Supplementary Information

Peptide-Functionalized Nanocapsules for Targeted Inhibition of β2-Microglobulin Amyloid Aggregation

Lin Tang,[‡a,b] Miao Sun,[‡c] Junnan Chen,[b] Qiong Dai,[b,c] Song Xue,*[c] Chaoyong Liu,*[b,c] and Ming Zhang*[d]

[a] Department of Medical Imaging, Qilu Medical University, 255100, P. R. China

[b] Beijing Advanced Innovation Center for Soft Matter Science and Engineering, Beijing University of Chemical Technology, Beijing 100029, P. R. China

[c] College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, P. R. China

Email: xues08@126.com; chaoyongliu@mail.buct.edu.cn

[d] Department of Pathology, Peking University International Hospital, Beijing 102206, P. R.China

E-mail: zhangming1@pkuih.edu.cn

‡ These authors contributed equally to this work.

1. Materials

All primers were purchased from BGI (China). DNA polymerase KOD FX (TOYOBO Life Science Department Inc.) was used for polymerase chain reaction (PCR). Gel purification was performed using kits from Axygen (China). Restriction endonucleases were obtained from New England Biolabs (NEB, USA). The plasmids pET-28a-EGFP and pET-28a were sourced from Miao Ling Plasmid (China), and amplified plasmids were purified using the Plasmid Mini Kit I (100) from OMEGA BIOTEK. Transetta (DE3) Chemically Competent Cells were provided by TransGen Biotech (China). Lysogeny broth (LB) was purchased from Beijing Aoboxing Biotech Co. Ltd. (China). Affinity chromatography resins, including NI-NTA and Glutathione resin, were obtained from GenScript. HEK293 cells were kindly provided by Y. Chen (Tsinghua University, China). Dulbecco's modified Eagle's medium (DMEM) 1× with glucose (4.5 g/L) and MEM Alpha 1× were purchased from Corning (USA). Fetal bovine serum (FBS) was sourced from Thermo Fisher Scientific, and penicillin/streptomycin was acquired from General Electric. Live cell dyes, including Hoechst (for nuclear staining) and DiI (for membrane staining), were obtained from Beyotime Biotechnology. Bovine serum albumin (BSA) was purchased from Solarbio. N-acryloxysuccinimide (NAS), acrylamide (AAM), N-(3-aminopropyl) methacrylamide (APM), N,N'-methylenebisacrylamide (BIS), ammonium persulfate (APS), and tetramethylethylenediamine (TEMED) were obtained from HEOWNS and used without further purification. The BCA Protein Assay Kit was purchased from Solarbio. Sepharose CL-4B was sourced from Sigma-Aldrich. Fluorescent dyes, including 5-TAMRA, SE (5-Carboxytetramethylrhodamine, succinimide ester) and FITC (Fluorescein Isothiocyanate), were provided by Thermo Fisher Scientific. Dimethyl sulfoxide (DMSO, 99.9%) and triethylamine (TEA) were obtained from SCRC. Unless otherwise specified, all other chemical reagents were purchased from Sigma-Aldrich.

2. Cell culture

HEK-293 cells were cultured in Dulbecco's modified Eagle medium (DMEM)

supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and passaged daily to ensure optimal growth conditions.

3. Instruments

Zeta potential and dynamic light scattering (DLS) measurements were conducted using a Malvern Laser Particle Size Analyzer. Transmission electron microscopy (TEM) imaging was performed on a JEM-1400Flash microscope at an acceleration voltage of 120 kV. UV-Visible spectra were obtained using a NanoDrop OneC spectrophotometer (Thermo Scientific, USA). The Atomic Force Microscopy (AFM) imaging was performed on Dimension ICON AFM (Bruker, USA). The fluorescence intensity was measured by FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, USA). The confocal imaging was performed on Leica TCS SP8-STED (Leica, Germany). Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) was performed on Autoflex III LRF 200-CID (Bruker, USA).

4. Synthesis of the peptide monomers

Peptides NH2-KDWSFYILAHTEF and NH2-KFETYYLLYFSDW were synthesized by SHENLANG BIOTECH. As illustrated in Figure S1, peptide monomers functionalized with acrylic groups were prepared via the reaction of the N-terminal amine or lysine residue amine groups with N-acryloxysuccinimide (NAS). Specifically, 6 mg of peptide and 120 μL triethylamine (TEA) were dissolved in 450 μL anhydrous dimethyl sulfoxide (DMSO), followed by the gradual addition of 30 μL NAS (100 mg/mL in DMSO) under stirring conditions. The reaction mixture was incubated at room temperature for 12 hours and subsequently dialyzed against PBS/DMSO (v/v, 50:1) to remove unreacted monomers. The successful modification of ACR-CF was confirmed by MALDI-TOF MS analysis, as shown in Figure S2.

5. Preparation of nanocapsules

5.1 Fluorescence-labeling of BSA

BSA was labeled with 5-Carboxytetramethylrhodamine succinimide ester (TAMRA)

and Fluorescein isothiocyanate (FITC) for imaging purposes. Initially, BSA was dissolved in water and dialyzed against PBS buffer (10 mM, pH 7.4) to remove residual ammonium sulfate. Following dialysis, the protein concentration was adjusted to 5 mg/mL using PBS buffer. TAMRA and FITC were dissolved in DMSO to prepare 10% (m/v) stock solutions. The dyes were then added to the BSA solution at a molar ratio of 5:1 (dye to protein). The reaction was carried out at 4 °C for 4 hours, followed by thorough dialysis in PBS buffer (10 mM, pH 7.4) to remove unbound dye.

5.2 Propylene acylation of BSA

The fluorescence-labeled BSA was conjugated with N-acryloxysuccinimide (NAS) to introduce acryloyl groups onto its surface. A NAS stock solution (10% in DMSO, m/v) was added to the BSA solution at a molar ratio of 10:1 (NAS to protein), and the reaction was conducted at 4 °C for 2 hours. The solution was then dialyzed against a PBS buffer to remove any unreacted NAS. The acryloylated protein solution was stored at -20 °C for subsequent experiments.

5.3 Synthesis of the nanocapsules

Polymerization was performed in the BSA solution using acrylamide (AAm) and ACR -CF as monomers, N,N'-methylenebisacrylamide (BIS) as a cross-linker, and tetramethylethylenediamine (TEMED) with ammonium persulfate (APS) as initiators. The reaction was maintained at 4 °C for 2 hours. The molar ratio of BSA: AAm: ACR-CF: BIS: APS was 1: 5000: 40: 600: 350, and the mass ratio of APS:TEMED was 1:2. Post-polymerization, the reaction mixture was dialyzed against PBS buffer (10 mM, pH 7.4) to remove unreacted monomers and by-products. The resulting solution was purified using a hydrophobic column (Sepharose CL-4B) to eliminate unreacted proteins, yielding the NC-CF product. A control nanocapsule (NC) was synthesized using the same procedure but without the addition of ACR-CF.

5.4 Characterization of the nanocapsules

The protein content in the nanocapsules was determined using the BCA Protein Assay Kit. In brief, BCA reagent and diluted nanocapsule solutions were mixed at a volume ratio of 10:1 and incubated at 60 °C for 15 minutes. After cooling to room temperature, absorbance at 562 nm was measured using a NanoDrop OneC spectrophotometer. BSA

solutions of known concentrations were used as standards. The size and zeta potential of the nanocapsules were evaluated using dynamic light scattering (DLS), transmission electron microscopy (TEM), and ZETAPALS/BI-200SM at room temperature.

5.5 Ultraviolent-visible (UV) spectra of nanocapsules

TAMRA-labeled BSA was used to normalize the peptide concentrations in NC-CF by measuring UV-Vis absorption at 555 nm. The absorbance at 555 nm for NC and NC-CF solutions was adjusted according to their BCA results. The peptide concentration was determined as the difference between NC-CF and NC, while the actual BSA concentration was calculated by subtracting the total protein concentration from the CF peptide concentration. The ratio of CF fragments per NC-CF was confirmed via the equation:

$$X=[CF]*\ {}^{M_{BSA}}/_{M_{CF}}*[BSA]$$

Where X is the rough ratio of CF fragments, [CF] is the concentration of CF peptide, and [BSA] is the actual concentration of BSA. M_{BSA} is the molecular weight of BSA, and M_{CF} is the molecular weight of the CF peptide.

6. Transmission electron microscope (TEM)

TEM measurements were conducted using a JEM-1400Flash electron microscope at an acceleration voltage of 120 kV. TEM samples were prepared by depositing 10 μL of the sample solution onto a carbon-coated copper grid (Beijing Zhongjingkeyi Technology Co., Ltd, China) for 2-3 minutes. Excess liquid was removed using dust-free paper along the edge of the grid. Negative staining was performed by applying 10 μL of 1% phosphotungstic acid for 1-2 minutes. Finally, the grid was washed with 10 μL of ddH_2O and dried overnight.

7. Expression and purification of β2m and EGFP-β2m

The expression and purification of β2m and EGFP-β2m were performed using an E. coli expression system. DNA fragments were amplified with primers and inserted into vector plasmids (Figures S7-S10). The recombinant plasmids (pET-28a-EGFP-β2m

and pET-28a-β2m) were introduced into BL21-DE3 competent cells. The expression and purification of β2m were carried out as previously described. For EGFP-β2m, cells were cultured in LB medium at 37 °C until OD600 reached 0.8-0.9, followed by induction with 0.5 mM IPTG and overnight incubation at 16 °C. Cells were harvested by centrifugation (4000 rpm, 20 min), and EGFP-β2m was purified using a Ni-NTA affinity column and desalted into PBS using Zeba Spin Desalting Columns (Thermo Fisher).

The β2m gene and GST tag were amplified and assembled into a long fragment (HBG), which was inserted into the pET-28a vector to generate the pET-28a-β2m-GST plasmid. The plasmid was introduced into BL21-DE3 cells, and expression was induced under the same conditions. The β2m-GST fusion protein was purified using a Glutathione Resin column and digested with precision protease overnight at 4 °C to remove the GST tag, yielding soluble β2m.

8. Thioflavin-T (ThT) fluorescence kinetics

The preparation of β2m seed fibrils and fiber growth assays were performed as previously described. NC-CF was added to a solution containing β2m (10 μM) and seeds (1 mg/mL), followed by the addition of ThT (5 μM final concentration). The reaction system was equilibrated at 37 °C, and ThT fluorescence emission at 485 nm (excitation at 440 nm) was monitored.

9. Atomic force microscopy (AFM) study

A 10 μL sample was applied to freshly cleaved mica and allowed to adsorb for 30 minutes. The mica was washed twice with 100 μL of ultra-pure water, dried using compressed air, and left to air dry overnight. The sample concentration was 5 μM based on monomer protein content, and imaging was conducted at a scan rate of 1 Hz.

10. Confocal imaging study

Fluorescence co-localization imaging was conducted using a laser confocal microscope. EGFP-β2m/NC-CF samples and control samples (EGFP-β2m or NC-CF alone) were incubated in PBS at 37 °C for 3 hours. Imaging was performed using inverted laser confocal microscopy in 96-well plates with transparent substrates. The concentrations of EGFP-β2m and NC-CF were 10 μM and 100 μg/mL, respectively.

11. Cell proliferation assay

HEK293 cells were seeded in 96-well plates and allowed to adhere for 12-24 hours. After removing the culture medium, cells were treated with various concentrations of NC-CF in a serum-free medium for 24 hours. Subsequently, 100 μL of 0.5 mg/mL MTT solution was added to each well and incubated for 4-6 hours. The resulting formazan was dissolved by adding a triple solution, and absorbance at 570 nm was measured. All MTT-related steps were performed under light-protected conditions.

HEK293 cells (2×10^6) were cultured in 3.5 cm confocal dishes and treated with EGFP-β2m and NC-CF for 3-5 hours for confocal imaging. Before imaging, nuclei and cell membranes were stained with Hoechst and DiI dyes, respectively, for 10-15 minutes. Cells were washed five times with PBS and analyzed by flow cytometry. The experiment included control (DMEM), EGFP-β2m only, and EGFP-β2m with NC-CF groups.

12. Cell viability assay

HEK293 cells were plated in 96-well plates at a density of 1×10^4 cells per well. NC-CF was diluted to concentrations of 0, 5, 10, 20, 30, and 60 μg/mL in serum-free DMEM and incubated at 37 °C with 5% CO_2 for 24 or 48 hours. Cell viability was assessed using the MTT assay, and absorbance was measured at 570 nm.

Supplementary figures

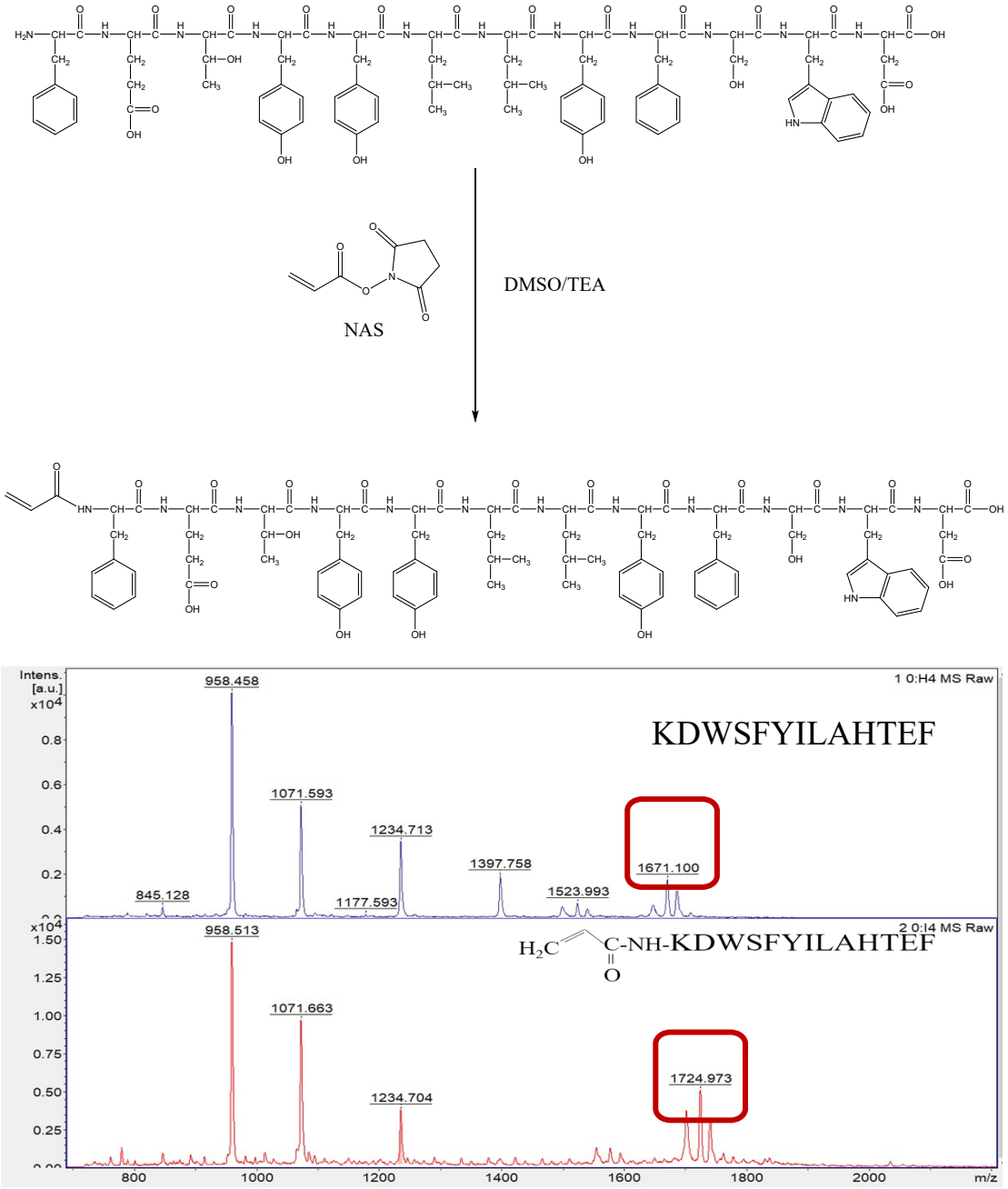

Figure S1. Schematic illustration and MALDI-TOF mass spectra of the synthesized peptide monomer. The CF peptide exhibited a molecular weight of 1671 Da, while the double bond-introduced peptide (ACR-peptide) showed a molecular weight of 1724.9 Da. The successful synthesis of the ACR-peptide monomer was confirmed by the observed mass increase.

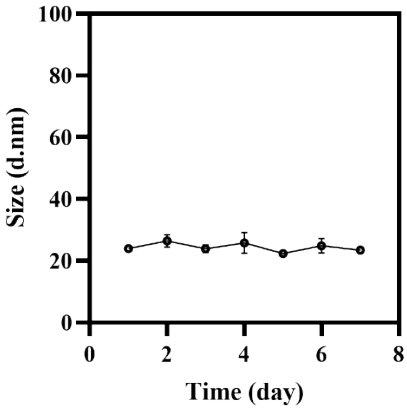

Figure S2. Particle size variation of NC-CF during one week. The size of the NC-CF (0.5 mg/mL) were evaluated using DLS.

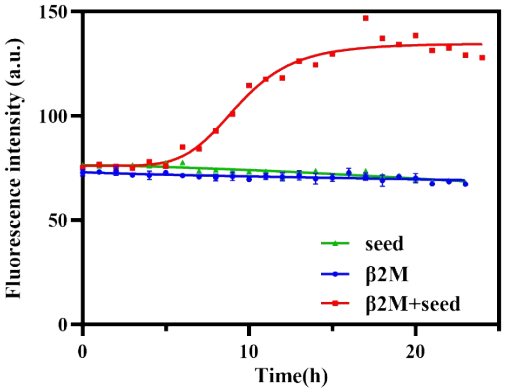

Figure S3. Aggregation kinetics of β2m after seeding.

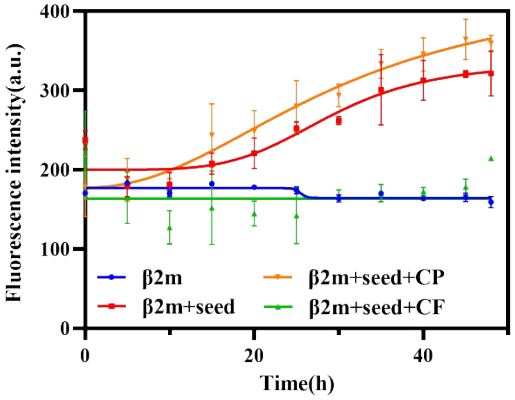

Figure S4. Aggregation kinetics of β2m incubated with CP/CF after seeding.

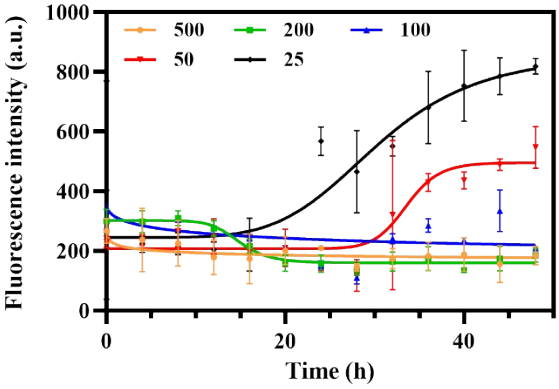

Figure S5. Aggregation kinetics of β2m incubated with NC-CF after seeding under different NC-CF concentrations (25, 50, 100, 200, 500 μg/mL).

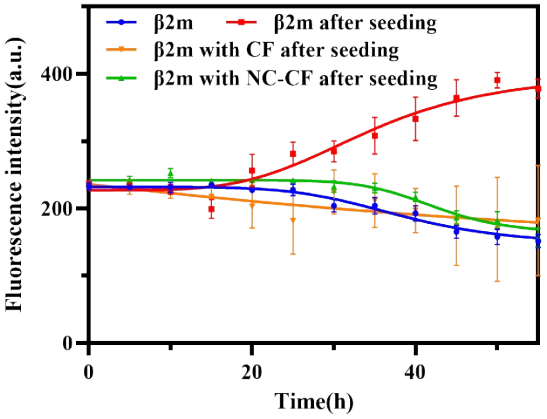

Figure S6. Aggregation kinetics of β2m incubated with CF/NC-CF after seeding in FBS.

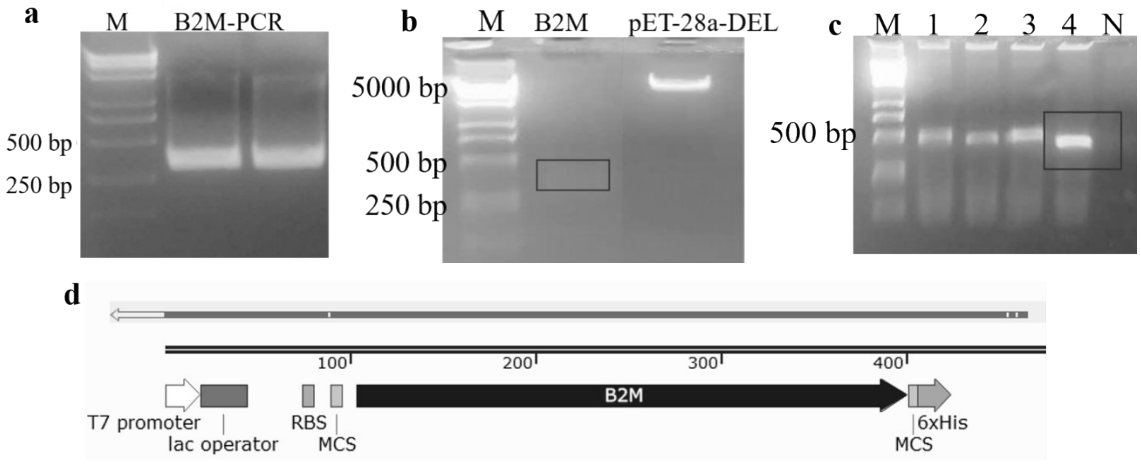

Figure S7. DNA agarose gel electrophoresis analysis for the construction of the recombinant plasmid pET-28a-β2m. (a) Amplification of the β2m gene, with a DNA sequence length of 318

bp. (b) Digestion of the β2m gene and vector plasmid. (c) Preliminary identification of the recombinant plasmid. The recombinant plasmid was introduced into E. coli, and the bacterial solution was plated. Single colonies were selected and directly amplified using gene-specific primers. Lane 'M' represents the DNA marker; lanes '1,2,3,4' correspond to amplified single colonies; lane 'N' represents the negative control. (d) Sequencing results confirming the successful construction of the recombinant plasmid.

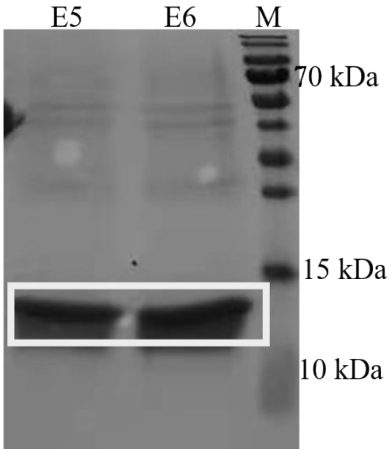

Figure S8. Expression and purification of fusion protein β2m-His by SDS-PAGE. The molecular weight of the protein was 13 kDa. Lane 'M' represents the protein ladder. Lanes ' E5' and ' E6' represent the fusion protein after purification.

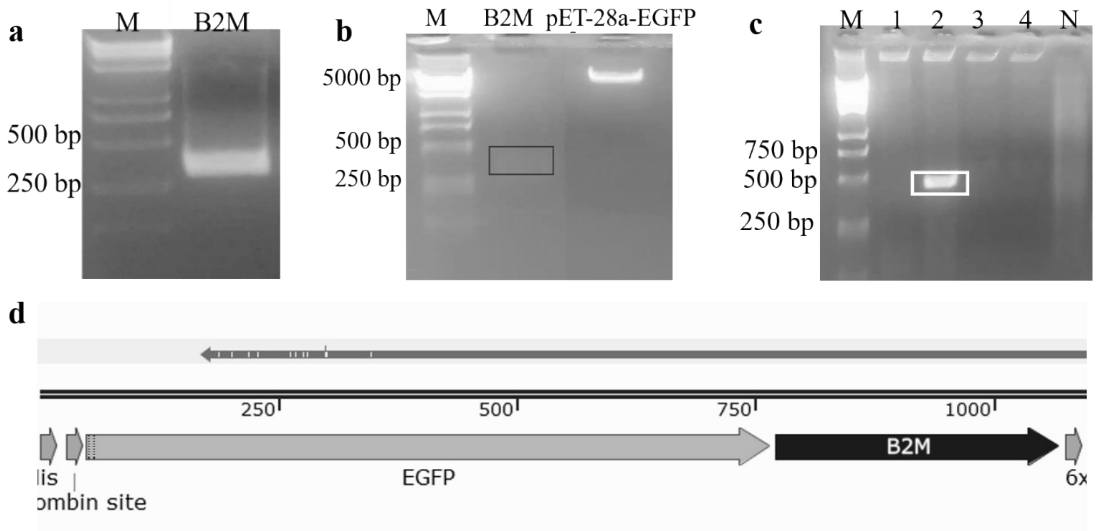

Figure S9. DNA agarose gel electrophoresis analysis for the construction of the recombinant plasmid pET-28a-EGFP-β2m. (a) Amplification of the β2m gene, with a DNA sequence length of 315 bp. (b) Digestion of the β2m gene and vector plasmid. (c) Preliminary identification of the recombinant plasmid. The recombinant plasmid was introduced into E. coli, and the bacterial solution was plated. Single colonies were selected and directly amplified using gene-specific primers. Lane 'M' represents the DNA marker; lanes '1,2,3,4' correspond to amplified

single colonies; lane 'N' represents the negative control. (d) Sequencing results confirming the successful construction of the recombinant plasmid.

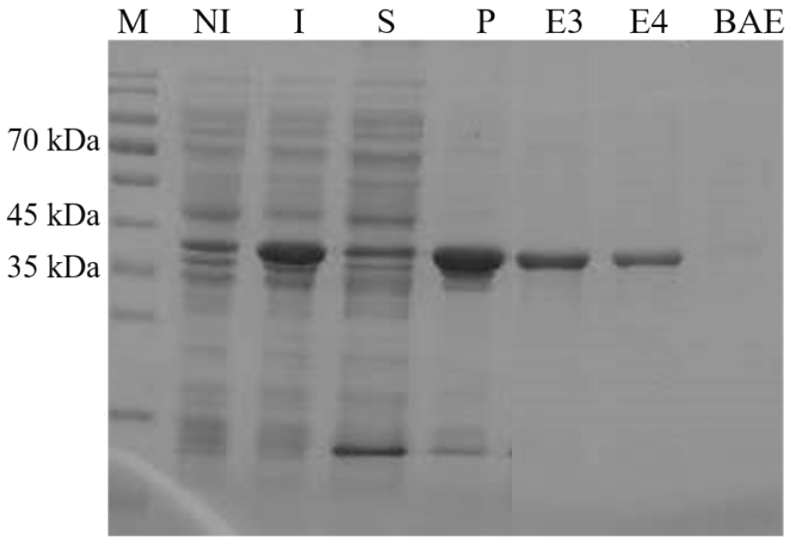

Figure S10. Expression and purification of the fusion protein EGFP-β2m analyzed by SDS-PAGE. The molecular weight of the target protein was 42 kDa. Lane 'M' represents the protein marker. Lane 'NI' shows the sample before induction, while lane 'I' represents the sample after induction. Lane 'S' corresponds to the supernatant obtained after cell lysis and centrifugation, and lane 'P' represents the pellet fraction. Lanes 'E3' and 'E4' contain purified fusion protein, and lane 'BAE' shows the beads after elution.

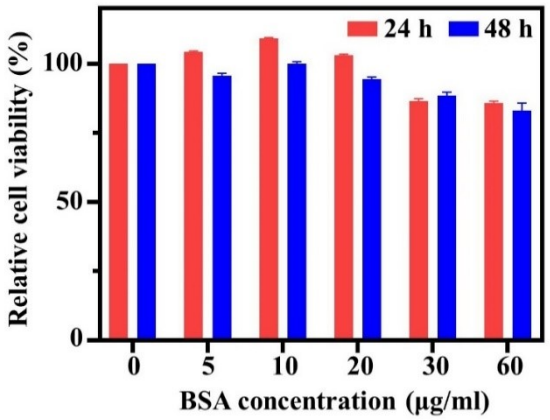

Figure S11. MTT assay results evaluating the cytotoxicity of NC-CF on HEK-293 cells. Cells were incubated with varying concentrations of NC-CF for 24 and 48 hours to assess cell viability. At concentrations up to 20 μg/mL, cell viability remained above 94% even after 48 hours of exposure. As the NC-CF concentration increased, cell viability remained relatively high, consistently exceeding 85%.