Stabilization of Supramolecular Membrane Protein-Lipid Bilayer Assemblies Through Immobilization in a Crystalline Exoskeleton

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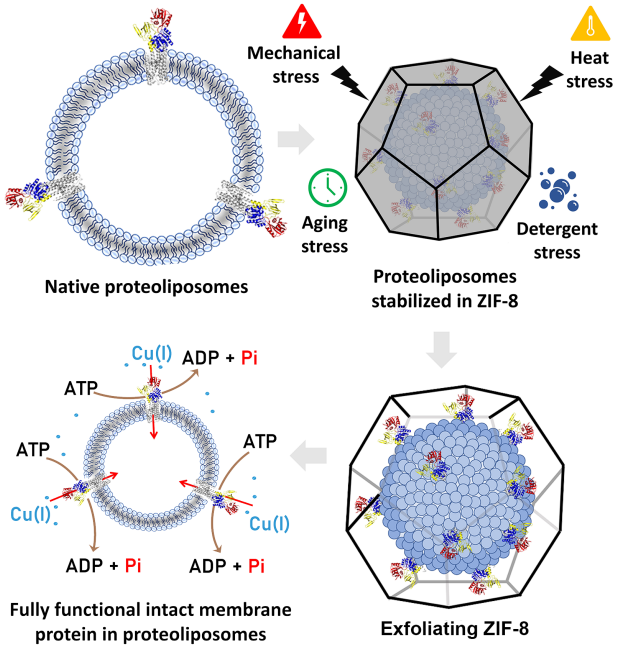
Abstract: Artificial native-like lipid bilayer systems constructed from phospholipids assembling into unilamellar liposomes allow the reconstitution of detergent-solubilized transmembrane proteins into supramolecular lipid-protein assemblies called proteoliposomes, which mimic cellular membranes. Stabilization of these complexes remains challenging because of their chemical composition, the hydrophobicity and structural instability of membrane proteins, and the lability of interactions between protein, detergent, and lipids within micelles and lipid bilayers. In this work we demonstrate that metastable lipid, protein-detergent, and protein-lipid supramolecular complexes can be successfully generated and immobilized within zeolitic-imidazole framework-8 (ZIF-8) to enhance their stability against chemical and physical stressors. Upon immobilization in ZIF-8 biocomposites, blank liposomes, and model transmembrane metal transporters in detergent micelles or embedded in proteoliposomes resist elevated temperatures, exposure to chemical denaturants, aging, and mechanical stresses. Extensive morphological and functional characterization of the assemblies upon exfoliation reveal that all these complexes encapsulated within the framework maintain their native morphology, structure, and activity, which is otherwise lost rapidly without immobilization.

Introduction

All living organisms, from prokaryotes to higher eukaryotes, rely on transmembrane protein systems for a variety of functions including signal transduction, substrate transport, and intramembrane enzymatic catalysis.[1] A significant fraction of polytopic transmembrane proteins act as transporters and are critical for the translocation of large and/or charged substrates in and out of the cell and understanding their function is imperative to understanding the etiology of many human diseases.[2-4] Purification of transmembrane proteins and subsequent reconstitution in artificial lipid bilayers—called proteoliposomes—generates metastable systems that are utilized for both structural analysis and functional investigations in which substrate transport can be studied on transporters embedded into their native–like environments.[5, 6] Unfortunately, owing to the patchwork of hydrophobic and hydrophilic surfaces of transmembrane proteins and the dynamic non-covalent nature of liposomes, these assemblies are intrinsically unstable and susceptible to denaturation, precipitation, and loss of function when left at room temperature for even a few hours. Despite years of effort toward stabilizing membrane-bound proteins, only a handful of approaches exist, and discovery of methods that protect them within their native bilayer environment remains an open challenge. Immobilization of

biomacromolecular motion by chaperone-like confinement in polymers has emerged as a way to stabilize soluble proteins, but the accessibility of addressable functional groups of transmembrane proteins is reduced from confinement in a lipid matrix and the poor stability of membrane-bound proteins further complicates bioconjugation.[7] Recently, polymeric excipients designed to match the natural distribution of polar residues on proteins[7] or stabilization in amphipathic copolymer-based membrane nanodiscs[8] have advanced the state-of-the-art; however, these coatings are neither removable nor scalable to protect a functional catalytic proteoliposome system. Specifically, a "sheddable" coating that avoids excipients or chemical functionalization of either protein or lipid would help advance work on these systems tremendously. To that end, we turned to metal-organic frameworks (MOFs), which are a class of crystalline porous coordination polymers constructed from the interlinking between metal centers and monomeric organic ligands.[9-11] Through thoughtful selection of the organic ligand and metal center, MOFs can be modulated sophisticatedly towards a wide array of applications[12] such as gas separation/sensing/storage,[13-15] catalysis,[16-18] and protein stabilization.[19-21] In recent years, several MOFs displaying zeolitic topologies have been reported,[22] the most ubiquitous being zeolitic-imidazole framework eight (ZIF-8), which consists of the coordination between Zn^{2+} and 2-methyl imidazole (HMIM).[23,24] ZIF-8 is unique in its ability to form thermodynamically stable crystalline shells by nucleating on biomacromolecules[25] and these systems can withstand high temperatures and pH[26] yet are kinetically labile around strong metal binding chelates.[27-29] ZIF-8 is well known for its ability to nucleate on the surface of colloidal and dissolved biomacromolecules forming a crystalline matrix shell.[30,31] We suspected that colloidal liposomes, proteoliposomes, and detergent solubilized transmembrane proteins would nucleate the growth of ZIF-8 over the surface, that would inhibit both protein denaturation and liposome degradation. Further, the kinetic lability of Zn-HMIM bond in the presence of strong metal chelators would allow us to recover the encapsulated systems without significant loss of protein function or sample homogeneity.



Scheme 1. Proteoliposome stabilization through biomolecular nucleation in ZIF-8. The scheme portrays the process by which the proteoliposome after encapsulation can resist different forms of stress. An 'exfoliating step' is then used to remove the shell and free the proteoliposomes when ready for analysis. Even after intense stressing, catalytic activity of transmembrane copper transporters remains very high.

In this study, we demonstrate a method for the thermal stabilization of (i) blank 200 nm liposomes, (ii) purified transmembrane proteins, and (iii) 200 nm transmembrane protein-liposome supramolecular complexes (proteoliposomes) against chemical and thermal stressors through biomolecular nucleation of ZIF-8. We selected two α-helical polytopic (8 transmembrane α-helices) iron and copper transporters— called IroT/MavN and CopA respectively— as representative examples of metal transport systems and virulence factors in bacteria that cause fatal human diseases.[32,33] Transition metal transporter proteins are the subject of ongoing research in a number of laboratories,[34-36] and their stabilization serves not only as a proof-of-concept but also in aiding and abetting research into these systems. We found that encapsulation of the proteoliposome complex generates thermodynamically stable bio-composites that can withstand exposure to high temperatures, aging, and common protein denaturants (Scheme 1). Further, the ZIF-8 coatings can be removed to afford pristine proteoliposomes, liposomes, and transmembrane protein micelle complexes of similar composition, morphology, structure, and catalytic activity to their native counterparts. Finally, our work demonstrates the generalizability and potential ZIF-8 scaffolds have for stabilizing highly sensitive and metastable systems.

Results and Discussion

Liposome Stabilization

We progressed systematically to demonstrate that each component—the as-prepared liposome, the detergent purified protein, and the proteoliposome system—could be encapsulated and protected by biomolecular nucleation. We prepared 200 nm liposomes by freeze-fracture and extrusion using mixtures of L-α-phosphatidylcholine and *E. coli* total polar lipid extracts, that allow the formation of native-like unilamellar lipid bilayers. While this composition was selected because it provides the best stabilization for our selected transmembrane proteins, the anionic nature of the formulated lipids are useful in promoting ZIF-8 formulation, as we and others have demonstrated.[37,38] Prior work with ZIF-8 synthesis has been done in pure water; however, the internal composition of the liposome lumen requires ideal buffering conditions to guarantee protein activity and stability when the proteoliposomes are generated and avoid osmotic bursting. Instead, we used a solution of 100 mM NaCl, 1 mM TCEP, and 20 mM MOPS buffered to a pH of 7.0 (M-buffer) as a solvent and systematically varied the concentrations of Zn salt and HMIM until liposomes (Lp) were quantitatively captured within ZIF-8 shells forming Lp@ZIF-8 (**Figure 1A**).

Our investigation uncovered two synthetic conditions that produced crystal encapsulated liposomes—M-buffer and 20 mM of zinc acetate dihydrate and 320 mM HMIM, which is an HMIM concentration 16-fold higher than the metal concentration (20×16) and 40 mM of $Zn(OAc)_2$ with 640 mM HMIM (40×16). Precursor solutions of zinc acetate dihydrate, HMIM, and 200 nm liposomes were mixed to immediately yield a white flocculate. Full crystal formation occurred following 18 h of incubation at RT and the artificial lipid bilayer-embedded ZIF-8 crystals were harvested by centrifugation, washed, and allowed to dry at RT for 12 h. No residual liposome formulations were found in the supernatants collected during washing steps per DLS. Crystals formed under the two different formulations were investigated by powder-X ray diffraction (PXRD) spectroscopy and SEM analysis (**Figures 1 B-D and Figures S1 A-B**). PXRD of the artificial lipid bilayer-embedded ZIF-8 and ZIF-8 controls was carried out and ZIF phase identification was performed using an online software program.[39] The 20×16 preparation is an 11% match with the well-known porous sodalite phase and an 89% match with the non-porous ZIF-C phase of ZIF-8. Unsurprisingly, nitrogen isotherms of both 20×16 Lp@ZIF-8 and 20×16 ZIF-8 control reveal low porosity (332 vs 418 m^2g^{-1} — **Figure S1 C and Table S1)**. In addition, there were several new reflections (9°,11°, 13°, and 28°), which we could not identify but likely are a new phase formed from use of M-buffer during encapsulation (**Figure 1 B**).[40] Indeed, energy dispersive X-ray (EDX) analysis shows sodium from M-buffer overlapping with Zn in these samples (**Figures S1 D-E**). In contrast, the higher metal-organic ligand ratio (40×16) formulation is intriguing. The PXRD is broad and matches poorly with the known polymorphs of ZIF-8. While the control liposome-free sample has high porosity (1569 m^2g^{-1}), curiously it shows a type II isotherm and microporosity. The surface area of 471 m^2g^{-1} for the Lp@ZIF-8 also indicates it is porous— **Figure 1 B, Figure S1 C, and Table S1**). Finally, thermogravimetric analysis (TGA) of 20×16 Lp@ZIF-8 and 20×16 ZIF-8 control indicates high thermal stability, with a ~15% mass loss of 20×16 Lp@ZIF-8 at 250 °C attributed to decomposition of lipids (**Figure S1 F**) whereas TGA of 40×16 reveals a gradual ~30 % mass

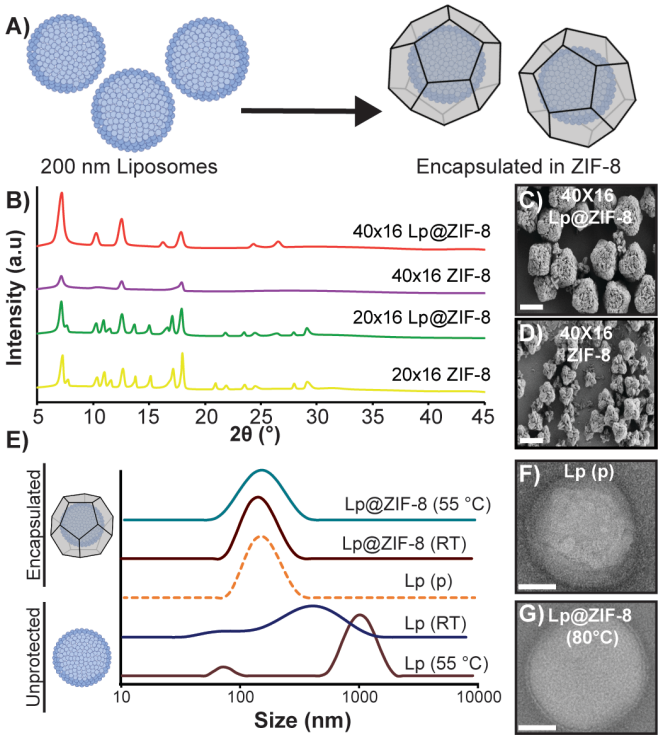


Figure 1. Characterization of artificial lipid bilayers embedded in ZIF-8. A) Biomolecular nucleation of liposomes in ZIF-8. B) PXRD spectra of ZIF-8 liposome complexes (Lp@ZIF-8) and ZIF-8 controls. SEM micrograph of C) 40×16 Lp@ZIF-8 and D) 40×16 pristine ZIF-8 (Scale bars = 1μm). E) DLS profiles obtained for immobilized liposomes exposed at high temperatures (after exfoliation) compared to native and non-encapsulated liposomes. F) TEM micrograph of pristine liposome and G) 40×16 Lp@ZIF-8 following exfoliation after exposure to 80 °C for 5 min (Scale bars = 50 nm).

loss starting at 200 °C, indicative of lipid decomposition (**Figure S1 F).** Curiously, despite the differences in morphology, both formulations appear to protect the liposomes equally well.

The protective capacity of the ZIF-8 coating toward the liposomes was evaluated on samples stressed at RT for 48 h and 55 °C for 15 min—this latter temperature being above the phase transition temperature of the liposomes. After stressing, the ZIF-8 shell was dissolved in EDTA (50 mM)—a process we refer to as exfoliation (**Scheme 1**)—and these solutions analyzed by dynamic light scattering (DLS) to assess liposomal size distribution. As controls, we stressed non-encapsulated liposomes following the same experimental conditions. As expected, the unprotected liposomes showed a significant increase in size and polydispersity (average diameter: 604.8 nm; PDI: 0.449; **Table S2**) resulting from membrane fusion and liposome aggregation (**Figure 1 E, lower two traces**) under both conditions. Conversely, we were happy to find that the monodisperse nature of freshly extruded blank liposomes (average diameter: 141.2 nm; PDI: 0.138) was retained for the ZIF coated composites (average diameter: 123.2 nm; PDI: 0.179; **Figure 1 E top two traces**). Preservation of the original liposomal morphology in ZIF-8 immobilized samples was further confirmed by transmission-electron microscopy (TEM). While the stressed ZIF-8 liposomes showed liposomes that were indistinguishable from freshly extruded pristine samples (**Figures S2 A-E**), unprotected liposomes showed altered morphology as a consequence of extensive membrane rupture and fusion (**Figures S2 F-G**). In light of the observed stabilization, Lp@ZIF-8 composites were thermally stressed at 80 °C and subsequent analysis by DLS and TEM imaging revealed that the original morphology and size distribution was largely retained (average diameter: 119.3 nm; PDI: 0.231; **Figures S2 A & D**). To test the ability of ZIF encapsulation in protecting from aging, Lp@ZIF-8 was dried and the Lp@ZIF-8 powder was left on the benchtop for 20 days at room temperature. Following exfoliation, TEM characterization revealed that the liposomes retained their original size distribution and morphology (**Figure S2 E**). In contrast, the non-encapsulated liposomes kept in solution aggregated within two days (**Figures S2 F-I**). Finally, both formulations discussed above (20×16 and 40×16) provided outstanding protection against thermal stress and aging following liposome immobilization in ZIF-8 composites (**Figure 1 and Figure S3**).

Stabilization of Purified Transmembrane Proteins

We selected two different transmembrane proteins, both of which are poorly stable at room temperature, to demonstrate the broad utility of our approach. IroT/MavN is a transmembrane protein found in *Legionella pneumophila* (*L. pneumophila*), a thin, flagellated, gram-negative bacteria responsible for Legionnaires' disease.[41] IroT meditates iron sequestration as an essential micronutrient from host cell, allowing for *L. pneumophila* to replicate in a host-derived vacuole within the infected macrophages.[42] IroT topology is characterized by 8 transmembrane (TM) helices and a long C-terminal domain.[43, 44] The structure and substrate translocation modality in IroT are active areas of research, but much has been gleaned from reconstituting IroT in artificial lipid bilayer systems and performing real-time transport assays.[44] IroT was shown to act as a Fe^{2+}/H^{+} antiporter that allows Fe^{2+} acquisition into the vacuole from the host cell for pathogen survival.[43] The second protein selected is a copper P_{1B}-type ATPase from *E. coli* (CopA), a transmembrane primary-active pump, and part of the P-type ATPase superfamily, that utilize energy generated by ATP hydrolysis to drive Cu^{+} transport across biological membranes against electrochemical gradients.[45,46] These catalytically driven pumps constitute an essential system to drive the selective translocation and export of Cu^{+} ions, thereby controlling the intracellular Cu^{+} levels.[47,48] Their activity tightly balances the biogenesis and integrity of copper centers in vital enzymes to non-toxic intracellular copper levels. The CopA structure is characterized by the existence of an 8 TM helices membrane domain (M-domain) connected to large cytosolic domains (N-, P- and A-domains) responsible for ATP hydrolysis, phosphorylation and energy transduction, allowing Cu^{+} translocation across the membrane.[35, 45, 47, 49] As a result of their critical involvement in essential iron and copper metabolism, both IroT and CopA homologues have been identified as key virulence factors in bacterial pathogens.[37,50]

Transmembrane proteins, including IroT and CopA, are commonly extracted from membranes and purified as detergent micellar complexes for solubilization in aqueous environments. The detergent molecules surround the hydrophobic regions of the protein in the micelles, which helps avoid aggregation, precipitation, and refolding in water. Though they are more stable, these proteinaceous assemblies still require unique environmental conditions to remain fully active—*e.g*. long-term storage at −80 °C, constant refrigeration for analysis *etc*. Since this strategy is employed in the typical workflow for incorporating transmembrane proteins in liposomes,[39,42] and naked transmembrane proteins are

extremely prone to denaturation, we suspected simply nucleating the ZIF-8 over the detergent-protein supramolecular complex would improve the likelihood of retaining protein function in high yields following exfoliation. We thus solubilized and purified IroT as in Cymal-7 (7-Cyclohexyl-1-Heptyl-β-D-Maltoside) micelles and CopA in micelles prepared with DDM (*n*-Dodecyl-β-D-Maltopyranoside) and applied our synthetic strategy, developed above, to produce ZIF-8 composites (**Figure 2A**). Crystals were isolated by centrifugation, washed, and allowed to dry at RT for 12 h. As-obtained crystals were characterized by SEM and showed a star-shaped morphology (**Figures 2 B-E and Figures S4 A and B**) yet, curiously, by PXRD both the 40×16 IroT@ZIF-8 and 40×16 control showed a 13% match with sodalite and 87% match with ZIF-C morphologies (**Figure 2 F**). TGA analysis of 40×16 IroT@ZIF-8 and 40×16 control revealed high thermal stability as shown in Figure S4, where the 40×16 IroT@ZIF-8 is characterized by an ~20% mass loss at 200 °C corresponding to protein-micelle decomposition (**Figure S4 C**). Further, nitrogen isotherms of 40×16 IroT@ZIF-8 reveal no measurable porosity, whereas the pristine material shows a BET surface of 993 m^2g^{-1} (**Figure S4 D**). Quantification of encapsulation efficiency was determined by SDS-PAGE gel densitometry. Supernatants obtained during the washing of ZIF-8 bio-composites and exfoliated protein-detergent complexes were run in tandem with either IroT or CopA pristine standards of varying concentrations. We found the encapsulation efficiency to be quantitative—no residual protein was found in the supernatant after the encapsulation procedure. Indeed, after encapsulation, isolation of the final protein@ZIF product, and subsequent exfoliation, resulted in almost 75% recovery, regardless of the protein or metal-to ligand ratio used (**Figure S5 A–C**).

To determine the integrity of IroT or CopA detergent-protein micelles after biomolecular nucleation, two properties were analyzed to benchmark the protective effect of immobilization: i) monodispersity analysis by size-exclusion chromatography (SEC), for IroT; and ii) catalytic metal transport activity assessed by metal-stimulated ATP-hydrolysis, for CopA. SEC is a good proxy for testing the stability of the generated IroT-detergent complexes as the absence of aggregation is an indicator of the integrity and stability of the protein-detergent assembly. On the other hand, for purified Cu^+ P-type ATPases, we could measure the rate of ATP hydrolysis in presence of the selected metal substrate as a means to show persistence of structure and function, as ATP hydrolysis and Cu^+ transport in CopA are tightly coupled. To verify the CopA functionality in detergent micelles (or upon incorporation in proteoliposomes) the Cu^+-dependent stimulation of ATPase activity was determined by photometric quantification of released inorganic phosphate generated by the catalytic ATP-hydrolysis using Malachite green. Upon stressing, immobilized samples were exfoliated and immediately characterized by either SEC or metal-dependent ATP-hydrolysis assays. The non-encapsulated IroT samples showed increased polydispersity when incubated at RT as evidenced by development of an asymmetric elution peak shoulder by SEC and almost complete aggregation after exposure to 55 °C and 80 °C for a few minutes (**Figure 2 G and Figure S6 A**). This is to be expected as these proteins are extremely prone to aggregation at even low temperature. In contrast, for the encapsulated samples, exposure to RT for 48 h and 55 °C for 15 min had little impact in the monodispersity of IroT. SEC analysis of the exfoliated IroT samples showed a single elution peak (elution volume = 12.0 mL), closely corresponding to non-stressed and

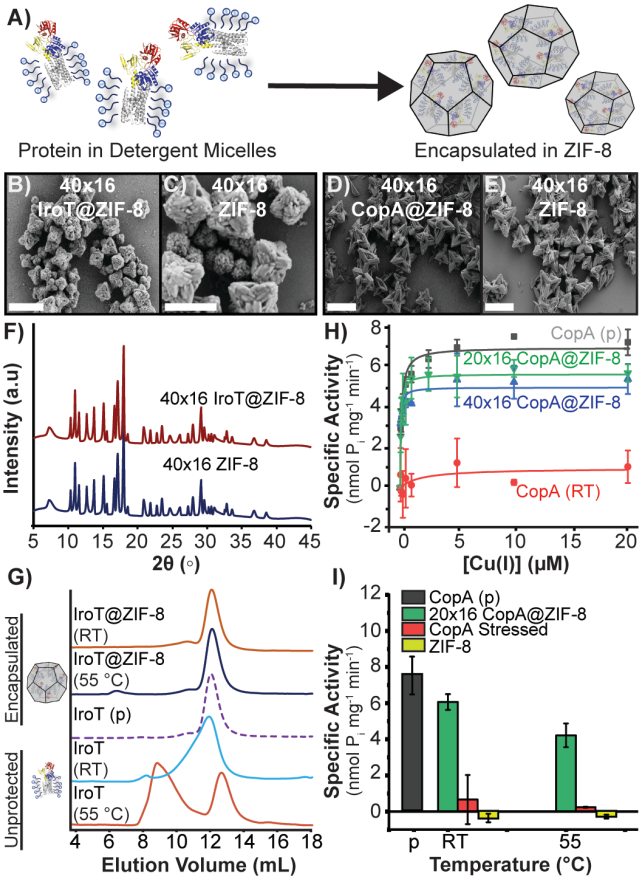


Figure 2. IroT@ZIF-8 and CopA@ZIF-8 characterization. A) Biomolecular nucleation of detergent stabilized CopA and IroT in ZIF-8. SEM micrographs of B) 40×16 IroT@ZIF-8 and C) 40×16 ZIF-8 control (Scale bars = 1μm). SEM micrographs of D) 40×16 CopA@ZIF-8 and E) 40×16 ZIF-8 control (Scale bars = 1μm). F) PXRD of IroT@ZIF-8 and pristine ZIF-8. G) SEC traces of heated IroT@ZIF-8 bio-composites against native and non-encapsulated IroT. H) ATPase activity analysis of CopA after stressing and exfoliation with unencapsulated control (red line). I) Comparison of maximal specific activity in CopA samples and controls.

refrigerated IroT controls (12.0 mL) as shown in **Figure 2 E**. Incredibly, exposure to 80 °C (5 min) also led to a minimal degree of aggregation (**Figure S6 A**).

Resilience to thermal stress was subsequently investigated on the CopA-DDM micelle complexes by analyzing the catalytic substrate-dependent ATP hydrolytic activity. Cu^{+}-dependent stimulation of ATPase rates for non-stressed CopA, in the presence of Mg^{2+}/ATP revealed characteristic catalytic hyperbolic Michaelis–Menten-type dependency as a function of Cu^{+}, confirming that the purification in detergent micelles maintains CopA in a functional form ($K_{M,\,Cu(I)}$ = 0.12 ± 0.02 μM; Vmax of 7.1 ± 0.2 nmol (mg min)$^{-1}$). However, upon thermal stress at RT, 55 °C and 80 °C, the CopA-DDM catalytic activity was completely abolished, with only < 10% residual activity at RT. In contrast to unprotected protein, CopA samples encapsulated in ZIF-8 and stressed retained the characteristic Michaelis-Menten dependency of their ATPase activity after thermal stress and exfoliation (**Figure 2H**). Analysis of CopA@ZIF-8 bio-composites ATPase activities values at saturating Cu^{+} concentrations revealed that CopA retained > 80 % (6.3 nmol P_i mg^{-1} min^{-1}) of its maximal ATPase activity upon stress at RT (**Figure 2I**), > 60% at 55 °C (4.7 nmol P_i mg^{-1} min^{-1}; **Figure 2I**) and at > 42% at 80 °C (3.1 nmol P_i mg^{-1} min^{-1};**Figure S5 D**). Accordingly, analysis of the $K_{M,\,Cu(I)}$ values at RT indicated that upon stress unaltered catalytic parameters are preserved by ZIF-8 encapsulation **(Figure S5 E).**

It is noteworthy that formulation conditions are an important aspect of stability. CopA was encapsulated under both metal-to-ligand ratios discussed above (20×16 and 40×16) and, interestingly, IroT-cymal-7 micelles showed better stabilization with the 40×16 formulation, while the 20×16 formulation was most effective at enhancing the thermal stability of CopA-DDM micelles. Thus, formulation optimization is an important parameter to be screened for optimal bio-composite protection depending on the protein topology and protein-detergent micelle structure **(Figure S5 D-F).**

Encapsulation of proteinaceous materials has been widely used to increase stability of moieties against chemical stressors, such as organic solvents and chaotropic agents.[33,34] Motivated by such reports, CopA@ZIF-8 and IroT@ZIF-8 were chemically stressed using SDS, a commonly used protein denaturant. Briefly, the samples were incubated for 30 min in a solution consisting of 0.1% SDS. Crystals were harvested by centrifugation, washed 5× with ultra-pure water, exfoliated, and immediately characterized by SEC analysis (IroT) or Cu^{+}-dependent ATP-hydrolysis assays (CopA). As shown in **Figure S6**, immobilization in ZIF-8 affords retention of monodispersity and activity for both encapsulated transmembrane proteins, while non-encapsulated control samples are fully denatured and inactive in the presence of 0.1% SDS. These results also suggest that a population of proteins are at least partly exposed to the MOF surface, accounting for the modest (∼15 %) loss of functionality (**Figure S6 B, C-D**). [36]

Proteoliposome Stabilization

Our analysis demonstrates that immobilization in new ZIF-8 composites allow for stabilization of both pristine liposomal vesicles as well as protein-detergent micellar complexes, providing a 3D scaffold that can morph around complex and chemically diverse biomolecular assemblies thereby providing protection against stressors. In light of the versatility of the approach we sought to determine if our ZIF-8 encapsulation strategy can be utilized to protect even more complex and metastable supramolecular entities such as proteoliposomes. Purified IroT and CopA were reconstituted in unilamellar liposomes via freeze–thaw and extrusion through 200 nm filters, followed by liposome destabilization by detergent addition and subsequent detergent removal by Biobeads resin. Protein incorporation was subsequently quantified by SDS-PAGE following removal of excess detergent solubilized protein from the proteoliposomes by ultracentrifugation, with subsequent protein quantification of the soluble and proteoliposome fractions conducted by gel densitometry. We subsequently immobilized in ZIF-8 proteoliposomes with IroT or CopA embedded in the lipid bilayer (**Figure 3A-C**). In a typical experiment, 6.25 mg mL^{-1} of proteoliposomes (with 0.25 mg mL^{-1} of either protein, TEM of typical samples shown in Figure 3B) were encapsulated at ambient conditions mixing the proteoliposome complexes with a solution of HMIM and zinc acetate dihydrate using M-buffer as solvent. Crystals were harvested by centrifugation after 18 h of aging at RT, washed with ultrapure water and methanol, and allowed to dry at RT for 12 h. SDS-page gel densitometry revealed protein recovery after exfoliation with no detectable traces of protein in the supernatants (**Figure S7 A**). As previously observed, in the presence of sodium chloride, TCEP or DTT, and MOPs the crystal morphology obtained gives rise to aggregates of star-like shape crystals (**Figures S7 B-H)** that displayed different ZIF-8 (U-14, ZIF-C, sodalite, and diamond) topologies by PXRD. The encapsulation and exfoliation process, which were optimized in the prior two studies, was very straight forward for proteoliposome assemblies to generate IroTPL@ZIF-8 and CopA-

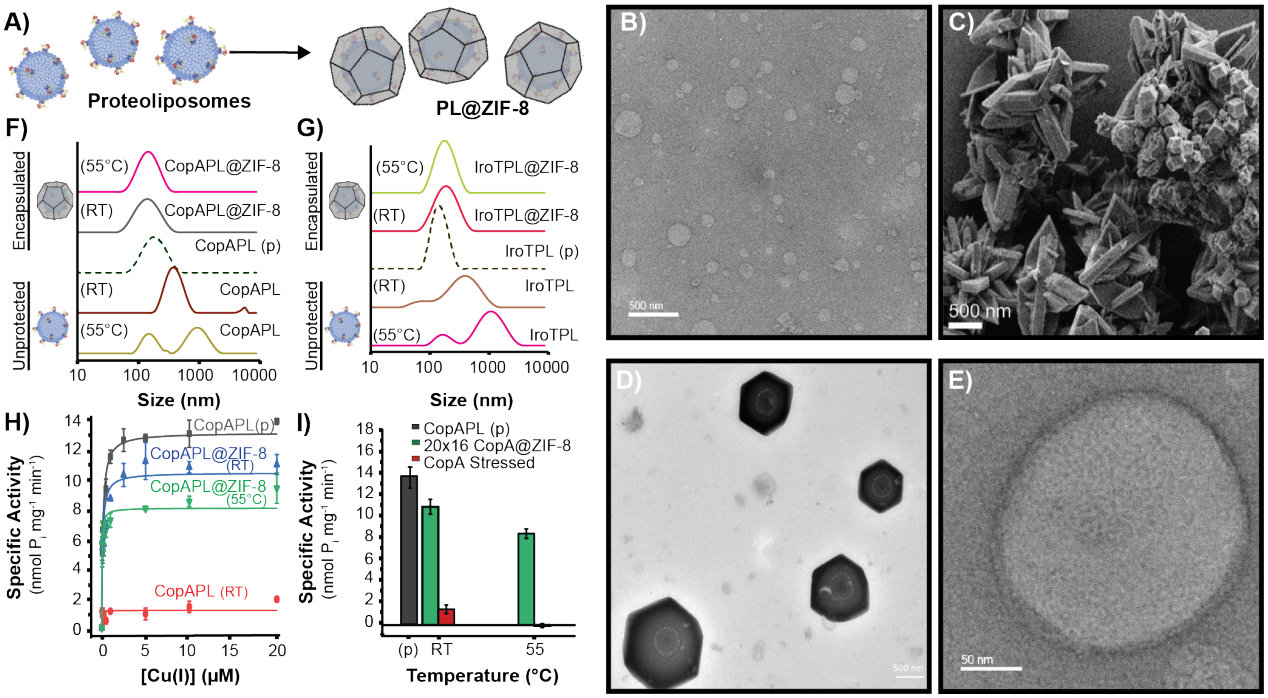


Figure 3. Characterization of proteoliposome@ZIF-8 bio-composites. A) Biomolecular nucleation of CopA-PL and IroTPL in ZIF-8. B) TEM micrograph of pristine CopA containing proteoliposomes. C) SEM micrograph of CopA-PL@ZIF-8 bio-composites. D) TEM micrograph of partially exfoliated CopA-PL@ZIF-8 bio-composites after addition of chelating agent, EDTA. E) TEM micrograph of a recovered fully functional CopA containing proteoliposome upon complete exfoliation. DLS of F) 20×16 CopA-PL@ZIF-8 bio-composites after thermal stressing followed by exfoliation compared to controls and G) 40×16 IroTPL@ZIF-8 bio-composites after temperature stressing followed by exfoliation compared to controls. H) Specific activity of 20×16 CopA-PL@ZIF-8 bio-composites stressed at RT and 55 °C. I) Comparison of maximal specific activity at saturating Cu^+ concentration of 20×16 CopA-PL@ZIF-8 bio-composites stressed at RT and 55 °C to the non-encapsulated and stressed CopA-PL

PL@ZIF-8 bio-composites. We found that brief exfoliation dissolved the U-14, ZIF-C and diamond topologies more quickly and left behind sodalite crystals that remarkably show single proteoliposomes inside these dodecahedral crystals (**Figure 3D**). The greater kinetic ability of these morphologies has been reported elsewhere.[39] Following exfoliation, we found the proteoliposome size and shape were not altered compared to unprotected proteoliposome by TEM and DLS analyses, even after heat exposures, regardless of the ZIF-8 formulation utilized for immobilization (**Figure 3E, Figure S8 and Table S4 and S5**). In addition to the size and morphology preservation, activity assays performed on the ZIF-8 immobilized CopA proteoliposomes confirmed that the MOF shell thermally enhances these delicate systems enabling them to resist temperatures that would otherwise promote loss of function (**Figures 3 F-I**). To demonstrate that encapsulated proteoliposomes not only resist prolonged periods of no refrigeration but also enhances stability towards physical/mechanical stressors we shipped a sample through the United States Postal Service. PL@ZIF-8 bio-composites were placed in a standard cushioned mailer and shipped across the United States, from Texas to Rhode Island and back again. They were then left at room temperature for two months following shutdown of our laboratories during the 2020 SARS-CoV-2 pandemic. After exfoliation, the catalytic activity and liposome morphology were similar to the pristine counterparts (**Figure S9**) in contrast to controls, which we know degrade within a day.

Conclusion

Lipid bilayers are the core building unit of cell membranes, which serve as the main line of action between the outside and the inside environments of the cells and organelles. Given their structural complexity, researchers have been motivated to develop simpler model systems to understand the molecular processes associated with cellular membrane dynamics and investigate protein-mediated solute translocation across lipid bilayers. Proteoliposomes are a powerful tool that mimic cellular membranes. By virtue of tuning the vesicle size and the lipid and protein composition, proteoliposomes have become instrumental to the study both prokaryotic and complex eukaryotic cell membranes, and proteins embedded into them, including transmembrane transporter proteins. Despite their utility, proteoliposomes are delicate systems that require unique conditions to maintain their functionality that have long imposed obstacles for their handling/transport and hindering their usefulness for the better understanding of the *modus operandi* of transmembrane proteins. Taking advantage of the high thermal and aqueous stabilities of ZIF-8, blank liposomes, detergent-

solubilized proteins, and proteoliposome complexes no longer require constant refrigeration and repeated extrusion to maintain their intrinsic structure, monodispersity and functionality over long incubation times. Further, we show that immobilization in ZIF-8 enables the as-prepared bio-composites to be exposed to chemical denaturants and temperatures above their lipid bilayer phase transition without structural and/or functional changes. Finally, we have shown that biomolecular nucleation is an effective process to preserve supramolecular membrane protein-lipid bilayer assemblies against conditions that, without encapsulation, would easily impair their structural and functional integrity.

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Author Contribution

§F.C.H., S.S.A, and N.S.A contributed equally to this manuscript. Primary manuscript writing and editing was done by F.C.H. G.M. and J.J.G. Blank liposome, IroT, and IroTPL encapsulation, stressing, was done by F.C.H. IroT expression, purification, proteoliposome preparation, SECs of IroT/IroTPL were performed by S.S.A. CopA expression purification, proteoliposome preparation, and ATPase activity assays were done by N.S.A. CopA/CopA-PL encapsulation and stressing were done by Y.H.W. SDS was done by F.C.H, S.S.A., N.S.A., and Y.H.W. DLS was done by F.C.H., S.S.A, and Y.H.W. TEMs were taken by O.R.B. SEMs were taken by M.A.L. and O.R.B. PXRDs were done by F.C.H and M.A.L. TGA was done by A.D.S. Nitrogen sorption and BET analysis were done by S.D.D. Funding was raised by R.A.S, G.M. and J.J.G.

Competing Interest

The authors declare no competing interest