Supporting Information for:

Amelioration of aggregate cytotoxicity by catalytic conversion of protein oligomers into amyloid fibrils

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Supplementary Methods

Mutant construction, protein expression, purification and labeling

Construction of the single cysteine mutant Ure2-S53C was described previously[1]. N-terminal His_6-tagged Ure2-S53C was expressed in *E. coli* C41 (DE3) cells and purified by nickel-affinity chromatography as described previously[2, 3]. The purified Ure2-S53C was dialyzed into 50mM Tris-HCl (pH 8.4) buffer containing 200 mM NaCl and 500 μM TCEP at 4 ℃. The purity of Ure2-S53C was checked by SDS-PAGE, and the protein concentration was determined by the absorbance at 280 nm using a molar extinction coefficient of 48,220 M^{-1} cm^{-1} ref. 2.

Fluorescence labeling of Ure2-S53C by Alexa Fluor 488 (AF488) or Alexa Fluor 647 (AF647) (Invitrogen) was carried out as described previously[1]. The unreacted free dye was removed using a PD-10 desalting column (GE Healthcare). The dye-to-protein labeling ratio was determined by its absorbance spectrum and calculated to be 85-95%. The labeled protein was flash frozen and stored at −80 ℃. Protein was thawed and centrifuged at 18000×*g* for 30 min to remove any small aggregates immediately before use.

The human Tau gene was a gift from Prof. Jianzhi Wang (Huazhong University of Science and Technology, China). The fibril core segment of Tau including N244-F378[4] plus initial M243 was constructed into the pET-28a vector. The segment of Tau was produced and purified as previously described with some modifications[5]. The pET-28a plasmid carrying the target gene was transformed into BL21(DE3) competent *Escherichia coli* cells. The cells were cultured in 2YT medium at 37 ℃ to reach OD_{600}=0.7-0.8. To induce protein production, 0.4 mM isopropyl β-D-thiogalactoside (IPTG) was added into the culture medium and the cells were incubated for a further 4 h. Cells were then centrifuged for 45 min at 4,000×*g*. Pellets were resuspended in 20 mM PIPES buffer (pH 6.5) containing 500 mM NaCl, 1 mM EDTA and 50 mM 2-mercaptoethanol, boiled at 100 ℃ for 15 min, and sonicated 12 times for 5 s each time in an ultrasonic cell disruptor (Scientz-IIIE). The cell debris was removed by centrifugation at 15,000×*g* for 45 min and the supernatant including Tau protein was precipitated with ammonium sulfate (60%, m/V). After incubation for 1 h on ice, the sample was centrifuged at 13,000×*g* for 12 min. The precipitate was resuspended in distilled H_2O containing 2 mM dithiothreitol (DTT) and filtered with a 0.22 μm filter membrane (Pall) before loading onto a Mono S cation exchange column (Bio-Rad). Protein was eluted with 20 mM PIPES buffer (pH 6.8) containing 0.5 mM EDTA and 2 mM DTT using a linear NaCl gradient (0.05-1.00 M). The purified N244-F378 segment of Tau was dialyzed into 30 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl and 2 mM DTT at 4 ℃. The protein purity was checked by SDS-PAGE and the protein concentration was determined by BCA assay kit (Thermo).

The α-synuclein gene was a gift from Prof. Hongyu Hu (Institute of Biochemistry and Cell Biology, CAS) and was subcloned into the pET28a-His_6-SMT3 vector. The pET28a plasmid carrying α-synuclein gene was transformed to BL21(DE3) competent cells. The cells were cultured in 2YT medium containing 50 μg/mL kanamycin at 37 ℃ until the OD_{600} reached 0.6-0.8. The cells were then induced with 0.2 mM IPTG and grown at 16 ℃ for 16 h. The harvested cells were lysed in 50 mM Tris-HCl, 300 mM NaCl, pH 7.5 and the His_6-Smt3-α-synuclein fusion protein was first purified by Ni affinity column. After the Smt3 tag was cleaved by Ulp1 protease, the α-synuclein protein was purified by Ni column again. The flow through fraction was dialyzed against pure water and then lyophilized and stored at −20 ℃. The protein powder was solved in 50 mM Tris-HCl, 150 mM KCl, 5 mM $MgCl_2$, pH 7.5 buffer and centrifuged at 13,000×*g* before use. The protein concentration was determined from the absorbance at 280 nm using the molar extinction coefficient

5960 $M^{-1}cm^{-1}$ ref. 6.

ThT assay of the fibril formation of Tau and α-synuclein

The concentrations of Tau(N244-F378) and α-synuclein in the aggregation reaction were 10 μM, 200 μM and 2.5 μM respectively in the presence or absence of 40 μM LQVNIGNR peptide. For Tau(N244-F378) aggregation, 10 μg/ml heparin was also added. For α-synuclein aggregation, a polystyrene bead was added to each well. The reaction solution was supplemented with 5 μM ThT and was pipetted into a 96-well clear bottomed plate (Costar). The aggregation reaction was carried out in a Fluostar Omega plate reader (BMG Labtech) with orbital shaking at 200 rpm at 37 ℃. The ThT fluorescence was recorded every 10 min with excitation at 450 nm and emission at 485 nm. At least three replicates were performed to obtain the mean and standard deviation.

Peptide preparation

Lyophilized synthetic peptides (Table S1) with purity of 95% were purchased from Scilight Biotechnology (Beijing, China). Peptides were dissolved in 50 mM Tris (pH 8.4) buffer containing 200 mM NaCl and the concentrations were confirmed using the BCA assay kit (Pierce). Samples were centrifuged at 18000×*g* for 30 min to remove any aggregates immediately before use.

Microfluidic device fabrication

The single-channel microfluidic devices with width of 100 μm and height of 25 μm were fabricated by soft lithography into polydimethylsiloxane (PDMS; Dow Corning) using SU-8 photoresist on silicon masters as described previously[7, 8]. The PDMS channels were treated with plasma cleaner (Harrick) and bonded to coverslips to create sealed devices. Each device was checked on a microscope (Nikon Ti-U), and only those without dust or aberrations were used in the following single molecule experiments. A pipette tip containing the detection sample was inserted into the sample inlet, and the outlet was attached to a syringe connected to a syringe pump to pull the sample through the microfluidic channel[8].

Measurement of microfluidic flow rates using fluorescence correlation spectroscopy (FCS)

FCS experiments were carried out using a home-built confocal microscope similar to the one described previously[9]. An inverted fluorescence microscope (Ti-E, Nikon) was equipped with a 100× objective (N.A. = 1.4, Nikon). The 488 nm laser (Coherent) was directed to the back port of the microscope for excitation with a power of 50 μW. The fluorescence emission was collected via the same objective, with a ET525/50 filter (Chroma) and a 50 μm pinhole, and split into two channels with a 50/50 splitter (Chroma) before being focused onto two avalanche photodiode detectors (SPCM-AQRH-14, Excelitas). The fluorescence of the labeled sample that flowed across the focal volume was simultaneously collected using two avalanche photodiode detectors and recorded using a two-channel FCS card (Flex02-01D, correlator.com) and the auto-correlation curves were obtained.

Alexa Fluor 488 (AF488) dye diluted to nanomolar concentration was pumped through the microfluidic channel at different speeds (0 μL/h, 10 μL/h, 20 μL/h, 50 μL/h, 100 μL/h and 200 μL/h) (Fig. S5). The auto-correlation curves obtained using the FCS card were fitted to the equation below, which contains flow, diffusion and triplet processes[10],

$$G(\tau) = \frac{1}{N} \cdot \left(1 + \frac{\tau}{\tau_{diff}}\right)^{-1} \cdot \exp\left(-\left(\frac{\tau}{\tau_{flow}}\right)^2 \cdot \left(1 + \frac{\tau}{\tau_{diff}}\right)^{-1}\right) \cdot \left(1 - K + K \cdot \exp\left(-\frac{\tau}{\tau_T}\right)\right) \qquad \text{Eq. S1}$$

where τ_{diff} is the characteristic diffusion time, τ_{flow} is the characteristic flow time and τ_T is the triplet lifetime. K represents the relative proportion of the triplet process. The fitting results under different flow rates are shown in Table S3. The value τ_{flow} represents the average residence time that a fluorescent molecule remains in the focal volume under fast flow conditions, so it is an important reference for selection of the bin time for smFRET experiments. At the 200 μL/h flow rate which was used in the smFRET study of oligomerization, the average τ_{flow} value obtained from the fitting was 27 μs. Therefore we chose a bin time slightly higher than this value (50 μs) for single molecule events.

Transmission electron microscopy (TEM)

A 5 μL volume of fibril or peptide sample was loaded onto carbon-coated copper grids for 1 min and stained with 1% uranyl acetate. A Phillips Tecnai20 electron microscope at 120 kV was used to observe the morphology of the Ure2 fibrils and assembly of peptides (LQVNIGNR, MQVNIGNR and RQVNIGNR).

Dynamic light scattering (DLS)

Dynamic light scattering (DLS) experiments were performed on a Wyatt NanoStar (271-DPN) with a wavelength of 660 nm. A 10 μL volume of freshly prepared sample solution (after centrifugation at 18000×*g*) was added into a high-quality quartz glass cuvette for DLS measurement at 25 ℃. Data were acquired and analysed using the software DYNAMIC 7.1.8.

Cell culture and MTT assay

Dulbecco's modified Eagle's medium (DMEM), streptomycin, penicillin, fetal bovine serum (FBS), 0.25% trypsin-EDTA and PBS were purchased from GIBCO. Human SH-SY5Y cells were cultured in DMEM, supplemented with 10% FBS, 100 ug/mL streptomycin and 100 U/mL penicillin. The cells were incubated at 37 ℃ in a humidified environment containing 5% CO_2.

To determine effects on cell viability, cell number was quantified using a standard colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay similar to that described before[11]. Cells were transferred to a 96-well plate (4 × 103 cells/well) and allowed to attach overnight. The Ure2 samples were incubated in a BMG plate reader at 37 ℃ with shaking at 200 rpm. The native Ure2, fibrillar Ure2 and the Ure2 sample taken at the late lag phase were each diluted into culture medium to reach 1 μM final concentration before being used in the assays. Cells were treated with the above Ure2 samples in culture medium for 24 h. Next, fresh culture medium was added with 10 μL of MTT (5 mg/ml stock in PBS) per well and the cells were further incubated for 4 h at 37 °C. Finally, the culture medium was discarded, and 100 μL of DMSO was added per well to dissolve the purple formazan crystals present. Absorbance was measured using a microplate reader (Molecular Devices) at 590 nm. Each assay was performed in triplicate.

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Table S1 Peptides designed according to the sequence of Ure2 fibril core.

Peptide	Residue number
QVSNL	8-12
SNALR	13-17
RQVNI	17-21
LQVNIGNR (“LQ”)	17-24 (R17 to L17)
MQVNIGNR (“MQ”)	17-24 (R17 to M17)
RQVNIGNR (“RQ”)	17-24
QVSNLSNAL (“QV”)	8-16
NLSNALQVN (“NL”)	11-20

Table S2. Fitting results of ThT curves of Ure2 fibril formation in the presence of different concentrations of peptide LQVNIGNR (LQ).

Ure2 dimer : LQ	$(k_+k_n) \times 10^{-12}$ ($M^{-1}s^{-2}$)	$(k_+k_-) \times 10^{-9}$ ($M^{-1}s^{-1}$)
1:0	3.8 ±0.3	8.5 ±0.4
1:6	16.4 ±1.4	8.5 ±0.4
1:8	36.7 ±2.7	8.5 ±0.4
1:10	55.9 ±2.0	8.5 ±0.4
1:12	101.4 ±4.4	8.5 ±0.4
1:16	387 ±16	8.5 ±0.4

Table S3. Fitting results of the combined smFRET/ThT results of Ure2 oligomer/fibril formation in the presence or absence of the LQVNIGNR peptide (LQ).

Rate constant	k_+ ($\mu M^{-1}\ h^{-1}$)	k_{oligo} ($\times 10^{-4}\ \mu M^{-1}\ h^{-1}$)	$k_c \times 10^{-3}$ (h^{-1})	k_d (h^{-1})	k_- ($\times 10^{-3}\ h^{-1}$)
Ure2-S53C	50	7.5 ±1.4	4.2 ±1.0	1.0 ±0.2	1.8 ±0.3
Ure2-S53C + LQ	50	7.5 ±1.4	47.2 ±8.1	1.0 ±0.2	1.8 ±0.3

Table S4. Fitting results of the FCS curves measured at different flow rates as shown in Fig. S1

Laser Intensity (μW)	Pull rate (μL/h)	τ_{diff} (s)	τ_{flow} (s)
50	10	1.88×10^{-4}	9.75×10^{-4}
50	20	1.88×10^{-4}	1.92×10^{-4}
50	50	1.88×10^{-4}	8.73×10^{-5}
50	100	1.88×10^{-4}	4.88×10^{-5}
50	200	1.88×10^{-4}	2.70×10^{-5}

Figure S1

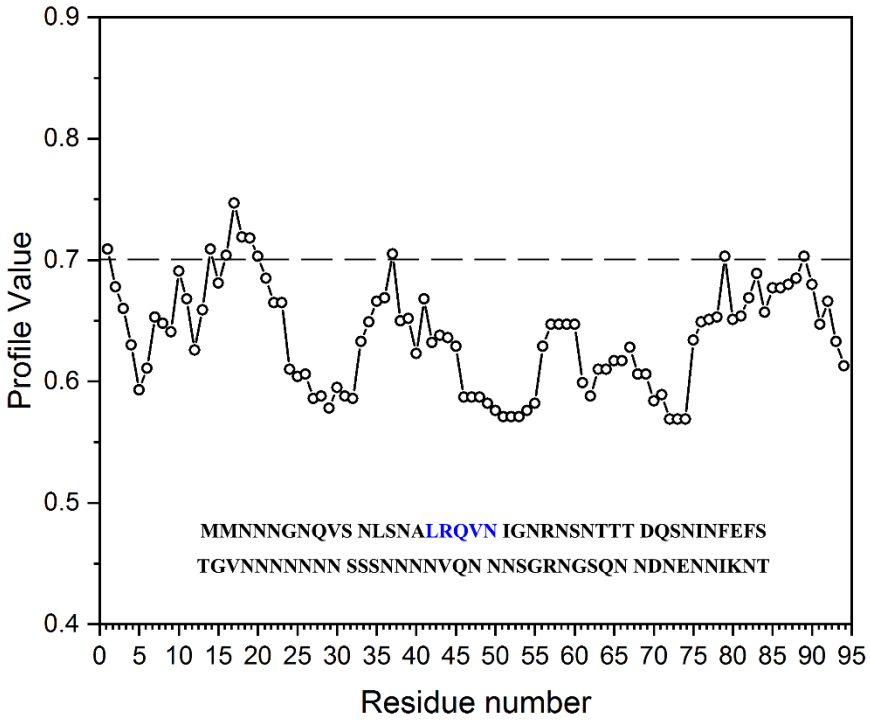

Fig. S1 The predicted amyloidogenic propensity of the N-terminal prion domain of Ure2. The amyloidogenicity of Ure2 residues 1-94 was calculated using the FoldAmyloid algorithm[12] using the online server (http://antares.protres.ru/fold-amyloid/), and the results indicate that residues 16-20 (LRQVN) has the highest aggregation propensity and is above the amyloidogenic threshold (dashed line).

Figure S2

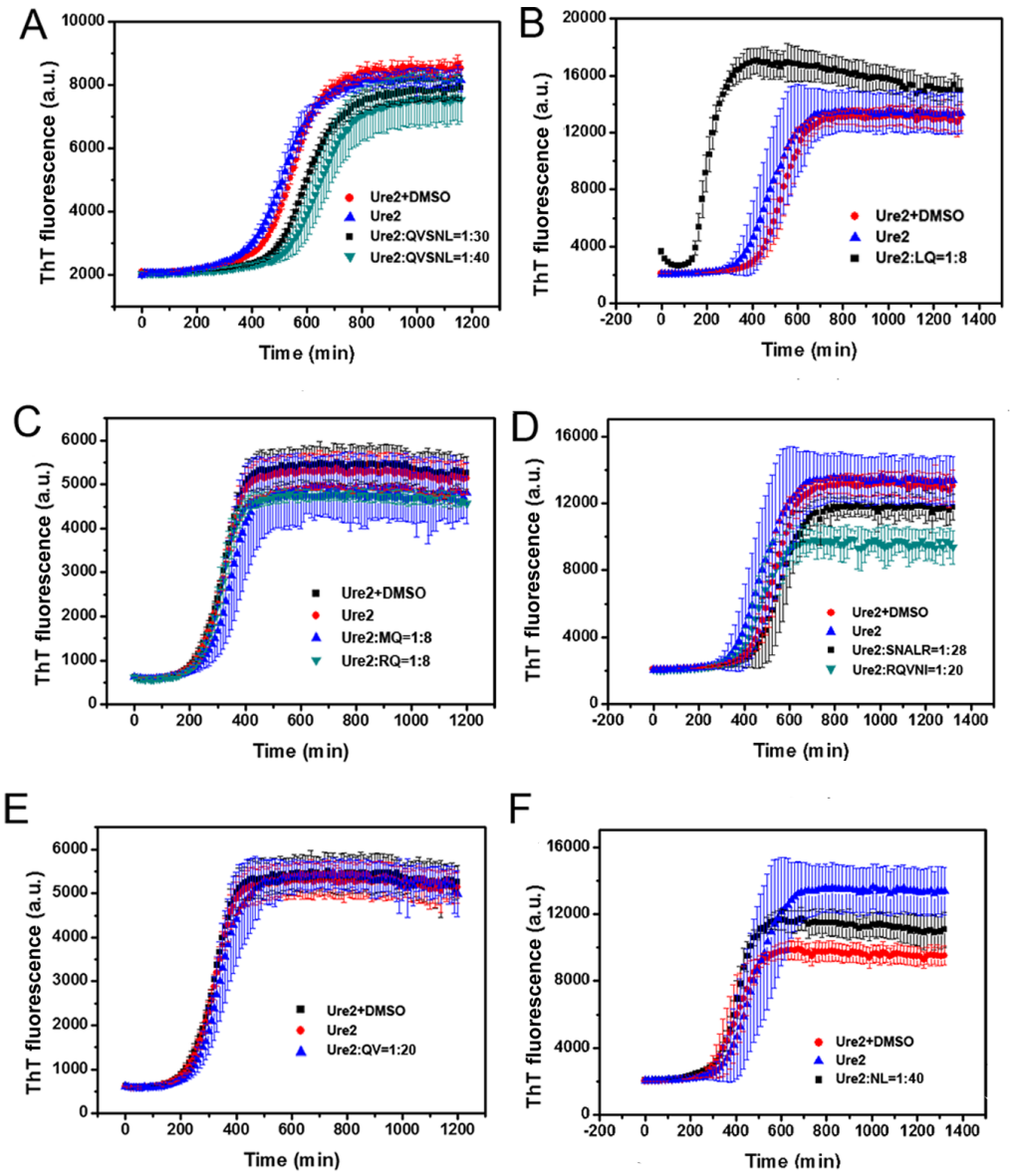

Fig. S2 ThT assay of Ure2 in the presence of different peptides. (A) QVSNL, (B) LQVNIGNR ("LQ"), (C) MQVNIGNR ("MQ") and RQVNIGNR ("RQ"), (D) SNALR and RQVNI, (E) QVSNLSNAL ("QV") and (F) NLSNALQVN ("NL"). The concentration of Ure2 was 5 μM and the concentration ratios of each peptide to Ure2 are indicated in the figure. The ThT curve of Ure2 with addition of DMSO instead of peptide is shown as a control to exclude a solvent effect. The data shown are the average of three replicates and the error bars represent the SD.

Figure S3

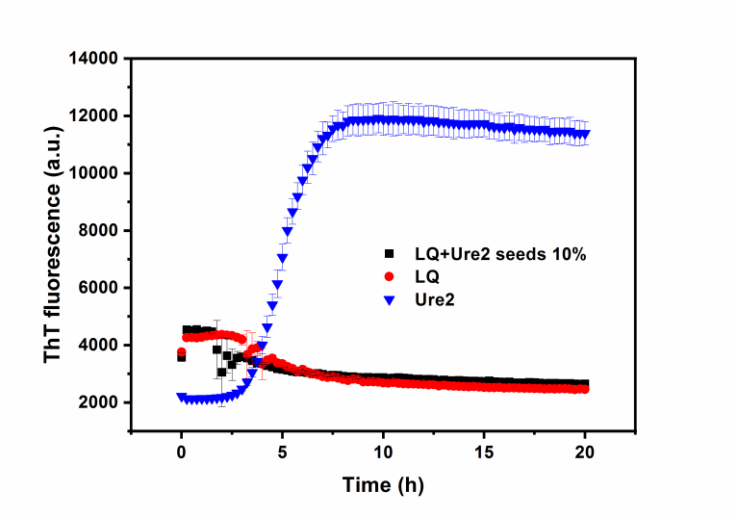

Fig. S3 Monitoring fibril formation of LQ by ThT fluorescence. ThT curves of 5 μM Ure2 protein (blue), 40 μM LQVNIGNR peptide alone (red) and in the presence of 5% Ure2 seeds (black). The data shown are the average of three replicates and the error bars represent the SD. Comparison with the turbidity measurements shown in Fig. 3B indicates that LQ aggregation is not readily detected by ThT fluorescence.

Figure S4

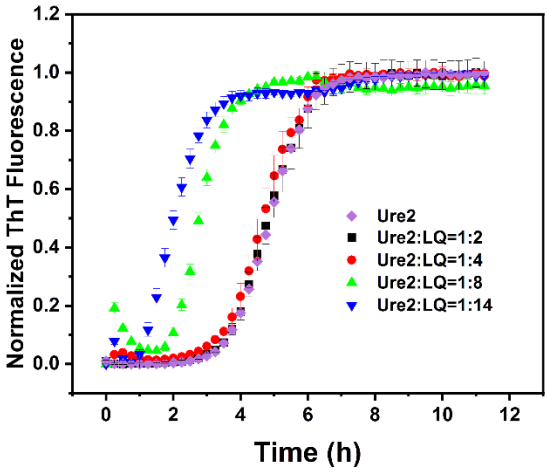

Fig. S4 ThT assay of Ure2 in the presence of LQVNIGNR peptide with concentrations lower and higher than the critical concentration. The concentration of Ure2 is 5 μM and the concentration ratios of LQVNIGNR peptide ("LQ") to Ure2 are 1:2 (10 μM), 1:4 (20 μM), 1:8 (40 μM) and 1:14 (70 μM) as indicated in the figure. The data shown are the average of three replicates and the error bars represent the SD.

Figure S5

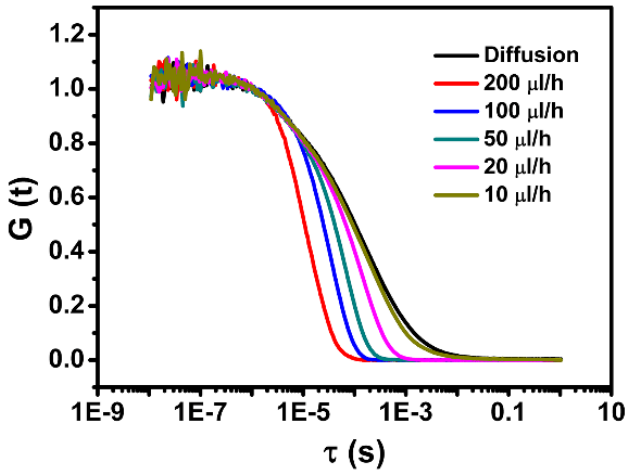

Fig. S5 FCS measurements of the characteristic time of Alexa Fluor 488 in the focus volume under fast-flow conditions using microfluidics. The curves were fitted to Eq. S1 and the fitting results are shown in Table S4. Fitting of the auto-correlation curve of a static AF488 sample (0 µl/h) provides the diffusion time (τ_{diff}), which is fixed in the fitting of data under fast-flow conditions.