Electronic Supplementary Information

Intrinsic Conformational Preference in the Monomeric Protein Governs Amyloid Polymorphism

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Experimental Methods

Materials. Ovalbumin (Chicken egg white albumin; A5503), sodium phosphate monobasic, disodium phosphate dihydrate, glycine, sodium chloride (NaCl), Thioflavin-T (ThT), 8-anilinonaphthalene-1- sulfonic acid ammonium salt (ANS) and 9-(2,2-Dicyanovinyl)julolidine (DCVJ), sodium dodecyl sulphate (SDS), Trizma® base, acrylamide/bis-acrylamide (30%) solution, N,N,N′,N′-tetramethyl ethylenediamine (TEMED) were purchased from Sigma-Aldrich (St. Louis, MO), whereas, ammonium persulfate, glycerol, Coomassie® brilliant blue R-250, bromophenol blue and β-mercaptoethanol were purchased from HiMedia, and used as received. The PageRuler Plus® pre-stained protein ladder was procured from Thermo Fisher Scientific. Methanol and acetic acid (glacial) were purchased from SD Fine-Chem Limited (SDFCL). All of the stock solutions involving buffers, ThT, ANS, and NaCl were prepared using Milli-Q water, while that of DCVJ was prepared in dimethyl sulfoxide. The pH of all the buffers was verified and adjusted using HCl or NaOH to a final value (±0.02) using a Cyberscan 510 pH meter (Eutech Pvt. Ltd.) at ~25 °C. Thereafter, the buffer stock solutions were stored at 4 °C and the respective working buffer solutions were always prepared freshly, followed by pH checking and adjustment, prior to the aggregation reactions. The stock solutions of ThT (1 mM), ANS (1 mM), DCVJ (2.5 mM) and NaCl (5 M) were stored at 4 °C. The 0.22 μm and 0.02 μm membrane filters were procured from Merck Millipore® and Whatman® (GE Healthcare Life Sciences), respectively.

Aggregation Reaction Conditions. The ovalbumin stock solution (1 mM) was prepared in 5 mM, pH 7 sodium phosphate monobasic buffer and was stored at 4 °C. The concentration of ovalbumin stock was determined spectrophotometrically (Shimadzu UV-2600) using the reported molar extinction coefficient of 30,957 M^{-1} cm^{-1} at 280 nm (Bhattacharya and Mukhopadhyay, *J. Phys. Chem. B* **2012**, *116*, 520-531). For carrying out all the aggregation reactions at 65 °C under quiescent conditions, the ovalbumin stock solution was diluted into freshly prepared pH 2.2, 20 mM Gly-HCl buffer, in the absence and in the presence of different concentrations of NaCl (25, 50, 75, 100, 150, 200, 300, 500 mM), to a final ovalbumin concentration of 100 μM. The reaction tubes/vials containing reaction mixture were then inserted into a pre-heated dry bath (Rivotek) whose temperature was set at 65±1 °C.

Steady-State Fluorescence Spectroscopy. The steady state fluorescence spectroscopic studies of ThT were carried out on LS55 Perkin Elmer spectrofluorimeter, and that of ANS and DCVJ

studies were performed on RF-6000 Shimadzu spectrofluorimeter at ~25 °C. A quartz cuvette of 10 x 2 mm was used for all the experiments. The reaction mixtures were monitored for 2 hours till an apparent saturation was reached. All of the kinetics experiments were repeated at least six times. The FL Winlab (Perkin Elmer) and LabSolutions RF (Shimadzu) software were used to acquire the fluorescence data that were replotted using OriginPro2021 version 9.8.0.200 and fitted using a mono-exponential function available in OriginPro. The apparent rate constants were then extracted and the fits with the best adjusted R^2 values (0.96-0.99) have been reported herein.

ThT Fluorescence. For ThT fluorescence kinetics measurements, aliquots from the aggregation reaction mixture were taken out at specific time-points and then diluted 10-fold using dilution buffer (50 mM sodium dibasic phosphate, pH 7.4) containing ThT (11.11 μM) to a final ThT concentration of 10 μM. The following parameters were used for collecting the ThT emission spectra: λ_{ex} = 450 nm, λ_{em} (range) = 460-520 nm, number of accumulations = 2, scan speed = 50 nm/min, excitation slit width = 2.5 nm and emission slit width = 4 nm. For collecting single-point emission intensities at 480 nm, following parameters were used: λ_{ex} = 450 nm, excitation and emission slit width = 2.5 nm and 4 nm, respectively, and an integration time of 5 sec. All of the kinetics experiments were repeated at least six times.

ANS Fluorescence. For recording the ANS fluorescence kinetics, aliquots were withdrawn in a similar manner as that mentioned for ThT fluorescence studies. The aliquots were then diluted 10-fold using Milli-Q water containing ANS (11.11 μM), to a final ANS concentration of 10 μM. The following parameters were used for collecting the ANS emission spectra: λ_{ex} = 350 nm, λ_{em} (range) = 400-600 nm, scan speed = 60 nm/min, excitation bandwidth = 3 nm and emission bandwidth = 5 nm. All of the kinetics experiments were repeated at least six times.

DCVJ Fluorescence. For recording the DCVJ fluorescence kinetics, 100 μM ovalbumin was incubated in the absence and in the presence of variable concentrations of NaCl at pH 2.2 for 3 hours at ~25 °C. The changes in DCVJ fluorescence were recorded on a kinetic mode whereby DCVJ was added into the reaction mixture, after 2 minutes, with a manual mixing dead-time of ~ 9 sec and the changes in DCVJ fluorescence as a function of time were recorded for a total of 35 minutes. The following parameters were used: λ_{ex} = 453 nm, λ_{em} = 500 nm,

excitation bandwidth = 3 nm, emission bandwidth = 5 nm and accumulation time = 10 ms/point.

Zeta (ζ) Potential and Dynamic Light Scattering (DLS) Measurements. Malvern Zetasizer Nano ZS (Malvern Instruments, UK), equipped with a He−Ne laser (632 nm), was used to measure the zeta potential and average hydrodynamic diameter of the protein samples (pre-heated and aggregates) in the absence and in the presence of variable NaCl concentrations at ∼25 °C. Prior to the measurements of pre-heated samples, the buffers, salt and ovalbumin stock solutions were filtered using 0.22 μm, followed by 0.02 μm Anodisc® 13 syringe filters. The zeta potential of the protein samples (100 μM), in the absence and in the presence of various concentrations of NaCl, was determined at ∼25 °C using the Smoluchowski method. For all of the DLS studies at ∼25 °C, the fluctuations in the scattering intensities were recorded in a quartz cuvette of 1 cm pathlength at a backscattering angle of 173°. The time-dependent changes in the average hydrodynamic diameters of the protein aggregates during the course of aggregation were monitored for 2 hours in the absence and in the presence of NaCl. Prior to setting up the aggregation reactions, the buffers and the protein as well as NaCl stock solutions were filtered twice in a similar manner, as that mentioned previously for the pre-heated samples. Typically, at first, ovalbumin reaction mixtures, in the absence and in the presence of a given concentration of NaCl, were prepared in a bulk volume of 20 mM Gly-HCl, pH 2.2 buffer. Thereafter, the reaction mixture was divided into smaller aliquots of same volume each and each of the aliquots was further transferred into separate reaction tubes that were kept simultaneously into a pre-heated dry bath pre-equilibrated at 65±1 °C. Following heating, the entire reaction mixture/aliquot in a given tube was taken out for the time-dependent DLS experiments at specific time-points. Prior to recording the scattering intensity fluctuations, the aliquots were quenched in ice and cooled to room temperature. Three scans were collected for each of the measurements and all of the experiments were repeated independently at least thrice. ZetaSizer 7.11 software was used to acquire the data that were replotted using OriginPro 2021.

Raman Spectroscopy. The Raman spectroscopic studies were carried out on an inVia Raman microscope (Renishaw, UK) at ~25 °C. The sample reaction mixture, after reaching an apparent saturation, was first centrifuged at 16,500 rpm for 30 min (5810R Eppendorf centrifuge, Germany) at ~25 °C. 3 μL of the resulting pellet was dropcast on a glass slide covered with an

aluminium foil, and it was then allowed to dry overnight at ~25 °C. An NIR laser (785 nm) with 10 sec exposure time and 500 mW (100%) laser power was used as an excitation source and a 100X long working distance objective (Nikon, Japan) was used for focusing. An edge filter of 785 nm was used to block the Rayleigh scattered light and a diffraction grating (1200 lines/mm) was used to disperse the collected Raman scattered light, further detected with the help of an air-cooled CCD detector. The number of spectral accumulations were 20 and spectral resolution was ~1 cm^{-1}. WiRE 3.4 software was used to acquire the data. The OriginPro2021 software was used for replotting the Raman spectra followed by baseline correction (spline interpolation method), and for deconvolution of the amide I (1600-1700 cm^{-1}) and amide III (1200-1300 cm^{-1}) regions to identify the peak positions and extract information on the presence of different secondary structural elements. Deconvolution was performed using Fit Peaks (Pro) of peak analyzer option, using the Gaussian function and formula

$$y = y_0 + \frac{Ae^{\frac{-4\ln(2)(x-x_c)^2}{w^2}}}{w\sqrt{\frac{\pi}{4\ln(2)}}}$$

whose bandwidth (FWHM) varied between 8-30 cm^{-1}. For deconvolution, user defined baseline detection algorithm and the local maximum method was used.

Attenuated Total Reflectance Fourier-Transform IR (ATR FT-IR) Spectroscopy. ATR-FTIR spectra were recorded on an IRTracer-100 Shimadzu spectrophotometer, equipped with QATR^{TM}10 single-reflection integration-type ATR accessory comprising a diamond crystal and an air-cooled Deuterated Lanthanum α-Alanine-doped TriGlycine Sulphate (DLATGS) detector. For preparation of the reaction mixtures (total volume: 2 mL), pH-adjusted milliQ-water (using 1 N HCl) was used whereby the pH of the solvent was checked using a Cyberscan 510 pH meter (Eutech Pvt. Ltd.) and set to 2.2 (±0.01) at ~25°C. The native ovalbumin solution at pH 7 was also prepared in a similar manner. 100 μM of ovalbumin was incubated at pH 2.2 in the absence and in the presence of varying NaCl concentrations at ~25 °C for 4-5 hours. Prior to recording the FTIR spectrum of a sample, the reaction mixture was concentrated 20-fold using a 10 kDa Amicon® Ultra-4 Centrifugal filter and centrifuged at 9000 rpm (SL8R ThermoFisher Scientific Centrifuge, Germany) for 10 minutes at ~25 °C. The final retentate volume was ~100 μL. A sample drop of 10 μL was used for recording the spectra in the range of 400-4000 cm^{-1} (full range) and 1500-1800 cm^{-1} involving amide I (1600-1700 cm^{-1}) and amide II (1500-1600 cm^{-1}) regions. All of the spectra were recorded at a resolution of 2 cm^{-1}

in the absorbance mode. The final spectrum was an average of 3 measurements and each measurement, involving 400 scans, was collected from a fresh drop of the respective sample. The crystal surface was cleaned with methanol every time before putting a fresh drop of sample onto it for a single measurement. Prior to recording a spectrum, a background run with 400 scans was performed and the absorbance of pH adjusted milliQ-water (i.e. solvent) was recorded under identical conditions and subtracted from the protein samples. All of these experiments were repeated thrice. LabSolutions IR (Shimadzu) software was used for baseline correction (using multi-point fit) and OriginPro2021 software was used for plotting and processing of the spectra. Fourier self-deconvolution with a bandwidth of 4 cm^{-1} followed by smoothening was applied to the solvent subtracted, baseline corrected spectra. The second-derivative of the amide I and II regions were utilized to identify and distinguish between various spectral components.

Transmission Electron Microscopy (TEM). The morphological characteristics of the protein aggregates, formed in the absence and in the presence of variable NaCl concentrations, were investigated using transmission electron microscopy on JEM-F200 (JEOL, Japan). The protein aggregates, formed after the reaction attained apparent saturation, were chosen for the TEM imaging. 3 μL of the sample reaction mixture was dropcast on a 300-mesh carbon-coated electron microscopy grid and incubated for 5 minutes at ~25 °C. Thereafter, the dropcast sample was stained using 1% uranyl acetate, followed by incubation for another 5 minutes at ~25 °C. The extra stain was gently removed using a Kimwipe tissue and the grid containing the desired sample was allowed to dry overnight at ~25 °C. TEM imaging was carried out using an acceleration voltage of 200 kV at a magnification that ranged between 4kX-80kX, and Gatan Microscopy Suite® (GMS) 3 software was used to capture the TEM images.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analyses. We utilized SDS-PAGE to analyze species of various molecular weight present in the native ovalbumin (pH 7) and ovalbumin aggregates, formed at pH 2.2 and pH 7, 65 °C, in the absence and presence of NaCl. For analyzing the native protein, the stock solution of the protein (~1 mM) was diluted 100-fold to a final concentration of 10 μM. For analyzing ovalbumin aggregates, aliquots (50 μL) were withdrawn from the reaction mixture at specific time-points, quenched in ice and cooled to room temperature and thereafter, used as such without any dilution. Prior to loading the samples on the gel, they were treated in a following manner:

Typically, 5 µL of the loading dye (pH 6.8, 250 mM Tris-Cl, 10% (w/v) SDS, 0.02% (w/v) bromophenol blue, 30% (v/v) glycerol, and 5% β-mercaptoethanol) was mixed with 20 µL (each) of the native protein and the undiluted aggregates. The samples were further heated at ~95 °C for 10 min on a pre-heated dry bath (Rivotek). 20 µL of the pre-heated (native and the aggregated) samples as well as 5 µL of the protein ladder (molecular weight marker) were loaded and analyzed on 15% gel. The gel was run on Bio-Rad PowerPac Basic setup at 75 V. After the run was complete, 0.25% Coomassie blue R-250 solution was used for staining the gel for 30 min and thereafter, destained (glacial acetic acid, methanol, and milli-Q water) for ~2 h. All of the SDS-PAGE experiments were repeated at least twice. The gel images were captured under white light on a UV transilluminator (DNR Bio-Imaging Systems, ISRAEL).

Figure S1

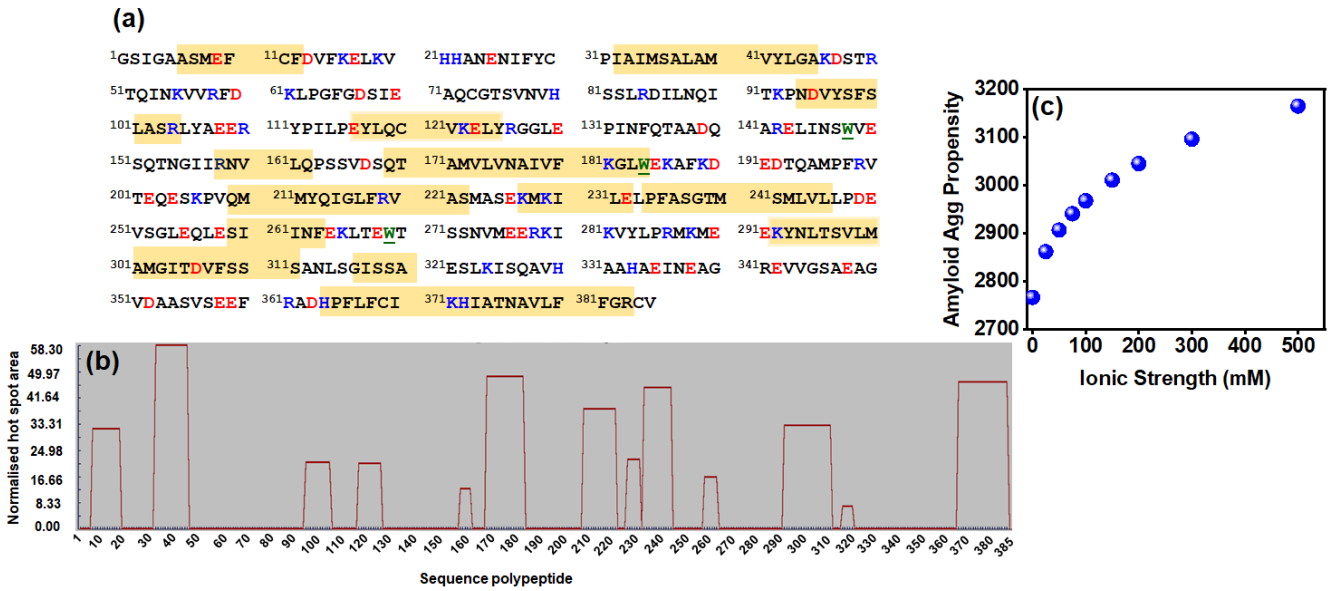

Figure S1. (a) The amino acid sequence of ovalbumin representing positively-charged (blue), negatively-charged (red), and tryptophan residues (green, underlined). The aggregation-prone regio-specific peptide segments present within ovalbumin are highlighted in yellow. (b) The aggregation hotspots of ovalbumin as a function of the protein sequence, obtained using AGGRESCAN, is shown here. (c) The aggregation propensity of ovalbumin (100 μM) at pH 2.2, 65 °C as a function of ionic strength, obtained using TANGO software, is depicted here.

Figure S2

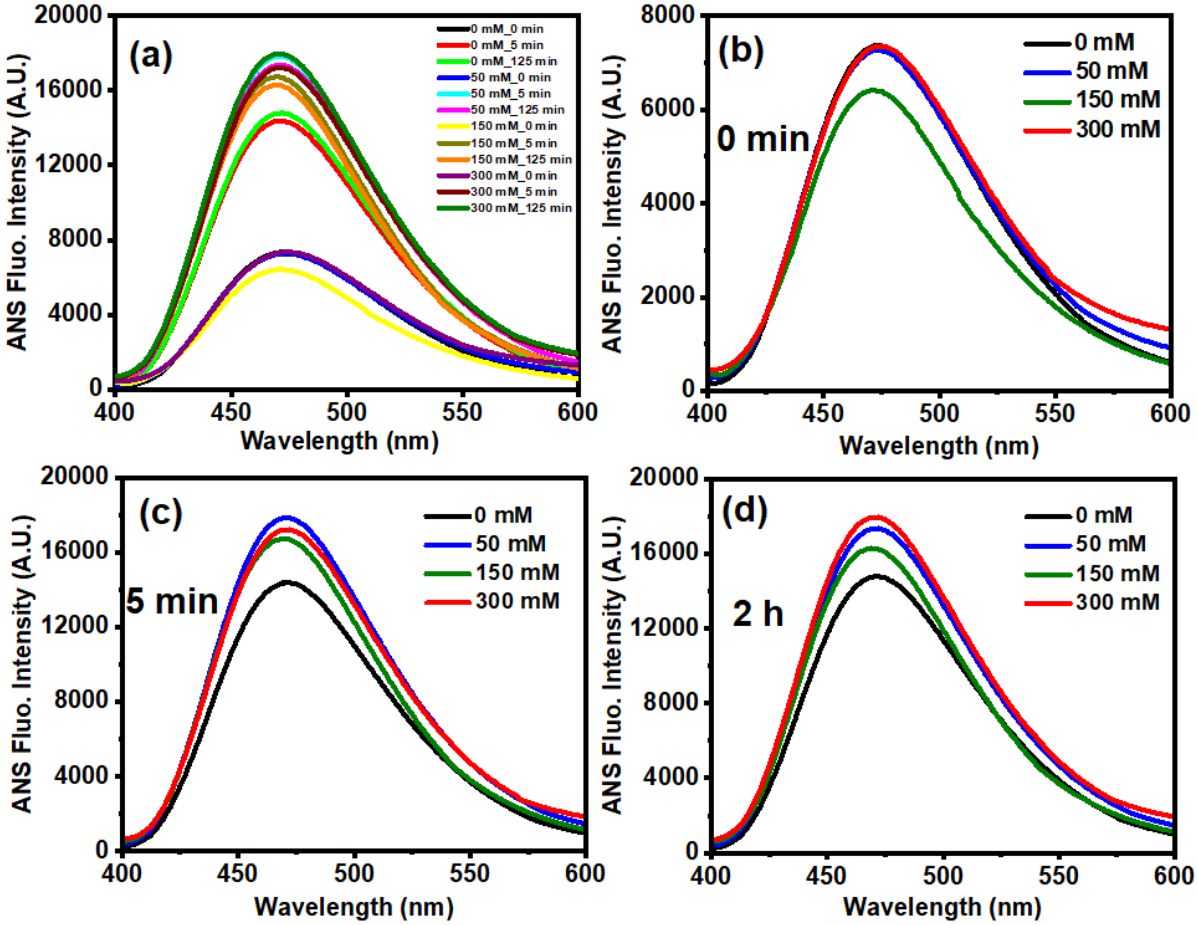

Figure S2. (a) Changes in ANS fluorescence spectra for pre-heated and incubated (65±1 °C) ovalbumin samples in the absence and presence of NaCl at different time-points. Alterations in ANS fluorescence emission in the absence and presence of NaCl at (b) 0 min i.e. pre-heated, (c) 5 min and (d) 2 hours after heating are shown for better clarity.

Figure S3

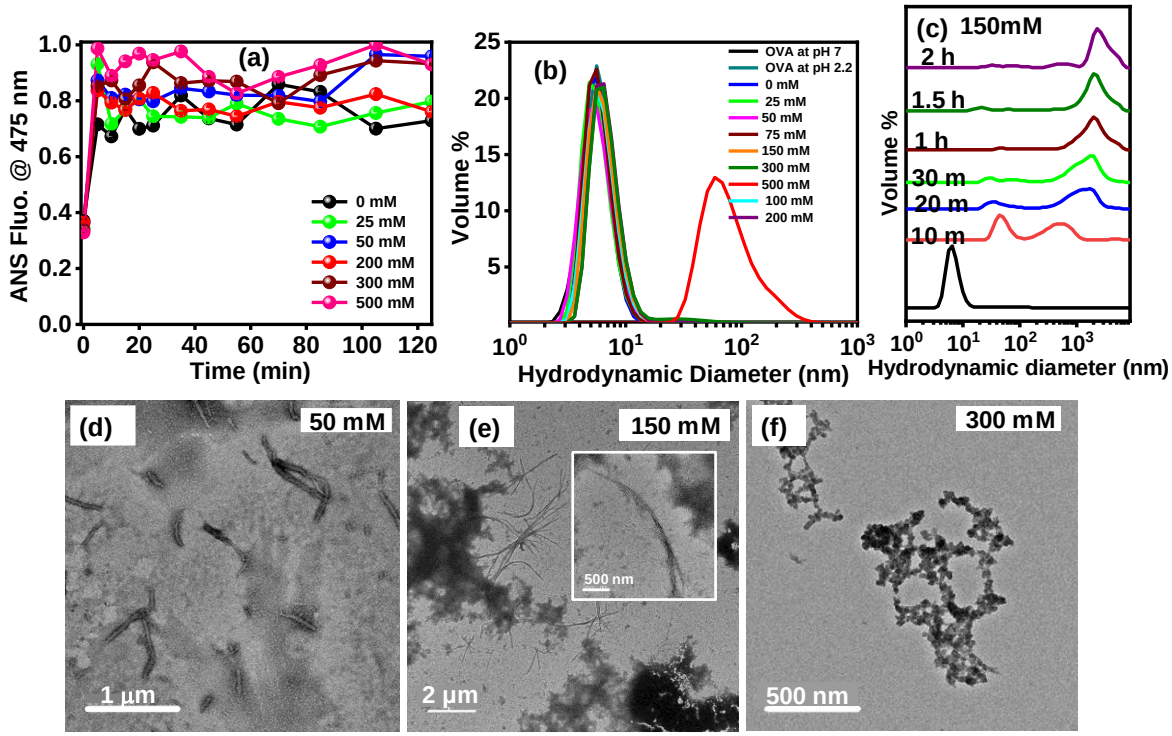

Figure S3. (a) ANS fluorescence kinetics, monitored at 475 nm, in the absence (black) and presence of variable concentrations of NaCl. (b) Changes in hydrodynamic diameter of native, and pre-heated ovalbumin samples in the presence and absence of salt, at ~25 °C. (c) Time-dependent changes in the hydrodynamic diameter of ovalbumin aggregates formed upon heating at 65 °C in the presence of 150 mM NaCl, and pH 2.2. (d-f) Transmission electron microscopy (TEM) images of ovalbumin aggregates formed in the presence of 50 mM, 150 mM, and 300 mM of NaCl, respectively. The inset in Figure S3e depicts long, curvilinear fibrils in addition to unstructured aggregates.

Figure S4

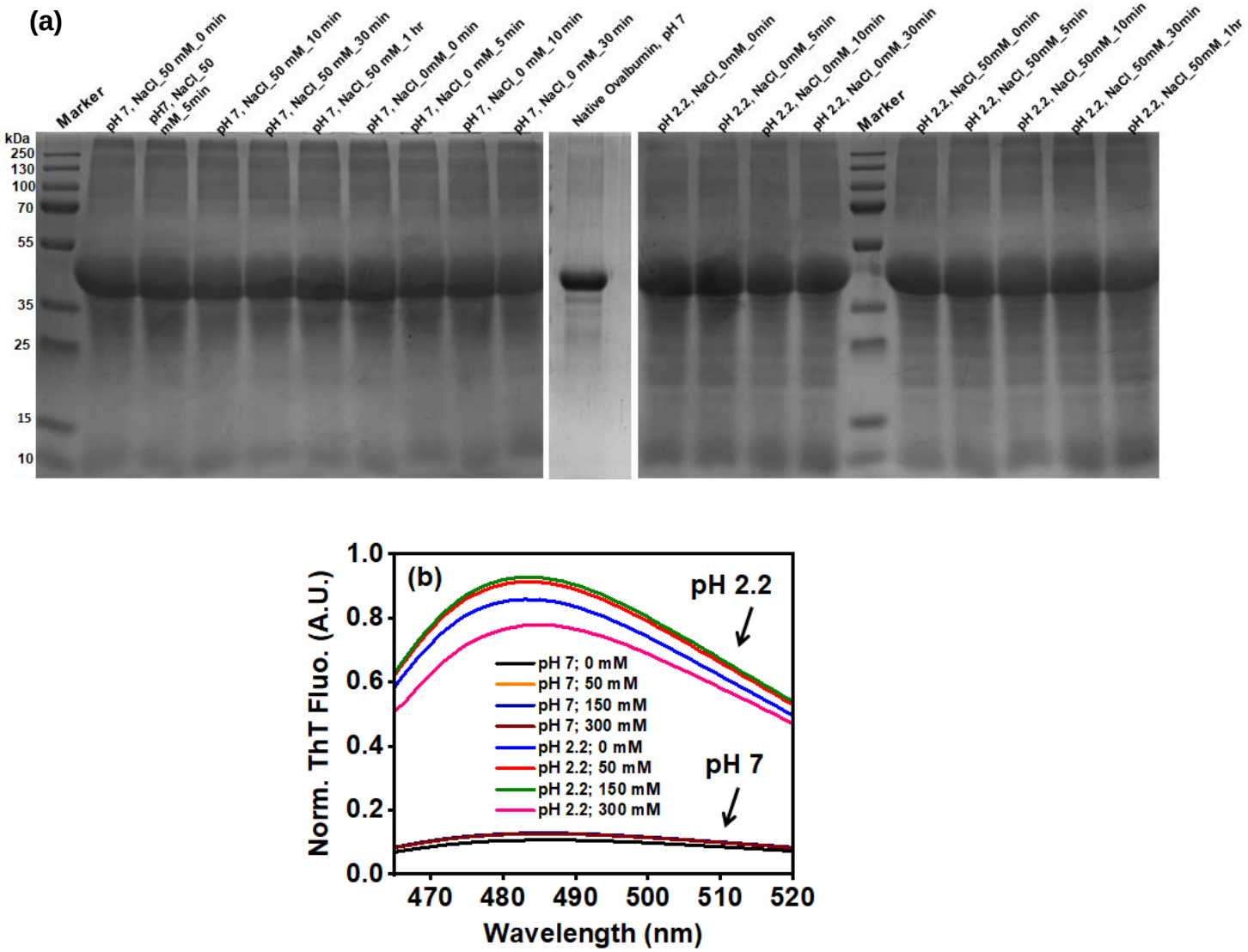

Figure S4. (a) SDS-PAGE results of native ovalbumin and ovalbumin aggregates formed at pH 7 and pH 2.2, 65 °C in the absence and in the presence of NaCl. (b) Normalized Thioflavin-T (ThT) fluorescence emission of ovalbumin aggregates formed at pH 7 and pH 2.2, 65 °C in the absence and in the presence of NaCl. The downward black arrows serve as eye-guides for the aggregates formed at pH 7 and pH 2.2. All of the spectra were collected at ~25 °C.

Figure S5

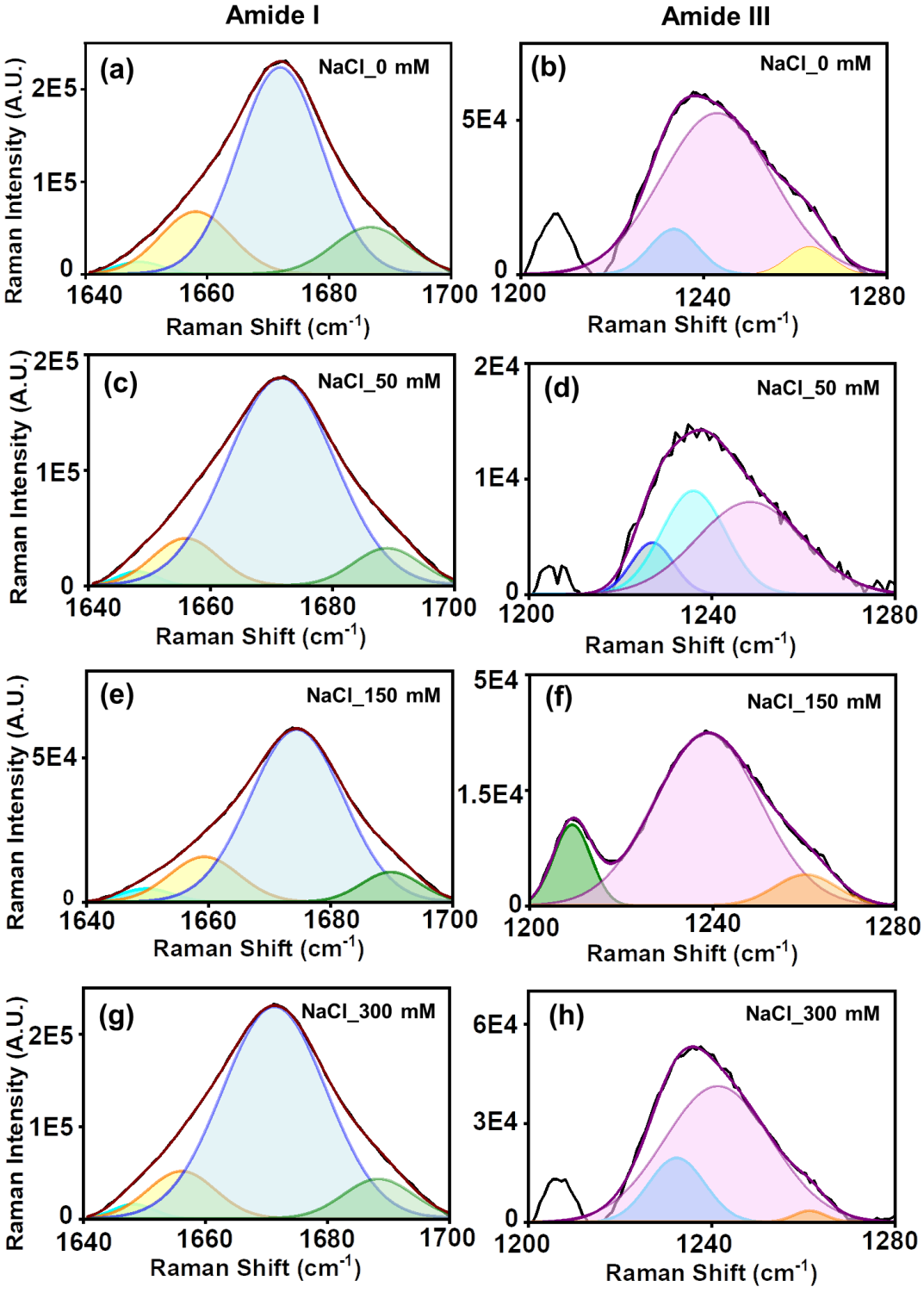

Figure S5. Representative deconvolutions of the amide I and amide III regions of ovalbumin aggregates formed after 2 hours at 65 °C (a,b) in the absence and (c-h) in the presence of varying concentrations of NaCl. The black lines in all of the spectra represent the actual data and the cumulative fits are denoted by the red (for amide I) and purple (for amide III) lines.

Figure S6

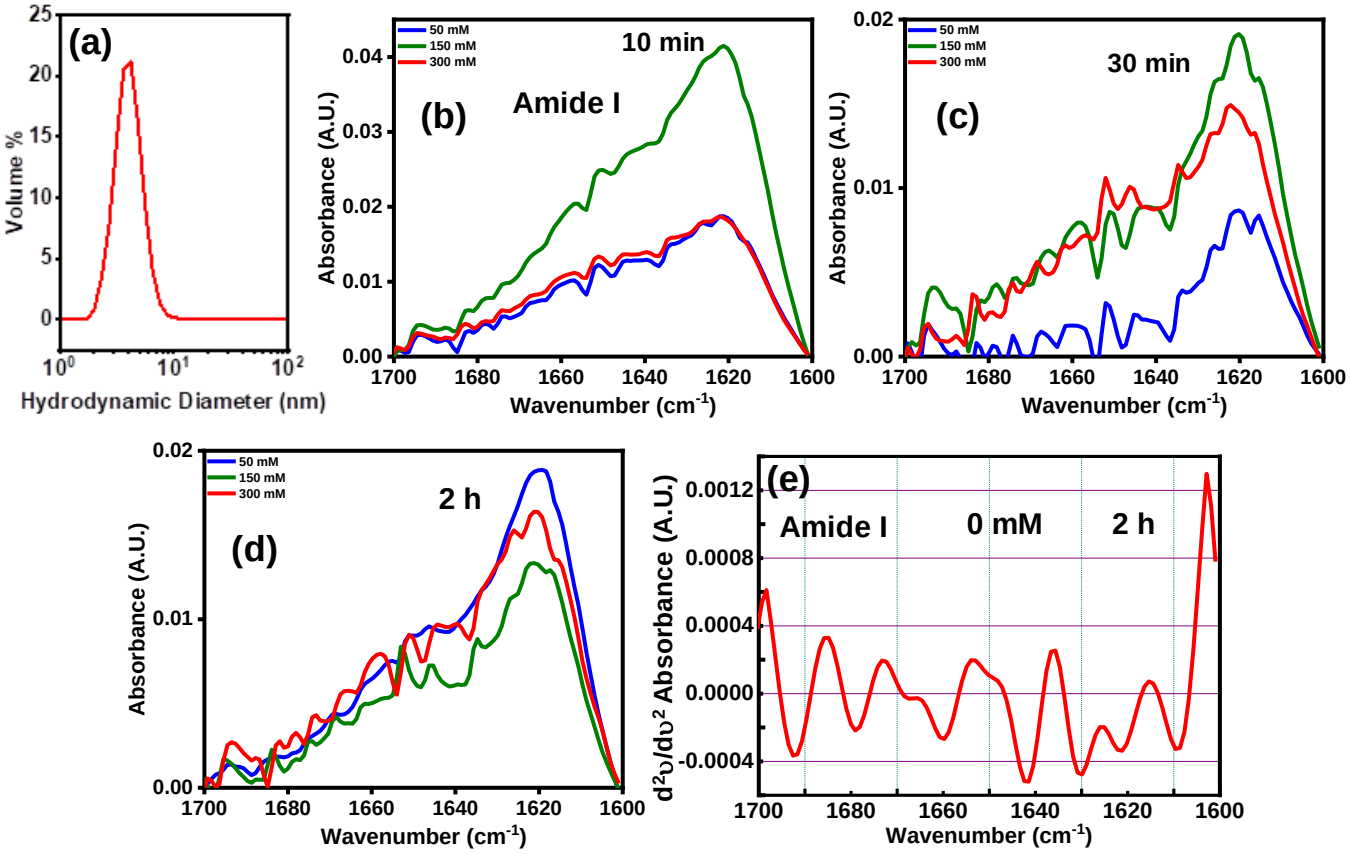

Figure S6. (a) Hydrodynamic diameter of ovalbumin at a concentration ≥1 mM estimated using dynamic light scattering (DLS) at ~25 °C prior to FT-IR measurements. (b-d) Temporal changes in the absorbance of the amide I region of ovalbumin aggregates formed at 65 °C in the presence of 50 mM (blue), 150 mM (olive), and 300 mM (red) of NaCl at (b) 10 min, (c) 30 min and (d) 2 hours. (e) The second derivative spectrum of the amide I region for the aggregates formed in the absence of salt.

Table S1

S. No.	Ionic Strength [NaCl] (mM)	Amyloid Marker Thioflavin-T (ThT)	Hydrophobic Marker 8-Anilinonaphthalene-1-sulfonic acid (ANS)
		Avg. k (min^{-1})	Avg. k (min^{-1})
1.	0	0.07 ± 0.02	0.41 ± 0.13
2.	25	0.1 ± 0.13	0.59 ± 0.08
3.	50	0.19 ± 0.18	0.43 ± 0.20
4.	75	0.13 ± 0.12	0.29 ± 0.08
5.	100	0.16 ± 0.22	0.44 ± 0.21
6.	150	0.14 ± 0.34	7.8 ± 0.12
7.	200	0.22 ± 0.07	0.50 ± 0.18
8.	300	0.18 ± 0.20	7.3 ± 0.59
9.	500	0.2 ± 0.06	0.20 ± 0.08

Table S1. The apparent rate constants, as a function of solution ionic strength, extracted from ovalbumin aggregation, monitored using changes in ThT and ANS fluorescence as a function of time. The kinetic traces were fitted using mono-exponential function (for details, see Experimental Methods). The error bars in the average rate constants represent the mean ± s.e.m. ($n \geq 6$).

Table S2

Amide – I deconvolution

(a)

[NaCl] (pH 2.2, 65 °C)	α-helix (%) (1645-1654 cm^{-1})	β-sheet (%) (1665-1674 cm^{-1})	Coils/Extended (%) (1660-1665 cm^{-1})
0 mM	19 ± 1.8	66 ± 1.3	14 ± 0.9
50 mM	11 ± 1.8	84 ± 4.7	9 ± 0.23
150 mM	3 ± 0.1	70 ± 1.5	27 ± 3.1
300 mM	13 ± 0.6	78 ± 0.7	9 ± 0.8

Amide – III deconvolution

(b)

[NaCl] (pH 2.2, 65 °C)	α-helix (%) (1264-1272 cm^{-1})	β-sheet (%) (1230-1240 cm^{-1})	Coils/Extended (%) (1245-1255 cm^{-1})
0 mM	7 ± 4.1	10 ± 1.5	82 ± 4
50 mM	–	52 ± 4.2	48 ± 4.2
150 mM	4 ± 0.1	51 ± 0.4	44 ± 0.1
300 mM	–	19 ± 0.9	81 ± 0.8

(c)

Residue	Intensity Ratio	[NaCl] 0 mM	[NaCl] 50 mM	[NaCl] 150 mM	[NaCl] 300 mM
Tyrosine	I_{852}/I_{827}	2.7	1.1	0.6	0.8
Tryptophan	I_{1360}/I_{1340}	0.3	0.3	0.5	0.3

Table S2. Percentage analysis of secondary structural contents of ovalbumin aggregates, formed at 65 °C in the absence and presence of salt and after reaching an apparent saturation, obtained upon deconvolution of the (a) amide I and (b) amide III regions. (c) Intensity ratios for tyrosine (I_{852}/I_{827}) and tryptophan (I_{1360}/I_{1340}) doublets, obtained from side-chain analysis of the Raman bands.

Table S3

S. No.	Solution Conditions (pre-heated; ~25 °C)	Amide Bands (cm^{-1})
1.	pH 7; 0 mM NaCl	1635, 1652, 1683, 1615, 1625, 1671, 1697
2.	pH 2.2; 0 mM NaCl	1620, 1628, 1648, 1639, 1660
3.	pH 2.2; 50 mM NaCl	1621, 1629, 1659, 1693, 1611, 1640, 1648, 1679
4.	pH 2.2; 150 mM NaCl	1620, 1628, 1658, 1693, 1647
5.	pH 2.2; 300 mM NaCl	1625, 1636, 1649, 1683, 1671, 1697

Table S3. FT-IR spectral analysis of amide I (1700-1600 cm^{-1}) region for native and oligomeric (in absence and presence of salt) ovalbumin, for pre-heated samples.