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Filamentous Chaperone Protein-Based Hydrogel Stabilizes Enzymes against Thermal Inactivation†

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Dawei Xu,[a] Samuel Lim,[a] Yuhong Cao,[b] Abner Abad,[a] Aubrey Nayeon Kang,[a] and Douglas S. Clark*[ac]

We report a filamentous chaperone-based protein hydrogel capable of stabilizing enzymes against thermal inactivation. The hydrogel backbone consists of a thermostable chaperone protein, the gamma-prefoldin (γPFD) from *Methanocaldococcus jannaschii*, which self-assembles into a fibrous structure. Specific coiled-coil interactions engineered into the wildtype γPFD trigger the formation of a cross-linked network of protein filaments. The structure of the filamentous chaperone is preserved through the designed coiled-coil interactions. The resulting hydrogel enables entrapped enzymes to retain greater activity after exposure to high temperatures, presumably by virtue of the inherent chaperone activity of the γPFD.

Hydrogels are cross-linked networks of polymers that contain a large amount of water dispersed throughout their three-dimensional structure. Their high-water content, tunable viscoelasticity, and biocompatibility render hydrogels particularly attractive for biotechnology applications.[1-5] Recently, protein-based hydrogels have gained increasing attention because they can be mass-produced from microbial hosts and their bulk properties can be conveniently engineered through genetic encoding[6-8]. Although a broad range of structural proteins such as collagen, gelatin, keratin, sericin, and fibroin have been previously employed to form hydrogels,[6, 9-16] the function of these proteins is limited to serving as the structural scaffold of the hydrogel. Thus, bioactive self-assembling protein templates offer the advantage of imparting inherent functions into the hydrogel backbone.

In this work, we created a novel protein-based hydrogel using a thermostable filamentous chaperone protein, gamma-prefoldin (γPFD) from the hyperthermophilic archaeon *Methanocaldococcus jannaschii*.[17, 18] γPFD subunits form filaments up to several μms in length through homomeric self-assembly of their beta-sheet domains, while the coiled-coil domains protrude outwards in a helical manner; these coiled-coils may interact with a broad range of protein substrates to stabilize them, allowing γPFD to function as a molecular chaperone.[19] Besides its inherent chaperone activity, the γPFD filaments offer several advantages as protein scaffolds including excellent thermostability (Tm=93°C), binding capacity for metals, and structural malleability.[20-23] In particular, because the helical assembly of γPFD exposes both termini of the protruding coiled-coils, γPFD can be genetically engineered into fibers displaying an array of functional moieties in a 360-degree pattern.[20, 22] Thus, introducing a cross-linking domain results in a γPFD-based protein hydrogel, which may retain the intrinsic ability of γPFD to stabilize proteins of interest.

The structural elements of coiled-coils are well understood to allow for highly specific and precise interactions.[24, 25] We genetically fused a helical coil consisting of positively- and negatively-charged segments to the C-terminal end of the γPFD monomer. These coils with charged segments should prefer to assemble with their counterparts in an antiparallel fashion. The subsequent coiled-coil interactions will favor the formation of inter-filament cross-links rather than intra-filament interaction between the coils bound to the same filament. In this way, the modified γPFD filaments will form a cross-linked hydrogel network. Using coiled-coil interactions to form a hydrogel avoids the need for chemical cross-linking reagents, and should also maintain the structure of the prefoldin filament, which is crucial for preserving its chaperone activity. Moreover, this hydrogel-forming variant of γPFD can be readily produced in an *E. coli* host without any need for post-translational modification, allowing for convenient production as well as potential *in vivo* applications.

In our design, formation of the γPFD-hydrogel network requires antiparallel coiled-coil interactions between neighboring fibers. To ensure this occurs, we genetically fused a 28-amino-acid coil comprising two positively charged coils (K-coils) and two negatively charged coils (E-coils) to the C-terminal end of the

[a] Department of Chemical and Biomolecular Engineering, University of California, Berkeley, CA 94720, USA. E-mail: dsc@berkeley.edu
[b] Department of Chemistry, University of California, Berkeley, CA 94720, USA.
[c] Address here Molecular Biophysics and Integrated Bioimaging Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA 94720, USA.
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γPFD (γPFD-K_2E_2). The fusion of the coils was confirmed by DNA sequencing and SDS-PAGE (**Figure S1).** As illustrated in Figure 1A, γPFD-K_2E_2 filaments should crosslink through antiparallel coiled-coil dimeric formations between the K_2E_2 domains. Conversely, the K_2E_2 domains on the same filament will repel each other, thereby avoiding intra-filament interactions. The crosslinked network of protein filaments subsequently forms a hydrogel once dissolved in water.

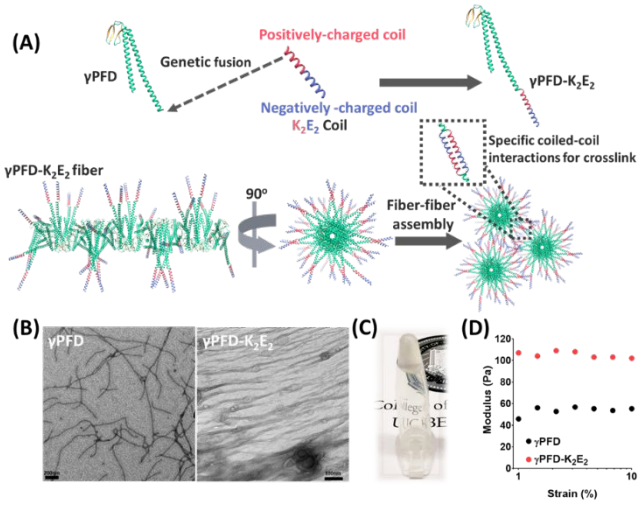

Figure 1. (A) Scheme of self-assembly of γPFD-K_2E_2 hydrogel. C-terminus of prefoldin is modified with alternating segments of positively charged (K-coil) and negatively charged (E-coil) peptides. The K_2E_2 coil will crosslink prefoldin fibers and form hydrogel. (B) TEM images of γPFD and γPFD modified with K_2E_2 coils. (C) Prefoldin hydrogel formed in a microcentrifuge tube; the gel does not flow in the inverted tube. (D) Amplitude sweep of γPFD and γPFD-K_2E_2 at 5%wt, measured at a fixed frequency of 1 Hz.

Transmission electron microscopy (TEM) images of γPFD-K_2E_2 and wildtype γPFD are shown in **Figure 1B**. The γPFD and γPFD-K_2E_2 were imaged on a carbon support at 0.5mg/ml to avoid protein gelation (the minimal concentration for gelation is 10 mg/ml). As expected, wildtype γPFD formed characteristically long and relatively straight filaments distributed in random directions. In similar fashion, γPFD-K_2E_2 assembled into long and relatively straight filaments. However, the γPFD-K_2E_2 filaments were aligned in parallel, indicative of the interactions between the genetically fused K-coils and E-coils.

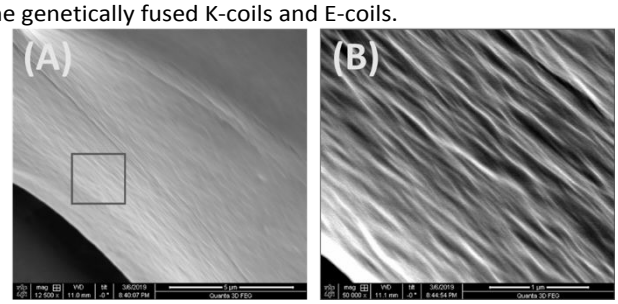

Figure 2. SEM images of γPFD-K_2E_2 hydrogel. (A) Images at 12500× magnification and (B) at 50000× magnification.

When dissolved in water, γPFD-K_2E_2 filaments should crosslink and align with one another through coiled-coil interactions. To study the crosslinked filament network, we flash froze the γPFD-K_2E_2 in its gel form, and the freeze-dried γPFD-K_2E_2 gel was characterized by scanning electron microscope (SEM). The SEM images (**Figure 2**) revealed the freeze-dried γPFD-K_2E_2 architecture comprising a crosslinked filament network. Consistent with the assembly pattern observed from TEM, the γPFD-K_2E_2 in high concentration aligns in an ordered and parallel manner through charged coiled-coil interactions. However, such a patterned assembly was not evident for wildtype γPFD with either TEM (**Figure 1B**) or SEM (**Figure S3**). In addition, oscillatory rheology experiments indicated that the ordered γPFD-K_2E_2 assembly resulted in gels with stronger mechanical properties compared to wildtype γPFD (**Figure S4**).

In nature, archaeal PFDs function mainly as "holdases," capturing nonnative proteins of varying size to stabilize them during the delivery to downstream chaperonins[26]. Thus, we hypothesized that our γPFD-K_2E_2 hydrogel could entrap proteins inside the cross-linked filament network, and the flexible helical arms of the γPFD could interact with proteins with a broad range of sizes. We therefore tested the release of protein from within the gel. Four model heme-containing proteins of various molecular weights (cytochrome C, 12.3kDa; horseradish peroxidase, 40kDa; hemoglobin, 64.5kDa; catalase, 250kDa) were examine for protein release. The experiment was conducted at pH 8 by measuring the increase of UV absorbance from heme in solution[27,28]. At room temperature, ~70% of the total heme proteins remained in the gel after 24 hours (**Figure S6**), and all four model proteins were released at similar rates, indicating that the flexible crosslinked prefoldin network could accommodate proteins of varying size to immobilize them without a need for additional reagents or chemical modification.

Gel stability is another important property when the hydrogel is used for protein encapsulation. The stability of the γPFD-K_2E_2 gel was investigated under aqueous conditions. Gels were cured for 6 hours and submerged in phosphate buffer to determine the degree of gel erosion based on the amount of protein released into solution. At room temperature, only ~30% of total γPFD was released after 24 hours (**Figure S5**). The results from the protein release test and gel stability test reveal that the γPFD-K_2E_2 gel can efficiently entrap proteins of various sizes with considerable gel stability.

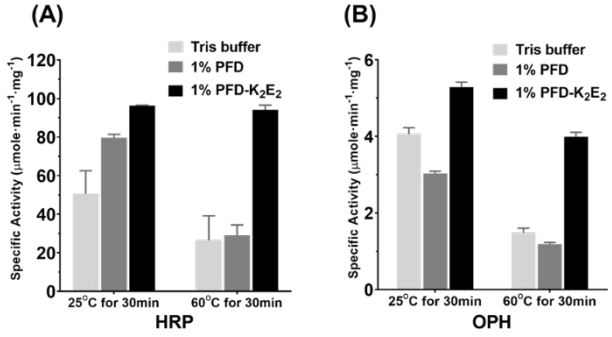

Figure 3. Thermal protection of enzymes by γPFD-K_2E_2 hydrogel. (A) Activity of horseradish peroxidase at 25°C after heat treatment. All samples were incubated at 25°C or 80°C for 30

min (n=3). (B) Activity of organophosphorus hydrolase at 25°C after heat treatment. All the samples were treated at 25°C or 60°C for 30 min(n=3).

We further speculated that the crosslinked hydrogel system engineered from a natural ultra-thermostable chaperone could stabilize entrapped proteins against thermal inactivation. Horseradish peroxidase and organophosphorus hydrolase were chosen as model enzymes to investigate the stabilizing effect of the γPFD-K_2E_2 hydrogel. As shown in **Figure 3** at temperatures of 80°C and 60°C, respectively, horseradish peroxidase and organophosphorus hydrolase lose substantial activity after 30 min in Tris buffer. However, when the enzymes were entrapped within the γPFD-K_2E_2 gel, ~95% (horseradish peroxidase) and ~74% (organophosphorus hydrolase) enzyme activity remained after 30 min.

Engineering thermostable variants of enzymes often requires multiple enzyme-specific designs and optimizations. In contrast, our strategy of entrapping enzymes in a chaperone-based hydrogel provides a cost-effective and practical method of protecting enzymes from thermal inactivation. Moreover, because the γPFD-K_2E_2 gel is a protein-based hydrogel, it could potentially be expressed together with other proteins *in vivo* to afford enzyme stabilization within whole cells.

In conclusion, we have developed a new type of hydrogel based on an ultrastable filamentous chaperone protein. The filamentous chaperone hydrogel network was formed through genetically-fused positively and negatively charged helical domains. This design allows gel formation without disrupting the filamentous protein structure and chaperone function. Proteins of varying size were stably immobilized into the chaperone-based gel network. Furthermore, the hydrogel showed excellent thermal protection ability for two model enzymes. As demonstrated in this report, this hydrogel is a promising material for stabilizing various enzymes and proteins against denaturation and inactivation at elevated temperatures, and possibly in the presence of other denaturants as well.

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Conflicts of interest

The authors declare no conflict of interest.

Notes and references

1. M. J. Webber, E. A. Appel, E. W. Meijer and R. Langer, *Nature materials*, 2016, **15**, 13-26.
2. C. Ghobril and M. W. Grinstaff, *Chem. Soc. Rev.*, 2015, **44**, 1820-1835.
3. L. S. Moreira Teixeira, J. Feijen, C. A. van Blitterswijk, P. J. Dijkstra and M. Karperien, *Biomaterials*, 2012, **33**, 1281-1290.
4. A. S. Hoffman, *Adv. Drug Deliv. Rev.*, 2012, **64**, 18-23.
5. C.-C. Lin and A. T. Metters, *Adv. Drug Deliv. Rev.*, 2006, **58**, 1379-1408.
6. A. M. Jonker, D. W. P. M. Löwik and J. C. M. van Hest, *Chemistry of Materials*, 2012, **24**, 759-773.
7. A. C. Schloss, D. M. Williams and L. J. Regan, *Adv Exp Med Biol*, 2016, **940**, 167-177.
8. P. Katyal, F. Mahmoudinobar and J. K. Montclare, *Curr. Opin. Struct. Biol.*, 2020, **63**, 97-105.
9. W. F. Daamen, J. H. Veerkamp, J. C. M. van Hest and T. H. van Kuppevelt, *Biomaterials*, 2007, **28**, 4378-4398.
10. L. Cen, W. Liu, L. Cui, W. Zhang and Y. Cao, *Pediatr. Res.*, 2008, **63**, 492-496.
11. A. J. Kuijpers, G. H. Engbers, J. Krijgsveld, S. A. Zaat, J. Dankert and J. Feijen, *J. Biomater. Sci. Polym. Ed.*, 2000, **11**, 225-243.
12. K. Y. Lee and D. J. Mooney, *Chem. Rev.*, 2001, **101**, 1869-1880.
13. U.-J. Kim, J. Park, C. Li, H.-J. Jin, R. Valluzzi and D. L. Kaplan, *Biomacromolecules*, 2004, **5**, 786-792.
14. G. H. Altman, F. Diaz, C. Jakuba, T. Calabro, R. L. Horan, J. Chen, H. Lu, J. Richmond and D. L. Kaplan, *Biomaterials*, 2003, **24**, 401-416.
15. S. Kapoor and S. C. Kundu, *Acta Biomater.*, 2016, **31**, 17-32.
16. A. Abaee, M. Mohammadian and S. M. Jafari, *Trends Food Sci. Technol.*, 2017, **70**, 69-81.
17. T. A. Whitehead, B. B. Boonyaratanakornkit, V. Höllrigl and D. S. Clark, *Protein Science*, 2007, **16**, 626-634.
18. B. B. Boonyaratanakornkit, A. J. Simpson, T. A. Whitehead, C. M. Fraser, N. M. A. El-Sayed and D. S. Clark, *Environ. Microbiol.*, 2005, **7**, 789-797.
19. D. J. Glover, L. Giger, J. R. Kim and D. S. Clark, *Biotechnol. J.*, 2013, **8**, 228-236.
20. Y. X. Chen, N. L. Ing, F. Wang, D. Xu, N. B. Sloan, N. T. Lam, D. L. Winter, E. H. Egelman, A. I. Hochbaum, D. S. Clark and D. J. Glover, *ACS Nano*, 2020, **14**, 6559-6569.
21. D. J. Glover and D. S. Clark, *FEBS J.*, 2015, **282**, 2985-2997.
22. S. Lim, G. A. Jung, D. J. Glover and D. S. Clark, *Small*, 2019, **15**, 1805558.
23. D. J. Glover, L. Giger, S. S. Kim, R. R. Naik and D. S. Clark, *Nat. Commun.*, 2016, **7**, 11771.
24. J. R. Litowski and R. S. Hodges, *J. Biol. Chem.*, 2002, **277**, 37272-37279.
25. Y. B. Yu, *Adv. Drug Deliv.*, 2002, **54**, 1113-1129.
26. S. Lim, D. J. Glover and D. S. Clark, *Adv. Exp. Med. Biol.*, 2018, **1106**, 11-23.
27. M. Fedurco, J. Augustynski, C. Indiani, G. Smulevich, M. Antalík, M. Bánó, E. Sedlák, M. C. Glascock and J. H. Dawson, *Biochim. Biophys. Acta.*, 2004, **1703**, 31-41.
28. H. M. Girvan, J. M. Bradley, M. R. Cheesman, J. R. Kincaid, Y. Liu, K. Czarnecki, K. Fisher, D. Leys, S. E. J. Rigby and A. W. Munro, *Biochemistry*, 2016, **55**, 5073-5083.