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Broad Stability and pH-Mediated Reversible Disassembly of *In Vitro* Assembled Bacterial Microcompartment Shells

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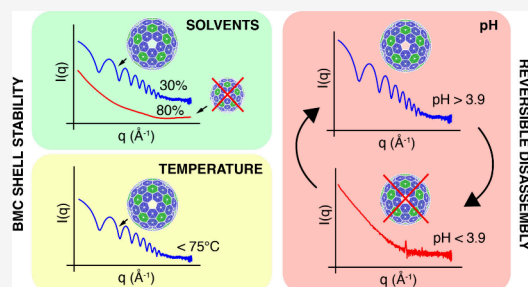
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Supporting Information

ABSTRACT: Bacterial microcompartments (BMCs) are self-assembling organelles that colocalize enzymes and metabolites of various biochemical pathways. Their selectively permeable shell is made entirely of protein, and the inherent properties of these systems make them attractive templates for bioengineering synthetic nanoreactors. Understanding the extent of shell stability in an extended range of conditions broadens the scope of chemistries that can be confined in these nanocompartments. Here we analyze the stability of *in vitro* assembled BMC shells and find that they retain their structure at high concentrations of various solvents and detergents, at temperatures up to 75 °C, and across a pH range from 3.9 to 11. Shell disassembly is observed below pH 3.9, but shells can be reassembled upon increasing the pH back to 8. These results expand the range of reactions that can be performed inside protein-based nanoreactors and underscore the potential of modular BMC shell proteins as versatile biomaterials.

KEYWORDS: Bacterial Microcompartment, Biomaterial, Stability, Reversible Disassembly



Nanoreactors are microscopic porous vessels that are used to confine catalysts and metabolites to enhance or alter chemical reactions.¹ Common nanoreactor frameworks are synthesized from compositions of inorganic material, crystalline material with an organic element, or self-assembling polymers.^{2,3} Bacterial microcompartments (BMCs) are self-assembling organelles composed entirely of protein,^{4–6} nature's biological polymer. These subcellular systems encapsulate enzymes and sequester metabolites for a variety of specialized biochemical pathways within a wide range of microbes. Separating chemical reactions from bulk environments aids in enzyme stabilization, colocalizes reactants, protects against competing metabolites, and prevents toxic intermediate spillage into the cytosol.^{5,7} The most well-known microcompartment systems are the carboxysome,^{8,9} the EUT¹⁰ (ethanolamine utilization), and the PDU¹¹ (propanediol utilization) microcompartments.

The inherent properties of these systems motivate the bioengineering of BMC shells that enhance reaction pathways¹² and create unique microenvironments that can house a broad range of biotic^{13–20} and abiotic catalytic cargo. The longstanding difficulties in constructing bioengineered shells using heterologous expression systems include minimal control over assembly and morphology, sparse cargo encapsulation,^{21–23} adventitious capture of undesirable cytoplasmic proteins,^{24,25} and restricted assembly conditions using an enzymatic assembly method.²⁶ We recently overcame many of these constraints by developing a chaotropic *in vitro* assembly

method with shell proteins from a well-characterized model system derived from the BMC of *Haliangium ochraceum* (HO).²⁷ Combining soluble hexameric (BMC-H) and trimeric (BMC-T) shell proteins while controlling chaotrope concentration rapidly and efficiently produces monodisperse ~38 nm wiffle (HT shell) icosahedral shells with gaps at the vertices^{27,28} (Figure 1). Given that chaotropic *in vitro* assembly is not restricted to conditions required to retain enzymatic activity,²⁶ our assembly method enables inclusion of a broader range of cargo types, namely abiotic catalysts.²⁹ Considering many abiotic catalysts require organic solvents or other solvation conditions that are typically incompatible with biomacromolecules, it is imperative to study shell stability in a variety of nonaqueous solvents and other noxious contexts to thoroughly assess the potential of BMC shells as a platform technology for an expansive scope of organizationally confined catalysis.

To date, BMC shell stability has been evaluated with native BMC systems or *in vivo* assembled synthetic shells using various biophysical techniques, as well as indirect analyses monitoring the activity of encapsulated enzymes.^{30–34} In this

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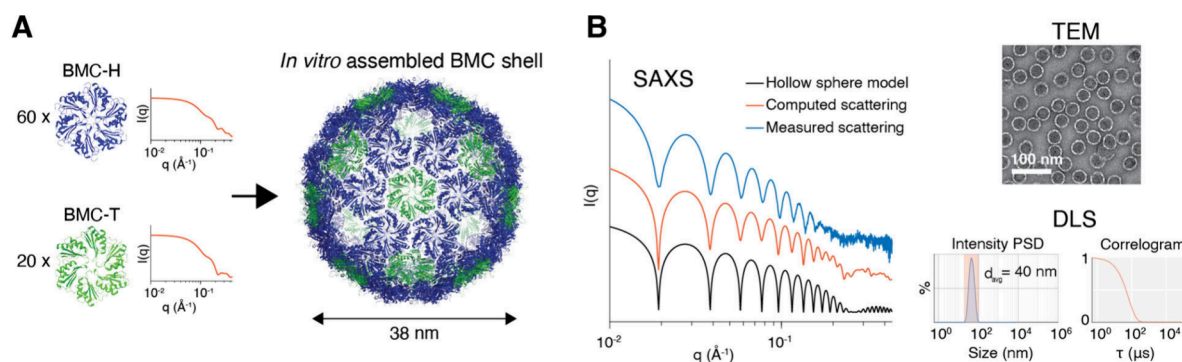


Figure 1. (A) Schematic of *in vitro* assembly of HT shells with computed SAXS profiles for soluble BMC-H (PDB entry 5DJB)³⁵ and BMC-T (PDB entry 5DIH)³⁶ proteins and a model of an HT shell (PDB entries 6MZX and 6N06).²⁵ (B) Computed and measured HT shell SAXS scattering profiles plotted alongside a hollow sphere model. Shown to the right are a TEM micrograph and a DLS measurement of assembled shells.

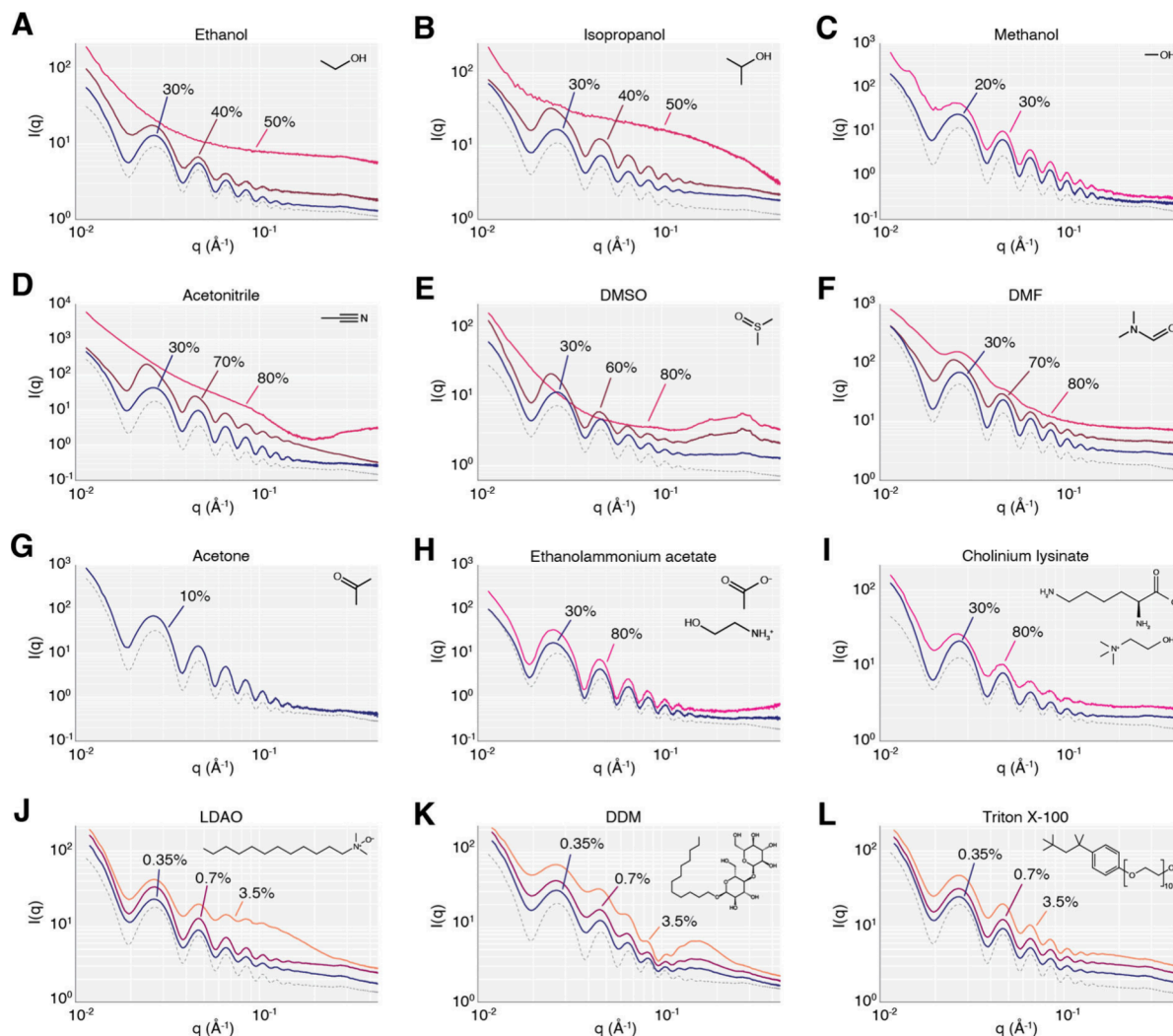


Figure 2. Shell stability in various solvents and detergents measured with SAXS. The dashed scattering profiles represent untreated HT shells. (A–G) Measured scattering profiles for HT shells in various concentrations (v/v) of organic solvents. (H, I) Measured scattering profiles for HT shells in various concentrations (v/v) of ionic liquids. (J–L) Measured scattering profiles for HT shells in various concentrations of detergents LDAO (w/v), DDM (w/v), and Triton X-100 (v/v).

study, we examine the stability of *in vitro* assembled HT shells (Figure 1A). We subjected shells to an extended range of physicochemical environments, including elevated concentrations of various organic solvents, ionic liquids, detergents, and across temperature and pH extremes. We monitored shell

stability using high-throughput small-angle X-ray scattering (SAXS), dynamic light scattering (DLS), and transmission electron microscopy (TEM). We find that shells retain their structure across many conditions typically destructive to proteins and show pH-mediated reversible shell disassembly.

Collectively, our findings expand the potential of BMC-based architectures that robustly self-assemble *in vitro* as synthetic nanoreactors for catalysis and chemistry, and further broaden their potential as versatile biomaterials.

We assembled HT shells *in vitro* by combining BMC-H and BMC-T shell proteins based on the protocol from Range, Chiang, and co-workers²⁷ (Figure 1A) and purified them by size-exclusion chromatography (SEC). Shell assembly was verified by DLS, TEM, and SAXS (Figure 1B). The scattering profile of a measured HT shell is shown in Figure 1B alongside the computed profiles from an atomic model (using FoXS)^{37,38} and a hollow sphere model with an inner radius of 151 Å and a shell wall thickness of 24 Å. All scattering profiles exhibit classical oscillatory features for hollow sphere geometries,^{39,40} and the experimentally measured scattering profile closely matches the computed profiles. Additionally, DLS and TEM of assembled shells show uniformity in both shape and size (Figure 1B). These results verify that shells assembled as expected and provide benchmark data for comparative stability studies.

We concentrated the purified shells, then diluted them in various conditions to a constant final shell concentration of 1 mg/mL. Shells were incubated in test solutions for 1–3 h before SAXS measurements were made. We tested shell stability in up to 80% v/v of several organic solvents, the ionic liquids ethanolammonium acetate and cholinium lysinate, up to 3.5% w/v lauryldimethylamine oxide (LDAO) and *n*-dodecyl- β -D-maltoside (DDM), and up to 3.5% v/v Triton X-100. All scattering curves at each solvent concentration tested are shown in Figures S2–S13. Scattering profiles of shell samples at increasing organic solvent concentrations are shown in Figure 2: 0%, 30% v/v (if tested), and the highest concentration at which shell features are still observed. Also shown are the scattering profiles at either the concentration at which shell features were lost, or the highest concentration tested if loss was not observed. The scattering profiles of shells in 30% v/v concentration across many of the tested organic solvents do not differ significantly from the control sample (0%). At this concentration, shells show high structural fidelity as verified by TEM (Figure S1). For comparison, shell stability in 30% v/v methanol exceeds what was previously reported by Tan et al. for an *in vivo* assembled α -carboxysome derived shell by 10%.³³ This difference may be attributed to variations in shell protein properties across BMC systems. We next tested cargo stability in an organic solvent solution with enzyme encapsulated HT shells. Using a modified trimer protein with a luminally fused SpyTag, we encapsulated a SpyCatcher-fused alcohol dehydrogenase (ADH) inside shells, previously shown by Retnadhas et al.¹⁷ We tested protein stability of the encapsulated ADH shell sample using circular dichroism (CD) spectroscopy (Figure S14A). The results indicate that at 30% v/v ethanol concentration, encapsulation inside HT shells may confer higher structural stability to the enzyme. At the same solvent concentration, free SpyCatcher–ADH undergoes a significant reduction in protein secondary structure (Figure S14B). Thus, the secondary structure of the encapsulated enzyme may be preserved because of its confinement inside a stable HT shell. Furthermore, we tested the catalytic activity of the encapsulated enzyme at 30% v/v ethanol. It is important to note that retained catalytic activity of encapsulated cargo in various conditions is due to enzyme-specific intrinsic properties and therefore, functional performance will need to be assessed on a case-by-case basis. Nevertheless, we demonstrate

a proof-of-concept example of confined catalysis at 30% v/v ethanol for encapsulated ADH. The enzyme remains catalytically active and able to reduce NAD⁺ to NADH as monitored by the absorbance at 340 nm, shown in Figure S15.

At higher solvent concentrations, there are noteworthy shifts in the symmetry of the form factor, and the scattering profiles begin to lose resemblance to the control. The upper concentration limit for shell stability varied by solvent. For example, SAXS profiles become increasingly dissimilar to the control in alcohol concentrations (ethanol and isopropanol) greater than 40% v/v, whereas larger shifts in scattering appear at higher concentrations (up to 70% v/v) of acetonitrile, dimethyl sulfoxide (DMSO), and dimethylformamide (DMF). Although it is unclear what explicitly causes these deviations, it is hypothesized that solvent-induced protein unfolding can arise from a change in the hydration layer around the surface of the protein or by direct solvent–protein contact, exposing hydrophobic side chains and triggering protein unfolding.^{41–43} Thus, it is plausible these shifts emerge from increased structural polydispersity due to disassembly or aggregation caused by protein–protein or protein–cosolvent interactions. The apparent tolerance of shells to this broad range of conditions is surprising, as a typical protein fold loses native conformation in more than 20% v/v organic solvent.⁴⁴ HT shell proteins in test solutions of 10–80% v/v ethanol concentration did not show significant protein unfolding, as shown by CD spectroscopy in Figure S16. This indicates that the individual shell proteins are remarkably robust, as secondary structure is retained at concentrations at which we observed loss of shells. Therefore, the impressive stability of the assembled shell may be rooted in the inherent properties of the proteins that compose it. The stability of shells in various organic solvents broadens the types of confined chemistries that can be conducted using enzymes and abiotic catalysts. BMC shell tolerance to solvent solutions not only increases the practicality of spatially organized catalysis for many industrial applications,^{45,46} but also enables the inclusion of solvents to act as stabilizers for catalysts and to potentially serve as active participants in the chemical reactions confined inside shells.^{47,48}

Shells diluted into ethanolammonium acetate and cholinium lysinate, ionic liquids chosen for their biocompatibility, catalytic, and bioprocessing applications,^{49–51} remain stable at considerably higher concentrations; increasing their potential use in the development of green methods to recover energy from waste biomass (Figure 2H,I). We verified shell stability and structural integrity in 30% v/v of both ionic liquids and 80% v/v of ethanolammonium acetate by TEM (Figure S1). Although not imaged due to incompatibility with TEM grids, the scattering profile for shells in 80% v/v cholinium lysinate is similar to the benchmark control and indicates shell stability. Ionic liquids are readily used in the biomass conversion industry as a green alternative to conventional solvents;^{52,53} therefore, shell stability in ionic liquids could potentially be exploited to enhance these conversion reactions with auxiliary confined catalysts. Additionally, we tested shell stability in zwitterionic detergent LDAO, and nonionic detergents DDM and Triton X-100 (Figure 2J–L), as shell stability in detergents expands confinement to membrane proteins⁵⁴ as well as various hydrophobic molecules. We tested stability up to 3.5% w/v LDAO and DDM and 3.5% v/v Triton X-100, concentrations higher than their reported critical micelle concentrations

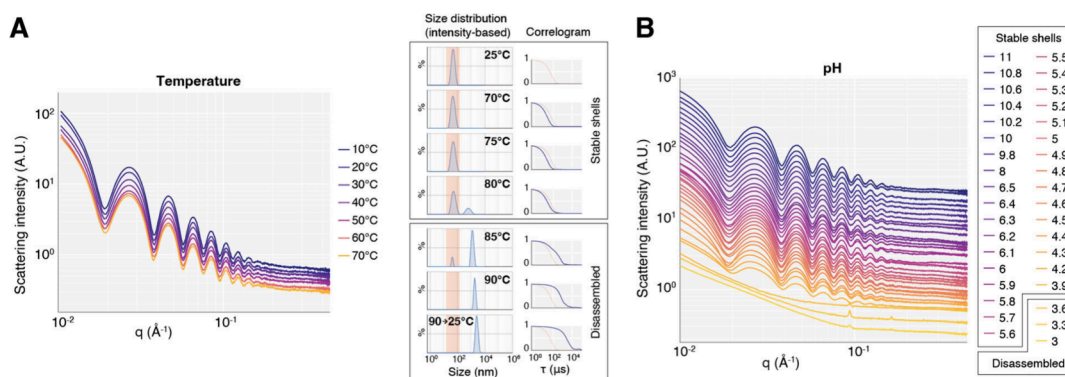


Figure 3. Stability in a wide range of temperature and pH. (A) SAXS curves of shell samples measured at temperatures between 10 and 70 °C (left) and DLS measurements of shells measured at temperatures between 75 and 90 °C (right). (B) SAXS curves of shells measured in pH conditions between 3–11. All SAXS curves have been scaled for clarity.

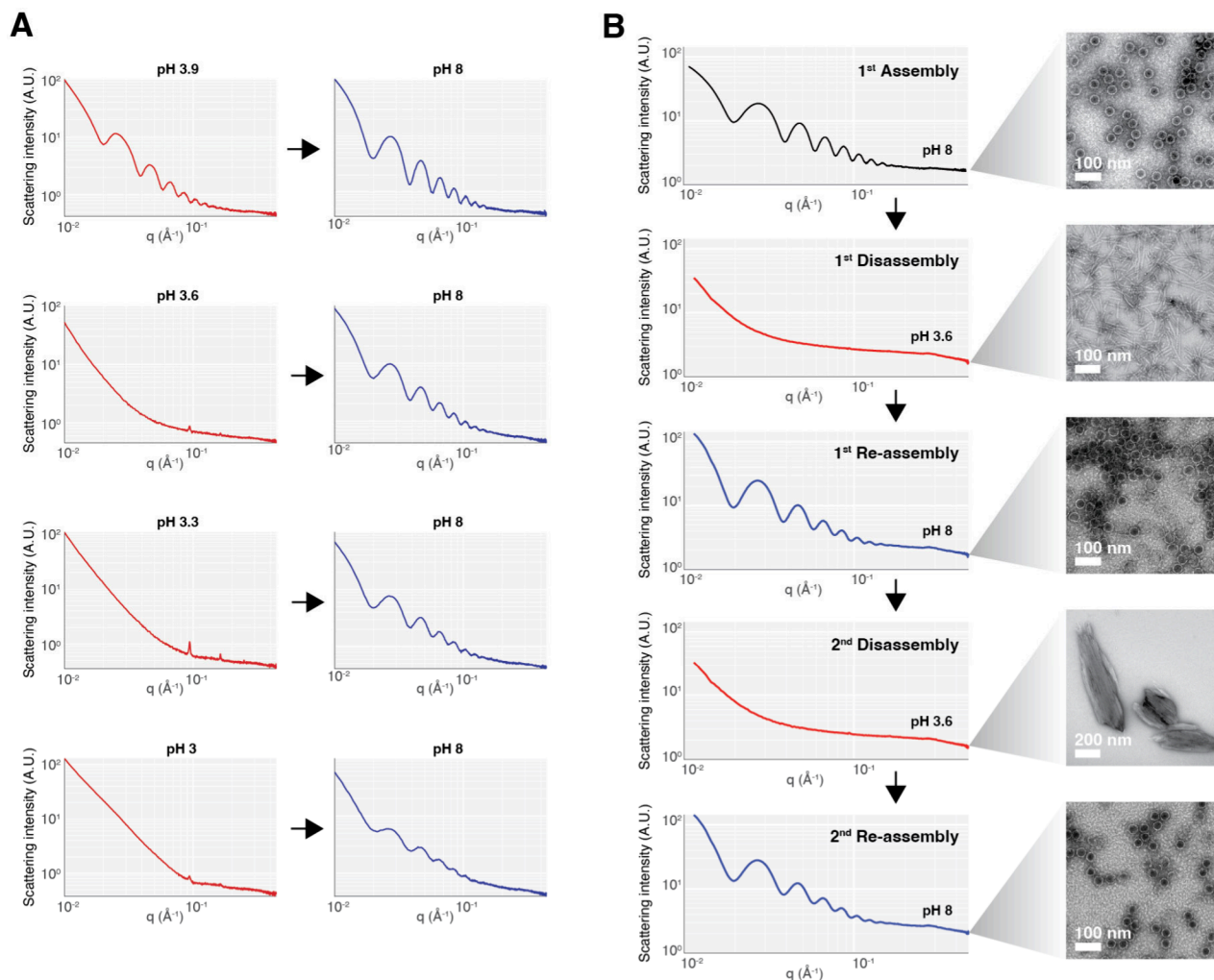


Figure 4. pH-mediated shell disassembly and reassembly. (A) HT shells in low pH conditions. Shell disassembly in sodium citrate buffer at pH 3.6, 3.3, and 3.0 and reassembly at pH 8 with a high concentration of Tris-HCl. (B) Multiple cycles of shell disassembly and reassembly. HT shells disassembled in sodium citrate at pH 3.6 and reassembled in TBS buffer at pH 8. TEM micrographs of assembled shells, disassembled shell proteins, and reassembled shells are shown at the right.

(CMCs) and typical membrane protein storage conditions.^{55–57} Shell oscillatory features remain present at all concentrations, but we hypothesize that deviations in the scattering profiles of LDAO and DDM arise from a mixture of detergent micelles and HT shells. The difference in size and concentration between the HT shells and the micelles makes it

possible to assess the presence of the shells, despite the contributions of micelles to the total measured form factor.

Next, we tested the stability of HT shells across a broad range of temperatures. Thermal measurements ranged from 10 – 90 °C. The scattering profiles, measured from 10 °C up to the limit of the SAXS temperature controller at 70 °C, are

consistent with the scattering profile of HT shells as typically prepared, shown in Figure 3A. We continued thermal stability analysis to higher temperature ranges, from 75 – 90 °C, using DLS. Up to 75 °C, shells remain monodisperse with an average diameter of approximately 40 nm. At 80 °C and above, formation of larger particle species occurs, perhaps due to agglomeration of shells, similar to thermal changes at 50 °C and above reported by Kim et al. with a PDU microcompartment shell encapsulating the reporter protein GFP,³⁰ or aggregation of thermally denatured shell proteins. At 90 °C, shell-sized particles are no longer detected, and only large species remain. This transformation does not appear to be reversible upon lowering the temperature back to 25 °C (Figure 3A). Additionally, we used differential scanning fluorimetry (DSF) and circular dichroism (CD) to assess protein temperature stability. The melting temperature of proteins in the shell sample is approximately 88.5 °C, as shown by DSF (Figure S17). Additionally, proteins remain folded from 25 to 80 °C, and proteins unfold at and above 85 °C, as shown by CD (Figure S18). Although neither technique provides structural information about the assembled shell, these results support the hypothesis that the stability of assembled shells may be a consequence of the intrinsic stability of the individual shell proteins.

Following temperature measurements, we tested shell stability across both acidic and basic pH conditions. We diluted shells into 50 mM sodium citrate buffer between pH 3.0–6.5 and 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer between pH 9.8–11.0 and measured the scattering profiles. We found that shells are stable in pH conditions between 3.9 and 11, as shell features remain at these conditions (Figure 3B). Shells lose stability at pH < 3.9, as indicated by the dramatic change in the scattering profile and loss of oscillatory features.

Native protein folds are typically stable within narrow pH and temperature ranges; therefore, the observed pH and thermal shell stability is advantageous, as these conditions can be fine-tuned⁵⁸ to enhance the performance of mesophilic and nonmesophilic cargo without detrimental effects to the protein shell. For example, encapsulating biotic catalysts specifically relating to reactions that employ extremozymes,⁵⁹ extremophilic enzymes, can be difficult to confine in a biological system such as a protein cage without destroying the structure due to harsh conditions required for optimal enzyme activity. For these reasons, BMC shells remaining stable in both acidic and basic conditions, at high temperatures, as well as in various water-solvent/detergent ratios, provide a means for encapsulating chemistries in far-from-physiological conditions.

Although thermal disassembly is apparently irreversible, we found that pH-disassembled shells can be reassembled by raising the pH back to assembly conditions. This phenomenon of pH-mediated shell reassembly has been observed for native PDU microcompartments by shifting conditions from pH 5 to pH 8,³⁴ and for synthetic protein-based shell architectures. Mak et al. reported pH-driven reassembly of a solid microparticle that formed from a carboxysome shell protein.⁶⁰ Here we show disassembly of *in vitro* assembled HT shells between pH 3.0–3.6. Disassembled shells were subsequently reassembled when we added a high concentration of Tris-HCl to raise the pH to 8, shown by the reappearance of oscillatory features in the scattering profiles in Figure 4A. Analysis of residues at tile interfaces, shown in Figure S19, leads us to hypothesize that pH-mediated shell disassembly below pH 3.9

is the result of changes in electrostatic interactions due to protonation of acidic residues at the hexamer–trimer and hexamer–hexamer interfaces. For example, Asp18 (BMC-T) and Lys28 (BMC-H) at the hexamer–trimer interface and Asp3 and Lys28 at the hexamer–hexamer interface form salt bridges at pH 8 (Figure S19). Lowering the pH below 3.9 would cause side chain protonation (pK_a of aspartate side chain is ~ 3.9) and a loss of those electrostatic forces, which could potentially lead to shell disassembly. When the pH is raised to 8, the reverse effect occurs: residues are deprotonated, and the electrostatic interactions necessary for shell assembly are reestablished. Protein cage disassembly triggered by changes in attractive and repulsive electrostatic forces has been demonstrated by Dalmau et al.⁶¹ and Kim et al.⁶² by engineering histidine residues at protein subunit interfaces. Both report disassembly of protein cages at pH 5, which is consistent with protonation of the side chain of histidine (pK_a value of ~ 6.0) and the introduction of destabilizing electrostatic repulsion forces. Because we observe disassembly of the shell at and below pH 3.6, we suggest that the electrostatic interactions of aspartate residues play the most prominent role in maintaining the HO BMC shell structure.

Lastly, we tested whether the pH-mediated reversible disassembly of shells could be repetitively cycled. We exchanged shells between sodium citrate buffer pH 3.6 and TBS buffer pH 8 twice (protein loss due to extraction for SAXS measurements and binding to the Amicon filter limited the number of successive cycles). The scattering profile for the shells at pH 3.6 indicated disassembly, while at pH 8, the shell structure is recovered as shown by the return of profile oscillations for each cycle. Shell reassembly was verified with TEM (Figure 4B). At pH values below 3.9, it is evident that the protein structure in all the samples was not reflective of a typically assembled HT shell. Interestingly, the presence of sharp diffraction peaks at high q , most evident in Figure 4A, indicates ordered aggregates of the hexameric and/or trimeric subunits. We analyzed the disassembled protein samples from the pH cycling experiments with TEM, and the results illustrate that the diffraction peaks correspond to fiber-like structures (Figure 4B). It is still unclear exactly what these aggregate(s) are but the nature of them will be investigated in a follow-up study.

Nevertheless, when considering the potential of bioinspired approaches for selective metal capture,⁶³ the pH stability and reversible disassembly of shells provide evidence that BMC protein-based architectures also have potential applications as biobased nanoextractors. In particular, critical mineral and rare earth element (CM/REE) recovery is of increasing global importance as these metals are important elements used in cell phones, batteries, and wind turbines.⁶⁴ Biohydrometallurgy techniques^{65,66} are multistep processes, and the typical leaching step can result in acidic leachate, with the need to raise the pH before protein-based extraction.^{67,68} Therefore, BMC shells remaining stable at acidic pH and their catalytic programmability facilitated by *in vitro* assembly make them candidates for use as protein-based metal adsorbents. The engineerability of shell proteins provides possibilities for facile introduction of specific metal-binding sites for selective capture, and the reversibility of BMC shell disassembly in low pH conditions further increases the likelihood that these systems, as opposed to many unrecyclable biomasses,⁶⁹ can be used as valuable biomaterials in acidic metallo-protein desorption without destruction of the protein.

Moreover, controlled disassembly also provides opportunities for controlled recovery or release of cargo in diverse applications, including advanced manufacturing and drug delivery. In contrast to reversible disassembly of protein cages constructed using environmentally sensitive linkers^{70,71} or targeted residue mutagenesis at the subunit interface,^{61,62} the disassembly and reassembly mechanism shown for HO BMC shells is generated by the properties of native or near-native shell proteins without targeted bioengineering. Thus, we expect that this mechanism can be applied to various modified shell proteins as well.

In conclusion, we systematically tested *in vitro* assembled BMC shell stability across a wide range of conditions using DLS, imaging, and high-throughput SAXS, which allowed us to monitor changes in structural fidelity. The results of this study broaden the experimental conditions for conducting spatially organized chemistry with biotic and abiotic catalysts using a biomimetic system, demonstrate controlled and reversible shell disassembly, and expand the potential of BMC shell protein-based systems as versatile biomaterials. BMC shell proteins from various systems form an array of architectures,^{35,72–75} and these results imply potential stability across many different organized structures, whether assembled *in vivo* or *in vitro*. Using biological scaffolds typically involves working within near-physiological environments, but the remarkable range of shell stability shown in this study increases the attractiveness of BMC systems as versatile confinement architectures and biomaterials.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are openly available in the Simple Scattering repository at <https://simplescattering.com/dataset/xsrdlayp> under ID codes XSKRO1ZA (Solvents and Detergents), XSYZZNCX (Temperature), and XSRDLAYP (pH).

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.nanolett.6c02629>.

Additional experimental details, materials, and methods, including SAXS profiles and transmission electron micrographs of HT shells in various tested conditions, CD, and DSF measurements (PDF)

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Notes

The authors declare no competing financial interest.

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