Electronic Supplementary Material (ESI) for Chemical Communications
This journal is © The Royal Society of Chemistry 2013

Supporting Information

Self-Assembly of Ferritin Nanocages into Linear Chains Induced by Poly(α, L-Lysine)

Rui Yang, Lingli Chen, Tuo Zhang, Senpei Yang, Xiaojing Leng, and Guanghua Zhao *

CAU & ACC Joint-Laboratory of Space Food, College of Food Science & Nutritional Engineering, China Agricultural University, Beijing Key Laboratory of Functional Food from Plant Resources, Beijing, 100083, China

E-mail: gzhao@cau.edu.cn

1. Sample Preparation.

Recombinant soybean seed H-1 ferritin (rH-1) and H-2 ferritin (rH-2) were cloned, expressed, and purified as previously described.[1,2] Recombinant heteropolymer rH-1H-2 was constructed by mixing dissociated H-1 and H-2 subunits in a 1:1 ratio and reassembled by slowly changing the pH to 7.0 according to reported methods.[3,4] The cDNA sequences of the H-1ΔE subunit and human H-chain with E-helix deletion (HuHΔE) were subjected to PCR with primers, N-term (5'-GGAATTCCATATGGCCTCAACGGTGCCTCTC-3') and C-term (5'-CGCGGATCCTCACCTTCTCAACTGAGCC-3') for H-1ΔE, N-term (5'-GGAATTCCATATGACGACCGCGTCCACCTCG-3') and C-term (5'-CGCGGATCCTTATCCCATCTTGCGCAAGTTG-3') for HuHΔE, respectively. Both of the amplified products were cleaved using *Nde I* and *BamH I*, and the resultant fragments were ligated into the *Nde I* and *BamH I* restriction sites of pET 3a vector (Novagen) to generate H-1ΔE and HuHΔE constructs, respectively. These two constructs were then, respectively, introduced into *E. coli* strain DH5α, cultivated for amplification, and the plasmids were extracted using SanPrep Column DNA Gel Extraction Kit (Sangon Biotech). These two extracted plasmids were then, respectively, introduced into *E. coli* strain BL21 (DE3), and the cultivation followed the same condition of the preparation of rH-2. The resulting inclusion bodies of the expressed rH-1ΔE

were denatured, refolded, and purified according to a reported method.[5] The recombinant human H-chain ferritin (rHuHF) and recombinant human H-chain ferritin with E-helix deletion (rHuHF∆E) were expressed and purified according to the reported method.[6] Reconstructed heteropolymeric rmSSF was prepared by mixing dissociated rH-2 and rH-1∆E subunits in a 1:1 ratio at pH 11.4, and the resulting solution was dialyzed against 50 mM 3-(N-morpholino) propanesulfonic acid (Mops) at pH 7.0, 4 °C in a dialysis bag (12-14 kDa MWCO). Reconstructed heteropolymeric rHuHF-HuHF∆E was conducted by mixing dissociated rHuHF and rHuHF∆E subunits in a 1:1 ratio at pH 11.4, and the resulting solution was dialyzed against 50 mM Mops at pH 7.0 at 4 °C in a dialysis bag (12-14 kDa MWCO).

cDNA cloning of *adzuki* bean seeds ferritin (ASF) was performed according to our reported method,[7] followed by subjection to PCR with primers N-term (5'-CCCATATGCGGAGTTGACGCGGAGA-3') and C-term (5'-CGGGATCCTCAAGCAGCATGTCCATC-3'). The following procedures including the ASF vector construction, the introduction into *E. coli* strain DH5α and BL21 (DE3), cultivation, and the denaturation and refolding of the resulting rASF inclusion bodies were conducted according to the method as rH-1∆E preparation. Reconstructed heteropolymeric rASF-H-1∆E was conducted by mixing dissociated rASF and rH-1∆E subunits in a 1:1 ratio at pH 11.4, and the resulting solution was dialyzed against 50 mM Mops at pH 7.0 at 4 °C in a dialysis bag (12-14 kDa MWCO).

The specific site-directed mutation was designed for substitution of the rH-2 His193 and His197 located at the E-helix of rH-2 with Glu (rH-2_H193E/H197E). Mutagenesis of the H-2 cDNA was performed with the description of the Fast mutagenesis system kit (TransGen Biotech, Beijing, China). The mutated H-2_H193E/H197E cDNA was then successively introduced into *E. coli* strain DH5α and BL21 (DE3), and the cultivation, expression and purification followed the same conditions of the preparation of rH-2. rmSSF_H193E/H197E was prepared by mixing dissociated rH-2_H193E/H197E and rH-1∆E subunits in a 1:1 ratio at pH 11.4, and the resulting solution was dialyzed against 50 mM Mops at pH 7.0 at 4 °C in a dialysis bag (12-14 kDa MWCO).

The molecular weights of all ferritin samples were estimated by native-PAGE using a 4–15% polyacrylamide gradient gel run at 5 mA for 15 h at 4 °C in Tris-HCl (25 mM, pH 8.3) as running buffer. SDS-PAGE was performed under reducing conditions using 15% minislab gels according to a reported method.[8] Gels were stained with Coomassie Brilliant Blue R-250. The concentrations of all types of ferritin were determined according to the Lowry method with BSA as a standard sample.

2. Self-Assembly of Linear Protein Cages induce by PLL_{15}.

All polypeptides used in this study are linear polymers where each amino acid residue participates in two peptide bonds and is linked to its neighbours in a head-to-tail fashion rather than forming branched chains. For linear protein assembly experiments, different volumes (0.1－1.0 μL) of PLL_{15} (500 μM) were mixed with 500 μL of 0.5 μM apo rmSSF solution (50 mM Mops, pH 7.0) to make a ratio of 0.2－2.0 PLL/ferritin in an eppendorf tubule. The reaction mixture was stirred for 10 min at room temperature ~ 25 °C. Then, a cultivation time of 0, 0.5, 1.0, 2.0, 3.0, 6.0, and 9.0 h at 25 °C was, respectively, conducted to obtain different morphologies of PLL_{15}-induced ferritin assemblies. PLL_2, PLL_5, and PLL_{50} were used as control samples to analyse the effect of PLL length on rmSSF linear assembly formation following the same procedures as that of PLL_{15}. Poly-L-arginine (PLR_{15}), poly-L-(lysine-glycine) ($PLLG_{15}$ where two amino acid lysine and glycine are arranged alternatively), and poly-L-glycine (PLG_{15}) were used as control samples to determine the effect of charge density on rmSSF linear assembly following the same procedures as that of PLL_{15}. For linear protein assembly of rASF-H-1ΔE and rHuHF-HuHFΔE induced by PLL_{15}, the reaction followed the same procedures for rmSSF self-assembly. All polypeptides used in this study are linear polymers where each amino acid residue participates in two peptide bonds and is linked to its neighbours in a head-to-tail fashion rather than forming branched chains.

3. Biotemplated Linear Array of Magnetic Fe_3O_4 Nanoparticles.

Magnetic Fe_3O_4 nanoparticles (diameter is 5 nm) (Galaxy Nano Tech., China) linear array was achieved by rmSSF_H193E/H197E trapping, followed by PLL_{15} induction. Specially, 1 mL of apo rmSSF_H193E/H197E (1.0 μM) was denatured at pH 11.4 for 3 h, at 25 °C, followed by addition of 2 μL of magnetic Fe_3O_4 nanoparticles (25 mg mL^{-1}). After stirring at 4 °C for 30 min, the protein and Fe_3O_4 nanoparticle mixtures were adjusted to pH 7.0 by adding 0.1 M HCl for trapping of magnetic Fe_3O_4 nanoparticles within the rmSSF_H193E/H197E cages. Then, the resulting supernatant was dialyzed against 50 mM Mops at pH 7.0, 4 °C in a dialysis bag (12-14 kDa MWCO) to remove the dissociative unpacked Fe_3O_4 nanoparticles. The concentration of the magnetic Fe_3O_4 nanoparticles was determined by measuring the protein concentration. Biotemplated linear array of magnetic Fe_3O_4 nanoparticles (0.5 mL, 0.5 μM) was conducted by adding 0.5 μL of PLL_{15} (500 μM) to solution of rmSSF_H193E/H197E trapped with magnetic Fe_3O_4 nanoparticles. The procedure was performed as the same procedure according to rmSSF linear self-assembly described above.

4. TEM Analyses.

Transmission electron microscope (TEM) experiments were carried out as following: Liquid samples were diluted with 50 mM Mops buffer (pH 7.0) prior to placing on carbon-coated copper grids and excess solution removed with filter paper, and then, proteins were stained using 2% uranyl acetate for 5 min. Transmission electron micrographs were imaged at 80 kV through a Hitachi H-7650 scanning electron microscope.

5. Fluorescence and UV/vis Titration.

Fluorescence titration experiments were performed using the Cary Eclipse spectrophotometer (Varian, USA). Measurements were carried out by an adding of 0—1.0 μL of PLL (500 μM) to 500 μL of the protein (0.5 μM) in 50 mM Mops, pH 7.0, followed by a reaction time of 10 min at 25 °C. Path lengths for excitation and emission were 1.0 and 0.2 cm at wavelengths of 290 and 330 nm, respectively. A slit width of 5 nm and 10 nm was set for excitation and emission, respectively. To obtain the stoichiometry and binding constant, the data were fitted to equation (1) for the binding of PLL_{15} to n independent binding sites on the protein.

$$I = I_0 - \left(\frac{I_0 - I_S}{2n[P]_0}\right)\left(\frac{1}{K} + [PLL_{15}]_0 + n[P]_0 - \left(\left(\frac{1}{K} + [PLL_{15}]_0 + n[P]_0\right)^2 - 4n[P]_0[PLL_{15}]_0\right)^{\frac{1}{2}}\right) \quad (1)$$

Here n is the binding site number and K is the apparent binding constant, $[P]_o$ and $[PLL_{15}]_o$ are the 24-mer protein (rmSSF) and PLL_{15} concentrations, and I_o and I_S are the relative fluorescence intensities in the absence and presence of PLL_{15} when the binding sites are fully saturated, respectively.

UV/vis titration was conducted on a Varian Cary 50 spectrophotometer (Varian, USA) under the same experimental conditions as the fluorescence titration.

6. Dynamic Light Scattering (DLS) Analyses.

DLS experiments were performed at 25 °C using a Viscotek model 802 dynamic light scattering instrument (Viscotek, Europe) as described previously.[9] The OmniSIZE 2.0 software was used to calculate the size distribution of prepared proteins. All samples, unless stated otherwise, the final concentration at 0.5 μM, were allowed to stand for 24 h prior to DLS measurement to ensure that the reactions were complete.

Supplementary figures (Figs. S1-11)

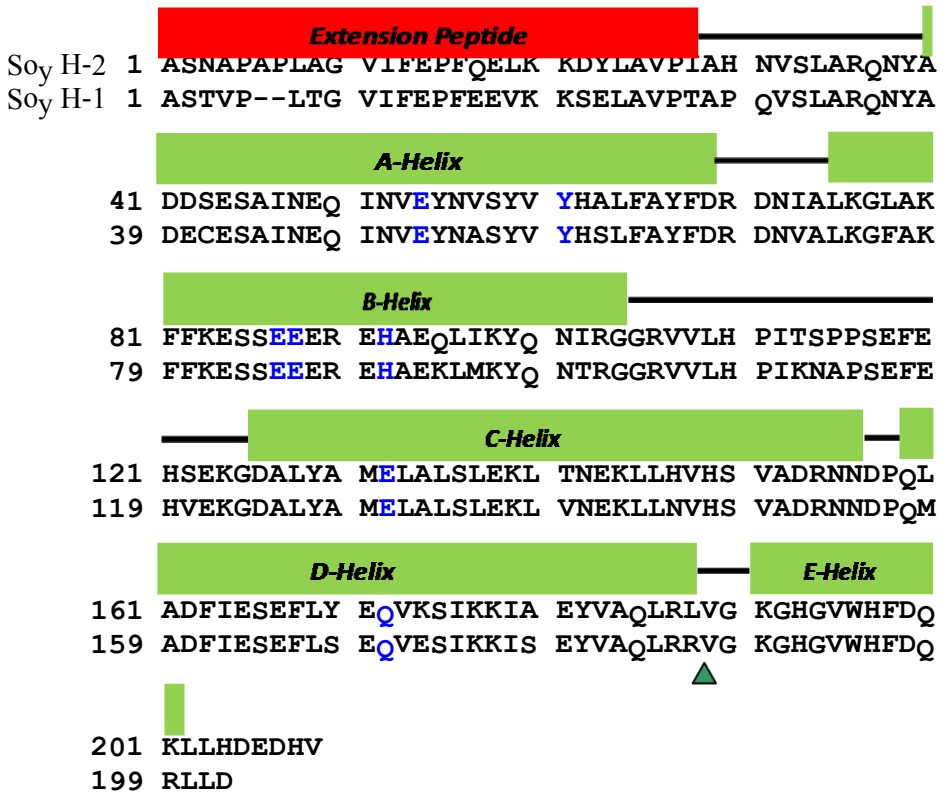

Fig. S1. Amino acid sequences of mature soybean seed ferritin subunits.[10] Top, amino acid sequence of the H-2 subunit. Bottom, amino acid sequence of the H-1 subunit. Conserved residues between the two subunits are shown in black. Residues in blue indicate the deduced ferroxidase center. The N-terminal EP residues of the H-2 subunit are shown boxed in red. The mature regions of both subunits are downstream from here. The cleavage site for conversion of the H-1 subunit is indicated by a green arrowhead.

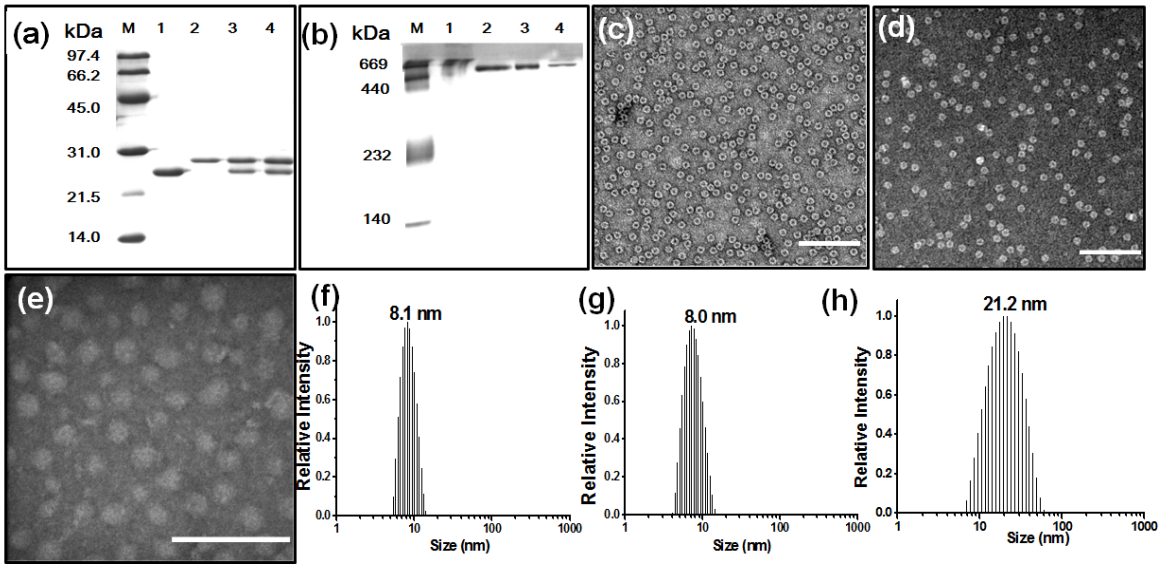

Fig. S2. Characterization of rmSSF cage. (a) SDS-PAGE and (b) Native-PAGE analyses of rH-1ΔE (lane 1), rH-2 (lane 2), mSSF (lane 3), and rmSSF (lane 4). (c-e) TEM images of rmSSF, rH-2 and rH-1ΔE, respectively. Scale bars represent 100 nm. (f-g) Relative scattered light intensity distribution of rH-2, rmSSF, and rH-1ΔE , respectively. [apo rH-2, rH-1ΔE, or rmSSF] = 0.5 μM, in 50 mM Mops (pH 7.0), 25 °C.

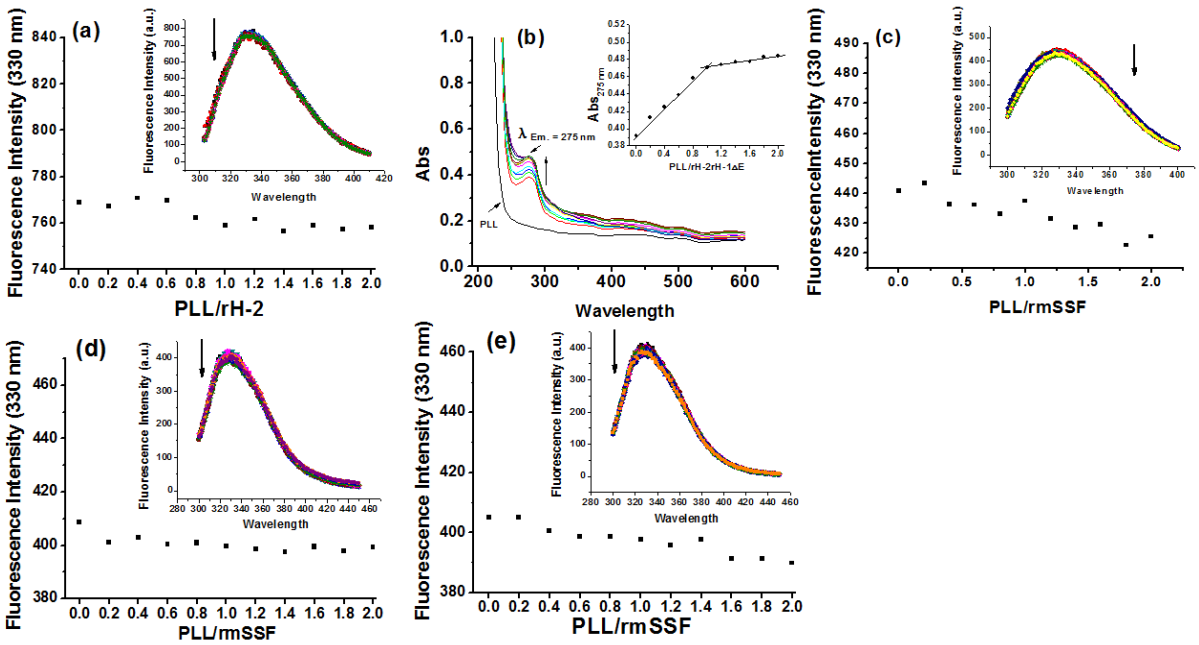

Fig. S3. (a) Fluorescence titration of rH-2 upon treatment with increments of PLL_{15}. (b) UV/vis and (c-e) Fluorescence titration spectras of rmSSF with addition of different amounts of PLL_{15} under different conditions. (a and b) [rmSSF or rH-2] = 0.5 μM in 50 mM Mops, pH 7.0, 25 °C. (c) apo rmSSF pretreated with 200 Ca^{2+}/ferritin shell at pH 7.0, [rmSSF] = 0.5 μM in 50 mM Mops, pH 7.0, 25 °C. (d) [rmSSF] = 0.5 μM in 50 mM Mops, pH 6.0, 25 °C. (e) [rmSSF] = 0.5 μM in 50 mM Tris, pH 9.0, 25 °C. For fluorescence titration experiment, $\lambda_{Ex.}$ = 290 nm, $\lambda_{Em.}$= 330 nm, slits for excitation and emission is 5 and 10 nm, respectively.

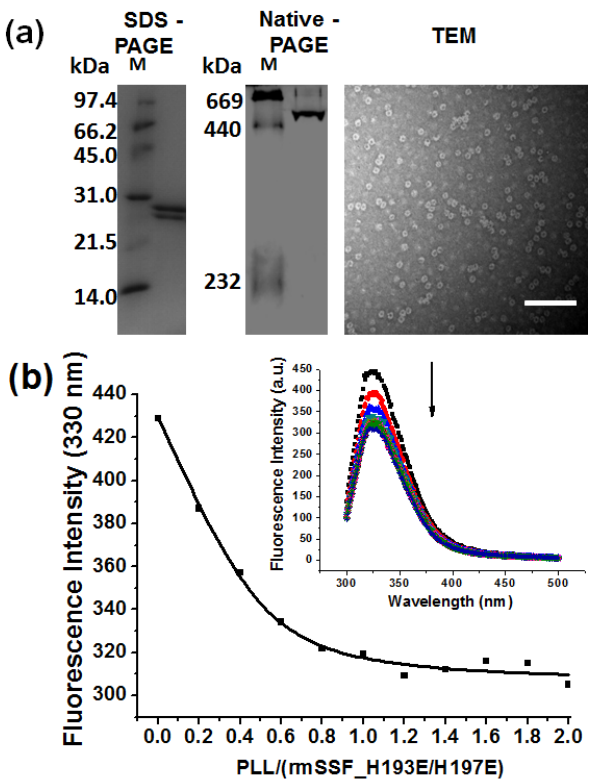

Fig. S4. (a) SDS-PAGE, Native-PAGE, and TEM of rmSSF_H193E/H197E. (b) Fluorescence titration of rmSSF_H193E/H197E at pH 6.0 upon treatment with increments of PLL_{15}. Average and standard deviation for three titrations were n = 1.06 ± 0.09, and K = (2.01 ± 0.05) × 10^5 M^{-1}. [apo rmSSF_H193E/H197E] = 0.5 μM, [PLL_{15}] = 0—1.0 μM, in 50 mM Mops (pH 6.0), 25 °C.

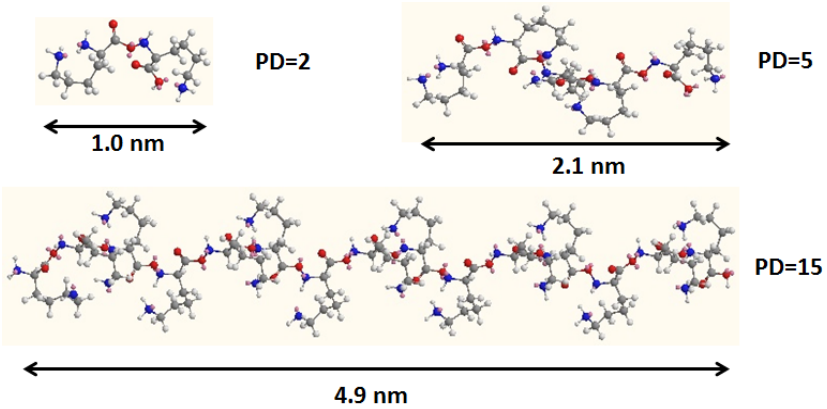

Fig. S5. Schematic diagrams of PLL with different polymerization degree (PD) represented by Chembiodraw Ultra 12 in a minimum energy state.

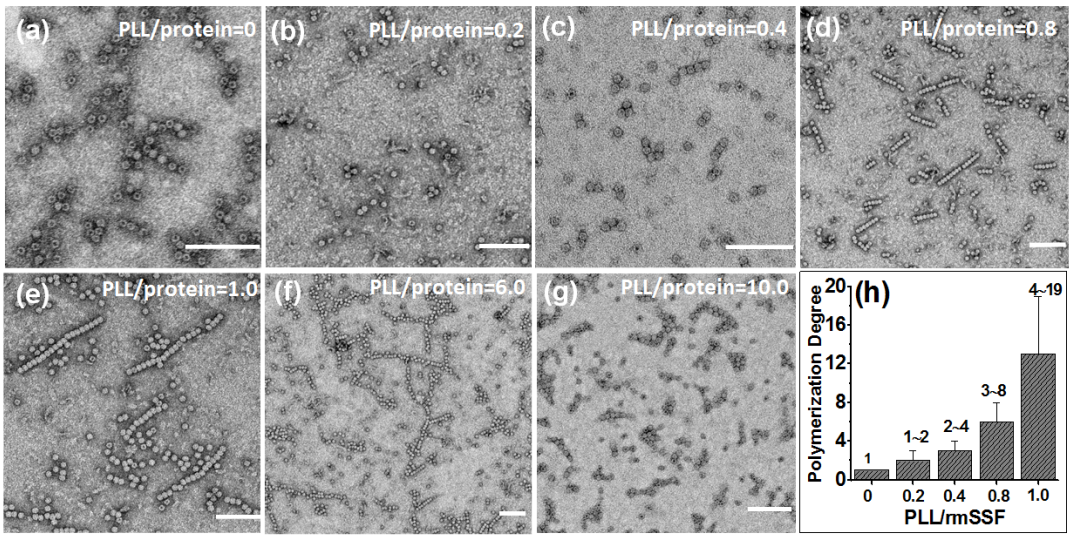

Fig. S6. (a–g) TEM images of rmSSF assemblies upon treatment with different amounts of PLL_{15} for 6 h. (h) The histogram shows the distribution of the polymerization degree of linear ferritin assemblies at different PLL/protein ratios (≤ 1). Scale bars represent 100 nm.

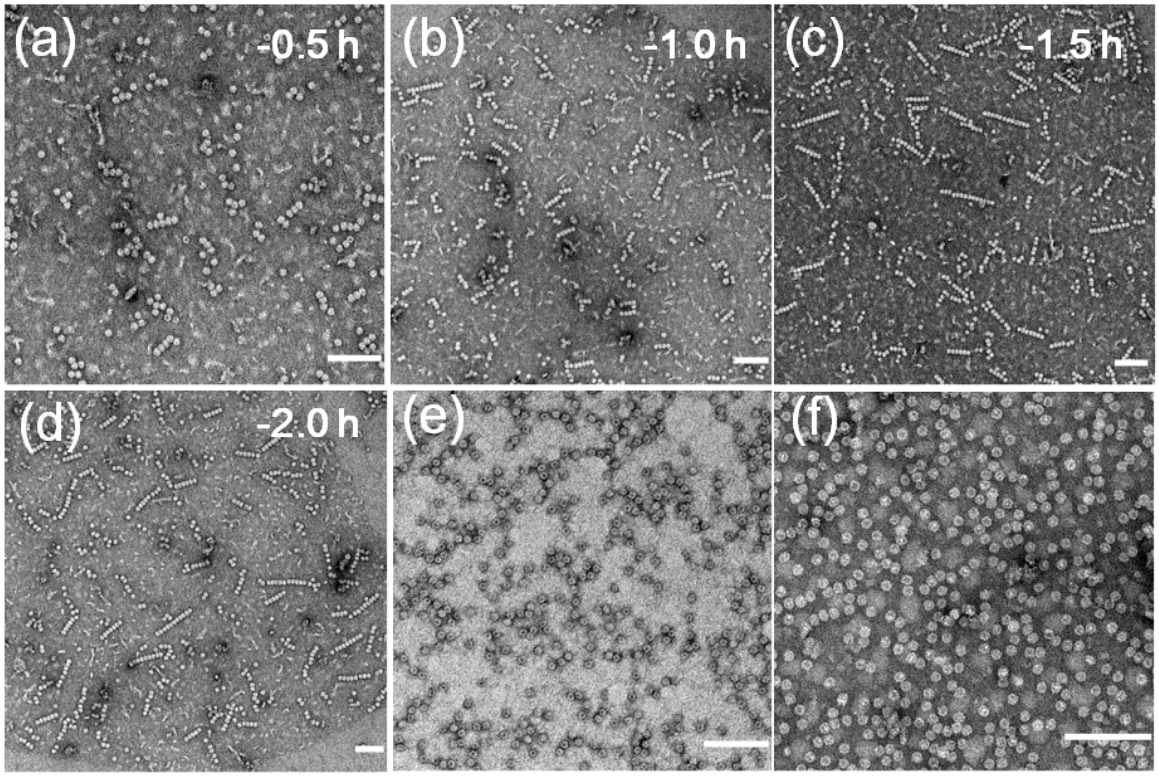

Fig. S7. TEM images of rmSSF assemblies induced by peptides with different charge density. (a-d) Time courses of TEM imaging of rmSSF assemblies induced by poly(α, L-arginine)$_{15}$, PLR$_{15}$. (e) TEM images of rmSSF induced by poly (α, L-lysine-glycine)$_{15}$, PLLG$_{15}$. (f) TEM images of rmSSF induced by poly(α, L-glycine)$_{15}$, PLG$_{15}$. [apo rmSSF] = 0.5 μM, [PLR$_{15}$, PLG$_{15}$, or PLLG$_{15}$] = 0.5 μM, in 50 mM Mops (pH 7.0), 25 °C. Scale bars represent 100 nm.

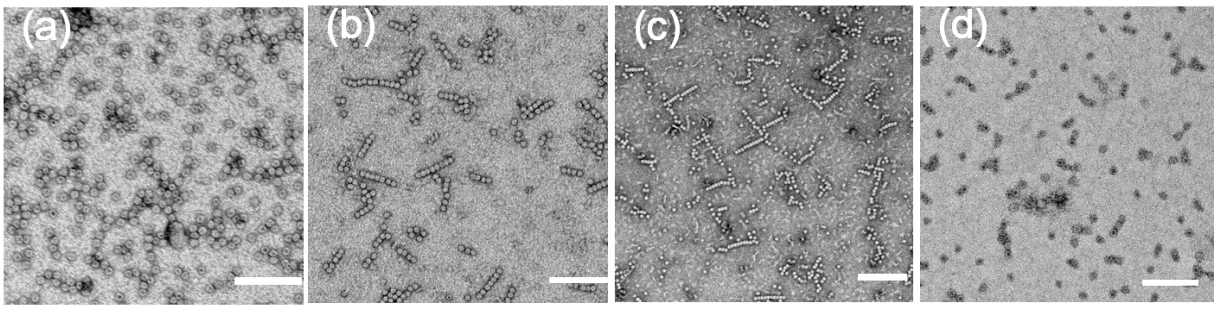

Figure S8. TEM images of rmSSF assemblies induced by PLL_{15} at different pH values. (a) pH 6.0, (b) pH 7.0, (c) pH 8.0, (d) pH 9.0. [apo rmSSF] = 0.5 μM, [PLL_{15}] = 0.5 μM, in 50 mM Mops (pH 6.0 and 7.0) or Tris-HCl (pH 8.0 and 9.0), 25 °C. Scale bars represent 100 nm.

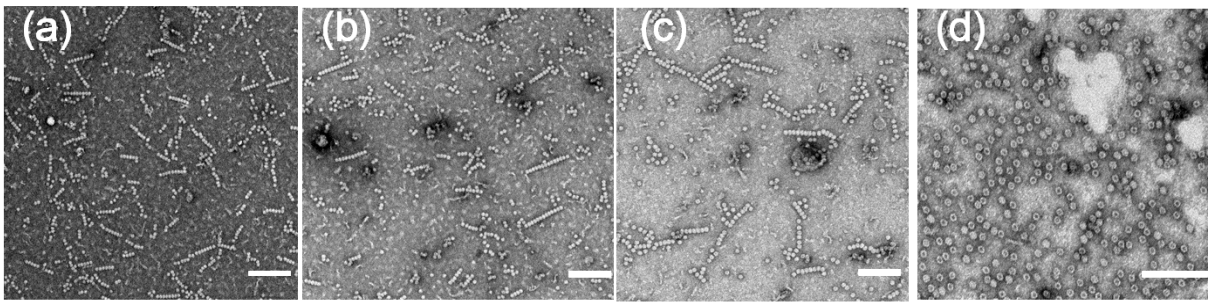

Figure S9. Effect of ion strength on TEM images of rmSSF assemblies induced by PLL_{15}. (a) 50 mM NaCl, (b) 150 mM NaCl, (c) 300 mM NaCl, (d) 500 mM NaCl. Conditions: [apo rmSSF] = 0.5 μM, [PLL_{15}] = 0.5 μM, in 50 mM Mops (pH 7.0), 25 °C. Scale bars represent 100 nm.

We found that PLL_{15} analogue, poly(α, L-arginine) (PLR_{15}) can also trigger nearly the same protein linear assembly as PLL_{15} does, but at a faster rate, while other PLL_{15} analogues with less charge density lose such ability (Fig. S7, ESI†)**.** Only at neutral pH (7.0 or 8.0) (Fig. S8, ESI†) and low or medium ionic strength (0–300 mM) (Fig. S9, ESI†), such linear array can be observed, again approving the electrostatic attraction between PLL_{15} and rmSSF.

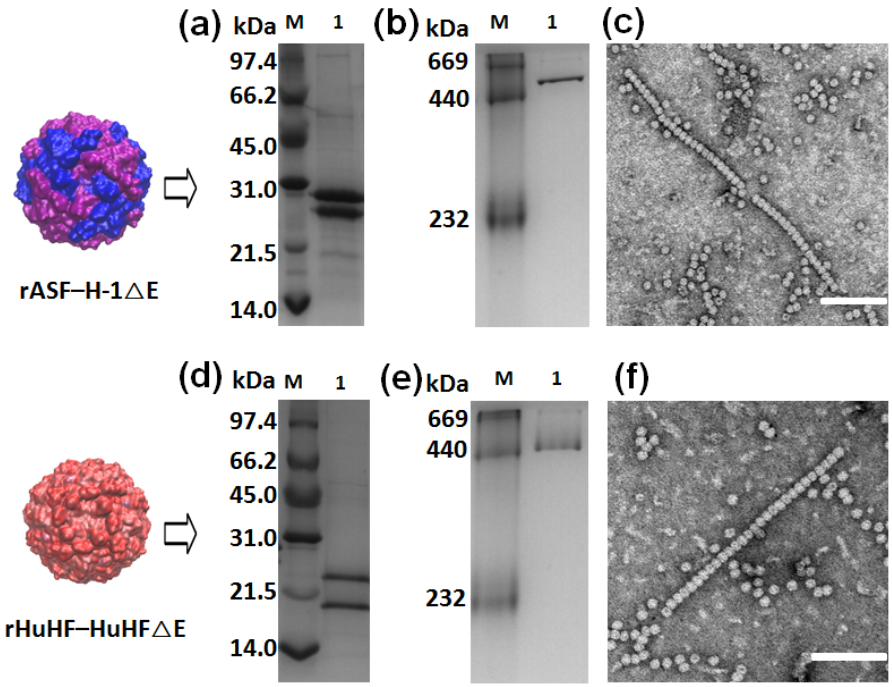

Fig.S10. Applicability of the channel-directed electrostatic interactions in other shell-like proteins. (a–c) show SDS-PAGE (a), native-PAGE (b) analyses of rASF–H-1△E, and TEM image (c) of linear rASF–H-1△E species induced by PLL_{15}, respectively. (d–f) show SDS-PAGE (d), native-PAGE (e) analyses of rHuHF–HuHF△E, and TEM image (f) of linear rHuHF–HuHF△E species induced by PLL_{15}, respectively. Scale bars represent 100 nm.

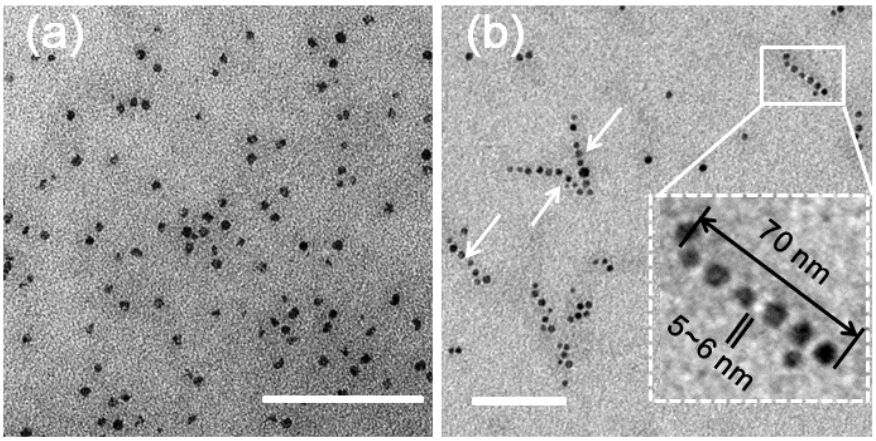

Fig. S11. TEM images of magnetic Fe_3O_4 nanoparticles (diameter is 5 nm) trapped by rmSSF_H193E/H197E upon untreatment (a) and treatment (b) with PLL_{15} in a 1:1 ratio without uranyl acetate stained. Arrows in (b) highlight the linear Fe_3O_4 arrays, and a low magnification image of the linear Fe_3O_4 nanoparticle array is shown as an inset. Scale bars represent 100 nm.

To broaden the applicability of such linear assembly, other two ferritins, namely hybrid plant ferritin rASF–H-1ΔE and mutant human H ferritin rHuHF–HuHFΔE, where their 4-fold channels are expanded were prepared in a similar way (Fig. S10a, b, d and e, ESI†). We found that PLL_{15} can also bridge both these two ferritins to generate linear species (Fig. S10c and f, ESI†). After treatment of Fe_3O_4-loaded rmSSF_H193E/H197E with PLL_{15} in a 1:1 ratio, magnetic Fe_3O_4 nanoparticles packed within protein shell arranged in a linear form (Fig. S11b, ESI†). Thus, such linear protein assembly could act as a template to fabricate linear inorganic nanomaterials.

References

1 C. Li, X. Fu, X. Qi, X. Hu, N. D. Chasteen, and G. Zhao, *J. Biol. Chem.*, 2009, **284**, 16743–16751.

2 T. Masuda, F. Goto, T. Yoshihara, T. Ezure, T. Suzuki, S. Kobayashi, M. Shikata, and S. Utsumi, *Protein Expr. Purif.*, 2007, **56**, 237–246.

3 S. Kang, L. M. Oltrogge, C. C. Broomell, L. O. Liepold, E. P. Prevelige, M. Young, and T. Douglas, *J. Am. Chem. Soc.*, 2008, **130**, 16527–16529.

4 R. Chiaraluce, V. Consalvi, S. Cavallo, A. Ilari, S. Stefanini, and E. Chiancone, *Eur. J. Biochem.*, 2000, **267**, 5733–5741.

5 O. W. Wuytswinkel, G. Savino, and J. F. Briat, *Biochem. J.,* 1995, **305**, 253–261.

6 T. Masuda, F. Goto, T. Yoshihara, and B. Mikami, *Biochem. Bioph. Res. Co.,*2010, **400**, 94–99.

7 M. Li, S. Yun, X. Yang, and G. Zhao, *Biochim. Biophys. Acta,* 2013, **1830**, 2946–2953.

8 U. K. Laemmli, *Nature,* 1970, **227**, 680–685.

9 H. Yang, X. Fu, M. Li, X. Leng, B. Chen, and G. Zhao, *Plant Physiol.*, 2010, **154**, 1481–1491.

10 T. Masuda, F. Goto, and T. Yoshihara, *J. Biol. Chem.*, 2001, **276**, 19575–19579.