Supporting Information

Self-assembly and Higher Order Structure Forming Triple Helical Polyproline-II like Protein as a Novel Biomaterial for Cell Proliferation

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Materials

Proline auxotroph *Escherichia coli (E. coli)* JW0233-28468-K12 was received from CGSC the coli genetic stock center (CT, USA). *E. coli* BL21 (DE3) was purchased from Novagen, USA. CLTP synthetic gene in the pMK-RQ vector was purchased from Invitrogen and the pQE80-L vector was purchased from Qiagen (Valencia, USA). All restriction enzymes and T4 DNA ligase were purchased from NEB, all amino acids, M9 salts were purchased from sigma and *Trans*-4-Hydroxyl proline was purchased from TCI chemicals. IPTG, Ampicillin were purchased from Himedia.

Construction of Plasmids and strains

The CLTP-pMK-RQ vector was transformed and isolated from *E. coli* BL21 (DE3) and restriction digested with *BamHI* and *HindIII*. Double digested CLTP gene was ligated with double digested pQE80-L vector and transformed into *E. coli* BL21 (DE3). Then CLPT-pQE80L was isolated from *E. coli* BL21 and transformed into proline auxotroph *E. coli* JW0233. The transformed proline auxotroph containing CLPT-pQE80L was inoculated from a single colony into LB broth containing ampicillin antibiotic (100 μ g/ml) and protein expression was induced with IPTG at 0.6 OD and incubated at 37 °C for 6 hrs. The whole cell lysate was prepared from the cell pellet and protein expression was analyzed in SDS-PAGE. The cell pellet was lysed in cell lysis buffer (10 mM Tris, 1 mM EDTA, 0.1% SDS, Lysozyme (0.2 mg/ml)) and centrifuged. The CLTP proteins in insoluble fractions and soluble fractions were analyzed in SDS-PAGE.



Figure S1a. Protein expression studies of CLTP protein, Lane (1) marker; (2) *E.coli* proline auxotroph cell lysate (3) Uninduced proline auxotroph cell lysate containing pQE80-L-CLTP; (4) IPTG induced proline auxotroph cell lysate containing pQE80-L-CLTP.



Figure S1b. CLTP protein solubility analysis. Lane1-Marker; (2) *E. coli* proline auxotroph lysate, (3) whole cell lysate (clone1- contains pQE-80L CLTP induced), (4) whole cell lysate (colne2- contains pQE-80L CLTP induced), (5) Proline auxotroph host cell lysate insoluble fraction, (6) cell lysate insoluble fraction (clone 1), (7) Cell lysate insoluble fraction (Clone 2), (8) Proline auxotroph host cell lysate soluble fraction, (9) Cell lysate soluble fraction (Clone1), (10) Cell lysate soluble fraction (Clone2).

Growth curve



Figure S2. Growth curve analysis *of Escherichia coli* JW0233-28468 K12 (*E. coli*- Proline auxotroph) in minimal medium contains various concentrations of proline (34.7, 0.01, 0.02, 0.03, 0.04, 0.05 mM).

Optimization of residue specific incorporation of *Trans*-4 Hydroxyproline incorporation into CLTP



Figure S3. Residue specific incorporation of Hyp in CLTP was optimized in *E.coli* proline auxotroph containing pQE-80L-CLTP vector. The protein expression was induced by 1mM IPTG and 500mM of NaCl was used to facilitate the intracellular accumulation of Hyp. **A)** Lane1- Marker, Lane2- *E.coli* proline auxotroph (Negative control), Lane3- Induced CLTP in the presence of proline, Lane4- Uninduced in the presence of 10 mM of Hyp. Lane5, 6, 7, and 8 - CLTHP expression in the presence of 10 mM, 20 mM, 25 mM and 30 mM Hyp respectively. **B)** Lane1- Marker, Lane2- *E. coli* pQE-80L (negative control), Lane3- Uninduced CLTP in the presence of proline, Lane4- Induced CLTP in the presence of proline. Lane5, 7, and 9 – Uninduced CLTHP in the presence of 50 mM, 60 mM, and 80 mM of Hyp respectively. Lane6, 8, and 10 – Induced CLTHP in the presence of 50 mM, 60 mM, and 80 mM of Hyp respectively.

Protein Purifications



Figure S4. Lane 1- Hyp incorporated purified protein (CLTHP), 2- Marker, 3- Purified CLTP protein.

Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) analysis



Figure S5. Total mass spectra of CLTP and CLTHP protein in MALDI-TOF using linear mode. The observed mass of CLTP is 34.530 kDa and CLTHP is 35.053 kDa. The mass shift of the CLTHP proteins confirms the hydroxyproline incorporation.

Spectrometry Quantification of Hydroxyproline incorporation in CLTHP



A Standard graph for Quantification of Hydroxyproline

Figure S6. A) Standard graph for quantification of hydroxyproline.

Table S1. Quantification of hydroxyproline present in collagen.

	RTT	CLTHP	CLTP
Amount of Hydroxyproline present	0.37 µg/mg	0.54 μg/mg	0.00



Figure S7. CD spectra of CLTHP and CLTP proteins in 10mM phosphate buffer at 25 °C (A), CD spectra of CLTHP and CLTP in 10mM phosphate buffer at 10 °C (B), CD of CLTP and CLTHP at 220 nm during cololing (refolding) from 50 to 10 °C at the rate of 1 °C/min after temperature dependent denaturation experiment (C) and the corresponding CD spectra of CLTP and CLTHP after refolding (D).



Figure S8. A) Fibril froming growth curve measurment of RTT collagen at physiological pH 7.4 by taking OD at 313 nm. B) Size distribution analysis of RTT by Dynamic light scattering.

Fibril formation turbidity analysis



Figure S9. Turbidity analysis to monitoring the fibril-forming ability of CLTP (A and C) and CLTHP (C and D) at immediately after initiating firbillation (A and B) and after 20 hrs (C and D) at 30 °C.

AFM analysis of RTT, CLTHP and CLTP protein fibrils

Α





Figure S10. Fibril formation analysis of RTT, CLTP and CLTHP proteins by using atomic force microscope. A & B) RTT fibrils image, C & D) CLTP image, E & F) CLTHP fibrils image.

TEM analysis of RTT, CLTHP and CLTP protein fibrils



Figure S11. Fibril formation analysis of RTT, CLTP and CLTHP proteins by using transmission electron microscope. A, B & C) RTT Collagen fibrils. D, E & F) CLTHP protein fibrils. G, H & I) CLTP protein fibrils.

Collagan fibrils average diameter distribution



Figure S12. TEM image of fibrils obtained from RTT (A) and CLTHP (B) and the corresponding average diameter distribution histogram of RTT (a) and CLTHP (b).

Fluorescence microscopy analysis of cell binding



BSA

RTT





CLTHP

Figure S13. The 3T3 fibroblast cells bound on protein coated wells were incubated for 6 hrs and stained with Acridine orange and viewed under fluorescence microscope. 1- BSA coated well (Negative control), 2- Rat Tail tendon (RTT) collagen (Positive control) coated well, 3 - CLTP coated well, 4- CLTHP coated well.