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Effect of C-terminus amidation of A β _{39–42} fragment derived peptides as potential inhibitors of A β aggregation†

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The C-terminus fragment (Val-Val-Ile-Ala) of amyloid- β is reported to inhibit the aggregation of the parent peptide. In an attempt to investigate the effect of sequential amino-acid scan and C-terminus amidation on the biological profile of the lead sequence, a series of tetrapeptides were synthesized using MW-SPPS. Peptide D-Phe-Val-Ile-Ala-NH₂ (12c) exhibited high protection against β -amyloid-mediated-neurotoxicity by inhibiting A β aggregation in the MTT cell viability and ThT-fluorescence assay. Circular dichroism studies illustrate the inability of A β ₄₂ to form β -sheet in the presence of 12c, further confirmed by the absence of A β ₄₂ fibrils in electron microscopy experiments. The peptide exhibits enhanced BBB permeation, no cytotoxicity along with prolonged proteolytic stability. *In silico* studies show that the peptide interacts with the key amino acids in A β , which potentiate its fibrillation, thereby arresting aggregation propensity. This structural class of designed scaffolds provides impetus towards the rational development of peptide-based-therapeutics for Alzheimer's disease (AD).

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First reported by Alois Alzheimer in 1906, Alzheimer's Disease (AD) is a progressive, neurodegenerative disorder with an irreversible decline in memory and cognition.¹ It is commonly seen in elderly populations and is marked by two major histopathological hallmarks, amyloid- β (A β) plaques and neurofibrillary tangles (NFT).² The number of patients suffering from AD has been increasing at an alarming rate. It is estimated that around 60 million patients will be suffering from AD by the end of 2020 and half of this patient population would require the care equivalent to that of a nursing home.³ Even after a century of its discovery, there has been no treatment that targets the pathophysiology of AD.⁴ The current treatment regimen includes acetylcholine-esterase inhibitors (AChEI) comprising of donepezil, rivastigmine and galantamine as well as *N*-methyl-D-aspartate (NMDA)-receptor antagonist, memantine. These provide only symptomatic relief to the patient. Most of the therapeutics that are currently being tested in clinical trials

couldn't proceed beyond phase II and phase III clinical trials due to lower efficacy in elderly patients, multiple side effects and limitations in their pharmacokinetic and pharmacodynamic profiles.^{5–9} This has created challenges on the societal and economic upfront.

Literature analysis reveals that the soluble oligomeric A β species is the culprit for neurotoxicity. It interrupts normal physiological functioning of the human brain. The central hydrophobic fragment A β _{16–22} (KLVFFAE) and the C-terminus region fragment A β _{31–42} (IIGLMVGGVVIA) are responsible for controlling the aggregation kinetics of the monomeric species, wherein the later still remains relatively less explored.^{8,9} Our group is focused on development of peptidomimetic analogues for preventing A β aggregation. Studies on a complete peptide scan on the C-terminus region regions has already been published previously.^{10–12} In an attempt to enhance the biological efficacy of the previously designed scaffolds,¹² we rationalized the use of sequential amino acid scan by modifying/replacing individual residues, as well as amide protection of the C-terminus on the lead tetrapeptide sequence (Val-Val-Ile-Ala) to enhance the proteolytic stability of the peptides.

C-terminus amidated peptides were synthesized by microwave-assisted Fmoc-solid phase peptide synthesis protocol. Scheme 1 shows the general route for the synthesis of peptides employing Rink amide resin (detailed methodology is mentioned in the ESI, Section 1†). These peptides were characterized using by analytical HPLC, ¹H and ¹³C NMR, APCI/ESI-MS and HRMS.

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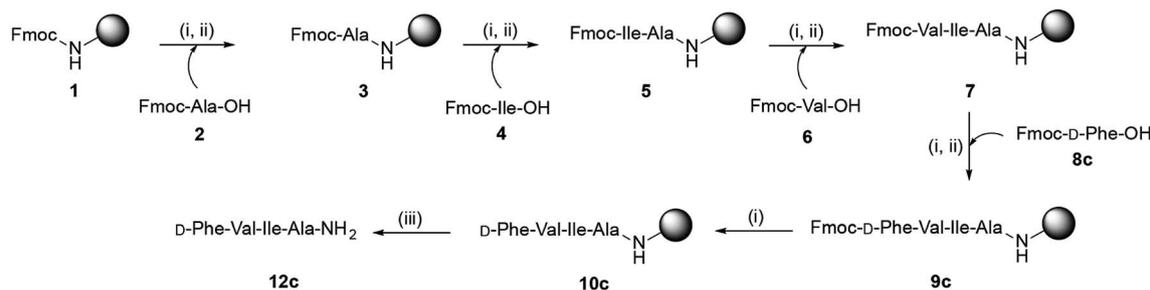
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Scheme 1 Synthesis of tetrapeptide **12c** using MW-assisted Fmoc-solid phase peptide synthesis protocol employing Rink amide resin. Reaction conditions: (i) 20% piperidine in DMF (7 mL), MW (40 W), 60 °C, 2 cycles – 1.5 & 3 min; (ii) 2, 4, 6, 8 (4 equiv.), TBTU (4 equiv.), HoBt (4 equiv.), DIPEA (5 equiv.), DMF (3.5 mL), MW (40 W), 60 °C, 13.5 min; (iii) TFA : TIPS : H₂O (95 : 2.5 : 2.5), rt, 2.5 h; reaction monitoring was done by: UV measurement: Fmoc deprotection-dibenzofulvene adduct, Kaiser test: 1° amines; acetaldehyde test: 2° amines.

Aggregation of A β ₄₂ results in accumulation of toxic species, which interact with neurons and hinder their functioning, leading to loss of memory and cognition. Inhibiting the process of A β ₄₂ aggregation would alleviate the neurotoxicity imparted in PC-12 cells; this was evaluated by MTT cell viability assay.¹³ Viability of untreated cells is considered 100%. Upon treatment with 2 μ M of A β ₄₂, only 74% cells were found to be viable. Out of the total tested peptides, seven tetrapeptides **12a**, **12c**, **12f**, **13e**, **14b**, **15b** and **15f** showed complete inhibition of A β ₄₂-induced toxicity by restoring the cell viability to 100%, at respective concentrations. Results for cell viability assay along with Thioflavin-T fluorescence assay for all the synthesized tetrapeptides has been summarized in Table 1.

The quantitative evaluation of β -sheet structures within the amyloid fibrils is accessed by the fluorescence of the Thioflavin-T dye.¹⁴ Inhibiting amyloid fibrillation would reduce or eliminate such an enhancement in fluorescence. This concept is utilized to evaluate compounds that would prevent A β from aggregating.^{15–17} ThT fluorescence in the presence of A β ₄₂ alone was considered 100% and % relative fluorescence unit (% RFU) values were calculated for A β ₄₂ co-incubated with the respective inhibitor peptides. ThT incubated alone, exhibited % RFU of nearly 53.3% as compared to control solution without dye. Complete data of % inhibition of A β ₄₂ by the test peptides has been summarized in Table 1.

The lead peptide **11** Val-Val-Ile-Ala-OH, was observed to mitigate the A β ₄₂ aggregation by 33.9, 60.6 and 66.2% at 2 μ M, 4 μ M and 10 μ M concentrations whereas peptide **11a** shows increased activity at 2 μ M but slightly less activity at higher dose concentrations in both cell viability and ThT-fluorescence assays.¹⁷ Out of all the tested peptides, peptides **12c** and **13e** showed minimal enhancement in ThT fluorescence when co-incubated with equimolar concentrations of A β ₄₂. Peptides **12f** and **12g** showed >90% activity at five-fold excess dose concentrations. A comparative bar graph representation for the four most active peptides **12c**, **12f**, **12g** and **13e** has been depicted in Fig. 1A. To understand the % RFU values indicating the relative fluorescence of ThT and % inhibition exhibited by the most active test peptides **12c**, **12f**, **12g** and **13e**, values have been summarized in ESI, Tables S1 and S2.† The observed RFU was close to that of the control wells where the dye incubated alone.

Negligible increment of ThT fluorescence when the test peptides are co-incubated with A β ₄₂ peptide clearly indicates the inhibition of fibrillation. It also provides further support to the inhibition of A β ₄₂-induced neuronal toxicity as studied in the MTT assay. The % inhibition of A β ₄₂ aggregation as depicted by the ThT fluorescence assay is in accordance with the % cell viability data obtained by the MTT assay. Exact correlation cannot be established between the two because of the differential behavior of A β ₄₂ in the presence of a cellular environment as well as the treatment/incubation time for both the experiments.

Peptides **12c** and **12f** that exhibited >98% cell viability at the lowest tested concentration of 2 μ M were then evaluated at lower dose concentrations of 1.0 μ M, 0.5 μ M and 0.1 μ M, against 2 μ M of A β ₄₂ maintaining the ratios of 1 : 2, 1 : 4 and 1 : 20 (test peptide : A β ₄₂), respectively. The graphical plot of dose dependent modulation of A β ₄₂ aggregation-induced-neurotoxicity in PC-12 cells is depicted in Fig. 1C. Peptide **12c** exhibited 78% inhibition of A β ₄₂ even at a lowest tested dose of 0.1 μ M. A graphical representation of the % decrease in RFU has been depicted in Fig. 1B and dose dependent % inhibition of A β ₄₂ aggregation has been depicted in Fig. 1D. Excellent activities were exhibited when the test peptide is present in equimolar concentrations of A β ₄₂, indicating 1 : 1 inhibition of the parent peptide.

Upon the basis of careful analysis of data reported herewith and results published earlier,^{10–12} a correlation between the amino acid residues within the tetrapeptide sequence and the exhibited activity was established. Replacement of the first residue, Val₃₉ with hydrophobic residues (Phe and D-Phe) exhibited >90% inhibition. The replacement of Val₄₀ with hydrophobic (Phe, D-Ile) or conformationally restricted amino acids (Pro, Aib) slightly enhanced the potency of the peptide. This holds true even in dual substitution at Val₃₉ and Val₄₀. Any replacement or modification yielded less active derivatives, indicating Ile₄₁ is critical for activity. Small analogous amino acid is preferred at the Ala₄₂ for retaining the activity. Amidation of the C-terminus results in enhancement of inhibition potential for some peptides. In some cases, a decrease in activity is also observed. A summary of the SAR is shown in Fig. 2. Understanding the sequential positioning of these residues



Table 1 % cell viability against A β ₄₂ aggregation-induced-neurotoxicity and % inhibition of A β ₄₂ aggregation exhibited by the test peptides

No.	Test peptide sequence ^a	MTT cell viability assay			ThT-fluorescence assay		
		Test peptide concentration range (A β ₄₂ : test peptide)					
		10 μ M (1 : 5)	4 μ M (1 : 2)	2 μ M (1 : 1)	10 μ M (1 : 5)	4 μ M (1 : 2)	2 μ M (1 : 1)
		% viable cells ^b					% inhibition ^d
11	Val-Val-Ile-Ala-OH (lead)	93.6	90.5	78.9	66.2	60.6	33.9
11a	Val-Val-Ile-Ala-NH ₂	82.2	89.3	86.4	52.1	52.7	58.8
12a	D -Val-Val-Ile-Ala-NH ₂	81.6	100.0	94.8	82.1	83.5	64.7
12b	Phe -Val-Ile-Ala-NH ₂	92.1	95.7	96.2	77.6	66.2	51.9
12c	D - Phe -Val-Ile-Ala-NH ₂	100.0	95.2	98.4	100.0	100.0	100.0
12d	D - Pro -Val-Ile-Ala-NH ₂	92.0	83.5	82.8	39.8	52.9	60.8
12e	Nva -Val-Ile-Ala-NH ₂	83.4	86.1	74.1	70.3	51.9	77.2
12f	Aib -Val-Ile-Ala-NH ₂	61.3	95.1	100.0	93.8	66.0	64.5
12g	Gly -Val-Ile-Ala-NH ₂	75.4	94.3	92.1	98.1	64.5	84.4
13a	Val- D -Val-Ile-Ala-NH ₂	87.6	75.2	74.7	15.7	46.5	89.3
13b	Val- D - Ile -Ile-Ala-NH ₂	94.6	92.8	90.3	49.4	35.1	65.6
13c	Val- Pro -Ile-Ala-NH ₂	83.1	96.0	93.0	18.5	16.3	31.8
13d	Val- Aib -Ile-Ala-NH ₂	100.0	93.0	75.1	45.3	50.0	52.1
13e	Val- Phe -Ile-Ala-NH ₂	93.3	100.0	79.3	57.2	61.7	100.0
13f	Val- D - Phe -Ile-Ala-NH ₂	99.7	92.9	94.2	62.5	66.2	75.8
14a	Val-Val- D - Ile -Ala-NH ₂	69.6	76.5	79.7	21.8	25.3	49.6
14b	Val-Val- Leu -Ala-NH ₂	86.3	100.0	83.8	0.0	16.5	42.2
15a	Val-Val-Ile- D - Ala -NH ₂	93.8	80.2	83.3	23.2	14.6	68.6
15b	Val-Val-Ile- Aib -NH ₂	99.1	100.0	71.9	35.1	42.0	24.9
15c	Val-Val-Ile- Gly -NH ₂	90.5	88.5	80.7	5.4	31.8	62.7
15d	Val-Val-Ile- Val -NH ₂	71.7	75.4	64.5	11.0	6.9	0.0
15e	Val-Val-Ile- Leu -NH ₂	70.6	71.7	89.4	31.2	30.0	18.7
15f	Val-Val-Ile- Ile -NH ₂	100.0	97.0	78.6	48.2	0.0	0.0
16a	Pro-Pro -Ile-Ala-NH ₂	77.7	60.7	74.6	14.2	18.3	24.7
A β ₄₂			74.05				
Control ^c			100.0				

^a Amino acid residue modified within the tetrapeptide sequence is indicated in bold. ^b Cell viability studies were performed using MTT cell viability assay against PC-12 cells. ^c The percentage of untreated cells was considered 100% (positive control); percentage cell viability was calculated for the cells incubated along with A β ₄₂ (2 μ M) in absence (negative control) and presence of the test peptides in respective dose concentrations for 6 h. % of viable cells was calculated by the formula as $100 \times [\text{A}\beta_{42} + \text{test peptide OD}_{570} - \text{A}\beta \text{ OD}_{570} / \text{control OD}_{570} - \text{A}\beta \text{ OD}_{570}]$. In a subset of triplicate wells, standard deviation values ranged 1.81–4.72. ^d Inhibition of A β ₄₂ aggregation was calculated by Thioflavin-T fluorescence assay. % relative fluorescence units (% RFU) exhibited by A β fibrils were considered as 100%. ThT dye incubated alone was considered as control and % RFU units were computed when A β ₄₂ was co-incubated with the test peptides for 24 h (λ_{ex} 440 nm, λ_{em} 485 nm). % inhibition of ThT fluorescence was calculated by using the formula: $100 \times [100 - (\text{A}\beta_{42} + \text{test peptide RFU}_{485} - \text{control RFU}_{485} / \text{A}\beta_{42} \text{ RFU}_{485} - \text{control RFU}_{485})]$. In a subset of triplicate wells, SD values ranged 1.22–4.83. Data for both the experiments was recorded for triplicate samples and the readings were averaged (<5% variation).

would help us further develop derivatives with enhanced potency to inhibit A β ₄₂ aggregation.

It is reported that there are two species of A β *i.e.* A β ₄₀ and A β ₄₂, present in the diseased brain.^{18–20} Evaluating the inhibitory activities of the test compounds on the aggregation of both the species would be of biological significance.²¹ Therefore, inhibitory potential of peptide 12c was evaluated on A β ₄₀. The relative increase in the ThT fluorescence when A β ₄₀ was incubated alone for 24 h was very less or similar to that of the control wells containing ThT (Fig. 3A). Further testing for 48 h and 72 h, respectively yielded no substantial results (ESI, Table S3†).

The minimal increase in fluorescence could be attributed to the slower nucleation rate and longer lag phase in the kinetics of A β ₄₀ fibril formation in comparison to A β ₄₂.^{22–24} Also, A β ₄₀ is relatively less neurotoxic and its aggregation propensity enhances in the presence of A β ₄₂, although the former is present in a ten-fold higher concentration. Thus, evaluation of

the inhibitory activity of test peptide 12c on the mixtures of A β ₄₀ : A β ₄₂ in the ratio of 10 : 1 was performed. On incubation of a mixture of 5 μ M of A β ₄₀ and 0.5 μ M of A β ₄₂ (ratio of 10 : 1) the % relative increase in ThT fluorescence was comparatively higher than when A β ₄₀ was incubated alone (Fig. 3B). When compared to that of A β ₄₂ incubated alone as analyzed in the previous experiments, the fluorescence intensities were less. This gave us a clear indication that the aggregation propensity of A β ₄₀ is amplified in the presence of 0.1 equimolar A β ₄₂. On co-incubation of the test peptide 12c with the 5 μ M mixture of A β ₄₀ : A β ₄₂ in the ratio of 10 : 1, substantial decrease in the fluorescence levels were observed (ESI, Table S4†).

As a prophylactic measurement, the potential ability of the test peptides to deform the aggregated A β ₄₂ was investigated.^{25,26} Monomeric A β ₄₂ was pre-incubated for a period of 24 h and fluorescence was measured. As anticipated, there was a marked increase in the fluorescence due the fibril state of



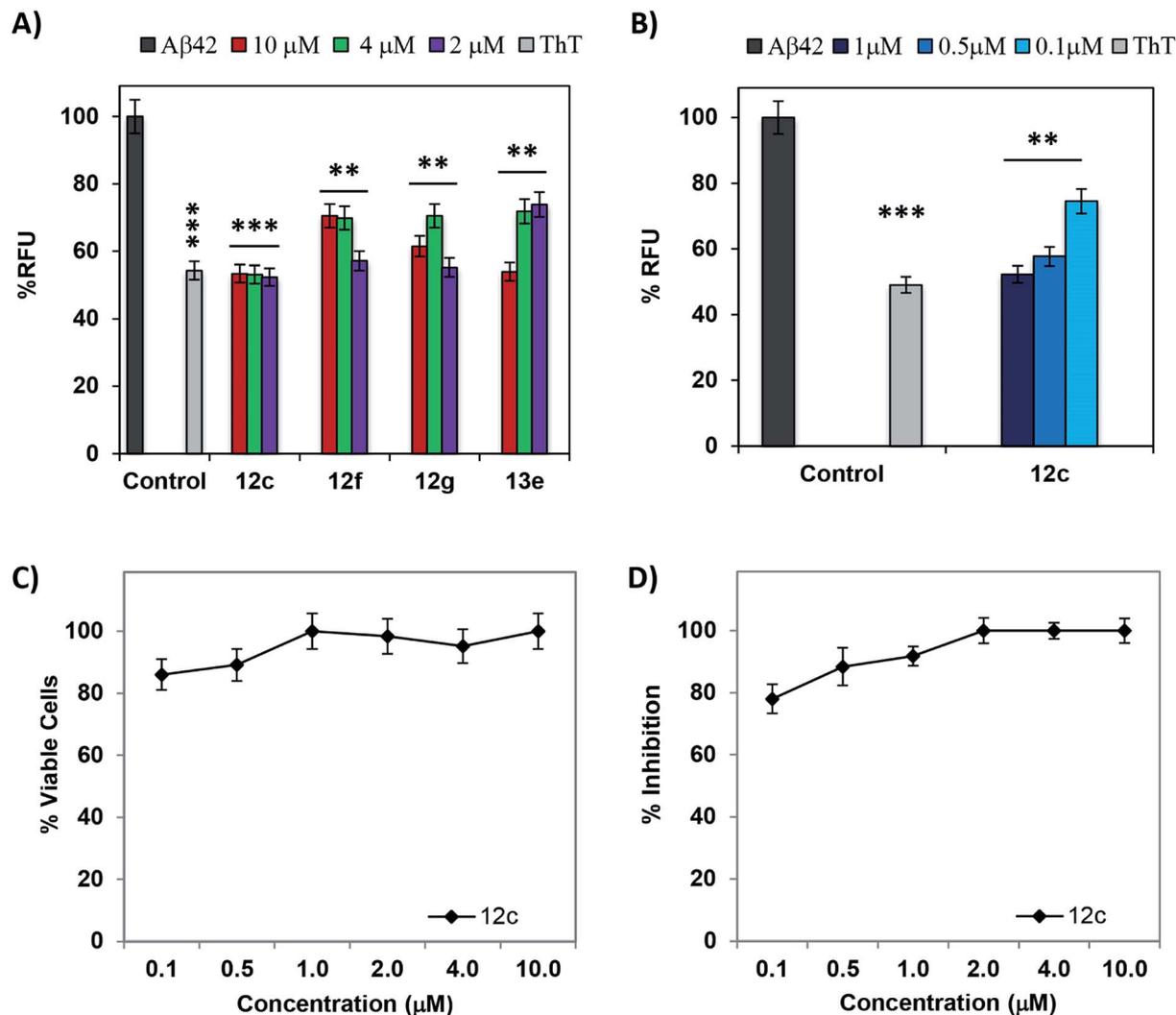


Fig. 1 Effect of most active test peptides on Aβ₄₂ aggregation: bar plots depicting the decrease in % RFU of ThT dye when Aβ₄₂ (2 μM) was co-incubated with test peptides at higher doses (A), and lower doses (B). Complete fluorescence was represented by the Aβ₄₂ peptide incubated along with the dye (black) and dye control (grey) represents the dye incubated alone. Subsequent bars represent Aβ peptide co-incubated with the varying concentrations of inhibitor peptides for 24 h. Significance values indicated with respect to the Aβ peptides, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (C) Dose dependent modulation of Aβ₄₂ aggregation-induced-neurotoxicity in PC-12 cells exhibited by the test peptide 12c (black). (D) Concentration dependent % inhibition on Aβ₄₂ aggregation mediated ThT fluorescence exhibited by the test peptide 12c (black). % inhibition of ThT fluorescence was calculated by using the formula: $100 \times [100 - (A\beta_{42} + \text{test peptide RFU}_{485} - \text{control RFU}_{485}) / (A\beta_{42} \text{ RFU}_{485} - \text{control RFU}_{485})]$. Readings (λ_{ex} 440 nm, λ_{em} 485 nm) was recorded for triplicate samples from three individual experiments and the readings were averaged (<5% variation). Error bars represent mean \pm SD ($n = 3$). Data were analyzed by one-way anova test.

Aβ₄₂. Test peptide was added to each of the wells at the respective dose concentrations and readings were recorded in a time dependent manner until 120 h of incubation. Time dependent % deformation of preformed fibrils in the presence of equimolar test peptide has been depicted in Fig. 4A. It could be observed that peptide 12c has the potential of deforming pre-aggregated Aβ₄₂ fibrils (>35%) until 48 h of incubation. Also, peptide 12c significantly reduced the fluorescence of Aβ₄₂ showing 57.5, 34.9 and 33.7% inhibition at 2, 1 and 0.5 μM, respectively after 24 h treatment. % inhibition and % RFU for time intervals of 24 h and 48 h has been provided in the ESI, Table S5.†

A time dependent ThT fluorescence assay was performed on the most active test peptide 12c at the similar concentrations of

2, 1 and 0.5 μM with Aβ₄₂ (2 μM) for a period of 7 days. Readings were recorded at regular time intervals of 24 h each. Fig. 4B shows the decrease in the RFU values when Aβ₄₂ was incubated in presence of peptide 12c. Aβ₄₂ on incubation alone with the ThT dye showed an enhancement in the fluorescence of about 57% that could be attributed to the aggregation of the Aβ₄₂ peptide. The fluorescence shown by the blank wells, wherein the dye incubated alone was considered as the control. Compared to the Aβ₄₂ sample, very low values of fluorescence were observed in the presence of peptide 12c. % inhibition of Aβ₄₂ aggregation exhibited by the test peptide has been summarized in ESI, Table S6.† It can be summed that peptide 12c exhibits activity on preformed Aβ₄₂ fibrils as well as inhibits Aβ₄₂ aggregation until 120 h of treatment.



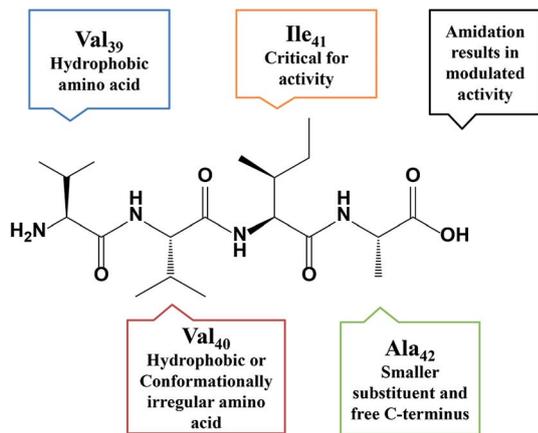


Fig. 2 Derived structure–activity-relationship of tetrapeptides.

ANS fluorescence assay was performed in complimentary to Thioflavin-T assay.^{27–29} The effect of test peptide inhibiting the process of A β ₄₂ aggregation as well as deformation of the pre-formed A β ₄₂ fibrils was evaluated. The relative fluorescence for A β ₄₂ fibrils was considered to be 100% and decrease in the % RFU when test peptides were co-incubated with A β ₄₂ was computed. The fluorescence emitted by binding of ANS to the test peptide itself was subtracted as the test peptide is hydrophobic. The results for relative decrease in fluorescence obtained in both the sub-experiments have been summarized in Fig. 5A. Upon co-incubation of A β ₄₂ and test peptide **12c** in ratios 1 : 1 and 1 : 0.5, a marked decrease in the fluorescence intensity was observed, in comparison to that of the lowest tested concentration of 0.5 μ M. These results are in accordance to the results seen in ThT fluorescence assay.

It is hypothesized that the aggregated soluble oligomeric form of A β ₄₂ interacts with the neuronal membranes by

hampering cellular processes and exhibiting neurotoxicity.⁷ The design of the experiment was similar to the MTT test conditions, wherein giant unilamellar vesicles (GUVs) with composition mimicking the rat neuronal myelin were prepared (ESI,† Section 5.1) and interaction of A β was evaluated by ANS fluorescence measurements.^{29–32} When A β ₄₂ was just added to the prepared GUVs ($t = 0$ h), ANS showed a good emission spectrum from 450 to 550 nm. After 24 h incubation of A β with the vesicles, no emission band was seen, indicating the absence of hydrophobic binding domain, thus no fluorescence.

This could be attributed that the hydrophobic region entered the vesicles, providing no binding site for the dye. Emission intensities obtained for the vesicles alone was considered as blank and was subtracted from the readings obtained for both the time points. To evaluate the effects of incubation of test peptides and its inhibitory effect on A β ₄₂ aggregation, A β ₄₂ and test peptide **12c** along with the GUVs was incubated for 24 h at 37 °C and the relative change in the fluorescence was observed. Readings for the test peptide incubated alone with the vesicles at the similar concentration were subtracted the final readings so as to obtain a comparable result for A β . On co-incubation of A β ₄₂ with **12c**, two distinct observations were seen, primarily a visible emission spectrum and secondly a blue shift with a slight increase in the emission intensity (Fig. 5B). The emission spectrum indicates that the hydrophobic region was available for binding to ANS, thus indicating that A β was available in its monomeric form itself. The increase in emission intensity and the blue shift does suggest certain interactions between **12c** and the full-length A β , which results into differential binding of ANS to the test peptide–A β complex (ESI, Fig. S2†).

Monitoring intrinsic Tyr fluorescence of A β ₄₂ during fibril formation and interaction with most active test peptides was also studied. Tyr has significantly lower quantum yield than Trp

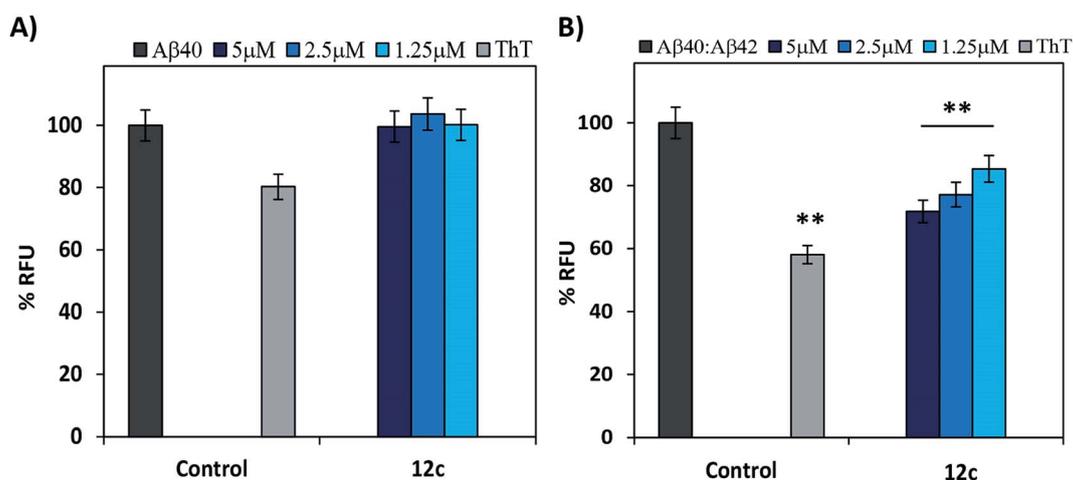


Fig. 3 Thioflavin-T fluorescence studies on A β species: % RFU exhibiting the effect of peptide **12c** on aggregation of (A) A β ₄₀ (5 μ M) and (B) A β ₄₀ & A β ₄₂ mixture (5 μ M) mediated ThT fluorescence. Complete fluorescence was represented by A β ₄₀ and the 10 : 1 mixture of A β peptides incubated along with the dye (black) and dye incubated alone (grey). Subsequent bars represent the respective concentrations of inhibitor peptide **12c** co-incubated with the corresponding A β peptides for 24 h. Readings (λ_{ex} 440 nm, λ_{em} 485 nm) was recorded for triplicate samples from three individual experiments and the readings were averaged (<5% variation). Error bars represent mean \pm SD ($n = 3$). Data were analyzed by one-way anova test. Significance values indicated with respect to the A β peptides, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.



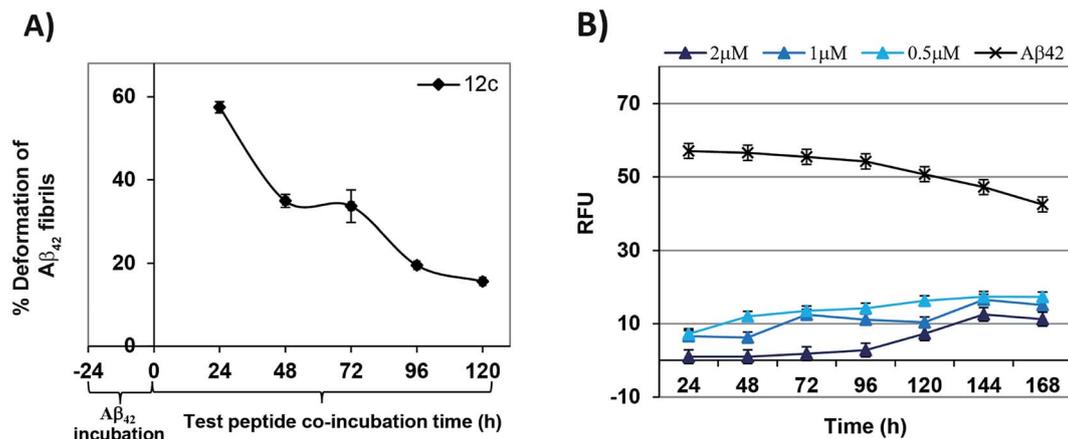


Fig. 4 Time dependent inhibition of Aβ₄₂: (A) % deformation or disaggregation of preformed Aβ₄₂ fibrils in presence of equimolar concentration of test peptide 12c as evaluated via ThT fluorescence assay. (B) Time and concentration dependent RFU comparison depicting the effect of individual tetrapeptide 12c on Aβ₄₂ mediated-ThT fluorescence. % inhibition of ThT fluorescence was calculated by using the formula: $100 \times [100 - (\text{A}\beta_{42} + \text{test peptide RFU}_{485} - \text{control RFU}_{485}) / \text{A}\beta_{42} \text{ RFU}_{485} - \text{control RFU}_{485}]$. Readings (λ_{ex} 440 nm, λ_{em} 485 nm) was recorded for triplicate samples and the values were normalized to the ThT dye control and averaged (<5% variation). Data was interpreted from three individual experiments. Error bars represent mean \pm SD ($n = 3$). Data were analyzed by one-way anova test.

and is usually only used as an intrinsic fluorescent probe in Trp-lacking proteins or peptides, since energy transfer to Trp residues usually quenches the Tyr fluorescence. Tyrosine shows a typical emission band at 305 nm, which is red shifted to 340 nm due to resonance of the phenol to phenolate ion. We hypothesized that in aggregated state of Aβ₄₂, stacking of phenolate ions of the tyrosine residues will produce slightly enhanced fluorescence in comparison to that of the monomeric

or non-aggregated state. It could be clearly seen, in the overlay of fluorescence spectrum for Aβ₄₂ incubated alone for 0 h (green) and 24 h (black), as well as in presence of 12c (blue, Fig. 5C) that the monomeric state of Aβ₄₂ was retained in presence of the test peptides. A comparative bar plot analysis (ESI, Fig. S3†) of the fluorescence response at 340 nm has been indicated to compare the change in observed fluorescence intensities.

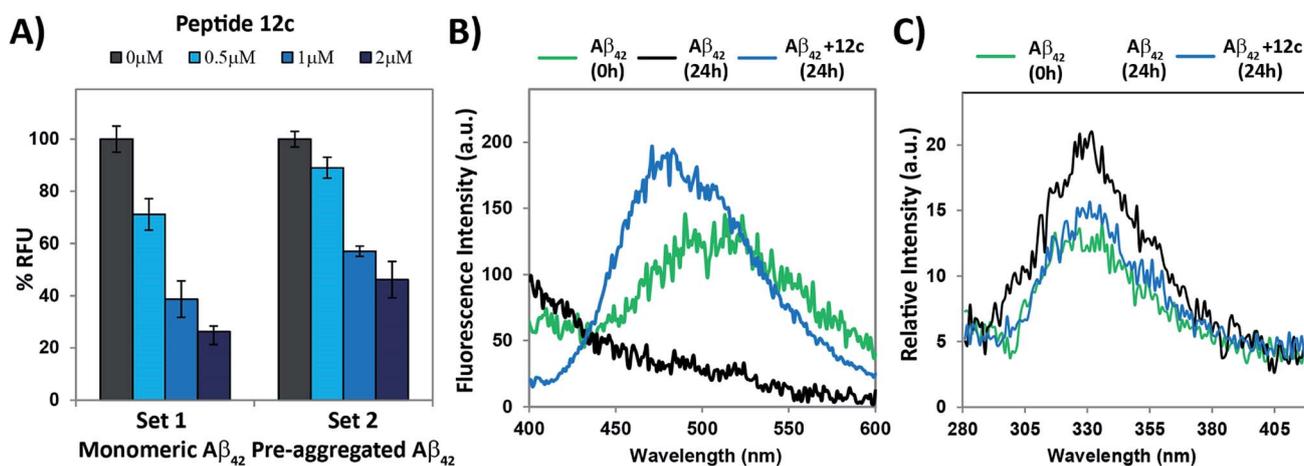


Fig. 5 Additional fluorescence studies: (A) effect of varying concentration of tetrapeptide 12c on inhibition of Aβ₄₂ fibril formation (Set 1) and on pre-aggregated fibrils of Aβ (Set 2). Complete fluorescence was represented by the Aβ₄₂ (2 μM) incubated alone, monomeric (Set 1) and pre-aggregated $t = 24$ h (Set 2) and in the presence of respective concentrations of the test peptide 12c after 24 h. ANS dye incubated alone was considered as control and % RFU units for individual samples were computed by normalizing to the ANS dye control (λ_{ex} 480 nm, λ_{em} 535 nm). Subsequent bars represent the % RFU of the respective concentrations of the inhibitor peptide 12c co-incubated with the differential states of Aβ₄₂ peptide (2 μM) for 24 h. (B) Fluorescence spectrum showing effect of test peptide on Aβ₄₂ aggregation and its interaction with GUVs. Fluorescence of Aβ₄₂ alone at 0 h (green), 24 h (black); along with test peptides 12c (blue) after 24 h in the presence of GUVs (λ_{ex} 480 nm, λ_{em} 400–600 nm). ANS dye incubated alone was considered as control and relative FL. Intensities for individual samples were computed by normalizing to the ANS dye control. (C) Intrinsic tyrosine fluorescence of Aβ₄₂ during fibrillation and inhibition by test peptide 12c (λ_{ex} 260 nm, λ_{em} 280–410 nm). Fluorescence of 5 μM Aβ₄₂ ($t = 0$ h, green), 5 μM Aβ₄₂ incubated alone ($t = 24$ h, black), Aβ₄₂ co-incubated along with 5 μM of the test peptides, 12c ($t = 24$ h, blue). Readings was recorded for triplicate samples from three individual experiments and were averaged (<5% variation). Error bars represent mean \pm SD ($n = 3$). Data were analyzed by one-way anova test.



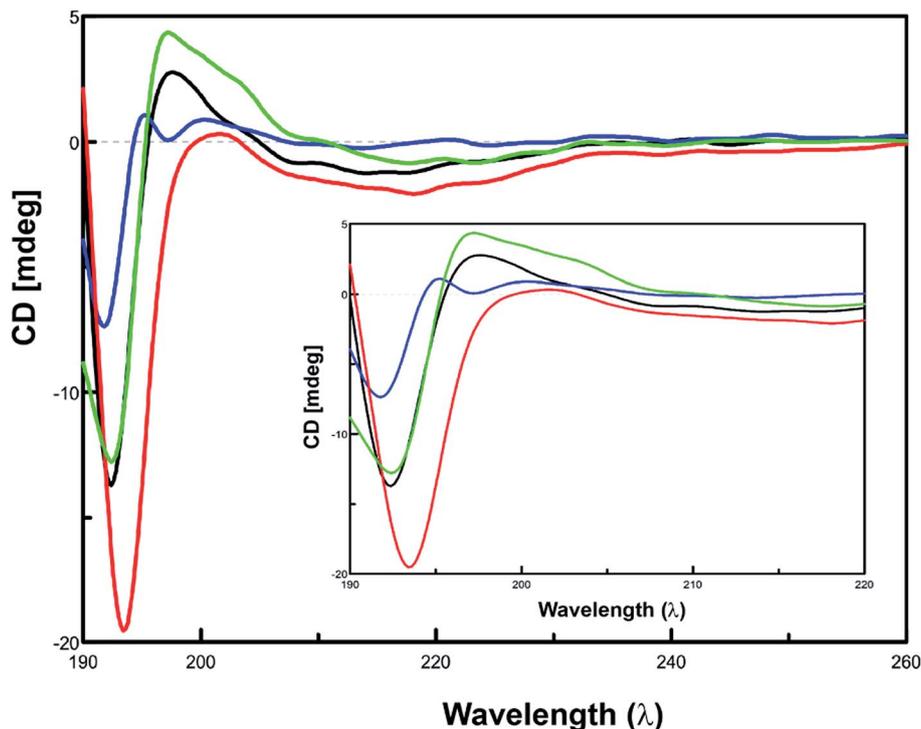


Fig. 6 Secondary structure analysis using CD: CD spectrum showing the conformational changes on $A\beta_{42}$ aggregation in the presence of active peptide **12c** and inactive peptide **13a**. $A\beta_{42}$ (10 μ M) at 0 h (black) and 24 h (blue), co-incubated individually with equimolar ratios inhibitor peptide **12c** (green) and inactive peptide **13a** (red) for 24 h.

Since amyloid- β aggregation is preceded by the conformational transition towards increasing β -sheet structure, monitoring the content of β -sheet formation would therefore depict the effect of inhibitors on the aggregation of $A\beta_{42}$.^{32,33} The effect of inhibitor peptides on the conformation of $A\beta_{42}$ was assessed *via* CD spectroscopy.^{34–36} Spectra of equimolar concentrations of the individual test peptides were subtracted from the corresponding spectra of inhibitor peptides co-incubated $A\beta_{42}$. Predicted values of conformation are summarized in ESI, Table S7.† When incubated alone, $A\beta_{42}$ exhibited a conformational transition from random coiling and turn to majorly β -sheet form. Initially, $A\beta_{42}$ peptide majorly comprised of 49.4% β -sheet conformation. At the end of 24 h incubation period, β -sheet content increased to 66.4%, with the α -helix content increasing from 6.3% to 17.0%. In the presence of inhibitor peptide **12c**, β -sheet form completely vanished and turns and random coiling

was seen to be present in 46.6 and 41.0% respectively. This clearly indicates that **12c** inhibits β -sheet formation propensity of $A\beta_{42}$. The spectral curve obtained for $A\beta_{42}$ ($t = 24$ h) shows the presence of a positive maxima at 195 nm (black), clearly indicating the conformation of the peptide in the β -sheet form, is absent in the former that is, $A\beta_{42}$ ($t = 0$ h), which clearly exhibits a positive maxima at around 205 nm, indicating larger proportion of turn type conformation to be present. No definitive negative minima on the curve were visible at 217 nm, but the shallow curves in the expanded region were indicative of the presence of smaller proportions of α -helix conformations. In the presence of **12c** reduction in β -sheet content can also be visualized by the complete absence of the positive maxima at 195 nm, wherein the spectrum follows the similar pattern to that of the $A\beta_{42}$ ($t = 0$ h). The prevention of conformational transition to β -sheet suggests the ability of **12c** to inhibit the

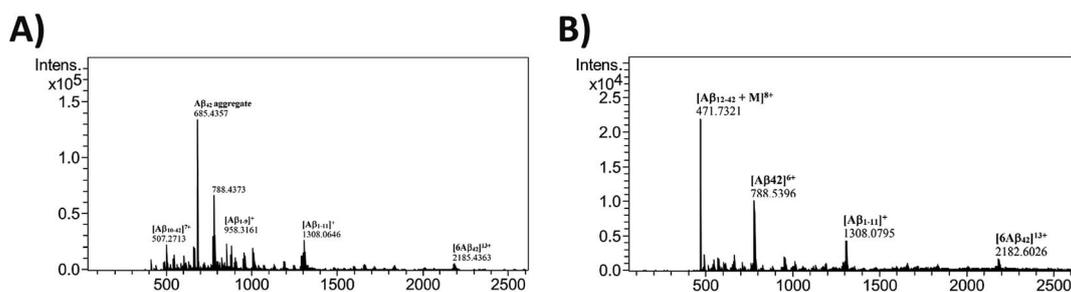


Fig. 7 HRMS Analysis: ESI-MS for $A\beta_{42}$ (10 μ M) incubated alone (A), in presence of equimolar ratios of test peptide **12c** (B) for 24 h.



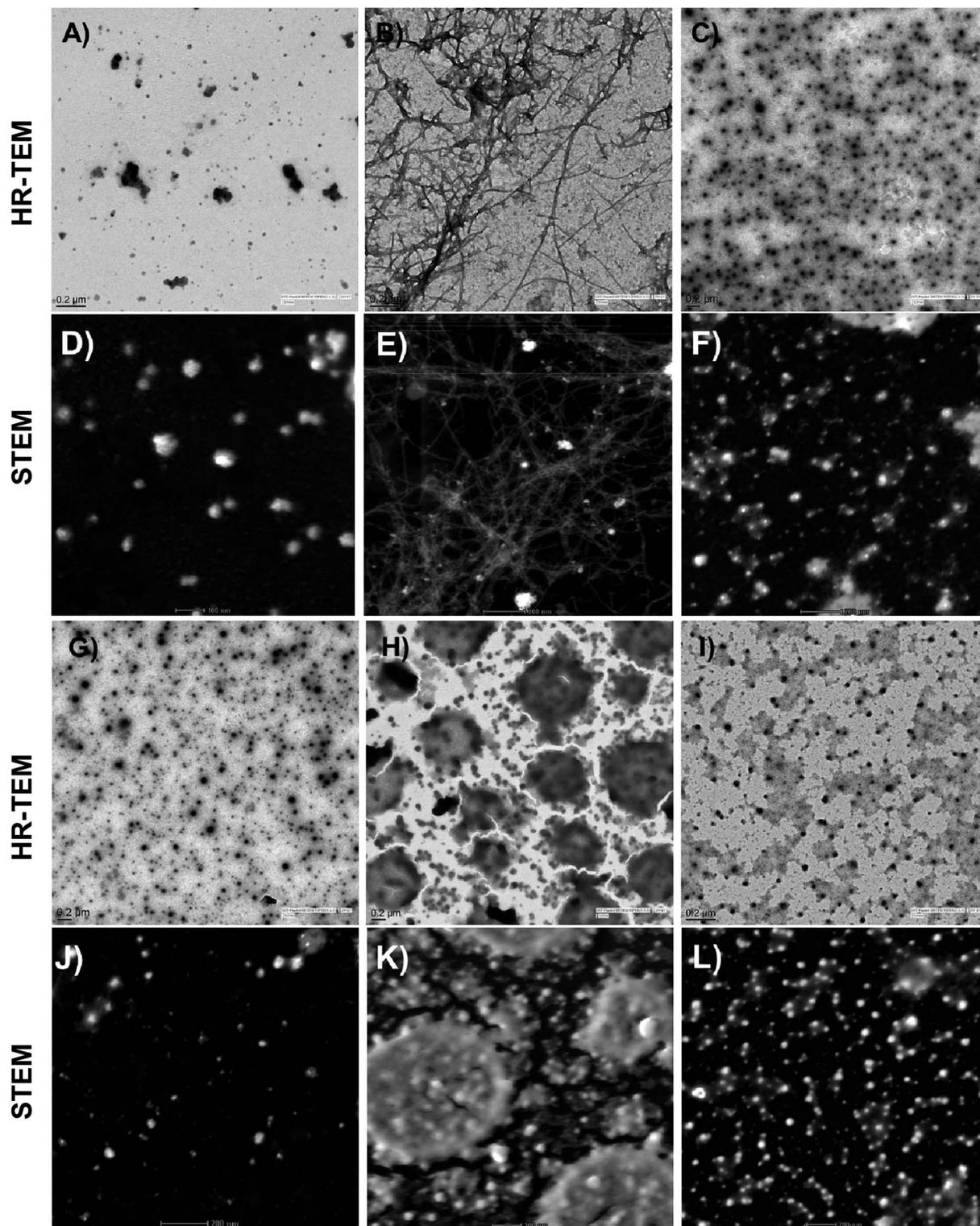


Fig. 8 Electron microscopy studies: HR-TEM and STEM images depicting the effects of active peptide 12c and inactive peptide 13a on the aggregation of A β ₄₂. A β ₄₂ (10 μ M) was incubated alone $t = 0$ h (A and D), $t = 24$ h (B and E); with equimolar concentrations of inhibitor peptide 12c (C and F); inactive peptide 13a (H and K) as well as peptide 12c (G and J) and inactive peptide 13a (I and L) incubated alone, respectively. (Additional images have been provided in the ESI,[†] Section 11.3).

fibrillation process. The positive curve shifts more towards 200–205 nm, indicating a larger proportion of the peptide to be present in the turn form. These observations coincide to the

predicted values by the Yang protocol. The effect of the presence of equimolar concentrations of inactive peptide 13a on the conformational changes on A β ₄₂ was evaluated. A positive



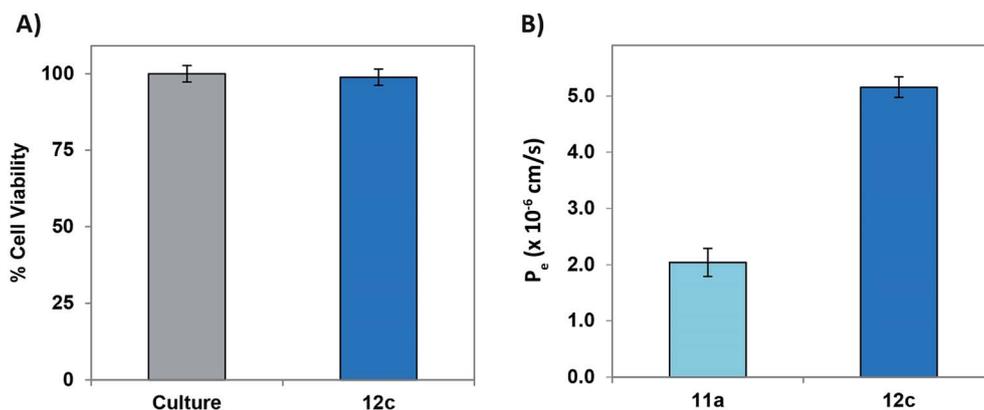


Fig. 9 Cytotoxicity and bioavailability study: (A) analysis of the cytotoxic effects of the peptide **12c** (20 mM) on the viability of PC-12 cells evaluated using MTT cell viability assay. The percentage of untreated cells was considered 100% (positive control) and presence of the test peptides in respective dose concentration for 6 h. (B) BBB-permeability of peptide **12c** in comparison to **11a**, as determined by the PAMPA-BBB assay. P_e was calculated by using the formula $V_d V_a / [(V_d + V_a) S t] \ln(1 - A_a / A_e)$, where V_d and V_a are the mean volumes of the donor and acceptor solutions, S is the surface area of the artificial membrane, t is the incubation time, and A_a and A_e are the UV absorbance of the acceptor well and the theoretical equilibrium absorbance, respectively. Data was recorded for triplicate samples in three individual experiments and the readings were averaged (<5% variation).

maxima at 195 nm is a clear indicative of higher proportions of β -sheet type of secondary structural conformation to be present. This indicates the inactiveness of the peptide **13a** and its inability to prevent aggregation of $A\beta_{42}$. Self-aggregation potential of peptide **13a** deters its potential to inhibit $A\beta_{42}$ aggregation.

A compiled CD spectrum recorded depicting a relative comparison of peptides **12f** and **13a**, incubated for 24 h at 37 °C in the presence and absence of equimolar concentrations of $A\beta_{42}$ has been presented in Fig. 6. Spectra of test peptides incubated alone were subtracted to obtain the final spectra for comparing the conformational state of $A\beta_{42}$. A comparison of the individual CD spectrums of the test peptides incubated alone to that incubated in the presence of equimolar ratio of $A\beta_{42}$ has been summarized (ESI, Fig. S4†).

To understand the process of inhibition of $A\beta_{42}$ in presence of **12c**, mass fragmentation techniques were employed.³⁷ The MS spectrum of full-length $A\beta_{42}$ (10 μ M) in the presence and absence of an equimolar amount of **12c** was recorded. Fig. 7, shows the ESI spectrum for $A\beta_{42}$ incubated alone (A), along with **12c** (B).

The spectrum for $A\beta_{42}$ incubated alone shows a major peak at m/z 685.4357, which may be attributed to aggregated $A\beta_{42}$ depicting higher mass-to-charge ratio. Further peaks at m/z of 788.4373 [$(A\beta_{42})^{6+}$], 507.2713 [$(A\beta_{1-9})^{1+}$], 958.3161 [$(A\beta_{10-42})^{7+}$] and 1308.0646 [$(A\beta_{1-11})^{1+}$] represent the $A\beta_{42}$ monomer and its specific fragments, respectively. Hexameric form of $A\beta_{42}$ with m/z 2185.4363 [$(6A\beta_{42})^{13+}$] is also observed in the spectrum.³⁸ On comparing the spectrum obtained for $A\beta_{42}$ incubated along with **12c**, and that of $A\beta_{42}$ incubated alone, additional signal peaks were seen. This suggested the occurrence of adduct between $A\beta_{42}$ and **12c**, as these peaks were not analogous to the molecular weight of native $A\beta_{42}$ or test peptides themselves. Analyzing the interactions of **12c** with that of $A\beta_{42}$, the mass spectrum shows a peak at m/z 471.7321 [$(A\beta_{12-42} + 12c)^{8+}$],

depicting 1 : 1 covalent interaction with $A\beta_{12-42}$ fragment of $A\beta_{42}$. The spectrum also shows signal peaks corresponding to that of $A\beta_{42}$, seen in the previous spectrum. It was clear from the above spectrum that the test peptide interacts with the monomeric unit of the $A\beta_{42}$, thereby preventing its aggregation.

Visual investigation of the effects of the peptide **12c**, on the morphology and abundance of $A\beta_{42}$ fibrils was performed by high resolution transmission electron microscopy (HR-TEM).³⁹ Shapes and morphology of the fibrils were also examined using scanning transmission electron microscope (STEM).⁴⁰ Inactive peptide **13a** was selected as a negative control. The control sample of $A\beta_{42}$ incubated alone at $t = 0$ h, where uniform distribution of smaller particles of $A\beta$ was seen (Fig. 8A, HR-TEM and Fig. 8D, STEM).

After an incubation span of 24 h, appearance of amyloid fibrils and an extensive network of long, straw-shaped fibrils were observed (Fig. 8B and E). In the presence of peptide **12c** (Fig. 8C and F), only smaller particulate aggregates were seen, indicating complete inhibition of the amyloid fibrils. On co-incubation of $A\beta_{42}$ with the inactive peptide **13a**, large aggregated structures (Fig. 8H and K) were observed. In order to visualize the aggregation of the peptide themselves, equimolar concentrations of peptides **12c** and **13a** were incubated alone under similar conditions and visualized. Very small granular structures were seen for the **12c** (Fig. 8G and J) whereas slightly larger and patchy aggregates were seen for inactive peptide **13a** (Fig. 8I and L).

In order to evaluate and understand the biosafety and pharmacokinetic profile of the test peptides, cell-cytotoxicity studies employing PC-12 cells was performed. Test peptide were tested up to a highest tested concentration of 20 μ M and none of the peptides exhibited undesirable cytotoxicity. Fig. 9A depicts a graphical representation of the % viable cells in presence of 20 μ M concentration of peptide **12c**.



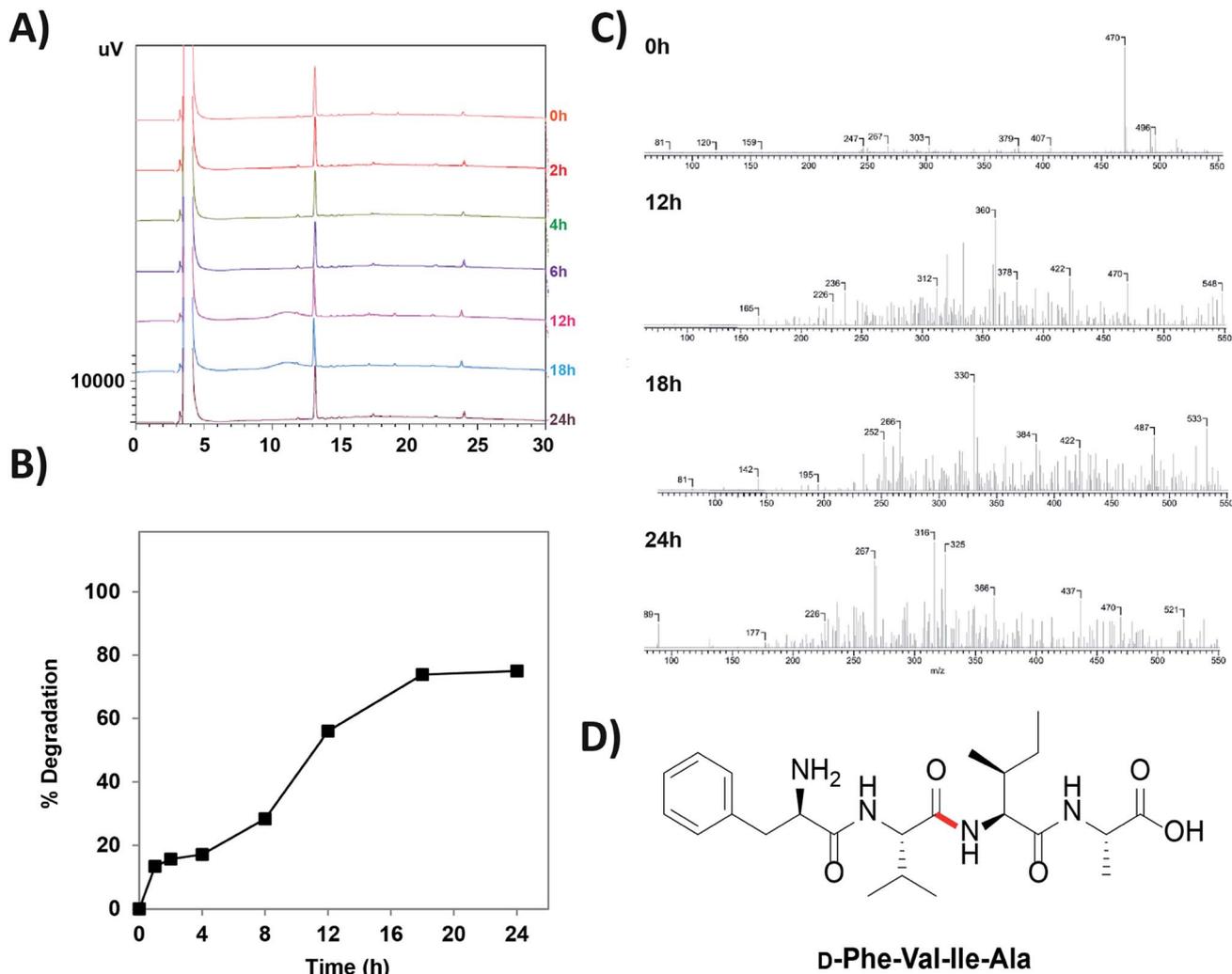


Fig. 10 Proteolytic stability study: (A) superimposed HPLC chromatograms of most active peptide 12c at time intervals of 0, 2, 4, 8, 12, 18 and 24 h after trypsin treatment; (B) graphical representation showing % degradation for peptide 12c on serum treatment. (C) Mass spectra for peptide 12c at 0, 12, 18 and 24 h of serum treatment. Analyzed by ACD-Mass Fragmenter tool. (D) Predicted susceptible cleavage sites for peptide 12c. Most susceptible peptide bond has been indicated in bold red.

A major challenge for peptide-based therapeutics is the BBB permeability and proteolytic stability against various enzymes within the body.⁴¹ *In vitro* BBB penetration of the most active peptide 12c as well as the lead peptide 11a using parallel artificial membrane permeation assay (PAMPA-BBB) was performed following the previously reported protocols.^{42–45} The UV/Vis absorptions of both the peptides was recorded after permeating through an artificial porcine polar brain lipid (PBL) membrane and the effective permeabilities (P_e) were calculated. As described in Fig. 9B, the P_e values of the most active peptide 12c was significantly higher than that of 11a, demonstrating enhanced permeability of the modified peptide.

Trypsin is the one of the most notorious endopeptidases and cleaves the amide bond next to a charged cationic residue.⁴⁶ Hence, in order to evaluate whether the synthesized peptides have incorporated the proteolytic stability properties, trypsin and serum stability studies on the peptide 12c was performed. The peptide was incubated with 100-fold excess of trypsin and

was subjected to analysis by RP-HPLC. Chromatograms depicted that the peptides exhibited intact integrity, having their retention time unaltered even after 24 h of trypsin treatment. It was observed that, there were no peaks seen before and after the main peak of the peptide indicating no fragment and/or other intermediate formation. Superimposed HPLC chromatograms of time point's intervals have been depicted in Fig. 10A.

Serum stability assay following similar protocol was performed. The chromatograms depicted that the peptides exhibited intact integrity, having their retention time unaltered up to 12 h of serum treatment. The peaks obtained for the peptides at 18 h and 24 h of serum treatment, were comparatively smaller to that of the previous peaks indicating slight degradation of the peptide. Superimposed HPLC chromatogram for peptide 12c has been provided in ESI, Fig. S6.†

To calculate the rate of degradation of the peptide on serum treatment, analysis and comparison the area under the curve of the peak of the respective peptide at their specific time



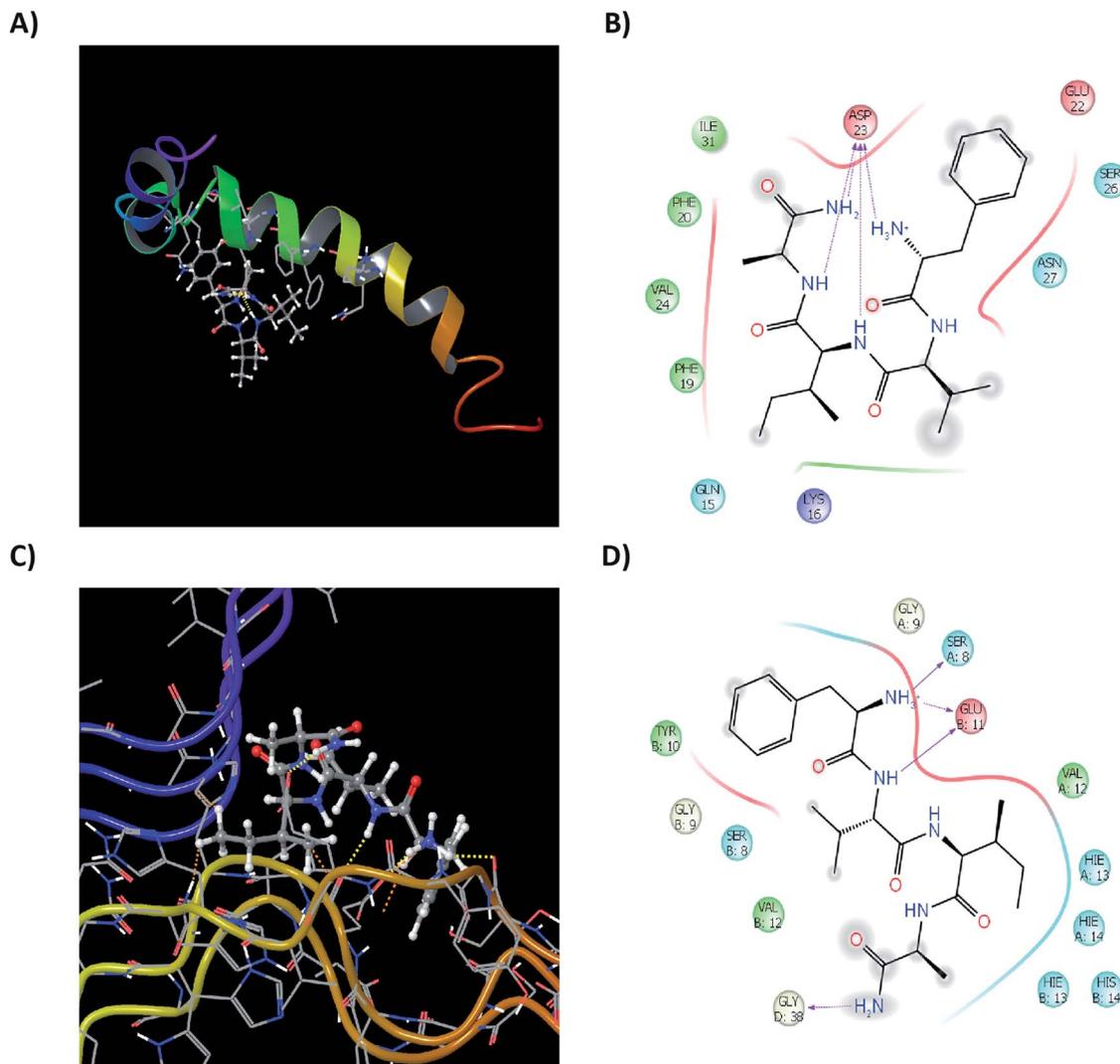


Fig. 11 *In silico* study: ligand interaction diagram showing interactions of the ligand with the residues of monomeric unit 1IYT-10 (A and B) and with the proto-fibrillar unit 2NAO-06 (C and D). 3D representation (Left) showed along with 2D representation (Right).

intervals.^{47,48} A comparative analysis depicted that around 20% of the peptide is present after 24 h of serum treatment. Fig. 10B indicates the % degradation of the peptide in serum over a period of 24 h. The initial calculation of % peptide present in the sample aliquot is indicated in the ESI, Fig. S7.† The extrapolated data helped us to determine the degradation rate of the peptide in serum (ESI, Table S8†).^{48–50} It can be concluded that approximately 50% of the peptide is stable until 12 h of serum treatment, following which it shows a decline in stability decreasing to about 22% until 24 h.

In order to study the mode of degradation of the peptide and the susceptibility of the peptide towards peptide degradation, mass spectroscopy was employed. The samples analyzed for peptide content on RP-HPLC were further subjected to LCQ analysis.^{51,52} At 0 h, the molecular ion peak (m/z 470) of the peptide corresponded to the molecular mass of the peptide itself. Sequential fragmentation pattern was minimal and a clear mass spectrum was seen. After 12 h, multiple

fragmentation peaks were seen indicating that the peptide has undergone cleavage at multiple sites. ACD-Mass Fragmenter tool was used to analyze the specific fragmentation pattern. Fig. 10C summarizes the mass spectrums and shows the specific fragmentation pattern. Based on the fragmentation pattern for the tetrapeptide sequence, the most susceptible bonds that could easily undergo cleavage were identified. ACD Mass Fragmenter tool was used to understand the peptide fragmentation pattern. Fig. 10D depicts the structure of the peptide 12c indicating the most susceptible peptide bond. This understanding provides impetus in site specific modification in improving the stability of the peptides.

Computationally understanding the binding mechanism and intra-residual interactions of the test peptides with the single monomeric as well as the proto-fibrillar unit of A β ₄₂ would prove to be useful. Various structures for the both the forms of A β ₄₂ are reported in the literature. A monomeric sequence bearing complete sequence of all 42 amino acids



residues was used (PDB Id: 1IYT; ESI, Fig. S8†). The structure comprises of α -helices and random coiling, similar to the data previously reported.⁵³ A proto-fibrillar unit comprising of 6 full length spatially arranged in a 'S' shaped manner recently reported by Colvin and co-workers⁵⁴ (PDB Id: 2NAO, ESI, Fig. S10†) was used for understanding the interaction of the test peptides on the proto-fibrillar unit of A β . Reactive site analysis of the pre-optimized framework of the monomeric A β ₄₂ showed that the hinge region is the most prone to aggregation due to the presence of reactive residues in that particular segment (Maestro, Bioluminate suite; ESI Fig. S9†). To understand the interaction of the test peptide with the full-length A β ₄₂, a grid incorporating the whole sequence was generated. Docking studies were performed with the peptides mentioned in this work along with a few molecules reported in literature^{10–12,55,56} for comparative analysis of the binding modes and interactions.^{57–59} Docking, glide and residual interaction energy scores for the molecules selected from literature and test peptides from the current study are summarized in ESI (Tables S9 and S10†). Although the analysis of the scores reveal that similar docking and glide scores were seen in both the cases. The energy of interaction of the test peptides with the monomeric unit proved to be a comparable factor to that of the literature reported ligands (ESI Table S11†).

Test peptides have shown to interact with Glu₁₁, His₁₃, His₁₄, Gln₁₅, Phe₁₉, Phe₂₀ and more specifically with Asp₂₃. These residues are involved to aid the aggregation of monomeric A β ₄₂ into the fibrillar species, as also seen in reactive residue analysis (ESI, Fig. S11†). Binding of the test peptides to these residues, block the free interaction of these residues to others, inhibiting their aggregation propensity. Ligand interactions of the most active test peptide **12c** with the monomeric unit, their respective ligand interaction diagrams (2D and 3D) have been summarized in Fig. 11A and B. The interaction energies are in accordance to those exhibited by the standard ligands indicating similar kind of interactions and thus reinforcing the results of studies carried out in this work. A structural framework 2NAO-06 of proto-fibrillar A β ₄₂ was rationalized to be the most optimal structure and was prepared for docking studies (ESI, Fig. S10†). Since it is a proto-fibrillar unit, the most reactive sites for the binding were identified. SiteMap Analysis feature identified 5 ligand-binding sites (ESI, Fig. S12†). The predicted sites did coincide with the predicted reactive residues, thus indicating certain interactions between those residues and the ligand to be feasible, which would inhibit the process of aggregation. To carry out docking studies, receptor grids at the predicted sites on the proto-fibrillar unit was generated. Since there has been no docking studies performed using 2NAO as the protein, validation of the use of 2NAO and the predicted sites, by docking standard ligands was performed (ESI, Tables S12 and S13†). Analysis of the docking studies revealed SiteMap-2 to be the most plausible site for action of an inhibitor. The molecule can interact with the residues that aid in aggregation. This would block the further attachment of another monomeric unit to the site-recognition units on the proto-fibrillar structure. Subsequent interaction with the neighboring residues of both

the chains destabilizes the preformed bonds, which hold the adjacent units together.

Docking studies of the peptides presented in this work was carried out and docking scores and the residue interaction energies obtained for the set of synthesized tetrapeptides for SiteMap-2 has been summarized in ESI, Table S14.† On careful analysis it can be seen that interaction with Met₃₅, Val₃₆, Gly₃₇ of chain D, as well as Glu₁₁, His₁₃, His₁₄ and Gln₁₅ of the neighboring chain A, show that the test peptide does interact with both the neighboring chains and especially with those amino acids that are solely responsible for maintaining the dimeric structure of the proto-fibrillar unit. To have a clear understanding of the interactions, ligand interaction diagrams for the test peptide **12c**, depicting its interaction with the specific residues of the proto-fibrillar unit have been summarized in Fig. 11C and D.

Conclusion

The peptides described herein show potent inhibition against amyloid aggregation and A β ₄₂ mediated neurotoxicity. Thioflavin-T, ANS and tyrosine fluorescence assays support the results and depict the inhibitory potential of the reported peptides. Inferences from CD studies show that peptide **12c** has β -sheet breaking ability, which was also visually inspected by the absence of A β ₄₂ fibrils in the electron microscopy experiments. Amidated C-terminus protection is a well utilized concept and has enhanced stability of peptide-based therapeutics. Improved BBB permeation along with proteolytic and serum stability parameters provide the designed compounds an attractive biological profile. Peptide **12c** bind to the parent peptide in its monomeric as well as proto-fibrillar form, which aids in inhibiting the process of aggregation as well as destabilization of the preformed fibrils as perceived by *in silico* studies. Excellent potency, enhanced permeability, negligible cytotoxicity along with optimal biological profile, dictate that peptide **12c** is an interesting lead as anti-Alzheimer's disease agent. This study provides further impetus to rational design and development of peptide-based therapeutics for AD.

Abbreviations

A β ₄₀	Amyloid- β _{1–40}
A β ₄₂	Amyloid- β _{1–42}
AD	Alzheimer's disease
Aib	α -Aminoisobutyric acid
APCI	Atmospheric pressure chemical ionization
CD	Circular dichroism
HRMS	High resolution mass spectrometry
HR-TEM	High resolution-transmission electron microscopy
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2'-5-diphenyltetrazolium] bromide
MW	Microwave
Nm	Nanometer
OD	Optical density



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PBS	Phosphate buffered saline
PC-12	Pheochromocytoma-12 cells
RFU	Relative fluorescence unit
RP-HPLC	Reverse-phase high performance liquid chromatography
rt	Room temperature
SAR	Structure–activity relationship
SD	Standard deviation
SPPS	Solid phase peptide synthesis
STEM	Scanning transmission electron microscopy
TBTU	O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
ThT	Thioflavin-T
TIPS	Triisopropylsilane
t _R	Retention time (in chromatography)
UV	Ultraviolet

Conflicts of interest

The authors declare no competing financial interest.

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