Electronic Supplementary Information (ESI)

Spatiotemporal control over 3D protein nanocage superlattices for hierarchical encapsulation and release of different cargo molecules

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Experimental Procedures

Protein production and purification

The relevant genes encoding of ferritin mutants ($^{H158/H160}$MjFer) and wild-type MjFer (*Marsupenaeus japonicus ferritin*) on the plasmids pET-3a were expressed in *E. coli* BL21 (DE3) cells. Then BL21 (DE3) cells were grown at 37 °C on LB-ampicillin (amp) plates overnight. A 5 mL starter culture containing LB-amp was inoculated with a single colony and grown at 37 °C overnight. 500 mL LB-amp were inoculated with the preculture and incubated at 37 °C and 200 rpm until an OD_{600} of 0.6 was reached. Protein production was induced by 1.0 mM isopropyl-β-D-1thiogalactopyranoside (IPTG). After introduction, the cultures were grown further at 37 °C for 12 h, after which the cells were harvested by centrifugation at 10000 rpm for 10 min and suspended in a buffer solution containing 50 mM Tris-HCl (pH 8,0), and disrupted by sonication. Subsequently, the supernatant collected was subjected to precipitation with 20% ammonium sulfate. The resulting precipitates were redissolved and dialyzed against 50 mM Tris-HCl (pH 8.0). Final protein purification was achieved by passing it through an ion-exchange column (Q-Sepharose Fast Flow, GE Healthcare) with a gradient elution from 0 to 1 M NaCl. The purity of purified protein was checked using SDS-PAGE (polyacrylamide gel electrophoresis) and concentration of protein was measured according to the Lowry method with bovine serum albumin (BSA) as standard.

Transmission electron microscopy (TEM) analysis

The morphology and structure of protein and their assembly state were characterized by transmission electron microscope. Protein samples (10 μL) were deposited on a 400-mesh holey copper grid surfaced-coated with a thin layer of amorphous carbon for 3 min, and excess solution was blotted away with filter paper. Then samples were negatively stained with 2% uranyl acetate for 5 min. TEM micrographs were obtained using a Hitachi H-7650 transmission electron microscope at 80 KV.

Dynamic light scattering (DLS) analysis

DLS experiments were performed at 25 °C by using a dynamic light scattering instrument (Viscotek, Europe, Viscotek model 802). The OmniSIZE 2.0 software was used to calculate the size distribution of samples, and Origin was used to present the collected data. The concentration of protein was 1.0 μM.

High-Resolution Gel Filtration Chromatography Analyses

High-resolution gel filtration chromatography analyses were performed using an ÄKTA pure system coupled to a Superdex 200 increase 10/300 column (GE Healthcare) in buffer (50 mM Tris, 100 mM NaCl, pH = 8.0) with a flow rate of 0.5 mL/min.

Calculation of efficiencies for the reconstruction of protein nanocages

To evaluate the efficiency of reconstituted protein nanocage, $^{H158/H160}$MjFer were disassembled by adjusting pH from 8.0 to 2.3 or 3.5, and then reconstructed by adjusting pH back to 8.0. As control, HuHF and ^{T158H}MjFer protein nanocages were reconstructed by adjusting pH

from 8.0 to 2.3, followed by adjusting pH back to 8.0, respectively. The dimers that did not participate in the reconstruction of nanocages were separated with ultrafiltration centrifugation (100 kDa, 4000 rpm, 15 min). The ~440 kDa ferritin nanocage did not go through a 100 kDa cut off ultrafiltration tube. The efficiency of reconstructed protein nanocage (%) was calculated according to the mass amount of reconstructed ferritin nanocage / the total mass amount of protein used × 100%. Protein concentrations were determined according to the Lowry method with bovine serum albumin (BSA) as standard.

Encapsulation of Rhodamine 6G (R6G) and Black hole quencher 2 (BHQ-2)

R6G was dissolved in ddH_2O to make a stock solution with a final concentration of 10.0 mM and stored in the dark at 4 °C. BHQ-2 was dissolved in methanol to form a stock solution with a final concentration of 10.0 mM and stored in the dark at -20 °C. For the encapsulation of R6G, the protein solution was prepared with a concentration of 2.0 μM in 50.0 mM Tris (pH 8.0) with a volume of 5 mL, and then solution pH was adjusted slowly to 3.5 with HCl (1.0 M) to disassemble ferritin into subunits. After 5 min, 200 μL of R6G stock solution was dropwise added to the protein solution with a $^{H158/H160}$MjFer/R6G ratio of 1 to 200 followed by stirring for 10 min, and then the pH value of the solution was adjusted to 8.0 with NaOH (1.0 M), followed by stirring for 30 min. The resultant mixture was allowed to stand at 4 ˚C for 24 h. Finally, resulting R6G and protein mixture was dialyzed (100 kDa) against 50.0 mM Tris (pH 8.0) four times at 6.0 h intervals to remove free R6G.

Then the above protein solution was used to encapsulate BHQ-2 in the pore of 3D protein superlattices. 20 μL of BHQ-2 stock solution was added to the protein solution with a

$^{H158/H160}$MjFer/BHQ-2 ratio of 1 to 200 followed by stirring for 10 min, and then 0.7 mM Ni^{2+} plus 500 mM NaCl was added to the solution to obtain 3D protein superlattices, and meanwhile embed BHQ-2 in the pore of protein superlattices.

Small angle X-ray scattering (SAXS) analysis

To prepare the experimental sample, 0.7 mM Ni^{2+} plus 500 mM NaCl was added into 5 mL 1.0 μM protein solution, and protein assemblies formed overnight. Then, protein assemblies were harvested by centrifugation, and sealed in a metal ring. Small angle X-ray scattering measurements were carried out using a Xeuss2.0 SAXS/ WAXS system (Xenocs, France). The distance between sample and director was 2.5 m, and samples thickness was approximately 1.5 mm. Initially, a silver behenate standard sample was used for calibration of the length of the scattering vector q. One-dimensional SAXS data were obtained by azimuthally averaging the 2D scattering data. The magnitude of the scattering vector q is given by $q = 4\pi \sin\theta/\lambda$, where 2θ is the scattering angle. The simulated scattering patterns were obtained with Scatter (version 2.5).

FRET measurements

Samples containing encapsulated R6G with and without co-encapsulated BHQ-2 as well as free protein mixtures were excited at 488 nm, and emission was observed from 500 nm to 650 nm. Fluorescence spectra were recorded on a F-7000 FL Spectrophotometer at 25 ˚C.

Protein crystallization, data collection and data processing

Purified $^{H158/H160}$MjFer was concentrated to about 10 mg mL^{-1} in 10 mM Tris-HCl (pH 8.0) buffer. Protein crystals were produced through hanging-drop vapor diffusion technique. 1.50 μL aliquots of the protein sample was mixed with an equal volume of mother liquid and the mixture was equilibrated against 500 μL mother liquid at 20 °C to prepare crystals. X-ray diffraction data were collected on beamline BL18U at Shanghai Synchrotron Radiation Facility (SSRF) with merging and scaling by HKL-3000 software[1]. Data processing statistics are summarized in Table S1. The structure of mutant was solved through molecular replacement using the Molrep program in CCP4 using the wild type *Marsupenaeus japonicus* ferritin as initial model (PDB code: 6A4U). Following refinement and iterative manual model building were conducted with software PHENIX[2] and COOT[3]. All Figures of the resulting structures were produced using the PyMOL[4] program and UCSF Chimera package.[5]

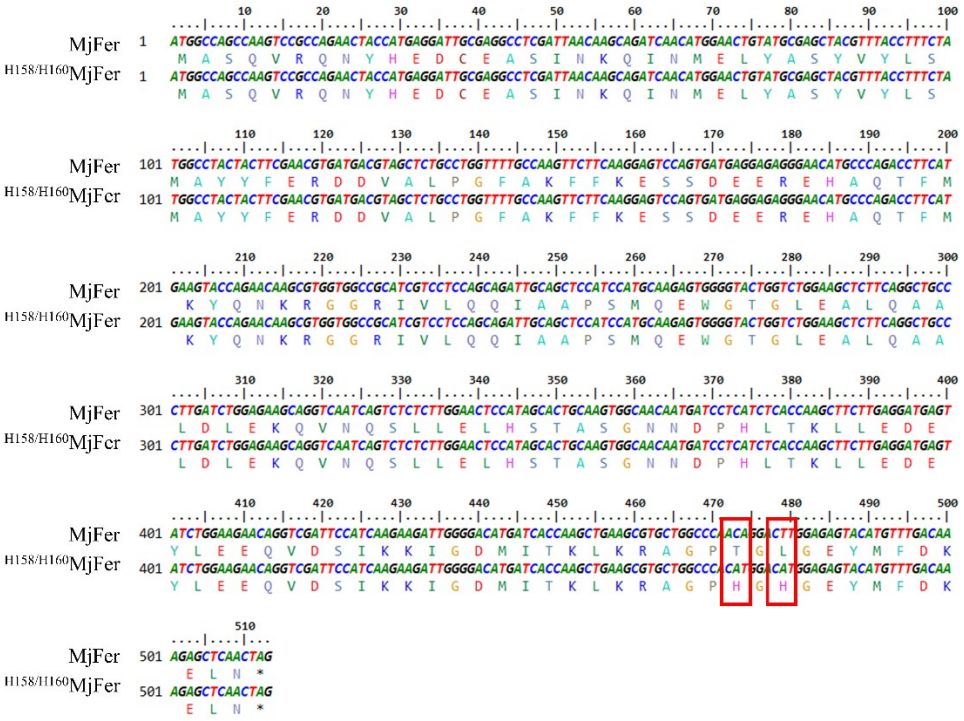

Figure S1. The amino acids sequence of MjFer (wt *Marsupenaeus japonicus* ferritin) and $^{H158/H160}$MjFer.

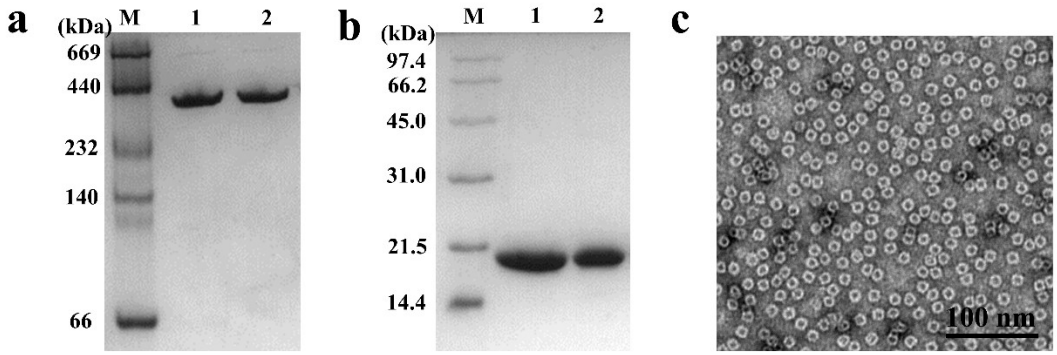

Figure S2. Characterization of $^{H158/H160}$MjFer after purification. Native-PAGE (a) and SDS-PAGE (b) analyses of purified $^{H158/H160}$MjFer with wt MjFer as control. Lane M, protein markers and their corresponding molecular masses; lane 1, wt MjFer; lane 2, $^{H158/H160}$MjFer. (c) TEM images of $^{H158/H160}$MjFer.

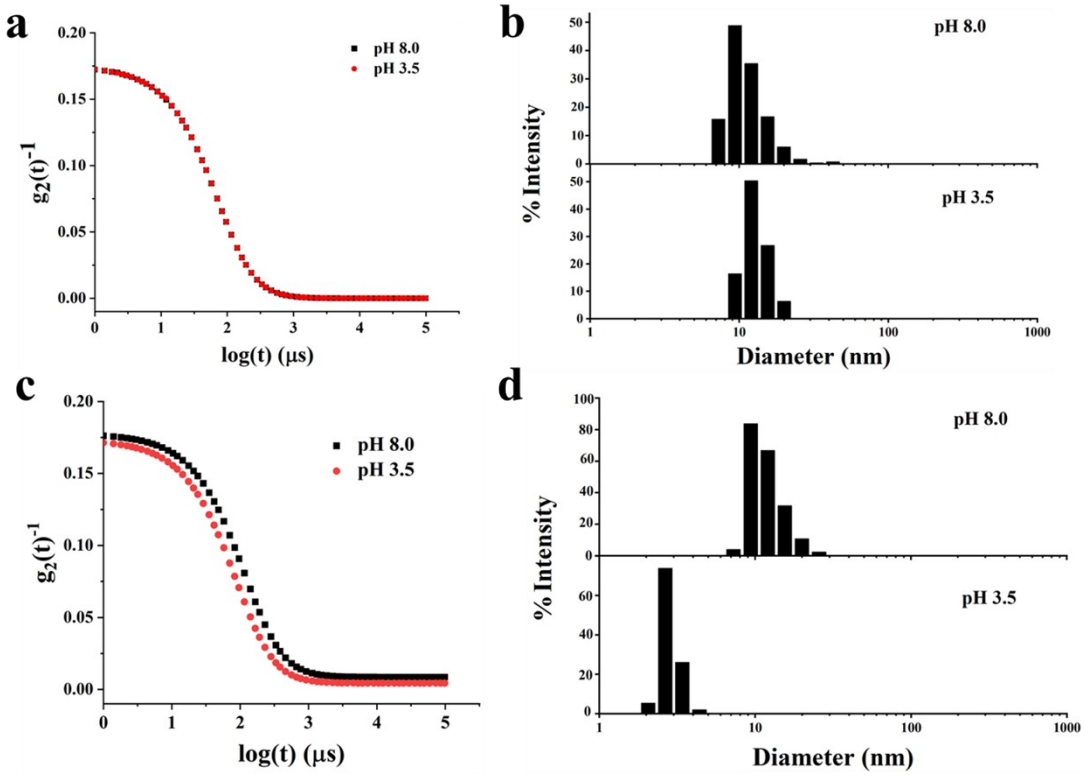

Figure S3. Normalized intensity autocorrelation functions of ^{T158}MjFer (a) and $^{H158/H160}$MjFer (c) and size distributions of ^{T158}MjFer (b) and $^{H158/H160}$MjFer (d) induced by pH.

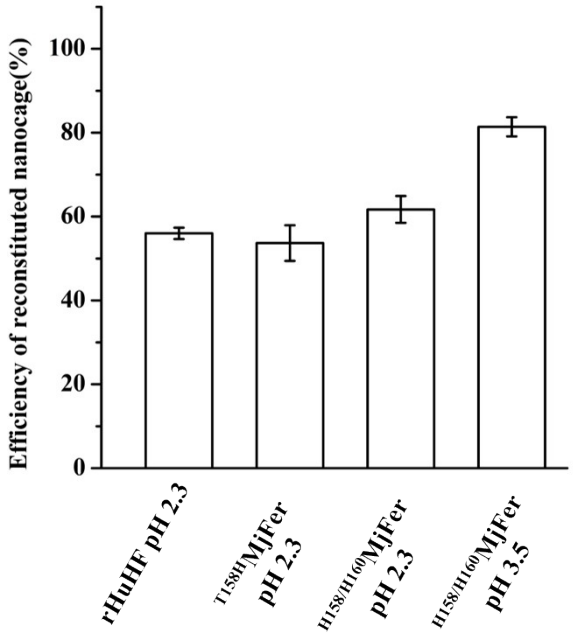

Figure S4. Efficiency of reconstituted HuHF (rHuHF), ^{T158H}MjFer, and $^{H158/H160}$MjFer at pH 8.0 upon pH-induced ferritin nanocage disassembly at pH 2.3 or 3.5.

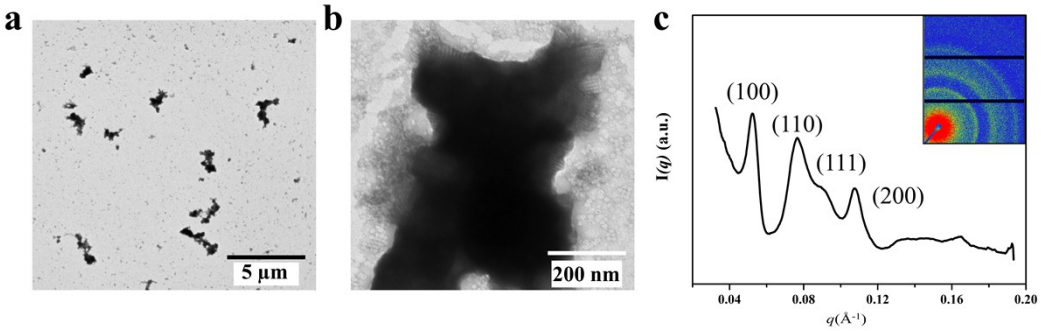

Figure S5. The 3D self-assembly of reconstituted $^{H158/H160}$MjFer nanocage induced by Ni^{2+}. (a) TEM images of the 3D assembly of reconstituted $^{H158/H160}$MjFer upon treatment with Ni^{2+}. (b) High-magnification view of (a). (c) SAXS analyses of 3D superlattices formed upon treatment of reconstituted $^{H158/H160}$MjFer with Ni^{2+}. The (*hkl*) values in radially averaged 1D SAXS data are labelled above the peaks. The inserted image in is the 2D SAXS pattern of $^{H158/H160}$MjFer assemblies.

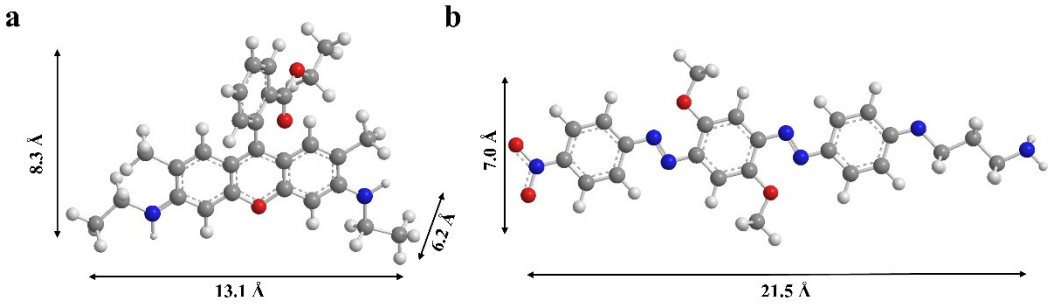

Figure S6. (a) Chemical structure of rhodamine 6G (R6G). (b) Chemical structure of BHQ-2.

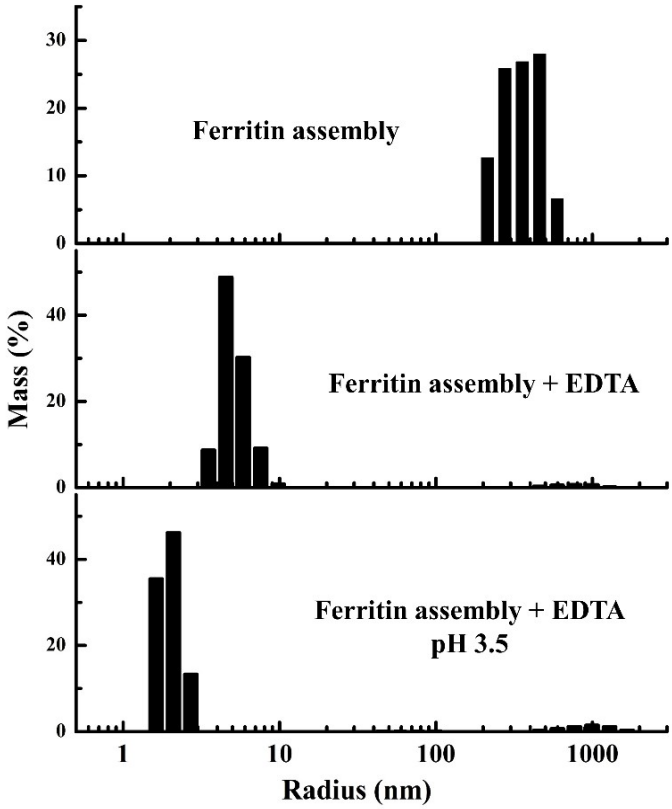

Figure S7. Dynamic light scattering (DLS) analyses of the R6G-loading and BHQ-2-loading 3D ferritin assemblies upon treatment with EDTA and acids to pH 3.5, respectively.

Table S1. Crystallographic properties and data collection and model refinement statistics.

Parameters	cubic-shaped crystal
Wavelength (Å)	0.979
Space group	P432
Resolution range (Å)	58.64-1.9 (1.968-1.9)
Unit cell	117.278, 117.278, 117.278 90, 90, 90
Unique reflections	21289 (1833)
Completeness (%)	95.12
Mean I/sigma (I)	4.1
Wilson B-factor	29.42
CC1/2	0.992
Reflections used in refinement	21287 (1833)
Reflections used for R-free	2000 (173)
R-work	0.1898 (0.2393)
R-free	0.2218 (0.2983)
Number of non-hydrogen atoms	1488
macromolecules	1389
ligands	4
Protein residues	169
RMS (bonds)	0.009
RMS (angles)	1.06
Ramachandran favored (%)	98.20
Ramachandran allowed (%)	1.80
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	1.33
Clashscore	2.56

Average B-factor	29.52
macromolecules	29.30
ligands	42.22
solvent	32.26

References

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