Electronic Supplemental Information

**Designing protein nano-construct in ionic liquid: A boost in efficacy of Cytochrome c under stresses**

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## Experimental Details

### Materials

Bombyx Mori's silk is obtained from Kanakapura, Karnataka, India (12° 81'N, 77° 57'E). Sigma-Aldrich from USA, supplied Cyt C from horse heart with >95% purity (CAS No. 9007-43-6), 2,2'-azino-bis(3-ethylbenzothiazoline-6 sulfonic acid) diammonium salt (ABTS) with >98% purity (CAS No. 30931-67-0), Choline hydroxide 46% in $H_2O$ (CAS No. 123-41- 1), Choline bicarbonate (CAS No. 78-73-9) and Trypsin (CAS No. 9002-07-7). Avra synthesis Pvt. Ltd., Hyderabad, India, supplied 30% (w/w) hydrogen peroxide solution ($H_2O_2$) in water (CAS No. 7722-84-1), guanidium hydrochloride (GuHCl) (CAS No. 50-01-1), Methanol (CAS No. 67-56-1). Rankem, RFCL Pvt. Ltd., from New Delhi, India, provided Orthophosphoric acid (CAS No. 7664-38-2) and Sodium bicarbonate (CAS No. 144-55-8). All of the compounds were utilised without further purification. Double distilled water from a laboratory was used throughout the operation.

### Preparation of SNFs

Bombyx mori silk was degummed two times in a hot solution of 1 wt% $Na_2CO_3$ for 30 minutes at 70 °C for removing sericin. [1] An experiment involved stirring 20 mg/mL of degummed silk fibres with 46 wt% aqueous choline hydroxide at 500 rpm for one hour at room temperature (ChOH). In addition, a 1:2 mixture of ice-cold methanol (MeOH) and the silk solution was added, and the IL/MeOH mixture and SNFs were separated by centrifugation at 10,000 rpm for 30 minutes (Scheme 1). The obtained precipitate was then washed using MeOH and double distilled water before being freeze dried. A fixed volume of SNF was diluted in distilled water for the subsequent steps.
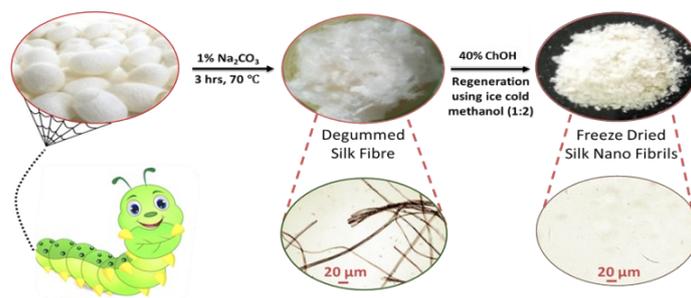


**Scheme.1** Schematic representing SNF preparation

## Characterization / Experimental methods

SNF, Cyt C, IL and their combinations were characterized using a variety of spectroscopic and analytical techniques. Activity studies and UV-Vis spectra were recorded using a Shimadzu UV-1900 spectrometer with resolution (1 nm) and quartz cuvettes of 1 cm path length. A Bruker instrument was used to collect FT-IR spectra in the range of wavelength of 4000 to 500 cm$^{-1}$. For each spectrum, 64 pictures were created at a resolution of 2 cm$^{-1}$. Dynamic Light Scattering (DLS) of Cyt C and SNF aqueous solutions was determined using an Anton Paar Particle Analyzer Litesizer 500 device. A Jasco-1500 spectrophotometer with temperature control Peltier system was used for CD spectroscopic experiments. With route lengths of 0.1 cm and 1 cm, far-UV CD spectra were observed in the range 190–250 nm, whereas soret spectra were recorded in the region 350–450 nm. Each sample spectrum was collected by taking average of three spectra after eliminating the relevant blank from the tentative spectrum.

## Peroxidase Activity of Cyt C

Cyt C's peroxidase activity was evaluated using ABTS as a substrate along with $H_2O_2$. The emergence of a green ABTS radical cation result from the oxidation of ABTS, which is catalyzed by the addition of $H_2O_2$.[2] To monitor the formation of the ABTS radical cation, the absorbance variation at 420 nm was tracked over the course of one minute. The reaction media contains 2.0 µM Cyt C, 1.0 mM $H_2O_2$, and 3.0 mM ABTS had their respective absorption spectra measured. To calculate the percentage relative activities with respect to enzymatic activity at room temperature, distilled water was always used. The activity of Cyt C was assessed prior to the procedure using the appropriate concentrations of SNF and IL solutions. The activities of bare Cyt C, Cyt C+IL, Cyt C+SNF and Cyt C+SNF+IL were performed under the identical conditions. The activity studies are also carried out by keeping concentration of SNF fixed, varying the concentration of IL and vice-versa. All the activity studies are repeated 3 times and the average value is represented by the bar graphs with error bar indicating standard deviation in the obtained values.

## Activity studies of Cyt C under different stress conditions

Utilizing ABTS as the substrate, Cyt C's peroxidase activity was assessed under a variety of conditions, including high temperature, variable pH, the presence of denaturants like GuHCl and trypsin, and the presence of SNF, IL, and SNF+IL. In all the activity studies, Cyt C was first interacted with IL and SNF for 10 min and further different stresses were induced. To test Cyt C's stability against temperature-

induced stress, its activity was measured at five different temperatures after a 30 minute incubation period: 25, 50, 70, 90, and 110 °C. Following a 15-minute incubation period, Cyt C activity was assessed with 6M GuHCl present. Trypsin was used to examine how SNF+IL affected Cyt C stability. Trypsin causes Cyt C to break down to form amino acids, that prevents it from exhibiting peroxidase activity.[3] 6μM trypsin was added into Cyt C+SNF+IL solutions at 37°C for 24 hours. Changes in the absorbance peak at 420 nm were used to track the generation of the ABTS cationic radical. Every activity study is performed three times, and the average value is displayed as a bar graph with error bars showing the standard deviation of the results.

**Molecular Docking studies**

Cyt C and Fibroin protonation states of titratable remains was calculated from ProteinPrepare[4] Cyt C PDB file (PDB: 2b4z) and Fibroin PDB file (PDB: 3ua0) were download from Protein Data bank and loaded in ProteinPrepare application. The pKa estimation was performed at pHs 7.0, without water molecules and ligands from input PDB file. After calculation performed, the protonated PDB file was downloaded and applied in protein-protein docking analysis. The complex of Cyt C with Fibroin was obtained using HDOCK server.[5] For this, the input files were: Cyt C as ligand (PDB:2b4z obtained from ProteinPrepare) and Fibroin as receptor (PDB: 3ua0 obtained from ProteinPrepare). The Cyt C-Fibroin complex with lower docking score and ligand rmsd[6] was downloaded and used to identify IL interactions. The interaction of Cyt C-Fibroin complex with the IL anion and cations were recognized using the AutoDock Vina 1.1.2 program.[7] The structure of Cyt C-Fibroin complex was gained from protein-protein docking analysis and applied for molecular docking. AutoDockTools (ADT)[8] was used to prepare the Cyt C-Fibroin complex input file. Ligands 3D atomic coordinates were shaped using Discovery Studio, v20 (Accelrys, San Diego, USA), registered to Chem3D-MM2 protocol for minimizing the energy, and ligand stiff root was made using AutoDockTools (ADT). The grid at the mass center of Cyt C-Fibroin complex was -1.255 × 33.150 × 12.696 in the x-, y-, and z-axes, respectively. The dimension of the grid was 20 Å × 20 Å × 20 Å to cover the Cyt C-Fibroin complex interface. For each of the ten ligands, the binding model with the lowest binding free energy was sought out. The results of protein-protein and protein- protein+IL ions were envisioned and analyzed using Discovery Studio, v20 (Accelrys, San Diego, CA, USA).
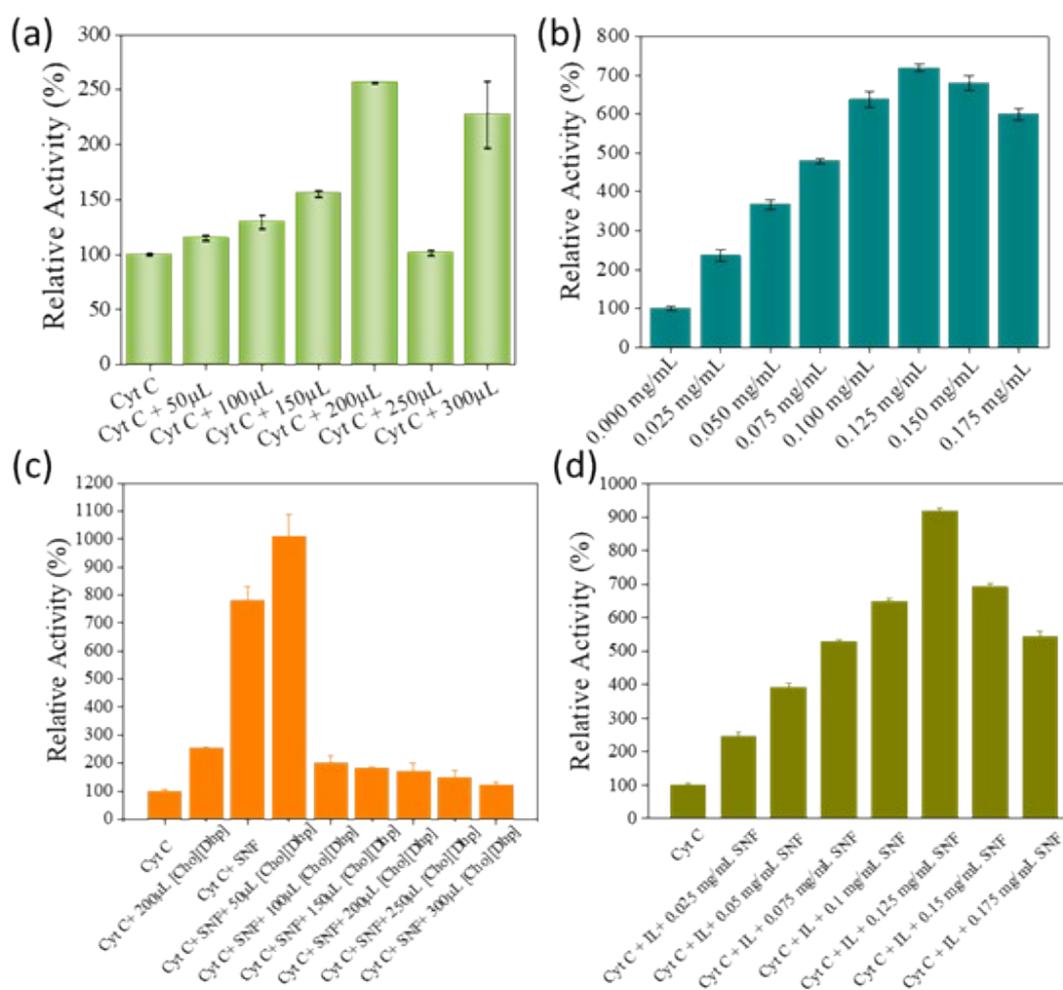
**Fig. S1** Effect of concentrations of (a) [Cho][Dhp] (stock solution is 50% [Cho][Dhp] in distilled water), (b) SNF (0.025 to 0.175 mg/mL stock solutions) , (c) SNF (0.125 mg/mL fixed) by varying the concentration of [Cho][Dhp] and (d) [Cho][Dhp] (50µL fixed) by varying the concentration of SNF on Cyt C's peroxidase activity.
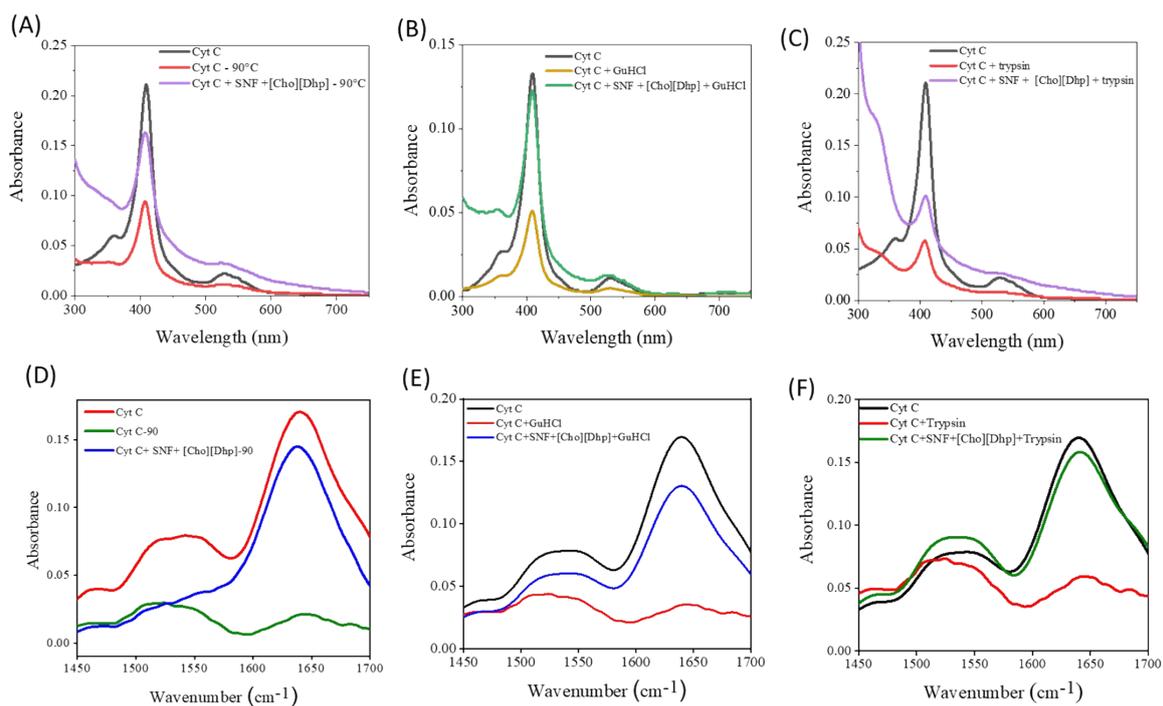
**Fig. S2** Stability studies of Cyt C using UV-Vis analysis in presence of (a) High temperature, (b) GuHCl and (c) Trypsin, Stability studies of Cyt C using ATR-FTIR analysis in presence of (d) High temperature, (e) GuHCl and (f) Trypsin.

**Table S1**. Interacting amino acids and nucleic acids predicted by HDOCK for CytC-Fibroin (A-B) complex.

| Type of interaction | From | To | Distance (Å) |
|---|---|---|---|
| Electrostatic | A:LYS87 | B:ASP91 | 3.56 |
| | B:ARG66 | A:PHE82 | 4.98 |
| Hydrogen Bond | B:ASN68 | A:GLU90 | 2.70 |
| | B:ASN93 | A:GLU90 | 3.30 |
| | A:GLN16 | B:THR36 | 3.38 |
| | A:CYS17 | B:ASP29 | 2.43 |
| Pi-Sulfur | A:MET80 | B:PHE31 | 4.70 |
| Hydrophobic | A:LYS27 | B:PHE26 | 3.94 |
| | B:PHE26 | A:LYS25 | 5.44 |
| | B:PHE31 | A:CYS17 | 3.66 |

**Table S2**. Docking affinity energy and linking atoms projected by AutoDock Vina for Cyt C‑Fibroin complex + Ligands (ILs ions).

| Compound | Affinity (kcal/mol) | Type of interaction | From | To | Distance (Å) |
|---|---|---|---|---|---|
| [Ch]+ | -3.1 | Hydrogen Bond | [Ch]+ | Cyt C:GLN16 | 2.30 |
| | | | | Cyt C:VAL11 | 3.69 |
| | | | | Fibroin: VAL37 | 3.62 |
| [DHP]- | -2.7 | Electrostatic | Fibroin:ARG66 | [DHP]- | 5.39 |
| | | Hydrogen Bond | Cyt C: GLN16 | [DHP]- | 3.40 |
| | | | [DHP]- | Fibroin:TYR30 | 2.45 |
| | | | | Fibroin:THR36 | 2.22 |
| | | | Fibroin:ARG66 | [DHP]- | 3.10 |

**References**

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