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Supporting Information

Coomassie Brilliant blue G-250 acts as a potential chemical chaperone to stabilize therapeutic insulin

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Table of Contents

Figures

I. Supplementary Methods:

1. Materials and sample preparation

Human HI (HI, 91077C), Thioflavin T (ThT), Coomassie brilliant blue G-250 (CBBG), NaCl and HCl were purchased from Sigma-Aldrich. Milli-Q water was used in the preparation of a buffer and stock solution of ThT dye. HI solution was prepared in 25 mM HCl containing 100 mM NaCl (pH ~1.6) to form a stock solution and then centrifuged at 15000 rpm for 10 min and passed through a 0.22 μm pore size filter to remove any insoluble aggregates. The stock concentration of the protein solution was determined through JASCO-600 UV-vis spectrophotometer, using the extinction coefficient value of the protein studied was as follow 6200 M^{-1} cm^{-1} at 276 nm.[1] For inducing amyloid fibrillation, freshly prepared HI (320 μM and 100 μM) solution in absence and presence of CBBG was incubated for several hours at 60 °C without agitation.

2. ThioflavinT (ThT) Fluorescence Assay

ThT fluorescence was recorded to investigate the kinetics associated with the fibril formation of human insulin in different solution conditions. ThT is a small dye molecule that becomes highly emissive upon binding to the cradle of the β-sheet present in amyloid fibrils and produces a typical fluorescence emission spectrum with a peak maximum at ~482 nm in an aqueous solution.[2] The growth curve (kinetics) was made by measuring the fluorescence intensity of ThT in the presence of a quantitative amount of HI samples (100, 320 μM) taken at different time points of incubation of the HI solution in the presence and the absence of CBBG. The samples were incubated at 60 °C in 25 mM HCl containing 100 mM NaCl (pH 1.6) or sodium phosphate buffer (pH 7.2), in the presence and absence of CBBG to confirm whether the interaction of CBBG indeed inhibits the HI self-assembly and fibrilization process. 4 μl of incubated HI solution (either in the absence or presence with CBBG) was pipette out, added to 500 μl solution of ThT (~22 μM) and mixed carefully for the acquisition of fluorescence emission spectra using a Cary Eclipse fluorescence spectrophotometer. The optical path length of the fluorescence cuvette was 10 cm. The fluorescence emission wavelength range was 450-600 nm (excitation 440 nm, emission peak maximum ~ 482 nm). ThT fluorescence peak intensity at 482 nm was plotted against time, analysed and fitted to the sigmoidal curve using equation 1.[3]

$$Y = y_i + m_i x + \frac{y_f + m_f x}{1 + e^{-\left[\frac{(x - x_0)}{\tau}\right]}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where *Y* is the ThT fluorescence intensity at a particular time (x), *x* is the incubation time, and x_0 is the time to reach 50 % of maximal fluorescence; other parameters are determined by the fitting. The lag time is defined by $x_0 - 2\tau$. The apparent rate constant ($1/\tau$), m_i *and* m_f are two constants (linear coefficients).

In order to analyse insulin fibril disintegration, one and two-fold molar excesses of CBBG (10 μM and 20 μM) were added to preformed matured HI fibrils (10 μM) and incubated for 3 h at 37 °C with agitation 200 rpm in the presence of 10 μM ThT. 10 μM matured HI fibrils were incubated in the presence of various ThT concentrations (10, 20, and 30 μM) with and without CBBG (20 μM) treatment for the ThT competition experiment. The ThT intensity were

measured in a 96-well black plate using a BMG LABTECH POLARstar Omega spectrometer (Ortenberg, Germany).

3. ^{1}H proton NMR

All NMR spectra were recorded on Bruker AVANCE III 700 MHz, equipped with RT probe and at 25 °C. All NMR data acquisition and processing were done by using Topspin v4.0.6 software (Bruker). The 1D NMR time kinetics of HI (100 µM) was performed in presence or absence of equimolar CBBG at 60 °C in 25 mM HCl buffer with 100 mM NaCl and 10% D_2O at pH ~1.6. Normalized peak intensity (I/I_0) was calculated by taking the total peak intensity of HI from aromatic and amide region with time in presence or absence of CBBG and normalized by the HI peak intensity at initial (I_0). The data was fitted using sigmoidal curve equation 1. To rule out the effect of ThT on HI fibril kinetics, we added equimolar ThT in HI and HI:CBBG (1:1 molar ratio) sample and incubated at 60 °C.

The interaction of CBBG dye with HI (100 µM) in 25 mM HCl buffer with 100 mM NaCl and 10% D_2O at pH ~1.6 was determined through a series of one-dimensional (1D) ^{1}H proton NMR spectra of HI in presence of 0, 0.1, 0.25, 0.5, 0.75 and 1 molar excess of CBBG dye (from a stock of 3 mM CBBG). The NMR signal intensity data were fitted by using equation 2:

$$Y = Y_O + A\left(\frac{X}{Kd+X}\right) \quad \text{(2)}$$

Here, Y is normalized signal intensity value, Y_O is initial signal intensity value, A is the fraction of fast exchange protons under our condition, X is the concentration of added CBBG dye (µM), and K_d is the apparent intensity decay rate constant or fast exchange rate constant between free and bound CBBG.

Parallelly, we also recorded 1D ^{1}H NMR of 500 µM CBBG with the treatment of 10 µM and 25 µM HI fibrils in 25 mM HCl containing 100 mM NaCl and 10% D_2O (pH ~1.6) at 25 °C.

HI (1 mM) was dissolved in 10 mM sodium phosphate buffer (pH 2.0) containing 10 mM NaCl and 10% D_2O, with or without the addition of CBBG at 1:1 molar ratio for kinetics experiment using NMR. One-dimensional ^{1}H NMR and two-dimensional homonuclear ^{1}H-^{1}H NOESY NMR spectra (400 ms mixing time) were taken before and/or after incubation of samples at 60 °C for 24 h.

At the same time, zinc-free HI was prepared by the addition of EDTA followed by extensive dialysis and lyophilization. The lyophilized sample was then dissolved in 20% acetic acid-d_4 and 10% D_2O (pH 1.9) where the insulin exits as monomer.[4, 5] NOESY spectra of 350 µM HI were performed in the presence or absence of equimolar dye with a mixing time 200 ms at 25°C. 512 increments in t_1 and 2048 data points in t_2 dimension along with excitation sculpting pulse sequence were used for water suppression for all NOESY spectra. All NOESY spectra of HI were also recorded at 25 °C with 64 scans and a spectral width of 12 ppm for both the dimension.

Normalized peak intensity (I/I_0) was measured by taken the intensity ratios of [HI]:[CBBG] (I) and HI control (I_0). The running average of I/I_0 was determined by calculating the average I/I_0

of $C_\alpha H$ intensity for three sequential residues. CSP of residues was determined using equation no 3.

$$CSP = \sqrt{(\Delta\delta_{\omega 1})^2 + (\Delta\delta_{\omega 2})^2} \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Here $\Delta\delta_{\omega 1}$ and $\Delta\delta_{\omega 2}$ represents ^1H chemical shift difference in ppm unit between HI alone and HI in presence of CBBG in $\omega 1$ and $\omega 2$ direction, respectively in 2D NOESY NMR.

4. **Atomic Force Microscopy (AFM)**

Morphologies of the insulin aggregates produced in the absence and presence CBBG were performed using Pico plus 5500 ILM AFM system (Agilent Technologies). Incubation and sample conditions were similar to sample prepared for ITC and ThT fluorescence assay measurement described earlier. Micro-fabricated silicon cantilevers (resonance frequency of 300 kHz and spring constant range of 21-98 N/m) was used derive the morphological features of the aggregates formed from the incubated samples. The aliquot taken at a defined time of incubation was diluted with water and drop-casting was made on freshly cleaved muscovite mica substrate. The solvent was removed by evaporation at room temperature in the open air. The images were captured with a scan speed of 0.5 lines/sec and processed using Pico view version 1.1 software (Agilent Technologies).

5. **Cell viability assay**

Rat pancreatic β-cells (RIN-5f) were acquired from National Centre for Cell Science (NCCS, India) and cultured in complete RPMI-1640 media (Gibco) supplemented with 10% FBS, penicillin-streptomycin (1 unit/ml), gentamycin (50 µg/ml), and amphotericin B (2.5 µg/ml) in a 5% CO_2 incubator at 37 °C. Experiments were conducted out at 70-80% cell confluency.

Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which depicts the mitochondrial activity of the living cells.[6] By using mitochondrial enzymes, live cells may convert MTT into purple formazan crystals, and the amount of formazan formed corresponds with cell viability. 1×10^4 cells/well were seeded in 96-well plate for 24 h. The media was exchanged with complete RPMI-1640 containing 50 µM aggregated containing HI fibrils and coincubated HI:CBBG (at 1:1 molar ratio) complex and incubated for 24 h. In a separate set of experiments CBBG were treated in concentrations of 10 µM, 20 µM, 40 µM, 80 µM and 100 µM and incubated for 24 h. In both set of experiments; 24 h incubation was allowed. After that at a final concentration of 0.5 mg/ml, MTT solution was applied to the wells, which were then placed in an incubator with 5% CO_2 humidity at 37 °C for 3.5 h. To dissolve the formazan precipitate 100 µl of dimethyl-sulfoxide (DMSO) was added in each well. At 570 nm, the absorbance was measured with a microplate reader. Results were compared and expressed as a percentage of control non-treated cell.

We prepared two set of samples for MTT experiment to measure the toxicity of disintegrated fibril. In first set of samples, we incubated the matured HI fibril (100 µm) in presence or absence of one and two fold molar excess of CBBG at 37 °C and 200 rpm for 3 h. The other set of samples were heated at 60 °C with no agitation. The cells were treated, with 50 µM sample and followed the above MTT method.

6. Scanning Electron Microscopy (SEM)

RIN5f cells were processed similar as cell viability assay procedure. 50 μM HI fibrils and/ or HI:CBBG (1:1) aggregated samples were added in RIN5f cells and incubated for 24 h. The cells were then prepared for SEM. Glutaraldehyde and formaldehyde were used as a primary fixative to stabilize the ultrastructure. Osmium Tetra oxide was used for secondary fixation. The samples were dehydrated using ethanol gradient. The drying process is then supplemented with HMDS and liquid CO_2. A sticky carbon disc was used to attach the specimen on the metal stub. Finally, conductive material was used for sputter coating and proceed for SEM.

7. Native PAGE

The HI aggregated solutions (100 μM, without or with CBBG) were centrifuged at 5000 rpm for 5 min. The supernatant was studied by 15% native PAGE in tris-glycine running buffer (pH 8.8) for 1.5 h at 100V, 80 A. Coomassie brilliant blue stain was used to detect the protein. The gels were imaged using ImageLab software in Bio-Rad ChemiDoc system. Using the ImageLab software, bands were quantified.

8. Mass spectrometry

1 μl HI sample in absence or presence of equimolar CBBG was spotted onto MALDI plate and dried it. The mass spectrometry was recorded using a MALDI-TOF spectrometer (Autoflex spped by Bruker Daltonics GmbH) in the positive ion reflector mode. MS data were processed by the use of Data Flex Analysis and Biotools 3.6 software. .

9. Isothermal Titration Calorimetry (ITC)

ITC measurements were carried out at 25 °C on a VP-ITC titration microcalorimeter (Micro Cal Inc., Northampton, MA). HI and CBBG samples were thoroughly degassed on a thermovac before the use in titration. For the correction of heat of dilution, the sample cell was loaded with buffer (pH ~1.6) and the reference cell was also filled with the same buffer solution. The solution in the cell was stirred at 90 rpm by the syringe filled with 0.4 mM HI in an identical buffer solution. Injections of 4 μl of the buffer in the syringe were started after baseline stability reached. 28 sequential such injections were made into the ITC cell containing buffer solution. The titration of the CBBG solution (0.01 mM) in the cell was followed by 28 sequential 4μl injections of 0.4 mM HI into the ITC cell containing 1.8 ml of CBBG solution. The protein and CBBG solution were made in identical buffer conditions (25 mM HCl containing 100 mM NaCl (pH ~1.6)). The raw calorimetric data profile (heat released) of interaction between CBBG and HI at 25 ^{o}C were collected automatically and subsequently fitted to a one-site binding model by the Microcal LLC Origin 7.0 software. After subtracting the heat of dilution, a non-linear least-squares algorithm was used to fit an equilibrium binding equation to the data points (heat flow per injection against the concentration ratio of HI and CBBG). This best fit provides the apparent binding stoichiometry (n), the change in enthalpy (ΔH), and the dissociation constant (K_d). The change in free energy (ΔG) and change in entropy (ΔS) for the binding reaction were analyzed by the important equations of thermodynamics.

$\Delta G = -RT\ln K$ …… (4)

$\Delta S = (\Delta H - \Delta G)/T$ …… (5)

10. Circular dichroism (CD) measurements

Far-UV circular dichroism (CD) spectra were recorded at 25 °C on a Jasco J-815 spectro polarimeter (Easton, MD). For CD analysis, freshly prepared HI (320 μM and 100 μM) solution in the absence and presence of CBBG was incubated for several hours at 60 ^{o}C without agitation. HI solutions were prepared in 25 mM HCl containing 100 mM NaCl (pH ~1.6). The spectra were measured using diluted aliquots (final concentration was ~ 15 μM of HI) at a different time point of incubation. 300 μl of the incubated protein solution was taken in 0.1 cm path length cuvette and scanned between 200–250 nm with a scanning speed 50 nm/min, resolution of 0.2 nm. For each sample, the representative spectrum was average of at least three individual scans. We used BeStSel webserver for the deconvolution in default mode for extracting the secondary structure content from CD spectra.

11. Methods for in vivo animal-based experiments

a. Animals

Male C57BL/6J mice (6-8 weeks old, healthy) weighing 20-30 g were obtained from the Centre for Translational Animal Research (CTAR), Bose Institute, Kolkata, India. All animals were maintained according to the guiding principle of the Institutional Animal Ethics Committee (IAEC) using the CPCSEA approved protocol wide IAEC approval No # IAEC/BI/011/2021 dt. 28/09.2021. Before conducting the experiment, all animals were acclimatized for at least two weeks along with constant 12 h light/dark cycle with water and food ad libitum. All experiments complied with the National Research Council's Guide for the Care and Use of laboratory Animals (NIH Publication No.8023, revised 1978, U.S.A). All experiments also complied with ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments; https://arriveguidelines.org/arrive-guidelines).

b. Design of Experiments

24 Male C57BL/6J Mice were randomly divided in Four (4) groups: Control, CBBG (1 mg), CBBG (5 mg), CBBG (10 mg). Each group contained Six (6) animals and administration was performed intraperitoneally with the following Schedule:

Control – Only PBS as vehicle.

CBBG (1 mg): 1 mg /kg body wt. of CBBG dissolved in PBS

CBBG (5 mg): 5 mg /kg body wt. of CBBG dissolved in PBS

CBBG (10mg): 10 mg /kg body wt. of CBBG dissolved in PBS

Mice were Injected Once per week for Four weeks i.e., a month of time and were sacrificed one week after completion of the last treatment. On the day of sacrifice blood was collected in previously marked vials for biochemical and hematological examinations. The liver, spleen and kidney were isolated very carefully for histopathological examination and evaluated for cytoarchitectural alterations after additional processing.

c. **Preparation of CBBG and Administration of CBBG Intraperitoneally**

The body weights of animals and calculated doses of CBBG to be injected were recorded for each animal before their treatment. 70% ethanol was applied to the abdominal cavity of the animal. A 26-gauge DISPOVAN needle of 1 ml capacity was inserted inside the peritoneal cavity. The needle along with the syringe were kept at a very suitable angle in respect to the body. The respective dosage of CBBG (1 mg/kg, 5 mg/kg and 10 mg/kg) and PBS for control group was injected inside the peritoneum cavity slowly. The needle was removed with utmost care and gentle compression was applied over the region. Animals were returned to their cages. After completion of treatment for 4 weeks body weights of animals were recorded.

d. **Examination of blood for biochemical and hematological parameters with respect to CBBG toxicity**

The various biochemical parameters including serum glutamic oxaloacetic transaminase, serum urea, serum glutamic pyruvic transaminase, and creatinine were analyzed for sub-chronic toxicity of CBBG by taking commercially available diagnostic test kits (ARKRAY Healthcare Pvt. Ltd.). Using standard kits by following the manufacturer's manual, blood parameters (red blood cells, white blood cells, hemoglobin, eosinophil, neutrophil, basophil, monocyte, and lymphocyte) were estimated.

e. **Histopathological examinations for sub-chronic toxicity studies**

The Liver, Kidney, Spleen were isolated after sacrificing the animals and individual weights were recorded for each organ. The preserved liver, kidney, and spleen tissues were histopathologically examined. CBBG treated mice and control mice were sacrificed by decapitation following 4-weeks of treatment regimen. The Liver, Kidney, Spleen were collected and fixed onto 10% neutral buffered formalin for approximately 48 h. The organs were then further immersed in paraffin wax. Tissue sections were carefully cut with a microtome with a thickness of 5 µm. Prepared Slides were stained with Hematoxylin and eosin (H &E staining), Gomori Trichrome and Periodic Acid-Schiff for histological examinations. Stained Slides were observed using the light microscope (NIKON T2, India) for histopathological alternation (if any), and pictures were taken at 10x and 40x magnification.

12. **Western Blot Analysis**

The expression of the proteins pAKT, AKT1, and β-Actin was measured by Western blot analysis. The cells were serum starved for 12 h in DMEM media without serum. Afterwards, the cells were treated with only media, native insulin, untreated aggregated HI and CBBG treated HI at 100 nM concentration. About 5× 106 cells were washed with cold PBS twice following 30-40 mins treatment, the cells were homogenized with ice-cold RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride (PMSF) along with protease inhibitor cocktail. (Roche) Cell lysates were cleared at 12,000 × g for 20 min at 4 °C. Protein were quantified using the Bicinchoninic acid (BCA) protein estimation kit (ThermoFisher). Electrophoresis were carried out on 10% (W/V) SDS- Polyacrylamide gel (MiniPROTEAN® tetra cell with mini-trans Blot®, Bio-Rad, USA) which was then subsequently transferred onto a nitrocellulose membrane (Pall corporation).[7] Next, incubation of the membrane was done with fresh blocking buffer containing 5% nonfat-dried milk for 1 h at room temperature and then probed with primary antibodies Phospho-AKT1-S473 Rabbit pAb(Abclonal :AP0140) , Anti β-Actin Rabbit pAb (

BioBharati: BB-AB0024), AKT1 Monoclonal Antibody (Elabscience: E-AB-22210) overnight. The following day, membranes were washed three times with TBST solution followed by incubation with HRP conjugated goat anti-mouse, (Jackson Biolab 115-03-003), goat anti-rabbit secondary antibody (Jackson Biolab 111-035-003) for 2 h at room temperature. After that the membranes were washed three times in the TBST buffer. Immunoreactive bands were seen using the ECL substrate solution. Image Lab analysis was used to quantify the protein bands, which were normalised against the corresponding β-actin band.

13. **Serum stability assay**

A method described by Jenssen and Aspmo that was slightly modified was used to measure serum stability.[8] It was warmed to 37 °C for 15 min before being added to 1 ml of RPMI 1640 supplemented with 25% (v/v) foetal bovine serum. To precipitate serum proteases CBBG was added at a final concentration of 100 µg/ml, and at the appropriate time periods, the reaction mixture (100 µl) was removed and mixed with 96% ethanol (400 µl). To pellet down the precipitated serum protein, the hazy reaction mixture was centrifuged at 18,000 g for 2 min after being cooled at 4 °C for 15 min. Reverse-phase HPLC (SHIMADZU, Japan) was used to evaluate the reaction supernatant using a 250 x 4.6-mm C18 column. Gradient elution from 0.1% TFA in 100% acetonitrile to 0.1% TFA in water was carried out at a flow rate of 1 ml/min for 30 min. The area under the peak at 610 nm was calculated using SPINCHROME CFR software.

14. **Protein solubility estimation by Bicinchoninic acid assay (BCA)**

HI sample (100µM) was incubated at 60 ^{o}C for 24 h in absence or presence of CBBG at molar ratio 1:1 [HI]/ [CBBG] in 25 mM HCl containing 100 mM NaCl (pH ~1.6). The aggregated HI samples were centrifuged at 5000 rpm for 5 min and the supernatant was collected. Protein solubility was measured using Pierce (Thermo Scientific #2328) BCA reagent A and BCA reagent B in ratio of [50:1]. The reagents were mixed in given proportion according to manufacturer's protocol. 1µl protein from each of sample of HI fibril, HI monomer and co-incubated HI+CBBG were mixed with BCA reagents. The mixture was incubated at 37 ℃ in dark conditions and O.D was taken using a microplate reader at 562 nm. The soluble protein content was estimated from the standard curve values.

15. **Insulin secretion assay**

RIN-5f cells were seeded in 12-well plate at 1x10 5 cells per well. Treatment was carried out the following day after the cells became adherent. CBBG were added to the cell at different concentrations (0.5, 1, 2.5, 5 and 10 µM) along with buffer and control. Quercetin (20 µM) was added as positive control.[9] 24h after treatment, cells were stimulated by exchanging media either with normal glucose RPMI-1640 [11 mM] or high glucose RPMI-1640 [22 mM]. After 24 h, aliquots from the wells were collected and determined the amount of secreted insulin in the cell media by Enzyme Linked Immunosorbent Assay (insulin ELISA kit, Ab100578, Abcam, Cambridge, UK) following the manufacturer's protocol. The amount of insulin secretion in the cell medium of treated cells was compared with the secreted insulin level of control cells.[10]

16. **Molecular docking**

a. **Protein Preparation**: Hexameric assembly of Insulin A and B subunits with pdb 2OM1 was utilized.[11] The protein assembly was checked for any missing atoms in residues

and overlapping hydrogen atoms were adjusted H-bond optimization and energy minimized using OPLSe force field in maestro - Schrödinger suite of Program.[12]

b. **Ligand preparation**: Using prepare ligand modules, the 2D coordinates of Coomassie blue (CBBG) were converted into 3D and energy minimized using smart minimizer for 2000 steps via minimize ligands tools in Discovery studio.[13]
c. **Docking**: Glide docking tool from Schrödinger suite of programs was utilized to dock the CBBG.[12] The NMR CSP data highlighted residues were used to define the grid point using default settings. Next, Standard precision docking protocol was used to dock the CBBG with default parameters was used to rank the poses.

SII Molecular interactions of CBGG

Exploiting this vital information from chemical shift perturbed residues as ambiguous interaction site points, we next evaluated the molecular interaction of CBBG on insulin. Our results suggested that the CBBG was docked onto the cavity formed between the A and B chains of insulin in one tetramer with a glide score of -6.7 kcal/mol. Chemically, CBBG bears a N,N-disubstituted tri-anilinyl-methane scaffold and with a IUPAC chemical name as 3-(((4-((4-((4-ethoxyphenyl)amino)phenyl)(4-(ethyl(3-sulfobenzyl)amino)-2-methylphenyl)methyl)-3-methylphenyl)(ethyl)amino)methyl)benzenesulfonate]. Among the three phenyl rings of CBBG, two of the phenyl rings are oriented towards the B subunit, while the 3rd phenyl ring orients toward the A subunit. Among the three aniline fragments on triphenyl rings of CBBG, one fragment - N-ethoxy-phen-4-yl-aniline is sandwiched between two B subunits and one A subunit. The N-ethoxy moieties in this fragment was engaged in hydrophobic interaction with L^{B11} / C^{B7} residues from one of the B subunits (3.9 Å /3.0 Å) and L^{B6} residues (3.6 Å) from another B subunit. Phenyl ring is juxtaposed with face to edge π- π interaction between H^{B5} (3.1 Å) one B subunit and hydrophobic interaction with A^{B14} (3.4 Å) from another B subunit. The phenyl ring (to which - N-ethoxy-phen-4-yl-aniline substitution) is positioned in the vicinity of H^{B5} (3.1 Å), C^{B7}(3.0 Å), L^{B17} (4.1 Å), and L^{A13} (3.9 Å) at the interface of two B subunits at the tetrameric interface. The N-ethyl-N-(phenyl-3-sulfonic acid)-2-methyl-aniline fragment on of the triphenyl rings was anchored to two B subunits. On one side, the SO_3 acid atoms on N-(phenyl-3-sulfonic acid moiety of this fragment was in H-bonding interaction with F^{B1} amino terminus (2.1 Å), while on the other side, the phenyl ring mediates cation interaction with K^{B29} residue (2.7 Å) from another B subunit. The-2-methyl-benzene ring was sandwiched in the vicinity between I^{A10} (3.4 Å) and E^{B21}-G^{B20} residues (3.3 -3.5 Å) from two B subunits. Lastly, the fragment- N-ethyl-N-(phenyl-3-sulfonic acid)-2-methyl-aniline was involved in hydrophobic interactions with two B subunit. The SO_3 acid atoms on N-(phenyl-3-sulfonic acid moiety of this fragment was engaged in H-bonded interaction with main chain atoms of I^{A10} residue (1.9 Å), while N-ethyl atoms on this fragment was in van der Vaal contacts with S^{A12} residue on A subunit.

Table S1. Biochemical Chart. Effect of CBBG on haematological and biochemical parameters in mice (n= 6 in each group). Haemoglobin (Hb); red blood cells (RBC); white blood cells (WBC); Packed Cell Volume (PCV); serum glutamic oxaloacetic transaminase (SGOT); serum glutamic pyruvic transaminase (SGPT) .

HAEMATOLOGICAL PARAMETERS	**Control Mice**	**CBBG (1 mg) Mice**	**CBBG (5 mg) Mice**	**CBBG (10 mg) Mice**	**Normal Range**
HAEMOGLOBIN %	11.3±2	10.5±1.5	9.6±2.5	12±3	6.1-19.73
Red Blood Cell Count/Lac (10^6)	4.1±0.5	3.9±0.6	3.8±0.3	4.5±0.5	3.57-11.7
PCV %	36±4	34.5±5	32.7±2	33.9±3.5	
PLATELETS COUNT/Lac (10^6)	3.5±1	2.4±0.7	1.8±1.2	2.6±1.5	0.59-6.75
W.B.C Total Count/ Cu mm	9100±700	6000±500	5500±1500	7100±1000	5500-12500
Neutrophils %	33±5	30±3	32±2	28±3	3-27
Lymphocytes%	50±10	40±5	38±3	60±15	12-41
Monocytes %	02±1	01±2	03±1	02±1	00-04
Eosinophils %	01±2	01±1	01±1	02±1	00-01
Basophil %	00±0	00±0	00±0	00±0	00-01
ESR mm/1^{st} hr	20±3	18±2	26±3	30±5	
BIOCHEMICAL PARAMETERS					
Serum SGOT (AST) IU/L	70±2.5	62±3.7	75±5.9	55±3.2	
Serum SGPT (ALT) IU/L	90±2.1	110±3.5	80±3.8	120±2.9	
Serum Creatinine mg/dl	0.86±0.10	0.86±0.05	0.79±0.15	0.92±0.03	
Blood Urea mg/dl	20±5	39±7	52±4	32±10	

Table S2. Lag time and growth rate constants from ThT experiments for Insulin amyloid fibril formation in the presence and absence of CBBG.

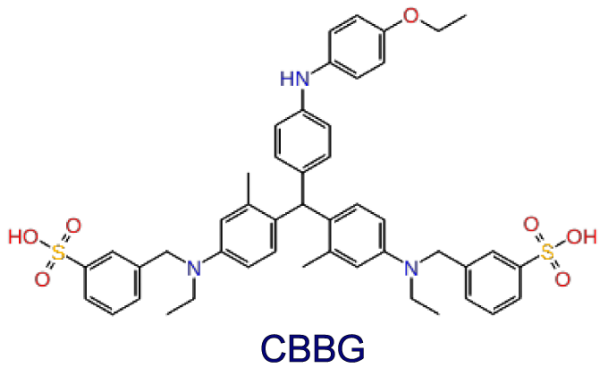
condition	reaction	lag phase	apparent rate (k_{app})
HI 100 μM, pH ~1.6	HI	1.8 h	0.83 h^{-1}
HI 320 μM, pH ~1.6	HI	1.6 h	0.95 h^{-1}
+CBBG 7.5 μM	HI/CBBG	3.06 h	0.42 h^{-1}
+CBBG 15 μM	HI/CBBG	3.32 h	0.42 h^{-1}
+CBBG 30 μM	HI/CBBG	3.68 h	0.32 h^{-1}
HI 100 μM, pH ~7.2	HI	1.6 day	0.88 day^{-1}

Table S3. Binding constants and thermodynamics of binding between CBBG and insulin as obtained from ITC and NMR experiments.

Complex	n	Method	K_d (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	-TΔS (kcal/mol)
HI-CBBG	2	ITC	12.5	-11.1	-57.2	46.1
		NMR	7.50 ± 1.5 (aliphatic region)			
			5.26 ± 1.6 (aromatic region)			
			7.54 ± 2.5 (amide region)			

Supporting Figures:

(A)



(B)

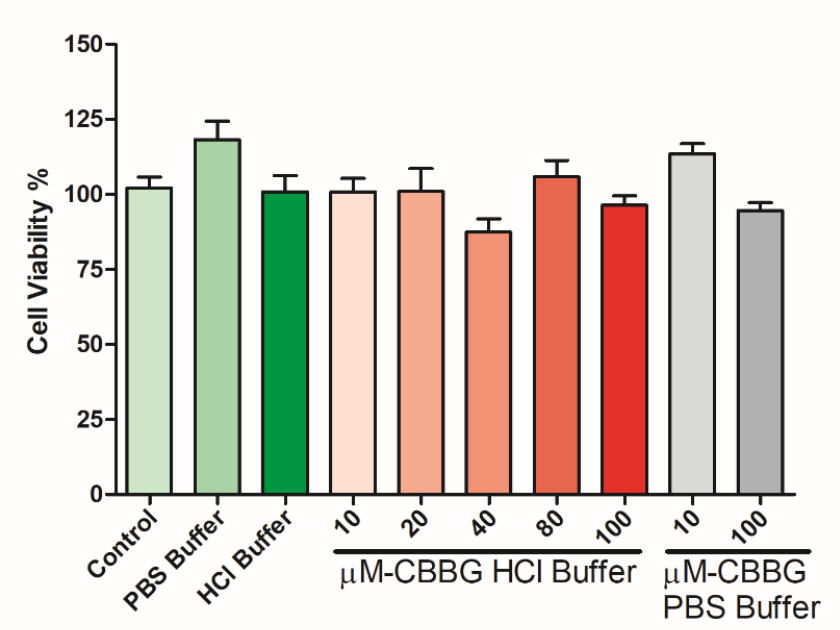


Figure S1. (A) The chemical structure of Coomassie Brilliant Blue G-250 (CBBG). (B) MTT assay showing cell viability of RIN5f cells in the presence of CBBG with concentrations of 10, 20, 40, 80 and 100 µM in HCl buffer (pH 1.6) with NaCl (0.1 M) and 10 µM and 100 µM in PBS buffer (pH 7.4). The values are shown as mean ± SEM, (for n=6).

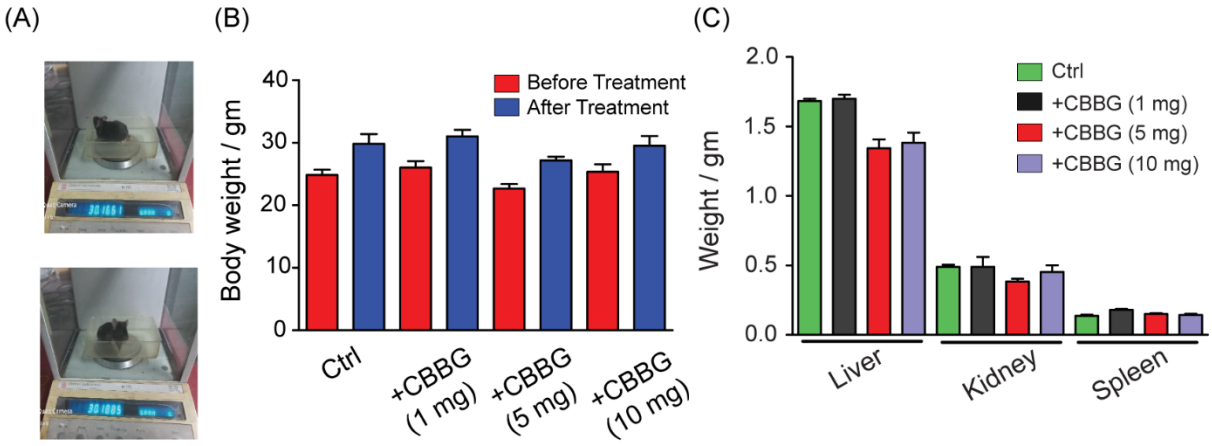


Figure S2. (A) The images of mouse (C57BL/6J) taken after treatment with CBBG. (B) Mice body weight before and after treatment with CBBG. (C) Mice organ weight of Liver, Kidney, and Spleen for control and CBBG treated group after sacrifice.

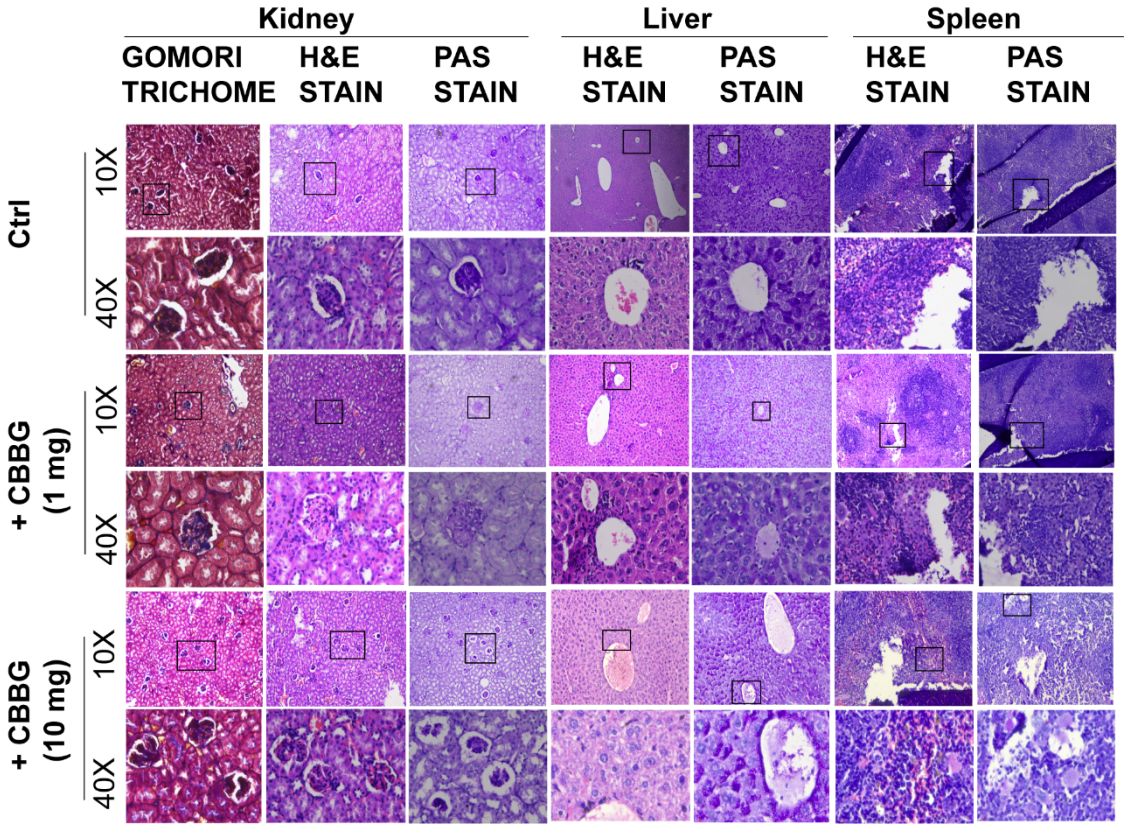


Figure S3. Histopathological examination of Kidney, liver and spleen of the mice for sub-chronic toxicity with treatment of 1 mg and 10 mg CBBG for per kg body weight of mice, respectively. The images S3 control (ctrl) have purposefully been maintained the same as in Figure 1A control, referring to the same composition. This makes reading easier and allows the supporting material Figure S3-control to compare with CBBG treated mice for histological studies, Haematoxylin and eosin (H and E staining), Gomori Trichrome and Periodic Acid-Schiff staining slides were shown under 10x and 40x magnification.

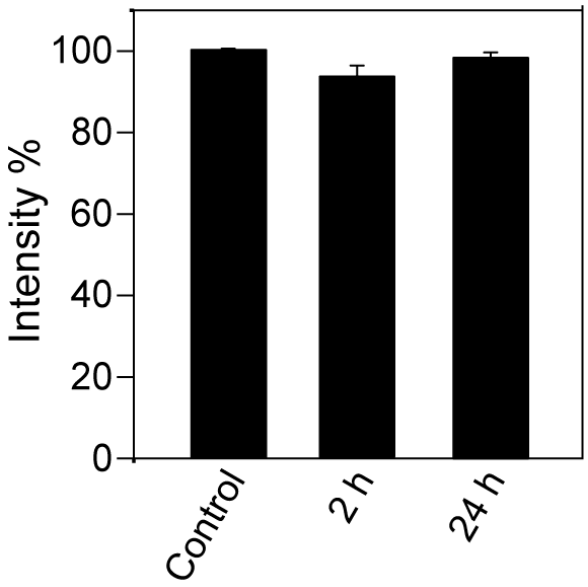


Figure S4. The stability of the CBBG in fetal bovine serum was determined using Reverse-phase HPLC at 2 h and 24 h.

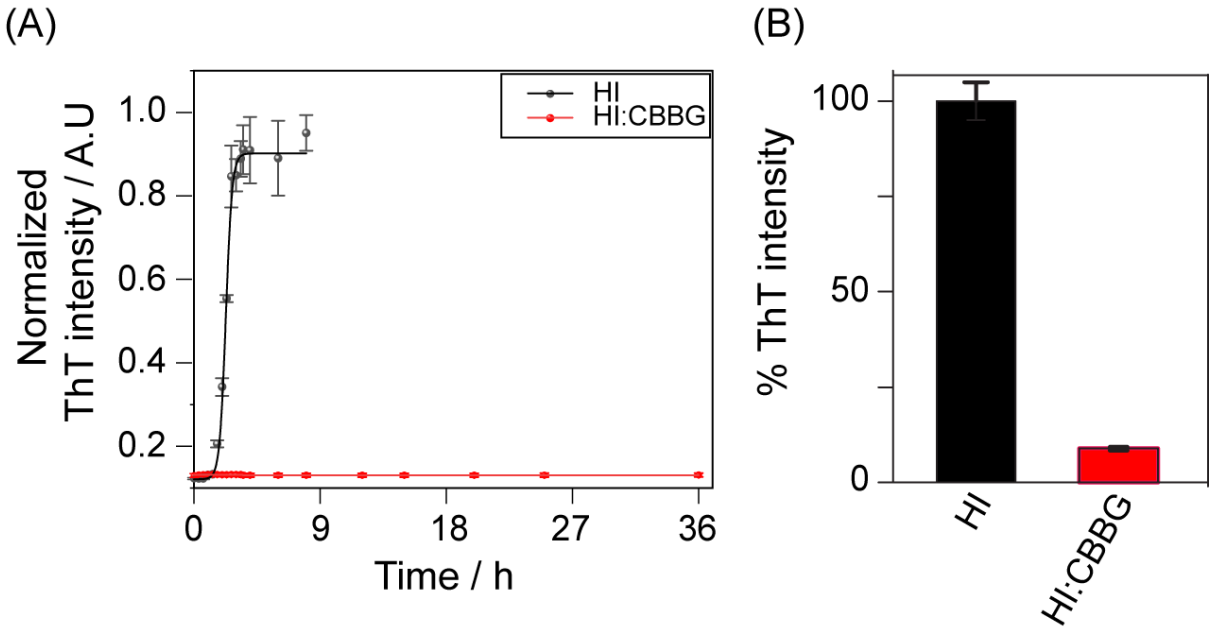


Figure S5. ThT fluorescence assay of HI fibrillation in presence and absence of CBBG. (A) ThT fluorescence intensity at 482 nm in the presence of the quantitative amount of incubated HI solution at a different time point of incubation. Incubation condition, 100 μM insulin, temperature 60 °C, 25 mM HCl, 0.1M NaCl, pH ~1.6, without CBBG (black trace) and in the presence of 100 μM CBBG (red trace). (B) Percentage of final ThT fluorescence intensity (at 482 nm) after five hours of HI incubation in absence and presence of equimolar CBBG. For the three independent experiments, the error bars show the standard deviation.

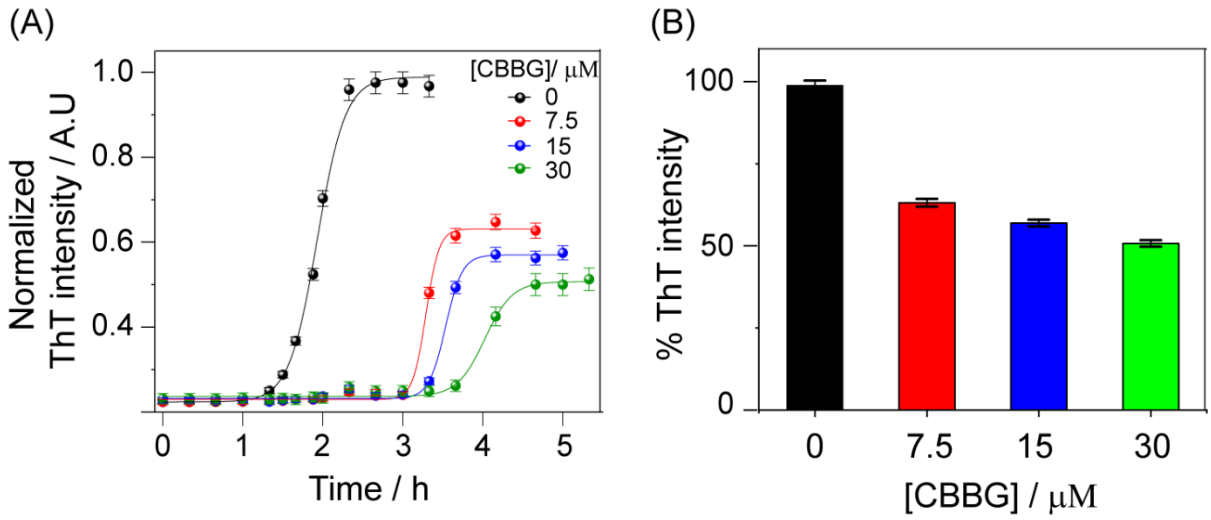


Figure S6. (A) ThT fluorescence intensity against time for HI incubated at higher concentration (320 μM) in the absence and presence of a different amount of CBBG (7.5, 15 and 30 μM). (B) Percentage of final ThT fluorescence intensity at saturation point of the samples described in (A) and marked as HI (black), HI + 7.5 μM CBBG (red), HI +15 μM CBBG (blue) and HI +30 μM CBBG (green).

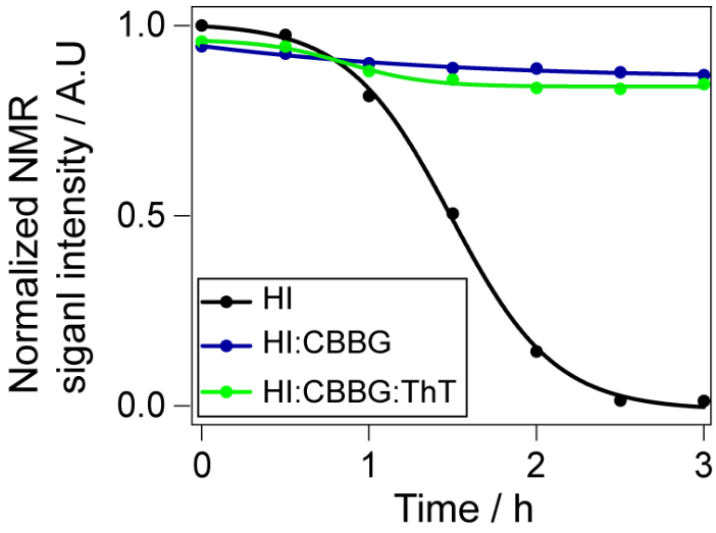


Figure S7. Time course HI aggregation by ^{1}H NMR and competition for HI binding between equimolar ThT and CBBG. Solid line represents by sigmoidal fits to normalized NMR signal intensity with time. The 1D ^{1}H NMR experiments of HI and HI+CBBG at 60 ^{o}C were performed in HCl buffer (25 mM) with 100 mM NaCl (pH ~1.6) in presence and absence of ThT on Bruker Avance III 700 MHz NMR.

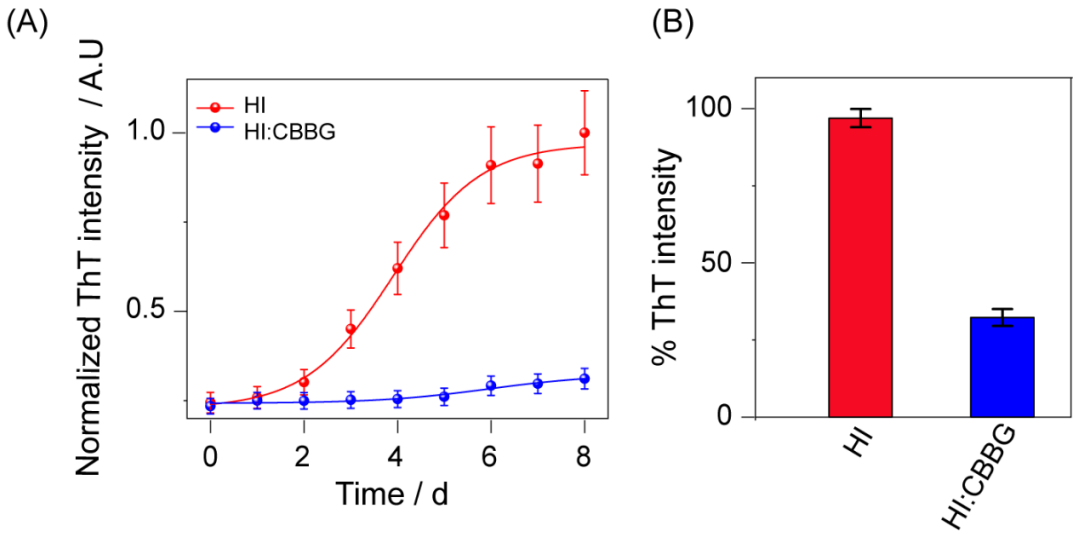


Figure S8. At pH 7.2, ThT fluorescence of HI aggregation and its inhibition by equimolar CBBG. (A) HI in absence (red trace) and presence (blue trace) of CBBG at HI:CBBG ratio of 1:1. HI (100 μM) incubated at 60 °C, phosphate buffer, 0.1 M NaCl, pH 7.2, for 8 days. (B) Percentage of final ThT fluorescence intensity of HI incubated in the absence and presence of CBBG after 8 days.

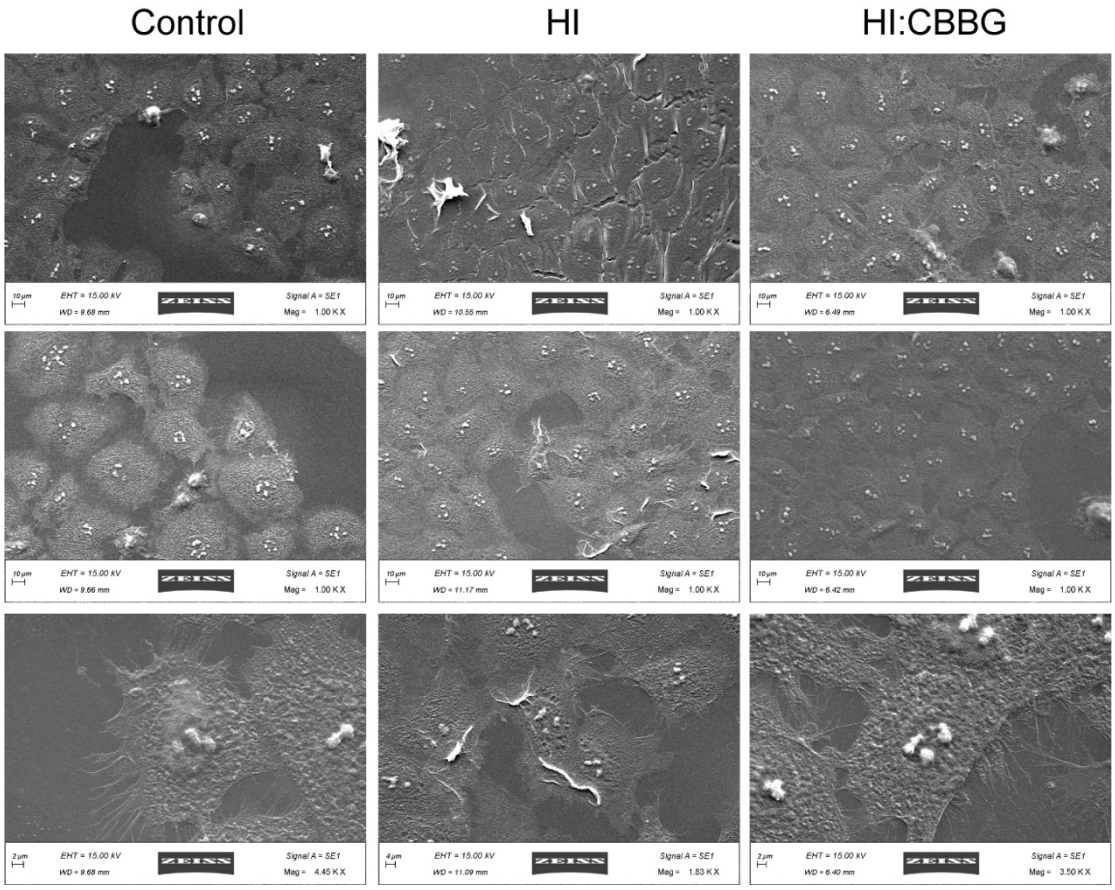


Figure S9: SEM images panel of RIN5f cell after the treatment of preformed HI fibrils and HI:CBBG complex (at 1:1 molar ratio) and compared the morphology of cell with the control cell.

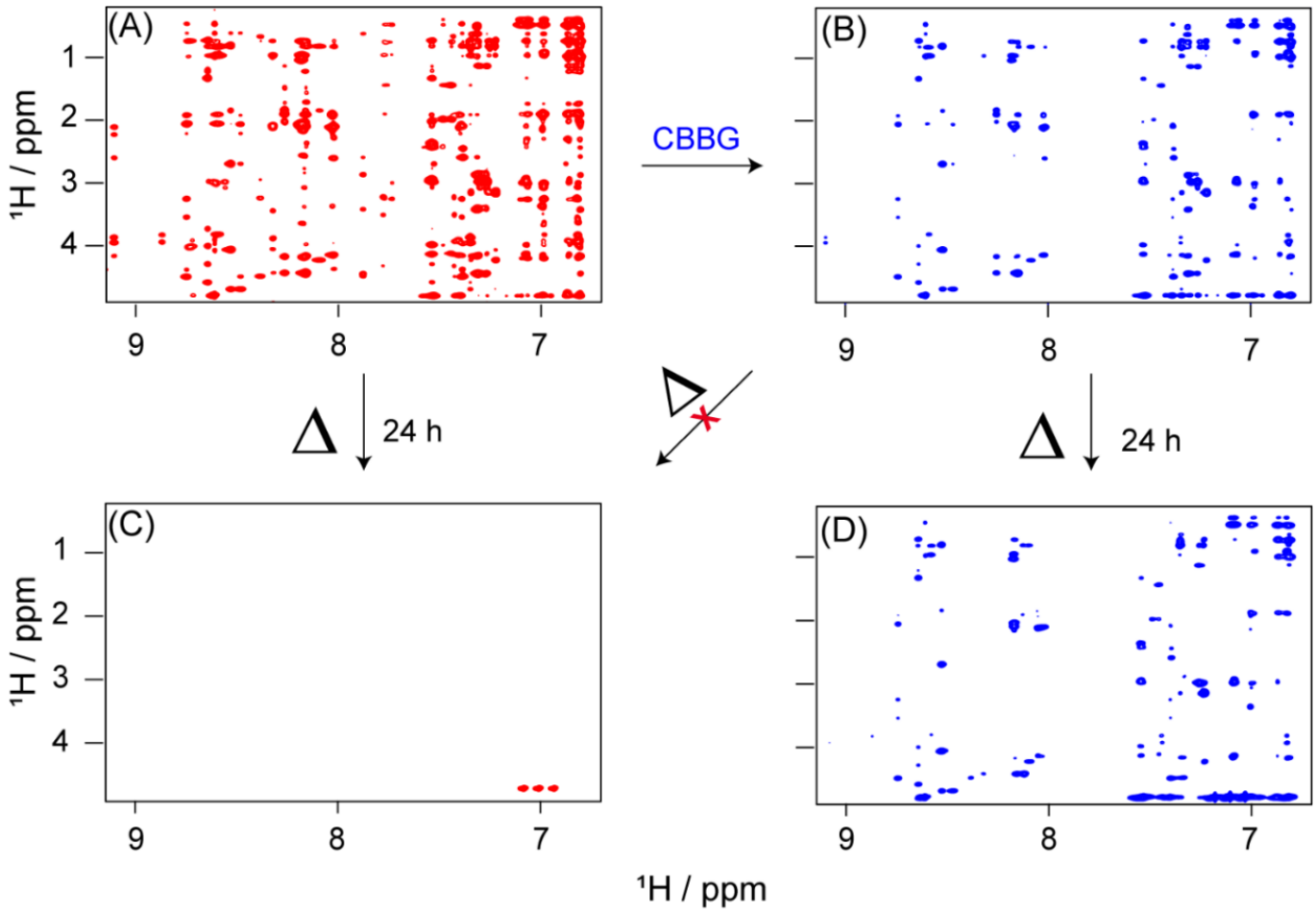


Figure S10. Inhibition of HI amyloid fibrillation by equimolar CBBG using NMR. Two-dimensional NOESY NMR spectra: (A) HI alone and (B) in the presence of CBBG at 1:1 molar ratio ([HI]/[CBBG] just before incubation; (C) HI solution after heating at 60 °C for 24 h shows no cross peak and (D) HI co-incubated with equimolar CBBG at 60 °C for 24 h. The experiment was performed in 10 mM sodium phosphate, 10 mM NaCl (pH 2.0) and 10% D_2O using Bruker Avance III 700 MHz at 25 °C.

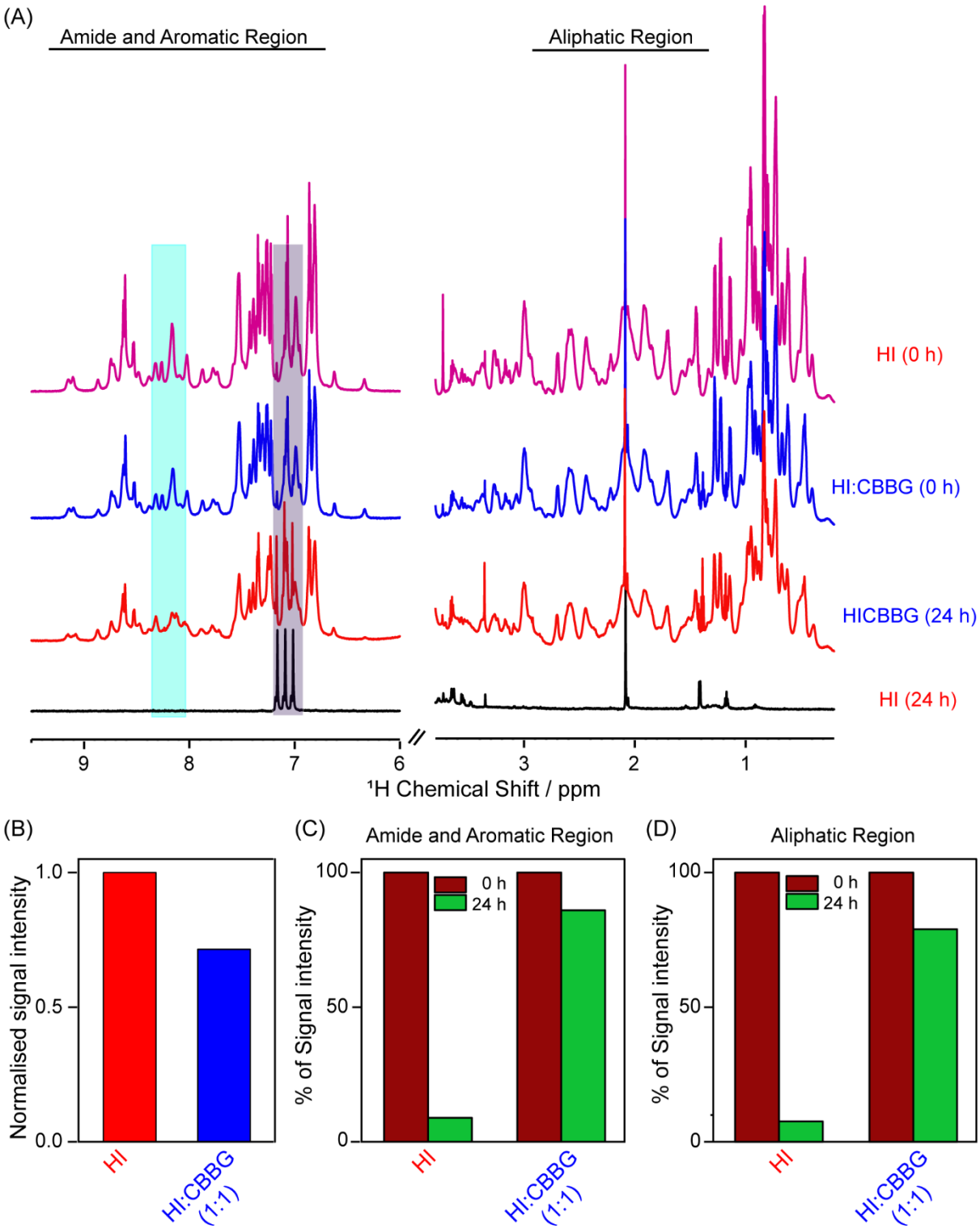


Figure S11. The aggregation kinetics of HI were acquired by one dimensional ^{1}H NMR. (A) Time-lapse ^{1}H NMR spectra of 1 mM HI and/or HI treated with equimolar CBBG in 10 mM sodium phosphate buffer (pH 2.0) with 10 mM NaCl. (B) Normalised signal intensity of HI upon addition of CBBG at 1:1 molar ratio. The percentage of ^{1}H NMR signal intensity of amide and aromatic region (C) and aliphatic region (D) of HI and/or HI+CBBG (1:1 molar ratio) for 0 and 24 h incubation at 60 °C, calculated from the integrated total peak intensities of corresponding regions. Appearance and disappearance of new peaks after heating are highlighted. All the spectra were recorded on Bruker Avance III 700 MHz and at 25 °C.

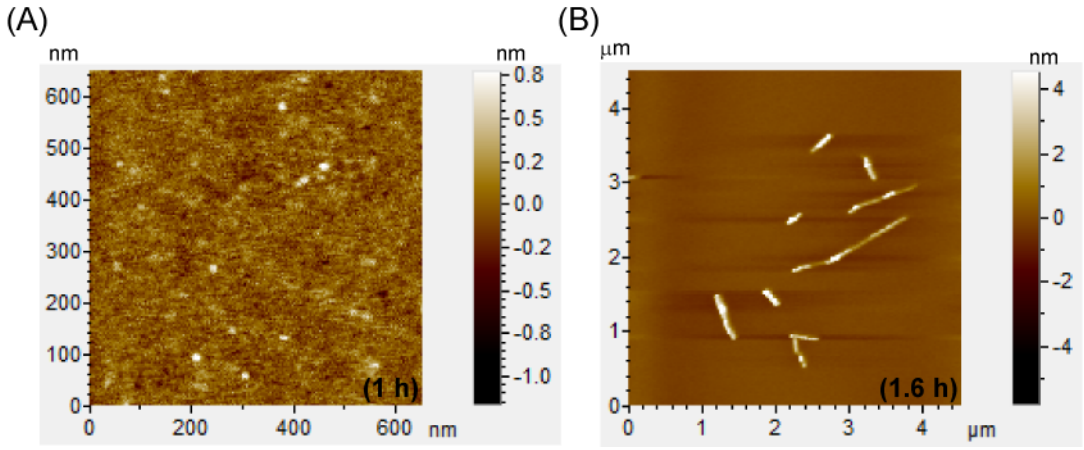


Figure S12: Representative AFM images of HI showing aggregate morphology of HI at several time point of incubation. (A) and (B) represent the surface morphology of the aggregates formed at 1 h and 1.6 h, respectively, of HI (100 µM) samples incubated (temperature 60 °C, 25 mM HCl, 0.1 M NaCl, pH ~1.6).

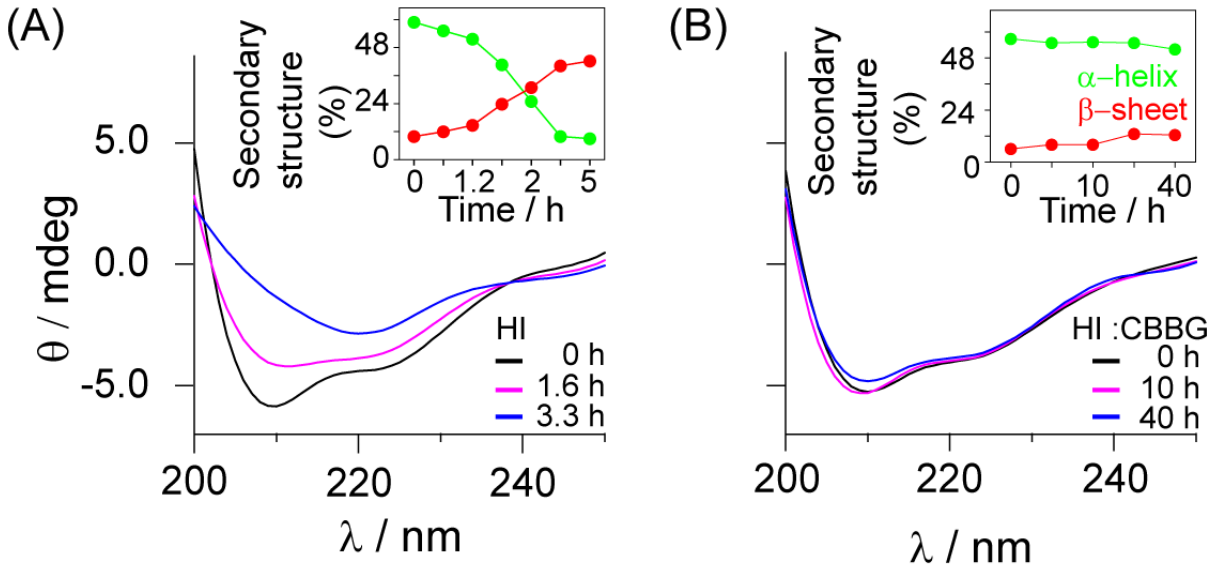


Figure S13. (A) CD spectra of HI (100µM) aggregation at different time of incubation. (T = 60 °C, 25 mM HCl, 100 mM NaCl, pH ~1.6); HI (black trace, 0 h, pink trace, 1.6 h, blue trace, 3.3 h). (B) CD spectra of HI in the presence of CBBG at 1:1 molar ratio of [HI]/ [CBBG] of selected time points: black trace (0 h), pink trace (10 h) and blue trace (40 h). The changes in the secondary structural component (%) against incubation time: α-helix (green) and β-sheet (red).

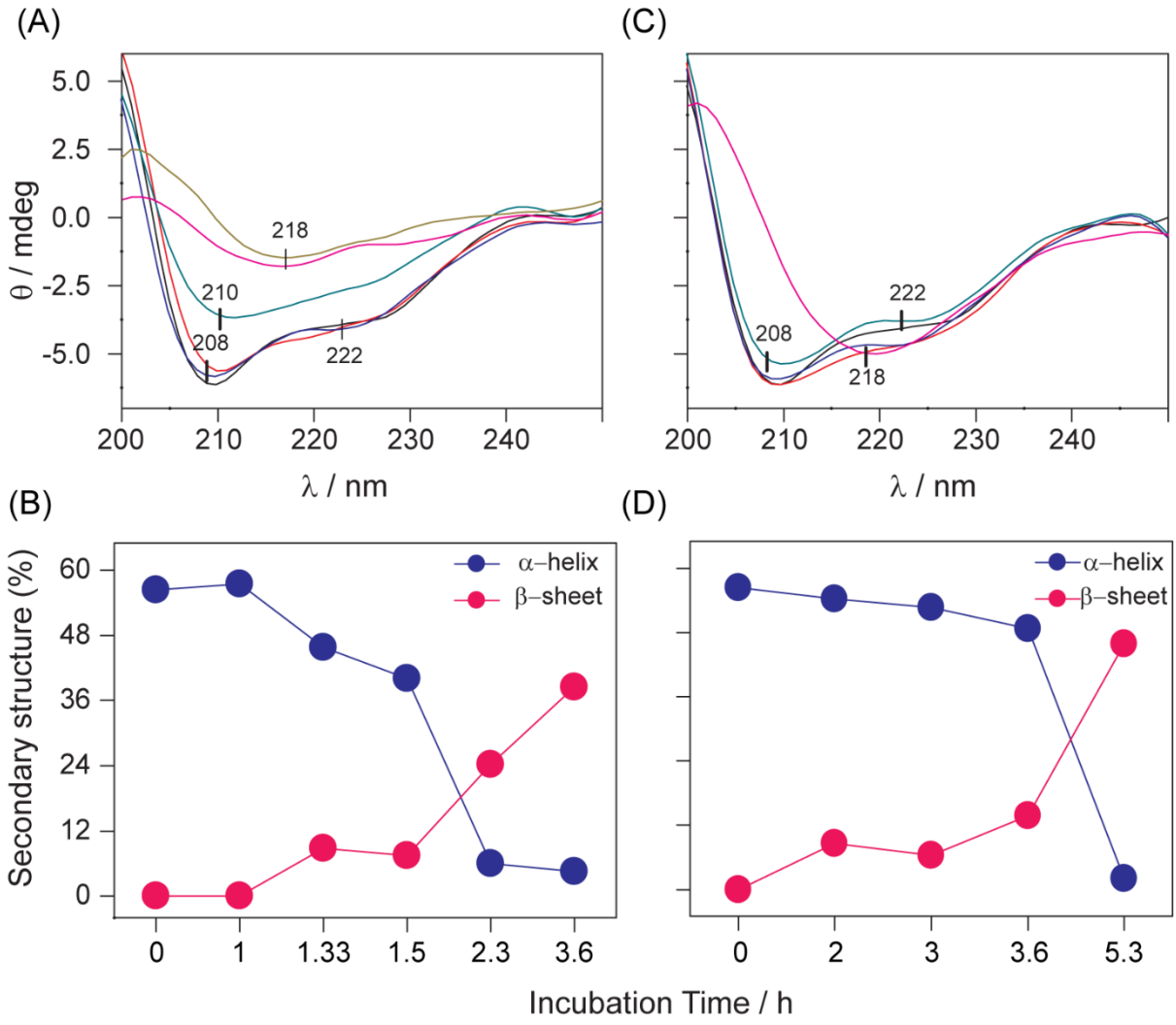


Figure S14. (A) CD spectra of HI solution at different time periods of incubation at high concentration (320 μM) in absence of CBBG: black trace (0 h), red trace (1 h), blue trace (1.33 h), green trace (1.5 h), pink trace (2.33 h) and yellow trace (3.6 h). (B) The changes in the secondary structural component (%) of HI against incubation time: α-helix (blue) and β-sheet (pink). (C) CD spectra of aggregating HI (320 μM) in the presence of lower dose CBBG (30 μM) of selected time points: black trace (0 h), red trace (2 h), blue trace (3 h), green trace (3.6 h) and pink trace (5.3 h). (D) The changes in the secondary structural component (%) of HI: CBBG (at 1: 0.093 molar ratio) against incubation time: α-helix (blue) and β-sheet (pink).

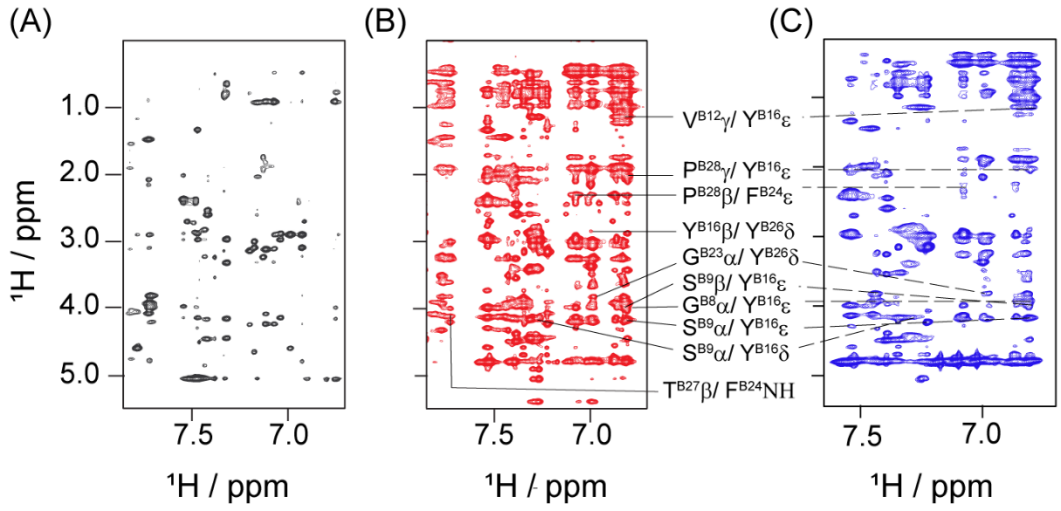


Figure S15: Structural difference between monomer and dimer HI using two-dimensional (2D) NMR. (A) 2D ^{1}H-^{1}H NOESY spectra of HI monomer (black) in 20% acetic acid-d_4 containing 10% D_2O (pH 1.9). (B) NOESY spectra of HI dimer (red) in 10 mM sodium phosphate buffer, 10 mM NaCl containing 10% D_2O at pH 2.0. (C) NOESY NMR spectra of HI in presence of CBBG (blue) at 1:1 molar ratio after heating at 60 °C for 24 h. Intermonomer NOEs were detected and assigned for HI dimer. The spectra were recorded at 25 °C on 700 MHz Bruker NMR spectrometer.

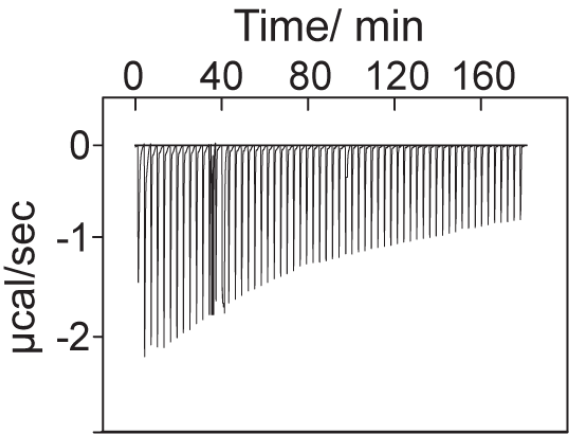


Figure S16: ITC thermogram profile for insulin binding to CBBG. The panel presents the raw heat effects against time for the titration of CBBG (0.01 mM) with insulin (0.4 mM) in aqueous HCl solution (25 mM HCl, 100 mM NaCl, pH ~1.6) at 25 °C.

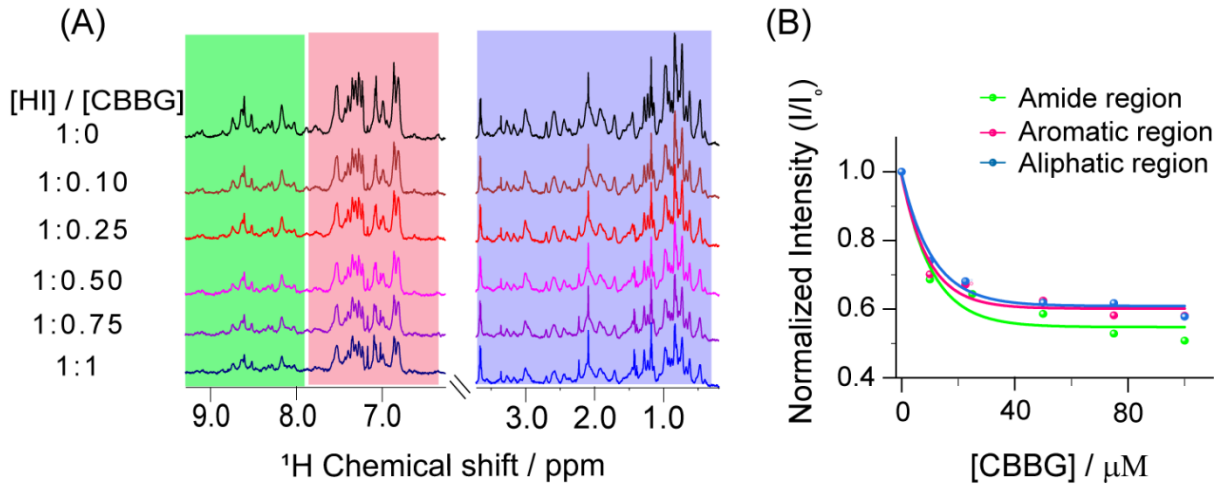


Figure S17. (A) ^{1}H NMR spectra of 100 μM HI in 25 mM HCl buffer with 100 mM NaCl (pH ~1.6, black curve) and the presence of a different concentration of CBBG at 25 °C. The concentration of CBBG were varied from 10 μM to 100 μM. Highlighted green for amide, red for aromatic and blue for aliphatic region of HI. (B) The NMR proton peak intensity decay of aliphatic (blue) aromatic (red) and amide (green) region of HI upon titration with CBBG. The solid lines represent at a particular CBBG concentration, the ^{1}H NMR signal intensity of insulin for corresponding region.

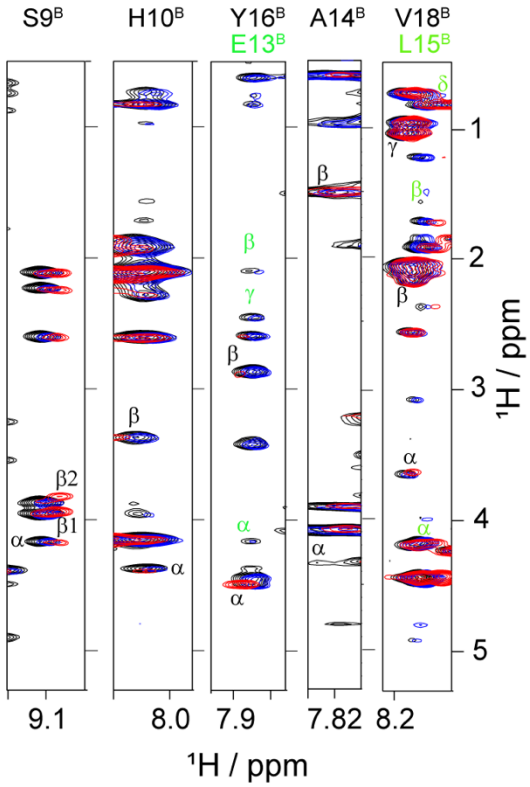


Figure S18. Strip plot from 2D NOESY spectra exhibiting the chemical shift perturbation and peak intensity broadening of insulin dimer from the B-chain domain in absence (black) or presence of CBBG at 1:1 molar ratio before (blue) and / or after (red) incubation at 60 °C. NOESY NMR spectrum were recorded at 25 °C in 10 mM sodium phosphate buffer (pH 2.0) containing 10 mM NaCl and 10% D_2O using a Bruker Avance III 700 MHz NMR spectrometer with a RT probe.

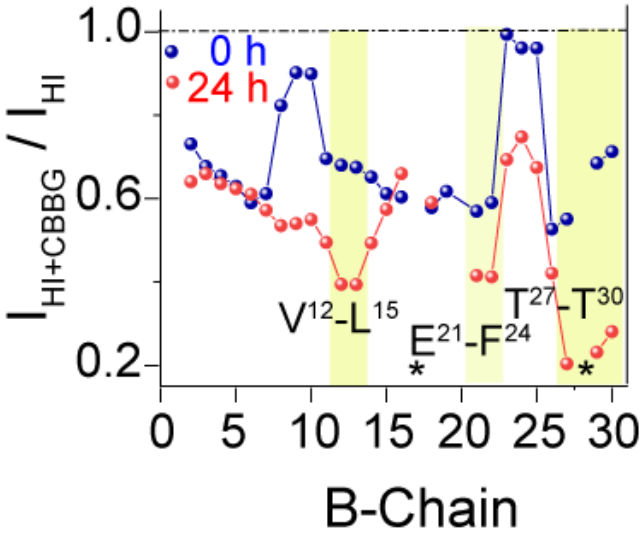


Figure S19: The running average of normalized NMR signal intensity of α protons in B-chain of HI dimer for three consecutive residues in presence of equimolar CBBG, before (blue ball) and after (red ball) heating of HI:CBBG complex at 60 ^{o}C for 24 h, respectively. Yellow background highlighted the important residues showing significant peak broadening. I_{HI} and $I_{HI+CBBG}$ represents NMR peak intensity of α protons of HI dimer residues in the absence and presence of equimolar CBBG, respectively. Asterisks (*) use here for overlapping and/or undetected residues. 2D NOESY NMR spectrum was recorded in Bruker Avance III 700 MHz NMR at 25 ^{o}C and pH 2.

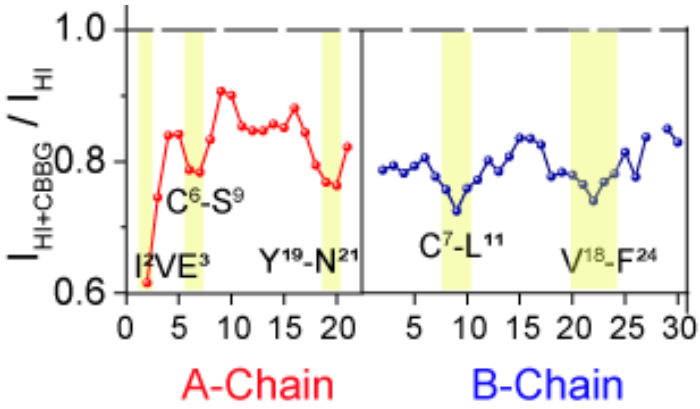


Figure S20: Running average NMR peak intensity (I/I_0) of α protons in HI monomer for three consecutive residues in A-chain (red) and B-chain (blue) in the presence of CBBG at a molar ratio of 1:1. I_{HI} and $I_{HI+CBBG}$ represents NMR peak intensity of α protons of HI monomer residues in absence and presence of equimolar CBBG respectively. The most broaden residual sequences are highlighted by yellow color strip. 2D NOESY NMR of HI monomer was performed in Bruker Avance III 700 MHz NMR using 20% D4-acetic acid buffer at 25 ^{o}C.

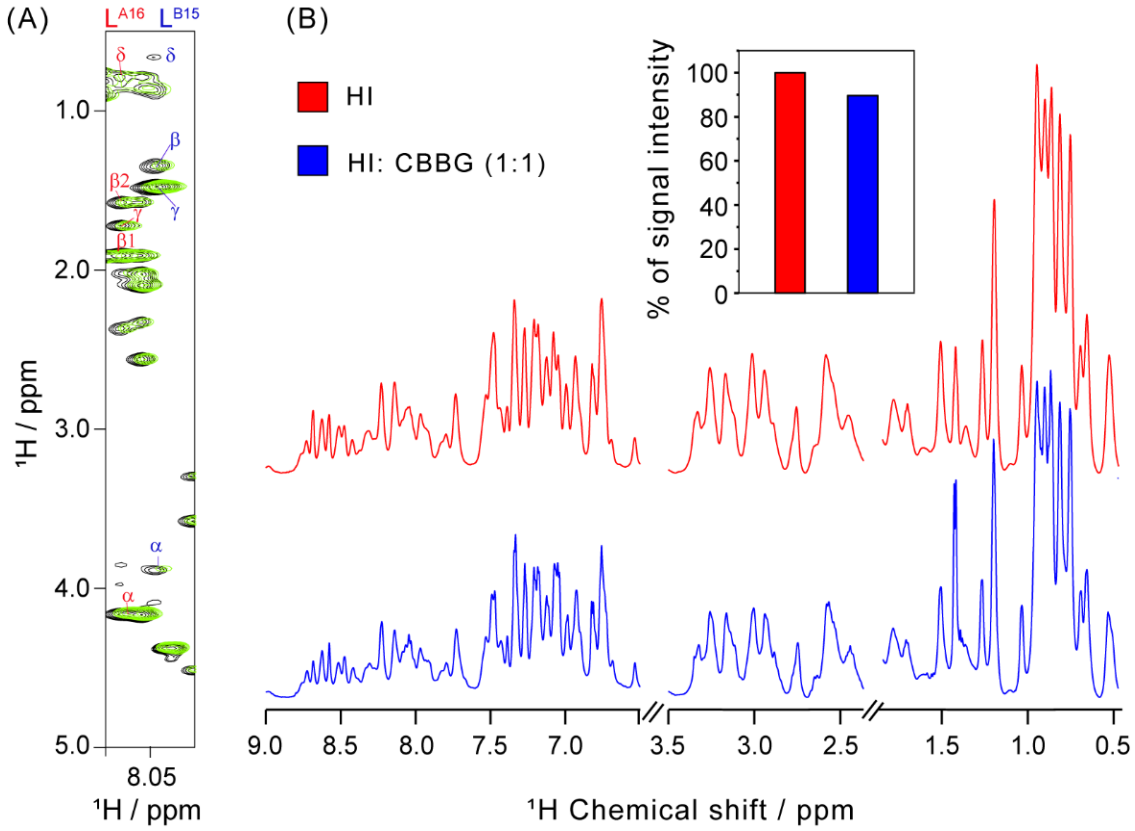


Figure S21: (A) Two dimensional NOESY Strip plot for Leu^{A15} (blue) and Leu^{B16} (red) residues from HI monomer displaying CSP and broadening in HI alone (black) and presence of 1:1 (green) molar ratio of [HI]/ [CBBG]. (B) One dimensional ^{1}H NMR spectra of HI monomer before (blue) and after (red) addition of equimolar CBBG in 20% acetic acid-d_4 and 10% D_2O at pH 1.9 (at 25 ^{o}C using Bruker Avance III 700 MHz NMR). ^{1}H NMR signal intensity (%) of selected region in HI reduces to 88% from 100% upon addition of CBBG at 1:1 molar ratio, is shown in inset.

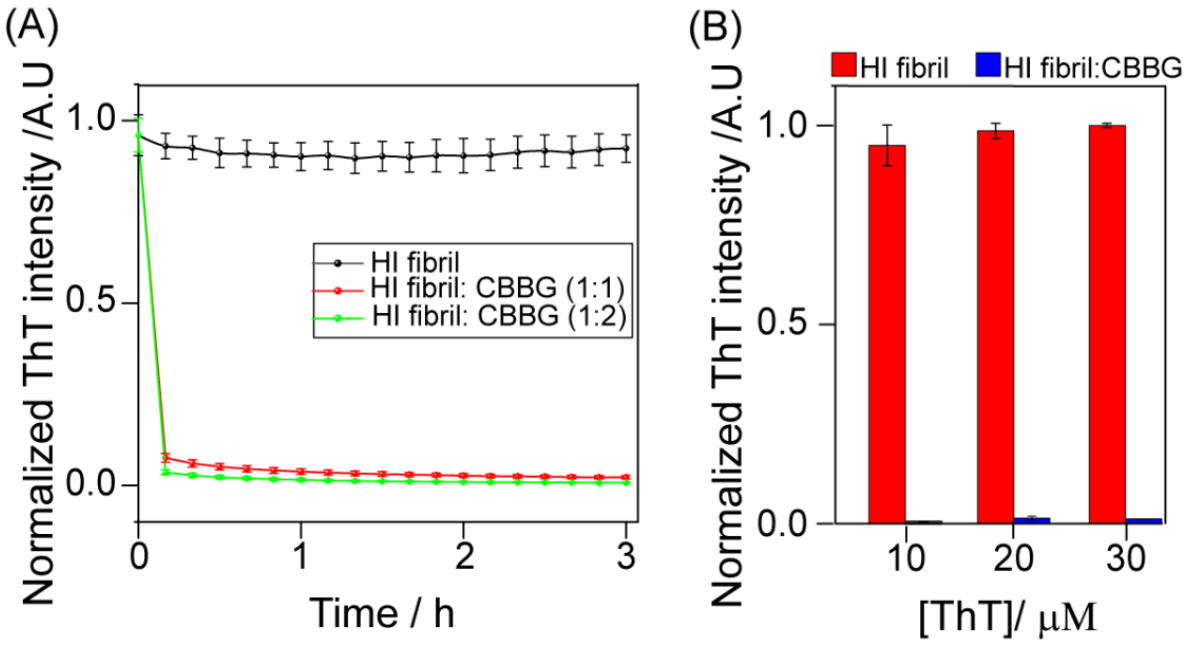


Figure S22: Disaggregation of HI fibril with treatment of CBBG. (A) Disassembly of preformed fibrils by using the one and two molars equivalent CBBG at 37 °C in HCl buffer with 100 mM NaCl (pH-1.6). (B) Final ThT intensity of HI fibril and disaggregation of fibrils upon addition of CBBG at 1:2 molar ratio in presence of different concentration of ThT (10, 20 and 30 μM). The standard deviation was represented by the error bar for three different experiments.

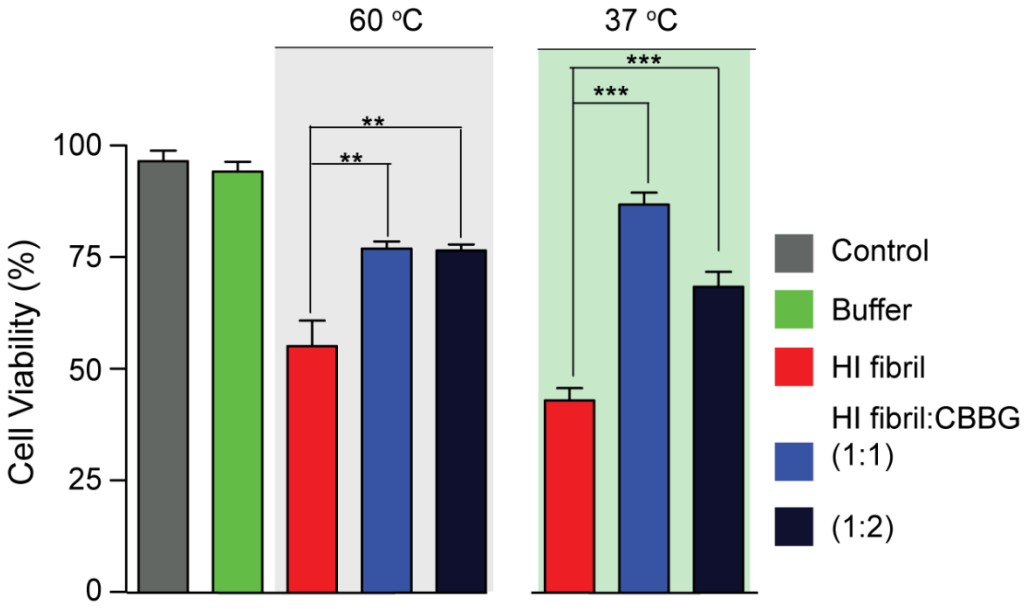


Figure S23: Relative cell viability measurements of RIN5F cell after addition of HI mature fibril (red) alone and co incubated in presence of CBBG at 1:1 (blue) and 1:2 (indigo) [HI fibril]/ [CBBG] molar ratio. The matured HI fibril with or without treatment of CBBG incubated for 3 h at 60 °C (no shaking) and 37 °C (with 200 rpm shaking) respectively in 25 mM HCl and 100 mM NaCl buffer (pH 1.6).

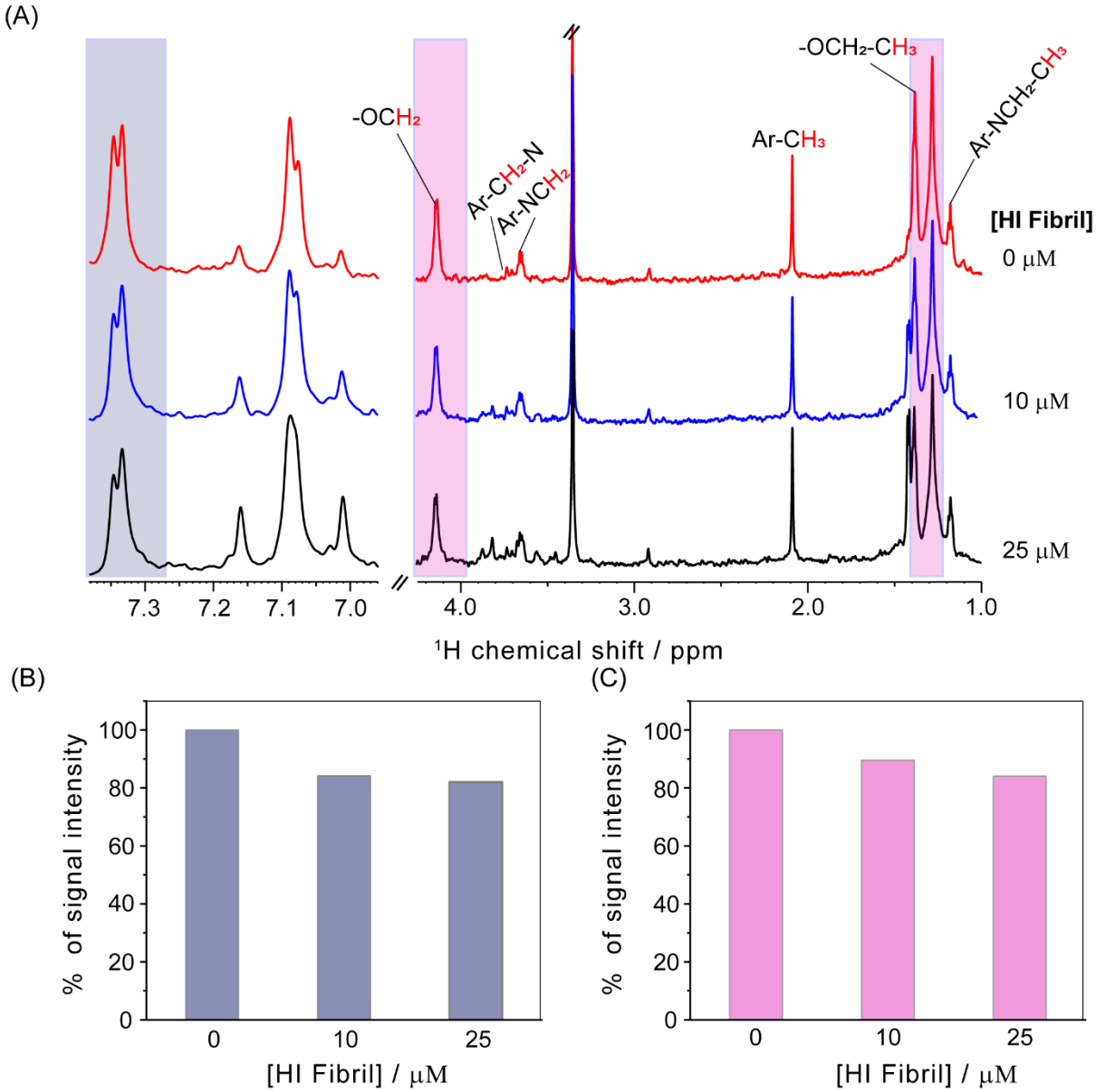


Figure S24: NMR signal intensities of the free and bound CBBG peaks upon titration with insulin fibril. (A) Selected section of 1D ^{1}H NMR spectra of CBBG (0.5 mM) acquired using Bruker Avance III 700 MHz at 25 °C showing the line width broadening with treatment of HI fibril. % of ^{1}H NMR signal intensity were calculated from the highlighted blue (B) and purple (C) colour regions as a function of HI fibril concentration (0, 10 and 25 μM). CBBG dissolved in 25 mM HCl, 100 mM NaCl buffer with 10% D_2O at pH1.6 at 25 °C.

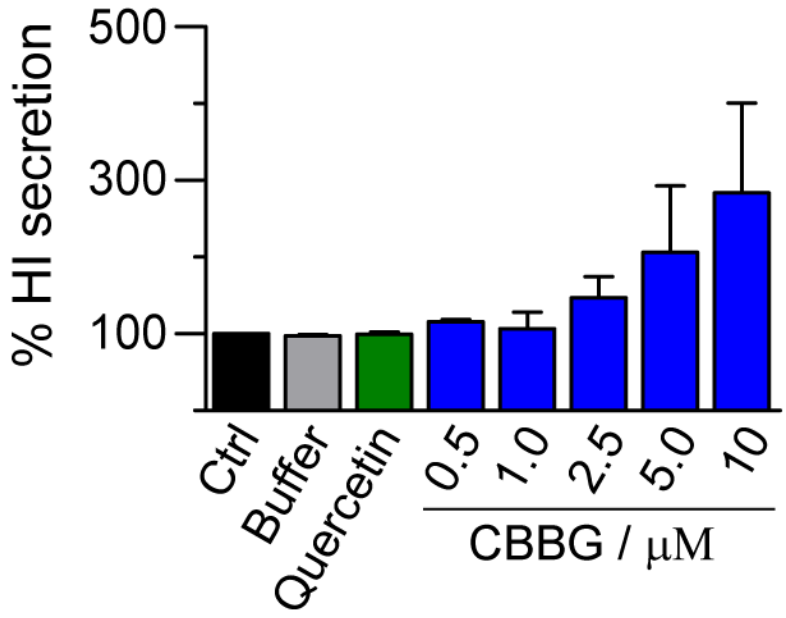


Figure S25: The effect of CBBG on release of insulin stimulated with normal glucose (11 mM). RIN5f cells were treated with buffer and various concentration of CBBG as indicated. Nontreated cells were taken as control. Quercetin was kept as a positive control following its role in stimulating HI secretion. Secretion of insulin (%) in respect to control was measured by insulin ELISA kit and plotted in Graphpad Prism. Values represented here are mean ± SEM, n=3.

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