Thermostable exoshells fold and stabilize recombinant proteins

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The expression and stabilization of recombinant proteins is fundamental to basic and applied biology. Here we have engineered a thermostable protein nanoparticle (tES) to improve both expression and stabilization of recombinant proteins using this technology. tES provides steric accommodation and charge complementation to green fluorescent protein (GFPuv), horseradish peroxidase (HRPc), and Renilla luciferase (rLuc), improving the yields of functional in vitro folding by ~100-fold. Encapsulated enzymes retain the ability to metabolize small-molecule substrates, presumably via four 4.5-nm pores present in the tES shell. GFPuv exhibits no spectral shifts in fluorescence compared to a nonencapsulated control. Thermostable proteins internalized by tES are resistant to thermal, organic, chaotropic, and proteolytic denaturation and can be released from the tES assembly with mild pH titration followed by proteolysis.

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he production and stabilization of recombinant protein remains a significant challenge for basic and industrial science. Technologies to improve protein folding have had varied success, ranging from chaperone coexpression to chemically engineered hydrogels\(^1\)–\(^3\). Methods to stabilize protein products, including chemical cross-linking, rational mutagenesis, and directed evolution approaches, have also found use in basic and industrial applications\(^1\)–\(^4\).

Thermophilic organisms have evolved unique solutions for protein folding and stabilization due to the extreme conditions in which they live. Although engineered thermostable proteins have had major impacts in basic and applied biology\(^5\)–\(^7\), this approach is limited by the observation that many protein functionalities found in higher-order organisms have no homologs within the biology of prokaryotic thermophiles. Thus, general technologies which can impart thermostable qualities, in particular improved folding and stability, to normothermic substrates remain highly desirable.

*Archaeoglobus fulgidus* is a hyperthermophilic halophile naturally found in hydrothermal vents and subsurface oil fields\(^6\),\(^7\). Both eukaryotic and *A. fulgidus* ferritins (AfFtn, PDB accession code: 1SQ3) contain 24 subunits, with each subunit containing a single four-helix-bundle motif. AfFtn is different from typical ferritins in two important ways. First, the unique, tetrahedral (2–3) symmetry of the AfFtn quaternary structure results in four ~4.5-nm pores, which connect the internal and external volumes of the shell (Fig. 1a)\(^8\),\(^9\). Second, while eukaryotic ferritin assemblies are stable in low-salt concentrations\(^10\), AfFtn disassociates in low-salt (NaCl) concentrations\(^8\).

To improve recombinant protein expression and product stabilization using a single technology, we have engineered the AfFtn assembly derived from *A. fulgidus*, to create a thermostable exoshell (tES). tES accommodates foreign proteins within an 8-nm aqueous cavity while, at the same time, allowing internalized enzymes to have an access to molecular substrates through four ~4.5-nm pores present in the native structure of the shell. We hypothesize that tight steric and electrostatic complementarity to an internalized substrate prevents aggregation during the folding process while stabilizing correctly folded tertiary structures (Fig. 1 and Supplementary Fig. 1). We also demonstrate that tES is protective against a wide range of denaturants, thus imparting thermostable qualities to internalized normothermic proteins.

**Results**

**Engineering of tES.** AfFtn subunits have unstructured negatively charged C-termini, which protrude into the central cavity of the 24-subunit AfFtn assembly. Together, these C-termini have a molecular mass of ~21 kDa, which would likely interfere with the internalized protein folding and function. Therefore, we created a
truncation mutant at residue 164 (Q164), to remove unstructured amino acids from the cavity and create the template for “tES.” Surprisingly, the modified AFtH assembly was stable in all salt concentrations tested (Fig. 1c). A circular dichroism study of tES (+), tES(−), and tES(+/−) shells showed stability of a secondary structure to 80 °C; however, partial denaturation was observed at 90 °C (Supplementary Fig. 2).

Given the proximity between the internal cavity surface and internalized recombinant peptides, we further modified the charge characteristics of the tES aqueous cavity to present either net positive [tES(+)], negative [tES(−)], or neutral [tES(+/-)] charge environments to an internalized protein of interest (POI) (Fig. 1b and Supplementary Fig. 1b). All tES constructs showed a high expression (Supplementary Fig. 3) with an estimated yield of 412, 402, and 237 mg/L (approximately 36, 35.23, and 20.76%, respectively). Because the truncation mutant of a native enzyme, there are no reports of successful in vitro refolding, its relatively high thermolability, and ease of the functional assay as a bioluminescent reporter enzyme. Finally, we chose Renilla luciferase (rLuc, 36 kDa) due to an absence of previous reports of successful in vitro refolding, its relatively high thermolability, and ease of functional measurement. We chose Renilla luciferase (rLuc, 36 kDa) due to an absence of previous reports of successful in vitro refolding, its relatively high thermolability, and ease of the functional assay as a bioluminescent reporter enzyme. Finally, we chose Renilla luciferase (rLuc, 36 kDa) due to an absence of previous reports of successful in vitro refolding, its relatively high thermolability, and ease of the functional assay as a bioluminescent reporter enzyme.3, 5

Effect of F116H mutation on shell assembly. Because the engineered tES assembly was highly stable in both low- and high-salt concentrations, we further engineered a pH-titratable mechanism to control tES assembly and release. F116 exists along a 3-fold symmetry axis and its substitution to histidine would be expected to create mutual charge repulsion with H104, located on a neighboring subunit, upon protonation (Supplementary Fig. 4a). As expected, tES(+)F116H, tES(−)F116H, and tES(+/-)F116H show a reversible dissociation at pH 5.8 and a stable assembly at pH 8.0 (Fig. 1c and Supplementary Fig. 4b, c).

Soluble expression of proteins in the presence of tES. To demonstrate the effect of tES on recombinant Escherichia coli expression and in vitro folding, we prepared genetic fusions (tES-POI) between a histidine-tagged tES monomer and three divergent POI (Supplementary Fig. 1c). Green fluorescent protein (GFPuv, 27 kDa) was chosen due to its highly variable expression in E. coli, prior use in the identification of productive folding environments, and ease of functional measurement. We chose Renilla luciferase (rLuc, 36 kDa) due to an absence of previous reports of successful in vitro refolding, its relatively high thermolability, and ease of the functional assay as a bioluminescent reporter enzyme. Finally, we chose Renilla luciferase (rLuc, 36 kDa) due to an absence of previous reports of successful in vitro refolding, its relatively high thermolability, and ease of the functional assay as a bioluminescent reporter enzyme.3, 5

To test the effect of coexpression with tES for the three fusion proteins, we developed a two-plasmid system. The tES was expressed using a high-expression T7 promoter (pRSF) and the tES-POI fusion subunit was expressed using L-arabinose induction from a highly titratable, complementary plasmid (pBAD). Induction was initially optimized with the tES(+)/tES-GFPuv fusion (Supplementary Fig. 1c). To demonstrate tES rescue of GFPuv expression, GFPuv was induced at 0.8 (O.D. 600 nm) due to its extremely low expression in late exponential phase. While leaving isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration constant (0.4 mM), L-arabinose was varied from 0.001 to 0.1%. The maximum functional and soluble expression of tES-GFPuv (Supplementary Fig. 3a) occurred at 0.01%. While the expression of all three POIs separate from tES showed a negligible soluble expression (with the exception of rLuc, which is previously reported to result in soluble expression in E. coli).
coexpression of tES-POI with tES resulted in easily visualized bands in the soluble fraction in all three cases (Supplementary Fig. 3b–d). The tES(+) shell resulted in the highest relative concentration of a soluble product, with yields of 79.5, 74, and 57 mg/L of GFPuv, HRPc, and rLuc, respectively, as analyzed by densitometry using ImageJ software. We hypothesize that this may be due to charge complementation between the net-negative surface charge of the POIs and the net-positive charge of the tES(+) internal surface. Assembly of tES (+)/tES-POI was confirmed by pull down of shell components by the histidine-tagged fusion subunit, followed by size-exclusion chromatography (SEC) to confirm tES/tES-POI assembly (Supplementary Fig. 3e, f). The ratios of tES-GFP, tES-HRPc, and tES-rLuc to encapsulating tES subunits were in approximate agreement with the expected value of 1:23 (1:32, 1:19, and 1:20, respectively), as estimated by gel densitometry of purified protein fractions.

Using coexpressed tES(+)/tES-POI, we optimized postexpression protocols for improved yield after in vivo assembly within E. coli. We focused on HRPc as it requires disulphide formation and a...
prosthetic group. We confirmed that only by adding the known cofactors heme and calcium, in addition to oxidizing conditions, does maximum functional yield result after harvesting soluble tES (/+)tES-HRPc from E. coli lysates (Supplementary Fig. 5a).

**Effect of different tES:tES(+)GFPuv ratios protein folding.** We tested the ability of tES to aid in vitro folding of proteins. The tES (/+)F116H assembly is highly stable at pH 8.0, as evidenced by similar elution profiles on SEC after treatment with 8 M urea or 6 M guanidinium hydrochloride (GuHCl), compared with PBS controls. Likewise, tES(+)F116H can reversibly associate and dissociate with pH titration with no observable precipitates (Supplementary Fig. 4c). We then hypothesized whether tES could functionally encapsulate substrate proteins under conditions that would denature the POI. tES fusion proteins expressed as inclusion bodies in the absence of coexpressed tES (Supplementary Fig. 3b–d). Thus, using tES(+)F116H, we tested the ratios of tES to tES-POI, using a pH shift from 5.8 to 8.0 to induce assembly of the shell. The addition of tES(+)F116H to tES-GFPuv resulted in an ~100-fold increase in functional yield, which was maximum at a 90:1 ratio of tES(+)F116H subunits to tES-GFPuv (Fig. 2a and Supplementary Fig. 5b, c). We found that heating the inclusion body suspension to 60 °C in the presence of 6 M GuHCl and 10 μM β-mercaptoethanol was critical for a maximum final yield.

Based on pH titration results (Fig. 2a), we developed a standard protocol with a 60:1 ratio of tES(+)F116H:tES-POI, starting with all components in 6 M GuHCl at pH 5.8. Protein-specific, posttranslationally required agents were added, and the solution was then dialyzed against GuHCl-free buffer at pH 8.0 (Supplementary Fig. 6). Using this protocol, we demonstrate a near-absoluate requirement for tES for functional in vitro folding of tES-GFPuv, tES-rLuc, and tES-HRPc. Under the same protocol, very little or no functional yield of GFPuv, HRPc, or rLuc, was observed in the absence of tES (Fig. 2b). A similar pattern of protein activity was seen in E. coli lysates with the exception of rLuc, which is known to be expressed as a soluble protein in E. coli (Fig. 2c).

**Iron uptake qualities of tES.** The native AfFtn shell is a physiologic iron-storage protein; we investigated whether tES variants also retain iron, either in our purified preparation or when exposed to an in vitro iron-loading protocol. Equimolar amounts (1 μM) of tES(+), tES(−), tES(+/−), and commercially available horse iron ferritin were compared, and we observed no detectable iron core in purified tES variants (Supplementary Fig. 7a). We then tested iron uptake using equimolar amounts (1 μM) of wild-type AfFtn, tES(+), tES(−), tES(+/−), tES(+)F116H, tES(+)F116H/tES-GFPuv, tES(+)F116H/tES-HRPc, and tES(+)F116H/tES-rLuc. Wild-type AfFtn showed the highest in vitro iron uptake, while empty tES shells had appreciable but lower iron accumulation, likely due to amino acid substitutions near the ferroxidase center (E128 and E131). A comparison of tES(+) and tES(+)F116H iron uptake was qualitatively similar, indicating that the F116H mutation had little effect on iron uptake. All three tES (+)F116H/tES-POI had greatly reduced (~1–2%) iron uptake when compared with wild-type AfFtn and empty tES variants (Supplementary Fig. 7b).

**POI stabilization with tES and release with pH titration.** We tested the ability of tES to impart thermostable qualities to internalized proteins. GFPuv is highly stable in its native form. We therefore tested rLuc and HRPc versus unencapsulated controls to determine the stabilizing effect of tES encapsulation. For both rLuc and HRPc, tES was protective from 0.4% trypsin, 30% acetonitrile, 20% methanol, 8 M urea, 6 M GuHCl, and thermal denaturation (Fig. 3a–g and SupplementaryFig. 7c).

Finally, we tested the encapsulated, functional proteins for their ability to be released from tES(+)F116H with mild pH titration (pH 5.8–6). All three proteins were easily released and purified under SEC, and POIs could be proteolyzed from the fusion tES subunit to produce a monomeric, soluble protein (Fig. 3h–k).

**Discussion**

The pathway from a nascent polypeptide to a functional protein structure is determined by a balance of factors, some working in favor of, and many against, the successful folding of the end product. Natural chaperones prevent the aggregation of unfolded or partially folded intermediates, undergo specific interactions that bias folding along productive energetic pathways, and exert kinetic effects that accommodate folding processes of widely varying time scales. In the setting of recombinant expression, the intrinsic chaperone availability in an E. coli cell may be overwhelmed by the bulk of a nascent, unfolded recombinant protein. In support of this hypothesis, the overexpression of native chaperones such as Hsp70 and GroEL/GroES complex has aided in recombinant protein expression. Chaperone overexpression has also shown limits in its application, as the coexpression of DnaJ, DnaK, and GrpE with HRP inhibited the growth of host cells and DnaK-assisted expression of GFP resulted in reduced yield and lower conformational quality of the target protein.

Studies using the P22 VLP bacteriophage have shown sequestration of a recombinant protein within the interior surface. However, the requirement of ~450 coat proteins, additional scaffold proteins, and the harsh conditions required to dissociate the capsid have limited the use of P22 in recombinant expression.

By using 23 copies of a single thermostable subunit to form a protective shell around internalized proteins, we report that tES can improve expression, in vitro folding, and product stabilization. tES(+)F116H can release the encapsulated protein with mild pH titration (pH 5.8–6.0) and the soluble protein fusions can be selectively proteolyzed to create monomeric protein products. A caveat of this study is that we use protein function as a surrogate for folding. Thus, to understand the precise effects of environmental engineering on the folding of protein substrates, further studies such as differential scanning calorimetry, deuterium exchange, and cryoelectron microscopy may be needed. Because each POI substrate is physically isolated from other unfolded proteins via the TES, we hypothesize that folding studies can be performed at a higher concentration without aggregation.

Although ~80% of translated proteins in the eukaryotic genome are 80 kDa or smaller, the uses of TES may be broadened by variants with larger internal volumes. This will be particularly important for POIs that require multimerization for optimum activity. The ability to stabilize thermostable substrates within TES may be helpful for a variety of applications in bionanotechnology and synthetic biology, including the production of difficult-to-fold proteins, using the shell as a mediator of cellular enzyme uptake, and exploiting the stabilized qualities of TES substrates in industrial settings.

**Methods**

**Plasmids and competent cells.** The pRBS1 vector expression vector (Merck) and pBAD/HisB vector (Life Technologies) were used for cloning. Chemically competent XL1 Blue E. coli cells (catalog #RH119; Simply Science) and BL21 (DE3) E. coli cells (catalog #RH217-J40: Simply Science) were used for transformation.

**Reagents.** The following reagents were used: restriction enzymes NcoI, EagI, and SpeI (New England Biolabs), ExoIII T4 DNA ligase (Life Technologies),

**References**
Cloning. A ΦTn was selected based on the sequence in GenBank AF_RS04235. The gene with mutations for C-terminus truncation, as well as altered charges was synthesized from Genscript. The mutated gene was digested using Nol and Spe restriction enzymes and cloned into pRSFb expression vector using Expresslink. The ligation reaction was transformed into chemically competent E. coli XL1 Blue (Stratagene, Santa Cruz Biotechnology), His-probe (H-3) HRP mouse monoclonal IgG1 (catalog 6sc-40 HRP, Santa Cruz Biotechnology), rabbit monoclonal GFP antibody (catalog #G10362; Invitrogen), and HRP-conjugated anti-rabbit IgG antibody (catalog #A10260; Invitrogen).

Protein expression in E. coli. For the expression of each clone, a single positive colony was selected from a freshly transformed plate and grown in 100-mL LB broth, with kanamycin (50 µg/mL), for a protein gene on pRSFb vector), ampicillin (100 µg/mL for a protein gene on pBAD/HisB vector), or both (cotransformation) used as selection markers. Following overnight incubation at 37 °C, 12.5 mL of the starter culture was used to inoculate a 500-mL LB broth and allowed to grow until an absorbance of (O.D600) of 0.4–0.5 was reached. Protein expression was then induced with 0.4 mM IPTG (pRSFb vector), 0.1% L-arabinose (pBAD vector), or both (cotransformation). We tested the role of a tightly controlled relative expression of the encapsulated POI by evaluating the effect of L-arabinose (0.01, 0.1, and 1% for the relative expression of 1, 10, and 100% of the wild type respectively). After 4 h of growth at 37 °C, cells were pelleted by centrifugation at 13,750 ×g for 10 min. The cell pellet was resuspended in a lysis buffer (25 mM Tris-HCl, 150 mM NaCl, pH 7.5), sonicated, and centrifuged to separate cell debris. The supernatant was purified using a two-step chromatography procedure. pRSF clones were subjected to hydrophobic interaction chromatography (HIC) using HisPrepTM Phenyl FF (low sub) 16/10 (GE healthcare), followed by SEC of a Superdex S-200 10/300 GL column (GE healthcare). pBAD clones were subjected to Ni-NTA (Expedeon) precipitation, followed by SEC using a Superdex S-200 10/300 column (GE healthcare). pBAD clones were subjected to Ni-NTA (Expedeon) precipitation, followed by SEC using a Superdex S-200 10/300 column (GE healthcare). pBAD clones were subjected to Ni-NTA (Expedeon) precipitation, followed by SEC using a Superdex S-200 10/300 column (GE healthcare). pBAD clones were subjected to Ni-NTA (Expedeon) precipitation, followed by SEC using a Superdex S-200 10/300 column (GE healthcare). pBAD clones were subjected to Ni-NTA (Expedeon) precipitation, followed by SEC using a Superdex S-200 10/300 column (GE healthcare). pBAD clones were subjected to Ni-NTA (Expedeon) precipitation, followed by SEC using a Superdex S-200 10/300 column (GE healthcare). pBAD clones were subjected to Ni-NTA (Expedeon) precipitation, followed by SEC using a Superdex S-200 10/300 column (GE healthcare). pBAD clones were subjected to Ni-NTA (Expedeon) precipitation, followed by SEC using a Superdex S-200 10/300 column (GE healthcare).
min. The TMB substrate was added for color development and the reaction stopped using 2 M H2SO4 after 5 min. Absorbance was recorded at 450 nm. All luciferase reactions took place at ambient temperature (24–27°C) in a 96-well plate with a white interior. Following chromatographic purification, equimolar concentrations (500 nM) of purified tES(+)F116H/tES-HRPc, tES-tLuc, and rLuc were evaluated for luciferase activity using Renilla luciferase kit (Promega) with some modifications in the manufacturer’s instructions. The reaction was initiated by injecting 50 µl of Renilla luciferase assay reagent (11,000 dilution of coelenterazine in the assay buffer). The assay reagent was protected from light at all times by covering the tubes with an aluminum foil. The signal was integrated for 1 min with a 2-s delay and was reported in RLU. All readings were recorded on a Perkin Elmer Plate reader. Appropriate controls were used in each case to minimize the background.

**Release of a functional protein.** Engineered pH-responsive tES(+)F116H shells containing the POI—GFPuv, HRPc, and tLuc—were subjected to cage break as described above. Following cage break, GFPuv, HRPc, and tLuc activity of each fraction was analyzed, as described earlier. The release of the functional POI from the tES subunit was studied by cleavage with bovine FXa/TEV protease. Briefly, the cage break fraction corresponding to the elution of tES-POI on SEC was subjected to FXa cleavage at 37 °C for 4 h (TEV cleavage at 34 °C for 5 h). The reaction mixture was run on an SDS gel and the separated POI band was analyzed through western blot.

**Thermostability and heat shock tests.** Concentrations of 0.5 μM tES(+)F116H/tES-HRPc or tES(+)F116H/tES-tLuc, 50 μM HRPc, and 800 μM tLuc protein samples were incubated in an assay buffer (25 mM Tris-HCl, pH 8.0) at 21.5, 37, 55, 65, and 75 °C for 15 min. Following incubation, samples were cooled down and their activities were evaluated. For a heat shock test, 0.5 μM tES(+)F116H/tES-tLuc and 800 μM tLuc protein samples were incubated in 25 mM Tris-HCl (pH 8) at 80 °C for 5 min, followed by cooling the protein samples at 0 °C for 5 min. The process was repeated for 5 and 10 cycles, followed by evaluation of protein activity.

**Trypsin digestion.** Concentrations of 0.5 μM tES(+)F116H/tES-HRPc or tES(+)F116H/tES-tLuc, 50 μM HRPc, and 800 μM tLuc protein samples were treated with 0.4% trypsin-EDTA solution at 37°C for 0, 30, 60, 90, and 120, and 150 min, followed by analysis of their activities.

**Analysis of protein stability.** The effect of urea, GuHCl, ACN, and MeOH on protein stability was evaluated by treating 0.5 μM tES(+)F116H/tES-HRPc or tES(+)F116H/tES-tLuc, 50 μM HRPc, and 800 μM tLuc protein samples with an assay buffer (pH 8) containing the POI—GFPuv, HRPc, and rLuc protein samples were treated with 0.4% trypsin-EDTA solution at 37°C for 0, 30, 60, 90, and 120, and 150 min, followed by analysis of their activities.

**Data availability.** All relevant data are available from the authors upon request.

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Author contributions
C.L.D.: Conceived the idea and was responsible for the overall project supervision. C.L.D. and J.M.C.: Analyzed the results. S.D., N.D.M., and V.M.G.: Designed and performed the experiments and wrote the manuscript together with C.L.D. S.D.: Designed, performed, and analyzed stability experiments. N.M.: Designed, performed, and analyzed the luciferase experiments. M.D.: Prepared the various clones, designed, and performed the GFP experiments. G.C. and S.D.: Designed, performed, and analyzed the HRPc experiments.

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