Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

Accelerating Protein Aggregation and Amyloid Fibrillation for Rapid Inhibitor Screening

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Experimental Procedures

Chemicals

The chemical reagents used in the experiments, including ammonium acetate, boric acid, sodium borate, Thioflavin T (ThT) fluorescent dye, etc., were all purchased from Aladdin (Shanghai, China). Myoglobin (1 μg/mL) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA), the β-amyloid 1-40 and 1-42 peptides derived from rats were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), and α-synuclein was obtained from rPeptide (Bogart, Georgia, USA). These proteins and peptides were used without further purification when using standard samples. Purified water was obtained from Watsons and used as the solvent for sample preparation. A 40 μM Thioflavin T (ThT) aqueous solution was prepared for specific binding to misfolded sites of amyloid-like proteins, followed by laser confocal imaging. Myoglobin was dissolved in a 50 mM ammonium borate aqueous solution, and the solution's pH was adjusted using ammonia and confirmed with pH test strips. Hemin, tramiprosate, epigallocatechin gallate (EGCG), quercetin, furosemide, and 3-ethoxysalicylaldehyde were all purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and used without further purification.

Microdroplet generation module

The microdroplet generation module employed a nano-electrospray ionization source (nanoESI). For each spray, a sample volume of 6 μL was used, and the applied voltage ranged from 3 kV to 4 kV, depending on the specific conditions. The platinum wires used had a purity of 99.99%, and an outer diameter of 0.5 mm. The capillary glass tubes used were borosilicate glass capillaries obtained from Sutter Instrument (USA) with an outer diameter of 1500 μm and an inner diameter of 860 μm. They were pulled using a Sutter Instrument P-1000 model puller, resulting in a final nano-electrospray capillary tip diameter of approximately 5 μm. Before each analysis, the capillary glass tubes were sonicated in a 50/50 (v/v) methanol-water solution for 30 minutes to remove impurities from both the inside and outside of the capillaries.

ThT specific dye and fluorescence imaging

Thioflavin T (ThT) is a specific dye used for detecting amyloid-like protein fibers. Studies have shown that the excitation spectrum of ThT shifts when it binds to β-sheets. Specifically, the excitation wavelength of the dye is 350 nm and the emission wavelength is 438 nm when it is not bound to β-sheets. However, when it binds to β-sheets in amyloid-like fibers, the excitation wavelength shifts to 450 nm, and the emission wavelength shifts to 482 nm. Research has demonstrated that ThT binding does not interfere with the continued assembly of peptides into amyloid-like fibers. Here, after the microdroplet reaction, microdroplets were stained with ThT fluorescence, incubated for 1 hour, and observed under a laser confocal microscope (FV3000, Olympus Corporation, Japan) with excitation at 445 nm. Clear filamentous fluorescence signals were observed in the vicinity of 482 nm.

Circular dichroism

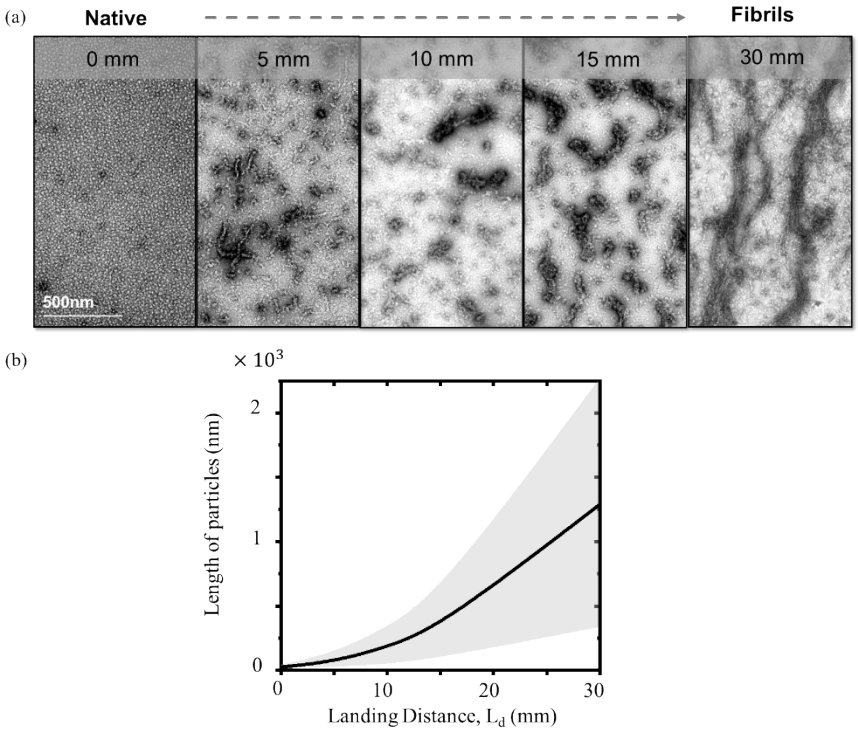
The circular dichroism (CD) analysis in microdroplet mode was performed using a Chirascan Plus circular dichroism spectrometer (Applied Photophysics Ltd., UK) with a wavelength range of 192-240 nm and ellipticity readings recorded every 1 nm. Data collection was conducted using a photomultiplier tube detector. In the microdroplet collection target plate, a 35 mm cell culture dish was placed, and 50 μL of buffer solution was pipetted into the center of the dish. High voltage was applied at the nano-electrospray end to sustain spraying for 10 minutes. The collected ~40 μL solution was transferred to a 1 mm quartz cuvette for subsequent circular dichroism spectral analysis. DichroWeb software was employed for further analysis of the circular dichroism spectral data, utilizing the ContinnLL analysis algorithm with reference set 4.

Transmission electron microscope and data analysis

The TEM grids with 200 mesh used for transmission electron microscopy (TEM) were purchased from Zhongjingke (Beijing Zhongjingkeyi Technology Co., Ltd.). The specific experimental procedure is as follows: before the experiment, a hydrophilic treatment was performed by placing the grids in a glow discharge instrument and subjecting them to low-pressure oxygen plasma treatment. Negative staining was carried out using standard procedures, and the TEM grids with deposited microdroplets were stained with uranyl acetate (2% w/w). Uranyl acetate (Sigma-Aldrich, St. Louis, MO) is a compound with mild radioactivity and toxicity, and it should be handled under safety regulations and stored in a light-protected manner. TEM images were obtained on an FEI Tecnai Spirit 120 kV transmission electron microscope (FEI, Hillsboro, OR, USA). The images were analyzed using ImageJ software to measure the length based on the characteristics of the fibers. To ensure objectivity, the reported values for each condition represent the average length of several hundred particles in the field of view, indicated as the mean ± standard deviation, with each condition repeated 3 times.

Mass spectrometry analysis

The mass spectrometry analysis was conducted as follows: The nano-electrospray ionization source (nanoESI) was mounted on an xyz three-dimensional translation stage, located in front of a cyclic IMS Q-TOF mass spectrometer (Waters Corporation, Wilmslow, UK), with an adjustable distance to the mass spectrometer inlet. The nanoESI source used was a commercially available source compatible with the instrument. To achieve high-performance analysis of protein oligomers, the instrument was configured within the following ranges and tested in Table S3.



Supplementary figures

Fig S1. (a) TEM images of the fibrillation process under different landing distance, Ld. The scale bar is 500 nm. (b) Length of the fibril as a function of the Landing Distance L_d (mm) in microdroplets. The error bars represent one standard deviation (SD) with 4 replicates.

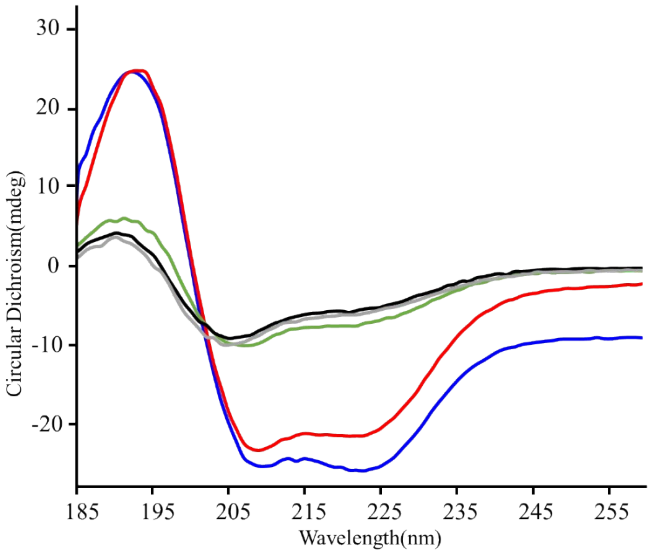


Fig S2. Circular dichroism of the control group: globular myoglobin in 50 mM sodium borate, pH 9.0 at 65℃ for different heating time: 0 h (blue), 0.5 h (red), 10 h (green), 12 h (black), 48 h(grey).

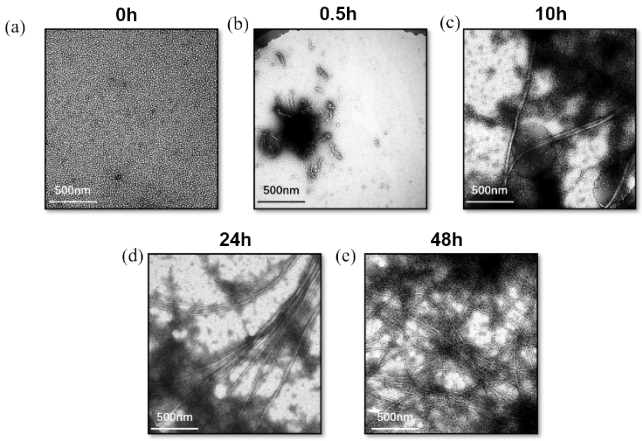


Fig S3. TEM images of the control group: globular myoglobin in 50 mM sodium borate, pH 9.0 at 65℃ for different heating time,**(a)-(e)** is 0 h, 0.5 h, 10 h, 24 h and 48 h, respectively.

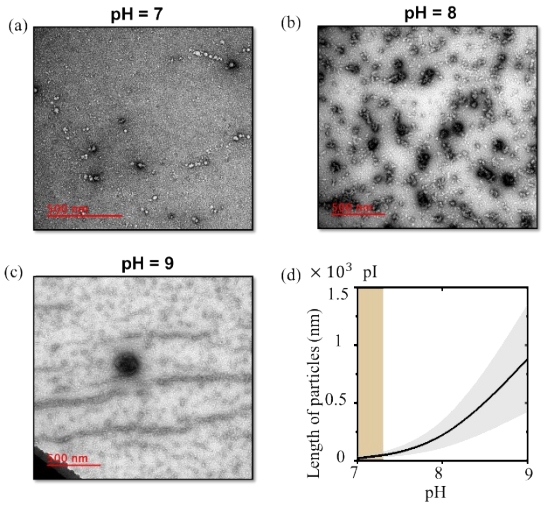


Fig S4. Characterization of pH value effects on the aggregation of myoglobin in microdroplets by TEM. Under the solution 50 mM NH_4AC, **(a)** pH=7, **(b)** pH=8, **(c)** pH=9. Under the solution 50mM NH_4AC, pH=9, there shows evident aggregation and fibers in the microdroplets. **(d)** Synthesis rate in microdroplet: pH-course change in the length of protein particles. The gray area represents the sample standard deviation (SD).

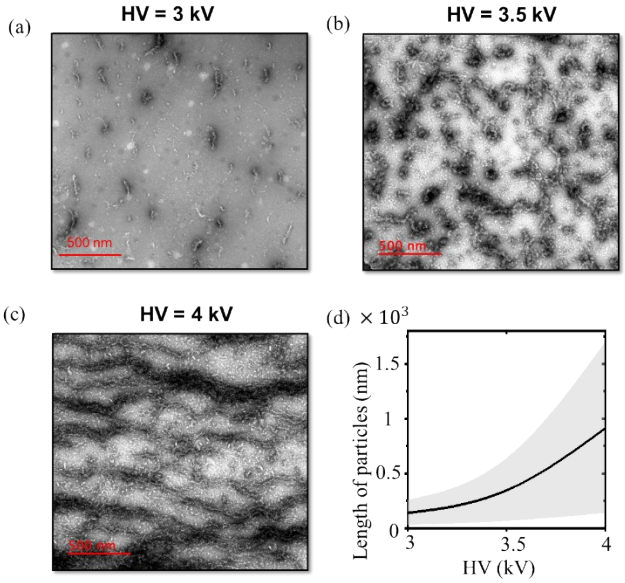


Fig S5. Characterization of ionization high voltage (HV) effects on the aggregation of myoglobin in microdroplets by TEM. Under the solution 50 mM NH_4AC, pH=9, **(a)** HV=3 kV, **(b)** HV=3.5 kV, **(c)** HV=4 kV. **(d)** Synthesis rate in microdroplet: HV-course change in the length of protein particles. The gray area represents the sample standard deviation (SD).

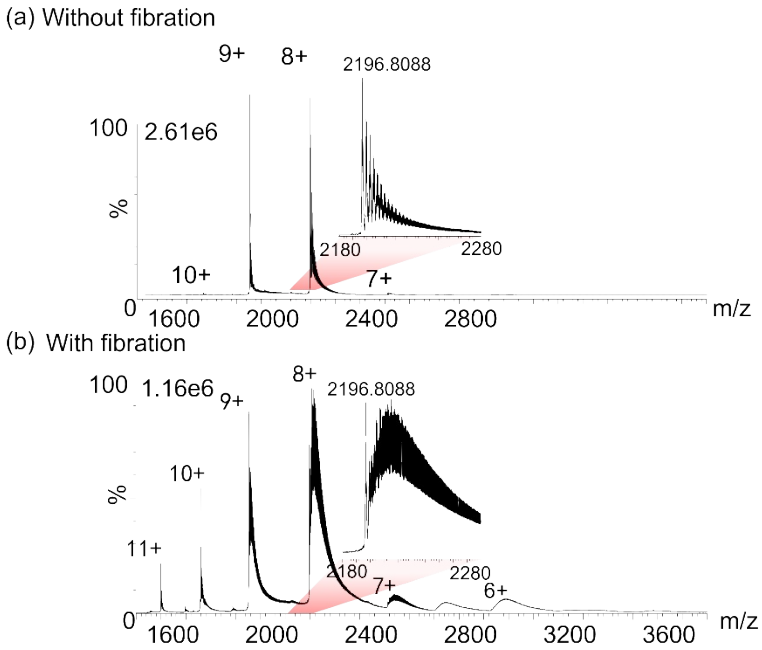


Fig S6. Myoglobin mass spectra of the control group (a) under the solution 50 mM NH4AC, pH=7, and the landed microdroplets state (b).

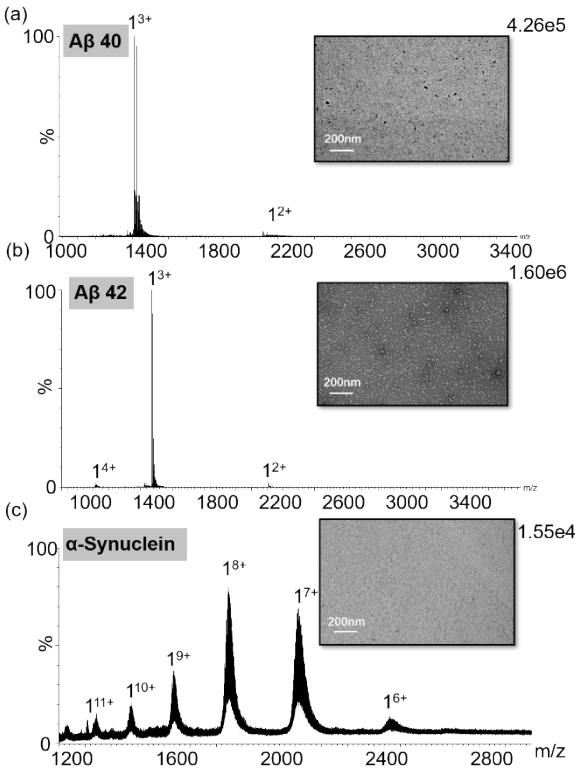


Fig S7. Negative control groups of peptides associated with clinical amyloidoses without fibration. Under the solution 50 mM NH_4AC, pH=7, there is no obvious polymers or fibers in the microdroplets. Here shows the evidence including the mass spectrometry, and inset TEM images of microdroplets of **(a)** Aβ40 **(b)** Aβ42 and **(c)** α-Synuclein.

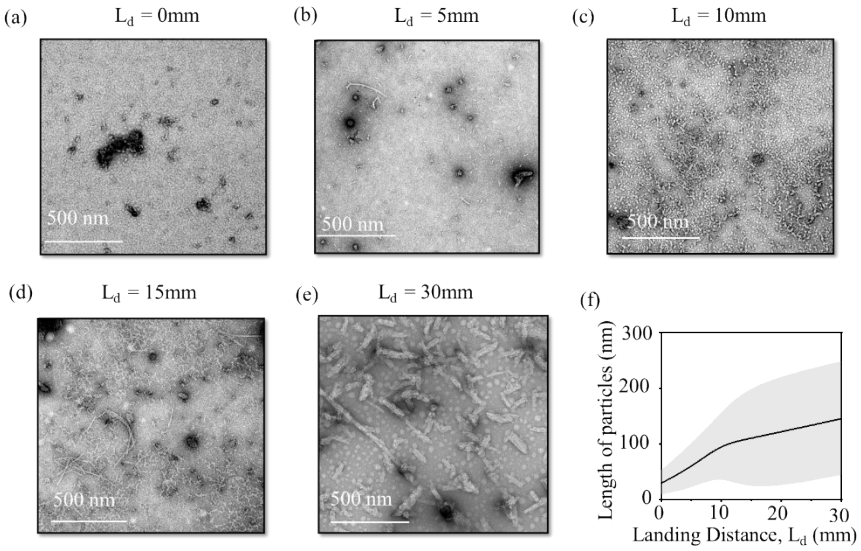


Fig S8. Synthesis of Aβ40 peptide in microdroplet. **(a-e)** TEM images of fibrillation at different L_d. Scale bar represents 500 nm. **(f)** Synthesis rate of Aβ40 peptide in microdroplet: L_d-course change in the length of protein particles, the gray area represents the sample standard deviation (SD).

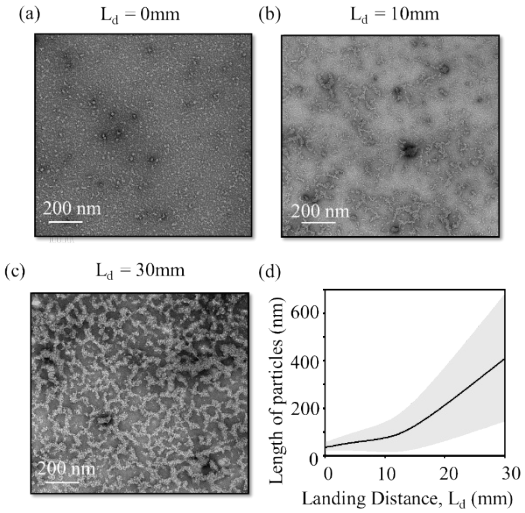


Fig S9. Synthesis of Aβ42 peptide in microdroplet. **(a-c)** TEM images of fibrillation at different L_d. Scale bar represents 200 nm. **(d)** Synthesis rate of Aβ42 peptide in microdroplet: L_d-course change in the length of protein particles, the gray area represents the sample standard deviation (SD).

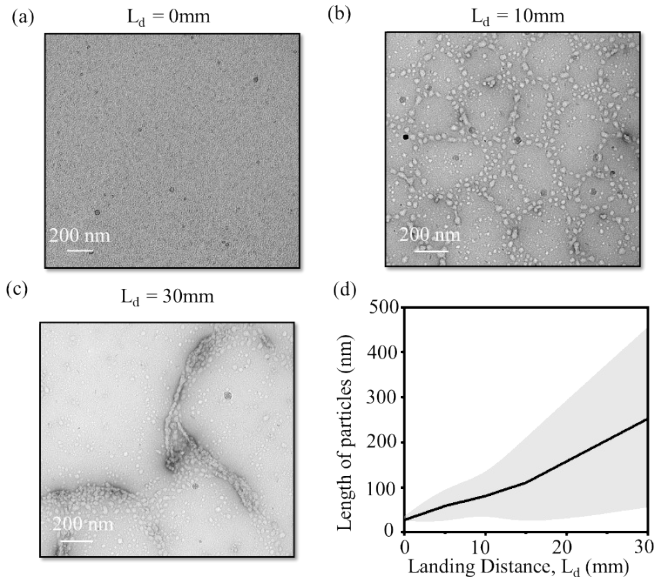


Fig S10. Synthesis of α-Synuclein in microdroplet. **(a-c)** TEM images of fibrillation at different L_d. Scale bar represents 200 nm. **(d)** Synthesis rate of α-Synuclein in microdroplet: L_d-course change in the length of protein particles, the gray area represents the sample standard deviation (SD).

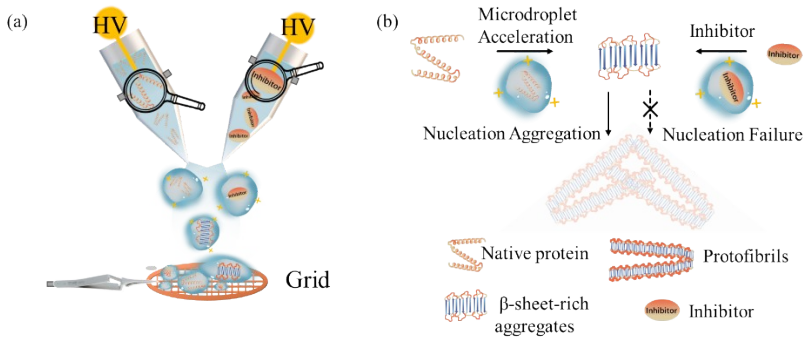


Fig S11. Schematic diagrams of (a) the microdroplet reaction system and (b) the mechanism of small molecule inhibitors acting on fibrotic proteins.

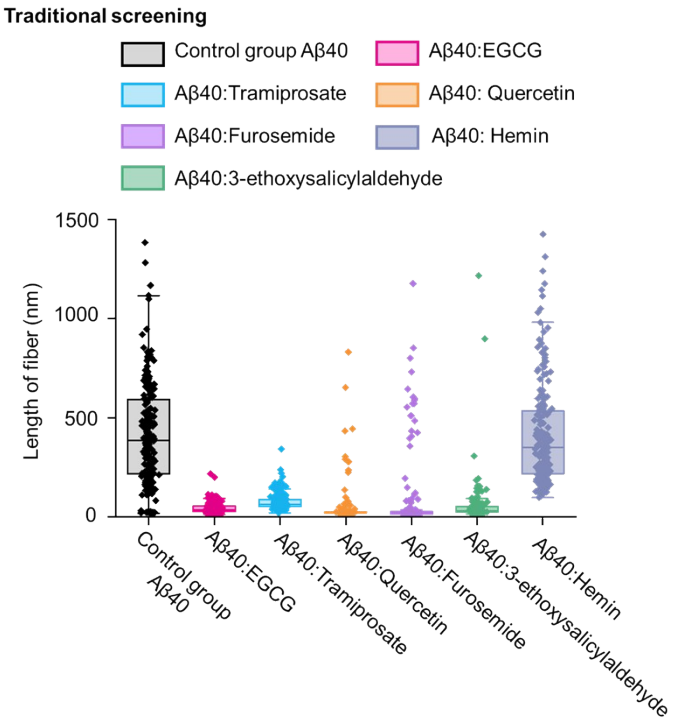


Fig S12. Statistical representation of fibrillation lengths in TEM images for inhibitors screening in traditional bulk phase. The central line in the box plot represents the median of the samples.

Supplementary tables

Table S1. Circular dichroism results for the percentages of different types of myoglobin secondary structures under different heating time via DICHROWEB[1]

Heating time	α-helix	β-sheet	Turns	Unordered
0 h	0.804	0	0.052	0.144
0.5 h	0.722	0	0.098	0.18
10 h	0.514	0.197	0.082	0.207
24 h	0.464	0.005	0.076	0.455
48 h	0.207	0.241	0.069	0.483

Table S2. Secondary structure analysis of myoglobin in different states using CD spectroscopy, including bulk phase, solution phase adding high voltage and landed microdroplets.

	α-Helix	β-Sheet	Turns	Other
Bulk phase without adding high voltage	0.917	0.023	0.052	0.008
Bulk phase adding high voltage	0.809	0.010	0.062	0.117
Landed microdroplets	0.153	0.251	0.154	0.443

Table S3. Mass spectrometer parameters of cyclic IMS-Q-TOF

List of parameters (unit)	value
NanoLockSpray Capillary（kV）	3
Cone（V）	80
Source Offset（V）	30
Source Temperature（℃）	100
Cone Gas（L/hour）	100
Purge Gas（L/hour）	200
Trap CE（V）	10
Transfer CE（V）	8
StepWave RF（V）	200
Ion Guide RF（V）	600
Trap RF（V）	500
Driftcell RF（V）	400
Pre/Post Array RF（V）	400
Cyclic RF（V）	250
Transfer RF （V）	200
Transfer RF Gain	5

References

[1] aL. Whitmore, B. Wallace, *Nucleic Acids Res* **2004**, *32*, W668-W673; bL. Whitmore, B. A. Wallace, *Biopolymers* **2008**, *89*, 392-400; cA. J. Miles, S. G. Ramalli, B. Wallace, *Protein Sci* **2022**, *31*, 37-46; dS. W. Provencher, J. Gloeckner, *Estimation of globular protein secondary structure from circular dichroism, Vol. 20*, ACS Publications, **1981**.