Enhancement of bioactivity, thermal stability and tumor retention by self-fused concatenation of green fluorescent protein

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ABSTRACT

The widespread application of protein and peptide therapeutics is hampered by their poor stability, strong immunogenicity and short half-life. However, the existing protein modification technologies require the introduction of exogenous macromolecules, resulting in inevitable immunogenicity and decreased bioactivity. Herein, we reported an easy but universal protein modification approach, self-fused concatenation (SEC), to enhance the *in vitro* thermal stability and *in vivo* tumor retention of proteins. In this proof of concept study, we successfully obtained a set of green fluorescence protein (GFP) concatemers, monomer (GFP 1), dimer (GFP 2) and trimer (GFP 3) of GFP, and systematically studied the effects of SEC on the biological activity and stability of GFP. Notably, GFP concatemers displayed remarkable improvement in *in vitro* bioactivity and thermal stability over the monomeric GFP. In a murine tumor model, GFP 2 and GFP 3 exhibited significantly prolonged duration, with increases of 220- and 381-fold relative to GFP 1 in tumor retention 4 h after administration. Furthermore, the biological activity, thermal stability and tumor retention can be enhanced by the concatenated number of self-fused proteins. These findings demonstrate that SEC may be a promising alternative to design advanced protein and peptide therapeutics with enhanced pharmaceutic profiles.

1. Introduction

Compared with simple chemical compounds, proteins and peptides have great potential in clinic for medical therapy due to their high specificity and low toxicity [1–5]. A variety of FDA-approved therapeutic agents (like Etanercept, Insulin glargine, Pegfilgrastim, Bivalirudin, Cyclosporine and Octreotide) have been applied in fields such as oncology, immunology, viral diseases and endocrinology [6,7]. However, when widespread application as therapeutic agents, the proteins and peptides are profoundly restricted due to their intrinsic disadvantages, such as poor stability, strong immunogenicity and quick clearance from circulation [1,8,9]. Therefore, frequent administrations of proteins/peptides are required to realize high level in circulatory system and achieve therapeutic effects, resulting in dramatically oscillating concentration in blood and serious adverse effects [10–12].

Numerous approaches have been developed to partially solve the obstacles above and increase the delivery efficiency of proteins/peptides [13–20]. For example, covalent conjugation of a nontoxic polymer, typically poly(ethylene glycol) (PEG) to a protein, named PEGylation, can effectively extend the circulating half-life and increase the stability of the protein [7,21,22]. Many PEGylated protein therapeutics, such as PEG-interferon α-2a (PEG-IFNα-2a), PEG-ʟ-asparaginase, PEG-adenosine deaminase, PEG-uricase and PEG-tumor necrosis factor alpha (PEG-TNFα), PEG-continuous erythropoietin receptor activator (PEG-CREA) have been applied in clinic and exhibit better pharmaceutical profiles compared to the unmodified forms [7,23,24]. Genetic fusion of a protein with long-lived human serum albumin (HSA) or Fc fragment is another successful method that has been used to increase the *in vivo*
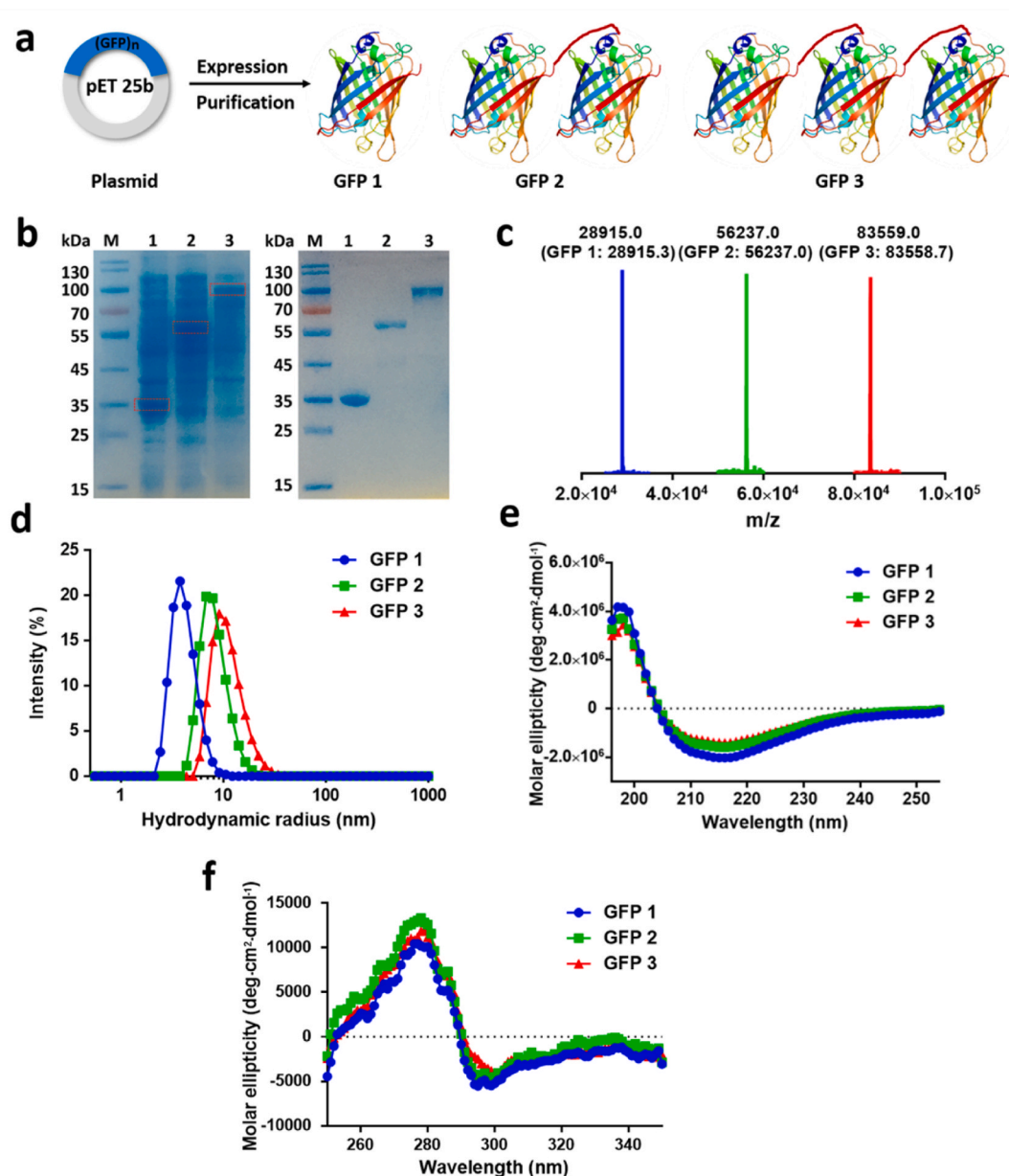
Fig. 1. Biosynthesis and characterization of GFP concatemers. a) Schematic illustration of the design and synthesis of GFP concatemers via SEC. b) SDS-PAGE analysis of GFP concatemers. Left: expression of GFP concatemers. Lane 1, crude *E. coli* lysate of GFP 1; lane 2, crude *E. coli* lysate of GFP 2; lane 3, crude *E. coli* lysate of GFP 3. Right: purified GFP concatemers. Lane 1: purified GFP 1; lane 2: purified GFP 2; lane 3: purified GFP 3. c) The LC-ESI-MS measurements of GFP concatemers. d) DLS analysis of GFP concatemers. e) CD spectra of GFP concatemers from 195 to 255 nm. f) CD spectra of GFP concatemers from 250 to 350 nm.

circulating half-life of IFNα, antihemophilic factor (AHF), recombinant factor IX (F9), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and anti-vascular endothelial growth factor (anti-VEGF) [7,25–28]. Most recently, we have developed two general approaches to prolong the circulating half-life of IFN via *in situ* growth of PEG-like polymer poly (oligo(ethylene glycol) methyl ether methacrylate) (POEGMA) or fusion of artificial biopolymer elastin-like polypeptide (ELP) to yield well-defined IFN-POEGMA conjugate [29] or IFN-ELP fusion protein [30,31], respectively. We call these two methodologies site-specific *in situ* polymerization (SIP) [29,32,33] and ELPfusion [30,31]. However, whether the commercialized techniques (PEGylation, HSA and Fc fusion) or the newly developed techniques (SIP and ELPfusion) require the introduction of exogenous macromolecules for modification, which leads to inevitable immunogenicity, decreased bioactivity and other potential toxic effects [23,33–39].

In this study, we introduce a new but universal protein modification methodology -self-fused concatenation (SEC)- to enhance the stability and extend the *in vivo* half-life of proteins (Fig. 1a). As the first case, we selected green fluorescent protein (GFP) as the model protein and designed a set of concatemers in high yield using this protein fusion technique. We systematically studied the effects of self-fused modification on the biological activity and stability of GFP. Interestingly, the tandem GFPs exhibited remarkable improvement in bioactivity, *in vitro* thermal stability and *in vivo* tumor retention over the monomeric GFP, and the results were further enhanced by increasing the concatenated number of self-fused GFP. Based on these exciting results, we believe that SEC may be a promising protein modification alternative to optimize the pharmaceutic profiles of protein and peptide therapeutics without the introduction of potential toxic effects.

2. Materials and methods

2.1. Materials

All biological reagents and chemical reagents, unless otherwise stated, were commercially obtained from Sigma Aldrich. C26 cells were purchased from the cell bank of the Chinese Academy of Medical Sciences. Female BALB/c-nude mice were purchased from Vital River Laboratories (Beijing, China).

2.2. Biosynthesis and purification of GFP concatemers

The details of construction, expression and purification of GFP concatemers were listed in the supplementary materials.

2.3. Liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS)

The proteins were separated by a 10 min gradient elution at a flow rate of 0.5 mL/min with the ACQUITY UPLC system, which was directly interfaced with a SYNAPT-G2-Si mass spectrometer (Waters, USA). Mobile phase A consisted of 0.1% formic acid aqueous solution, and mobile phase B consisted of 100% acetonitrile and 0.1% formic acid. The analytical column was a Protein BEH C4 silica capillary column (2.1 mm ID, 100 mm length; Made in Ireland) packed with C-4 resin (300 Å, 1.7 μm; Waters, USA). Aliquots of 2 μL analytes were loaded into an autosampler for nanoelectrospray ionization. Samples were analyzed on a Q-TOF mass spectrometer (SYNAPT G2-Si; Waters, USA) instrument optimized for high-mass protein analysis. The measurements were performed with capillary 3000 V and data were collected over the expected m/z range. Once having acquired raw native electrospray mass spectra, the raw spectrum can be deconvoluted by MaxEnt 1 (Waters, USA) to generate a spectrum (relative intensity versus mass) where all the charge-state peaks of a single species have been collapsed into a single (zero-charge) peak.

2.4. Dynamic light scattering (DLS)

The hydrodynamic radius (R_h) of proteins were assessed by DLS using a Malvern Zetasizer Nano-zs90 with a laser wavelength of 633 nm and a scattering angle of 90° at 25 °C. Samples were treated with a filter of 0.22 μm pore size before analysis. Data were analyzed using Zetasizer software 6.32.

2.5. Circular dichroism (CD)

The secondary structures of proteins analysis were performed on a Pistar π-180 (Applied Photophysics Ltd, UK) instrument with a far-UV region from 195 to 255 nm. Proteins were diluted in deionized H$_2$O (a concentration of approximately 0.18, 0.25 and 0.28 mg/mL for GFP 1, GFP 2 and GFP 3, respectively) and placed in quartz cuvettes (Hellma, Germany) of 1 mm path length during detection. The tertiary structures of protein were also examined in a near-UV region (wavelengths between 250 and 350 nm). The proteins were diluted in 50 mM Tris•HCl, 150 mM NaCl, pH 7.4 at a concentration of approximately 0.4, 0.45 and 0.50 mg/mL for GFP 1, GFP 2 and GFP 3, respectively, and analyzed under 10 mm path length. Data were analyzed using CDNN V2.1.

2.6. UV–vis absorption spectra

UV–vis absorption spectra (230 nm–600 nm) were recorded on a Varioskan Flash Microplate Reader (Thermo Scientific, USA) at room temperature at the same protein mass concentrations (a concentration of 2.4 mg/mL for GFP concatemers) and the same molar concentrations (a concentration of 25 μM for GFP 1, GFP 2 and GFP 3).

2.7. Bioactivity retention quantification

The fluorescence spectra (480 nm–570 nm) were performed on a Varioskan Flash Microplate Reader (Thermo Scientific, USA) at room temperature at the same mass concentration (a concentration of 0.25 mg/mL) and the same molar concentration (a concentration of 10 μM), excited at 460 nm. The fluorescence was evaluated with the excitation wavelength at 460 nm and the emission wavelength at 507 nm. The fluorescence retention per GFP of proteins was calculated by comparing the fluorescence of GFP concatemers with that of monomeric GFP at the same mass concentration, as the fluorescence intensity of GFP in GFP 1 was defined as 100% retention. The relative number of GFP per protein was calculated by comparing the fluorescence of GFP concatemers with that of monomeric GFP (GFP 1) at the same molar concentration, as the number of GFP in GFP 1 was considered as one equivalent.

2.8. In vitro thermal stability

For *in vitro* thermal stability assays, the proteins were simply denatured at 90 °C for 2 min and renatured at room temperature. Before heating, the fluorescence concentrations of samples were adjusted to the same (a final protein fluorescence concentration of 2500 determined by microplate reader, equivalent to approximately 10, 7 and 5 μM of GFP 1, GFP 2 and GFP 3, respectively) before analysis. The recovered fluorescence values of proteins were quantified at given times (1, 5, 10, 15, 20, 25, 30, 45, 60 and 120 min) as described above. For the plasma stability assays, the proteins of same fluorescence concentrations were incubated with plasma at a ratio of 1:1 (v/v) at 65 °C and the fluorescence were quantified at given times (5, 10, 15 and 30 min, 1, 2, 4, 6, 8, 12, 24 and 36 h). The plasma were obtained from the orbit of rats under standard protocols.

2.9. In vivo tumor retention

All animal experiments applied in this study were performed strictly with the approval of Peking Union Medical College Hospital Institutional Animal Care and Use Committee (IACUC). The protocols of *in vivo* tumor retention assay were employed as previously described with slight modifications [32]. Briefly, C26 cells were cultured in RPMI-1640 complete medium containing 10% fetal bovine serum (FBS), 4.5 g/L D-glucose, 10 mM HEPES, 1 mM sodium pyruvate, 1 mM nonessential amino acid (NEAA) and 1% penicillin/streptomycin at 37 °C in 5% CO$_2$ humidified atmosphere. Cells were harvested by trypsinization, washed and resuspended in fresh RPMI-1640 without any additives. Female BALB/c nude mice of eight weeks old received subcutaneous injections of 2.5×10^6 C26 cells in the flank of left hind legs. When the tumor volume reached 100–150 mm^3, mice were randomly assigned into 3 groups and intratumorally injected with 50 μL of GFP 1, GFP 2 and GFP 3. Proteins were adjusted to similar fluorescence concentration before dosing (the fluorescence concentration was 3500 determined by microplate reader, equivalent to approximately 15, 9 and 7 μM of GFP 1, GFP 2 and GFP 3, respectively). Tumor volume was calculated using the formula: volume = (width × width) × length)/2. The fluorescence values of proteins in tumors were carried on an IVIS Lumina II *in vivo* imaging system (Caliper Life Sciences, USA) at given times (1, 5, 10, 15, 20, 30, 45 min and 1, 1.5, 2,3, 4, 6, 8, 12 h). The images were analyzed by Living Image 4.2 software.

2.10. Statistical analysis

Data were performed using GraphPad Prism software 8.0 and were shown as the mean ± standard deviation. Comparisons of the data including bioactivity retention quantification, *in vitro* thermal stability and *in vivo* tumor stability were analyzed using Student's *t*-test and one-way ANOVA. A *P* value less than 0.05 was considered significant. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.
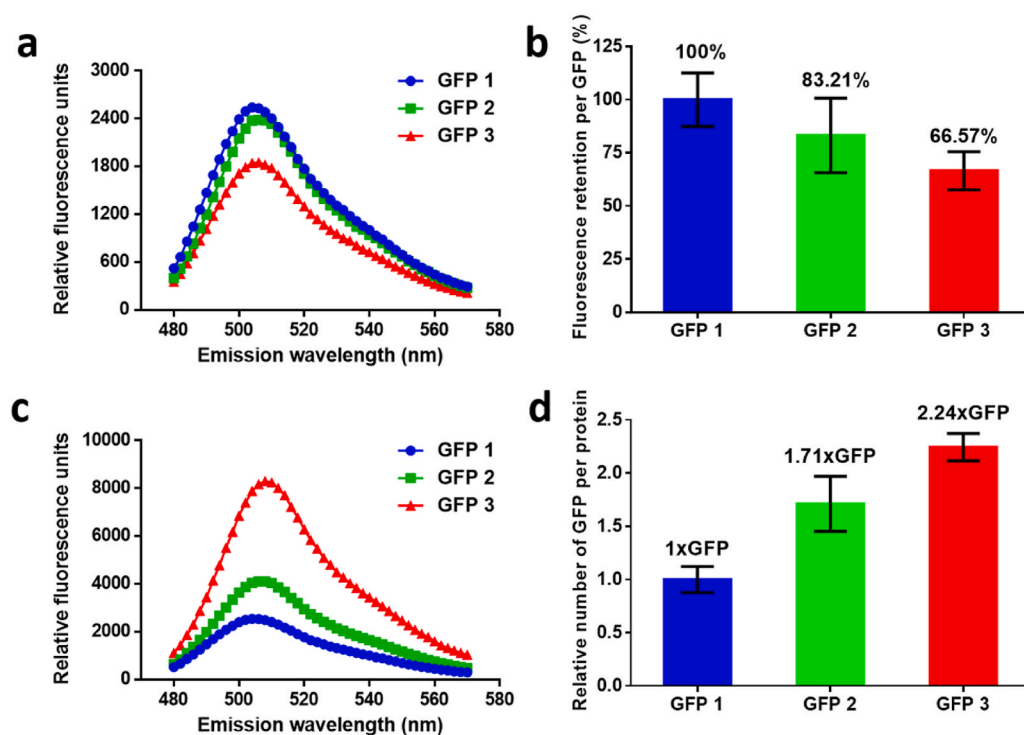
Fig. 2. The fluorescence of GFP concatemers with the excition wavelength at 460 nm. a) The fluorescence spectra of GFP concatemers at the same mass concentration. b) The fluorescence retention of concatemers per unit of GFP. Data are shown as the mean ± standard deviation (n = 3, $P < 0.05$ for GFP 3 vs GFP 1). c) The fluorescence spectra of GFP concatemers at the same molar concentration. d) The relative number of GFP per protein. Data are shown as the mean ± standard deviation (n = 3, $P < 0.05$ for GFP 3 vs GFP 1 and GFP 2 vs GFP 1).

3. Results

3.1. Biosynthesis and physicochemical characterization of GFP concatemers

To access concatenated GFPs, we constructed three recombinant plasmids that can express monomer (GFP 1), dimer (GFP 2) and trimer (GFP 3) of GFP, with a 6 × His tag (H6) fused at the C-terminus for purification; the subunits of concatemers were spaced by the flexible linker GGGGS (Fig. 1a). Herein, we chose these three macromolecules with the molecular weights (MWs) below, around and above 60 kDa, since the cut-off for glomerular filtration is approximately 60 kDa [40, 41]. After overexpression in *Escherichia coli (E. coli)* and rapidly purified with a high yield of ~100 mg/L (Figs. S1–S3), the proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results showed only a single band around the MW position of approximately 30, 60 and 90 kDa that corresponded to GFP 1, GFP 2 and GFP 3, respectively (Fig. 1b). Liquid chromatography-electrospray ionization mass spectrometry (LC-E-SI-MS) further confirmed the MWs of GFP 1, GFP 2 and GFP 3 were 28915.0, 56237.0 and 83559.0 Da, which were consistent with the theoretical MW of 28915.3, 56237.0 and 83558.7 Da, respectively (Fig. 1c). The data above showed that we had successfully obtained the high quality of concatenated GFPs with one, two and three repeats of GFP.

The hydrodynamic radius (R_h) of GFP 2 and GFP 3 evaluated by dynamic light scattering (DLS) were 6.85 and 9.62 nm, which were 1.83- and 2.57-fold larger than that of GFP 1 (3.75 nm) (Fig. 1d). The secondary structures of GFP concatemers analyzed by circular dichroism (CD) spectroscopy in the "far-UV" region displayed the typical signature of β-barrels, with minima at 217–218 nm and maxima at 195–198 nm (Fig. 1e). Moreover, the spectra of GFP 2 and GFP 3 were in accordance with GFP 1 in the "near-UV" region (Fig. 1f), which roughly suggested that the tertiary structure did not change significantly. The CD results indicated that self-fused modification had no obvious influence on the conformation of the GFP. The UV–vis absorption spectra of GFP 1, GFP 2 and GFP 3 quantified at similar mass (Fig. S4) or molar (Fig. S5) concentrations were overlapped and exhibited two absorption peaks at 280 and 478 nm, suggesting the well retained photophysical properties of GFP.

3.2. Bioactivity retention

To characterize the biological activity of proteins, the fluorescence spectra were quantified at similar concentrations. The mass or molar concentrations of GFP concatemers were confirmed by UV–vis absorption at 280 nm (Fig. S6 and Fig. S7). The fluorescence spectra of concatenated GFPs (GFP 2 and GFP 3) and monomeric GFP (GFP 1) were coincident with each other, and the emission maxima was at 507 nm (Fig. 2). As expected, the monomeric GFP possessed the highest fluorescence intensity per unit of GFP, whereas the fluorescence retention in concatemers decreased with the increasing concatenated number due to the steric hindrance by the fused GFP subunits (Fig. 2a), with a retention activity of 83.21% and 66.57% for GFP 2 and GFP 3, respectively (Fig. 2b). However, the fluorescence intensity per protein was significantly increased with the increasing MW of GFP concatemers (Fig. 2c). Particularly, GFP 2 and GFP 3 had an equivalent of 1.71x and 2.24x GFP per protein after calculation (Fig. 2d). We regarded that the increased activity caused by the number of fused GFP outperformed the reduced activity due to the steric hindrance, thus resulting in the improved bioactivity in concatemers. Note that the general principle of the existing techniques (including the commercialized PEGylation, HSA and Fc fusion, and the newly published SIP and ELPfusion) was to introduce inactive macromolecules (such as nontoxic polymers or long-lived proteins) on the surface of proteins, thus leading to significantly reduced biological activity, and sometimes the biological activity can be decreased to only 1% comparing to the unmodified proteins [42,43]. To sum up, the results suggest that SEC can remarkably enhance the *in vitro* bioactivity of proteins via genetic fusion of active proteins themselves.

3.3. In vitro thermal stability

How to increase the stability of engineering proteins and peptides is a difficult but very important challenge in practical application. We
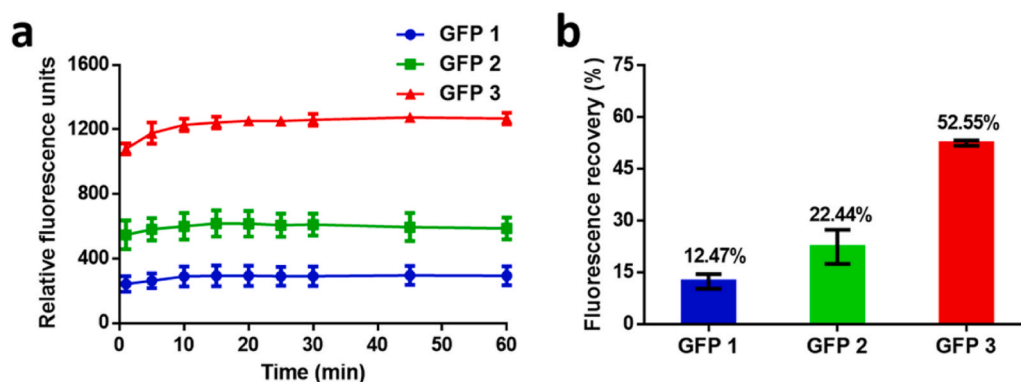
Fig. 3. Thermal stability of GFP concatemers after denaturation at 90 °C for 2 min. a) Fluorescence recovery of GFP as a function of time. Data are shown as the mean ± standard deviation (n = 3, *P* < 0.0001 for GFP 2 and GFP 3 vs GFP 1). b) The fluorescence recovery percentage of GFP concatemers after denaturation at 90 °C for 2 min. Data are shown as the mean ± standard deviation (n = 3, *P* < 0.05, **P* < 0.01 and ****P* < 0.0001 for GFP 2, GFP 3 vs GFP 1).
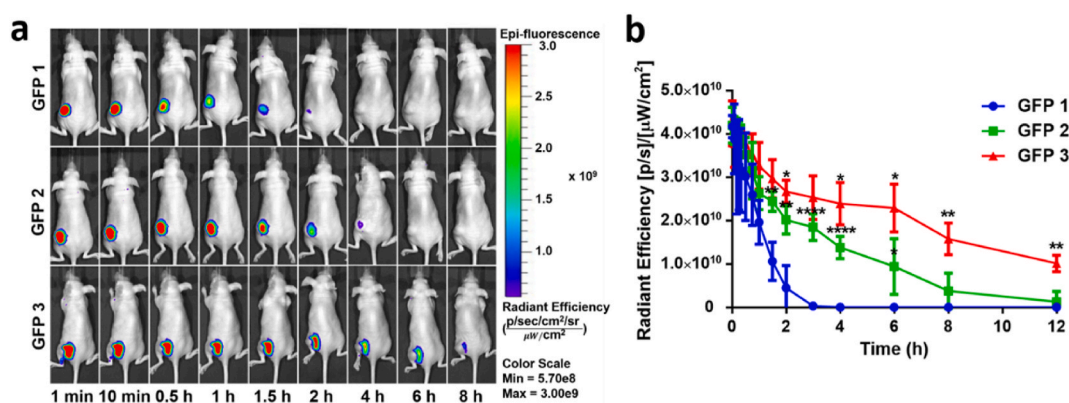

Fig. 4. Fluorescence retention of GFP concatemers in tumors. a) Dynamic observation of *in vivo* fluorescence imaging of GFP concatemers in mice. b) The fluorescence of GFP concatemers in tumors as a function of time. Data are shown as the mean ± standard deviation. (n = 4, *P* < 0.05 and **P* < 0.01 for GFP 3 vs GFP 2, **P* < 0.01 and ****P* < 0.0001 for GFP 2 vs GFP 1.)

quantified the fluorescence recovery after denaturation at 90 °C for 2 min. Before heating, we adjusted the fluorescence concentrations of samples to be consistent. Obviously, GFP 3 showed a much quicker fluorescence recovery than GFP 2, and GFP 2 exhibited a much quicker recovery than GFP 1 immediately (Fig. 3a and Fig. S8). Two hours after heating, the recovery fluorescences of GFP 3 (53%) and GFP 2 (22%) were 4.2- and 1.8-fold higher than that of GFP 1 (12%), respectively (Fig. 3b). The data suggest that the thermal stability of GFP concatemers is positively correlated with the concatenated number of proteins. We have also tested the stability of GFP concatemers in plasma and they showed similar behaviors of fluorescence declined, indicating the well retained stability in plasma (Fig. S9).

3.4. In vivo tumor retention

One of the major problems in protein delivery is their poor *in vivo* stability. Oligomerization can effectively increase the MW and enlarge the size of protein, resulting in low permeability of glomerular cut-off pore sizes and better tissues retention. Therefore, we supposed that SEC can well extend the *in vivo* stability and retention time. In order to verify our hypothesis, we investigated the *in vivo* stability of GFP concatemers in the tumor using the C26 mouse model. BALB/c nude mice with a mean tumor size of ~100 mm³ were intratumorally administrated with GFP 1, GFP 2 and GFP 3 at a dosage of the same initial fluorescence value. The change of fluorescence was measured by *in vivo* mice imaging. GFP 1 was quickly cleared from the tumor and the fluorescence could seldom be detected within 2 h, while GFP 2 exhibited a

slower clearance and the fluorescence was rarely detected until 4 h (Fig. 4a). In contrast, the fluorescence of GFP 3 was significantly prolonged and could still be observed even after 6 h (Fig. 4a). Particularly, the fluorescence values of GFP 2 (2.01 × 10¹⁰ photons) and GFP 3 (2.67 × 10¹⁰ photons) in tumors were 4.45- and 5.91-fold higher than that of GFP 1 (4.52 × 10⁹ photons) 2 h after dosing, and the fluorescence values of GFP 2 (1.38 × 10¹⁰ photons) and GFP 3 (2.39 × 10¹⁰ photons) increased by 220- and 381-fold as compared to GFP 1 (6.27 × 10⁷ photons) 4 h after injection (Fig. 4b). Taken together, these data indicate that increasing the MW of GFP concatemers can remarkably extend the tumor retention and enhance the *in vivo* stability of GFP due to the increased hydrodynamic size and thermal stability.

4. Discussion

Herein, we report an easy but universal protein modification strategy -self-fused concatenation (SEC) to improve the *in vitro* biological activity, thermal stability and enhance the *in vivo* stability of proteins without the introduction of extra macromolecules. As the first case, we successfully expressed a series of GFP concatemers in prokaryotic *E. coli* system and systematically investigated the effects of SEC modification on the bioactivity and stability of GFP. Notably, concatenated GFPs displayed significantly increased bioactivity and thermal stability relative to the unmodified GFP. Furthermore, GFP concatemers could remarkably extend the tumor retention and enhance the *in vivo* stability of GFP, resulting in 220.23- and 381.29-fold increases in tumors for GFP 2 and GFP 3 compared to GFP 1 after injection for 4 h. Our results also

display a MW rationale for the design of advanced protein/peptide therapeutics, that is the increases of *in vitro* bioactivity, thermal stability and *in vivo* tumor stability of GFP concatemers are positively correlated with the concatenated numbers. This proof-of-concept study demonstrate that SEC may be a promising modification alternative to optimize the pharmaceutic profiles of protein and peptide therapeutics without the introduction of potential toxic effects.

Authorship contribution

J. H. conceived the project. J. H., J. S. and Y. Y. performed most of the experiments and data analysis. J. H., J. S., Y. Y., S. Li and B. Z. purified the proteins and performed characterization. J. H., J. S., and H. D. performed the animal studies. J. H. wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101112.

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