Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Display of Functional Proteins on Supramolecular Peptide Nanofibrils Using a Split-Protein Strategy

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ELECTRONIC SUPPLEMENTARY INFORMATION

Contents:

Experimental Details

Peptide Synthesis

Ac-$(FKFE)_2$-NH_2, S15, FP1S15, GFP 11, and G11P1F were synthesized as *C*-terminal amides using Rink amide resin (Advanced ChemTech) using Fmoc synthesis on a microwave equipped CEM Liberty Peptide synthesizer utilizing DIC/HOBt activation. S15 was synthesized as the *C*-terminal acid using Wang resin (Advanced ChemTech) using Fmoc synthesis also on the CEM Liberty with DIC/HOBt activation. Peptides were acetylated (except S15) using 20% Ac_2O. Peptides were cleaved and deprotected from resin using standard cleavage conditions of trifluoroacetic acid, triisopropyl silane, and water (95 : 2.5 : 2.5). The collected supernatant was evaporated to roughly 0.5 mL and precipitated using cold (-78 ˚C) diethyl ether. The resulting solid was pelleted by centrifugation and the supernatant was decanted and the resulting peptide pellet was dissolved in DMSO and purified via preparatory HPLC. Peptides were characterized via MALDI-TOF-MS and purity was assessed by analytical HPLC.

Peptide Purification and Characterization

Purification of peptides was performed by reverse phase HPLC with a Shimadzu 6AD, equipped with a Phenomenex Gemini 10 micron C18 Axia column, 250 x 21.5 mm with a guard column. A gradient of water and acetonitrile (0.1% TFA) was used at a rate of 10 mL/min and eluent was monitored via UV at 215 and 254 nm. Collected fractions were assessed with analytical HPLC using a Shimadzu 2010A, equipped with a Phenomenex Gemini 5 micron C18 column, 250 x 4.6 mm with a guard column. Pure fractions were analyzed by MALDI-TOF-MS to determine correct products. Pure peptides were frozen and lyophilized to yield powders.

Cleavage of RNase A to yield S protein

A 2% solution of RNase A from bovine pancreas (Type 1-A, powder, ≥60% RNase A basis, Sigma-Aldrich) was prepared in 100 mM Tris (pH = 8). The solution was chilled to 0 ˚C and 40 μL a 1% solution of protease from *Bacillus licheniformis* (subtilisin) (Type VIII, lyophilized powder 7 units/mg solid, Sigma-Aldrich) in the same buffer was added dropwise. This was allowed to stir at 0 ˚C for 15 hours. The reaction was quenched with enough 3M HCl to bring the pH to 4. The resulting solution was immediately purified via RP HPLC.

Peptide Assembly and Concentration Determination

S15 concentration was determined using UV absorbance and the extinction coefficient 299 M^{-1} cm^{-1} at 280 nm. Concentration of S-pro was determined using UV absorbance and an extinction coefficient of 9055 M^{-1} cm^{-1} at 280 nm. Concentrations of synthesized peptides were determined by correlation to an HPLC concentration curve calibrated by amino acid analysis (AIBioTech, Richmond, VA) after being dissolved in a mixture of acetonitrile/water. Assembly of Ac-$(FKFE)_2$-NH_2 was induced via dissolution of lyophilized powders in unbuffered water (for use in CD or IR experiments) or 20 mM Bis TRIS with 1 mM EDTA (pH = 6.01) (for use in TEM and kinetics experiments). To coassemble FP1S15 with Ac-$(FKFE)_2$-NH_2, peptides were mixed in acetonitrile/water at varying mole percents of FP1S15 (0.1%, 0.5%, 1.0%, 2.5%, 5.0%, and 10%) relative to Ac-$(FKFE)_2$-NH_2 and then lyophilized to powders and then dissolved in either unbuffered water (for use in CD or IR experiments) or 20 mM Bis TRIS with 1 mM EDTA (pH = 6.01) (for use in TEM and kinetics experiments) to induce assembly. To coassemble G11P1F with Ac-$(FKFE)_2$-NH_2 peptides were mixed in acetonitrile/water at varying mole percent of G11P1F (5%, 10%, 15%, 20%, 25%, and 50%), lyophilized, and then dissolved in unbuffered water to assemble.

Preparation of S Protein from RNase A

A 2% solution of RNase A from bovine pancreas (Type 1-A, powder, ≥60% RNase A basis, Sigma-Aldrich) was prepared in 100 mM Tris (pH = 8). The solution was chilled to 0 ºC and 40 μL of a 1% solution of protease from *Bacillus licheniformis* (subtilisin) (Type VIII, lyophilized powder 7 units/mg solid, Sigma-Aldrich) in the same buffer was added dropwise. This was allowed to stir at 0 ºC for 15 hours. The reaction was quenched by addition of 3 M HCl to adjust the pH to 4. The resulting material was immediately purified via reverse phase HPLC to provide the S-protein.

Preparation of GFP 1-10

A pET-15b plasmid encoding for N-terminal histidine tagged GFP 1-10 was provided by Steven G. Boxer and coworkers from Stanford University.[1] GFP 1-10 was expressed in *E. coli* using a BL21(DE3) cell line and purified through a Ni-NTA affinity column (Fisher Scientific) by anion exchange chromatography using a BioRad DuoFlow FPLC system with UV/Vis detector and automated fraction collector. The purification was performed under denaturing conditions (8 M urea, 5 mM DTT) using a wash buffer containing 20 mM imidazole and an elution buffer containing 300 mM imidazole. Fractions containing purified protein were filtered using a Milipore Amicon Pro Affinity Concentrator (10 Kd cutoff) and stored in a 1.5 mL Eppendorf tube at -80°C until needed. The stock concentration was determined by absorbance at 280 nm using Beer-Lambert's law (where l = 1 cm and $\Delta\varepsilon_{280}$ GFP 1-10 = 17,545 cm^{-1} M^{-1} as calculated by ExPASy ProtParam tool). SDS-PAGE was used to confirm the expression of GFP 1-10 (26,486 g/mol) and run against a dual color precision plus protein standard (BioRad).

(1) Kent, K. P.; Childs, W.; Boxer, S. G. *J. Am. Chem. Soc.* **2008**, *130*, 9664.

Fourier Transform Infrared Spectroscopy

To remove residual TFA after HPLC purification, counterion exchange was performed by dissolving peptides in 40 : 90 : 1 mixture of acetonitrile : water : HCl solution, samples were frozen and then lyophilized. The resulting powders were twice dissolved in D_2O and relyophilized to remove any residual water. The dry powders were dissolved in D_2O and allowed to stand for 2 hours to ensure complete fibril formation. FTIR spectra were obtained with a Shimadzu 8400 Fourier transform-infrared spectrophotometer. The spectra were obtained with a 2 cm^{-1} resolution, Happ-Ganzel apodization, and 512 in a 0.1 mm pathlength CaF_2 cell.

Transmission Electron Microscopy

10 μL of sample was pipetted onto a 200 mesh carbon coated copper grid and allowed to stand for 1 minute. Residual solvent was wicked off via capillary action with filter paper and the grids were quickly washed for 1 second with unbuffered water, which was then wicked away by capillary action. Grids were then stained with 10 μL of uranyl acetate for 1 minute, which was wicked away with capillary action. Grids were then allowed to air dry for 5 minutes. Images were obtained on a Hitachi 7650 transmission electron microscope with an accelerating voltage of 80 kV.

Hydrolysis of cCMP by RNase S´

Kinetic data were measured by following the hydrolysis of cytidine 2´, 3´-cyclic monophosphate (cCMP, Sigma-Aldrich) on a Shimadzu UV-2401PC spectrometer. Fibril was mixed with cCMP and the appropriate amount of 20 mM Bis-Tris, 1 mM EDTA, thus [fibril] = 100 μM and [cCMP] = 1 mM, 2 mM, 3 mM, 4 mM or 5 mM. To this solution, S-Pro in the same buffer solution was added to give [S-Pro] = 1 μM. Immediately following addition of S-Pro, the

solution was transferred to a 1 mm quartz cuvette and UV absorption at 286 nm was recorded every 0.4 second for 10 minutes. Fibrils used were Ac-$(FKFE)_2$-NH_2, 0.1% FP1S15, 0.5% FP1S15, 1.0% FP1S15, 2.5% FP1S15, 5.0% FP1S15, and 10.0% FP1S15. Control experiments were also performed in which the hydrolysis of cCMP was observed with just Ac-$(FKFE)_2$-NH_2 and FP1S15 functionalized fibril in the absence of S-Pro. These data were not subtracted from the presented data because there was no observed hydrolysis.

Fluorescence Spectroscopy

Fluorescence intensity was monitored for solutions of G11P1F at varying mole percent (5%, 10%, 15%, 20%, 25%, and 50%) relative to Ac-$(FKFE)_2$-NH_2 using a Tecan Infinite M1000 plate reader. G11P1F/Ac-$(FKFE)_2$-NH_2 solutions (total [fibril] = 130 μM) were aliquoted from acetonitrile/water and frozen and lyophilized. The resulting powder was reconstituted in 37.5 μL of unbuffered water and allowed to assemble for 24-48 hours to ensure fibril formation. To this solution, 37.5 μL of naturing buffer was added (50 mM Tris·HCl, 100 mM NaCl, 10% glycerol) bringing total [fibril] = 65 μM followed by addition of 1.2 mole equivalent GFP 1-10 relative to G11P1F. Stocks of GFP 1-10 were stored in denaturing buffer (8 M urea and 5 mM DTT) after protein purification and stored at -80°C. This solution was allowed to thaw (on ice) and dilutions were made as necessary to ensure a 25 times volume dilution of denaturing buffer upon mixing with G11P1F solutions. The GFP 1-10 was then allowed to complex to G11P1F for 24 hours before measuring GFP fluorescence. A control was prepared using equimolar GFP 1-10 relative to the 50% G11P1F solution in 50% H_2O/naturing buffer containing no G11P1F. A second control was prepared using equimolar GFP 1-10 relative to the 50% G11P1F solution followed by addition of excess synthetic GFP 11 (not attached to Ac-$(FKFE)_2$-NH_2 fibirls). The solutions were prepared directly in a 96 well plate (Greiner, 96 well, flat bottom, black) and the

fluorescence measurements were taken using the following parameters: excitation = 400 nm, emission scan = 410-600 nm, bandwidth = 5 nm. Solutions were prepared the same way using 200 µL volumes directly into half dram glass vials for imaging purposes.

Immunogold Functionalization/Microscopy

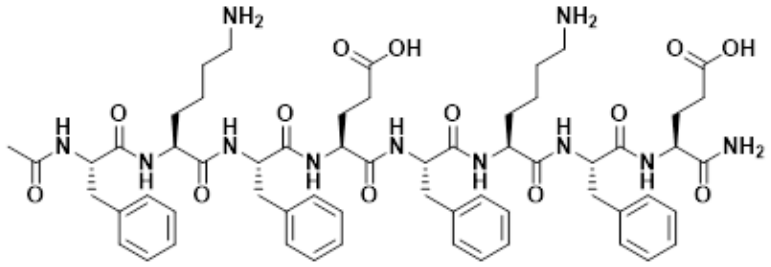
Rabbit anti-ribonuclease A antibody (Abcam, US) was functionalized using a Gold ABConjugation Kit (AppliChem, Germany). Briefly, 20 mL of "conjugation buffer" was placed in a 50 mL centrifuge tube. To this 2.5 mL of the gold nanoparticle (AuNP's) solution was added. 2 µL of the 0.2 mg mL^{-1} stock of IgG anti-RNase A antibody was diluted to 200 µL with conjugation buffer to make a solution with [antibody] = 20 µg/mL and this was added to the mixture of conjugation buffer and AuNP's. This solution was carefully mixed by inversion 3x and allowed to incubate at room temperature for 15 minutes. 1.25 mL of "blocking solution" was added and this was mixed and again allowed to incubate for 15 minutes. 250 µL of "stabilization solution" was added and mixed; this was allowed to incubate for 30 minutes at room temperature. To this, 375 µL of "neutralization solution" was added and mixed and the solution was allowed to incubate for 5 minutes. Finally, 0.75 mL of 30% glycerol in water was added and mixed into the solution. The solution was then centrifuged for 30 minutes, at 7000 g and 20 ˚C. The supernatant was carefully removed and the pellet was suspended in 1 mL of "storage buffer." Each of the previously mentioned buffers is included in the kit.

Goat anti-GFP antibody (Abcam, US) was functionalized with 40 nm gold particles using a Gold Conjugation Kit (Abcam, US). After the kit components warmed to room temperature, a 0.1 mg/mL antibody stock was prepared using the "gold antibody diluent". To a 1.5 mL Eppendorf tube 42 µL of "gold reaction buffer" was added followed by 12 µL of the antibody stock. The solution was mixed thoroughly by gentle inversion. Next, 45 µL of this mixture was

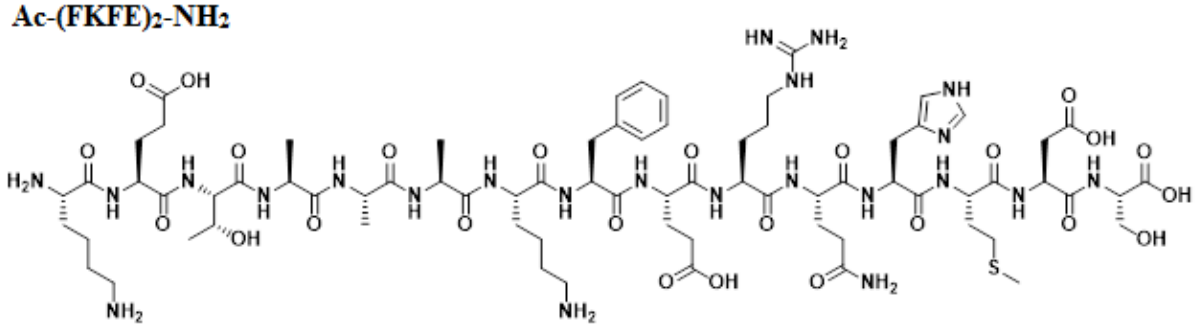
added to one vial of freeze dried gold and the powder was reconstituted by pipetting up and down. This mixture sat at room temperature for 10 minutes. Finally, 5 μL of "EL gold quencher" was added to the solution and mixed gently by pipetting up and down to provide 50 μL of 20 OD AuNP-antibody conjugate.

To attain micrographs of fibrils treated the antibody functionalized gold nanoparticles (AB-AuNP's), 30 μL solutions of 200 μM fibril with 20 μM S-Pro were prepared. The fibrils were allowed to grow for 1 day to ensure mature fibril growth, and the S-Pro was allowed to complex for 30 minutes. 10 μL was then taken and spotted on a TEM grid as described in the previous section. To the remaining 20 μL, 1 μL of AB-AuNP's were added carefully mixed via pipette. This solution was allowed to stand for 20 minutes, and then spotted as previously described. 100 μM Solutions of G11P1F/Ac-$(FKFE)_2$-NH_2 were assembled for 1-2 days to ensure mature fibril growth followed by GFP 1-10 complexation for 1 day. 1 μL of the GFP AB-AuNP stock was added and TEM spotting proceeded as previously described.

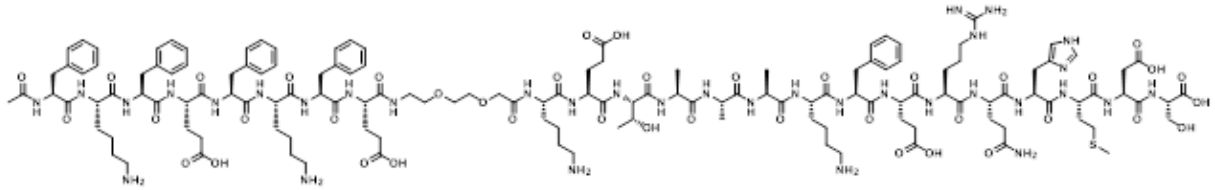
Figure S1 Primary Structure of Peptides synthesized via SPPS.



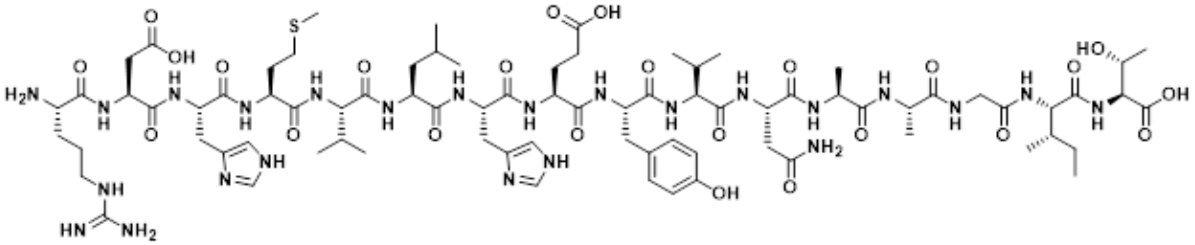
Ac-(FKFE)$_2$-NH$_2$



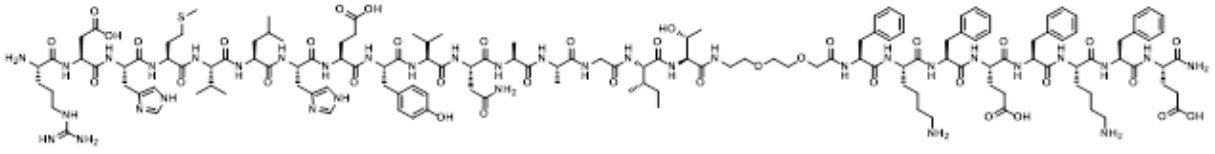
S15



FP1S15



GFP 11



G11P1F

Table S1 Analytical HPLC purification conditions.

Peptide	**R_t (min)**	**Gradient** (solution **A**: water/0.5% TFA; solution **B**: acetonitrile/0.5% TFA)
Ac-$(FKFE)_2$-NH_2	12.076	Isocratic 5% **B** 5 min, 5–95% **B** over 10 min, 95% **B** 5 min
S15	10.632	Isocratic 5% **B** 5 min, 5–95% **B** over 10 min, 95% **B** 5 min
FP1S15	12.100	Isocratic 5% **B** 5 min, 5–95% **B** over 10 min, 95% **B** 5 min
S-protein	11.521	Isocratic 5% **B** 5 min, 5–95% **B** over 10 min, 95% **B** 5 min
GFP 11	11.113	Isocratic 5% **B** 5 min, 5–95% **B** over 10 min, 95% **B** 5 min
G11P1F	11.725	Isocratic 5% **B** 5 min, 5–95% **B** over 10 min, 95% **B** 5 min

Figure S2 Analytical HPLC trace (215 nm) of Ac-$(FKFE)_2$-NH_2

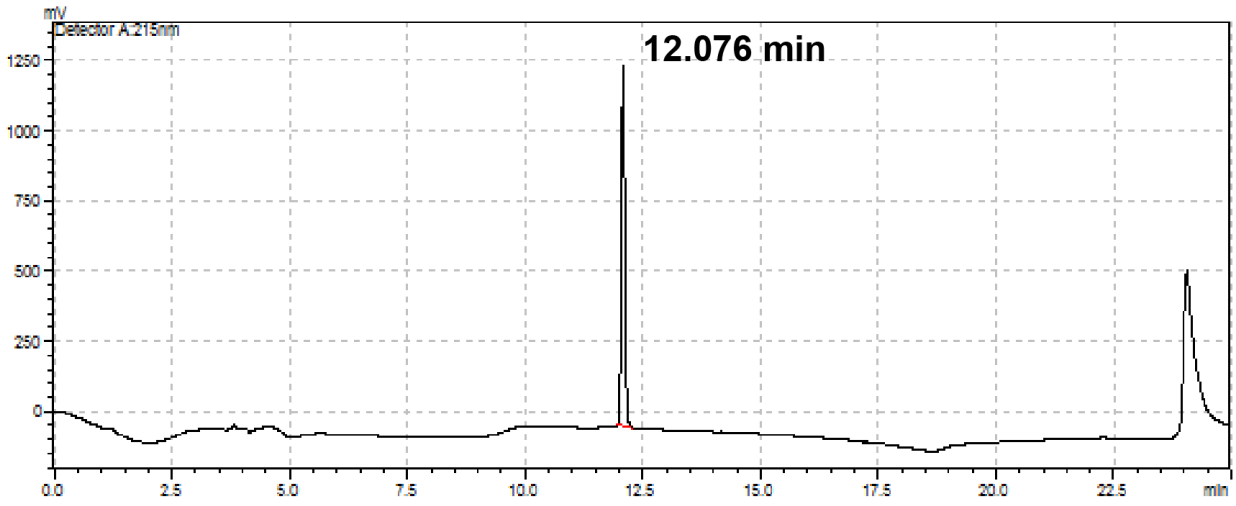


Figure S3 Analytical HPLC trace (215 nm) of S15

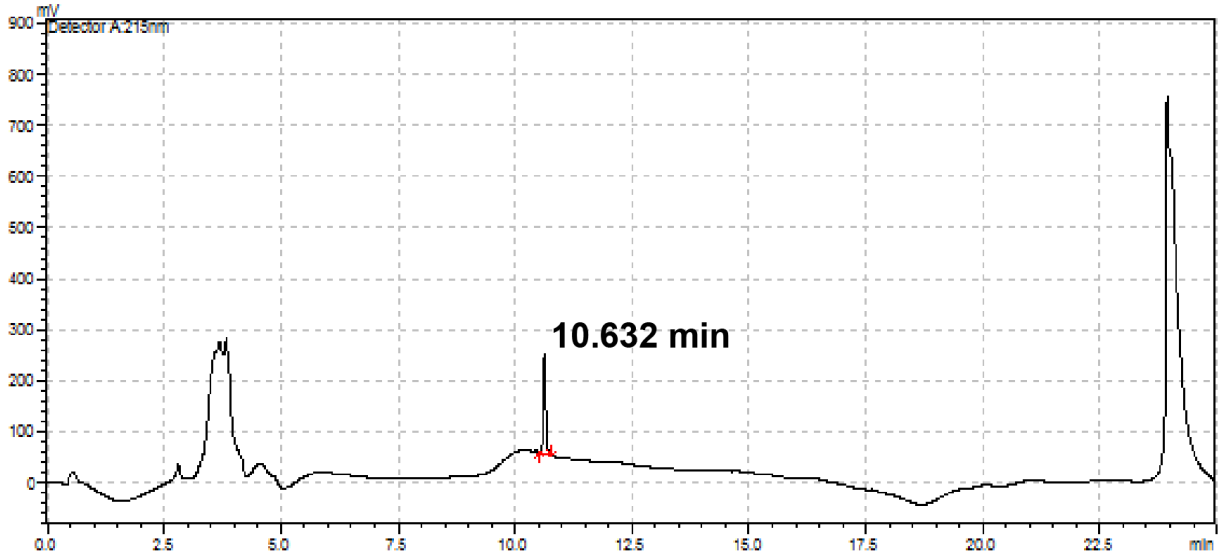


Figure S4 Analytical HPLC trace (215 nm) of FP1S15

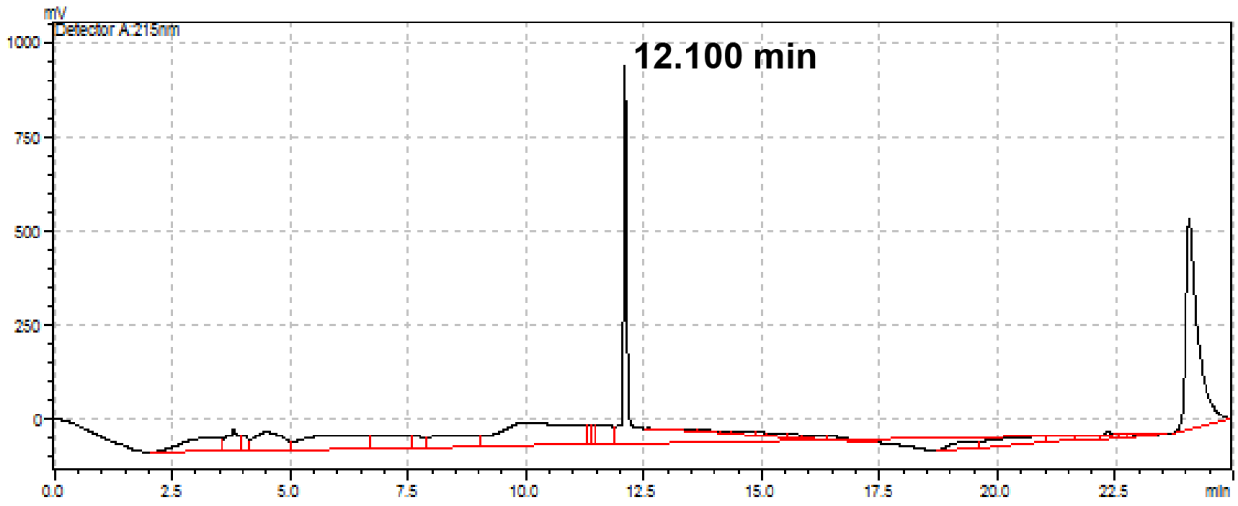


Figure S5 Analytical HPLC trace (215 nm) of S-Protein

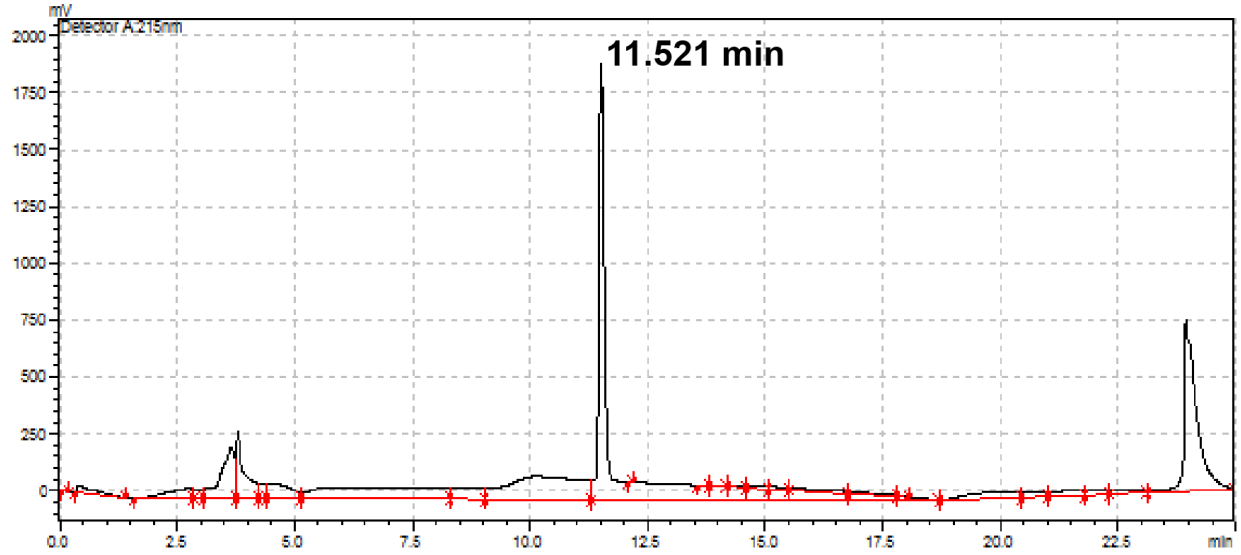


Figure S6 Analytical HPLC trace (215 nm) of GPF 11

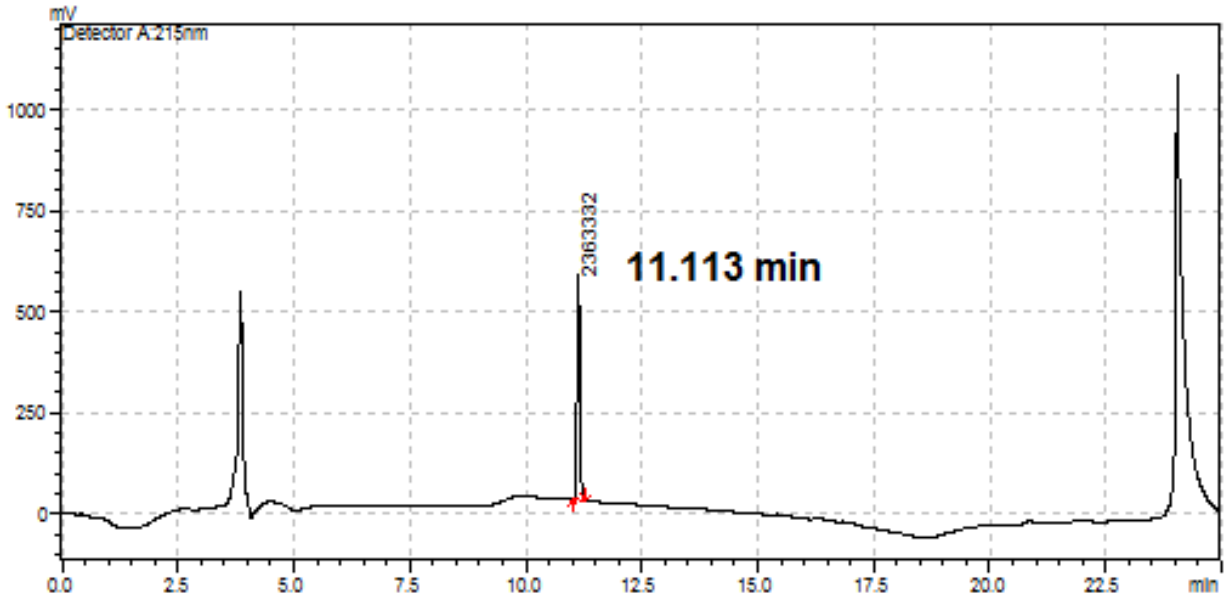


Figure S7 Analytical HPLC trace (215 nm) of G11P1F

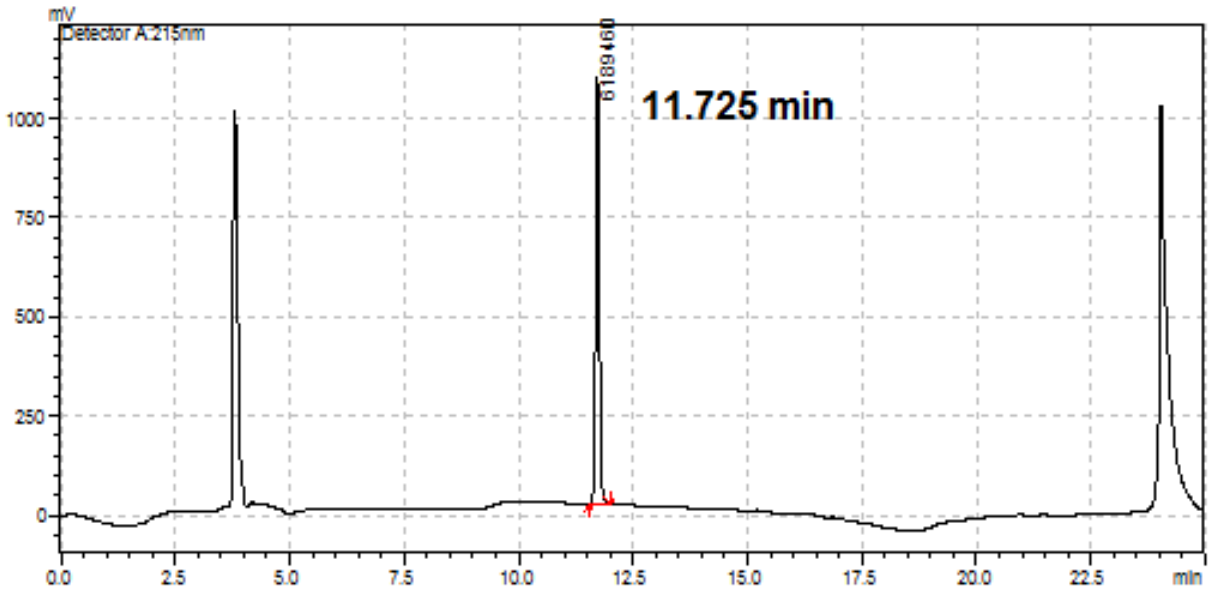


Table S2 Calculated and observed *m/z* for all peptides by MALDI-TOF-MS

Peptide	**Calc [MH^+]**	**Obs [MH^+]**	**Calc [MNa^+]**	**Obs [MNa^+]**
Ac-$(FKFE)_2$-NH_2	1162.59	1162.981	1184.58	1184.966
S15	1748.83	1747.84	1770.82	1769.911
FP1S15	3037.46	3040.968	3060.45	--
S-protein	11535.32	11527.436	11557.31	--
GFP 11	1825.89	1826.269	1847.88	--
G11P1F	3072.53	3072.248	3094.52	3092.349

Figure S8 MALDI-TOF-MS spectrum of Ac-$(FKFE)_2$-NH_2

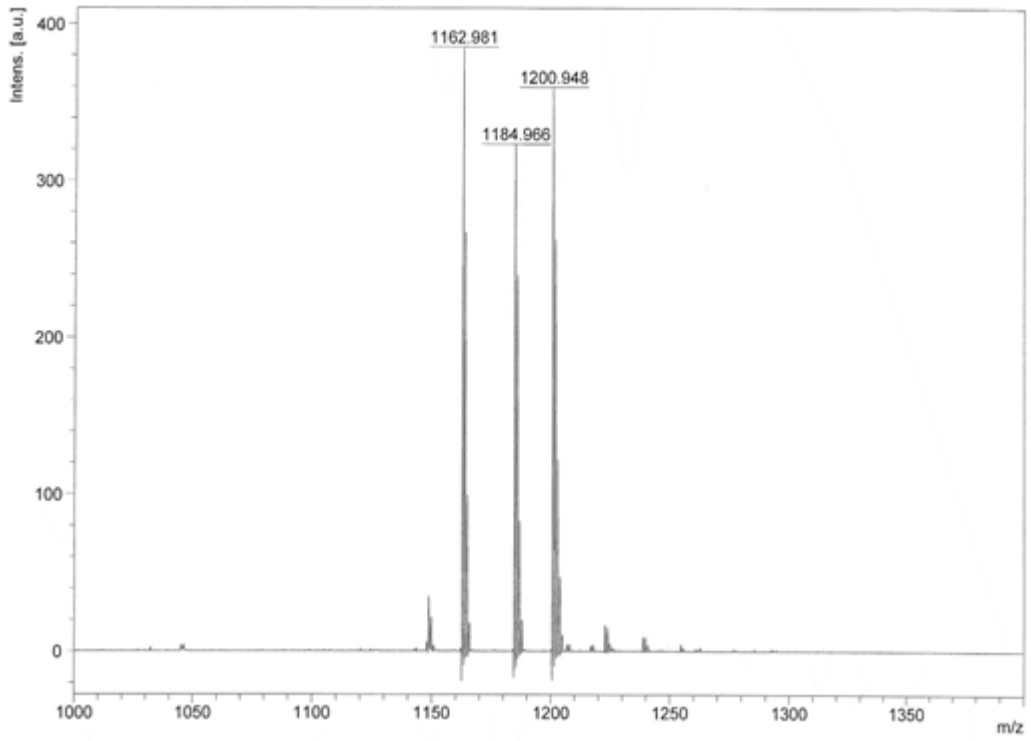


Figure S9 MALDI-TOF-MS spectrum of S15

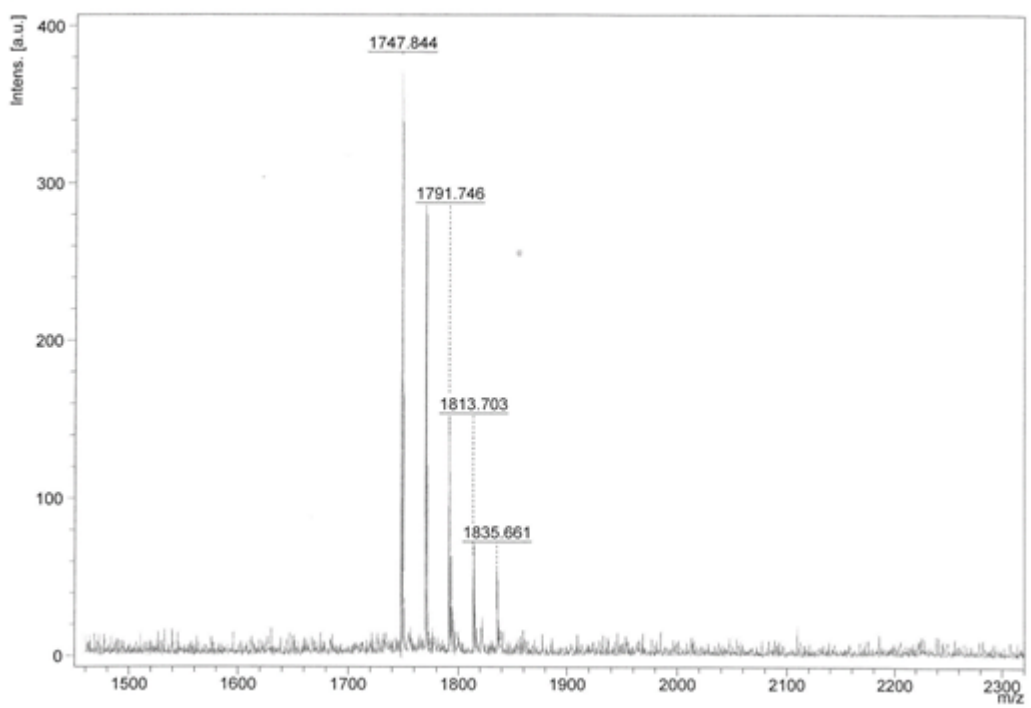


Figure S10 MALDI-TOF-MS spectrum of FP1S15

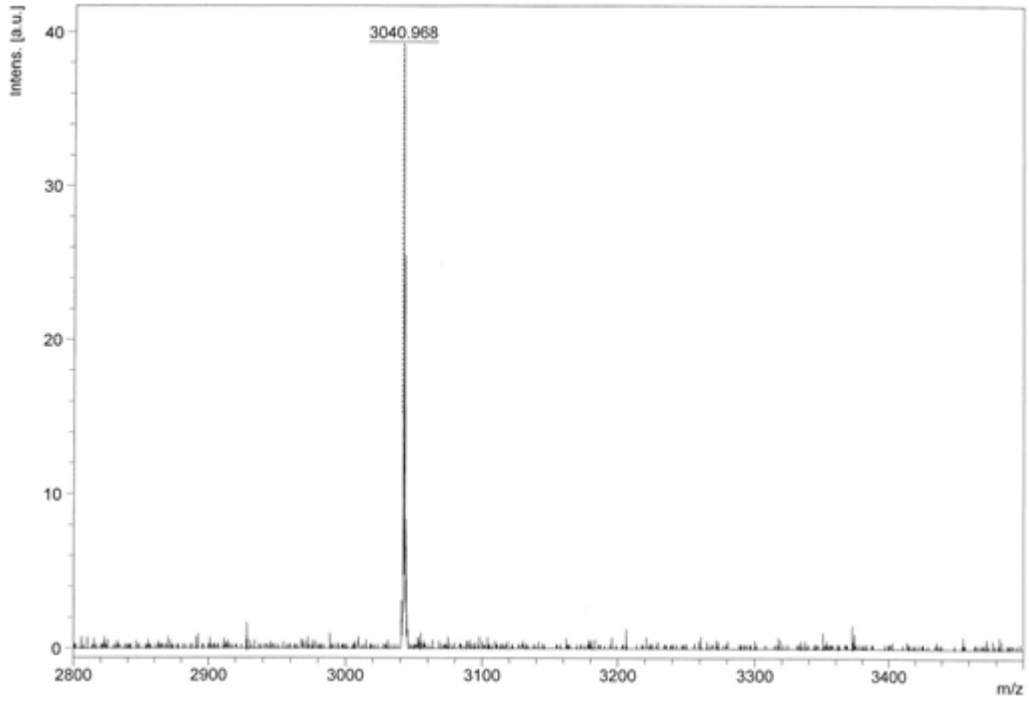


Figure S11 MALDI-TOF-MS spectrum of S-Protein

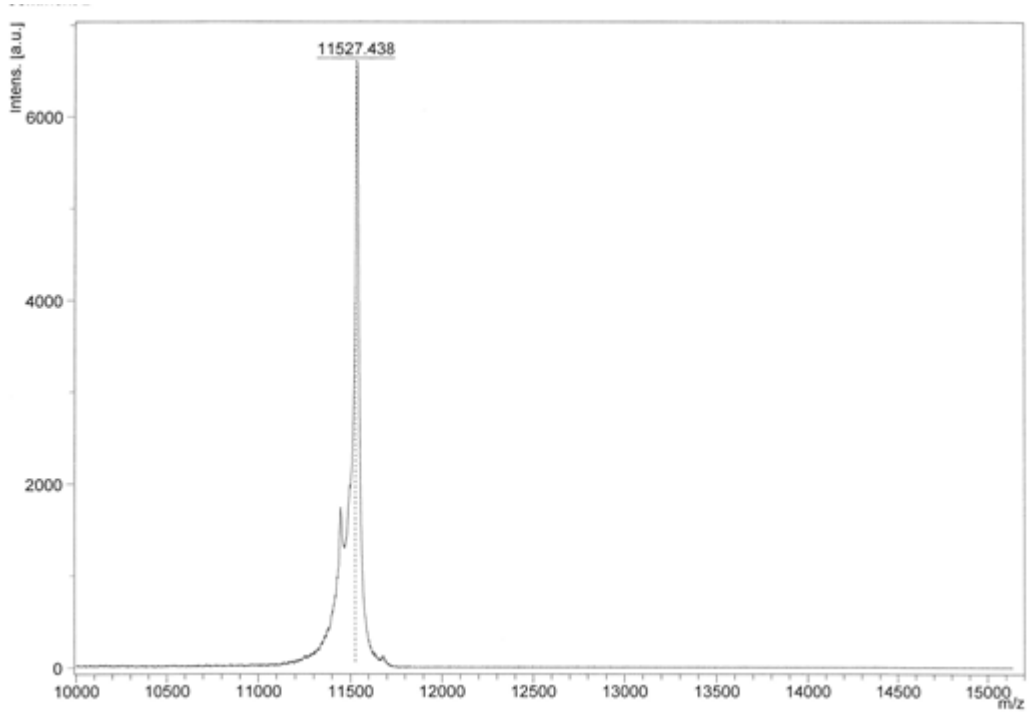


Figure S12 MALDI-TOF-MS spectrum of GFP 11

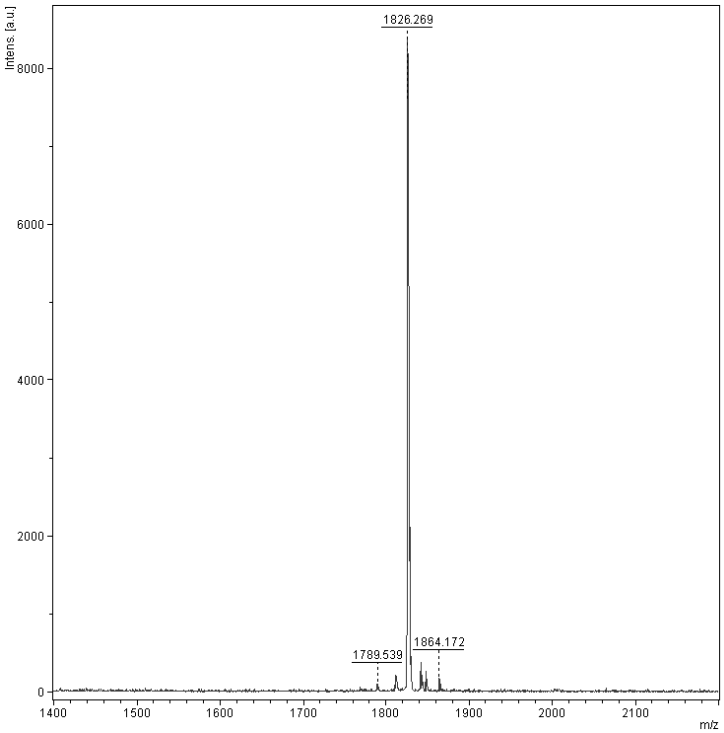
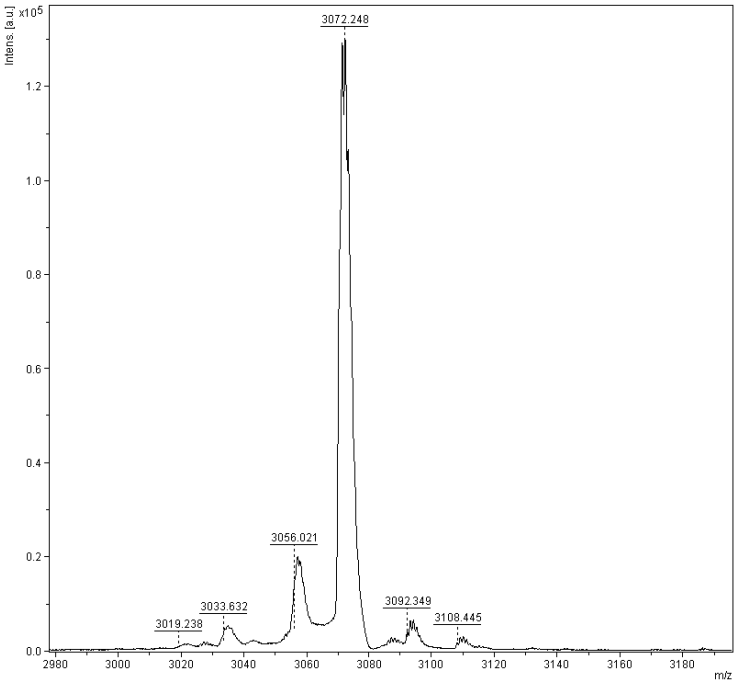


Figure S13 MALDI-TOF-MS spectrum of G11P1F



Protein Sequences

S-Protein:
SSSNYCNQMMKSRNLTKDRCKPVNTFVHESLADVQAVCSQKNVACKNGQTNCYQSYS
TMSITDCRETGSSKYPNCAYKTTQANKHIIVACEGNPYVPVHFDASV

GFP 1-10:
GSSHHHHHHSSGLVPRGSHMGGTSSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEG
DATIGKLTLKFICTTGKLPVPWPTLVTTLSYGVQCFSRYPDHMKRHDFFKSAMPEGYVQ
ERTISFKDDGKYKTRAVVKFEGDTLVNRIELKGTDFKEDGNILGHKLEYNFNSHNVYIT
ADKQKNGIKANFTVRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQTVLSKDP
NEKGT

Figure S14 SDS-PAGE to confirm expression of GFP 1-10 with mass of 26,486 g/mol

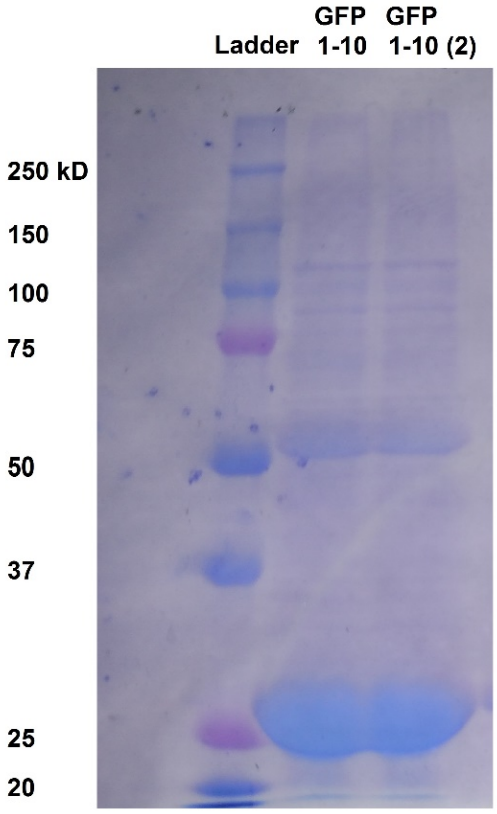


Figure S15 IR data of FP1S15/Ac-(FKFE)$_2$-NH$_2$ cofibrils where [peptide] = 500 µM. (**A**) Ac-(FKFE)$_2$-NH$_2$ (**B**) 0.1% FP1S15 (**C**) 0.5% FP1S15 (**D**) 1.0% FP1S15 (**E**) 2.5% FP1S15 (**F**) 5.0% FP1S15 (**G**) 10.0% FP1S15

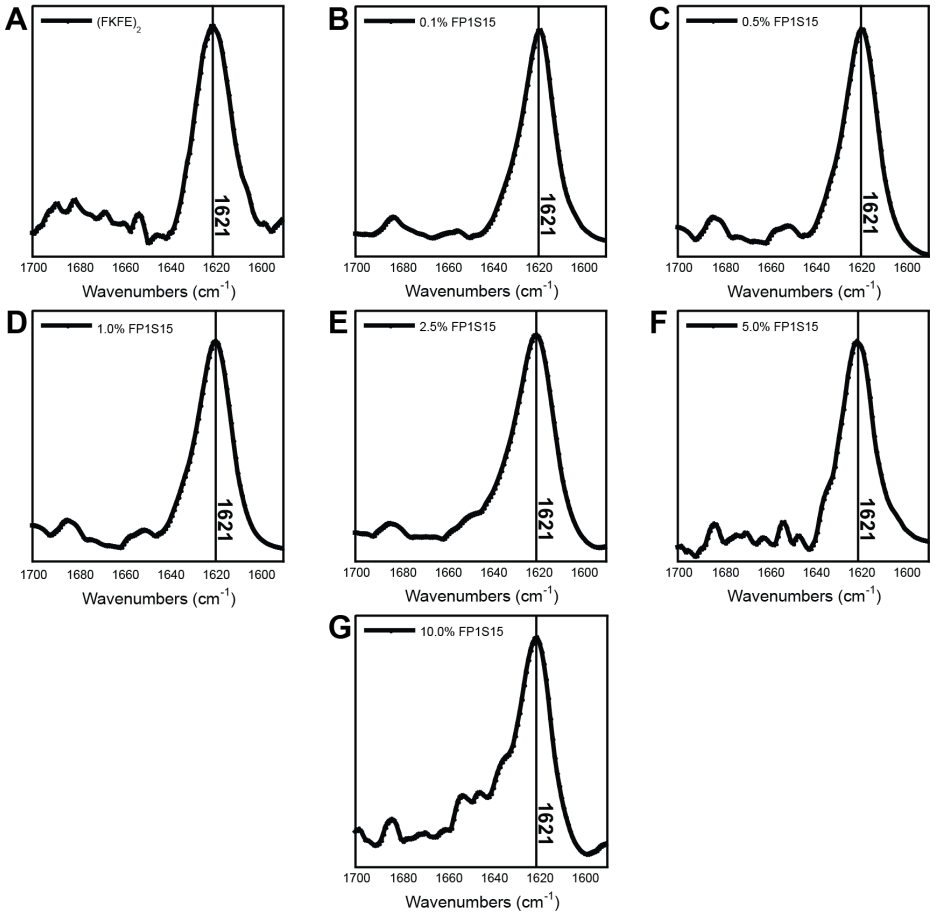


Figure S16 TEM images of fibrils. [total peptide] = 250 µM. (A) Ac-$(FKFE)_2$-NH_2 **(B)** 0.1% FP1S15 **(C)** 0.5% FP1S15 **(D)** 1.0% FP1S15 **(E)** 2.5% FP1S15 **(F)** 5.0% FP1S15 **(G)** 10.0% FP1S15

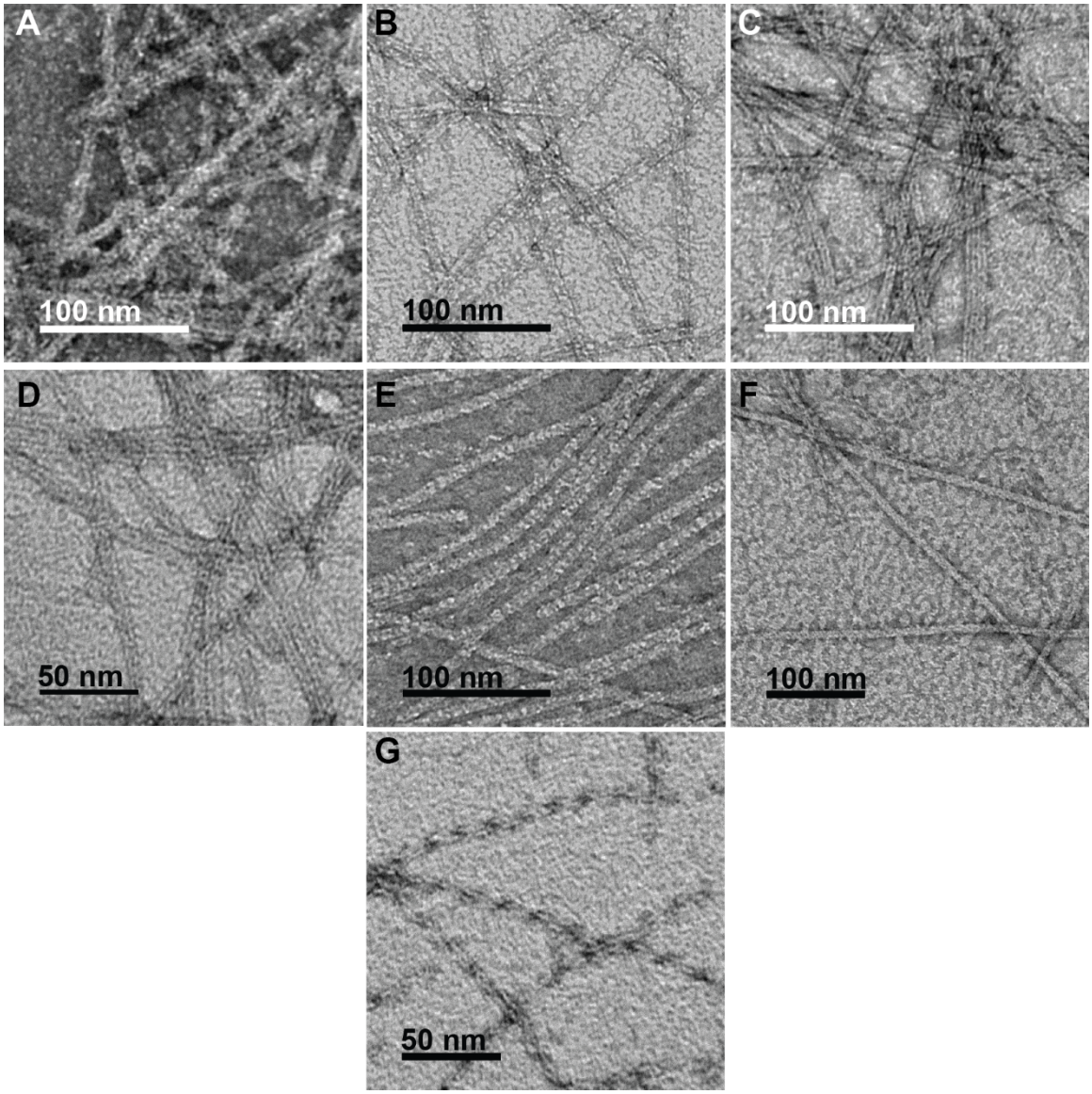


Figure S17 TEM micrographs of fibrils treated with S-protein. [Total peptide] = 200 µM, [S-Pro] = 20 µM (A) Ac-$(FKFE)_2$-NH_2 (**B**) 0.1% FP1S15 (**C**) 0.5% FP1S15 (**D**) 1.0% FP1S15 (**E**) 2.5% FP1S15 (**F**) 5.0% FP1S15 (**G**) 10.0% FP1S15

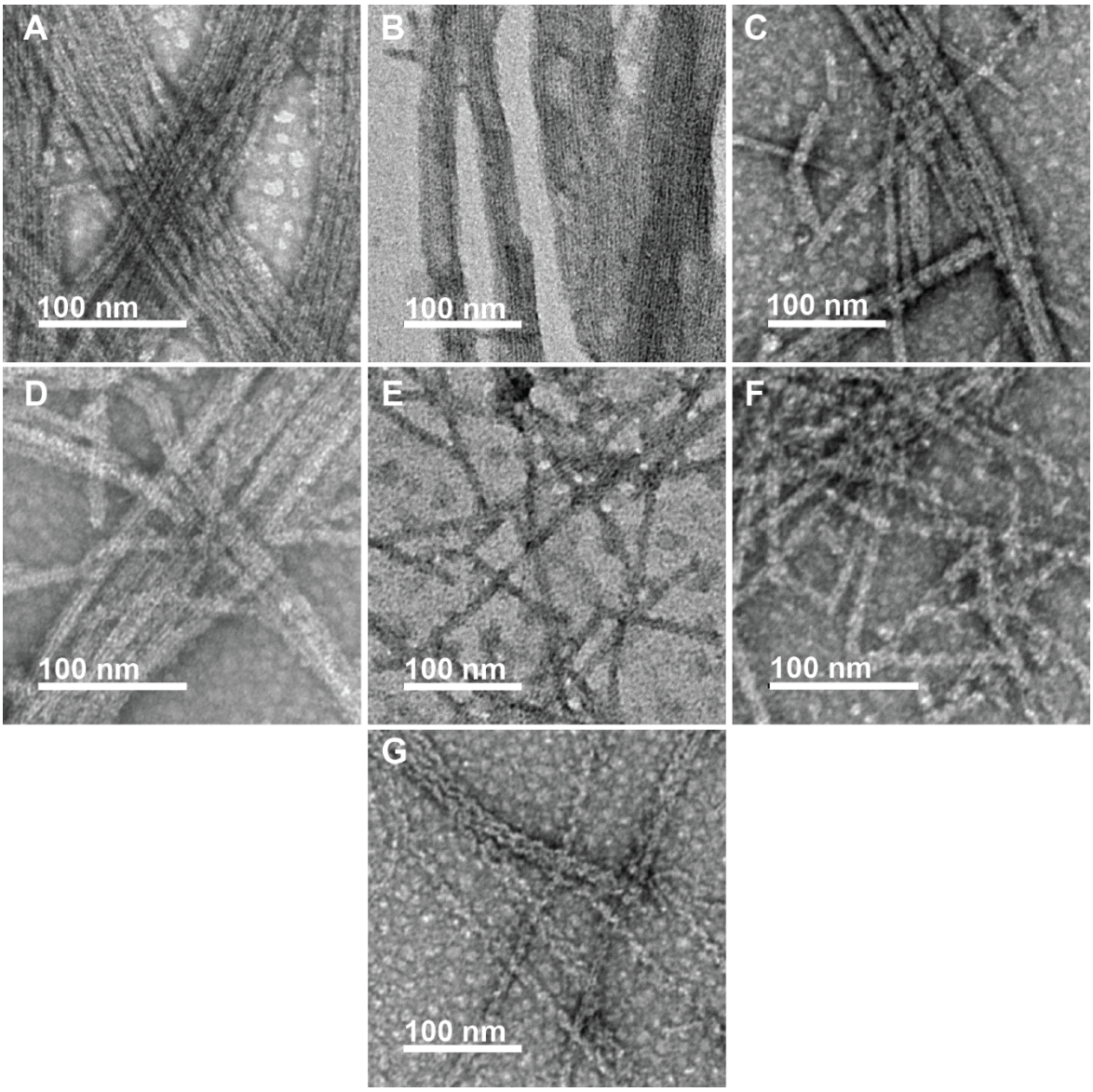


Figure S18 Kinetics of hydrolysis of 1 mM cCMP. Total [fibril] = 100 μM, [S-protein] = 1 μM, [RNase S´] = 1 μM

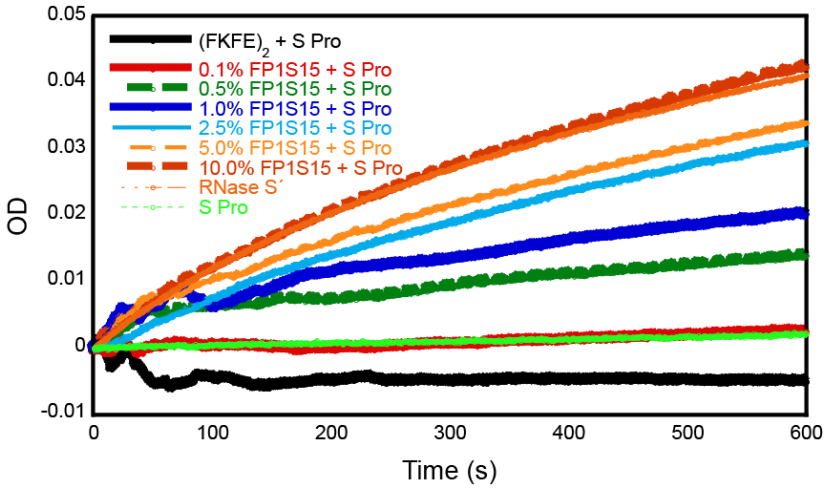


Figure S19 Kinetics of hydrolysis of 2 mM cCMP. Total [fibril] = 100 μM, [S-protein] = 1 μM, [RNase S´] = 1 μM

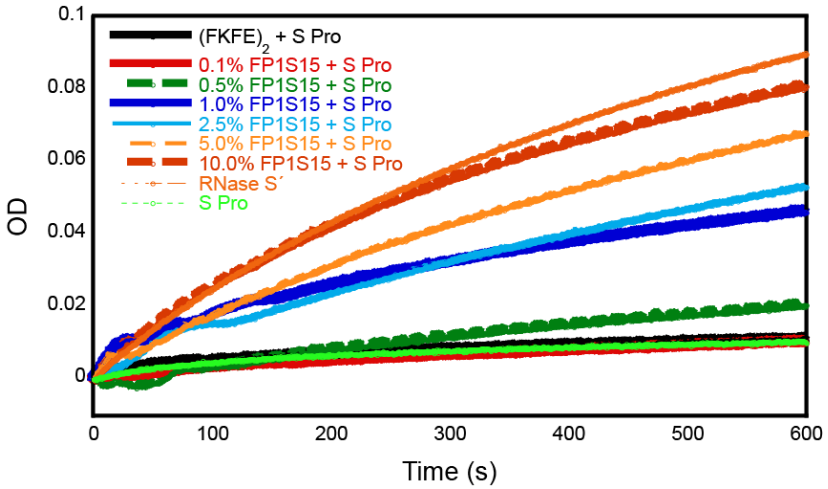


Figure S20 Kinetics of hydrolysis of 3 mM cCMP. Total [fibril] = 100 μM, [S-protein] = 1 μM, [RNase S´] = 1 μM

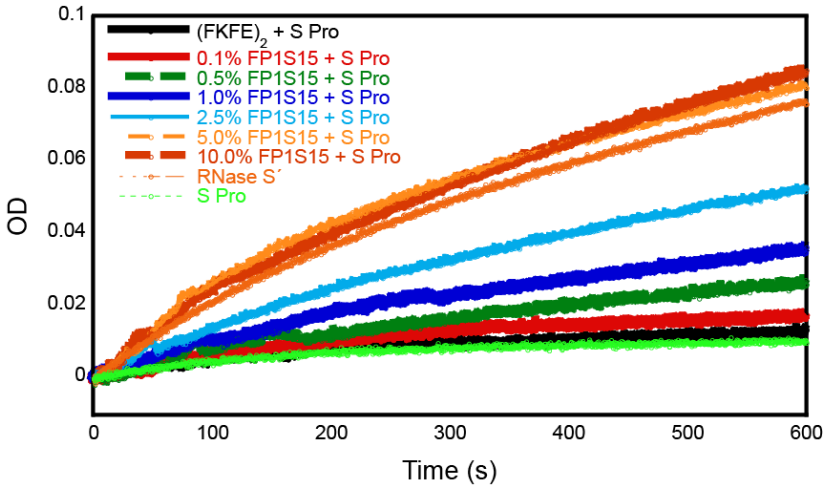


Figure S21 Kinetics of hydrolysis of 4 mM cCMP. Total [fibril] = 100 μM, [S-protein] = 1 μM, [RNase S´] = 1 μM

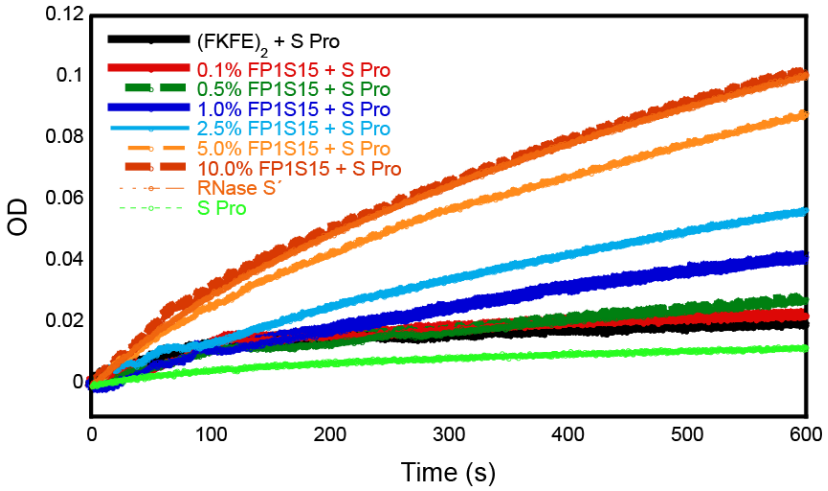


Figure S22 Kinetics of hydrolysis of 5 mM cCMP. Total [fibril] = 100 μM, [S-protein] = 1 μM, [RNase S´] = 1 μM

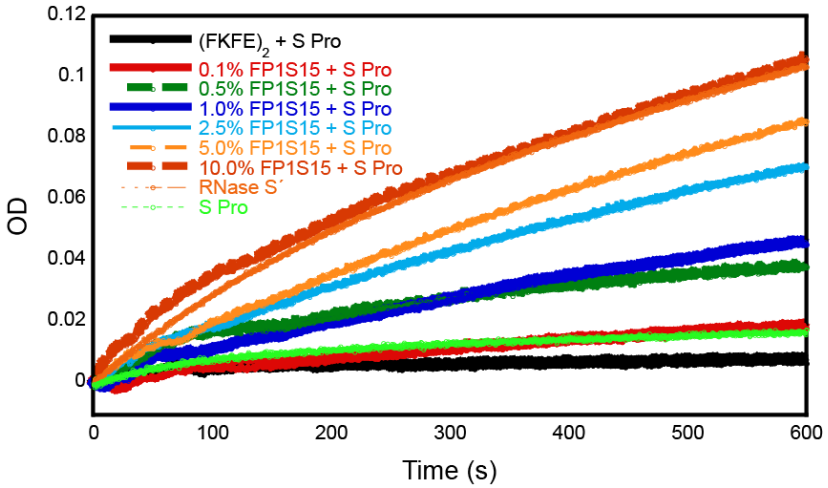


Figure S23 Kinetics of hydrolysis of cCMP by 1 μM RNase S′

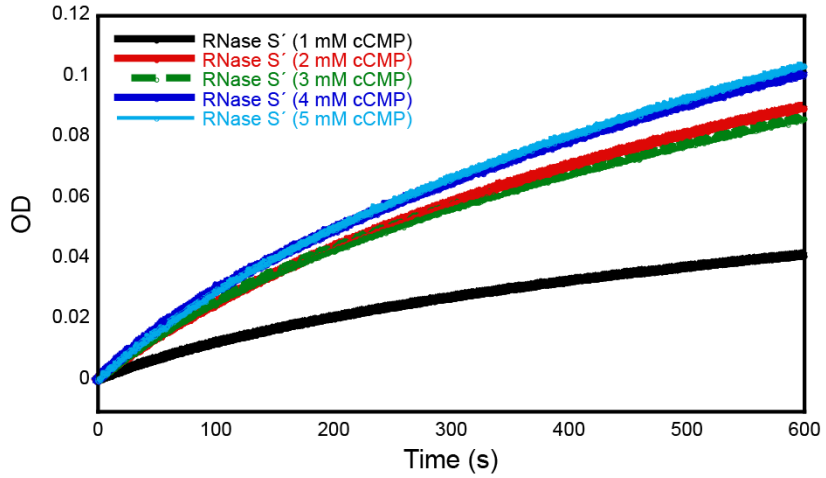


Figure S24 TEM micrographs of fibrils with S-protein and AB-AuNP. [Total peptide] = 200 µM, [S-Pro] = 20 µM, AB-AuNP = 1 µL (A) Ac-$(FKFE)_2$-NH_2 (**B**) 0.1% FP1S15 (**C**) 0.5% FP1S15 (**D**) 1.0% FP1S15 (**E**) 2.5% FP1S15 (**F**) 5.0% FP1S15 (**G**) 10.0% FP1S15

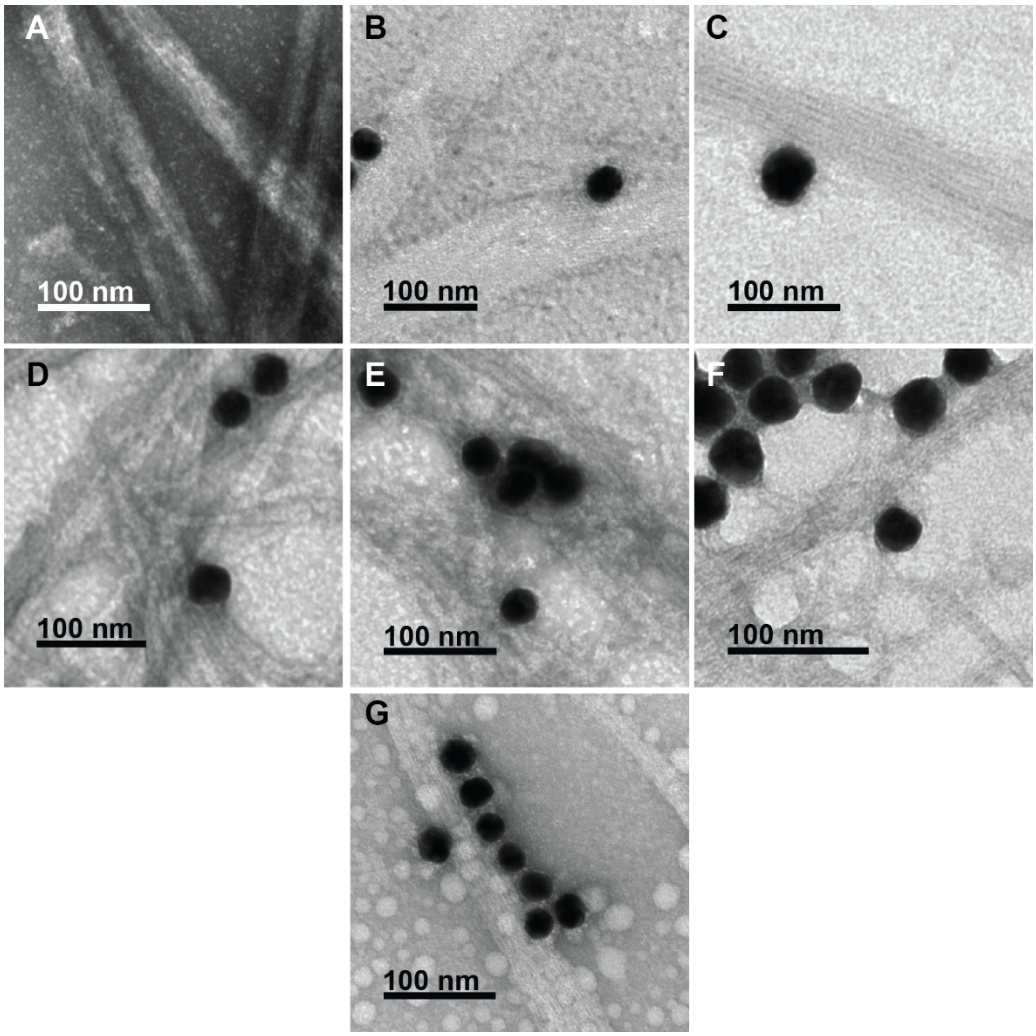


Figure S25 Representative TEM micrographs of G11P1F fibrils treated with GFP 1-10. [Total peptide] = 65 µM (A) 15.0% G11P1F (**B**) 25.0% G11P1F

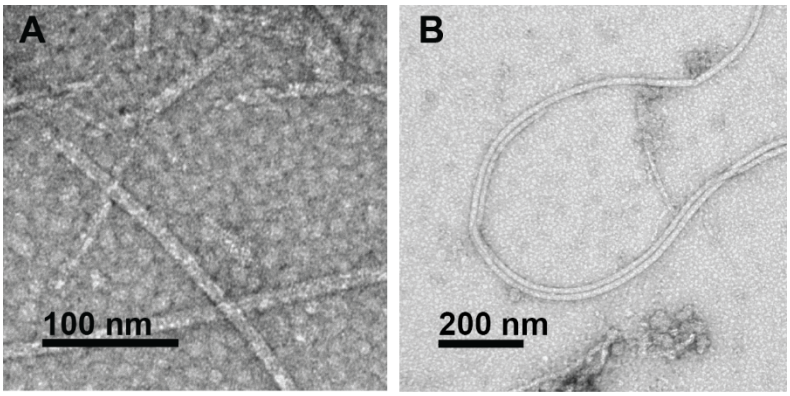


Figure S26 TEM micrographs of fibrils with GFP 1-10 and AB-AuNP. [Total peptide] = 65 µM, AB-AuNP = 1 µL (A) Ac-$(FKFE)_2$-NH_2 (**B**) 20.0% G11P1F (**C**) 25.0% G11P1F