Supplementary Information (SI) for Journal of Materials Chemistry B.

Intervention of hIAPP amyloid aggregation by smart post-transmuting anti-amyloidogenic peptidomimetics

Altaf Hussain Kawa,[a] Biswadeepak Satapathy,[a] Shinjini Bhattacharya,[b] Sachin Kumar,[b] and Bhubaneswar Mandal[a]*

[a]Department of Chemistry, [b]Department of Bioscience and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, 781039 Assam, India., Assam - 781039, India. Email: bmandal@iitg.ac.in

Table of Contents:

1. Reagents and Solvents

Rink Amide MBHA resin (0.07 mmol/100mg loading), all Fmoc-protected amino acids (with OBzl for aspartic acid side-chain protection) were obtained from GL Biochem (Shanghai). Coupling reagent Ethyl 2-cyano-2-(2-nitrobenzenesulfonyloxyimino) acetate (o-NosylOXY) was synthesized in the laboratory. Diisopropylethylamine (DIPEA) was procured from Spectrochem Pvt. Ltd. (India). 5(6)-Carboxyfluorescein was acquired from Sigma. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and Ganglioside GM1 were procured from Avanti Polar Lipids, Inc. Cholesterol (99%), trifluoroacetic acid (TFA, extrapure grade), acetic anhydride (synthesis grade), and N-methyl-imidazole (extrapure grade) were obtained from SPL (India). Acetonitrile (HPLC grade), dimethylformamide (DMF, extrapure grade), and dichloromethane (extrapure grade) were supplied by Merck (India). Milli-Q water (18.2 Ω) was used for all experiments.

2. Peptide synthesis

All peptides were manually synthesized using the standard Fmoc/tBu solid-phase peptide synthesis (SPPS) method with Rink Amide MBHA resin as the solid support on a Stuart blood tube rotator. The resin was first swollen in DCM for 3 hours, followed by DMF for 1 hour, inside a 5 mL frit-fitted plastic syringe. Coupling reactions were performed using 2 equivalents of Fmoc-protected amino acids, 2.5 equivalents of the coupling reagent (o-NosylOXY), and 5 equivalents of DIPEA as the base. The progress of each coupling step was monitored using the Kaiser test. If incomplete coupling was detected, the cycle was repeated, followed by acetylation (capping) with Ac2O/NMI (1:2) in DCM for 1.5 hours. Fmoc deprotection was carried out using 20% piperidine in DMF for a total of 21 minutes (3 cycles of 7 minutes each). After each Fmoc removal, the resin was washed alternately with DCM and DMF for a total of 8 washes. The relevant amino acid was then coupled. The process was repeated until the peptide sequence was completed. The final peptide sequence was tested for cleavage to characterise it. After confirmation that the peptide was complete, it was cleaved from the resin by treating the resin with 80% TFA, 15% DCM, and 5% H_2O for 3h. The crude peptide was obtained as a precipitate in diethyl ether and purified by HPLC. Finally, the peptide was lyophilised to get the final dry product.

3. Instrumentation

3.1. High-Performance Liquid Chromatography (preparative/analytical)

Crude peptides were dissolved in a CH_3CN/H_2O mixture and purified using reverse-phase HPLC (RP-HPLC, Thermo Scientific) on a C18-Bondapak column (250 × 10 mm, particle size 12 µm, pore size 175 Å) at a flow rate of 4 mL/min. A binary solvent system was used, with solvent A (0.1% TFA in H_2O) and solvent B (0.1% TFA in CH_3CN). The purification was carried out over a total run time of 20 minutes, using a UV detector set at 214 nm and 254 nm. A linear gradient of 5–95% CH_3CN over 18 minutes was followed by 5% CH_3CN for 19 minutes.

The purity of the peptides and all the analytical studies were performed using an RP-HPLC (Agilent 1260 infinity II) Analytical System equipped with a C18 analytical column (Poroshell 120, EC C18, 4-µm, 4.5×100 mm). The conversion studies were performed at a flow rate of 1 mL/min with the same gradient conditions and detection wavelengths (214 nm and 254 nm) over a total run time of 12 minutes.

3.2. MALDI-TOF Mass Spectrometry

All the peptidomimetics were mixed with α-cyano-4-hydroxycinnamic acid (CHCA) matrix in a 1:1 ratio and analysed for characterisation using a Bruker Daltonics MALDI-TOF mass spectrometer with flexAnalysis software.

3.3. Thioflavin T (ThT) Fluorescence Assay

Thioflavin T (ThT) was obtained from Sigma-Aldrich, and a 50 μM stock solution was prepared in PBS (50 mM, pH 7.4) and stored at 4°C, protected from light. $hIAPP_{1-37}$ and purified peptides were dissolved in PBS (50 mM, pH 7.4) containing 3% DMSO to prepare stock solutions of 400 μM for peptide samples and 40 μM for $hIAPP_{1-37}$. For dose-dependent studies, different breaker peptide samples were added to $hIAPP_{1-37}$ solutions in molar ratios of 1:0.5, 1:1, and 1:2, with the final volume adjusted to 800 μL using PBS.

For the fluorescence experiment, 40 μL of peptide solutions were mixed with 160 μL of PBS (50 mM, pH 7.4), followed by the addition of 200 μL of ThT solution. Fluorescence measurements were conducted using a Fluoromax-4 Horiba Fluorospectrometer with excitation at 440 nm and emission at 485 nm. Each scan was repeated three times using a 5 nm slit width. Data were transferred to an Excel file and plotted in OriginPro 8. Three independent readings were recorded for each data point, and the average fluorescence intensity was plotted on the y-axis against time (h) on the x-axis, with the standard deviation as the y-error.

Normalization was performed using the equation:

$$relative\ \%\ of\ amyloid = \frac{flourescence\ observed\ in\ presence\ of\ designed\ peptidomimetic - 1}{flourescence\ observed\ in\ absence\ of\ designed\ peptidomimetic - 1} \times 100$$

where 1 served as a normalization factor, ensuring fluorescence intensity was expressed as F/F0 (F: sample fluorescence, F0: reference fluorescence), with the minimum F/F0 value set to 1.

3.4. Transmission Electron Microscopy (TEM)

After six days of peptide incubation, a 5 μL aliquot from each solution was drop-cast on the dark side of a carbon-coated copper grid and left to adhere for 2 minutes. Excess solution was soaked away by seeping the grid with a fine tissue paper. Finally, a 5 μL drop of 2% uranyl acetate was also drop-cast for 2 minutes. After removing excess stain, the samples were dried overnight in an incubator and stored in a desiccator. Images were captured using a JEOL JEM-2100F Field Emission Electron Microscope at 200 kV.

3.5. Congo-Red Stained Birefringence

Congo red was obtained from Sigma-Aldrich, and a saturated solution was prepared in 80% ethanol and then filtered. After 6 days of peptide incubation, 5 μL aliquots were placed on clean glass slides, dried overnight, and stained with 5 μL of Congo red solution. Samples were analyzed using a Leica ICC50 HD polarizable microscope.

3.6. Circular Dichroism (CD) Spectroscopy

CD analysis was conducted after six days of peptide incubation. $hIAPP_{1-37}$ (40 µM) was used without further dilution. Stock peptide solutions containing breaker peptides were diluted in PBS (50 mM, pH 7.4) to final concentrations of 20 µM, 40 µM, and 80 µM, corresponding to molar ratios of 0.5, 1, and 2, respectively. A 400 µL sample volume was placed in a 1 mm cuvette, and three scans were recorded from 190 to 260 nm using a JASCO J-815 spectrometer. Observed ellipticity (mDeg) was converted to mean residue molar ellipticity using:

$$\theta(\text{deg. cm}^2\text{. dmol}^{-1}) = \frac{\text{Ellipticity (mdeg)} \times 10^6}{\text{Pathlength (mm)} \times [\text{Protein} (\mu M)] \times N}$$

where [Protein] is the peptide concentration (µM), and N is the number of amide bonds in the sequence.

3.7. Fourier Transform Infrared (FT-IR) Spectroscopy

FT-IR spectra were recorded after six days of incubation. A 15 µL aliquot of each stock solution was mixed with KBr to form pellets. Background spectra were subtracted from the sample spectra. Data were analyzed using OriginPro 8.

3.8. Dynamic Light Scattering (DLS)

DLS measurements of $hIAPP_{1-37}$alone (at different time intervals) and in the presence of 2 equivalents of peptide samples were performed at 25°C using a 633 nm He-Ne laser on a Zetasizer Nano-ZS90 (Malvern Instruments). $hIAPP_{1-37}$ (40 µM) was prepared in PBS (pH 7.4) with 3% DMSO, followed by adding peptide solutions. Samples were incubated at 37°C, and 200 µL aliquots were analyzed at various times. Before scanning, the system was equilibrated for 60 seconds. Each measurement represented an average of seven scans, recording intensity percentage and size distribution. Data from Zetasizer software were plotted in OriginPro 8 and finalized in Adobe Illustrator.

3.9. Isothermal Titration Calorimetry (ITC)

The ITC experiment was performed using a Nano-ITC instrument from MicroCal to determine the formation constants and thermodynamic parameters for the binding of the designed peptidomimetics to the target peptide (NAc-NFGAIL-NH_2). A 40 µM solution of peptide AH_4 (NAc-EEENFG-D(OBzl)I-L-NH_2) in buffer (PBS, pH 5.8) was injected, dropwise, into the 4 mM solution of the core amyloidogenic sequence of amylin (NAc-NFGAIL-NH_2). Each injection was 1.3 µL and performed at 2-minute intervals using a 40 µL microsyringe with constant stirring (500 rpm) at 298K. All the solutions were degassed prior to titration. The same experimental process was repeated with the control peptide AH_2 (NAc-NFGPIL-NH_2). The ITC showed a better binding affinity for peptide AH_4 than that of control peptide AH_2.

4.0. Large Unilamellar Vesicles (LUVs) Preparation

LUVs were prepared using DPPC, Cholesterol, and GM1 in a 68:30:2 molar ratio. Lipids were weighed, dissolved in chloroform/methanol (2:1), and dried under nitrogen to form a thin lipid film. A 200 μM carboxyfluorescein dye solution was prepared in 500 μL of PBS (50 mM, pH 7.4) and used to hydrate the lipid film. The mixture was vortexed for 30 minutes and subjected to five freeze-thaw cycles using liquid nitrogen and a 60-70°C water bath. The solution was centrifuged at 15,000 rpm, and excess dye was removed. The lipid pellet was resuspended in 500 μL of PBS and vortexed to obtain a homogeneous 2 mM lipid suspension. LUV formation was confirmed by TEM, and dye leakage assays were performed using a Fluoromax-4 Horiba Fluorospectrometer.

5.0. Cell penetration and cytotoxicity study

A chromophore (FITC) was attached to one of the designed peptidomimetics for the cell penetration study. FITC conjugation was performed on the following peptide to assess its cell-penetrating ability. Briefly, BHK-21 cells were seeded at a density of 1 × 104 cells per well in a 96-well plate and incubated overnight for attachment. The cells were then treated with 10−100 μM concentrations of amylin (AM) and amylin in combination with the AH4 compound (AM: AH4). After incubation for 24 h, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (SRL, India) was added to each well and then incubated at 37◦C for another 3 h. The MTT reagent was removed from the wells, and formazan crystals were dissolved with dimethyl sulfoxide (Sigma, USA). The absorbance was measured at 570 nm using Multiskan Go (Thermo Scientific, USA). To check the internalization of the AH4 compound, BHK-21 cells were further treated at 40 μM, 80 μM, and 100 μM for 24 h. For quantitative assessment of the internalization of the AH4 compound, BHK-21 cells were seeded, and similar experimental groups were set up. Cells were then harvested at 24 hours post-treatment. Cell suspension was made in PBS and analyzed using a CytoFLEX flow cytometer (Beckman Coulter, USA). A minimum of 10,000 events was acquired per sample. The statistical analyses for the cytotoxicity studies were conducted with GraphPad Prism software, and results are reported as mean ± standard deviation (SD). All the data have been gathered from at least three independent experiments. The significance of the results was determined using a one-way ANOVA test in GraphPad Prism. Significant differences between groups were marked as *, **, *** and **** at $p < 0.05$, $p < 0.01$, and $p < 0.001$, and 0.0001, respectively.

6.0. Characterisation of the designed peptidomimetic and the characterization of the *in situ* generated peptides.

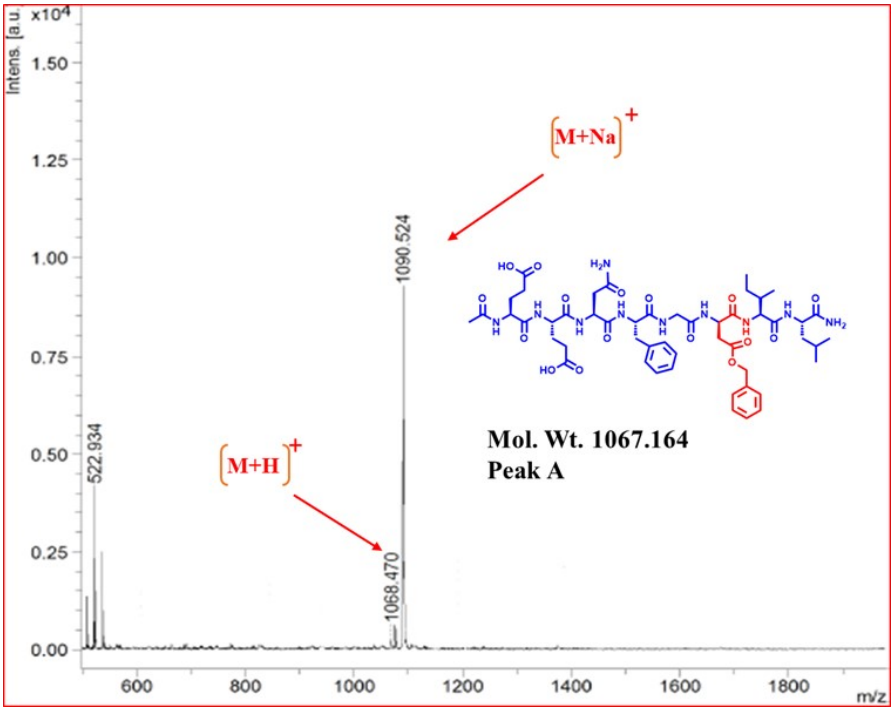

Fig S1: Maldi mass spectrum of peak A, representing the designed peptidomimetic.

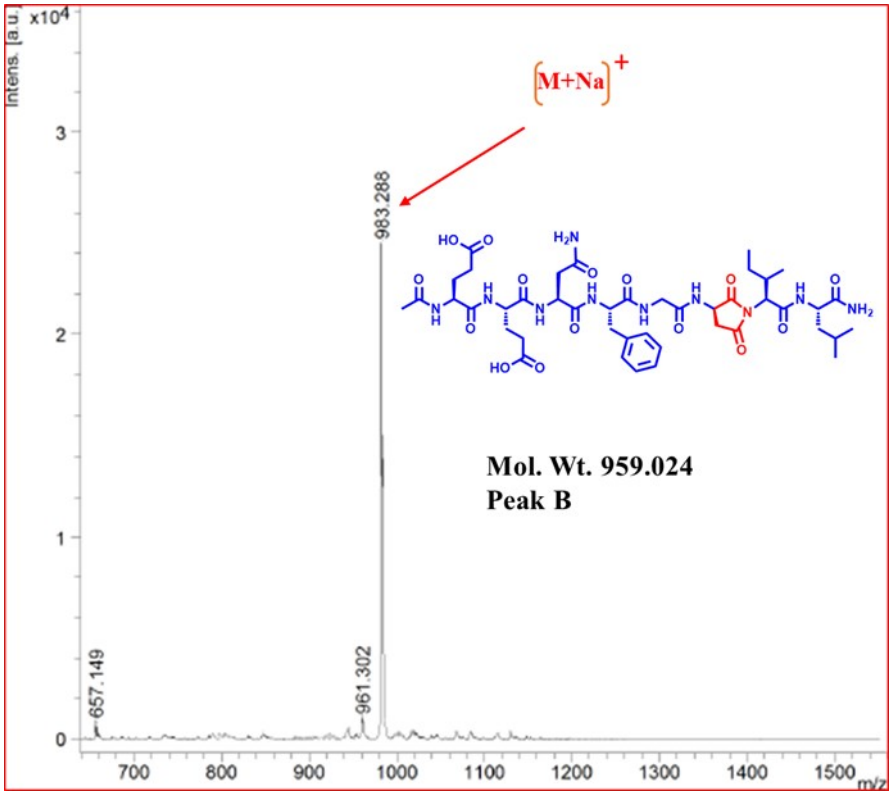

Fig S2: MALDI mass spectrum of peak B characterising the aspartimide-borne peptide

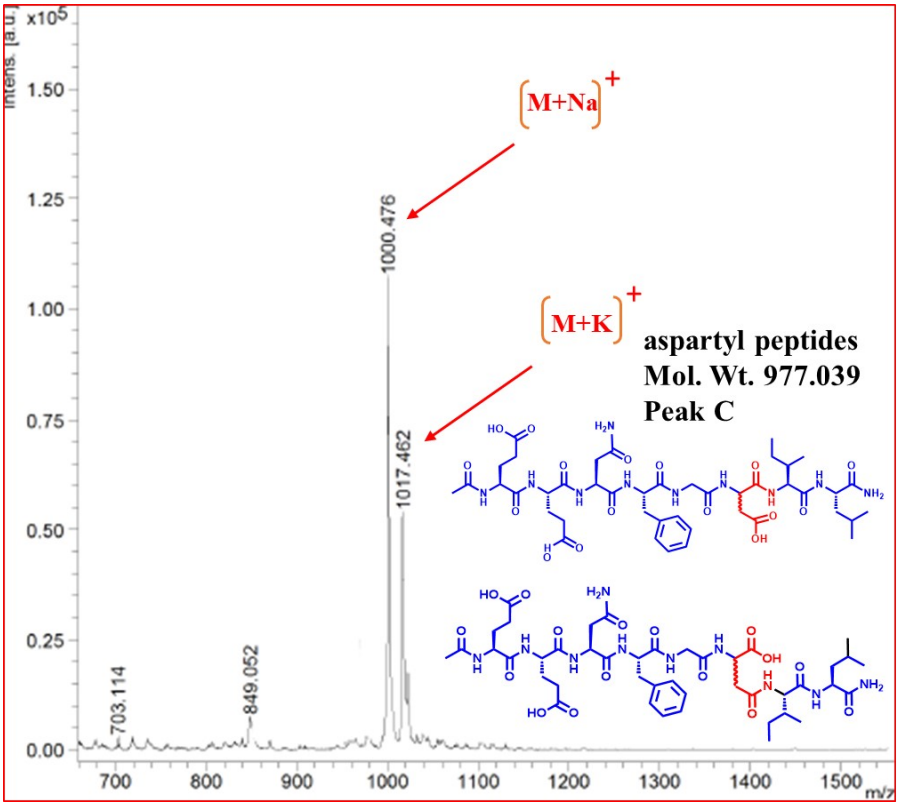

Fig S3: MALDI mass spectrum after hydrolysis, characterising newly generated aspartyl peptides and aspartyl isopeptides.

7.0. Characterisation of the designed peptidomimetic AH1 and AH3 and the characterization of their newly generated peptides.

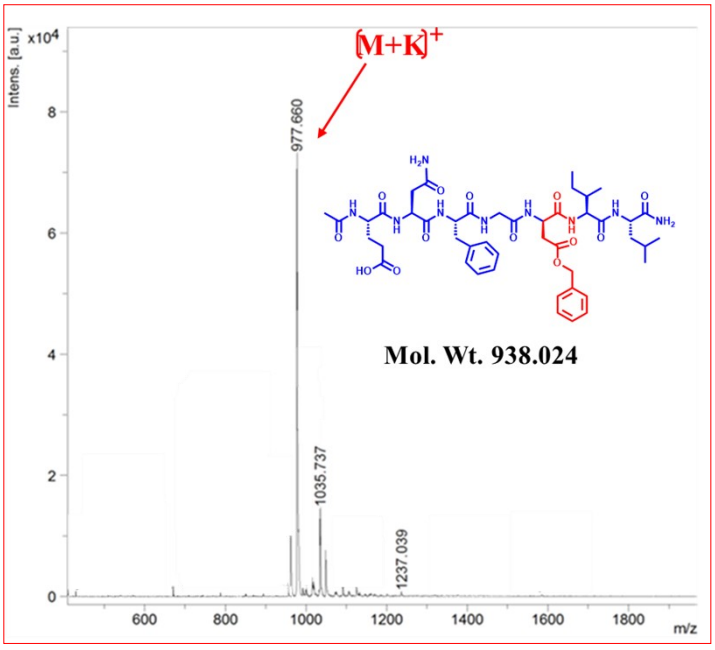

Fig S4: MALDI mass spectrum of designed peptidomimetic AH1.

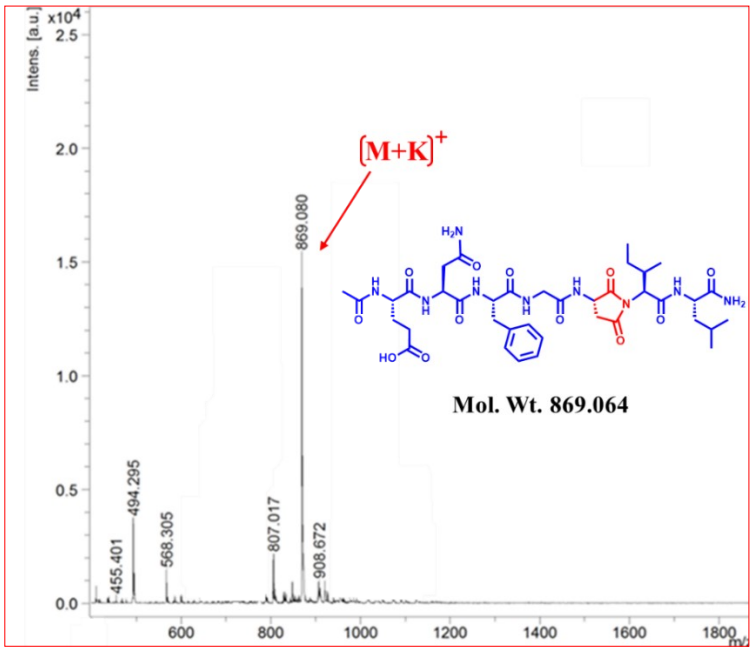

Fig S5: MALDI mass spectrum of designed peptidomimetic AH1 after the formation of the aspartimide.

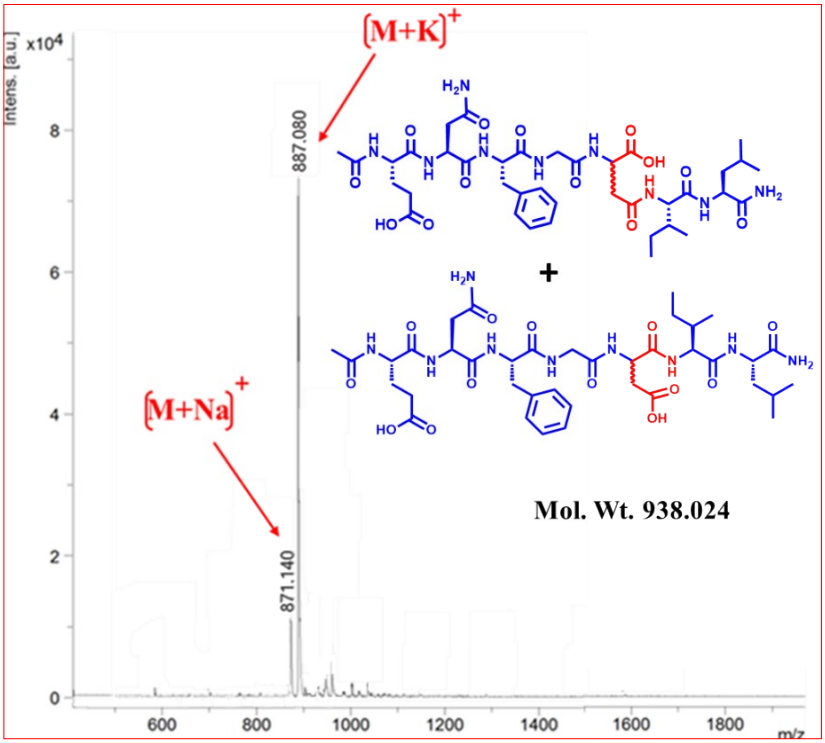

Fig S6: MALDI mass spectrum of designed peptidomimetic AH1 after the hydrolytic ring opening of the aspartimide.

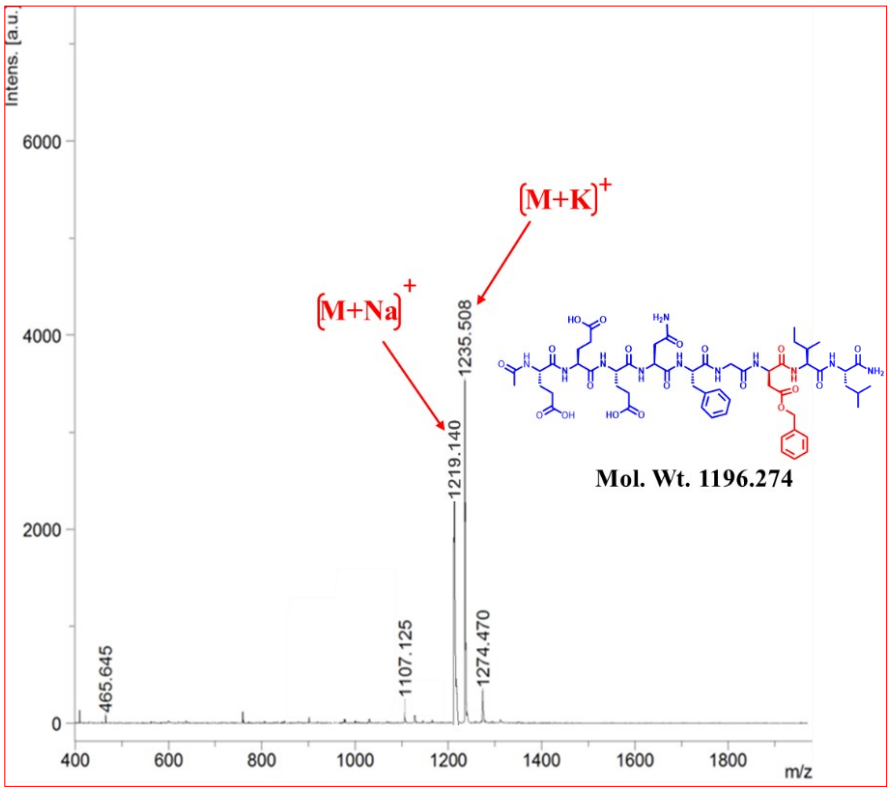

Fig S7: Maldi mass spectrum of designed peptidomimetic AH4.

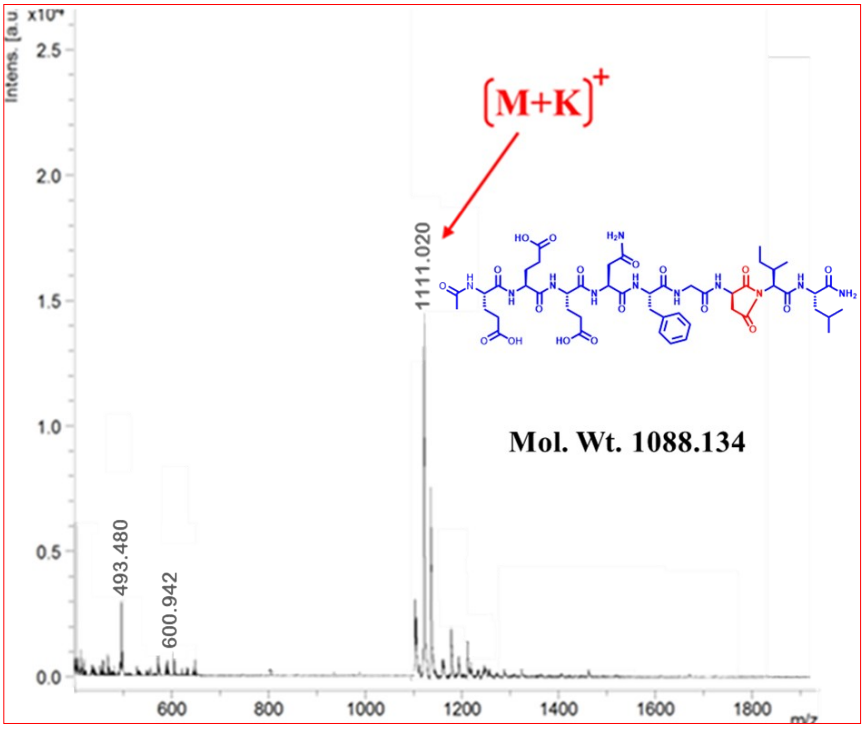

Fig S8: MALDI mass spectrum of designed peptidomimetic AH4 after the formation of the aspartimide.

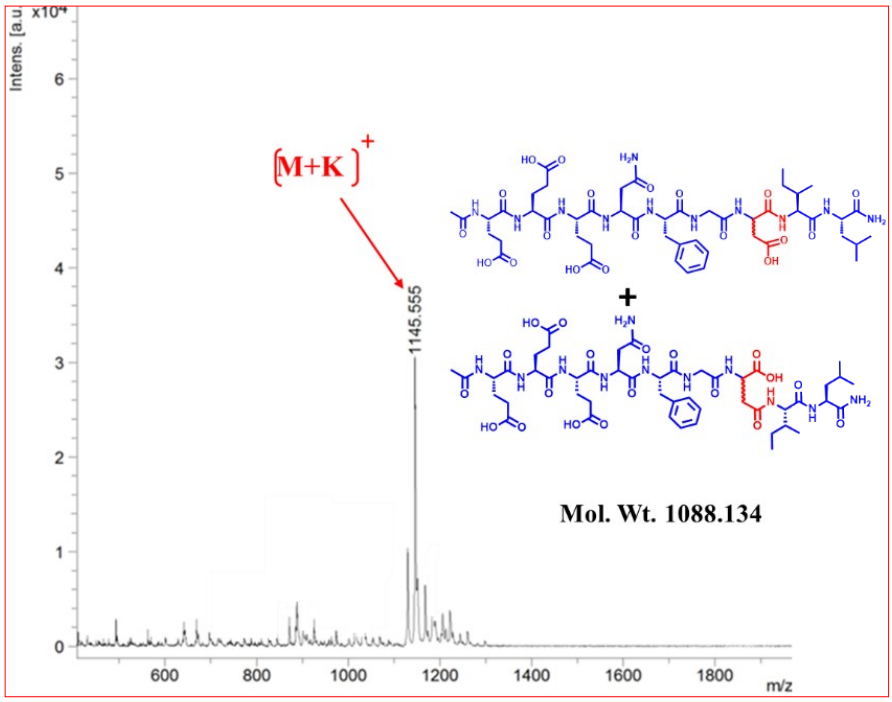

Fig S9: MALDI mass spectrum of designed peptidomimetic AH4 after the hydrolytic ring opening of the aspartimide.

8.0. Characterisation of the FTIC bound peptide.

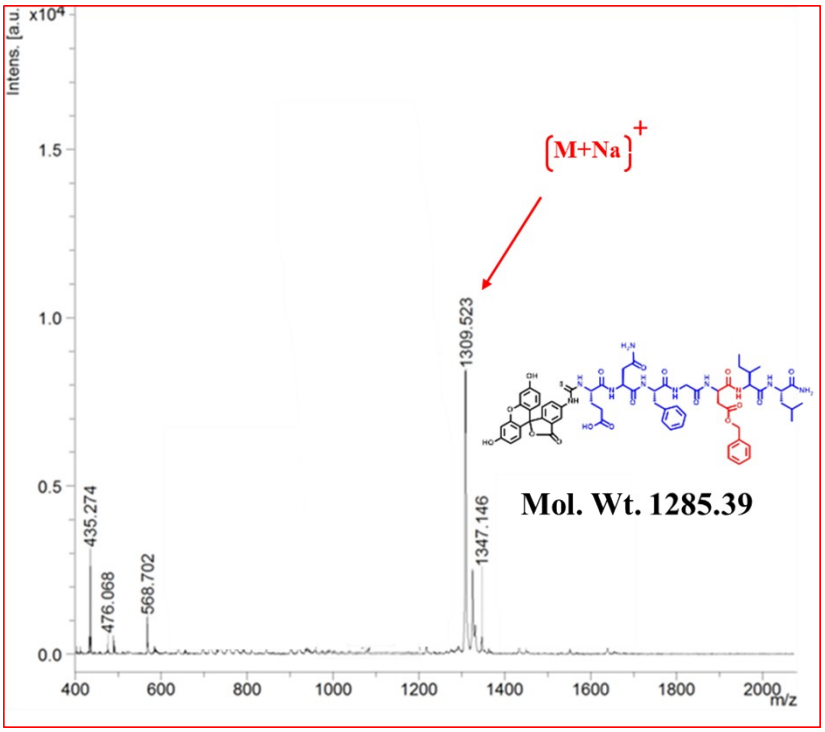

Fig S10: MALDI mass of the fluorescein isothiocyanate dye-bound peptide.

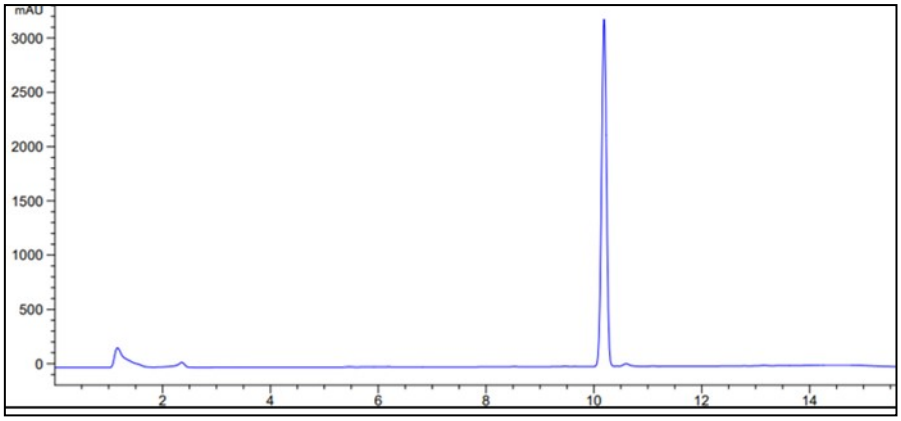

Fig S11: HPLC profile of the purified FTIC-bound peptide

9.0. Mechanism of chemical transformation of designed peptidomimetic AH1, also modelling AH3 and AH4.

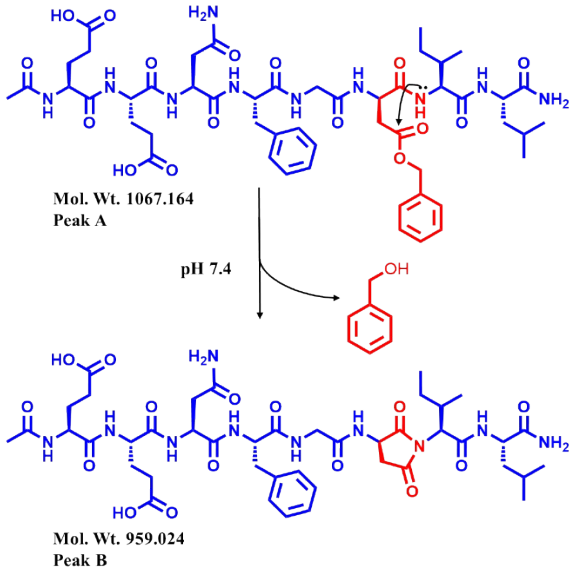

Fig S12: Aspartimide formation with the release of benzyl alcohol.

At a basic pH, the newly generated peptide undergoes racemization, producing another D-aspartimide containing peptide.

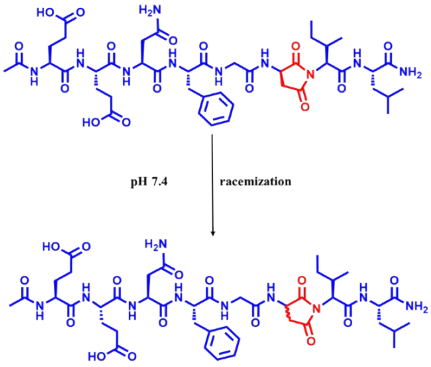

Fig S13: racemization of the aspartimide-borne peptide.

There is further hydrolysis of this aspartimide-borne peptide that produces D-/L- α-aspartyl peptide and D-/L- β-aspartyl peptide. These chemical transformations were monitored by analytical HPLC (Agilent 1260 Infinity II) using a Poroshell 120 EC-C18, 4 µm, 4.6×100mm column. All of these peptides were characterised by the MALDI mass instrument.

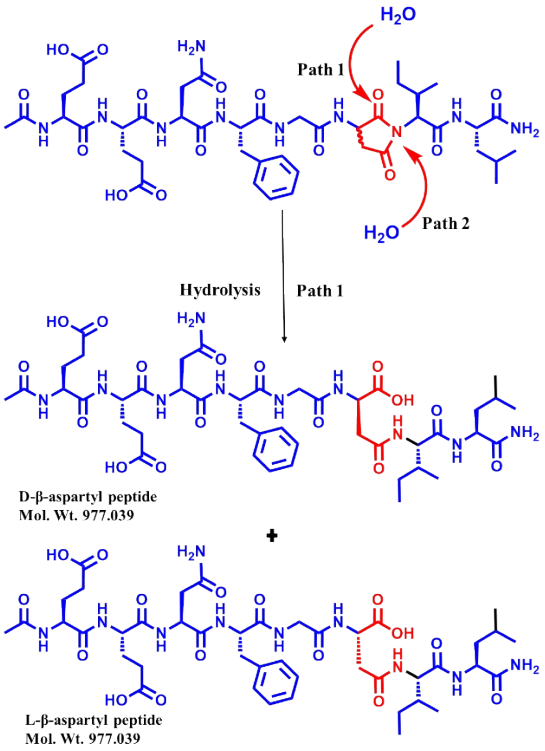

Fig S14: hydrolysis of the aspartimide-borne peptide through path 1, generating L/D-β-aspartyl peptides.

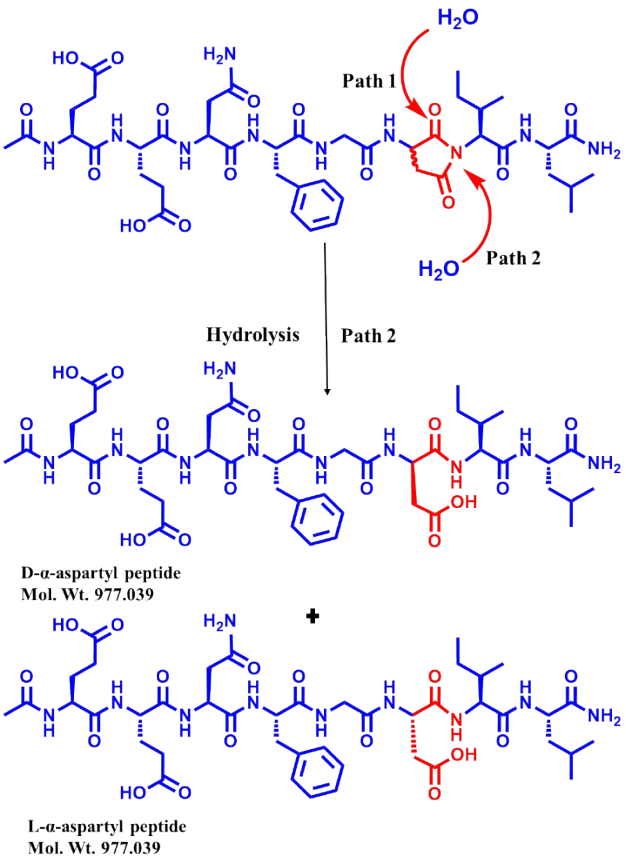

Fig S15: hydrolysis of the aspartimide-borne peptide through path 2, generating L/D-α-aspartyl peptides

10.0. Kinetics of these chemical transformations as analysed through the analytical HPLC.

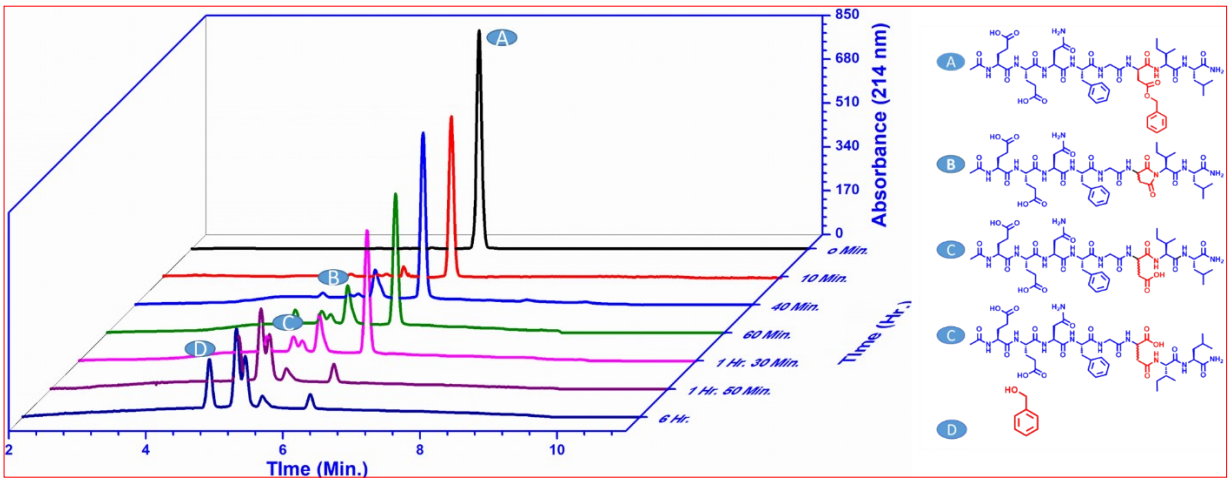

Fig. S16: HPLC profile of peptide AH1 when incubated at 37 ºC in PBS buffer of pH 7.4. **(Peak A)** designed peptidomimetic AH1. **(Peak B)** aspartimide borne peptide; racemised. **(Peak C)** D-, α- and β- aspartyl peptides and L-, α- and β- aspartyl peptides. **(Peak D)** benzyl alcohol.

11.0. Morphology of the self-assembly of the peptide AH_4

As we mentioned in the manuscript, because the peptide is stable at pH 5.8 (PBS), it does not undergo any aspartimide formation. As such, the peptide maintains backbone linearity and begins to self-assemble at pH 5.8. (PBS). The kinetic Th-T study showed a fluorescence plateau after 48 hours of incubation. At this point, the morphology of the peptide was analysed. A rod-like morphology was observed.

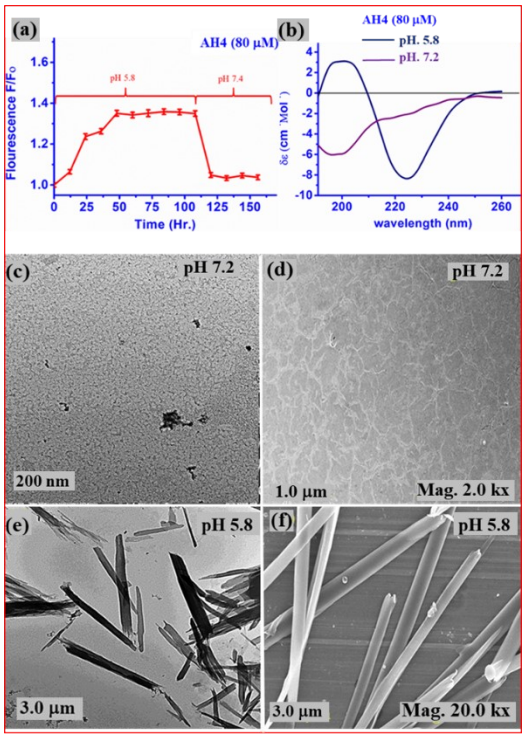

Fig.S17: (a) Th-T kinetics of AH4 in the pH 5.8 (PBS) and in the pH 7.2 (PBS), (b) CD profile of the peptide in the pH. 5.8 (deep blue) and pH. 5.8 (purple); (c) and (e) FETEM images of the peptide AH4 in pH. 7.2 and 5.8 (rod-like morphology), respectively, (d) and (f) FESEM images of the peptide AH4 at pH 7.2 and 5.8 (rod-like morphology), respectively.

At basic pH, the peptide undergoes aspartimide formation, and the peptide backbone develops a kink that distorts the beta-sheet alignment and breaks off self-assembly; hence, the Th-T fluorescence decreases. The anti-amyloidogenic activity of the peptide is studied at pH. 7.2. Therefore, the peptide is not expected to stay aggregated. Thus, the peptide doesn't contribute to the Th-T in the basic pH. of 7.2. The peptide transiently co-aggregates with amylin and then undergoes various aspartimide-driven chemical transformations.