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	Sigma/Himedia
Lysozyme	Sigma/Himedia
Insulin	Himedia
Cytochrome *c*	Himedia
Myoglobulin	Himedia
L-Phenylalanine	Himedia
L- Proline	Himedia
L-Tyrosine	Himedia
L-Tryptophan	Himedia
L-Glutamine	Himedia
L-Alanine	Himedia
L-Arginine	Himedia
L-Hydroxyproline	Sigma–Aldrich
Phosphate Buffer Saline (PBS)	Himedia
Water (HPLC-Grade)	Merck
Leishman Stain	Merck
Methanol	Merck
Ethanol	Merck
Sodium Hydrogen Phosphate	Merck
Di- Sodium Hydrogen Phosphate	Merck
Sodium Chloride (NaCl)	Sigma–Aldrich
Sodium Hydroxide (NaOH)	Sigma-Aldrich
Silver Nitrate ($AgNO_3$)	Sigma-Aldrich
Osmium Tetrachloride	Merck
Sodium Dodecyl Sulphate	Merck
Potassium Hydroxide (KOH)	Sigma-Aldrich
Ammonium Persulphate	Sigma-Aldrich
N,N,N′,N′-Tetra methyl ethylenediamine	Sigma-Aldrich
Commasie Brilliant Blue R-250	Himedia
Glycine	Sigma–Aldrich
Bromophenol Blue	Himedia
Tris Buffer	Himedia
Glycerol	Himedia
Acrylamide	Himedia
Bis-acrylamide	Himedia

Capsaicin	Sigma–Aldrich
Thioflavin-T	Sigma–Aldrich
Piperine	Sigma–Aldrich
Micrococcus lysodeikticus (Lyophilized)	Sigma–Aldrich
Sodium Borohydrate	Sigma–Aldrich
Trisodium Citrate	Sigma–Aldrich
Acetic Acid	Sigma-Aldrich
Hydrochloric Acid (HCl)	Himedia
Tetrachloroauric Acid ($HAuCl_4$)	Sigma-Aldrich
Type I Collagen (Rat Tail Tendon)	Obtained from Dr. B. Madhan's Laboratory at CLRI, Chennai
Type I Collagen (Calf skin)	Sigma-Aldrich

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

Amyloid aggregation kinetics of proteins was monitored using a ThT fluorescence assay that shows increased fluorescence intensity of ThT as it detects formation of amyloid fibrils. All experiments were performed in an assay buffer (10 mM phosphate buffer saline, pH 7.4 at 37°C) and concentration of ThT was maintained at 30 μM (Morozova-Roche et al. 2000). The ThT fluorescence was recorded as a function of time under ambient conditions, using a Perkin Elmer LS 55 fluorescence spectrometer, with a 440-nm excitation filter and a 495-nm emission filter.

A.2.2 Protein-Ligand Binding Assay

The interaction between the protein and ligand can be analyzed by fluorescence quenching experiment. The interaction between piperine coated gold nanoparticles and the bovine insulin was recorded with the help of fluorescence spectrophotometer and analyzed quantitatively. The concentration on insulin was kept constant ~9μM with varying the concentration of piperine capped gold nanoparticles from 0.4μM to 6μM. The samples were prepared in 0.1 M PBS at pH of 7.4. Insulin samples were excited at 276 nm, and the slit width (band width) was fixed to 10 nm for excitation and emission data. The emission spectra were collected from 280 to 350 nm at room temperature. Binding constants were calculated using fluorescence intensity values obtained at the emission maximum of 305 nm. From these values, the Stern-Volmer constant was calculated. The nature of binding, binding constant and then the number of binding sites were also predicted.

The insulin molecule was considered as a fluorophore and piperine as quencher. Insulin molecule when excited at 276 nm (due to the presence of four aromatic tyrosine molecule) gives its emission peak at 305nm. The concentration of the insulin was kept constant at ~9 μM for all the experiments while the concentration of the quencher was varied from 0.4 to 6 μM. The obtained fluorescence spectra for the insulin were analyzed by using Stern Volmer equation:

$$\frac{F0}{F} = 1 + Ksv[Q]$$

F_0 = Fluorescence intensity in the absence of quencher.
F = Fluorescence intensity in the presence of quencher.
Q = Concentration of the quencher.

The data were plotted. The slope value of the linear fit gives the Stern Volmer constant [K_{sv}] which is ~$4.48 \times 10^2 M^{-1}$. From this one can predict whether the binding of ligand with the molecule is static or dynamic. This can be calculated using the formula Ksv = τ_o[Kq] where τ_o is

the average life time of any biomolecule which is 10^{-8} seconds. The results revealed the binding value [Kq] was $4.48x10^{12}M^{-1}$. Since any biomolecule with the Kq value greater than $2.0x10^{-10}M^{-1}$ has a static binding property, this study indicated a static binding between piperine and insulin

A2.3 Calculation of binding constant (K_a) and binding site (n).

The binding site and the binding constant of piperine to insulin were calculated by the formula as mentioned below.

$$\log\frac{Fo-F}{F} = \log(Ka) + nlog[Q]$$

F_0 = Fluorescence intensity in the absence of quencher
F = Fluorescence intensity in the presence of quencher
Q = Concentration of the quencher
K_a= binding constant
N= number of binding sites

The values of log (F_o-F/F) were calculated and a graph was plotted against the calculated log [Q] values and was fitted linearly. The (ln) of slope is the binding constant which is approximately $4.23x\ 10^2$±1.511. The slope value was 0.61 which indicated single binding site for piperine.

A.3 AMYLOID FIBRIL FORMATION OF PROTEINS

A.3.1 Understanding Amyloid fibril formation:

In chapter 3, amyloid fibril formation process in proteins was studied in an *in vitro* environment. Aggregation of phenylalanine monomers (1mM to 3mM) was monitored in both water and PBS at room temperature as well as at 37°C. Aggregation of all the protein samples were carried out at a concentration of: ~15µM for BSA, ~174µM for insulin, ~70µM for lysozyme, ~57µM for myoglobin, ~82µM for cytochrome *c*. For the sample containing a mixture of protein monomers, equimolar concentration all proteins at ~2µM. Phenylalanine-induced aggregation was carried out in PBS at 37°C. Preformed phenylalanine aggregates (~15 % w/w) were used as seeds for conducting all seed-induced aggregation reactions of protein samples.

A.3.2 Understanding single molecule aggregation:

In chapter 3, aggregation kinetics of phenylalanine (~1mM) and a mixture of amino acids (proline, tryptophan, tyrosine, glutamine, alanine and arginine in equimolar concentrations of 0.1mM) were carried out in PBS (pH 7.4) at 37°C. Phenylalanine sample and the sample containing mixture of amino acids was incubated in the presence and in the absence of phenylalanine-fibrils (used as seeds at ~15% w/w). Then turbidity of the samples was monitored at 450 nm at different time points using UV-1800 Shimadzu spectrophotometer.

A.3.3 Amyloid aggregation studies of BSA with Silver nanoparticles:

In chapter 4, amyloid aggregation of BSA was studied by incubating the monomer samples (~5µM) in 1X PBS at ~70 °C (Chatani et al. 2015)(Holm et al. 2007)(Krebs et al. 2004) in the presence and in the absence of capsaicin, control silver nanoparticles (AgNPs) and capsaicin capped silver nanoparticles ($AgNPs^{Cap}$). Small aliquots of these aggregating samples were taken out at regular intervals and their Thioflavin T fluorescence intensities were recorded. The molar concentration of ThT was ~60µM in the final 1ml volume prepared for ThT readings. The same concentration of AgNPs and $AgNPs^{Cap}$ was maintained in all the experiments to make sure the number of nanoparticles remains same.

A.3.4 Amyloid aggregation of Insulin and Insulin with Gold nanoparticles:

In Chapter 4 Amyloid fibril formation was achieved by incubating the protein monomer samples BSA and Insulin in PBS (pH 7.4) at ~70°C in the presence and absence of $AuNPs^{piperine}$

piperine and citrate coated gold nanoparticles. For conducting seed-induced aggregation of proteins, preformed amyloid fibrils (~15 % weight/weight) of the respective proteins were used as seeds.

A.4 CIRCULAR DICHROISM

For the CD experiments, JASCO CD Spectro polarimeter (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. All CD signals in results are the average representative of three acquisitions.

In the third chapter, CD was employed to check the structural changes in the protein samples incubated with and without phenylalanine aggregates by monitoring the CD scans before and after aggregation, under ambient conditions. For all the amyloid related experiments, the protein concentrations in CD samples were maintained in between 0.25 $mg.ml^{-1}$ to 0.8 $mg.ml^{-1}$.

In chapter 5, the structural changes of insulin samples undergoing aggregation in the presence and in the absence of $AuNPs^{piperine}$ and piperine were monitored using CD.

A.5 ACTIVITY ASSAY OF LYSOZYME

The activity of lysozyme was determined against *M. lysodeikticus* using the turbidimetric 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) 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. Activity was measured in the presence of $AuNPs^{piperine}$ at 1:10 and 1:20 molar ratio values of protein: ligand. The lysozyme sample (100 µl from 1 $mg\ ml^{-1}$ stock) was incubated with $AuNPs^{piperine}$ (400 µl and 600 µl for maintain 1:10 and 1:20 respectively from 200 µM stock) for ~5 min. The same amount of $AuNPs^{piperine}$ was added to the reference cell. All experiments were repeated at least three times.

A.6 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.7 DYNAMIC LIGHT SCATTERING (DLS) AND ZETA POTENTIAL MEASUREMENTS

The hydrodynamic size (diameter) measurements of the synthesized nanoparticles were performed on a Malvern Zetasizer Nano ZS (Malvern Instruments, Southborough, Massachusetts) equipped with a back-scattering detector (173 degrees). Samples were filtered through a pre-rinsed 0.2-µm filter before a minimum of three measurements per sample were made. Zeta potential measurements were carried out using a Nano ZS (Malvern) and a titrator MPT-2. An aqueous suspension of silver nanoparticles was filtered through a 0.45 µm PTFE membrane before measurement. The value of zeta potential is useful for understanding and predicting interactions between particles in suspension.

A.8 ATOMIC FORCE MICROSCOPY (AFM)

Conventional atomic force microscopy measurements were performed in the air by using XE-70 Park Systems. For AFM measurements, respective nanoparticles 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.9 SCANNING ELECTRON MICROSCOPY (SEM)

A Carl Zeiss EVO18 SEM was used throughout this study.

A9.1 SEM of Amyloid and collagen fibers

The amyloid fibers and collagen fibers generated were observed in SEM. The aggregate samples were centrifuged for 10,000 rpm for 10 minutes at 4°C. The obtained pellet was then resuspended in milliQ deionized water to remove the salts. Then the samples washed five times by centrifugal separation. Then obtained pellet was resuspended in water and then casted over silver stubs and was sputtered with gold/ palladium for 180 seconds. Thus, the prepared samples were imaged in SEM at a constant voltage of 20 kV.

A9.2 Scanning Electron Microscopic studies of RBC's

The exact morphology of RBC was confirmed by Scanning Electron Microscopy. The cell pellets washed in PBS and was fixed by incubating with 1% of glutaraldehyde for 2 hours after that the samples are treated with 2% osmium tetrachloride for an hour. The cell was then washed thrice with 10% ethanol and dehydrated by incubating with 10%, 20%, 30%, 50%, 70% and 80% ethanol for an hour each and then desiccated (Yildirim et al. 2013). Thus, obtained RBC pellet was sputtered with gold/palladium and viewed under compound microscope and then in SEM.

A.10 TRANSMISSION ELECTRON MICROSCOPY (TEM)

Transmission electron microscope (HR-TEM JEOL JEM-2100 and JEOL 1010) is used to examine mature amyloid fibrils and, all the silver and gold 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.11 FLUORESCENCE MICROSCOPY

The fluorescence microscopic images of Thioflavin T stained amino acid co-aggregates and insulin fibrils in presence and absence of inhibitors, by using FLoid cell imaging station Life Science.

A.12 DARK FIELD MICROSCOPY

Dark field microscopy was used to visualize the morphological changes of RBC's in the presence and absence of phenylalanine aggregates.

A.13 UV-VISIBLE SPECTROSCOPY

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

A.13.1 Collagen Fibril Formation

The process of collagen fibril formation, in the absence and in the presence of different compounds (piperine, AuNPs^{piperine}, citrate capped gold nanoparticles, proline, hydroxyproline, phenylalanine, tryptophan, AuNPs^{PHE}, AuNPs^{PRO}, AuNPs^{HYP}, AuNPs^{TRP}), was carried out by monitoring the absorbance of collagen samples at 313 nm at 37°C using a UV-visible spectrophotometer (nanodrop thermo). Collagen stock solution was prepared by dissolving appropriate amount of lyophilized collagen in 0.2 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. All turbidity curves shown in results are the average representative of three acquisitions.

A.13.2 Protein Concentration Measurement

UV-visible spectrophotometer (Varian Cary-4000 and Shimadzu UV-1800) was used to measure the concentrations of protein samples. 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 278 nm for insulin and 28 $mM^{-1}.cm^{-1}$ at 550 nm for cytochrome *c* and 12.8 $mM^{-1}.cm^{-1}$ at 450 nm for myoglobulin.

A13.3 Characterization of nanoparticles

The synthesized nanoparticles ($AgNPs^{Cap}$, $AuNPs^{piperine}$, citrate capped gold nanoparticles, $AuNPs^{PHE}$, $AuNPs^{PRO}$, $AuNPs^{HYP}$, $AuNPs^{TRP}$) were centrifuged at 10000 rpm and the obtained pellet was washed thrice with water. Then the pellet was re-suspended in water Initial characterization of both the control and capsaicin coated silver nanoparticles were examined by measuring the absorption spectra (280nm-750nm) by a UV Spectrophotometer (Shimadzu Japan UV-1800).

A.14 NATIVE GEL ELECTROPHORESIS

Native (non- denaturing) polyacrylamide gel electrophoresis for the aggregates was performed in a 15% the acrylamide gel was run at a constant voltage of 10 Amp with a mini-PROTEIN II Bio-Rad electrophoresis system using a Tris-HCl polyacrylamide gel at 4°C. The main objective of this experiment was to check the formation of higher order entities but not the molecular weight. The gels were then developed by silver staining. The stained gels were visualized by Biorad gel documentation unit and the images were processed by image lab software (Biorad)

A.15 SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS.

To check the released surface proteins from RBCs SDS gel electrophoresis was done. The lysate sample obtained after the hemolysis assay was centrifuged at 15000 rpm for 20 minutes at 4°C the resultant pellet was washed in sterile PBS buffer thrice and the final pellet was again resuspended in PBS. The protein samples were then mixed with Laemmli buffer (4% sodium dodecyl sulfate [SDS], 20% glycerol, 10% 2 β-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris-HCl). The mixture was then heated using boiling water bath for ~5 minutes and the samples were loaded on a 12.5% polyacrylamide gel (operated in ambient conditions at ~ 30 Amp). Silver staining was performed and the stained gels were visualized by Biorad gel documentation unit.

A.16 SYNTHESIS OF NANOPARTICLES

Silver nanoparticles were synthsized by boiling 1mM silver nitrate solution in a rapid-stirring condition for about an hour (Amruthraj et al. 2015). A solution of ~17 μM of capsaicin (dissolved in 50 % ethanol) was added slowly to the silver nitrate solution under stirring condition till the end of the reduction process. To synthesize control silver nanoparticles without capsaicin, sodium borohydrate reduction method(Mulfinger et al. 2007) was used. Freshly prepared 2 mM of sodium borohydrate was rapidly stirred in an ice bath. To this sample, 1mM of silver nitrate was added drop wise (maintaining 1 drop per second). The reaction mixture was then stirred vigorously on a magnetic stirrer at least for 3 minutes. Reaction parameters were carefully controlled to obtain stable yellow colloidal silver sample.

The synthesis of piperine functionalized gold nanoparticles was carried out by dissolving in chloroform, which was then diluted in water to make a stock solution.

Approximately 0.2 mM of piperine was boiled under stirring condition with 1 mM KOH(Selvakannan et al. 2013). To this boiling sample, ~0.2 mM of $AuCl_4$ was added and the mixed sample was then kept under stirring condition till the end of the reaction.

The control gold nanoparticles used in this experiment were prepared by citrate-capped method (Gangwar et al. 2012). A 0.2 mM $HAuCl_4$ was boiled and stirred under the reflux condition for 30 min to this boiling solution a 7.76 mM of freshly prepared aqueous Tri Sodium citrate ($Na_3C_6H_5O_7$) was added directly into the boiling solution. The reaction was continued for ~20 to 30 min to attain the maximum reduction. These synthesized gold nanoparticles were aged for 7 days.

To synthesize phenylalanine, proline, hydroxy proline and tryptophan amino acid-functionalized gold and silver nanoparticles, aqueous solutions of 1 mM KOH containing 0.2 mM aminoacid were seperately allowed to boil under vigorous stirring experimental condition (Selvakannan et al. 2013). In the stirring boiling solutions, $[AuCl_4]^-$ ions were added to obtain functionalized gold nanoparticles, respectively. The total concentrations of the $[AuCl_4]^-$ ions were kept constant at 0.2 mM in all the reactions.

All the solutions 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.17 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.17.1 Molecular Docking Studies

Molecular docking studies were performed using Discovery studio 4.0 (DS4). The first step was preparation of the ligand and the protein molecule. The structure of ligand (eugenol) was obtained from PubChem (CID 3314) and was prepared using 'prepare ligand wizard' of DS4 where a pH based ionization method was followed (pH ranging from 6.5 to 8.5). X-ray crystal structures of insulin (PDB ID: 4I5Z) and BSA (PDB ID: 4F5S) (Pechkova et al. 2014) were obtained from Protein Data Bank (PDB). Initially the protein molecule was cleaned by removing water and heteroatoms leaving behind the nascent molecule. This nascent molecule was then prepared through 'prepare protein wizard' of DS4 with an inbuilt algorithm which protonates and preprocesses the protein molecules by predefined parameters at an ionic strength of 0.145 and at pH 7.4. The solvent was handled by explicit method in each case. Finally, these protein molecules were typed with CHARMm force field(Brooks et al. 2009b). The processed protein molecules were then used for further docking studies. Next, the ligand with the processed protein was docked using CDocker docking tool. CDocker is a powerful CHARMm based docking method that has been shown to generate highly accurate docked poses(Wu et al. 2003). The input ligand molecule (one tautomer and one stereoisomer) was docked with a stable static protein conformation. During docking the ligand molecule will be dynamic (100 ligand poses with a pose cluster of 0.1Å – predefined in CDocker protocol with 100 dynamic steps, 100 ligand orientation having 2000 heating and 5000 cooling steps while docking with the nascent protein structure). Each orientation is subjected to simulated annealing molecular dynamics protocol. After successful docking, the CDocker ranks the poses based on CHARMm energy and highest interaction energy scores (considering more negative as more favorable binding). The list of molecules with PDB IDs are shown in the following table. All these computational studies were triplicated, analyzed for its reproducibility and then reported.

MOLECULES	PDB-IDs
Insulin	PDB ID: 4I5Z
BSA	PDB ID: 4F5S
Lysozyme	PDB ID: 193L
Myoglobulin	PDB ID:1DWR
Cytochrome C	PDB ID:1HRC
Insulin	PDB ID: 4I5Z
BSA	PDB ID: 4F5S
Lysozyme	PDB ID: 193L
Myoglobulin	PDB ID:1DWR
Cytochrome C	PDB ID:1HRC
capsaicin	PubChem ID:1548943
Piperine	PubChem ID: 638024
Collagen Peptide $X(GPO)_2GFOGER(GPO)_3X$	PDB ID: 1Q7D
Tryptophan	PubChem ID: 6305
Phenylalanine	PubChem ID: 6140
Proline	PubChem ID:145742
Hydroxy proline	PubChem ID: 5810

A.17.2 Molecular Dynamics

In order to check the self-association and interaction between individual phenylalanine molecules the molecular dynamics studies were performed. The phenylalanine molecules were initially processed and packed in a cubic box model by using PACKMOL(J. M. Martínez and Martínez 2003). The mixture model protocol was selected for the phenylalanine and water molecules(L. Martínez et al. 2009). Thus, the obtained solvated molecules were used for further simulation studies. The MD simulations were carried out by Discovery studio 4.0 (DS4). Charmm36 force field was applied to the solvated cubic box. Simulations were performed at 273 to 310 K. The minimization of the molecules was done by steep descendent algorithm. The minimization was done twice. The obtained molecules were then re-equilibrated for 10,000 picoseconds to a target temperature of 310K. Leap frog verlet algorithm with shake constraints are used to fix the chemical bonds between the atoms of the molecule. To maintain a constant temperature and pressure for various components langevien dynamics and Berendsen pressure with MOLLY and Impulse algorithm was used. The molecular dynamics studies were performed with Discovery Studio 4.0 on a 16 core Dell Precision 5610 Workstation. All these studies were triplicated, analyzed for its reproducibility and then reported.

A.18 *In Vitro* HEMOLYSIS ASSAY

The human blood sample was collected from a volunteer donor all methods were carried out in accordance with relevant guidelines and regulations of Ethics Committee of Indian Institute of Technology Jodhpur, India. All the experimental protocols used for this study were approved by Ethics Committee of Indian Institute of technology Jodhpur (Approval letter no IITJ/EC/2016/02-D). Informed consent was obtained from all the subjects involved in this study prior to conducting these experiments. Packed Red Blood Cells (RBCs) were separated out from the whole blood by spinning the blood sample at 1500 rpm for 10 minutes. The pellets of RBCs obtained were washed five times with phosphate buffered saline (PBS) and then diluted. Briefly, from 25 % V/V of diluted RBC a 100µl was added to the samples (Yildirim et al. 2013)(Dobrovolskaia et al. 2008). All the samples and incubated at 37°C for four hours and was vortexed slightly for every half an hour. RBC`s incubated with deionized water and PBS were used as positive and negative controls respectively. After incubation, the samples are

vortexed again and centrifuged at 1600 rpm for 10 minutes. The obtained pellet was saved for microscopic observation. The supernatant that contains the lysate was separated out to check the absorbance. All the absorbance data shown are the average representative of three acquisitions.

A.18.1 Calculation of the percentage of lysis

To compute the lysis percentage. The supernatant of the blood samples was scanned by UV 1800 Shimadzu spectrophotometer. The percentage lysis was calculated by the given formula.

$$Percentage\ of\ Hemolysis = \frac{(A\ sample - A\ Negative\ control)}{(A\ positive\ control - Anegative\ \ control)} \times 100$$

A Sample- Absorbance of RBC supernatant in sample
A Negative control- Absorbance of RBC supernatant in PBS (pH ~7.4)
A positive control - Absorbance of RBC supernatant in water

A.19 LEISHMAN STAINING AND VISUALIZING OF RBCs BY COMPOUND MICROSCOPE

The cell pellet that has the RBC cells was examined initially by Leishman staining procedure for this the RBC were placed over clean glass slide and smeared. The smear was first washed with 100% methanol for 30 seconds. The Leishman stain was added to the fixed smear and was left undisturbed for 15 minutes. Then the slides were washed immediately by water and viewed under Leica microscope and then imaged.

A.20 Isothermal Titration Calorimetry (ITC)

The binding studies of AuNPsHYP inhibitors with soluble triple-helical collagen model peptides of (Pro-Hyp-Gly)10 were performed using NanoITC (TA Instruments, USA) at 298 K (Shea, K. J. et.all.,2012)). ITC titration of 10 μM of peptide sample with 50 μM nanoparticle was performed. All solutions were degassed under vacuum to eliminate air bubble formation inside the calorimeter cell. The reference cell was filled with double distilled and degassed water. During titration 1 μl of the AuNPsHYP (50μM) was injected to (POG)10 containing sample cell at an interval of 300 s. To correct the heat effects of dilution and mixing, control experiments were performed at the same concentrations of the collagen peptide and the nanoparticle sample and the obtained data were subtracted from the respective (Pro-HypGly)10 peptide-AuNPsHYP titrations. The data were analyzed and best-fitted using Nano Analyze 3.6 software to extract the thermodynamic parameters of the protein ligand interaction

A.21 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 analyzed by OPUS 6.5 software (Bruker Co., Germany).

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