Supporting information

Amyloid nanospheres from polyglutamine rich peptides: assemblage through an intermolecular salt bridge interaction

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Methodology

(M1) Peptide synthesis and solubilization: The peptides were synthesized by solid phase method and obtained in crude form, from Keck Biotechnology Centre at Yale University. These were purified using reverse-phase HPLC, lyophilized and stored at -80°C. Purified peptides were then disaggregated in 1:1 ratio of Trifluoroacetic acid (TFA) and Hexafluoroisopropanol (HFIP) (Sigma-Aldrich). TFA and HFIP solvents were evaporated using gentle stream of nitrogen gas forming peptide film around the glass vial. The glass vial is further placed in a desiccator for two hours to remove any remaining volatile solvent. Peptide film was then dissolved using water-TFA pH 3 and centrifuged (Thermo Scientific Sorvall MTX 150 Micro-ultracentrifuge) in polypropylene micro vials, using S80AT2 rotor at 50000 rpm for four hours. This ensures pelleting of undissolved peptide material. Supernatant containing soluble peptide fraction was collected gently for concentration determination and setting up of the aggregation reaction.

(M2) Transmission electron microscopy: 5 µl of final aggregate generated from the reaction mixture of PepQ, PepK, PepE and mixture (PepK+PepE) were adsorbed for 2 minutes on the carbon-coated 200 mesh Copper grids (Electron microscopy). Grids were washed with deionized water and peptide was stained with 0.5% uranyl acetate, and air-dried before imaging on TECNAI 200 kV TEM (Fei, Electron Optics) instrument. Dimensional analysis of fibres and nanospheres was done using ImageJ software version 1.47 from different grids imaged.[1]

(M3) Atomic force microscopy: 8µl of final aggregates were deposited on freshly cleaved mica sheet. Sample was allowed to get adsorbed on the sheet for about 5 minutes. Aggregates were washed with MiliQ water and dried overnight before used for imaging under Multimode VIII Scanning Probe Microscope (Bruker, USA) using Peak Force-QNM mode.[2] Dimension analysis of AFM images was conducted using Nanoscope analysis software. Diameter of nanospheres in the AFM images was measured in the X- axis direction because that is able to give the exact dimension of a single sphere as compared to Y- or Z- axis. Moreover, because nanospheres are stacked with one another in both these cases, measuring dimensions for individual spheres was difficult.

Table 1. Dimensions of aggregates obtained by TEM and AFM analysis

	TEM measurement (nm)		AFM measurement (nm)	
Peptides	Average length	Average diameter	Average length	Average diameter
PepQ	114±28	23±6	316±102	73±22
PepK	98±24	17±6	188±45	127±19
PepE	373±168	52±24	290±100	125±28
PepK+PepE (nanospheres)	N.A.	18±3	N.A.	38±3

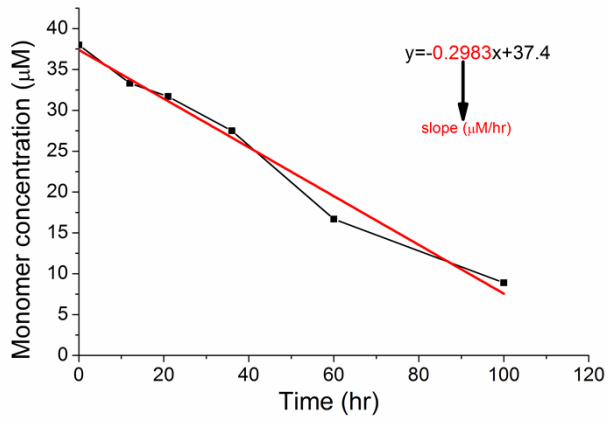
N.A: not applicable. Number of fibers and nanospheres analyzed for image analyses are shown in bracket; TEM measurement: PepQ (6), PepK (12), PepE (15) and PepK+PepE (50). AFM measurement: PepQ (11), PepK (10), PepE (11) and PepK+PepE (22).

(M4) Spontaneous aggregation kinetics: It was monitored using RP- HPLC (Agilent C-18 column on Agilent 1100 infinity instrument). Supernatant collected after centrifugation as explained in M1 was subjected to concentration determination, using a standard curve. A standard curve was generated by injecting different known concentrations of peptides in HPLC and measuring their corresponding peak area at 215 nm.[3] For setting up the spontaneous aggregation reaction, desired amount of peptide in the monomeric form were mixed with PBS (pH 7.2) and final reaction volume was made up using water-TFA pH 3. Sodium azide (0.05 %) was added to the reaction mixture before setting up the final volume to prevent any microbial contamination in the reaction mixture. Finally the reaction set up was kept at 37ºC for aggregation monitoring as described previously.[3] Starting concentration of all the peptides was kept 40 µM.

To set up the spontaneous aggregation reaction of PepK, PepE and PepQ at 40 µM for the 500 µl reaction volume, 100 µl of each peptide from their respective stock (200 µM) was added in different tubes and then volume was made to 500 µl with PBS and water-TFA.

In order to set up the aggregation reaction for the mixture of PepK and PepE, equimolar amount (40 µM each), 100 µl of each peptide from 200 µM of respective stock concentration were added to a single tube and made up to 500 µl with PBS and water-TFA. This corresponds to 80 µM of effective polyglutamine concentration. The same protocol was followed for PepQ and PepE mixture. To set up 80 µM PepK spontaneous aggregation reaction, 200 µl of PepK from its 200 µM stock concentration was used and final 500 µl reaction volume was made up using PBS and water-TFA.

To monitor the aggregation reaction an aliquot of an on-going reaction was centrifuged for 30 minutes at 50000 rpm, and supernatant concentration was measured at 215 nm by RP-HPLC using standard curve obtained for each peptide. PepE and PepK shows differential hydrophobicity, thus different retention time provided an independent way for monitoring their aggregation reaction and in the mixture (PepK+PepE) by RP-HPLC sedimentation assay.[3] For mixture (PepK+PepE) combined monomer peak was taken to calculate its aggregation rate. Aggregation rates (µM/hr) were obtained by calculating the slope of the curve fitted between monomer concentration and time as shown below in the representative figure.

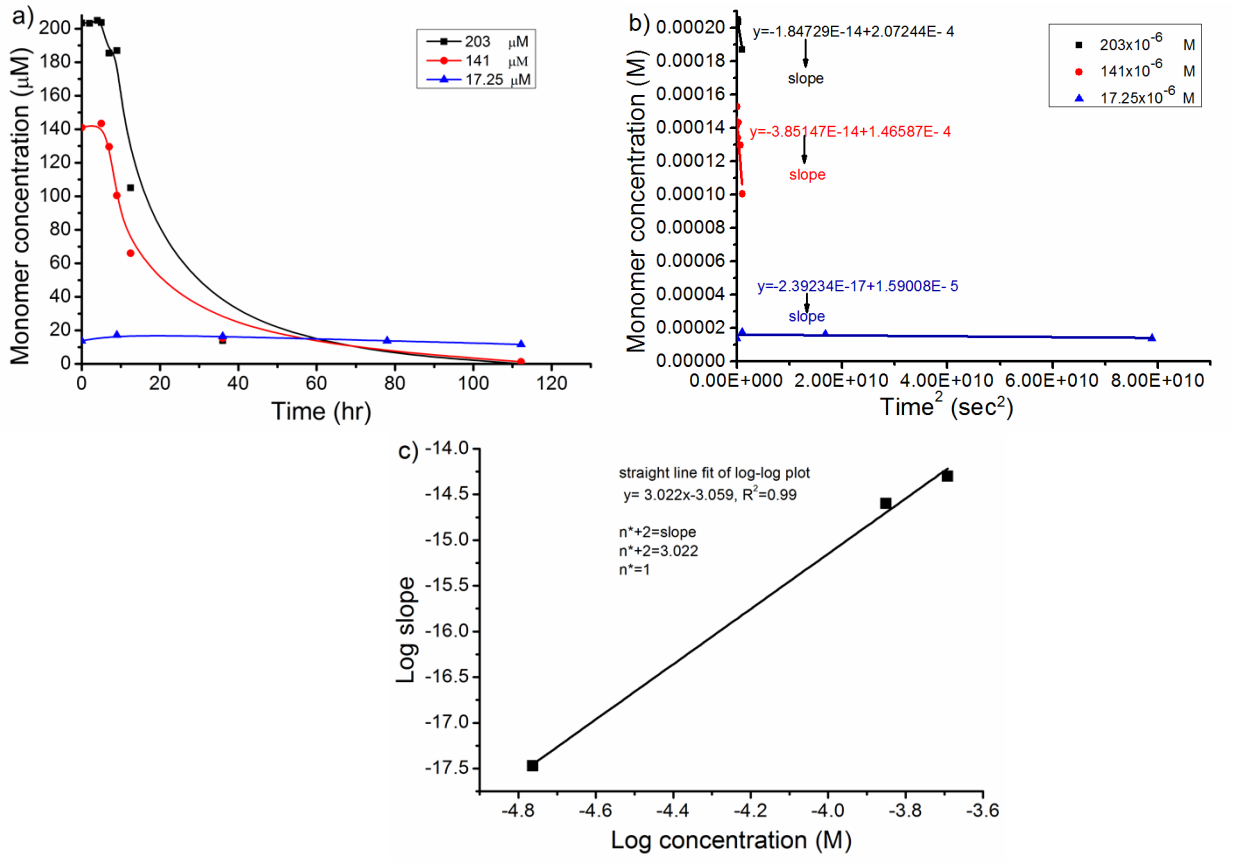


For pH dependent experiment to maintain pH 3, aggregation reaction using glycine buffer was set up such that it contains finally 50 mM glycine and 140 mM NaCl.[4, 5] In figure 3D the aggregation percentage was calculated by assuming, at the start of aggregation reaction, 100 percent monomer is present and as the reaction proceeds with time, monomer amount decreases and aggregates percentage increases. At 141 hours, PepE completely aggregates, representing 100% aggregation.

(M5) Thioflavin T binding assay: 20 µl ThT from 2.5 mM stock was added to 600 µl of ongoing aggregation reaction of PepQ, PepE, PepK and mixture (PepK+PepE) at different time points and aggregation kinetics was monitored using Perkin Elmer LS55 fluorescence spectrophotometer. The excitation wavelength used was 450 nm, with slit width 5 nm and emission was measured at 489 nm with slit width 10 nm. Photomultiplier tube voltage was kept constant at 600 throughout the experiment.[6]

(M6) Fourier Transform Infrared spectroscopy: Final aggregates were washed thrice with fresh 1X PBS buffer and their FTIR spectra were collected on Bruker Tensor 27 instrument using BioATR accessory with liquid nitrogen cooled MCT detector. Spectra were measured with 4 cm^{-1} resolution at 25 °C and represent the average of 120 scans. Spectra were corrected after subtraction from the buffer spectra using OPUS software.

(M7) Nucleation kinetics: The initial nucleation event in polyglutamine aggregation can be represented by rate equation $\Delta=1/2\ JJ^*c^*t^2$ (equation 1). Δ represent amount of monomers undergone into aggregation, J represent elongation rate of aggregates, J* elongation rate of nucleus and c* is the concentration of nucleus at time t. Elongation rate of aggregates (J) can be represented by k_+c, where k_+ is the forward elongation rate constant and c is monomers in bulk concentration. Concentration of the nucleus can be replaced through c*= Kn*cn*, where n* depict the number of monomers involved in generating the critical nucleus and K_n* represent equilibrium constant between monomer and nucleus. By substituting all this in equation 1 it becomes $1/2\ k_+^2\ K_n^*\ c(n^{*+2})t^2$ with an assumption that elongation rate constant of aggregates k_+ equals the elongation rate of nucleus k_+* . Plotting curve between Δ and t^2 will generate slope (A) = $1/2\ k_+^2\ K_n^*\ c(n^{*+2})t^2$. Taking log on both sides will give the expression; log (slope A) =log ($k^2_+ K_n^*$) + (n* + 2) log (c). From this expression a plot of log (slope A) and log c will generate slope B of n*+2. Now equating n*+2 against the slope value of log–log plot, will provide the n* (critical nucleus size) values for different peptides.[3, 7] Only the representative example, with some concentrations is shown below.



(M8) Seeding aggregation reaction: Seeds of PepE were prepared by setting up spontaneous reaction at 40 μM concentration. After all the monomers have been converted to aggregates, they were harvested through centrifugation at 50000 rpm and washed thrice before use with 1X PBS (pH 7.2). The aggregate concentration was determined by solubilizing them in 70% formic acid and injecting in RP-HPLC. The peak area was used to calculate the concentration from the standard curve as mentioned in M4 method. Seeds were sonicated for 5 cycles with 20 seconds ON and 30 seconds OFF operation. 4% w/w of them were added to a fresh reaction mixture of 40 μM of PepE and PepK peptide monomers.[8] The reactions were monitored as described in M4 method.

Figures:

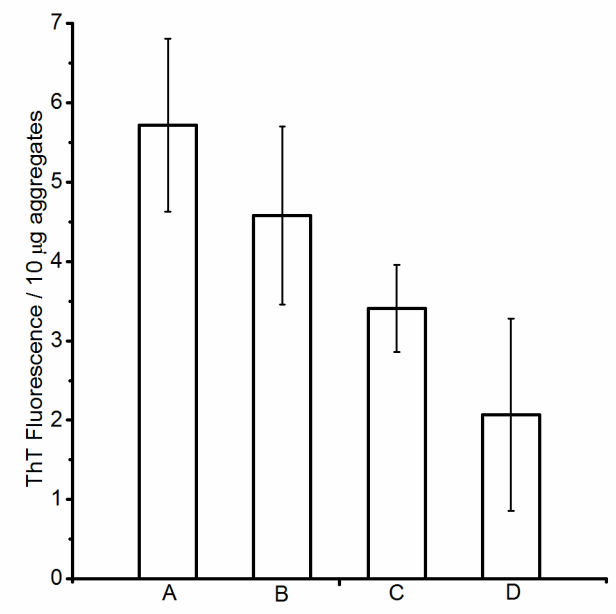


Fig. S1: ThT fluorescence of 10 μg aggregates of A (PepQ), B (PepE), C (PepK+PepE) and D (PepK). Error bars represent standard error of mean for minimum n=2.

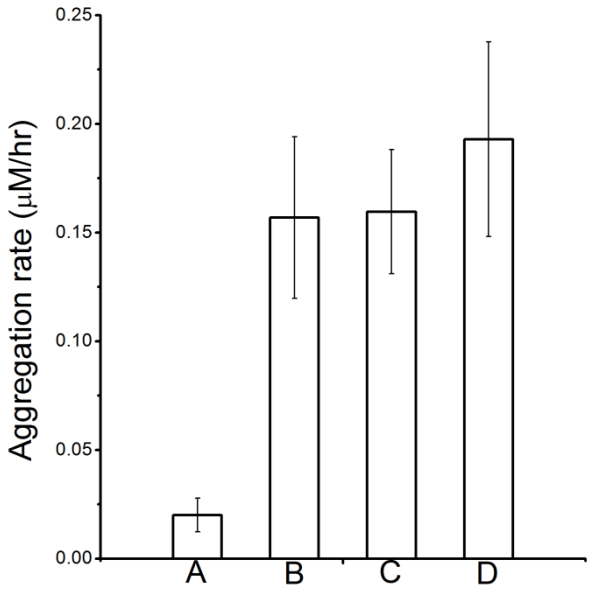


Fig. S2: Elongation rates: A (PepK alone), B (PepK in PepE), C (PepE alone), D (PepE in PepK), in PBS pH 7.2. Error bars represent standard error of mean for minimum n=5.

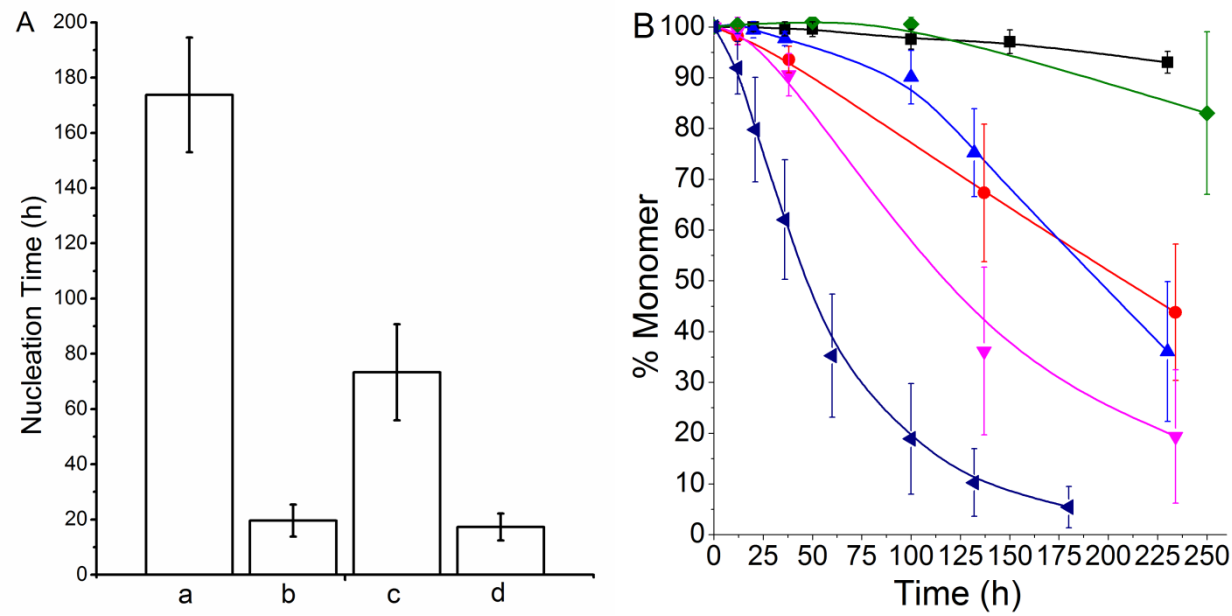


Fig. S3: A) Nucleation time: a (PepK alone), b (PepK in mixture), c (PepE alone) and d (PepE in mixture). B) Spontaneous aggregation kinetics of PepK (■), PepK in mixture (●), PepE (▲), PepE in mixture (▼), PepK 80 µM (♦) and PepQ (◄) . Error bars represent standard error of mean with minimum n=4 except for PepK 80 µM where n=2.

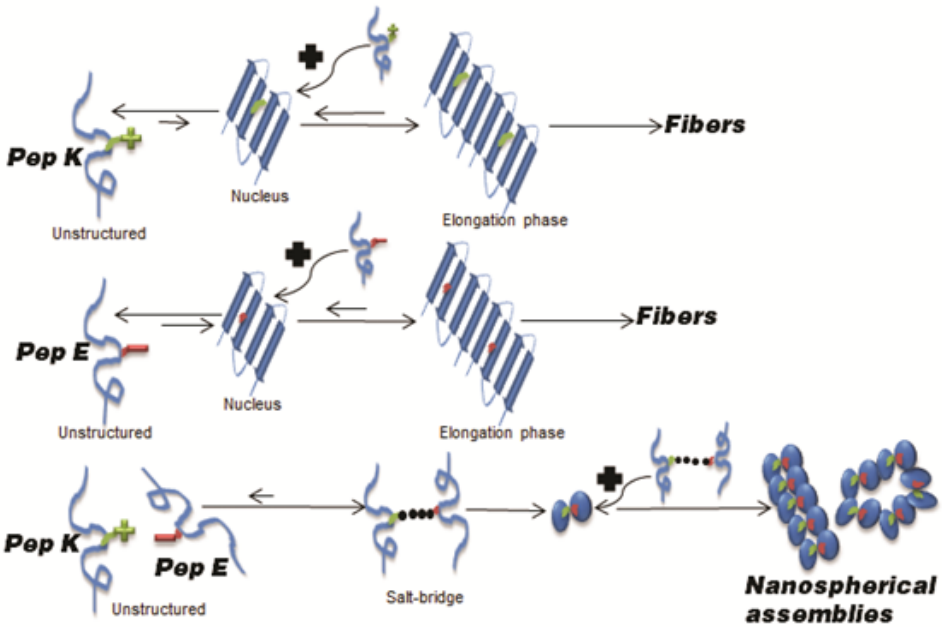


Fig. S4: The schematic model of fiber and nanospherical assembly formation at pH 7.2. Initially monomers of PepK and PepE remain unstructured and resist aggregation mainly due to charge effect present on the side chains of glutamate and lysine. With time, they attain a stable nucleus followed by elongation phase and give rise to fibrous structures. However, mixing of PepK and PepE generates nanospherical assemblies via a non-nucleated mechanism governed by salt bridge interactions.

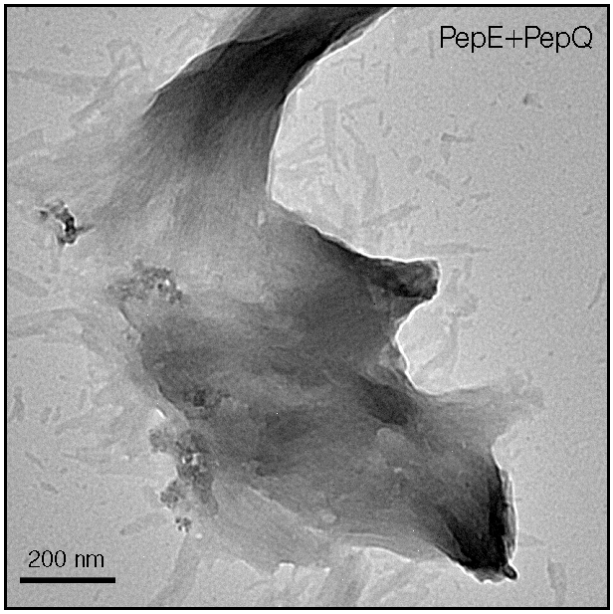


Fig. S5: TEM image of aggregates obtained from PepE (40 μM) and PepQ (40 μM) mixture.

References

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