Electronic Supplementary Material (ESI) for Chemical Communications

Covalently reactive group-modified peptide that specifically reacts with lysine16 in amyloid β

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Supporting Information

Contents:

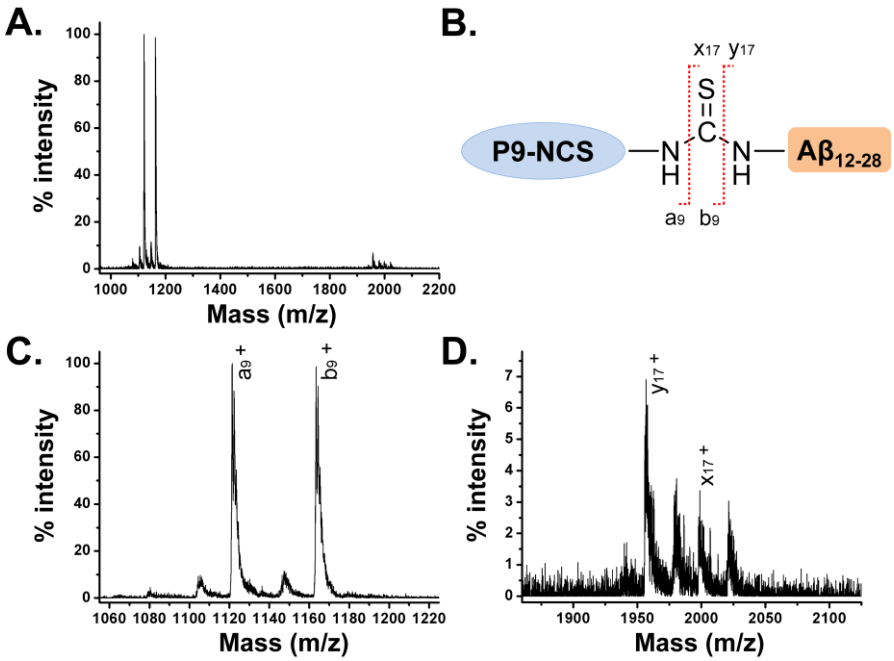

Fig. S1 P9-NCS can covalently react with $A\beta_{12\text{-}28}$ forming thiourea bond. 20 μM of $A\beta_{12\text{-}28}$ was incubated with 60 μM of P9-NCS at 37℃ in PBS buffer (pH 7.4) for 12 hrs. The sample was desalted by Zip Tip-C18 column prior to analysis. (A) MS/MS spectrum of covalent product of P9-NCS and $A\beta_{12\text{-}28}$ was examined by MALDI-TOF MS/MS analysis. MALDI-TOF mass spectrum of the covalent product has been shown in Figure 1D. (C) and (D) were zoom scan MS of (A). Schematic MS/MS fragmentation profile of the covalent product was shown in (B).

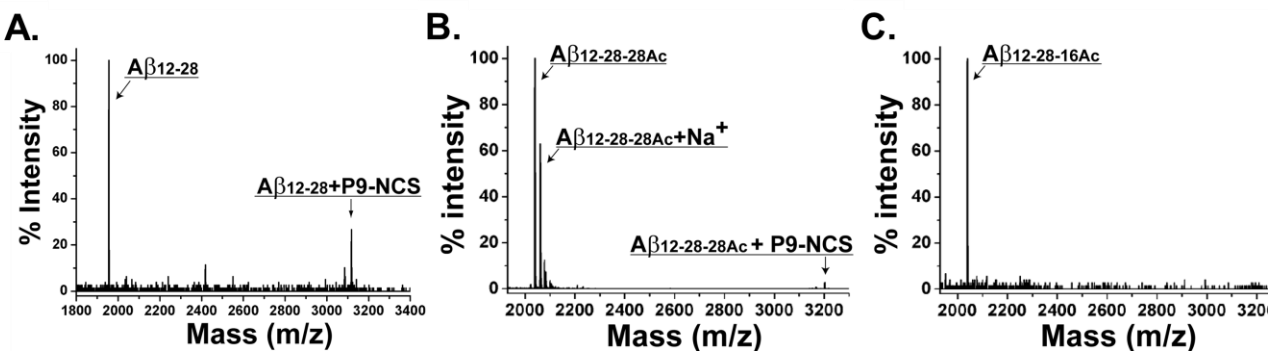

Fig. S2 P9-NCS specifically reacts with Lys16 of Aβ in reflect mode MALDI-TOF analysis for more accurate molecular mass data. (A) $A\beta_{12\text{-}28}$ (20 μM), (B) $A\beta_{12\text{-}28\text{-}28Ac}$ (20 μM) or (C) $A\beta_{12\text{-}28\text{-}16Ac}$ (20 μM) was incubated with P9-NCS (60 μM) at 37℃ in PBS buffer (pH 7.4) for 12 hrs. Samples were desalted by Zip Tip-C18 column prior to analysis. The covalent product of P9-NCS and $A\beta_{12\text{-}28}$ at the peak of 3116.39 (m/z), the calculated result is 3116.63 (m/z). We found the covalent product of P9-NCS and $A\beta_{12\text{-}28\text{-}28Ac}$ at the peak of 3200.52 (m/z), the calculated result is 3200.65 (m/z).

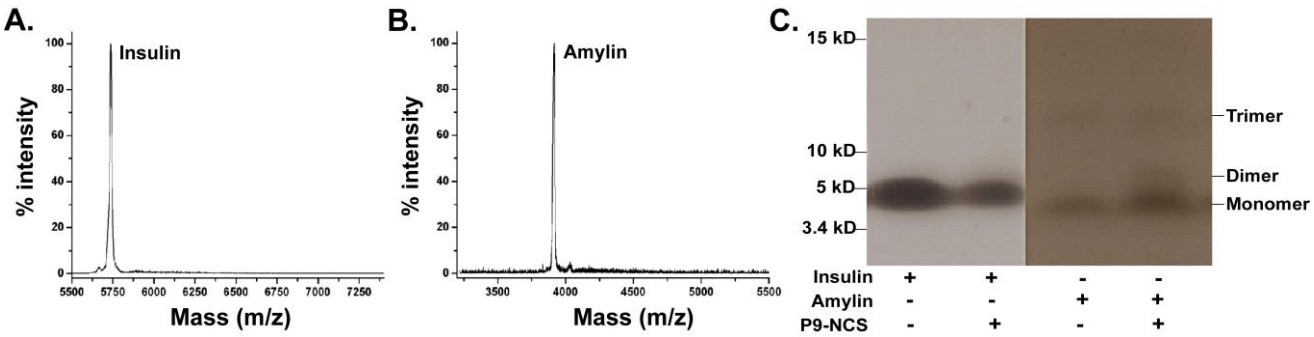

Fig. S3 P9-NCS cannot covalently react with Insulin and amylin. With MALDI-TOF mass analysis, 20 μM of insulin (A) and 20 μM of amylin (B) was separately incubated with 60 μM of P9-NCS at 37℃ in PBS buffer (pH 7.4) for 24 h. In the mass spectra, covalent product cannot be found. (C) After culturing at 37℃ in PBS buffer (pH 7.4) for 24 hrs, the mixture of insulin (10 μM) and P9-NCS (30 μM) did not appear covalent adduct in SDS-PAGE gel except insulin monomer. In the mixture of amylin (10 μM) and P9-NCS (30 μM), we could find the monomer, dimer, trimmer of amylin, but no covalent product was discovered.

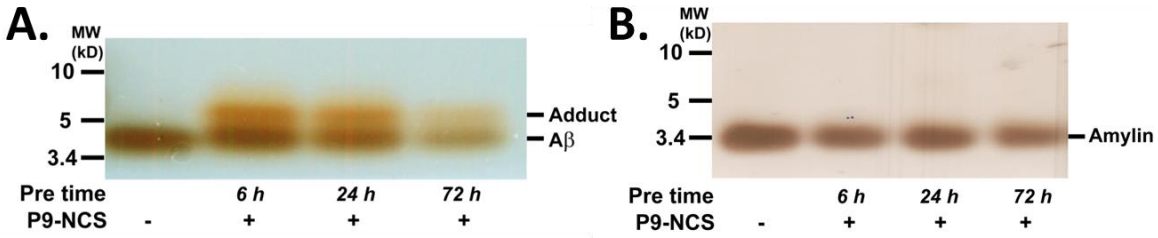

Fig. S4 P9-NCS can covalently react with aggregated Aβ but not aggregated amylin. Firstly, 10 μM of Aβ (A) or amylin (B) was incubated at 37℃ in PBS buffer (pH 7.4) for 6 hrs, 24hrs, 72hrs respectively. Then 20 μM of P9-NCS was added to each sample for another 36 hrs incubation. In SDS-PAGE gels, (A) covalent product can be found in the mixtures of aggregated Aβ and P9-NCS. (B) However, P9-NCS cannot react with aggregated amylin peptide.

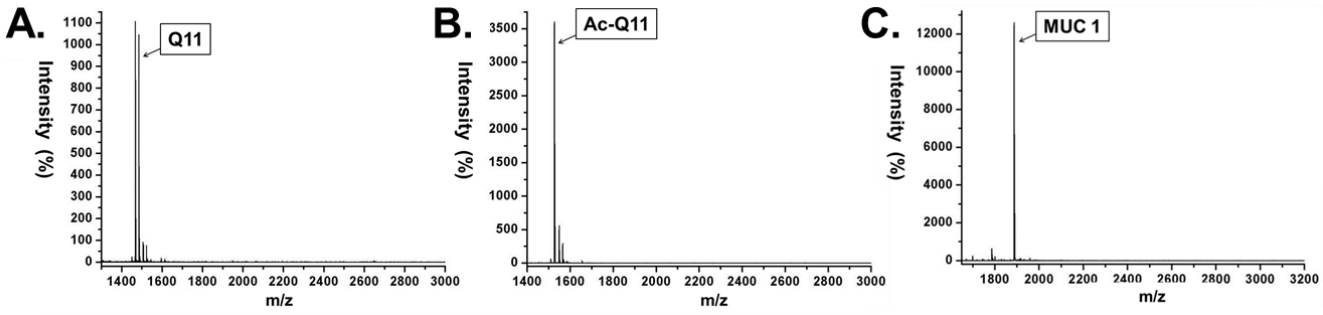

Fig. S5 P9-NCS cannot covalently react with simple peptides. 10 μM of (A) Q11, (B) Ac-Q11 or (C) MUC1 peptide was incubated with P9-NCS (30 μM) for 24 hrs in PBS buffer (pH=7.4) at 37℃, and then samples were analyzed by MALDI-TOF MS. No covalent product was found except original peptides themselves. Q11 peptide was detected at m/z 1484.66, which was calculated as m/z 1484.73. Ac-Q11 was found at m/z 1526.68, which was calculated as m/z 1526.74. MUC 1 was found at m/z 1886.90, which was calculated as m/z 1886.94. The amino acid sequences of these three simple peptides were shown as below. Q11：H- QQKFQFQFEQQ -NH_2. Ac-Q11：Ac- QQKFQFQFEQQ -NH_2. MUC1：H- HGVTSAPDTRPAPGSTAPPA –OH.

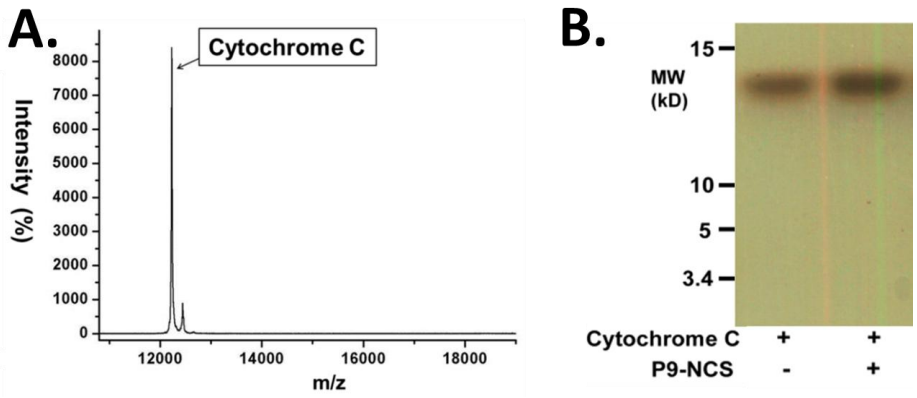

Fig. S6 P9-NCS cannot covalently react with Cytochrome C. Cytochrome C (10 μM) was incubated with P9-NCS (30 μM) at 37℃ in PBS buffer (pH=7.4) for 24 hrs. (A) With MALDI-TOF analysis, Cytochrome C can be found at m/z 12228, which molecular weight should be around 12.2 kD labeled on the commercial package. But no covalent product was found in the mass spectrum. (B) In SDS-PAGE gel, no new product was found except Cytochrome C itself. Therefore P9-NCS cannot covalently react with Cytochrome C although Cytochrome C contains many reactive lysine residues.

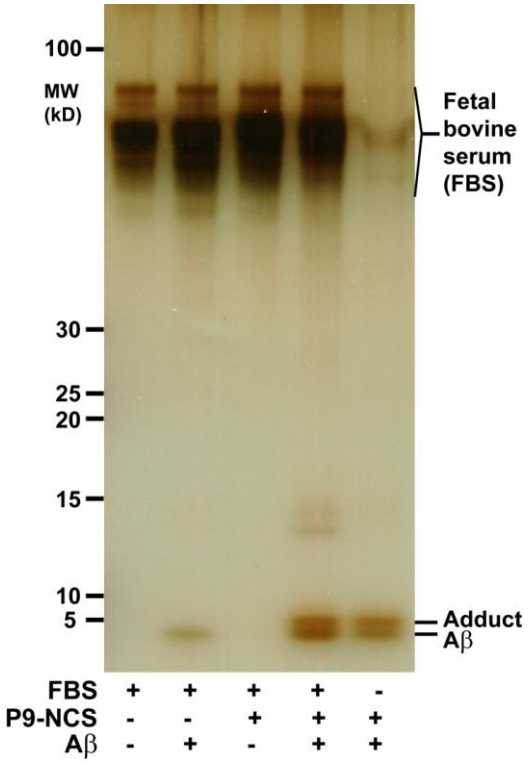

Fig. S7 P9-NCS scarcely react with fetal bovine serum. Firstly, 30 μM of P9-NCS was incubated in cell culture medium with 1% fetal bovine serum (FBS) at 37℃ for 24 hrs. Then 10 μM of Aβ peptide was added into the sample and incubated for another 24 hrs. After analyzing by SDS-PAGE, comparing with the sample incubated in PBS solution without FBS, the yield of adduct in cell culture medium with FBS is similar with the adduct produced in PBS solution. Therefore this result indicated that P9-NCS can specifically react with Aβ peptide.

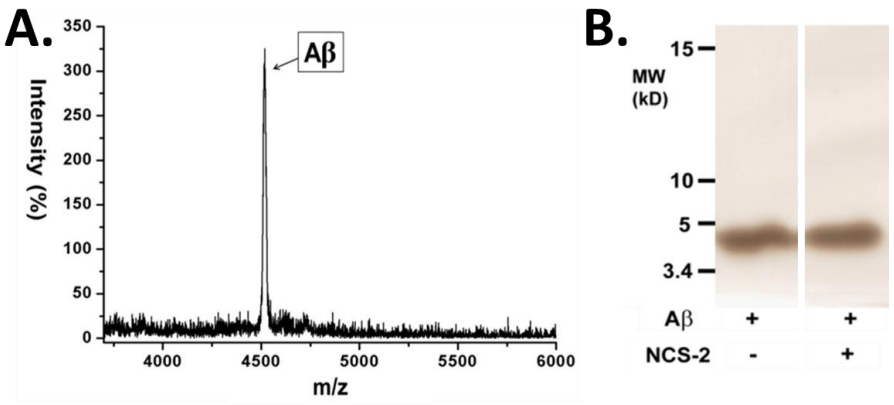

Fig. S8 Aβ peptide cannot be modified by NCS-2 pepitde. NCS-2 peptide was designed based on Tau protein but not Aβ peptide. Molecular weight of NCS-2 peptide is about 2262 m/z. 10 μM of Aβ peptide was incubated with 30 μM of NCS-2 peptide at 37℃ in PBS buffer (pH=7.4) for 24 hrs. (A) In the MALDI-TOF mass spectrum, no covalent product can be detected. (B) In the SDS-PAGE analysis, no adduct was found in the gel except Aβ peptide itself. The sequence of NCS-2 peptide is Ac-VQIVYK(NCS)GGSGGDRHDSGLDSM-NH_2.

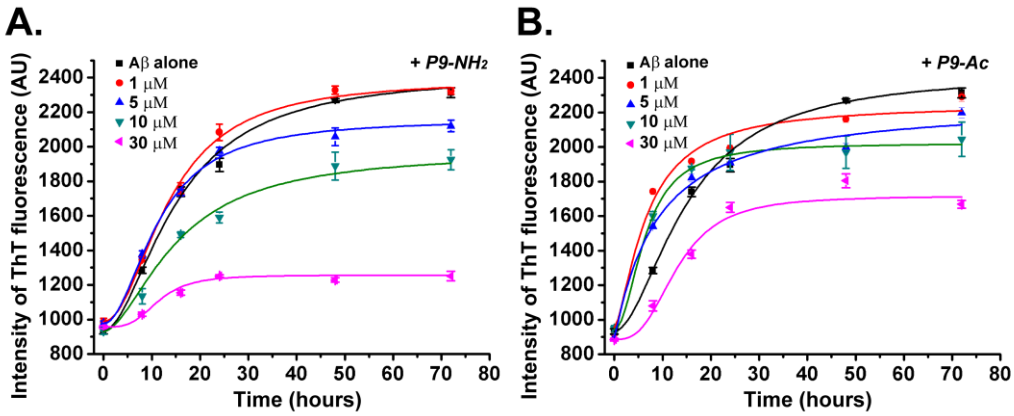

Fig. S9 Control peptides can inhibit β-sheet structure of Aβ formation in dose-dependent manner. In the ThT fluorescence assay, we mixed Aβ (10 μM) with different concentrations of (A) P9-NH_2 or (B) P9-Ac. All samples were agitated at 37℃ in PBS buffer (pH 7.4) for 72 hrs. The data were shown as the mean ± SD of 3 replicate groups.

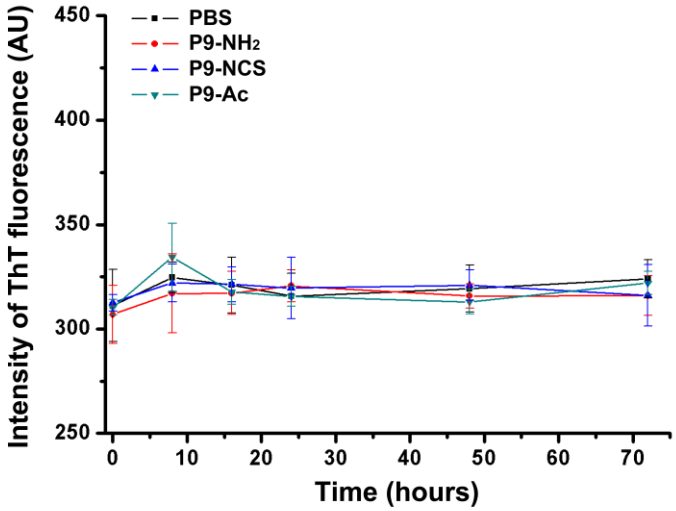

Fig. S10 P9-NCS, P9-NH_2 and P9-Ac alone can not increase ThT fluorescence. In the ThT fluorescence assay, 30μM P9-NCS, P9-NH_2 or P9-Ac was incubated in the absence of Aβ at 37℃ in PBS buffer (pH 7.4) for 72 hrs. The data were shown as the mean ± SD of 3 replicate groups.

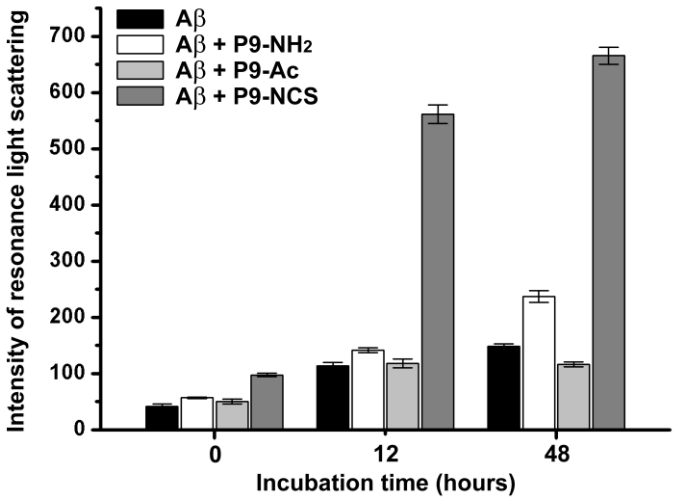

Fig. S11 P9-NCS can promote Aβ aggregation. We separately incubated different samples at 37℃ in PBS solution (pH 7.4), including Aβ (20 μM) alone, Aβ (20 μM) and P9-NH_2 (30 μM), Aβ (20 μM) and P9-NCS (30 μM), Aβ (20 μM) and P9-Ac (30 μM). After incubating for 48 hrs, we examined all these samples in resonance light scattering assay. The data were shown as the mean ± SD of 3 replicate groups. Both the excitation and emission wavelengths were set to 405 nm (excitation slit width = 1 nm, emission slit width = 2.5 nm).

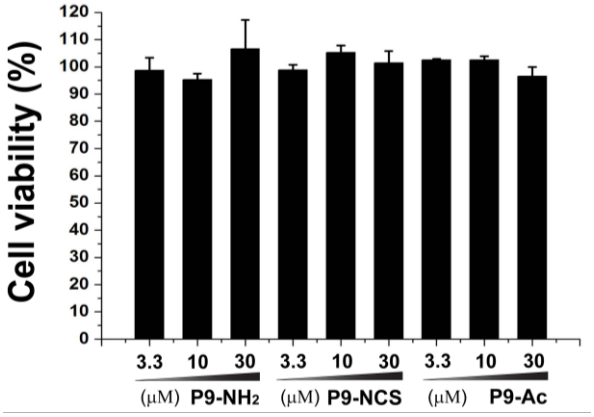

Fig. S12 P9-NCS and its control peptides alone have no neurotoxicity. With different concentrations of inhibitors (3.3 μM, 10 μM, 30 μM), all these peptides were mixed in the cell medium and then quickly diluted in each well of 96-well plate pre-cultured with N2a cells. The cell viability was detected by MTT analysis. The data were shown as the mean ± SD; n=5.

Experimental Section:

Peptide synthesis

P9-NCS peptide was synthesized at a 0.067mmol scale using the standard Fmoc SPPS (the solid phase peptide synthesis) strategy as described recently.[1] In brief, in the coupling step, a mixture of 4-fold excess of

amino acid, 3.6-fold excess of HATU and 6-fold excess of DIEA was dissolved in DMF with Rink-Amide resin (0.5mmol/g). A coupling reaction was carried out in 30 min at room temperature. After detecting the reaction activity by Kaiser test, the peptide was capping with AcOEt/pyridine/DMF(2:1:3) in 30 min. Then N-Fmoc deprotection reaction was achieved by 20% piperidine in DMF in 5 min at first and 20 min as following. After deprotecting Fmoc group of the last arginine residue in this peptide, we acetylated the N terminal of this fragment using the capping method as described above. In order to synthesize the isothiocyanate modified peptide, firstly we used $Pd(PPh_3)_4$ (0.1 equiv) and $PhSiH_3$ (12 equiv) in DCM to deprotect Alloc group, the side chain protecting group of lysine residue, in 30 min at room temperature. Then we achieved the isothiocyanate modification by TCD (4 equiv) in DMF for 4 h at room temperature. The final cleavage step was carried out by TFA/TIS/water (95: 2.5:2.5) mixture in 40 min and collect the part of solution. Then we repeat this cleavage step again to reduce the by product and promote the yield of main product.

For P9-NH_2, P9-Ac, $Aβ_{12-28}$, $Aβ_{12-28-28Ac}$, $Aβ_{12-28-16Ac}$, they were all synthesized by standard Fmoc SPPS strategy as described above. Because N-terminal amino group was located near the NCS modified lysine residue, the acetyl protecting group was induced to prevent self-reaction between these two parts.

The crude peptides were purified by reverse phase HPLC with a C18 column. The purification was achieved in a linear gradient of 30-80% aqueous acetonitrile containing 0.06% TFA over 40 min at a flow rate of 7mL/min. The identification of peptides was verified by MALDI-TOF mass spectrometry. Pure peptides were lyophilized to dry powder, and stored at -20℃ preparing for following experiments.

After MALDI-TOF mass spectrometry analysis, we got the results as below. P9-NCS: $[C_{54}H_{83}N_{17}O_{10}S + H]^+$, calculated (m/z): 1162.6308, found (m/z): 1162.6307. P9-NH_2: $[C_{53}H_{85}N_{17}O_{10} + H]^+$, calculated (m/z): 1120.6744, found (m/z): 1120.6677. P9-Ac: $[C_{55}H_{87}N_{17}O_{11} + H]^+$, calculated (m/z): 1162.6849, found (m/z): 1162.6869. $Aβ_{12-28}$: $[C_{89}H_{135}N_{25}O_{25} + H]^+$, calculated (m/z): 1955.0139, found (m/z): 1955.0106. $Aβ_{12-28-28Ac}$: $[C_{93}H_{139}N_{25}O_{27} + H]^+$, calculated (m/z): 2039.0351, found (m/z): 2039.0367. $Aβ_{12-28-16Ac}$: $[C_{93}H_{139}N_{25}O_{27} + H]^+$, calculated (m/z): 2039.0351, found (m/z): 2039.0106.

MUC1 is a kind of mucin glycoprotein, which is over-expressed on many epithelial tumor cells. Here we used a 20-amino acid peptide fragment from extracellular domain of MUC 1 protein, which can be used as B-cell epitope with the sequance of H- HGVTSAPDTRPAPGSTAPPA –OH.[2] In this work, we called this peptide as MUC 1 for short, and employed it in experiments as the model peptide of simple peptides.

Q11 is a self-assemby peptide with the sequence of H- QQKFQFQFEQQ -NH_2.[3] Here we used it as the model peptide of short-simple peptides.

Preparation of $Aβ_{42}$ solutions

$Aβ_{42}$ peptide was synthesized and purified by Dr. James I. Elliott at Yale University (New Haven, CT). To disrupt any aggregation, $Aβ_{42}$ was dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) (Alfa Aesar, USA) to 1mg/ml, and the solution was incubated with shaking overnight at room temperature and then stored at -20℃. To prepare the fresh $Aβ_{42}$ monomer solution, HFIP in the stocking solution was evaporated off under N_2 and oil pump in vacuum. Then we added DMSO (Sigma, USA) to dissolve the $Aβ_{42}$ to 1mM, sonicating the solution for 1 min. For the preparation of 10μM Aβ solution, the $Aβ_{42}$ DMSO stocking solution was diluted in PBS buffer (phosphate-buffered saline, containing NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 2 mM, pH 7.4).

SDS-PAGE analysis and silver stain

We used NuPAGE® 4-12% Bis-Tris gels, precasted by Invitrogen (USA). In order to verify that P9-NCS can covalently react with $Aβ_{42}$, samples containing $Aβ_{42}$ (10 μM), P9-NCS (60 μM), P9-NH_2 (60 μM), P9-Ac (60 μM) alone were prepared. And $Aβ_{42}$ (10 μM) separately incubated with 5 μM, 10 μM, 30 μM, 60 μM of P9-NCS in PBS solution (pH 7.4) for 24 hrs at 37℃ with shaking. Additionally, samples contained P9-NH_2 (60 μM) or P9-Ac (60 μM) peptides alone, and $Aβ_{42}$ (10 μM) incubated with P9-NH_2 (60 μM) or P9-Ac (60 μM) in PBS solution (pH 7.4) for 12 hrs at 37℃. In the experiment of determining whether P9-NCS could react with insulin and Amylin, samples contained insulin (10 μM) alone, amylin (30 μM) alone, insulin (10 μM) incubated with P9-NCS (30 μM), amylin (30 μM) incubated with P9-NCS (90 μM). For other experiments with SDS-PAGE

assay, experimental details have been demonstrated in each description of figure.Loading samples, containing 9 μl of peptide incubated mixtures in PBS buffer (pH 7.4) and 3 μl of NuPAGE® sample buffer, were heated at 70℃ for 10 min and injected into each well of precasted gels. The NuPAGE® SDS-PAGE gel system was monitored with MES running buffer with the manufacturer's recommendations. After electrophoresis, the gels were fixed in ethanol/acetic acid/water (5:1:4, v/v) solution overnight at room temperature. Next, silver stain kit (Beyotime Company, China) was used to demonstrate each peptide band.

MALDI-TOF mass spectrometry

P9-NCS (60 μM) was separately incubated with 20 μM of $A\beta_{42}$, insulin, amylin and $A\beta_{12-28}$ (and it analogues). Additionally, 30 μM of P9-NCS was cultured with 10 μM of Q11peptide, Ac-Q11 peptide, MUC 1 peptide, Cytochrome C and 1% fetal bovine serum. Meanwhile, another modified peptide, NCS-2 peptide (30 μM), was incubated with $A\beta_{42}$ (10 μM). All this samples were cultured at 37℃ in phosphate buffer (pH 7.4) . Before analyzed by MALDI-TOF mass analysis, we desalted all the mixtures with a reverse phase ZipTip-C18 column (Eppendorf, Germany). 1 μL desalted sample was spotted and dried on the target plate of MALDI-TOF mass spectrometry. Then samples were analyzed on 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems, USA).

Thioflavin T (ThT) fluorescence assay

To compare the amount of β-sheet structure from each sample, ThT fluorescence assay was operated with a black 96-well plate (Corning Costar Corporation, USA) in a Synergy 4 multi-mode microplate reader (Biotek Company, USA). For each inhibitors (P9-NCS, P9-NH_2, P9-Ac), we incubated $A\beta_{42}$ (10 μM) with 1 μM, 5 μM, 10 μM, 30 μM of inhibitors in PBS buffer (pH 7.4) at 37℃ during 72 h. For ThT analysis, 15 μl of a sample for ThT assay was injected into 185 μl of ThT solution (10 μM ThT in 12 mM phosphate buffer, pH 7.4) within a well of plate. Each sample was placed into 3 wells to replicate in the plate. After shaking the plate for 3 min, ThT fluorescence was measured at 485 nm which was excited at 440 nm.

Transmission electron microscopy (TEM)

To detect the inhibitors' effect on $A\beta_{42}$ fibril formation, we used TEM to observe the peptide morphology. Firstly, we made the samples by mixing $A\beta_{42}$ (10 μM) with inhibitors (30 μM) in PBS buffer (pH 7.4) at 37℃ After 48 h incubation, we drew 8 μl solution from each sample to the surface of 300 mesh formvar-coated copper grids for 1.5 min. And then the sample solution was drained by filter paper, which was followed by negatively staining with 1% (w/v) fresh tungstophosphoric acid for 1.5 min. The rest of staining solution was drained by filter paper and these copper grids were placed on filter paper until completely dried. At last, the sample was observed by H-7650B electron microscope (Hitachi, Tokyo, Japan) at 80 kV.

Measurement of cell viability

We employed the neurotoxicity assay to evaluate effect of different inhibitors on mouse neuroblastoma N2a cells. N2a cells were cultured in the medium at 37℃, containing 47.5% minimal essential medium (MEM) (GIBCO, Invitrogen, USA), 47.5% Dulbecco's modified eagle medium (DMEM) (GIBCO, Invitrogen, USA) and 5% fetal bovine serum. For neurotoxicity assay, cells (about 5000 cells per well) were seeded in 96-well plates (Corning Costar Corporation, USA) in the medium, 200 μl per well as described above. After culturing the cells for 24 h, we changed the original medium to the cell medium without fetal bovine serum (50% MEM, 50% DMEM), and added various peptides into different groups of wells. The volume of culture solution is 100 μl in total each well with the same amount of DMSO (0.16% v/v). The final concentration of $A\beta_{42}$ in each well was 4 μM. And each inhibitor was added at different concentration. After 24 h cell culture at 37℃, 20 μl of MTT (3-[4,5-dimethyl-2-thiazoyl]-2,5-diphenyltetrazolium bromide) (AMRESCO, USA) buffer (5mg/mL in PBS buffer) was added into each well and incubated for 4 h at 37℃. Then the cell medium was replaced with 150 μl of DMSO per well to dissolve the formazan slat. Finally, we measured the absorbance of each well at 570 nm in a Synergy 4 multi-mode microplate reader (Biotek Company, USA).

Resonance light scattering assay

This assay can reveal the relationship between the size of aggregates and the intensity of resonance light scattering (RLS). RLS of each sample was measured by Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan) in a 1 cm path length quartz cell at room temperature. The wavelengths of excitation and emission were both set to 405 nm (excitation slit width = 1 nm, emission slit width = 2.5 nm). We separately incubate different samples containing $A\beta_{42}$ (20 μM) alone, $A\beta_{42}$ (20 μM) and P9-NH_2 (30 μM), $A\beta_{42}$ (20 μM) and P9-NCS (30 μM), $A\beta_{42}$ (20 μM) and P9-Ac (30 μM). After incubating for 48 h, we took 700 μl of each sample into quartz cell, and then we measured intensity of light scattering. The data are shown as the mean ± SD; n=3.

Additional references:

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