (K_d) for α SN-RR-BA complexation, we performed steady-state analysis by plotting the average response at the end of the association phase as a function of RR-BA concentration (Fig. S13B†). The fitting analysis exhibited that RR-BA interacted with α SN with a K_d value of 168 μ M, suggesting that RR-BA moderately interacts with α SN.

MD simulation is a powerful technique for visualising and providing atomistic information on intermolecular interactions.⁴⁵⁻⁴⁸ Therefore, we explored the potential binding model of RR-BA to α SN using MD simulations. MD simulation of the molecular interaction between RR-BA and α SN was initiated by locating two RR-BA conjugates in the C-terminal part of α SN based on the NMR results (Fig. 3A). Of note, because monomeric α SN lacks a well-defined 3D conformation in an aqueous solution, a micelle-bound structural model of α SN was used here.³⁴ This model comprises a broken helix in the N-terminal and NAC regions, while the C-terminal region remains mostly unstructured. Therefore, although the initial structure of α SN requires careful attention, our computational results still provides valuable insights into the interaction between RR-BA and the disordered C-terminus of α SN.

As the charged state in α SN and RR-BA could not be applied, we focused on the polar interactions between α SN and RR-BA. After MD simulation for 100 ps, one of the two RR-BA conjugates remained attached to α SN by interacting

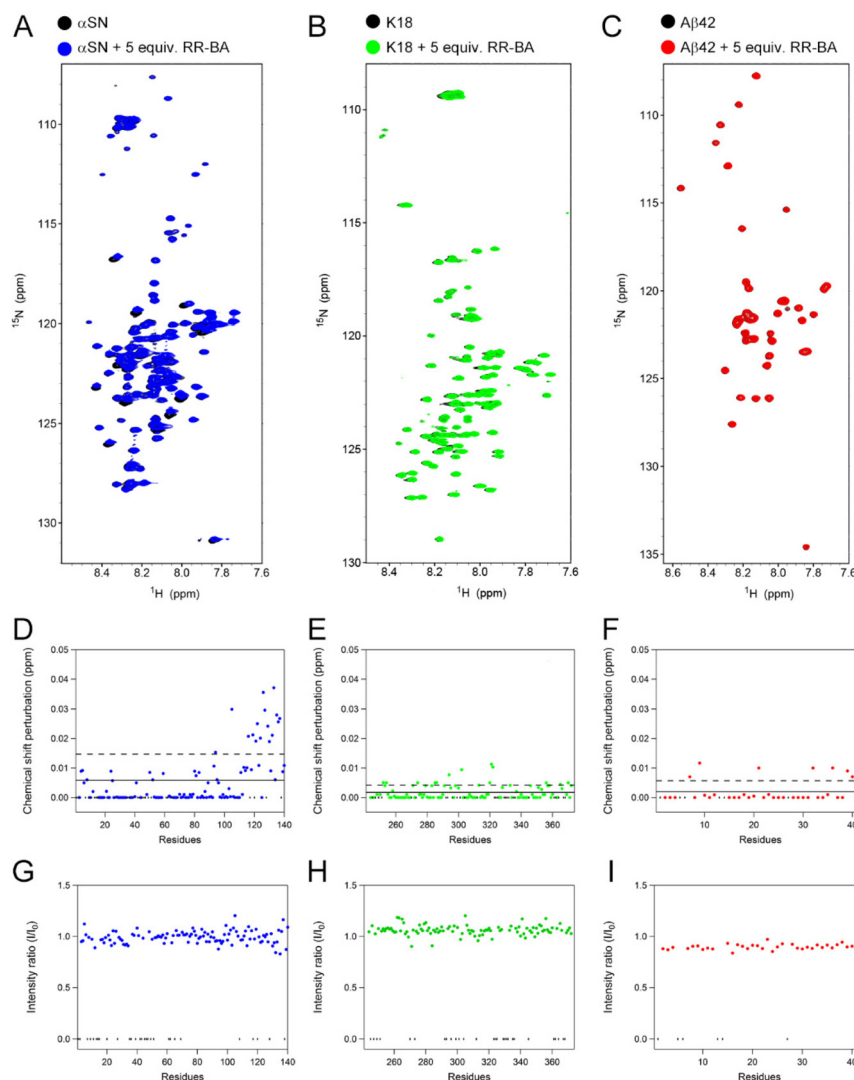


Fig. 3 Molecular interactions between RR-BA and three types of amyloidogenic proteins examined using NMR spectroscopy. 2D ^1H - ^{15}N HSQC NMR spectra of ^{15}N -labelled αSN (A), K18 (B), and A β 42 monomers (C) in the absence and presence of 5 molar equivalents of RR-BA. CSPs and peak intensity ratio of αSN (D and G), K18 (E and H), and A β 42 (F and I) upon the addition of RR-BA. Two horizontal lines in D–F represent the average chemical shift (solid line) plus one standard deviation (dashed line). The amino acid residues that could not be analysed because of overlapping chemical shifts and unassigned residues are indicated by black diamonds.

with the C-terminal residues (Fig. 4A). This attachment lasted for ~ 200 ps, after which dissociation occurred without any subsequent rebinding until the end of the simulation (500 ps). We monitored the time evolution of the interaction energies between RR-BA and αSN within the first 200 ps. Negative interaction energies of RR (-100 to -200 kcal mol $^{-1}$) were much larger than those of BA (~ -25 kcal mol $^{-1}$) (Fig. S14A †), indicating the more favorable binding of RR to αSN than BA. The contribution of electrostatic interactions between RR and αSN to complex formation was large while electrostatic energies coming from BA- αSN were minimal (Fig. S14B †), consistent with the NMR analysis (Fig. 3A and S12 †). There was no appreciable van der Waals interactions for complexation (Fig. S14C †). Based on the 2D interaction diagram, the hydrophilic moiety of RR-BA interacted with E126, M127, P128, and

S129 (Fig. 4B). E126 interacted with a guanidinium group in the side chain of the RR moiety of RR-BA through conventional hydrogen bonding and van der Waals interactions. M127 and S129 formed conventional hydrogen bonds with a nitrogen atom in the amide bond and a carbonyl oxygen in the RR moiety of RR-BA, respectively. P128 formed a carbon-hydrogen bond and an alkyl bond with RR-BA. These results indicate that the hydrophilic moiety in RR-BA plays a decisive role in interacting with the C-terminal region of αSN , mostly through polar interactions such as conventional hydrogen bonds. Thus, the results of MD analysis supported those of NMR analysis and enabled a comprehensive characterisation of the molecular interactions between αSN and RR-BA.

Next, intermolecular interactions between RR-BA and K18 in the absence and presence of heparin were investigated

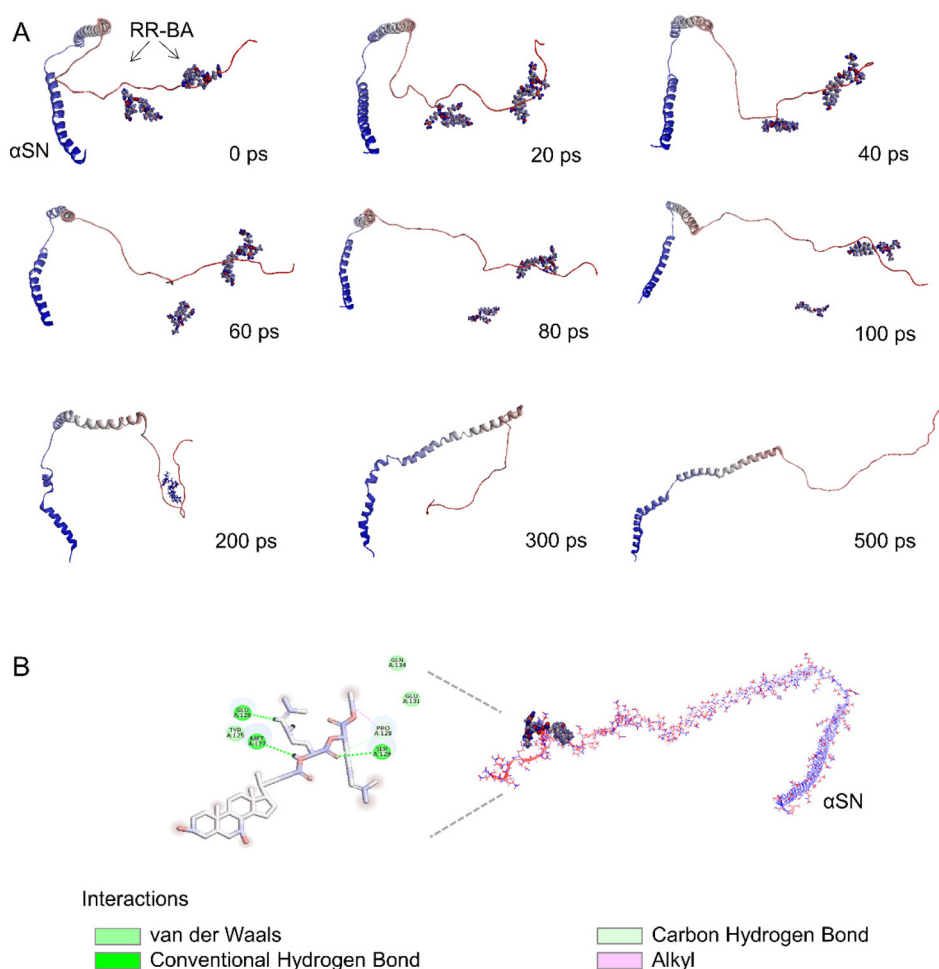


Fig. 4 MD simulation analysis of the interaction between α SN and RR-BA. (A) Snapshots of the interaction between α SN and RR-BA at different time points. The N- and C-terminal regions of α SN protein are represented in blue and red, respectively. (B) Intermolecular interactions between the C-terminal region of α SN and RR-BA at 100 ps are shown (right) and magnified (left). Information on the intermolecular interaction is shown.

using 2D ^1H - ^{15}N BEST-TROSY NMR. As observed for α SN, the NMR peaks of ^{15}N -labelled K18 showed a narrow distribution in the spectra with and without RR-BA, indicating the intrinsically disordered character of K18 (Fig. 3B). Based on the NMR chemical shifts, the CSPs of K18 with RR-BA were calculated without heparin (RR-BA:K18 = 5:1) (Fig. 3E). The following residues showed small CSPs throughout the K18 molecule: D252, L253, K254, G271, K274, N279, D283, N286, C291, K294, K298, G302, V313, S320, C322, G333, F346, L357, D358, G365, K370, and E372. The peak intensity of the K18 residues did not change upon incubation with RR-BA (Fig. 3H). Thus, RR-BA is likely weakly and non-specifically interacted with the whole region in K18.

In contrast to RR-BA, significant CSPs were detected for K18 mixed with an equal molar ratio of negatively charged heparin (Fig. S15A†). The I277, I278, N279, L282, D283, and L284 residues, which are at the beginning of the R2 domain (Fig. 1D), exhibited the largest CSPs (Fig. S15A,† lower panel), which is consistent with the results of previous studies.^{43,49} For example, Wang and coworkers observed the largest CSPs

for I278, K281, L282, and D283 of K18 upon the addition of heparin.⁴⁹ Considering that the hexapeptide motif in R2, which spans V275-K280, is important for tau aggregation, binding of heparin to this region may change the conformation and/or solubility of K18 to promote amyloid formation. Next, to determine whether RR-BA influences K18-heparin interactions, RR-BA was added to the K18-heparin mixture (RR-BA:K18 = 5:1) (Fig. S15B†). V256, Q276, I278, L282, D283, C291, G292, G302, T319, S320, G333, and K353 exhibited small CSPs (Fig. S15B,† lower panel). These results demonstrate that RR-BA had a minimal influence on K18-heparin complex formation.

As observed previously,^{50,51} A β 42 showed a pattern of largely disordered proteins and peptides (Fig. 3C). Similar to K18, NMR analysis revealed that the interaction between RR-BA and A β 42 was weak and non-specific, and large aggregates were not formed (Fig. 3C, F and I). When RR-BA was added to ^{15}N -labelled A β 42 (RR-BA:A β 42 = 5:1), small CSPs were observed in several residues: D7, G9, A21, I32, V36, V39, and V40 (Fig. 3F). Moreover, the overall peak intensity of ^{15}N -

labelled A β 42 did not notably change in the presence of RR-BA (Fig. 3I).

Impacts of RR-BA on the neurotoxicity of α SN

To investigate the effect of RR-BA on the neurotoxicity of α SN, we incubated α SN with and without 5 molar equivalents of RR-BA for 12 h at 37 °C (Fig. 5A). The resulting ThT data revealed that α SN monomers formed amyloid fibrils with a significantly enhanced ThT intensity after 12 h incubation with RR-BA, while ThT intensity remained low for α SN without RR-BA (Fig. S16†). Such results suggested that the amount of α SN aggregates in the sample grown without RR-BA was much less than that with RR-BA after 12 h incubation. We then treated primary mesencephalic neurons with α SN incubated with and without RR-BA to compare their neurotoxic effects (Fig. 5A). After 48 h incubation, the number of TH⁺ neurons, the length of total neurite outgrowth, and the total number of processes and branches of TH⁺ neurons were measured to monitor the effects of those α SN on neuron viability and neurite integrity (Fig. 5B–F). Of note, RR-BA itself has no effect on neuron viability and neurite integrity (Fig. 5B–F). Importantly, α SN incubated with RR-BA induced more cell death compared to that incubated without RR-BA (Fig. 5C). Moreover, neurons treated

with α SN incubated with RR-BA showed much less neurite outgrowth and a decreased number of processes and branches than those treated with α SN incubated without RR-BA (Fig. 5D–F), suggesting that α SN incubated with RR-BA is more neurotoxic than the one incubated without RR-BA. Taken together, our data demonstrate that RR-BA promotes the aggregation of α SN, leading to the earlier onset of the neurotoxic effect against primary mesencephalic neurons.

Discussion

Amyloid formation is closely related to diverse human diseases.^{2,3} The development of effective strategies against disease-related amyloid pathology remains challenging, primarily because of the complexity of amyloidogenesis. For decades, various key concepts have been suggested to understand the mechanism of amyloid formation: primary and secondary nucleation, amyloidogenic structure, breakage, maturation, and adaptation of amyloids, polymorphism, Ostwald ripening, solubility, supersaturation, *etc.*^{8,37,52–59} Therefore, the overall process of amyloid formation is highly susceptible to ambient conditions. Recently, it has been suggested that

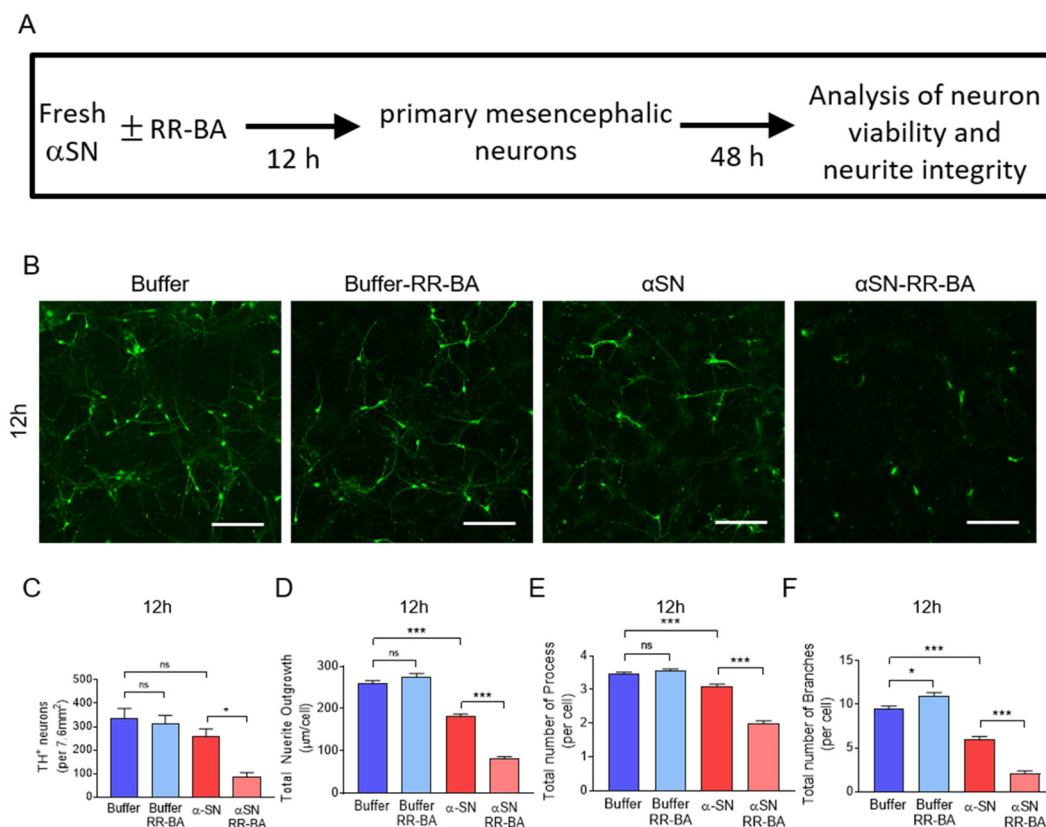


Fig. 5 Effect of RR-BA on the neurotoxicity of α SN. (A) Overall scheme of the experiments. (B) Representative images of neurons after 48 h incubation with vehicle, RR-BA, and α SN which was incubated with and without RR-BA. Analysis of the number of TH⁺ neurons (C), the length of total neurite outgrowth (D), and the total number of processes, (E) and the branches of TH⁺ neurons (F). Conditions: [α SN] = 30 μ M; [RR-BA] = 150 μ M; incubation for 48 h. Scale bars = 200 μ m. Data are represented as the mean $\pm$ s.e.m. Significance was determined using a one-way ANOVA and Tukey's multiple comparisons test. * P < 0.05; *** P < 0.001; groups with no significant differences are noted as ns.

bio-inspired and artificial nanomaterials have possibilities to control protein aggregation and combat amyloid pathogenesis based on their wide range of chemical components, surface properties, and architectures.²³ Diverse nanomaterials have been reported to modulate the amyloid fibrillation of various amyloidogenic proteins, including A β and α SN.^{24,25,60–63} However, the comprehensive molecular mechanisms of nanomaterials for modulating amyloidogenesis remain largely unclear.

We designed the amphiphilic sub-nanosized material RR-BA by conjugating positively charged RR to a hydrophobic BA (Fig. 1A) and investigated its impact on the aggregation process of three different amyloidogenic proteins, α SN, K18, and A β 42. RR-BA exhibited moderate and specific interactions with the C-terminal region of α SN, whereas its binding to K18 and A β 42 was mostly weak and non-specific (Fig. 3). Considering the positive charges in RR-BA and many negative charges, *i.e.*, a negatively charged cluster, in the C-terminus of α SN (Fig. 1C), attractive electrostatic interactions are fundamental for complex formation between two molecules. Although acidic amino acid residues also exist in K18 (D252, E264, D283, D295, D314, E338, E342, D345, D348, D358, and E372) and A β 42 (D1, E3, D7, E11, E22, and D23), these two proteins do not contain a negatively charged cluster (Fig. 1D and E).

As presented in the Das-Pappu diagram which indicates physicochemical properties including the charge cluster (Fig. S17†), a high population of negatively charged residues ($\sim 40\%$) was observed in the C-terminal region of α SN. In contrast, neither K18 nor A β 42 possesses a region with a high population of negatively charged residues. These results suggest that a specific charge cluster is important for determining the intermolecular interactions with increased strength of electrostatic interactions. Previous studies supported the key role of charged clusters in stabilising the protein-protein complex for biological functions. The negatively charged cluster in electron-transferring ferredoxin plays a critical role in interacting with the positively charged clusters in ferredoxin NADP⁺ reductase^{64,65} and sulphite reductase.^{66,67} Cytochrome *c* uses its positively charged cluster for electrostatic interactions with a negatively charged cluster in cytochrome *c* oxidase.⁶⁸

Our kinetic investigations showed that RR-BA significantly accelerated the amyloid formation of α SN, but not of K18 and A β 42 (Fig. 2). This finding suggests that the binding properties of RR-BA were responsible for the amyloid formation of the three proteins examined. In accordance with our results, previous studies reported that strong and specific binding to membranes caused dramatic changes in α SN aggregation behaviours, whereas only minimal effects were detectable for

Electrostatic binding-hydrophobic condensation model

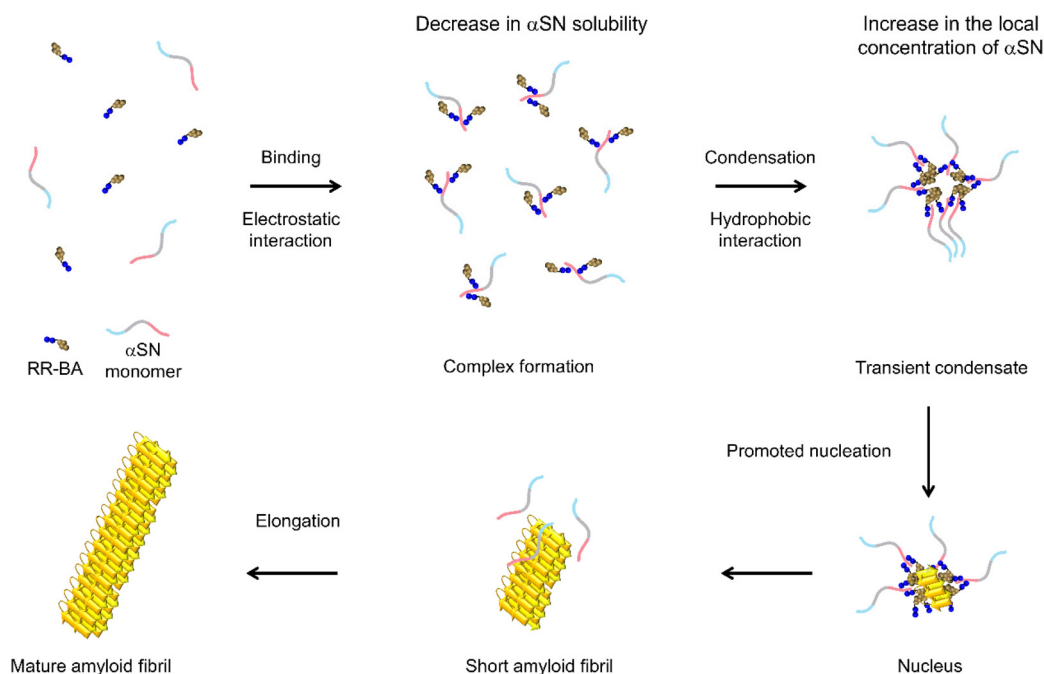


Fig. 6 An electrostatic binding-hydrophobic condensation model for the acceleration of α SN amyloidogenesis by RR-BA. Molecular mechanisms of RR-BA-induced α SN amyloid formation are schematically shown. Free α SN monomers and RR-BA conjugates (upper left), α SN-RR-BA complexes (upper middle), condensed α SN-RR-BA complexes (upper right), nucleus (lower right), short amyloid fibrils with α SN monomers (lower middle), and mature amyloid fibrils (lower left) are illustrated. The N-terminal region, NAC region, and C-terminal region of α SN are represented in blue, grey, and red, respectively. The positively charged RR and the hydrophobic BA of RR-BA are shown in blue and brown, respectively.

weak and non-specific interactions with membranes.^{17,18,20} In addition, although the positively charged dipeptide RR also interacted with the negatively charged C-terminus of α SN (Fig. S12†), it was insufficient to effectively promote α SN amyloid fibrillation (Fig. S9A†). Thus, BA may play a pivotal role in α SN amyloid aggregation (see below).

Based on the kinetic and binding results, we propose an electrostatic binding-hydrophobic condensation model that accounts for RR-BA-induced α SN amyloid aggregation (Fig. 6). First, RR-BA selectively binds to an α SN monomer through long-range electrostatic interactions between the positively charged RR and the negatively charged cluster in the C-terminus of α SN. The hydrophobic nature of BA in RR-BA contributes to the condensation of the α SN-RR-BA complex. Increases in the local concentration of α SN around the BA clusters promote primary nucleation, thereby accelerating amyloid fibrillation with a decreased energy barrier (Fig. 6). An elevated local concentration of proteins has been suggested to promote amyloidogenesis in diverse systems,^{17,18,20,69,70} supporting our molecular model. Specifically, at moderate lipid concentrations, α SN bound multiply with the negatively charged membranes using its positively charged N-terminal region, increasing the protein concentration at the membrane surface and consequently boosting amyloid formation.^{17,18,20} Similarly, air-water interfaces generated by mechanical forces with vortex and ultrasonication condense proteins at the interface to promote amyloidogenesis.^{8,37,71} Notably, the attractive electrostatic interaction between α SN and RR and the attachment of BA to α SN upon α SN-RR-BA complexation may decrease the solubility of α SN because of the loss of charges and the gain of hydrophobicity. Thus, the loss of solubility of α SN following complex formation may explain the intensified amyloidogenicity of α SN, as observed in β 2-microglobulin and lysozyme.^{37,55} In addition, previous studies reported that partially helical or β -structure-rich α SN facilitates its amyloid formation.^{72–74} However, we observed that no difference in the secondary structure contents of α SN monomers with and without RR-BA (Fig. S7A and Table S1†). Thus, it is obvious that the promotion of α SN amyloidogenesis observed here is not ascribed to RR-BA-induced conformational changes in α SN.

Collectively, these results provide a general mechanistic understanding of the effect of amphiphilic RR-BA on protein amyloid fibrillation. Electrostatic forces from RR are critical for targeting proteins, whereas hydrophobic forces from BA play a crucial role in expediting amyloid aggregation by elevating the local concentration and reducing the solubility of precursor proteins. So far, most investigations have primarily focused on the inhibition and disaggregation of amyloid fibrils, while the study and development of modulators that accelerate amyloid formation remain largely limited. This work provides a novel model that rationalizes how an amphiphilic modulator promotes amyloid aggregation. Recent studies have suggested that oligomers that appear during amyloid formation may be more toxic than amyloid fibrils,^{75–78} providing an additional strategy for suppressing

the pathogenesis of aggregation-related diseases by boosting rapid amyloid formation without oligomerization. Indeed, star-shaped poly(2-hydroxyethyl acrylate) nanostructures were developed to ameliorate amylin-elicited toxicity in pancreatic beta cells by promoting the amyloid formation of amylin.⁷⁹ Accelerating amyloid formation using a conjugate may also be useful for producing aggregate-based functional materials and developing diagnostic methods against aggregation-induced protein misfolding diseases. Our electrostatic binding-hydrophobic condensation model will contribute to the rational design and development of effective molecules for modulating amyloid formation.

Author contributions

Yuxi Lin: conceptualization, methodology, investigation, formal analysis, visualization, validation, writing – original draft, and writing – review and editing. So Hyeon Park: resource, methodology, investigation, and validation. Eugene Bok: methodology, investigation, and visualization. Yunseok Heo: investigation, formal analysis, visualization, and validation. Seong-Bin Yang: investigation and validation. Yoon-Sun Yi: methodology and investigation. Jun-Hyuck Lee: methodology and investigation. Donghyun Seo: resource and methodology. Eunae Jo: investigation. Sungsu Lim: resource. Yun Kyung Kim: resource. József Kardos: resource. Kyoung-Seok Ryu: resource. Jaekwang Kim: resource and funding acquisition. Jooho Park: conceptualization, funding acquisition, project administration, and writing – review and editing. Young-Ho Lee: conceptualization, funding acquisition, project administration, supervision, writing – original draft, and writing – review and editing.

Conflicts of interest

There are no conflicts of interest to declare.

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