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pH and Electrolyte Impact on the Secondary Structure Conformations of Amyloid Beta42

Subhajit Dasgupta[1,2,*], Mausumi Bandyopadhyay[1,2,3,4], Kumar Sambamurti[5]

[1]Regenerative Neuro Immune Research Institute of South Carolina, Charleston, South Carolina
[2]NeuroDrug Research LLC., Charleston, South Carolina
[3]Biology, Trident Technical College, Charleston, South Carolina
[4]Biology, College of Charleston, South Carolina
[5]Department of Neurosciences, Medical University of South Carolina, Charleston, SC

Abstract Amyloid beta42 (Aβ42) is a 4.5 kDa truncated protein from its transmembrane precursor. It is not clear if changes of pH or altered potassium and sodium ions exert an impact on release of Aβ42 from plasma membrane and promote aggregation with altered structural conformation during Alzheimer's Disease. The computer guided experiments by using Preddimer software demonstrate interaction between Aβ42 peptide at pH5, 7 and 8 generates different structural forms with variable dimer Packing value (DPV) and hydrophobicity. The Abeta42_1-21 generated stable dimeric structures with lower DPV and higher hydrophobicity. The Abeta42_22- 42 demonstrates maximum three compatible structures to generate stable dimeric forms with higher DPV and relatively lower hydrophobicity value. The aggregation kinetics demonstrate significant difference (2-folds increase) ($p<0.05$) in thioflavin (THT)-relative fluorescence unit (RFU) at pH5.5 than at pH7.4 (1.2-folds), pH8.5 (1.3-folds) as compared in between the pH groups and buffer control. The increasing potassium chloride (KCl) concentration from 5-100 mM shows moderate increase in THT-RFU (2.1-2.3 folds) with Aβ42 aggregation from 30 minutes till 2460 minutes at pH5.5. The presence of 0.1M NaCl in addition to 0.1M KCl in the reaction mixtures demonstrates an overall increase in Abeta42 aggregation (1.5- 2.1) ($p<0.05$). The circular dichroism (CD) polarimeter demonstrates 0.98% alpha helix, 46.3% beta strand and 53% random coil or irregular structures at 0 hour, pH5.5. More beta-strands and random coils are found with the CD results for 24-48 hours. The presence of KCl, NaCl in the reaction mixture demonstrates marginal increase in alpha helix conformation of Aβ42 aggregates at pH5.5 along with beta-strand structures.

Keywords Amyloid beta42, Protein structure, Electrolyte, Aggregation

1. Introduction

Amyloid beta 42 (Aβ42) is a 4.5 kDa truncated peptide derived from its precursor protein by the aspartyl protease enzyme Gamma Secretase. The peptide is generated from the C-terminal site of amyloid precursor protein (APP) [1-3]. The amphiphilic Aβ42 peptide (42 amino acid residue) is embedded within plasma membrane of neurons in human brain. The deposition of Aβ42 in brain is critical pathology as it forms senile plaques and initiates cognitive impairment leading to dementia in long run. Not only, the amyloid inclusion bodies are found in Parkinson's Disease (PD) and Kreutz-Feld Jacob Disease (KJD)- prion (PrP^{sc}) brain [4-6]. Recent investigations also suggest the role of amyloid protein in aging process of non-Alzheimer's normal brain [7-9]. The structural analysis of amyloids demonstrated formation of altered secondary structures either as soluble or plasma membrane bound forms including oligomers in AD brain [10-12]. The possibility is that the amyloid precursor protein (APP) is synthesized throughout life as monomer. However, release of the Aβ42 from membrane results in extracellular deposition as at least dimeric and later as polymeric structures. It is unclear yet if pH alteration or excess salts, potassium chloride and sodium chloride influence release of Aβ42 from membrane and facilitate aggregation to polymeric structures during AD.

Shea *et al.* [13] demonstrated the presence of non-stranded alpha-sheet secondary conformation in soluble amyloid beta oligomer as toxic component at its early lag phase of aggregation than late beta sheet conformation. In presence of lipids, aggregation of amyloid beta (1-42) (in reaction mixture) to fibrils and oligomers has been found much faster at pH7.4 *in vitro* [14]. Different laboratories suggested that alteration of secondary conformations of amyloid beta at pH 5 are found causing fibrillation and tetramerization of hydrophobic fragments [15] [16] [17]. Wang *et al.* [18] demonstrated the presence of electrostatic as well as hydrophobic interactions

* Corresponding author:
subhajitdasgupta@outlook.com (Subhajit Dasgupta)
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within the self-assembled monolayers facilitate aggregation of amyloid beta on cell membrane. The impact of micelle formation at the monomer level has been reported to facilitate oligomerization and fibril formation with elongation of amyloid beta (Aβ42 and 40 including Aβ39-43 residue fragments) [19].

The computer guided experiments on the ability between Aβ42 peptides for homodimers demonstrate overall low dimer packing quality ($F_{SCOR} \leq 2.0$) indicating the closest structures are packed together to the weak homodimer forms. The cell free reaction mixtures demonstrate an increase in relative fluorescence unit (RFU) of THT incorporation in Aβ42 with time corresponds to the increase in pH and salt concentration in the reaction mixture.

We herein demonstrate that different pH ranges from acidic and alkaline conditions with presence of excess salts-sodium and potassium chloride, facilitate Amyloid beta42 aggregation in a time dependent manner and alter secondary structure conformation in cell free reaction mixture containing potassium phosphate buffer.

2. Materials and Methods

Reagents. Amyloid beta (Aβ42 20 mg) was purchased from ERI Amyloid Laboratory LLC. (USA) (Dr. James I. Elliott, Ph.D and Margaret M. Elliott, M.S.). Potassium dihydrogen phosphate (KH_2PO4) and dipotassium hydrogen phosphate (K_2HPO4) were purchased from Fisher Scientific, (USA); Thioflavin-T was purchased from Merck (USA).

Analysis of Amyloid beta42 (Aβ42) sequences to form dimeric complex by computer modeling. The ability of Amyloid beta42 amino acid sequences to form dimeric complex were examined by using Preddimer software [20] [21]. The Dimer Packing Quality values (index of stable dimeric structure of alpha helix conformation) (F_{SCOR}) were determined from the amino acid sequences using the software automated calculation mode including angle of torsions and number of molecules to form stable dimers. The hydrophobicity (Kcal/mol) and isoelectric pH point (Ip) with net charges of the Amyloid beta amino acid sequences were determined by PepDraw peptide sequence analysis software.

Amyloid beta42 (Aβ42) suspension. The Aβ42 peptide was dissolved (15 µl/vial) in Hexafluoro isopropanol (HFIP) (Sigma Aldrich, US), air dried and preserved in aliquots (2 mg/ vial) in low binding tubes at -20℃. The working samples were prepared following reconstitution of peptides in 1.0 ml 10 mM NaOH solution in deionized water at room temperature followed by brief sonication (10 minutes per sample on ice with 1 minute burst). The stock Aβ42 suspension (2mg/ml of 10mM NaOH 1.0 ml solution, 446.06 mM) was aliquoted (15 µl/ vial) in low binding tubes and stored at -20℃ until use.

Potassium phosphate buffer and salts, potassium, and sodium chloride stock solutions. The stock potassium phosphate buffer was prepared from 1M each of K_2HPO_4 and KH_2PO_4 buffer stock solutions in deionized water. The sub stock 0.1M buffer was prepared by proportionate mixing of 8.5 ml 1M K_2HPO_4 solution with 91.5 ml KH_2PO_4 solution for pH5.8; 80.2 ml K_2HPO_4 solution with 19.8 ml KH_2PO_4 solution for pH7.4; 94.0 ml K_2HPO_4 solution with 6.0 ml KH_2PO_4 solution for pH8.0. The 10M stock solutions of potassium chloride (KCl) and sodium chloride (NaCl) in deionized water were prepared as excess salt sources. The buffer and salt solutions were stored at room temperature, 25℃.

Preparation of reaction mixture. The reaction mixtures were prepared separately by using 0.1 M potassium phosphate buffer (pH 5.8, 7.4, 8.0) and 1M sodium chloride (NaCl), 1M potassium chloride (KCl) working solutions. Aβ42 (60 µg/500 µl: 26.58 µM reaction mixture) were added to each tube to a final volume of 500 µl). The reaction mixtures were incubated for 0, 24 and 48 hours at 28-30℃. Following incubation, the reaction mixture samples were transferred in a 0.1cm quartz cuvette and analysed for polarization of far UV spectra in circular dichroism for characteristics of protein structures. The tubes containing buffer and salt solutions were used for blank control set up for CD Spectropolarimeter (Jasco J-810).

Fluorescent spectrometry. The rate of increase in Thioflavin-T (THT-T) fluorophore absorbance value (Relative Fluorescence Unit, RFU) in the cell free reaction mixture containing Aβ42 is an index for aggregation. To assess, each well of a 96 well transparent flatbottom, low binding microtiter plate (Coaster3884, Corning) was filled up with reaction mixture containing Aβ42. The aggregation kinetics were determined by THT-fluorescent spectrophotometry (Instrument: SpectraMax M5, Molecular Devices, US) at wavelength range of 440nm (excitation), 485 nm (emission) with cutoff at 475 nm for 70-hour time with 30 minutes interval at 30℃. The reaction mixture (35 µl) contains 32 mM Abeta42 in 10X (0.1 M) KH_2PO4/K_2HPO4 buffer separate for each pH 5.5, 7.4, 8.5 solution. The salt KCl (5mM, 10 mM and 100 mM) were added separately to each reaction mixture with or without 15 mM NaCl and 5.0 µl deionized water to make up total volume. The fluorophore Thioflavin-T (THT-T) (5.0 mM) was added to each of the reaction mixtures to attain final concentration 100 µM per sample in each well. The reaction mixture was equally distributed (35 µl/well) in each well of the microtiter plate, sealed at the top by transparent sheet to avoid loss due to evaporation and placed in the spectrophotometer (Molecular Devices, USA) programmed with intermittent mixing at 30℃.

Circular dichroism (CD). In a separate set of experiment, the reaction mixtures for CD experiments were prepared by adding 250 µl 10 mM KCl and 10 mM sodium chloride (NaCl) to the reaction mixtures (final concentrations was 5mM for each salt solution in deionized water) prior to Aβ42 in 500 µl buffer with and without unrelated protein bovine serum albumin (BSA; final concentration 1mg/ ml) and incubated for the time points 0, 24 and 48 hours. Following each incubation time point, the reaction was stopped by keeping the tubes at -20℃. The reaction mixtures (500 µl) containing amyloid beta42 suspension (in 10mM NaOH in

potassium phosphate buffer) at different pH and 5mM NaCl, 5mM KCl salt solution were tested for alterations in secondary protein structures by Circular Dichroism (CD) machine (Jasco J-810; at a wavelength range of 190 - 300 nm) at room temperature using 0.1 cm quartz cuvette (Starna Cells Inc. USA). The samples were analysed for the presence of alpha helix (negative peak 222 and 208 nm; positive peak 190 nm); beta sheet (negative peak 218 nm; positive peak 195 nm) and random coil/ irregular conformations (negative peak 200 nm: 197-205 nm). The far UV spectrum was used in the CD experiment to identify the strong pi-electron shift at 190 nm and weaker broader peak at wavelength range 210-220 nm. The CD data (ellipticity in milli-degrees) versus absorbance (in nm) were collected by machine in five accumulations. The CD data (milli-degrees) were converted into mean residual ellipticity values (θ, in deg*cm2 / dmol) by using equation with respect to mean residual weight [n = (Molecular weight)/ (Number of amino acids) -1)] of the peptide. The results were analysed by using CAPITO software for CD data and plot [22]. The CD plots are defined as molar CD data (θ, degree -cm^2 / dmol) versus wavelength (nm) [23], [24].

3. Results

Determination of the ability of Amyloid beta42 peptide to form homodimer at different pH range in cell free digital mode of reaction conditions.

The aggregation of Aβ42 in human brain is critical for onset of neurological disorders leading to dementia. To determine the ability of Aβ42 peptides to form homodimers, we performed computer guided experiment by using Preddimer software. The feasibility of formation of homodimer structures depends on the closest alignments of the peptide structures measured by increasing Dimer Packing Quality (F_{SCOR}) values. Results presented in the Table 1 show differential binding abilities of Aβ42 peptides at different pH ranges. At pH5, the fragment of Aβ42 peptide: DAEFRHDSGYEVHH QKLVFFA shows low mean F_{SCOR} value 1.67 ± 0.35 for seven different homodimer forms (n= 7). The F_{SCOR} is 45.25% less ($p< 0.5$) than that of residual peptide sequence: EDVGSNKGAIIGLMVGGVVIA (F_{SCOR} = 3.05 ± 0.502). The observations indicate overall presence of Aβ42 weak dimeric structures in the package of stable conformations formed by DAE---VFFA peptide as compared with relatively stronger dimeric structures formed by EDV---VIA peptide fragment. At pH7 reaction condition, DAE—VFFA peptide fragment of Aβ42 is shown to form weak dimeric structure as its mean F_{SCOR} value (1.55± 0.378; n=8) is 49.16% less ($p<0.5$) than that of the peptide fragment EDV---VIA (F_{SCOR} = 3.049 ± 0.502; n=3) of Aβ42. However, at pH8 reaction condition, the Aβ42 peptide fragment DAE VFFA has been demonstrated mean F_{SCOR} value, 1.349 ± 0.271 (n=8).

The value is 29% less than the residual peptide fragment EDV---VIA. Thus, the reaction mixture with pH8 buffer demonstrates relatively unfavourable condition for maintaining stable aggregated structure for Aβ42. The hydrophobicity value has been found higher for DAE--- VFFA peptide fragment of Aβ42 (A beta42_1_21: +29.41 kcal/ mol) than EDV---VIA peptide (A beta42_22_42: +18.91 Kcal/ mol) which explains the stable package with higher F_{SCOR} value for EDV---VIA fragment of Aβ42 that contains a smaller number of hydrophobic amino acids (Table 1). As found, Amyloid Precursor Protein (APP) amino acid sequence 672-713 (truncated Aβ1-42 sequence) demonstrates hydrophobicity value +40.42 Kcal/ mol.

Table 1. Dimer Packing Quality (F_{SCOR}) values of Amyloid beta 42 amino acid sequences to form homodimer structures

Position of amino acid sequences in Amyloid beta42	Amyloid beta42 Sequences*	FSCOR values†	‡Mean ± s.d.	pH
A beta42_1_21	DAEFRHDSGYEVHHQKLVFFA	1.967, 1.785, 1.772, 1.734, 1.724, 1.064, 1.008 (n=7)	1.67 ± 0.35 (p<0.5)	pH5
A beta42_22-42	EDVGSNKGAIIGLMVGGVVIA	3.675, 3.028, 2.445 (n= 3)	3.05 ± 0.502	
A beta42_1_21	DAEFRHDSGYEVHHQKLVFFA	2.129, 1.89, 1.697, 1.678 1.593, 1.358, 1.282, 0.812 (n= 8)	1.55 ± 0.378 (p<0.5)	pH7
A beta42_22-42	EDVGSNKGAIIGLMVGGVVIA	3.675, 3.028, 2.445 (n= 3)	3.049 ± 0.502	
A beta42_1_21	DAEFRHDSGYEVHHQKLVFFA	2.129, 1.89, 1.697, 1.678 1.593, 1.358, 1.282, 0.812 (n= 8)	1.349 ± 0.271 (p>0.5)	pH 8
A beta42_22-42	EDVGSNKGAIIGLMVGGVVIA	2.464, 1.796, 1.419 (n= 3)	1.893 ± 0.432	

*Amyloid beta42 sequence A beta42_1-21: Isoelectric point (Ip): 5.78 (net charge: -2) and hydrophobicity: +29.41 Kcal/ mol.
Sequence A beta42_22-42: Isoelectric point (Ip): 4.00 (net charge: -1); hydrophobicity: +18.91 Kcal/ mol.
†Preddimer software is used to determine Dimer Packing Quality (FSCOR). Optimum stability of a homodimer depends on FSCOR values calculated from crossing and rotational angles for each valid position of monomers. FSCOR ≥ 2.0 indicates stronger homodimers; 0 <FSCOR ≤ 2.0 values indicate weak dimeric structures.
‡ Level of significance (p-value) is determined by t-test.

Determination of the rate of aggregation of Amyloid beta 42 (Aβ42) at different pH in cell free reaction mixture by thioflavin-T fluorescent spectrometry.

The computer guided experiments determined relatively stable weak aggregated structures formed individually by Aβ42 fragments (1-21 and 22-42 amino acid sequences) to generate homodimers showing low to moderately higher F_{SCOR} value but less hydrophobicity value at different pH in the reaction mixtures. One reason is low hydrophobicity facilitates formation of hydrogen bonds and dipole interactions between intermediate structures to generate stable aggregate structures at least in their dimeric forms at different pH.

In order to determine rate of self-aggregation of Abeta42, we used potassium phosphate buffer (described in Materials and Methods) at different range of pH: 5.5, 7.4, 8.5 in the reaction mixture and incubated Aβ42 peptides at 28-30℃ temperature for aggregation kinetics. Here we hypothesize that, change in pH could alter zwitter ionic structural conformation of amino acid constituents of Aβ42 leading to alteration in self-aggregation. However, the mechanism of self-aggregation of Aβ42 can be more explained by cell free fluorescence spectrometry experiments using fluorophore Thioflavin-T (THT) as probe (excitation at 440 nm; emission at 485 nm).

The 96-well low binding microtiter plate (Coaster 3884, USA) was used for the fluorescence spectrometry. Each well of the plate was filled up with 35µl reaction mixture containing 29.53 µM Aβ42 suspension and Thioflavin-T (5 mM, 1000X, final concentration 1X in each well). The open wells of the plate were then covered by transparent sealer to avoid evaporation. The fluorescence spectrometer (SpectraMax M5, Molecular Devices, USA) was preset at temperature 30℃ to assay Aβ42 aggregation kinetics. The sealed microtiter plate was incubated in the spectrometer for 70 hours programmed for intermittent shaking to attain homogeneous reaction condition during incubation. The results presented in Figure 1 demonstrated aggregation of Aβ42 started at 0 time point with initial THT fluorescence 296, 347, and 352 RFU in the reaction mixture with pH5.5 (Fig.1A), pH7.4 (Fig.1B) and pH8.5 (Fig. 1C) respectively. The blank buffer control (Fig.1D) shows 205 – 188 RFU within the kinetics time range of 30 -2670 minutes at 30℃. The short exponential increment period of THT fluorescence was found to reach 314.12 RFU at the time range 0- 240 minutes (4.0 hours) for reaction mixture with pH5.5; 381 RFU at 0-210 minutes (3.5 hours) for reaction mixture with pH7.4 and 361 RFU at 0- 210 minutes (3.5 hours) for reaction mixture with pH8.5. After, the aggregation kinetics demonstrate to increase at moderate rate at least 2640 minutes (44 hours). Overall, the results demonstrated weak to moderately increase in aggregation kinetics of Aβ42 at pH5.5 while relatively slow Aβ42 aggregation was demonstrated at pH 7.4 and 8.5 as compared with control (Fig.1D). The observation shows significant differences ($p<0.05$) in between the RFU values in experimental pH groups as well as compared with buffer control (Blank).

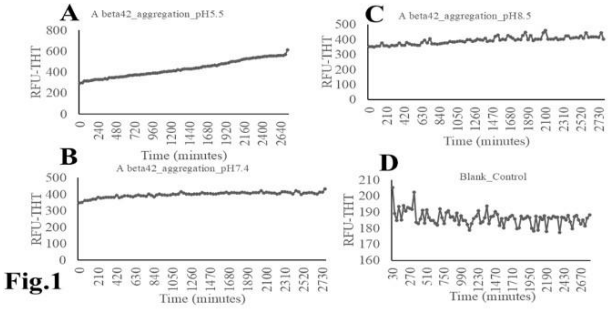


Fig.1

Time course of amyloid beta 42 aggregation at different pH under cell free reaction condition by thioflavin-T Fluorescence spectrometry. Aggregation of amyloid beta 42 (Aβ42) with time was determined under cell free reaction condition as described in the Materials and Methods. The observations (RFU) in the reaction mixtures in the wells for 3600 minutes (60 hours) to 4200 minutes (70 hours) were analyzed in the Excel plot (average of the RFU values) for **(A)** pH 5.5, **(B)** 7.4 (**C)** 8.5 reaction condition, **(D)** blank buffer control for background fluorescence of Thioflavin-T. (ANOVA two factor with replication; $p<0.05$).

Figure 1

Determination of the influence of potassium and sodium chloride salt concentrations on Amyloid beta42 aggregation in cell free reaction mixture by fluorescence spectrometry.

Alteration in sodium ion versus potassium ion concentration in human brain causes ion imbalance and changes membrane potential of cells in the tissue leading to unconsciousness to death. Whether increase in sodium and potassium ion concentration including chloride ion can influence aggregation of Aβ42 is unclear yet. We herewith determined influence of the presence of excess sodium chloride and potassium chloride on Aβ42 aggregation.

The Aβ42 reaction mixtures at pH5.5 (Fig.2), pH7.4 (Fig.3) and pH 8.5 (Fig.4) contain excess salts as 5 mM , 10 mM, and 100 mM KCl solution (described in the Materials and Methods). The results demonstrated an increase in THT fluorescence level in the Aβ42 reaction mixture (pH5.5) from basal level 426.0 RFU (Fig.2A), 362.0 RFU (Fig.2B) and 355.0 (Fig.2C) at 30 minutes time point to 886.0 RFU (2670 minutes), 1060.0 RFU (2670 minutes) and 908.0 RFU (2670 minutes) respectively. The RFU range (205- 188) is shown in the Figure 2D for blank buffer control. The results show significant ($p<0.05$) level of differences between the experimental groups with increasing KCl concentration and with respect to buffer control.

The Aβ42 aggregation kinetics with respect to the THT fluorescence value (RFU) at pH7.4 potassium phosphate buffer reaction mixture (Fig. 3) is comparatively less in presence of 5mM (Fig. 3A) and 10 mM KCl (Fig. 3B) than the corresponding RFU at pH5.5 reaction mixtures (Fig. 2).

The reaction mixture containing 100 mM KCl influences increase in THT fluorescence by 1.2 folds (from 434.0 RFU at 30 minutes time point to 503.0 RFU at 2670 minutes (Fig.3C). The fluorescence level maintained a less increment of RFU till 2670 minutes. The results show presence of increasing KCl concentrations facilitate lower rates of Aβ42 aggregation with respect to the RFU values at pH7.4 ($p>0.05$). Though, the differences between Aβ42 aggregation profiles in the reaction mixtures with KCl at pH 7.4 and pH5.5 are found significant ($p< 0.05$, ANOVA two factors with replication).

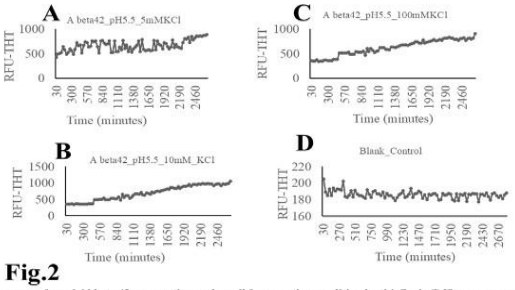


Fig.2

Time course of amyloid beta 42 aggregation under cell free reaction condition by thioflavin-T Fluorescence spectrometry at pH5.5 and presence of increasing concentrations of potassium chloride (KCl). HFIP solubilized Aβ42 in 10 mM NaOH solution (0.443 mM Aβ) sub stock was used to reconstitute the reaction mixtures (30 µl per well of a 96 well microtiter plate) as mentioned in Materials and Methods. The observations (RFU) in the reaction mixtures incubated in the wells for 3600 minutes (60 hours) to 4200 minutes (70 hours) were analyzed by the Excel plot (average of the RFU values) for **(A)** 5mM KCl, **(B)** 10 mM KCl, **(C)** 100 mM KCl and **(D)** blank buffer control for background fluorescence of Thioflavin-T. (ANOVA two factor with replication; $p<0.05$).

Figure 2

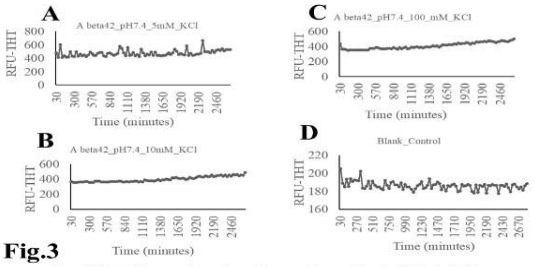


Fig.3

Time course of amyloid beta 42 aggregation under cell free reaction condition by thioflavin-T Fluorescence spectrometry at pH7.4 and presence of increasing concentrations of potassium chloride (KCl). HFIP solubilized Aβ42 in 10 mM NaOH solution (0.443 mM Aβ) sub stock was used to reconstitute the reaction mixtures (30 µl per well of a 96 well microtiter plate). The plate was then sealed and placed in a spectrometer (SpectraMax M5, Molecular Devices) programmed at 440 nm excitation, 485 nm emission wavelength, 30^{0}C reaction temperature to run for 72 hours with 30 minutes interval. The accumulated observations (RFU) in the reaction mixtures incubated in the wells for 3600 minutes (60 hours) to 4200 minutes (70 hours) were analyzed by the Excel plot (average of the RFU values) for **(A)** 5mM KCl, **(B)** 10 mM KCl, **(C)** 100 mM KCl, **(D)** blank buffer control for background fluorescence of Thioflavin-T. (ANOVA two factor with replication; $p<0.05$).

Figure 3

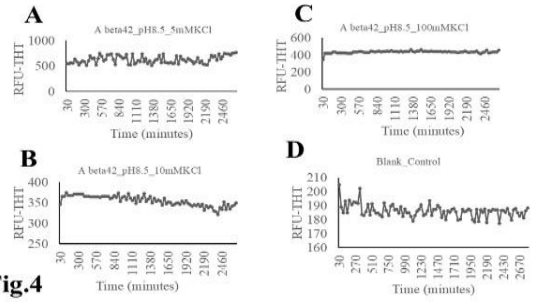


Fig.4

Time course of amyloid beta 42 aggregation under cell free reaction condition by thioflavin-T Fluorescence spectrometry at pH8.5 and presence of increasing concentrations of potassium chloride (KCl). The reaction mixtures (30 µl per well of a 96 well microtiter plate) were distributed in the wells as mentioned in the Materials and Methods. The plate was then sealed and placed in a spectrometer (SpectraMax M5, Molecular Devices) at 440 nm excitation, 485 nm emission wavelength, 30^{0}C reaction temperature to run for 72 hours with 30 minutes interval. The observations (RFU) in the reaction mixtures incubated in duplicate wells for 3600 minutes (60 hours) to 4200 minutes (70 hours) were analyzed by the Excel plot (average of the RFU values) for **(A)** 5mM KCl, **(B)** 10 mM KCl, **(C)** 100 mM KCl, **(D)** blank buffer control for background fluorescence of Thioflavin-T. (ANOVA two factor with replication; $p<0.05$).

Figure 4

The insignificant ($p>0.05$) increase of Aβ42 aggregation in the reaction mixture is demonstrated in pH 8.5 (Fig. 4). While there is 29% increment of Aβ42 rate of aggregation at pH8.5 with 5mM KCl as observed from 30 minutes to 2670 minutes reaction kinetics with change in RFU values from 546.039 to 764.886 (Fig. 4A), we found a marginal ($p>0.5$) decline of Aβ42 aggregation in presence of 10 mM KCl at pH8.5 reaction condition (Fig. 4B). The presence of 100 mM KCl in the reaction mixture at pH8.5 demonstrated 25% increase in THT fluorescence from initial 346.0 RFU at 30 minutes time point to a steady rise till 457.0 RFU up to 2670 minutes thus observed a low level of Aβ42 aggregation without any increase (Fig. 4C) ($p>0.5$). However, we found significant ($p<0.05$) difference in the overall rate of Aβ42 aggregation at pH8.5 in presence of different KCl concentrations as determined by ANOVA two factors in between the experimental groups as well as by comparing the results with the blank buffer control (Fig. 4D).

The presence of sodium chloride (NaCl) alters the aggregation kinetics of Aβ42 (Fig. 5). The combinations of 0.1M sodium chloride and 0.1M potassium chloride salts were found to increase aggregation of Aβ42 in the reaction mixtures at pH 5.5 (Fig.5A), pH 7.4 (Fig.5B) and pH8.5 (Fig.5C) as compared with blank buffer control (Fig. 5D). The THT fluorescence value (RFU) increases by 2 folds at pH5.5 (Fig. 5A), pH 7.4 and pH8.5 within reaction time of 30 minutes to 2550 minutes at reaction temperature, 30℃ ($p<0.05$).

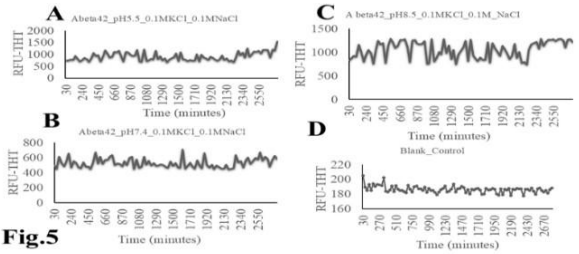


Fig.5

Time course of amyloid beta 42 aggregation under cell free reaction condition by thioflavin-T Fluorescence spectrometry at pH5.5 and 7.4 in presence of increasing concentrations of the mixtures of potassium chloride (KCl) and sodium chloride (NaCl). The reaction mixtures (30 µl per well of a 96 well microtiter plate- same as described above) were distributed in duplicate wells. The plate was then sealed; placed in a spectrometer (SpectraMax M5, Molecular Devices) at 440 nm excitation, 485 nm emission wavelength, 30^{0}C reaction temperature for 72 hours with 30 minutes interval. The observations (RFU) in the reaction mixtures incubated in duplicate wells for 3600 minutes (60 hours) to 4200 minutes (70 hours) were analyzed by the Excel plot (average of the RFU values) for **(A)** pH5.5 reaction mixture with 5mM KCl, 5mM NaCl, **(B)** pH5.5 reaction mixture with 10 mM KCl, 10 mm NaCl and **(C)** pH7.4 reaction mixture with 5 mM KCl, 5mM NaCl, **(D)** Blank control. (ANOVA two factor with replication; $p<0.05$).

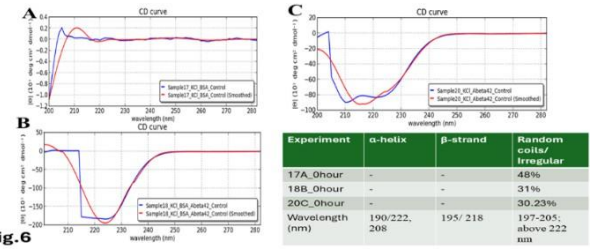
Figure 5

Influence of pH on secondary structure conformation of Aβ42 peptide at different time points of aggregation reaction kinetics.

We wanted to determine whether pH and presence of excess KCl and NaCl salts demonstrate any influence on Aβ42 secondary structure during aggregation?

To find answer, we prepared reaction mixtures including Aβ42 in potassium phosphate buffer with different pH 5.5, 7.4 and 8.5 with excess of KCl, NaCl and analysed the secondary structures of Aβ42 in the reaction mixtures at different time of incubation by circular dichroism (CD) experiments. For this, reaction mixtures containing Aβ42 (26.578 µM) at different pH with excess salts (10mM KCl and 1.0 mM NaCl solution in deionized water) (described in the Materials and Methods), were incubated at 28-30℃ temperature for 0, 24 and 48 hours. Following incubation, the reaction mixtures were analysed individually by CD Spectropolarimeter (Jasco J-810) for demonstration of secondary conformations (alpha helix, β-sheet/ β-coils, Irregular structures) of Aβ42 in the reaction mixtures (wavelength range 190-220 nm) in the experimental samples with reference to buffer and

salt solutions as blank control.

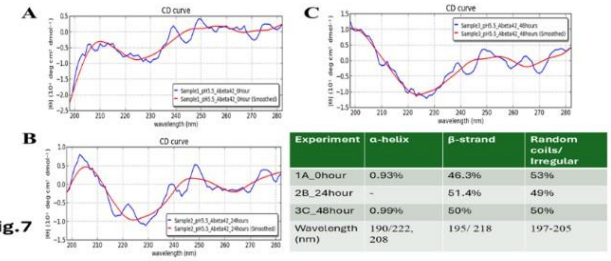


Experiment	α-helix	β-strand	Random coils/ Irregular
17A_0hour	-	-	48%
18B_0hour	-	-	31%
20C_0hour	-	-	30.23%
Wavelength (nm)	190/222, 208	195/ 218	197-205; above 222 nm

Fig.6

Determination of the effect of potassium chloride and unrelated protein bovine serum albumin (BSA) on the secondary structure conformation of Aβ42 during aggregation over time in the reaction mixture by circular dichroism (CD) polarimeter. The reaction mixtures (500 µl) for the CD polarimeter experiments include Aβ42 (26.578 µM/ 1.0 ml 10 mM NaOH in deionized water), unrelated protein bovine serum albumin (BSA) (15.05 µM/ 1.0 ml), potassium phosphate buffer pH7.4, 10mM KCl solution. The Mean Residual Weight for Aβ42 is 110 [n= Mol. wt./ (number of amino acid – 1)] and BSA is 114. The results were analyzed for the presence of secondary structure conformation, alpha helices, beta strand/ beta sheet and random coils/ irregular structures in the reaction mixtures by CAPITO software (as described in the Materials and Methods). Aβ42 mean residual weight (n=110). **(A)** CD curve showing BSA peak in presence of 10 mM KCl **(B)** CD curve showing Aβ42 peak in presence of BSA and 10mM KCl, **(C)** CD curve showing Aβ42 peak with 10 mM KCl in the reaction mixture.

Figure 6



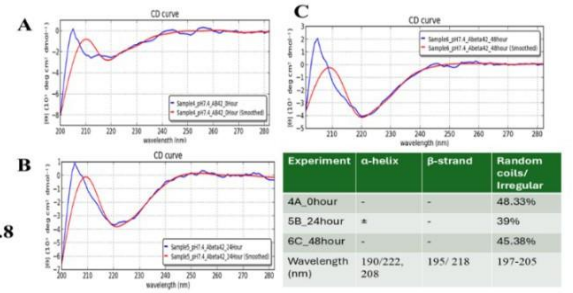
Experiment	α-helix	β-strand	Random coils/ Irregular
1A_0hour	0.93%	46.3%	53%
2B_24hour	-	51.4%	49%
3C_48hour	0.99%	50%	50%
Wavelength (nm)	190/222, 208	195/ 218	197-205

Fig.7

CD curve demonstrates effect of acidic pH5.5 on the secondary structure conformations of Aβ42 in a time dependent course of aggregation. The reaction mixtures were prepared similarly as described above but without BSA. The Mean Residual Weight for Aβ42 is 110 [n= Mol. wt./ (number of amino acid – 1)]. The reaction mixtures were incubated for 0, 24 and 48 hours at 28- 30 ^{0} C temperature before analyzing by CD Spectropolarimeter. The results were analyzed for the presence of secondary structure conformation, alpha helices, beta strand/ beta sheet and random coils/ irregular structures in the reaction mixtures by CAPITO software (as described in the Materials and Methods **(A)** CD curve showing Aβ42 in the 0 hour reaction mixture with buffer pH5.5, **(B)** CD curve showing Aβ42 peak in the 24 hour reaction mixture with buffer pH5.5, **(C)** CD curve showing Aβ42 peak in the 48 hour reaction mixture with buffer pH5.5.

Figure 7

The results presented in Fig.6 demonstrated CD spectra of purified bovine serum albumin (BSA) (15.05 µM) (control unrelated protein with respect to Aβ42) in potassium phosphate buffer (pH7.4) and 10 mM KCl reaction mixture as 0 hour control without Aβ42 (Fig.6A). The other controls were reaction mixture containing Aβ42 in potassium phosphate buffer pH7.4 including 10 mM KCl and 15.05 µM BSA (0 hour control) (Fig. 6B). The third control was reaction mixture including Aβ42 in potassium phosphate buffer (pH 7.4) and 10 mM KCl solution in deionized water (Fig. 6C). The continuous scanning mode of the polarimeter (speed 10-100nm/ minute) with five accumulations per data point generates the spectra from components of reaction mixtures within 0.1 cm cuvette located at 0.1 cm path from the UV light source. The spectral data were analysed by CAPITO software (mentioned in the Materials and Methods). The red line in the figures shows the smooth spectra line surrounding original spectra (blue line). The X axis of the CD plots is wavelength (nm) while the Y axis is defined by degree of ellipticity [θ] (10 ^{3}deg cm^{2} / dmol). The 0 hour control experiment Figure 6 demonstrated random coil (48%) structure of BSA at pH7.4. The defined wavelength is 195-205 nm for the positive peak degree of ellipticity (Fig. 6A). The control CD spectra plot presented in the Figure 6B demonstrated negative peak above 222 nm thus showing random coils and/or irregular secondary (31%) structures in the reaction mixture. The third control demonstrates composite curve with negative peak at 210 nm and another peak is beyond 222 nm wavelength (Fig. 6C). The CAPITO CD spectra analysis demonstrated 30.23% random coils/ irregular structures for Aβ42. The degree of ellipticity with wavelength regions beyond 199 nm/206 nm/ 220 nm are not analysed by CAPITO software.

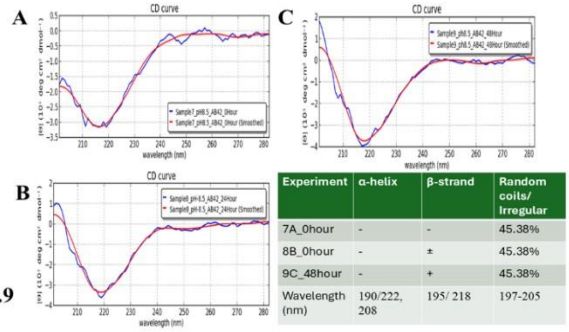


Experiment	α-helix	β-strand	Random coils/ Irregular
4A_0hour	-	-	48.33%
5B_24hour	*	-	39%
6C_48hour	-	-	45.38%
Wavelength (nm)	190/222, 208	195/ 218	197-205

Fig.8

CD curve demonstrates effect of physiological pH7.4 on the secondary structure conformations of Aβ42 in a time dependent course of aggregation. The reaction mixtures were prepared similarly as described above. The Mean Residual Weight for Aβ42 is 110 [n= Mol. wt./ (number of amino acid – 1)]. The results were analyzed for the presence of secondary structure conformation, alpha helices, beta strand/ beta sheet and random coils/ irregular structures in the reaction mixtures by CAPITO software (as described in the Materials and Methods). In the figure, CD curve showing Aβ42 in the **(A)** 0 hour, **(B)** 24 hour and **(C)** 48hour reaction mixtures.

Figure 8



Experiment	α-helix	β-strand	Random coils/ Irregular
7A_0hour	-	-	45.38%
8B_0hour	-	±	45.38%
9C_48hour	-	+	45.38%
Wavelength (nm)	190/222, 208	195/ 218	197-205

Fig.9

CD curve demonstrates effect of physiological pH8.5 on the secondary structure conformations of Aβ42 in a time dependent course of aggregation. The reaction mixtures were prepared similarly as described above. The analysis is made on the basis of Aβ42 mean residual weight (n=110). In the figure, CD curve showing Aβ42 in the **(A)** 0 hour, **(B)** 24 hour and **(C)** 48hour reaction mixtures.

Figure 9

The CD curve in Figure 7 presented the secondary structure conformations of Aβ42 in reaction mixture in pH5.5 incubated for different time periods. The CD curve for the reaction mixture at 0 hour demonstrates 53% random coils or irregular structural conformation and 46.3% beta-strand conformation. The reaction mixture also contains 0.93% alpha helices (Fig. 7A). The Aβ42 reaction mixture incubated till 24 hours demonstrates relatively less (49%) random coils or irregular structure conformation and increase in (51.4%) beta strand while no alpha helix conformation was noticed (Fig.7B). At 48 hours incubation period, the Aβ42 reaction mixtures exhibit 50% each of random coils/ irregular structures and beta strand with 0.99% alpha helices (Fig. 7C). At pH 7.4, the CD curves (Fig. 8) demonstrate all random coils or irregular structure conformations of Aβ42. The marginal reduction in the contents were found from 0 hour (48.33% random

coils/irregular structures, Fig. 8A) to Aβ42 at 24 hours reaction mixture (39% random coils, Fig.8B) and 48 hours reaction mixtures show 45.38% random coil structures of Aβ42 (Fig. 8C). The CD curves presented in the Figure 9 demonstrate presence of random coils/ irregular structures (45.38%) at incubation time 0 hours (Fig.9A), 24 hours (Fig. 9B) and 48 hours (Fig. 9C).

Determination of the influence of excess potassium chloride (KCl) and sodium chloride (NaCl) on secondary structures of Aβ42 in the reaction mixtures.

We found alterations in the degree of ellipticity peaks at wavelengths 190/222, 208 nm, 195/218 nm, and 197- 205 nm and between these defined wavelengths. The observations exhibited not only defined alpha helix, beta strand present at different pH in the Aβ42 reaction mixtures at different time point of incubation, there are intermediate structure conformation (irregular/ random coils) which are not defined by CD data analysing software CAPITO.

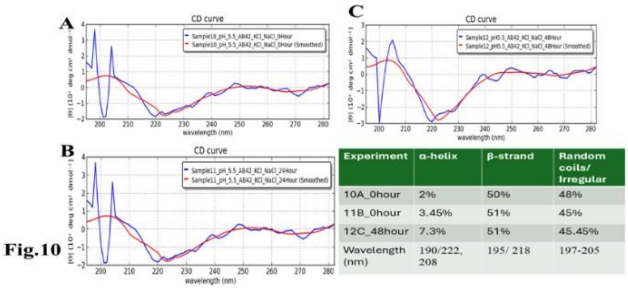


Fig.10

Experiment	α-helix	β-strand	Random coils/ Irregular
10A_0hour	2%	50%	48%
11B_0hour	3.45%	51%	45%
12C_48hour	7.3%	51%	45.45%
Wavelength (nm)	190/222, 208	195/ 218	197-205

Reaction mixtures (500 µl) contain Aβ42 (26.578 µM/ 1.0 ml 10 mM NaOH in deionized water), potassium phosphate buffer pH5.5 along with separately added 10mM KCl and 1mM NaCl were incubated for 0, 24 and 48 hours at 28- 30 ^{0} C temperature before analyzing by CD Spectropolarimeter. The Mean Residual Weight for Aβ42 is 110 [n= Mol. wt./ (number of amino acid – 1)]. The results were analyzed for the presence of alpha helices, beta strand/ beta sheet and random coils/ irregular structures in the reaction mixtures by CAPITO software (as described in the Materials and Methods). The analysis is made on the basis of Aβ42 mean residual weight (n=110). The CD curve shows Aβ42 in the **(A)** 0 hour, **(B)** 24 hour and **(C)** 48hour reaction mixtures.

Figure 10

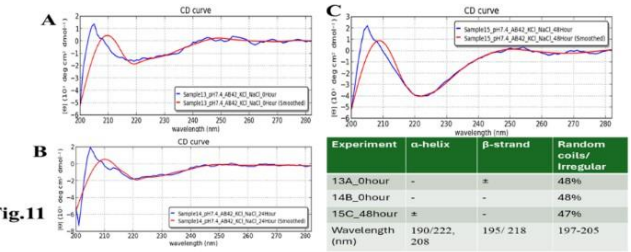


Fig.11

Experiment	α-helix	β-strand	Random coils/ Irregular
13A_0hour	-	*	48%
14B_0hour	-	-	48%
15C_48hour	*	-	47%
Wavelength (nm)	190/222, 208	195/ 218	197-205

CD curve demonstrates effect of pH7.4 and presence of excess KCl, NaCl mixture on the secondary structure conformations of Aβ42 in a time dependent course of aggregation. Reaction mixtures (500 µl) contain Aβ42 (26.578 µM/ 1.0 ml 10 mM NaOH in deionized water), potassium phosphate buffer pH7.4 along with separately added 10mM KCl and 1mM NaCl were incubated for 0, 24 and 48 hours at 28- 30 ^{0} C temperature before analyzing by CD Spectropolarimeter. The Mean Residual Weight for Aβ42 is 110 [n= Mol. wt./ (number of amino acid – 1)]. The results were analyzed for the presence of alpha helices, beta strand/ beta sheet and random coils/ irregular structures in the reaction mixtures by CAPITO software (as described in the Materials and Methods). The analysis is made on the basis of Aβ42 mean residual weight (n=110). The CD curve shows Aβ42 in the **(A)** 0 hour, **(B)** 24 hour and **(C)** 48hour reaction mixtures.

Figure 11

We wanted to determine influence of excess salts KCl and NaCl on secondary structures of Aβ42 at different pH ranges. The Figure 10 demonstrates the influence of pH5.5 and 10mM KCl, 1mM NaCl salt solutions in the Aβ42 reaction mixture. The results show 0 hour incubation generates 50% beta strands, 48% random coil/irregular and 2% alpha helix conformation in the Aβ42 reaction mixtures (Fig. 10A). At 24 hours incubation, the reaction mixtures demonstrate decrease in random coils/ irregular structures to 45% and marginal increase in beta strand to 51% with 3.45% alpha helices (Fig. 10B). The reaction mixtures incubated for 48 hours demonstrate 45.45% random coils/irregular structures, 51% beta strand and 7.3% alpha helix structures (Fig.10C). The presence of excess KCl and NaCl in reaction mixture at pH7.4 demonstrates only minimal influence on Aβ42 secondary structure conformation (Fig.11). Thus, at 0hour incubation (Fig.11A), the Aβ42 reaction mixture contains 48% random coils/irregular structures along with transitional beta strands (undefined) but no alpha helix structures (Fig.11A). At 24 and 48 hours of incubation, the Aβ42 reaction mixtures contain 48% and 47% random coil/irregular structures respectively but no defined beta strand or alpha helices (Fig.11 B and C). Alteration in sodium ion versus potassium ion concentrations are critical for ion channel alterations found in rat brain coronal astrocytes in presence of amyloid beta [25]. The ion-imbalance also promotes fatal clinical condition in human brain. The results indicate acidic pH with altered sodium and potassium chloride concentrations facilitates Aβ42 aggregation and change in secondary conformations more towards mixtures of beta-strand/ sheet and random coil/ irregular structures (Fig. 10).

4. Discussion

The computer guided experiments on the ability to form Aβ42 homodimer structures demonstrate overall low Dimer Packing Quality (F_{SCOR} < 2) value for Fragment1-21 as compared with Fragment22-42 at pH5 and pH7. However, at pH8, the F_{SCOR} values for the Fragment22-42 is decreased and become comparable to that obtained from Fragment1-21. The observation suggests differential ability of the two fragments within Aβ42 (1-42 amino acid) peptide structure to form stable homodimer, as, Fragment22-42 can generate comparatively stable homodimer (F_{SCOR} = 3) than Fragment 1-21 (F_{SCOR} = 1.6) at pH5, 7. At pH8, dimer formation ability is lower for both the fragments of Aβ42. The alterations in the physical properties (pKa, net charge of functional groups in amino acids present in Aβ42) and ability to form Hydrogen bonds are critical with change in pH thereby influence the formation of aggregated Aβ42 structures.

We found that the reaction mixture at pH 7.4 demonstrated a decrease in Aβ42 aggregation. The presence of potassium chloride facilitates only marginal aggregation but moderate level of Aβ42 aggregation is observed when sodium chloride is added to the reaction mixture. On the other hand, the acidic pH range (pH 5.5) facilitates Aβ42 aggregation. The Aβ42 reaction mixture, in presence of excess KCl and/or NaCl, not only shows increase in RFU with time as higher rate of Aβ42 aggregation but alters secondary structure conformation predominantly to beta sheet/ beta strand from the transient intermediate random coil/ irregular structures. In contrast, the alkaline pH (pH8.5), in presence and absence of salts (10

mM KCl and 1mM NaCl mixture), influences only marginal aggregation and alterations of transiently generated random coils but does not alter secondary structure conformations.

Altogether, the results from our cell free experiments demonstrate low to moderate increase in aggregation with respect to Thioflavin-T fluorescence within a time limit of 0-70 hours at 28-30℃. The reaction mixture with buffer pH5.5 demonstrated alterations in secondary structures of aggregated Aβ42 in 0, 24 and 48 hours preincubated samples. Though, random coils and/or irregular structures are present in the reaction mixtures with variable extent in all experimental conditions irrespective of the presence of KCl and NaCl. These random/ irregular coils are not considered as conventional secondary structures but transient structures with less stability on the defined wavelength as alpha helix is defined by positive peak at 190 nm, negative peak at 222, 208 nm wavelength. The β-sheet and β-strand secondary structures are defined by the wavelength at 195 nm for positive peak and 218 nm for negative peak. The random coils and irregular structures show negative peak at 200 nm.

The findings are partially consistent with the observations reported by different laboratories on Stefin A and B (Cystatin) and other amyloidogenic proteins which, in acidic pH 3-4, switch to its fibrillar structures through change in secondary conformations [15,16,26]. The explanations remain in the amino acids within the fragments 12-24 and 37-42 of 1-42 Aβ42 peptide. Hilbich et al. [27] demonstrated the chemical nature of aggregated amyloid beta A4 peptides in human brain as hydrophobic, fibrillar structure due to presence of hydrophobic amino acids which form anti-parallel beta sheet structures in the fragment containing amino acid sequence 10-43 in contrast to the purified amyloid beta which is water soluble. Barrow et al. [28] demonstrated characteristics of amino acids in sequence beta 1-39 and beta 1-42 of A beta42 are critical for amyloid deposition. Alpha helices (monomeric structures) are unfolded with increasing temperature at acidic pH1-4 and neutral pH7-10. However, beta sheet (oligomeric) structure is not sensitive to temperature and stable at pH4-7.

5. Conclusions

Overall, the results demonstrate weak to moderate increase in Aβ42 aggregation kinetics at acidic pH (pH5.5) as compared to that in pH7.4 and 8.5. Our observations also demonstrate an increase in beta-sheet/ beta strand secondary conformations with irregular/ random coil conformations of Aβ42 at pH5.5 and in presence of salts, KCl, NaCl as compared with the observations at pH7.4 and 8.5. The presence of increasing concentrations of potassium and sodium chloride together was also found to increase Aβ42 aggregation at pH5.5 than at pH7.4 and 8.5 in cell free reaction mixtures.

Conflict of Interest Statement

Authors declare no conflict of interests.

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M.B. reviewed the manuscript and provided critical suggestions.

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