Annexure A
Materials and Methods

A.1 REAGENTS

The chemicals and reagents that were procured from different sources to conduct all the studies in this thesis work are mentioned below:

Chemicals/Reagents	Make/Source
Bovine serum albumin (Lyophilized)	Sigma/Himedia
Lysozyme (Lyophilized)	Himedia
Insulin	Himedia
Cytochrome *c*	Himedia
Type 1 Collagen (Rat Tail Tendon)	Obtained for Dr. B. Madhan's Lab at CLRI, Chennai
Type 1 Collagen (Calf skin)	Sigma-Aldrich
Thioflavin-T	Sigma–Aldrich
Micrococcus lysodeikticus (Lyophilized)	Sigma–Aldrich
Eugenol	Sigma–Aldrich
Capsaicin	Sigma–Aldrich
Dihydro-capsaicin	Sigma–Aldrich
Type IA Collagenase	Sigma–Aldrich
Glycine	Sigma–Aldrich
Sodium Chloride (NaCl)	Sigma–Aldrich
Phosphate Buffer Saline (PBS)	Himedia
Guanidine Hydrochloride	Sigma–Aldrich
Hydrochloric Acid (HCl)	Himedia
Acetic Acid	Sigma-Aldrich
L-tryptophan	Sigma-Aldrich
L-tyrosine	Sigma-Aldrich
Sodium Hydroxide (NaOH)	Sigma-Aldrich
L-phenylalanine	Himedia
Tetrachloroauric Acid ($HAuCl_4$)	Sigma-Aldrich
Silver Nitrate ($AgNO_3$)	Sigma-Aldrich
Potassium Hydroxide (KOH)	Sigma-Aldrich
Ammonium persulphate	Sigma-Aldrich
N,N,N′,N′-Tetramethyleethylenediamine	Sigma-Aldrich
Commasie Brilliant Blue R-250	Himedia
Bromophenol Blue	Himedia
Tris Buffer	Himedia
Glycerol	Himedia
Acrylamide	Himedia
Bis-acrylamide	Himedia

A.2 FLUORESCENCE MEASUREMENTS

A Perkin Elmer LS 55 fluorescence spectrometer was used to carry out all the fluorescence experiments in the present thesis work, as mentioned below:

A.2.1 Thioflavin-T Assay

Thioflavin T binding assay was used to monitor the amyloid formation of proteins by recording Thioflavin T fluorescence. Thioflavin-T was excited at 440 nm with a slit width of 5 nm and the emission was observed at 490 nm with a slit width of 5 nm (Morozova-Roche, Zurdo et al. 2000). This assay was used to study both protein amyloid formation and disassembly of amyloid fibrils experiments.

A.2.2 Protein-Ligand Binding Assay

To study the molecular interactions between eugenol and insulin, intrinsic fluorescence property of eugenol was used. For monitoring the quenching of eugenol in the presence of insulin, fluorescence emission of the eugenol sample was measured in the presence of different concentrations of insulin. The excitation maxima (λ_{ex}) and emission maxima (λ_{em}) used for eugenol was 262 nm and 325 nm respectively (Bi, Yan et al. 2012). To obtain fluorescence emission spectra of eugenol, for data analysis, the spectra of insulin sample were subtracted from the respective spectra obtained for [insulin+eugenol] samples. (Morozova-Roche, Zurdo et al. 2000; Dubey, Anand et al. 2014). In this study eugenol concentration was kept constant, i.e. 1.65 μM whereas insulin concentrations were 1 μM, 2 μM, 7 μM, and 11 μM. It was also observed that insulin alone was also gives fluorescence emission when excited at 262nm. Therefore fluorescence spectra of insulin alone were subtracted from the eugenol-insulin spectra, to obtain fluorescence spectra of eugenol alone. Hence it was observed that eugenol fluorescence was significantly quenched by insulin, and there was a gradual decrease in eugenol fluorescence by increasing insulin concentration. This quenching of eugenol fluorescence in presence of insulin clearly indicates that there must be some molecular interactions taking place between them.

(a) Stern-Volmer Constant

The fluorescence quenching can be classified into static quenching and dynamic quenching process (Fujisawa, Kashiwagi et al. 1999). To calculate the magnitude and nature of quenching phenomenon, the fluorescence emission spectra was analyzed using **Stern-Volmer equation** (Fujisawa and Masuhara 1981; Fujisawa, Kashiwagi et al. 1999; Bi, Yan et al. 2012).

$$\frac{Fo}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{sv} [Q] \tag{A.1}$$

where, F_0 are the intensities of eugenol fluorescence in the absence of insulin, F are the fluorescence intensities of eugenol in the presence of the insulin, [Q] is the different concentration of insulin, K_q is the biomolecule quenching rate constant, τ_0 is the average lifetime of eugenol in the absence of insulin and its value is ~ 10^{-8} s (Bi, Yan et al. 2012), and K_{sv} is Stern-Volmer quenching constant. The maximum dynamic rate constant of various quenchers with the biopolymer is 2.0×10^{10} L mol^{-1} s^{-1} (Fujisawa and Masuhara 1981; Varman and Singh 2012). The Stern-Volmer plot was plotted against F_0/F vs [Q], the values of K_{sv} and K_q were 2.445×10^5 L mol^{-1} and 2.445×10^{13} L mol^{-1} s^{-1} respectively. It is observed that the value of K_q is greater than 2.0×10^{10} L mol^{-1} s^{-1}, which indicates that the quenching process of eugenol by insulin was static.

(b) Binding constant and binding site

Since the type of molecular interaction between eugenol and insulin was static, the binding constant (K_a) and binding site (n) parameters were calculated by using the following equation (Bi, Yan et al. 2012):

$$log\frac{F_0 - F}{F} = logK_a + \frac{1}{n}\ log\left([B_t] - \frac{1}{n}\frac{F_0 - F}{F_0}[D_t]\right) \quad (A.2)$$

Where, $[D_t]$ is the total eugenol concentration and $[B_t]$ is the total insulin concentration. On the assumption that 1/n in the bracket was equal to 1, the curve between $\log\frac{F_0-F}{F}$ versus $\log\left([B_t] - \frac{1}{n}\frac{F_0-F}{F_0}[D_t]\right)$ was plotted and fitted linearly. Then slope (1/n) was then calculated using the equation A.2, if the obtained slope was not equal to 1/n, then it was substituted into the bracket and the curve $\log\frac{F_0-F}{F}$ versus $\log\left([B_t] - \frac{1}{n}\frac{F_0-F}{F_0}[D_t]\right)$ was drawn again (Bi, Yan et al. 2012). This process was repeated till the same value of 1/n was not obtained. The final curve was plotted and binding constant (K_a) was measured to be ~5.75043 L mol^{-1}; and the number of binding sites (n) were 1.5, i.e. ~1.

A.2.3 Intrinsic Fluorescence Property of Proteins

A fluorescence spectrometer was used to measure the intrinsic tryptophan fluorescence of lysozyme. All measurements were carried out at 25 ºC. The lysozyme samples, in the presence and absence of collagen, were exposed to an excitation wavelength of 295 nm and emission spectra were recorded for further analysis. All the spectra were baseline subtracted.

A.3 AMYLOID FIBRIL FORMATION OF PROTEINS

Amyloid formation process in proteins was studied in an *in vitro* environment. To carry out protein aggregation, protein samples were incubated in two different conditions such as,

(i) In chapter 1, lysozyme (10 $mg.ml^{-1}$) samples were prepared in 10 mM glycine–HCl buffer at pH 2.0 containing 130 mM NaCl (Morozova-Roche, Zurdo et al. 2000; Kar and Kishore 2007), and were incubated at 58 ºC ;

(ii) For all the other aggregation studies in this thesis, amyloid formation of all the protein monomers individually (~ 0.3 $mgml^{-1}$) and in the presence of different molecules were achieved by incubating the samples in 10mM PBS, pH7.4, and at 70°C (Krebs, Morozova-Roche et al. 2004; Holm, Jespersen et al. 2007; Chatani, Imamura et al. 2014).

To study spontaneous amyloid formation in proteins, small aliquots from incubated samples were taken out at regular intervals to conduct the Thioflavin-T binding assay. Here the compounds studied against protein aggregation were type I collagen, eugenol, L-tryptophan, L-tyrosine, L-phenylalanine, $AuNPs^{Tyr}$, $AuNPs^{Trp}$ and $AgNPs^{Tyr}$ nanoparticles.

For conducting seed-induced amyloid formation reactions, preformed amyloid fibrils (~5-15 % weight/weight) were used as seeds. These preformed fibrils were incubated initially with protein monomers and the aggregation kinetics was carried out. To study cross-seeded amyloid formation reactions, preformed amyloid fibrils (~15 % weight/weight) of respective proteins were used as seeds.

For fibril-disassembly experiments, a suspension of mature protein amyloid fibrils was prepared and Thioflavin-T signal of the sample was recorded at different time intervals in the presence and absence of respective compounds such as, eugenol and $AuNPs^{Tyr}$.

All the measurement represents an average value of three data points and each experiment is repeated at least twice.

A.4 AMYLOID FIBRILS STABILITY STUDIES

Guanidine hydrochloride (chemical denaturant) was used to check the stability (Narimoto, Sakurai et al. 2004) of amyloid fibrils formed through individual aggregation as well as co-aggregation reactions. Thioflavin T signals of the samples containing suspension of amyloid fibrils in the presence and absence of GnHCl. The concentration of GnHCl was maintained at 1M and the incubation time was ~30 minutes at room temperature. Data presented were the % of Thioflavin T signal of the initial sample without GnHCl treatment. This experiment was repeated two times.

A.5 CIRCULAR DICHROISM

For the CD experiments, JASCO CD spectropolarimeter (model J-815-150 L and 715 CD) with attached Peltier temperature controller was used. The sample cell used was having path length of 2 mm.

For all the amyloid related experiments, the protein concentrations in CD sample were maintained in between 0.25 $mg.ml^{-1}$ to 0.8 $mg.ml^{-1}$. The structural changes in the protein sample were observed in the presence and in the absence of type1 collagen, eugenol, $AuNPs^{Tyr}$ and $AuNPs^{Trp}$; by monitoring the CD scan at different time points at 70 ^{o}C. Each plot represented here is an average of three accumulated plots. Thermal unfolding experiments of protein samples were carried out in the presence and absence of inhibitors by continuously recording the CD spectra at 222nm while the samples were heated from 15 ^{o}C to 90 ^{o}C with scanning rate of 2 ^{o}C min^{-1}.

In another study CD spectropolarimeter was used to understand the effect of capsaicin on the conformational properties of type I collagen. CD scans of collagen samples were obtained in the presence of different concentrations of capsaicin (1:10, 1:25, 1:50 and 1:100 molar ratios of collagen to capsaicin). Concentration of collagen solution was maintained at 0.5 $mg.ml^{-1}$. Thermal unfolding experiments of collagen samples were performed by monitoring the CD signal at 222 nm as the temperature of the sample was increased from 20°C to 60°C at a scan rate of 2°C per minute.

A.6 TRANSMISSION ELECTRON MICROSCOPY (TEM)

Transmission electron microscope (HR-TEM JEOL JEM-2100 and JEOL 1010) is used to examine mature amyloid fibrils and, $AuNPs^{Tyr}$, $AuNPs^{Trp}$ and $AgNPs^{Tyr}$ nanoparticles respectively. Mature amyloid fibril samples were spotted on a carbon-coated grid for ~2 mins and the samples were then washed with water. The samples were then stained with 1% (w/v) aqueous uranyl acetate solution for ~2 min followed by another washing step. Air-dried grids were then examined.

A.7 ATOMIC FORCE MICROSCOPY (AFM)

Conventional atomic force microscopy measurements were performed in the air by using XE-70 Park Systems. For AFM measurements, respective protein aggregate samples and collagen fibril were diluted (10 folds) in ultrapure water and then 20µl aliquot of was kept on freshly cleaved mica and samples were allowed to air dry. The dried samples were then washed drop-by-drop with ultrapure water and again allow to air dry. Images were taken immediately using tapping mode (NC-AFM) with a resonance frequency of 300 Hz. All AFM images were captured under ambient condition.

A.8 ATR-FTIR SPECTROSCOPY

FTIR spectra of of tryptophan and tyrosine coated gold and silver nanoparticles were recorded in DRS mode using Perkin-Elmer D100 spectrophotometer with a resolution of 4 cm^{-1}.

Bruker Vertor 70 spectrometer (equipped with silicon carbide source and MCT detector) was used for obtaining FTIR spectra of mature amyloid fibrils. OPUS 6.5 software (Bruker Co., Germany) was used for data processing. All original spectra of amyloid fibrils of different proteins formed in the presence and absence of eugenol were processed for baseline correction between 1700 $cm^{-1}$and 1600 cm^{-1} for further analysis.

A.9 ACTIVITY ASSAY OF LYSOZYME

The activity of lysozyme was determined against *M. lysodeikticus* using the turbidometric method (Kar and Kishore 2007). The decrease in turbidity of a 1 ml bacterial cell suspension (0.3 mg ml^{-1}) in different buffers (50 mM phosphate buffer at pH 6.5 and 10 mM glycine–HCl buffer at pH 2.0) was monitored after the addition of 0.1 ml of an appropriately diluted lysozyme solution. To the reference cell, 0.1 ml of lysozyme solution was added. The decrease in absorbance was monitored every 1s during a total incubation of 3 min at 450 nm using a UV-visible spectrophotometer. Activity of the enzyme was measured in the presence of collagen and all experiments were repeated at least three times.

A.10 UV-VISIBLE SPECTROSCOPY

Different UV-visible spectrophotometric techniques were used in this work to conduct some important studies, as described below:

A.10.1 Collagen Fibril Formation

The process of collagen fibril formation, in the absence and in the presence of different compounds (capsaicin and lysozyme), was carried out by monitoring the absorbance of collagen samples at 313 nm using a UV-visible spectrophotometer (Cary 100 and Cary 4000, Agilent Technologies). Collagen stock solution was prepared by dissolving appropriate amount of lyophilized collagen in 50 mM acetic acid solution, whereas capsaicin stock was prepared in ethanol and lysozyme stock was prepared in 10 mM glycine–HCl buffer. The concentration of collagen sample was maintained at 0.3 $mg.ml^{-1}$ and fibril formation measurements were recorded in PBS at pH 7.4 and at temperature 37°C. In the two different studies collagen sample was incubated with appropriate volume of capsaicin and lysozyme for ~5-30 minutes. Appropriate volume of 10X PBS was added to the sample just before the kinetic measurements. The reference solution for every experiment was prepared with appropriate amount of water, compound (capsaicin or lysozyme) and buffer without collagen to normalize the background.

A.10.2 Disassembly of Collagen Fibrils

For disassembly experiments, a suspension of mature collagen fibrils was prepared and its optical density at 313nm was monitored in the presence and in the absence of capsaicin. To the reference cell the same amount of capsaicin was added to do baseline correction. All the experiments were repeated at least two times.

A.10.3 Protein Concentration Measurement

UV-visible spectrophotometer (Varian Cary-4000 and Shimadzu UV-1800) was used to mmeasure concentrations of protein samples (23) and to obtain UV-vis spectra of tryptophan and tyrosine coated gold and silver nanoparticles. The protein concentrations were determined spectrophotometrically by using extinction coefficients as follows: 2.63 $g.l^{-1}.cm^{-1}$ at 280nm for lysozyme , 43284 $M^{-1}.cm^{-1}$ at 280 nm for BSA, 6080 $M^{-1}cm^{-1}$ at 277 nm for insulin and 28 $mM^{-1}.cm^{-1}$ at 550 nm for cytochrome *c*.

A.10.4 Sedimentation Assay

In the BSA and cytochrome *c* coaggregation and cross seeding experiments, sedimentation assay method was employed to monitor the loss of cytochrome *c* monomer concentration during the aggregation process. In this protocol, an aliquot of the coaggregation sample was taken out and centrifuged at ~14000 rpm for ~35 min. The concentration of the soluble cytochrome *c* monomer was determined by examining the supernatant using a UV-Vis spectrophotometer (Babul and Stellwagen 1972).

In chapter 4, sedimentation assay was carried out to monitor the concentration of eugenol in the sample that was containing a mixture of eugenol and amyloid fibrils. Eugenol and amyloid fibrils were incubated for 20 minutes and then centrifuged at 15000 rpm. The concentration of eugenol in the supernatant was then measured using UV-visible spectrophotometer (SHIMADZU-1800). By varing the concentrations of protein aggregates while keeping the concentration of eugenol constant for all the samples. All data were base line corrected.

A.11 ENZYMATIC DEGRADATION OF COLLAGEN TENDONS

The enzymatic degradation of native and capsaicin-treated rat tail tendons (RTT) by collagenase was analyzed by estimating the amount of hydroxyproline released from the sample after hydrolysis reaction. RTT fibres were treated with capsaicin (1µM to 100 µM) for 24 hour at 27ºC before the addition of collagenase. Native RTT and capsaicin treated RTT were treated with type IA collagenase in a 0.04 M $CaCl_2$ solution (0.05 M tris-HCl buffer at pH 7.2). The ratio of collagen to enzyme was maintained at 50:1 and enzyme treated samples were incubated at 37ºC for ~96 hrs. The degradation of RTT was monitored by measuring soluble hydroxyproline released from insoluble collagen (Ryan and Woessner 1971). Aliquots of 750 µl of the supernatant were withdrawn after centrifuging the sample at 10,000 rpm for 10 min. The collagenase hydrolysate was hydrolyzed in the sealed hydrolysis tubes with 6N HCl at 120ºC for 16 hrs. The hydrolysates were evaporated in a porcelain dish using a water bath to remove excess acid. The residue free of acid was made up to a known volume and the percentage (%) of hydroxyproline was determined using the Woessner's method (Woessner 1961).

Hydroxyproline is a unique amino acid for collagen and it acts as a useful marker for identifying collagen in the presence of non-collagenous proteins. Woessner method of determining hydroxyproline involves the oxidation of hydroxyproline to pyrrole-2-carboxylic acid, which complexes with p-dimethylaminobenzaldehyde exhibiting maximum absorbance at 557 nm.

$$\% \text{ Hydroxyproline} = [\text{concentration in } \mu g \,/\, \text{weight of the sample}] \times \text{dilution factor}$$

$$\% \text{ Soluble collagen} = \% \text{ Hydroxyproline} \times 7.4$$

A.12 HYDROTHERMAL SHRINKAGE MEASUREMENT OF TENDONS

Micro-shrinkage tester technique was employed to measure the shrinkage temperature of native RTT fibers in the presence and in the absence of capsaicin. After treatment incubation, a small strip of RTT fiber was employed on a saline-grooved microscope slide. The temperature of collagen fibers in saline was raised till the onset of shrinkage is observed. Saline filled grooved microscopic slide was mounted on a heating stage with a constant heating rate of 2°C.min^{-1}. One-third shrink from original size was taken as the shrinkage temperature in this study.

A.13 NATIVE GEL ELECTROPHORESIS

Native (non-denaturing) polyacrylamide gel electrophoresis was performed at a constant voltage of 25 V with a mini-PROTEIN II Bio-Rad electrophoresis system using a Tris-HCl polyacrylamide gel. The gels were then were stained with Commassie Blue protein gel stain and the stained gels were visualized by Biorad gel documentation unit.

A.14 SYNTHESIS OF AMINO ACID-CONJUGATED GOLD AND SILVER NANOPARTICLES

To synthesize tryptophan and tyrosine amino acid-conjugated gold and silver nanoparticles, 100 ml aqueous solutions of 1 mM KOH containing 0.5 mM tryptophan and 0.1 mM tyrosine were seperately allowed to boil under vigorous stirring experimental condition (Selvakannan, Ramanathan et al. 2013). In the stirring boiling solutions, $[AuCl_4]^-$ or Ag^+ ions were added to obtain tryptophan or tyrosine amino acid-conjugated gold and silver nanoparticles, respectively. The total concentrations of both the $[AuCl_4]^-$ or Ag^+ metal ions were kept constant at 0.2 mM in all the reactions. Through this process two different gold ($AuNPs^{Tyr}$, $AuNPs^{Trp}$) and two silver nanoparticles ($AgNPs^{Tyr}$, $AgNPs^{Trp}$) solutions were obtained. All these nanoparticle solutions thus obtained were cleaned from the unreduced metal ions and unbound amino acids by dialyzing these solutions against deionized water using 12 kDa dialysis membranes for 3 hours followed by overnight dialysis. Even after dialysis, all these gold and silver nanoparticles solutions remained stable without showing any sign of aggregation, indicating that these nanoparticles were strongly capped with amino acids.All the solutions of tryptophan and tyrosine amino acid-conjugated gold and silver nanoparticles were found to be stable under standard laboratory storage conditions at room temperature for more than 6 months, and used as such for characterisation and biological studies.

A.15 COMPUTATIONAL STUDIES

Apart from biophysical methods some *in silico* approaches were employed to get a better understanding towards the molecular interactions that are taking place during protein-ligand complex formation.

A.15.1 Sequence Similarity Analysis

While examining coaggregation phenomenon among globular proteins, sequence similarity search was carried out among all the globular proteins (as mentioned in table below) used in this study by using BLAST-p tool available in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein structures and sequences in FASTA format were obtained from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). The proteins and their FASTA sequences that were used for similarity search are summarized below:

Globular Proteins	PDB ID
Bovine Serum Albumin	4F5S
Lysozyme	193L
Cytochrome *c*	1HRC
Insulin	4I5Z

A.15.2 Molecular Docking Studies

Molecular docking studies were performed between protein and ligand using Discovery studio 4.0 (DS4). The ligand structures were prepared using 'prepare ligand wizard' of DS4. X-ray crystal structures of proteins were obtained from Protein Data Bank (PDB) and prepared through 'prepare protein wizard' of DS4. The structures were cleaned by removing

water and heteroatoms leaving behind nascent molecules. The pre-processing and protonation were carried out using CHARMm force fields (Brooks, Brooks et al. 2009). The ligand and protein were then docked using a blind approach (undefined active site) with 100 conformations to choose and 100 orientations to refine by following CDocker protocol (Wu, Robertson et al. 2003). The complex was then typed with CHARMm force field, and was subjected to molecular dynamics simulation for 5000 picoseconds under NTP (normal temperature and pressure) and distance-dependent dielectrics (implicit solvent model as a crude approximation of polar solvent). The simulations were executed in a Dell precision T5610 workstation with 16 processors and 32 GBs of RAM. All the details regarding to the x-ray crystallographic structures of protein and the natural compounds that were used in distinct docking studies are summarized below:

Insulin-Amino acid Interaction	
Insulin	PDB ID: 4I5Z
L-Tryptophan	PubChem ID: CID 6305
L-Tyrosine	PubChem ID: CID 6057
L-Alanine	PubChem ID: CID 5950
Protein-Eugenol Interactions	
Insulin	PDB ID: 4I5Z
BSA	PDB ID: 4F5S
Eugenol	PubChem ID
Collagen Peptides-Capsaicin Interactions	
Collagen Peptide $(POG)_4POA(POG)_5$	PDB ID: 1CAG
Collagen Peptide $(POG)_4EKG(POG)_5$	PDB ID: 1QSU
Collagen Peptide $(POG)_4LOG(POG)_5$	PDB ID: 2DRT
Collagen Peptide $X(GPO)_2GFOGER(GPO)_3X$	PDB ID: 1Q7D
Capsaicin	PubChem ID: CID 1548943

A.15.3 Decoy Analysis

Molecules structurally similar to capsaicin were identified from the ZINC database (Irwin and Shoichet 2005) by using a similarity score of 50 %. Of the best matches, 10 molecules (ZINC7911905, ZINC7911909, ZINC35317807, ZINC38032190, ZINC44869451, ZINC44869455, ZINC48325216, ZINC50950504, ZINC60968773, and ZINC78400413) were selected as decoys such that they, while having high structural similarity to capsaicin, were dissimilar with regard to the substructures reported to be involved in interactions with the molecule. These decoys were subjected to docking with the same procedure that was used for capsaicin. All docking studies were performed with Discovery Studio 4.0 on a 10 core Dell Precision 5610 Workstation.

A.15.4 Insulin Dimer Analysis

Visualizations were performed using DS4.0, for the analysis of crystal structure of bovine insulin multimers. All the insulin dimers that were used in this study were obtained from PDB. Out of the four multimeric bovine insulin crystal structures, two structures were hetero 12-mers (PDB IDs: 2A3G and 2INS) and two structures were hetero 6-mers (PDB IDs: 2ZP6, 4IDW) (Smith, Duax et al. 1982; Smith, Pangborn et al. 2005; Margiolaki, Giannopoulou et al. 2013). The interface of insulin structures was analyzed to identify the key amino acids involved in multimer formation using DS4. The structures were cleaned by removing water and heteroatoms leaving behind nascent molecules. Here those regions were analyzed where two insulin monomers were interacting with each other.

A.16 DATA-PROCESSING

Error bars are standard deviation from analysis in either duplicate or triplicate. The kinetic data points were fit in Origin v2015 software (Origin Lab). All the aggregation data as shown in figures were connected through the b-spline line using the ORIGIN program. FTIR data were analysed by OPUS 6.5 software (Bruker Co., Germany).

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