Spontaneous and reversible self-assembly of a polypeptide fragment of insulin-like growth factor binding protein-2 into fluoroscent nanotubular structures

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Supporting Information

Over-expression of IGFBP-$2_{249\text{-}289}$:

Cloning of IGFBP-$2_{249\text{-}289}$ [Cys^{281}] as a GST-fusion construct has been described earlier.[1] Plasmid DNA containing the clone was transformed into BL21 (DE3) strain of *E. coli* and incubated at 37 ^{0}C in 10ml of Luria-Bertani (LB) medium containing 100 µg/ml of ampicillin. The cells were grown till the optical density (O.D) reached 0.8 (i.e., O. $D_{600\ nm}$ ~0.8). After a 100-fold dilution into fresh LB medium containing 100 µg/ml of ampicillin, cells were re-grown at 37 ^{0}C to mid-log phase ($O.D_{600\ nm}$ ~0.6) at which point the expression of the protein was induced by addition of 0.5mM isopropyl β-D-thiogalactoside at 30 ^{0}C for 4 hours. Cells were then harvested by centrifugation at 4000 rpm for 30 min followed by re-suspension in a lysis buffer consisting of phosphate buffered saline (PBS), pH 7.5. Cell lysis was carried out on ice by sonication in four steps of 30-s cycles each with an intervening period of 2.5 min. Insoluble materials were removed by centrifugation (twice) at 9000 rpm for 45 min at 4^{0}C and the supernatant was taken for further processing.

Purification of IGFBP-$2_{249\text{-}289}$:

Supernatant obtained after centrifugation of cell lysate was pre-equilibrated at 4^0 C for 2 hrs with PBS buffer containing 50% slurry of Glutathione-Sepharose beads (Merck Bioscience). The fusion protein-bound to the matrix to the matrix was collected by

centrifugation at 4000 rpm for 5 min and washed three times with 10 bed volumes of phosphate-buffered saline. It was further washed three times with 10 bed volumes of high (25 mMHEPES, 0.05% NaN_3, 0.5 M NaCl, and 0.1% Triton X-100, pH 7.5) and low (25 mM HEPES, 0.05% NaN_3, 0.1 M NaCl, and 0.1% TritonX-100, pH 7.5) salt. Elution of the protein bound to the beads was carried out at 4^0C in 4 rounds with an intervening incubation period of 2 hrs, using for each round 2 ml of 25mM glutathione solution (pH 8.0). The glutathione thus present in the eluted fractions was removed by washing the sample several times with HRV-3C Protease enzyme cleavage buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5) using a 15 ml centricon tube (Millipore) containing a membrane with a cut-off of 5kDa and spun at 4000 rpm. The sample was concentrated down to 1ml. Next, for each milliliter of glutathione-sepharose bed volume, 10µl (20 units) of HRV-3C Protease (cleaves at Q-G bonds, Merck Bioscience) was mixed with 490 µl of cleavage buffer and added to the fusion protein eluted from glutathione-sepharose beads. It was then nutated at 4^0C for 12hrs. After completion of cleavage of IGFBP-$2_{249-289}$ from the fusion protein, β-mercaptoehanol was added to the solution to a concentration of 5 mM followed by was passing the solution through a centricon tube having a membrane with cut-off at 30 kDa and spun at 4000 rpm, thereby allowing only IGFBP-$2_{249-289}$ to flow-through the membrane and retaining the larger size proteins such as the free GST, any un-cleaved fusion protein and the added protease in the tube. The flow-through was collected and concentrated down to 1ml by ultra-filtration (an unit consisting of a stirred cell purged continuously with pure N_2 gas) using a membrane with cut-off at 1kDa. Protein samples were analysed by polyacrylamide gel electrophoresis and quantified by UV spectrophotometer. MALDI-TOF spectroscopy was used for further confirmation of

quality of the sample, which gave a single peak under reducing conditions at the expected molecular weight of ~5.6 kDa for the unlabeled sample and ~6.0 kDa for uniform $^{13}C/^{15}N$ isotope labeled sample prepared for NMR studies (see below). As part of the cloning strategy, a linker containing the amino acids GPLGS was added to the expressed protein.[1] In addition, extra amino acids, PGIRGS were added at the N terminus during the cloning process.

Preparation of IGFBP-$2_{249\text{-}289}$ nanotubes:

The IGFBP-$2_{249\text{-}289}$ nanotube was prepared at room temperature (25^0C) by slow removal of β-mercaptoethanol (added during purification) using an ultra-filtration cell (stirred cell purged continuously with pure N_2 gas) containing a membrane with cut-off of 1 kDa.

Transmission Electron Microscopy:

All samples were made in 10mM Tris-buffer (pH 7.0). Droplets containing samples were placed on a carbon coated copper grid (200 mesh), air dried and desiccated for 12h . The obtained specimens were examined in Technai F30 Transmission electron microscope (TEM) at an accelarating volatage of 200kV

Circular Dichroism (CD) measurements:

CD experiments were carried out at 25^0C with a sample concentration of ~200 μM on a JASCO Spectropolarimeter J-810 in a 1 cm cell. Spectra were obtained from 190-250 nm and all spectra were solvent subtracted.

NMR Sample Preparation

For $^{13}C/^{15}N$ labeling of IGFBP-$2_{249-289}$, transformed cells were incubated at 37 ^{0}C in 100ml of Luria-Bertani (LB) medium containing 100 μg/ml of ampicillin and allowed to grow overnight. The cells were then harvested by centrifugation at 4000 rpm and re-suspended in 1 Litre of 1X M9 minimal medium[2] containing $^{13}C_6$ –glucose (4gm/Litre) and $^{15}NH_4Cl$ (1gm/Litre) as the sole source of carbon and nitrogen, respectively. Following this, protein induction and purification was carried out as described above. In the final step, the sample was exchanged with Phosphate buffer (pH 6.0) for NMR studies.

NMR Spectroscopy

All NMR experiments were performed at 25 ^{0}C with samples containing 90% H_2O/10% ^2H_2O. NMR data for the monomer (~1 mM concentration) were acquired on a Bruker Avance 500 MHz NMR spectrometer, whereas data for the sample containing the nanotube were acquired on a Bruker Avance 700 MHz NMR spectrometer equipped with a cryoprobe. The acquisition parameters of the various NMR experiments are shown in Table S1. The spectra were processed using the software NMR-PIPE[3] and analyzed using XEASY.[4] Sequence specific backbone resonance assignments was carried out using 3D HNCACB and 3D CBCA(CO)NH spectra in concert[5] and using the methodology of TATAPRO[6] for spin system type identification. The rotational correlation time in the monomer state was obtained by measuring ^{15}N T_1 and T_2 relaxation rates.[5] Secondary structure estimation was carried out using the methodology of CSSI-PRO[7] by recording a GFT (3,2)D HA(CA)CO(N)H experiment.[7]

Table S1: Acquisition parameters of NMR experiments used for resonance assignments of IGFBP-2 $_{249\text{-}289}$ monomer and nanotube.

Sample	**Experiment Name**	**Indirect dimension: t_{max}(ms); Complex points; Digital Resolution (Hz/Pt)[a]**	**Measurement time (in hrs)**
Monomer	2D [^{1}H-^{15}N] HSQC	ω_1(^{15}N): 66.8; 128; 29.93	0.3
	3D HNCO	ω_1(^{13}C): 21.2; 32; 2.95 ω_2(^{15}N): 18.94; 24; 19.8	4.0
	3D HNCACB	ω_1(^{13}C): 6.73; 55; 15.96 ω_2(^{15}N): 15.78; 20; 19.8	23.6
	3D CBCA(CO)NH	ω_1(^{13}C): 6.72; 55; 15.96 ω_2(^{15}N) : 15.78; 20; 19.8	6.0
	GFT (3,2)D HA(CA)CO(N)H	ω_1(^{13}C'): 18.17; 64; 6.87	4.0
Nanotube [b]	2D [^{1}H-^{15}N] HSQC	ω_1(^{15}N): 52.2; 100; 29.93	2.0
	3D HNCO	ω_1(^{13}C): 12.98; 32; 4.81 ω_2(^{15}N): 15.38; 24; 24.39	4.0
	3D HN(CO)CA	ω_1(^{13}C): 6.05; 32; 10.32 ω_2(^{15}N): 16.1; 24; 23.28	4.0
	3D CBCA(CO)NH	ω_1(^{13}C): 6.06; 80; 25.77 ω_2(^{15}N) : 18.79; 28; 23.28	12.0
	3D HNCA	ω_1(^{13}C): 6.05; 32; 10.32 ω_2(^{15}N): 16.1; 24; 24.39	8.0
	(3,2)D HA(CA)CO(N)H	ω_1(^{13}C'): 18.17; 64; 6.87	24.0

[a] **Direct dimension**: ω_2/ω_3(^{1}H) : 512; 73; 6.9

[b] All experiments on the nanotube sample were carried out on a 700 MHz NMR spectrometer equipped with a cryogenic probe

Substitution of Cys281 (mutant) with Arg281 (wild type) by Site-directed mutagenesis:

The additional cysteine [Cys^{281}] in hIGFBP-$2_{249-289}$ in the current study was changed to Arg^{281} as in wild-type hIGFBP-2 by site-directed mutagenesis. PCR was performed using the plasmid containing GST-IGFBP-$2_{249-289}$ [Cys^{281}] construct (see above) as a template and *Pfu Ultra*™ High-Fidelity DNA Polymerase (Strategene) with primers described below. The following conditions were used: Initialization at 95^0C for 30 s followed by 12 cycles each at: 95^0C for 30 s, at 55^0C for 1 min, and at 68^0C for 15 min. The following primers were used:

5'-G CAG CAG GAG GCT **CGC** GGT GTG CAC AC-3' (coding)

3'-C GTC GTC CTC CGA **GCG** CCA CAC GTG TG-5' (non-coding)

The highlighted nucleotides represent the sequence that was mutated to encode arginine. Following PCR, the reaction was digested with Dpn1(NEB) enzyme and transformed into DH5α *E. coli* The resulting colonies carrying the mutant plasmid were screened by DNA sequencing.

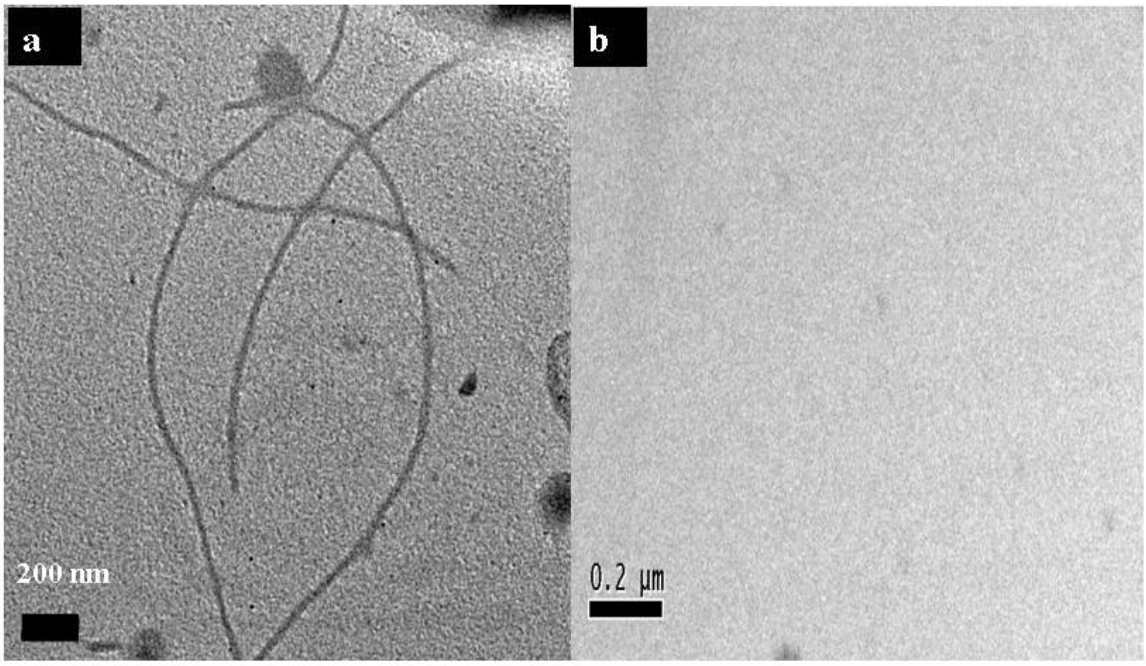

Fig. S1 TEM images of IGFBP-$2_{249\text{-}289}$ [Cys ^{281}] samples: (a) nanotube and (b) monomer (under reducing conditions)

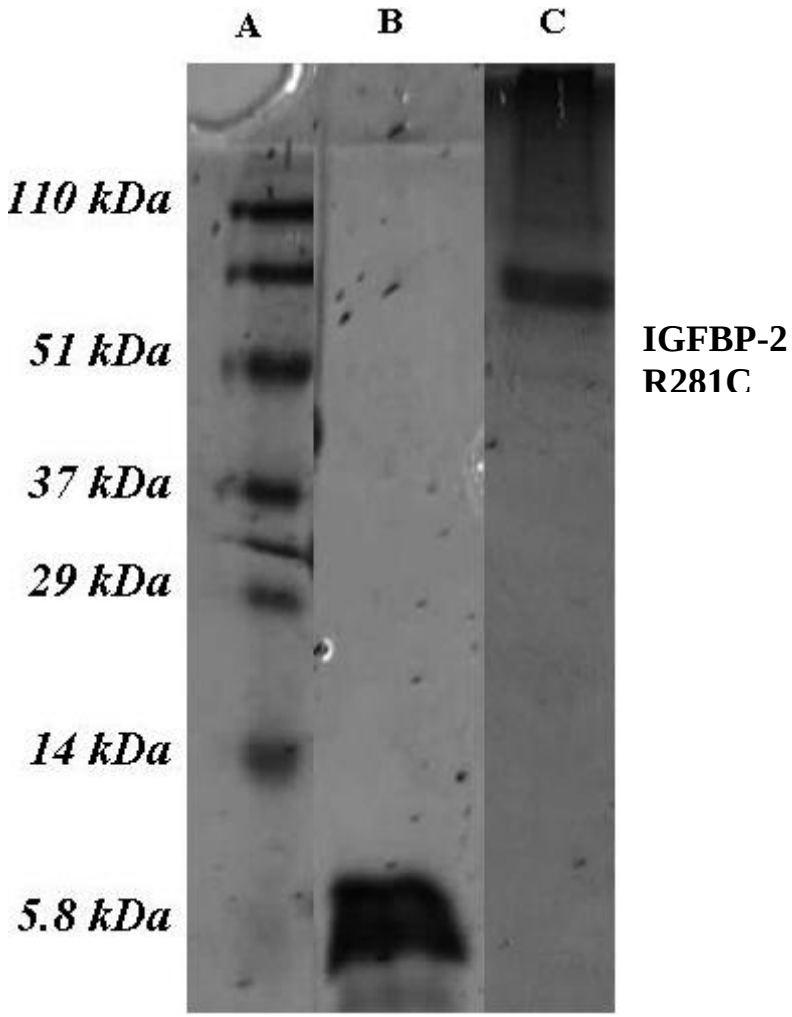

Fig. S2 SDS-PAGE: (a) molecular weight markers, (b) hIGFBP-$2_{249\text{-}189}$ [Cys^{281}] monomer in the presence of β-mercaptoethanol and (c) hIGFBP-$2_{249\text{-}189}$ [Cys^{281}] in absence of β-mercaptoethanol. The nanotube samples (shown in (c)) were pre-heated to 95^0C for 20 minutes before loading.

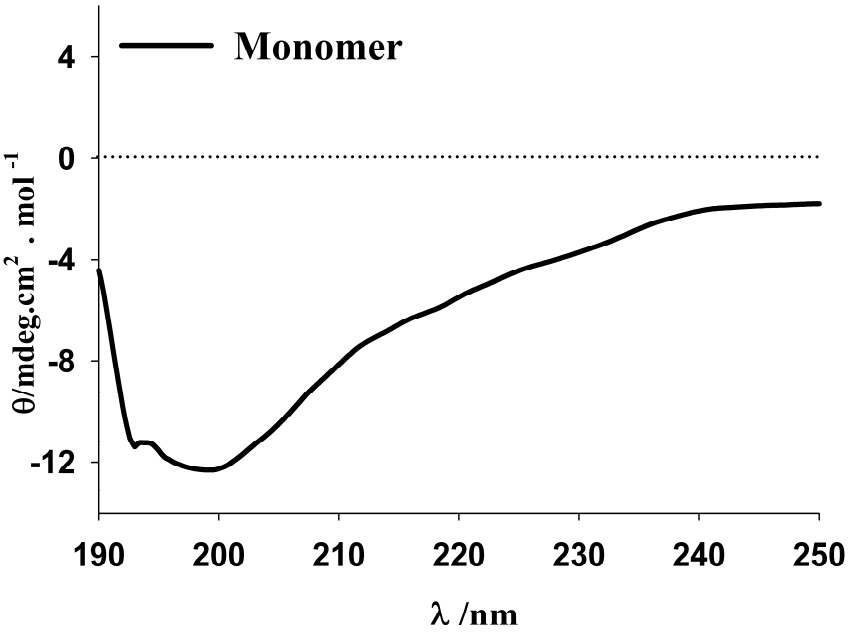

Fig. S3 CD spectrum of Monomer IGFBP-$2_{249\text{-}289}$

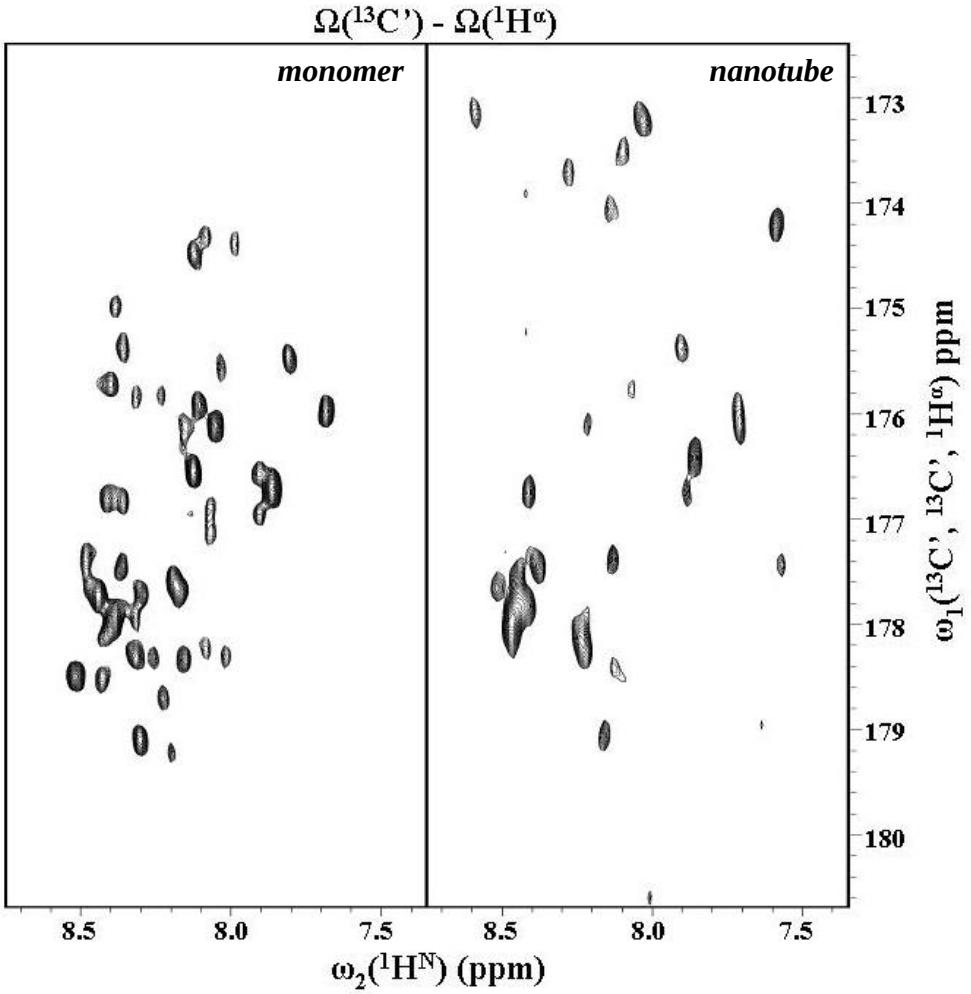

Fig. S4 GFT (3,2)D HA(CA)CO(N)H spectra[7] acquired for IGFGBP-$2_{249\text{-}289}$ [Cys^{281}] (a) monomer and (b) nanotube. This experiments helps in identifying the different secondary structural elements in a protein. The details of the experimental set up is described is Ref. 7. Briefly, peaks with chemical shifts in the range: 175-179 ppm corresponds to random coil, while those with shifts downfield or upfield of this range belong to residues in α-helical and β-strands, respectively. Thus, in (a), the monomer is seen to be consisting largely of random coil. In (b), peaks upfield of 175 ppm (i.e., < 175 ppm) could correspond to β-strands. However, their exact location in the protein could not be determined due to lack of complete resonance assignments arising from line broadening.

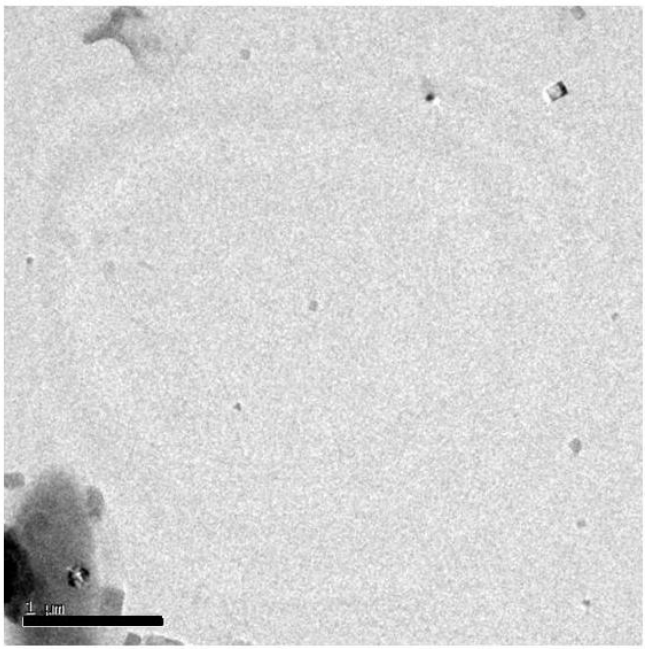

Fig. S5 TEM images of IGFBP-$2_{249\text{-}289}$ [Arg ^{281}] samples indicating absence of nanotubes. The removal of an extra cysteine at 281 by mutagenesis results in an even number of cysteines in the protein and nanotubes are not formed.

References

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