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A Robust Yet Simple Method to Generate Fluorescent Amyloid Nanofibers

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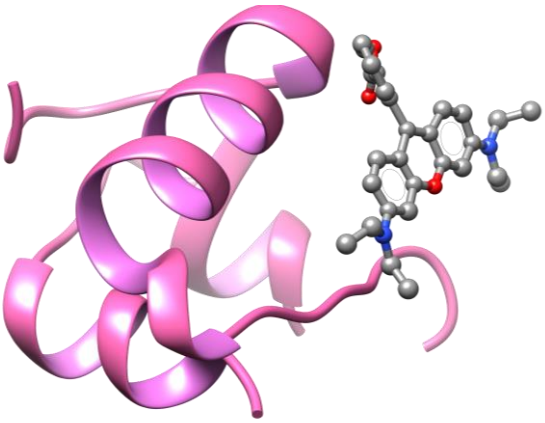
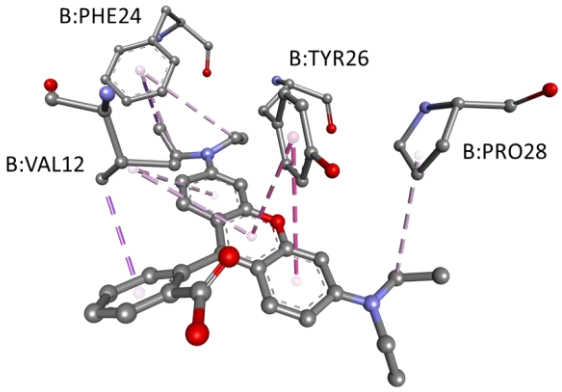
Insulin + Rhodamine B

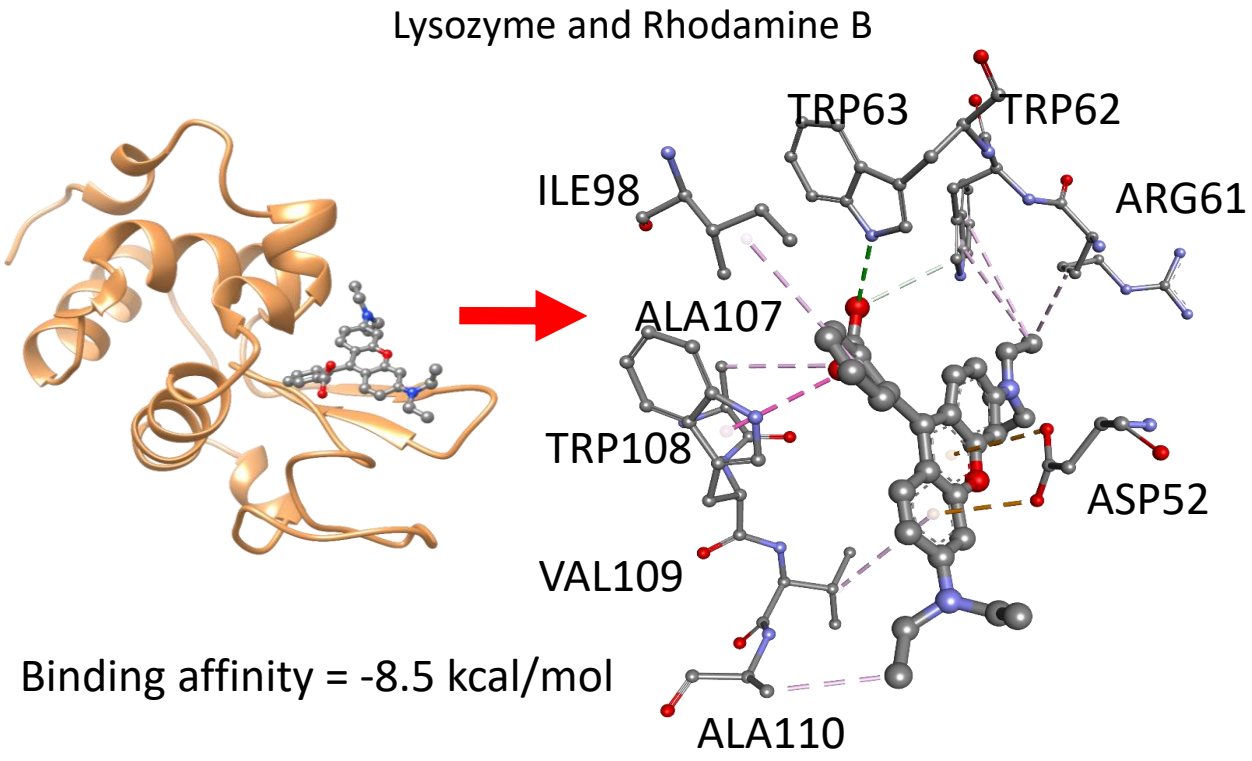




Binding affinity = -5.7 kcal.mol^{-1}

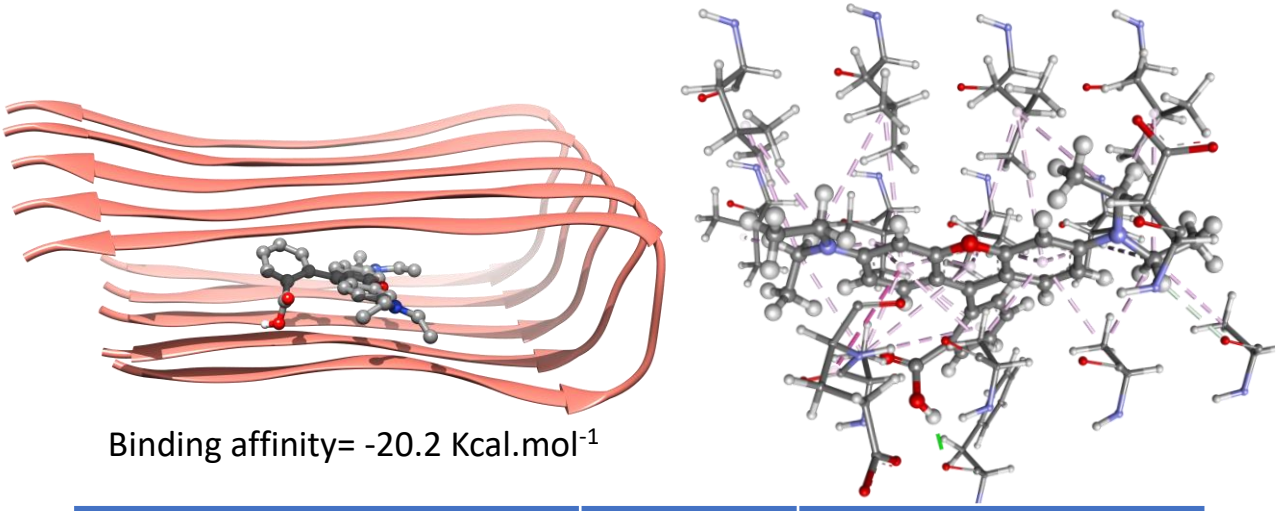
Interacting Residues	Bond length (Å)	Type of interactions
B:VAL12:CG1 - :RhoB	3.7	Hydrophobic
B:VAL12-:RhoB	5.2	Hydrophobic
B:VAL12-:RhoB	4.8	Hydrophobic
B:PHE24 - :RhoB	4.3	Hydrophobic
B:PHE24-:RhoB	3.7	Hydrophobic
B:PHE24 - :RhoB	5.1	Hydrophobic
B:TYR26 - :RhoB	4.6	Hydrophobic
B:TYR26 - :RhoB	5.2	Hydrophobic
B:PRO28-:RhoB	4.8	Hydrophobic

Figure S1. Molecular docking studies on Insulin-Rho-B interaction. Table given below lists the interacting residues.



Interacting Residues	Bond length Å	Type of interaction
A:TRP63:NE1 - RHO:O3	3.3	Hydrogen Bond
A:TRP62:CD1 - RHO:O3	3.6	Hydrogen Bond
A:ASP52:OD2 - RHO	3.5	Electrostatic
A:TRP108 - RHO	4.8	Hydrophobic
RHO - A:VAL109	4.6	Hydrophobic
RHO - A:ILE98	5.0	Hydrophobic
RHO - A:ALA107	4.5	Hydrophobic

Figure S2. Molecular docking studies on lysozyme-RhoB interaction. Table given below lists the interacting residues.



Binding affinity= -20.2 Kcal.mol^{-1}

Interacting residues	Bond Length (Å)	Type of interactions
R:RhoB:H65 - B:PHE19:O	1.9	Hydrogen Bond
R:RhoB:H22 - D:ALA21:O	2.8	Hydrogen Bond
A:ALA21:C,O;GLU22:N - R:UNK1	4.3	Hydrophobic
A:ALA21 - R:RhoB:C21	4.2	Hydrophobic
C:ALA21 - R:RhoB:C23	3.9	Hydrophobic
D:ALA21 - R:RhoB:C23	4.4	Hydrophobic
R:RhoB:C21 - A:LEU34	4.7	Hydrophobic
R:RhoB:C21 - A:VAL36	4.0	Hydrophobic
R:RhoB:C21 - B:VAL36	3.9	Hydrophobic
R:RhoB:C22 - A:LEU34	4.1	Hydrophobic
R:RhoB:C22 - B:LEU34	4.4	Hydrophobic
R:RhoB:C23 - D:LEU34	4.9	Hydrophobic
R:RhoB:C23 - D:VAL36	5.2	Hydrophobic
R:RhoB:C24 - C:LEU34	4.4	Hydrophobic
R:RhoB:C24 - D:LEU34	3.6	Hydrophobic
R:RhoB - A:ALA21	4.9	Hydrophobic
R:RhoB - B:VAL36	5.0	Hydrophobic
R:RhoB - C:LEU34	5.4	Hydrophobic
R:RhoB - C:VAL36	3.6	Hydrophobic
R:RhoB - A:ALA21	3.4	Hydrophobic
R:RhoB - B:ALA21	3.9	Hydrophobic
R:RhoB - B:LEU34	5.3	Hydrophobic
R:RhoB - B:VAL36	3.6	Hydrophobic
R:RhoB - C:VAL36	4.6	Hydrophobic
R:RhoB - B:ALA21	3.7	Hydrophobic
R:RhoB - C:ALA21	3.9	Hydrophobic
R:RhoB - C:LEU34	5.1	Hydrophobic
R:RhoB - C:VAL36	4.3	Hydrophobic
R:RhoB - D:VAL36	4.9	Hydrophobic
R:RhoB - A:ALA21	5.3	Hydrophobic
R:RhoB - B:VAL36	5.3	Hydrophobic

Figure S3. Molecular docking of cross-β structures of Aβ1-42 peptide (PDB ID: 2BEG) with Rhodamine B. Binding affinity = -20.2 Kcal.mol^{-1}.

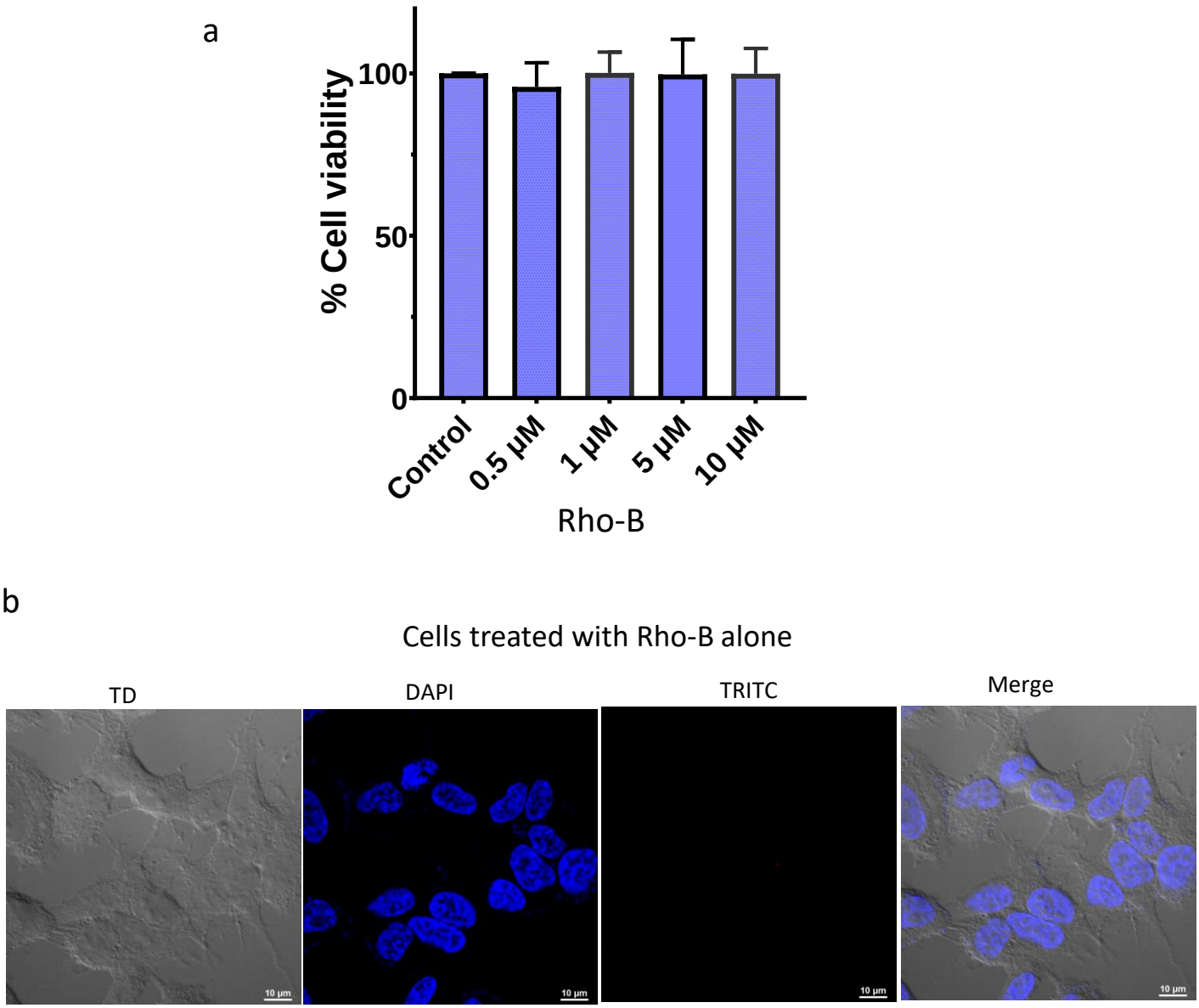


Figure S4. a, MTT assay showing the effect of only Rho-B on SH-SY5Y cells. ***b,*** Confocal microscopy of SH-SY5Y cells treated with only Rho-B (10 µM) at 24 h: (i)TD image; (ii) nucleus staining via DAPI (blue); (iii) TRITC visualization indicating no red puncta; (ii) Merged view of (i), (ii) and (iii).

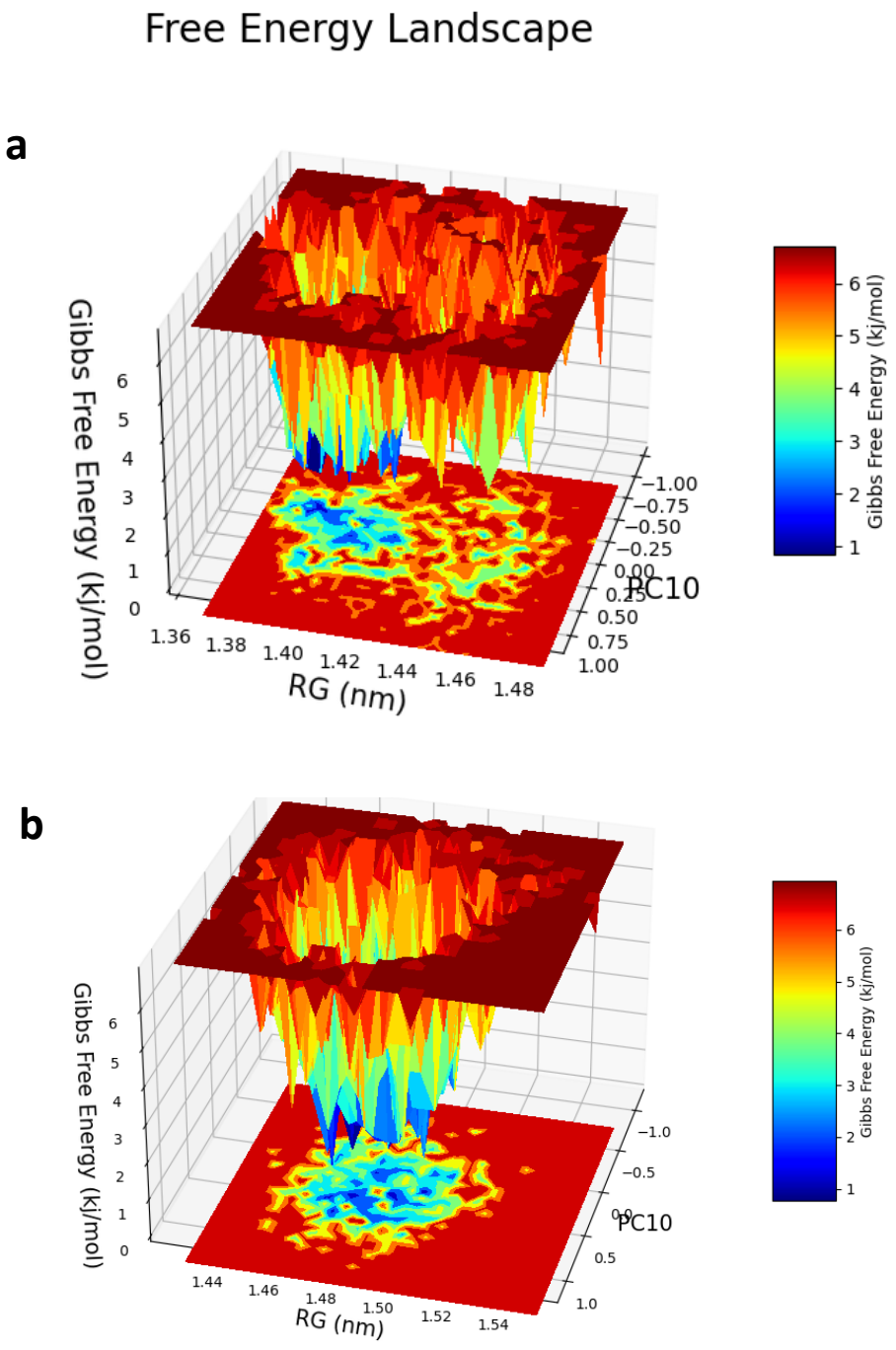


Figure S5. Free energy landscape from simulation of Rhodamine B and Aβ1–42-cross-β complex. ***a,*** only Aβ1–42 ; ***b,*** Aβ1–42-Rhodamine B complex.

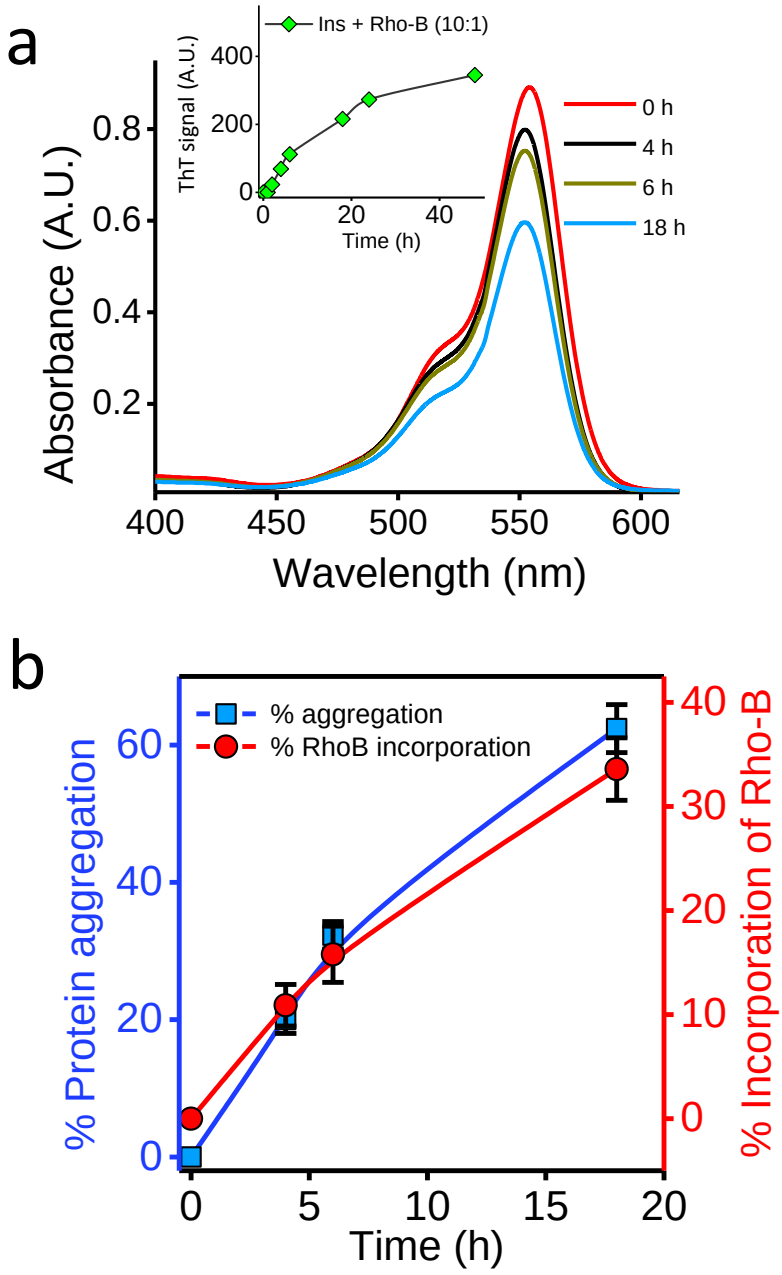


Figure S6. ***a,*** Rho-B absorbance in the supernatant of [100 µM ins + 10 µM Rho-B] sample during aggregation. Inset shows rise in the ThT signal. ***b,*** Correlation between %insulin aggregation and the decrease in the Rho-B absorbance for [100 µM Ins + 10 µM Rho-B] sample.

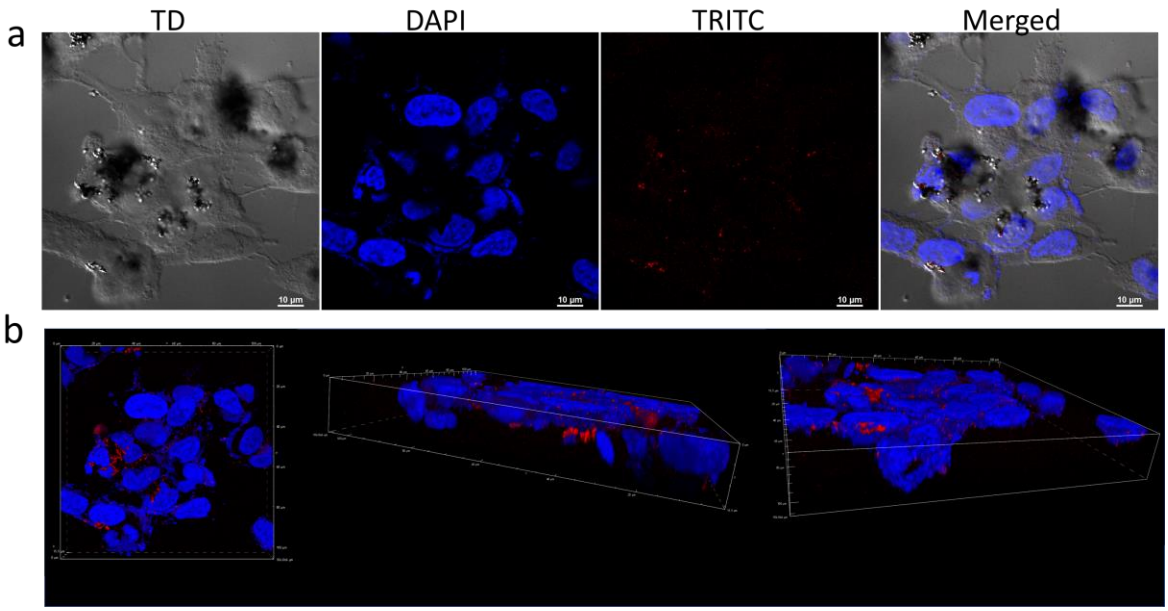


Figure S7. **Confocal microscopy shows cellular-internalization of Rho-B incorporated amyloid-mimicking dopamine-nanostructures.** ***a,*** Confocal imaging of SH-SY5Y cells treated with Rho-B incorporated dopamine fibrils at 24 h: (i) TD ; (ii) DAPI (blue); (iii) red puncta (TRITC), indicating the internalized Rho-B-incorporated dopamine aggregates; (iv) Merged view of (i), (ii) and (iii). ***b,*** 3D constructs view of panel ***a*** in different orientations, built from Z-stack confocal imaging, revealing internalization of dopamine nanostructures.

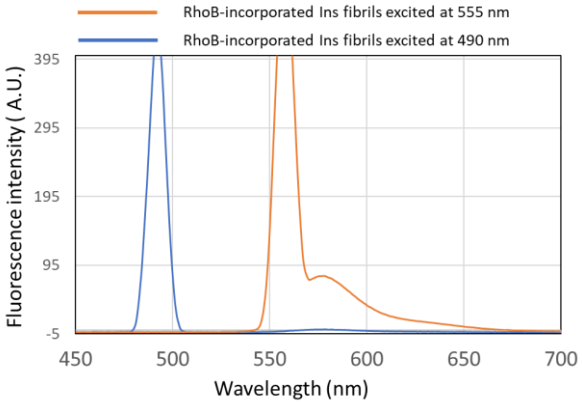


Figure S8. Fluorescence emission of Rho-B-incorporated insulin fibril sample at different excitation wavelengths: ▬ emission profile at excitation wavelength of 490 nm; ▬ emission profile at excitation wavelength of 555 nm, as labeled. There was no emission from Rho-B when it was excited at Fluorescein specific 490 nm.

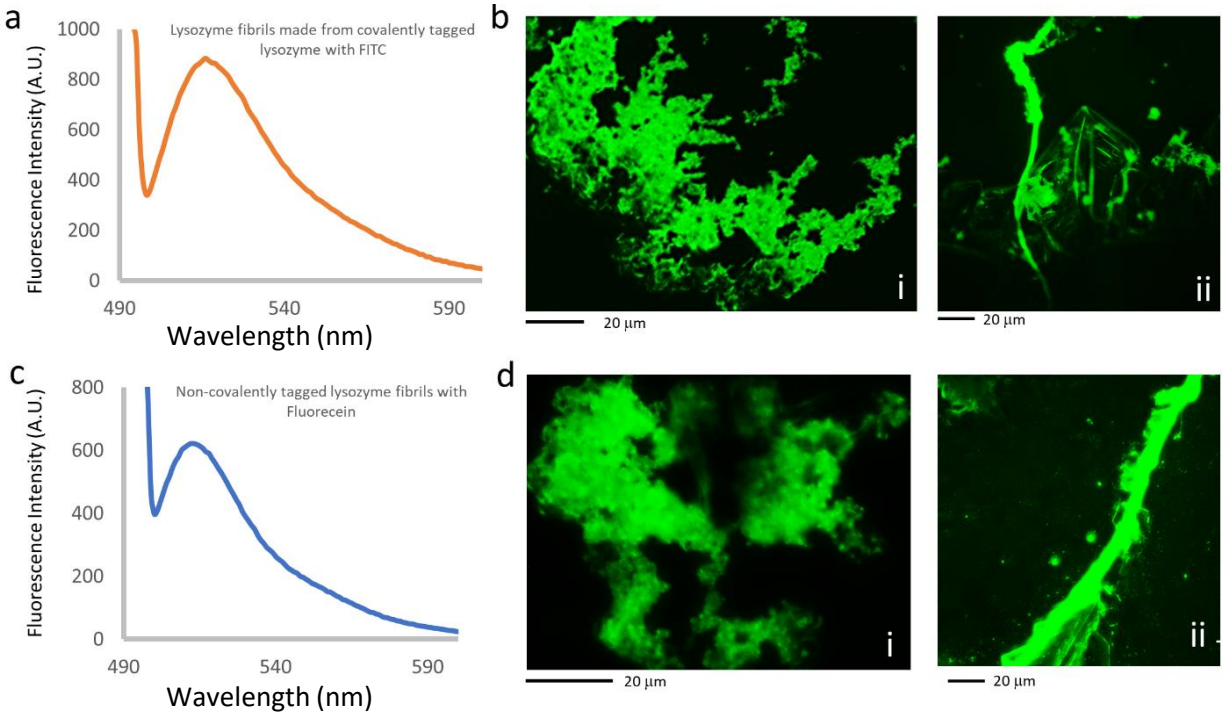


Figure S9. **Comparison of fluorescence properties of lysozyme fibrils made from two different sources:** a) Fluorescence emission from covalently tagged (*13*, *14*)(with fluorescein) lysozyme fibrils (λ_{ex} = 490 nm); b) Fluorescence microscopic images of lysozyme fibrils made from lysozyme monomers which are covalently tagged with FITC (i) and (ii). c) Fluorescence emission from lysozyme fibrils (with noncovalent incorporation of Fluorescein) (λ_{ex} = 490 nm); d) Fluorescence microscopic images of lysozyme fibrils fluorescein with noncovalently incorporated fluorescein: (i) and (ii). Scale bar 20µm.

MATERIALS AND METHODS

Reagents — All proteins and fluorophores were procured from either from Sigma- Aldrich or HIMEDIA, and the concentration was measured using Shimazdu UV-1900 spectrophotometer. Extinction coefficient values used were as follows: 6080 $M^{-1}cm^{-1}$at 278 for insulin (source-human recombinant), 38940 $M^{-1}.cm^{-1}$at 280 nm for lysozyme(source-hen egg white), 106,000 M^{-1} cm^{-1} at 555 nm for Rhodamine-B, 76,900 $M^{-1}cm^{-1}$ at 490 nm for Fluorescein.

Sample preparation — Stock solutions were prepared as follows: Stock solution of Rhodamine-B (RhoB) was prepared by dissolving in PBS buffer (pH-7.4) and Fluorescein was prepared by dissolving in 0.1 M tris-HCl (pH-8.0). Stock solutions of the proteins were prepared by dissolving them in PBS buffer, pH-7.4.

Aggregation studies of proteins in the presence of Rho-B and Fluorescein — Thioflavin T, a fluorescent dye that specifically binds to amyloids aggregates, was used for aggregation studies. Aggregation kinetic studies of protein samples in the presence and absence of fluorophores were performed in PBS buffer, pH 7.4 at 65 °C. ThT concentration was maintained at 30 μM, and the fluorescence intensity of ThT at different time points was recorded using a Shimazdu fluorescence spectrophotometer (RF-5300, Japan). The rise in ThT was detected at 490 nm by exciting the ThT molecule at 440 nm. Aggregation reaction of all the protein samples were carried out at 100 μM for insulin, 50 μM for lysozyme, with Rhodamine-B and Fluorescein at different molar ratio formulations (1:100, 1:20, and 1:10). The aggregation of $A\beta_{42}$ peptide was performed in PBS buffer, pH 7.4 at 37 °C.

Incorporation of fluorophores into Protein fibrils — Fluorophore absorbance spectra of the supernatant of the aggregating protein solutions having fluorophores at different time points was recorded using Shimazdu UV-1900 spectrophotometer, after centrifugation at 15,000 rpm for 10 min. Next, washing of the pellet was performed to remove excess fluorophores in the soluble form. For this, all the solutions after 72 hours were washed three times using PBS buffer, each wash involving centrifugation at 15000 rpm for 10 min and resuspension of the pellet into 1 ml PBS buffer. For stable incorporation of fluorophore into protein aggregates again after 2-day centrifugation of solution was done and absorbance spectra of supernatant was obtained, and pellet was resuspended into 1 ml PBS buffer. Detailed protocol is given below.

Fluorescence microscopy — The fluorescence microscopy was conducted using a fluorescence microscope (Nikon). 20 μl diluted aggregate sample was smeared over a glass slide, dried, and then stained with ThT and imaged, FITC filter was used for ThT imaging and TRITC used for Rhodamine-B and Fluorescein imaging. The cellular imaging studies were also performed using the same Nikon 90i microscope.

Cell Culture studies —The human neuroblastoma cell line (SH-SY5Y) and Lung cancer A549 cell lines were obtained from the American Type Culture Collection (ATCC, CRL-2266). The cells were grown in DMEM/ F12 (Gibco Life Technologies, USA) medium supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco Life Technologies, USA) and 50 U/ml penicillin, 100 μg/ml streptomycin (HIMEDIA). Cells were maintained in the incubator at 37 °C with 5% atmospheric CO_2 and 95% humidity. The medium was changed after 48 h. Description of methods for MTT assay and live cell imaging studies using confocal and fluorescence microscopy is given below.

MTT assay

The established MTT assay (*1, 2*) protocol was performed for cellular effect of only fluorophores. SH-SY5Y cells were seeded in 96-well plates (5 x10^3 cells/well) in triplicates in the culture medium for 24 h. The confluent cells were then treated with different doses (0.5 μM, 1 μM, 5 μM and 10 μM) of RhoB only for 24 h. Next, these cells were incubated with MTT for 4 h, and the change in the color was measured using spectrophotometer at 570 nm using a microtiter plate reader (ThermoFisher VARIOSKAN microplate reader).

Fluorescence microscopy and live cell imaging

Internalization of the RhoB incorporated amyloid fibril was monitored by fluorescence microscopy. Cells (SH-SY5Y and A549) were grown in six-well plates containing glass coverslips. When cells become confluent about 70% then the cells were treated with 5 and 10 μM of RhoB tagged amyloid fibrils. Treated and non-treated cells were incubated in CO_2 incubator at 37 °C for 24 h. After 24 h, the cells were washed with 2 mL of PBS followed by fixation of the cells with 2 mL of 4 % paraformaldehyde for 30 min at RT. Next, paraformaldehyde was removed, and cells were washed three times with 2 mL of PBS. Further, the SH-SY5Y cells were mounted with Vectasheild with DAPI (Vector Laboratories H-1200) on glass slides. Nikon AIR HD Fluorescent Confocal Microscopy was used to visualize the internalized RhoB-tagged amyloid aggregates and their uptake by the cells, Z-stack images of internalized fluorescent amyloid fibrils in cells were captured at 60 x. To further monitor the internalization process of these fluorescent labelled amyloid fibril we performed the live cell imaging of SH-SY5Y cells treated with RhoB-incorporated insulin aggregates by using confocal microscopy, cells were grown on confocal dish with glass bottom in CO_2 incubator, till the 70-80% confluency. Next, we monitored the internalization for 1.5 h after the treatment in live cell via confocal microscopy and each snapshot was captured in every 10 s. Inbuilt NIS element software was used for data acquisition, image analysis and live cell imaging. We also visualized A549 cells treated with RhoB-incorporated lysozyme aggregates. After fixation and washing step, the cells were mounted on glass slide. The images were captured at 20 x under Nikon TiE fluorescence microscope, analyzed by in-built NIS4.00.00 software.

Circular Dichroism (CD)

The structural changes during the conversion of soluble protein monomers into beta-sheet rich amyloid aggregates were studied using Chirascan™ qCD, attached to a Peltier temperature controller. PBS was used as the reference solution and the CD spectra were recorded at room temperature using a cuvette of 2 mm path length. Data presented in the text are the average of three independent measurements.

Fluorescence resonance energy transfer (FRET) assay

We performed FRET experiment using Shimazdu fluorescence spectrophotometer (RF-5300, Japan. For this specific assay two known FRET pair fluorophore dyes which are rhodamine B (acceptor) and fluorescein (donor) were selected. Both the dyes were incubated with soluble insulin at 65 ℃ for 72 h maintaining the concentration at 1:20 molar ratio of dye:protein. After incubation the sample was subjected to centrifugation (15000 rpm) and washing steps three times to prepare the suspension of fluorophore incorporated protein aggregates. Next, using fluorescence spectroscopy, FRET signal was obtained by exciting the sample at donor's excitation wavelength (490nm). Two distinct emission peaks (specific for both RhoB and fluorescein) were recorded.

Molecular docking studies

Molecular docking study was conducted by Autodock Vina using PyRx open-source software (GUI version 0.8)(*3*, *4*). We obtained the structure of Rhodamine B from PubChem (https://pubchem.ncbi.nlm.nih.gov/) (PubChem CID:6694). PDB structures of the studied proteins were obtained from RCSB (Lysozyme PDB ID: 5WRA(*5*), Insulin PDB ID 3I3Z(*6*), $A\beta_{42}$ fibril PDB ID: 2BEG (*7*)). Prior to docking, pre-processing and protonation of receptor molecules were performed by removing the unwanted ligand and water molecules and by adding polar hydrogen atoms. For docking, AutoDock Vina used a search space of grid box size (x = 24 Å, y = 24 Å, z = 24 Å) for 3I3Z, (x = 24 Å, y = 24 Å, z = 24 Å) for 5WRA and (x = 22 Å, y = 13 Å, z = 16 Å) for 2BEG. The results obtained were analyzed based on the binding affinity energy (kcal mol^{-1}) parameters linked to the respective protein−ligand complexes. The protein−ligand docked complex with the lowest energy was chosen for further analysis. Docked complexes were visualized and analyzed by using Discovery Studio visualizer (v 16.1.0153) and Chimera 1.15.

Molecular dynamics simulations

We performed MD simulations using the GROMACS 2020.6 (https://doi.org/10.5281/zenodo.4576060)(*8*) after docking of protein and RhoB. Here, we performed MD simulation of the complex between amyloid beta fibril and RhoB. Topologies for protein and protein–ligand complexes were produced using the CHARMM 36 force field(*9*). The complex and single protein structures were solvated in the water model after the topology file was created, and structures were neutralized by adding ions. After that, these structures were relaxed using an energy minimization approach involving the steepest descent Algorithm and the Verlet cut-off scheme that was run for 50,000 cycles at 10 kJ/mol. The equilibration step of protein and ligands complex was performed on NVT (constant volume) as well as NPT (constant pressure) for 1000 ps trajectory period. After equilibration step, the simulation analysis was calculated at 300 K temperature and 1 atm pressure using 2 fs time step for a 50 ns. The obtained trajectory files were used to visualize the deviation of protein and complex to determine the system's stability in a water environment. To investigate the deviation between protein and ligand complexes we use Root mean square variance (RMSD), Radius of gyration (RG), and Principal component analysis (PCA). Further, we calculated the interaction energy between protein and ligands to calculate the strength between protein and ligand. Furthermore, Molecular Mechanics Poisson–Boltzmann Surface Area (gmx_MMPBSA)(*10*) method was used to calculate the total binding free energy (equation 1) using gmx_mmpbsa package (*11*) in GROMACS software, the free solvation energy (polar + non-polar solvation energies), and potential energy (electrostatic + Van der Waals interactions) of each protein–ligand complex for last 100 frames

$$\Delta G_{bind} = G_{complex} - (G_{Rec} + G_{Lig}) \quad \text{(equation 1)}$$

The free energy landscape of protein folding on the RhoB bound complex was measured using geo_measures v 0.8. Geo_measures include a powerful library of g_sham and form the MD trajectory against RMSD and Radius of gyration (Rg) energy profile of folding recorded in a 3D plot using matplotlib python package (*12*).

Covalent tagging of Fluorescein isothiocyanate (FITC) with proteins:
To compare the fluorescence properties of our non-covalently tagged amyloid fibrils with that of covalently tagged amyloid fibrils, we have compared Fluorescein incorporated lysozyme fibrils with lysozyme fibrils made from FITC-attached lysozyme monomers. For covalent tagging FITC with lysozyme we followed the established protocol as reported in previously published studies (*13,14*). The FITC-tagged lysozyme was purified using sephadex-25G column and was lyophilized for storage. The stock solution of pure FITC-tagged lysozyme was prepared in the PBS buffer (pH 7.4) for both spectrofluorimetric and fluorescence microscopic studies.

DETAILED PROTOCOL FOR MAKING FLUORESCENT AMYLOIDS NANOFIBERS
Protein solution preparation (day-1) timing 1-3 hr
•For preparation of protein stock solution, insulin and lysozyme were weighed 6mg each.
•Both insulin and lysozyme are dissolved into 3ml each of 1x PBS buffer pH-7.4
Critical step- The volume and concentration of stock solution can vary according to need for the experiment.
•Stock solution is filtered using 0.22 μm filter, so that only soluble protein remains and also removal of any contamination that could enhance or interfere in aggregation.
Critical step- Filter of pore size 0.22 μm was used as through its pore soluble proteins of our choice can pass whereas no oligo-aggregates will be able to pass.
•Concentration of both the proteins were determined using UV-spectrophotometer, extinction coefficient values used as follows: 6080 M^{-1} cm^{-1} at 278 for insulin, 36 mM^{-1} cm^{-1} at 280 nm for lysozyme.
Critical step – the proteins were diluted 10 times while taking absorption, so that their absorption reading remains below 1 to measure concentration of stock solution accurately.
Protein aggregation day (1 to 3) timing 0.5-1 hr
•2 ml solution of Working concentration of 100 μM and 140 μM was prepared from stock solution using 1X PBS buffer pH-7.4 for insulin and lysozyme respectively.
•4.12 μl and 5.76 μl Rhodamine-B from 2.43 mM stock solution was added to the working concentration of protein solution of insulin and lysozyme respectively.
Critical step – the concentration of Rhodamine-B added to the protein solution is such that the ratio of protein to Rho-B should remain 20:1.
Caution – Rhodamine-B is light sensitive and to avoid its photobleaching experiments should be done dark condition.
•Now the Eppendorf of both insulin and lysozyme are kept in dry bath/heat block at 70°C for proteins to aggregate.
Critical step – We decided to perform protein aggregation at 70°C based on previous studies but aggregation can be done under range of temperature condition, and it should be decided accordingly.

Kinetics of aggregation and rhodamine incorporation day (1 to 2) timing 0.5-1 hr

•Thioflavin T readings were taken by exciting at 440 nm and rise was detected at 490 nm at 0,1,2,4,6,18,24,48 hr in which 50 µl of protein aggregates and 30 µl Thioflavin T from stock solution of 1.5mM dissolved into 470 µl of 1x PBS buffer pH-7.4.

Critical step – Readings were taken by keeping slit width of fluorophotometer at 5 and 10 if slit width will be kept at 3 and 5 then value of readings will change accordingly. Three reading were taken for each sample at a time point.

•Absorption spectra of Rhodamine-B with peak at 553-555nm using spectrophotometer were taken at 0,2,4,6 and 18 hr after centrifugation at 15,000 rpm for 10 min.

Caution – Both the steps should be done in dark condition to prevent the photobleaching of ThT and Rho-B.

Removal of excess rhodamine after aggregation of protein day (3) timing 0.5- 1 hr

Critical step – The washing should be done with extra caution only supernatant in every washing step should be removed to get read of any soluble fluorophore which can hinder in further studies and the pellet should remain undisturbed.

•After 72 hr of keeping insulin and lysozyme in dry bath these protein aggregates were washed 3 times using 1X PBS buffer pH-7.4. In each wash the solution was centrifuged at 15,000 rpm for 10 min and the supernatant was discarded while the pellet was resuspended into 2 ml 1x PBS buffer by vertexing.

Fluorescence microscopy day (3) timing 2-3 hr

Critical step – Preparation of sample side and observation under fluorescence microscope should be done under dark condition. Sample should be properly smeared and dried before staining with Thioflavin T.

•20 µl of 10 times diluted aggregated sample was smeared over a glass slide and completely dried.

•Then the smeared sample was stained using 20 µl Thioflavin T, after ThT was dried the slide was observed under fluorescence microscope. Thioflavin T staining was observed under FTIC filter and the incorporated Rhodamine-B was observed under TRITC filter.

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