RESEARCH ARTICLE

Controlling Protein Enrichment in Lipid Sponge Phase Droplets using SNAP-tag Bioconjugation

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Abstract: All cells use organized lipid compartments to facilitate specific biological functions. Membrane-bound organelles create defined spatial environments that favor unique chemical reactions while isolating incompatible biological processes. Despite the fundamental role of cellular organelles, there is a scarcity of methods for preparing functional artificial lipid-based compartments. Here, we demonstrate a robust bioconjugation system for sequestering proteins into zwitterionic lipid sponge phase droplets. Incorporation of benzylguanine (BG)-modified phospholipids that form stable covalent linkages with an O^6-methylguanine DNA methyltransferase (SNAP-tag) fusion protein enables programmable control of protein capture. We show that this methodology can be used to anchor hydrophilic proteins at the lipid-aqueous interface, concentrating them within an accessible but protected chemical environment. SNAP-tag technology enables the integration of proteins that regulate complex biological functions in lipid sponge phase droplets, and should facilitate the development of advanced lipid-based artificial organelles.

Introduction

Lipid compartments play a key role in governing essential cellular processes, including molecular segregation,[1,2] protein sorting,[3,4] and trafficking.[5] Membrane-bound organelles coordinate complex synthetic reaction networks by regulating the spatial organization of molecules anchored to their membrane.[6] Moreover, a large number of membrane-based organelles can exhibit a convoluted morphology, in which lipid bilayers form non-lamellar three-dimensional periodic structures.[7–10] However, the majority of studies in bottom-up synthetic biology have used closed spherical lipid vesicles to mimic organelle structures and functions.[11–13] Therefore, there is considerable interest in exploring new artificial lipid architectures capable of recreating the physiological environment of natural organelles. The complex topology observed in lipid mesophases could allow them to serve as a generic model system of the convoluted membrane architecture in membrane-bound organelles that would help to elucidate the functional roles of non-lamellar structures in biology.[14] Lipid mesophases consist of a highly curved continuous bilayer that encompasses an interconnected aqueous channel network.[15,16] Given the utility of such bicontinuous architectures for protein crystallization,[17–19] drug delivery,[20–22] and as food emulsifiers,[23] the applications of lipid mesophases in mimicking organelle function is expanding.

A lipid mesophase that could be potentially used as a synthetic organelle is the lipid sponge phase. For instance, we recently reported a novel lipid sponge phase system using a combination of *N*-oleoyl β-D-galactopyranosylamine (GOA) and a commercially available nonionic surfactant,

octylphenoxypolyethoxyethanol (IGEPAL).[24] When combined in a 4:3 molar ratio, these amphiphilic surfactants form a dispersion of lipid sponge phase droplets with L_3 phase.[24] We further demonstrated that such a mesophase could be programmed using interactions such as biotin/streptavidin to concentrate proteins capable of catalyzing biochemical reactions.[24] Unfortunately, this approach presents several limitations, such as a lack of covalent bond formation, challenges in controlling protein stoichiometry, and crosslinking during enrichment in the sponge droplet due to the multivalency of streptavidin. Therefore, developing straightforward conjugation methodologies based on stable covalent bond formation with the lipid membrane would enable proteins of interest to be efficiently incorporated into well-defined lipid sponge phase droplets.

Here, we present a one-step bioconjugation strategy for the covalent linkage of proteins to the lipid bilayer of lipid phase sponge droplets formed by self-assembly of the zwitterionic gemini surfactant **1**, which contains an anionic phosphodiester and a cationic quaternary ammonium separated by two methylenes. A C8 and C10 alkyl tail is linked to the phosphodiester and the ammonium groups, respectively (Figure 1A,B).[25,26] Compound **1** was reported to spontaneously self-assemble into structures with a sponge morphology characterized by a three-dimensional network of phospholipid bilayers.[25,26] Although the formation of sponge phases generally requires the combination of multiple lipids with specific physical properties,[27,28] compound **1** is notable in that it is capable of forming these structures without additional components.[25,26] Because at least one other lipid bearing a group that can undergo the functionalization reaction must be present, we chose to use compound **1** in order to reduce the total number of necessary components for lipid sponge phase formation and functionalization. Our approach takes advantage of an O^6-methylguanine DNA methyltransferase (SNAP-tag) fusion protein, which enables covalent linkage of proteins to benzylguanine (BG)-modified phospholipids embedded in the membrane of lipid sponge phase droplets.

We show that the SNAP-tag bioconjugation can be used to control sequestration of hydrophilic proteins into lipid sponge phase droplets. This bioconjugation method serves as a versatile tool to program protein enrichment into non-lamellar lipid phases. We demonstrate that the SNAP-tag reactive lipid system can cooperate with the complex biomolecular machinery required for *in vitro* protein expression, enabling protein partitioning of *in situ* expressed proteins. The developed SNAP-modification method for lipid sponge droplets provides a valuable model system to more closely mimic the ability of natural membrane-bound organelles to spatially control protein organization.

Results and Discussion

We first investigated lipid phase sponge droplets by characterizing their structural and physical properties. Gemini surfactant **1** (Figure 1A) was synthesized as previously reported (Scheme S1).[25,26] To obtain a dispersion of lipid sponge phase droplets, a thin film of compound **1** was deposited on the walls

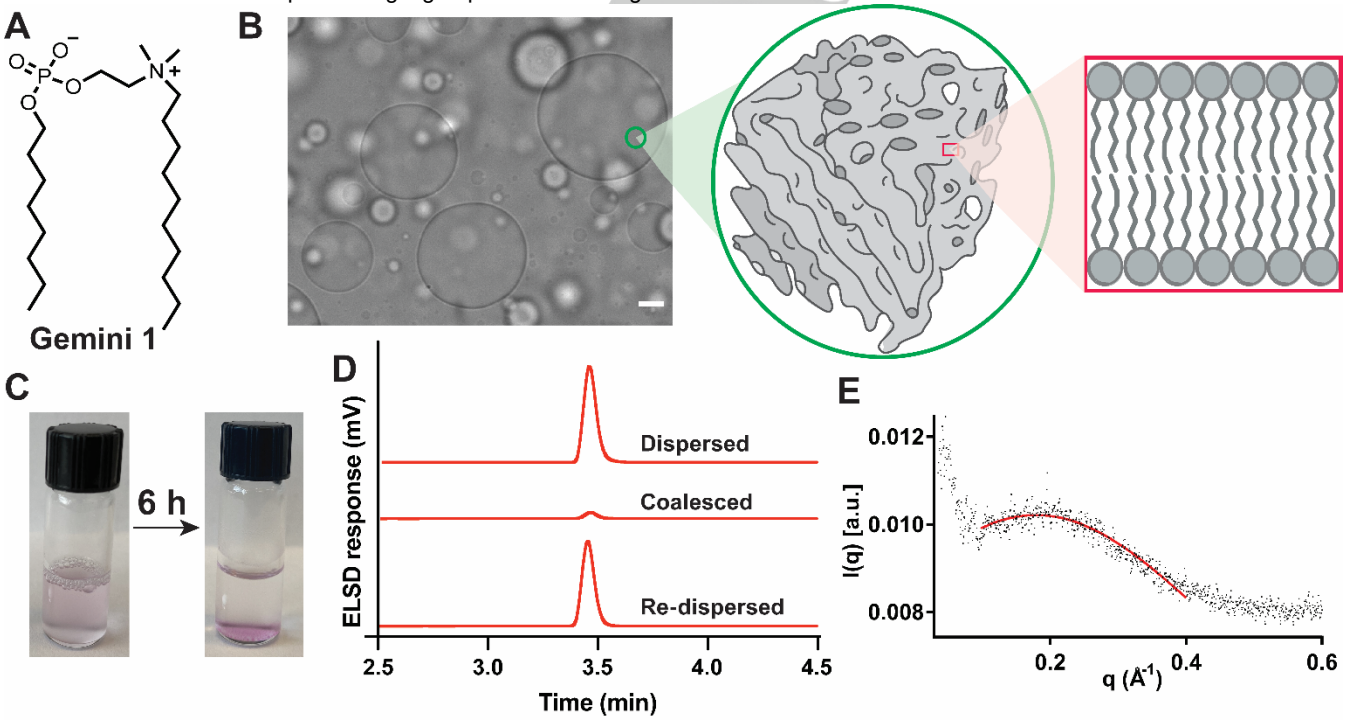

Figure 1. Structural and physical characterization of lipid sponge phase droplets. (A) Chemical structure of the gemini lipid **1**. (B) Phase contrast microscopy of lipid sponge phase droplets (Scale bar: 10 µm), and illustration of their porous (highlighted in the green circle), bilayer-rich (highlighted in the red rectangle) nanostructures. (C) Gemini **1** (5 mM equivalent concentration) (stained with Nile red 0.01 mol%) in PBS buffer pH 7.4 right after hydration, showing the formation of a distinct dispersion *(left)*. After 6 h, a coalesced phase at the bottom of the vial was formed *(right)*. (D) HPLC-ELSD traces of a dispersion of compound **1** (5 mM equivalent concentration) in PBS buffer pH 7.4. Immediately after vortexing, compound **1** *(Dispersed)* could be detected throughout the mixture, but extensively partitioned into the coalesced phase within 6 h *(Coalesced)* of being allowed to settle. Compound **1** could then be re-dispersed into a uniform dispersion by vortexing *(Re-dispersed)*. (E) Synchrotron SAXS intensity profile (dotted black) and fitting (solid red) from a dispersion of compound **1** (30 mM equivalent concentration) in PBS buffer pH 7.4. The horizontal scale is the wave vector transfer q, which is inversely proportional to the length scale being measured.

of a glass vial, hydrated with an aqueous solution such as water or PBS (pH 7.4), and vortexed to obtain a uniformly turbid dispersion. Phase contrast microscopy analysis showed that the dispersion consisted of micrometer-sized spherical droplets (Figure 1B). The droplets were observed within a wide range of gemini **1** concentrations (0.5 to 5 mM equivalent concentration) and pH (2 to 10) (Figure S3). Over time the droplets tended to coalesce into a separated phase at the bottom of the vial (Figure 1C). The lipid phase separation was confirmed using high performance liquid chromatography-mass spectrometry-evaporative light scattering detection (HPLC-MS-ELSD) (Figure 1D). Analysis of the supernatant of a 5 mM gemini **1** dispersion over time showed that a large portion of compound **1** partitioned into the coalesced phase within 6 h. A uniform dispersion was reobtained by simple vortexing (Figure 1D). For a better assessment of droplet stability in the dispersion, the droplet coalescence was monitored by turbidimetry analysis, measuring the optical density (OD) over time. The constant decrease of OD showed in the results (Figure S6) indicated that droplet coalescence occurs in a slow and continuous manner, requiring more than 15 h to complete rather than by an abrupt aggregation event.

Thermogravimetric analysis (TGA) of the coalesced phase revealed a gradual 67% mass loss from 30 to 150 °C (Figure S4), presumably deriving from the loss of water, which suggests the presence of an aqueous-accessible environment within the lipid nanostructure. To further probe the droplet environment, we measured the fluorescence emission of the solvatochromic dye Laurdan added to a dispersion of compound **1**. The fluorescence emission peak of Laurdan is generally centered at 440 nm in apolar environments such as in oil droplets, while emission profile undergoes a red shift when embedded into more polar environments, such as at a lipid-water interface.[24,29] For our lipid sponge phase droplets, we observed a maximum fluorescence emission at 490 nm (Figure S5), suggesting that the assembled lipid structures have a water accessible environment.

To determine the nature of the lipid phase structure in droplets, we performed small-angle X-ray scattering (SAXS) measurements on dispersions of compound **1** at the Advanced Photon Source synchrotron at 25 ºC. SAXS is a well-established and direct technique to study and characterize lipid self-assembly in aqueous environments. The fitting of SAXS patterns enables determination of the average lipid structural parameters and allows extrapolation of information on the lipid self-assembly structure. Each lipid mesophase exhibits a characteristic 2D SAXS pattern, which is converted to a 1D plot represented by the intensity [I(q)] of the lipid dispersion against the scattering vector (q). For instance, a lamellar phase will present Bragg peaks distanced in a 1:2:3:4:5 ratio.[30] The broad peak observed for dispersions of compound **1** (Figure 1E) corresponded to a fluid isotropic phase that fits with a sponge bicontinuous phase (L_3). The L_3 is a non-lamellar phase presenting a 3D lipid bilayer structure surrounded by interconnected water channels. Despite the similarities between the architectures of an L_3 phase and an ordered cubic phase, the L_3 sponge structure is much more fluid, which is evidenced by the absence of well-defined Bragg peaks. Thus, the sponge phase has also been described as a “melted” cubic phase.[31] An average periodic repeat distance can also be determined, which is related to the average pore diameter size in the sponge phase. To obtain the periodic repeat distance (L), the droplets broad peak was fitted as follows:[32]

$$I(q) = \frac{c}{\xi^{-2}+(q-q_c)^2} \quad \text{(eq. 1)}$$

where c refers to the intensity normalization constant, ξ to the correlation length and q_c to the peak center. The correlation length ξ estimates the root mean square of the fluctuations in the interpore separations and gives qualitative information about the disorder of the system. The sponge phase characteristic distance, corresponding to the average interpore separation, is defined by:

$$L = \frac{2\cdot\pi}{q_c} \quad \text{(eq. 2)}$$

The calculated repeat distance L for our lipid sponge phase droplets was 3.5 nm. The average pore diameter can then be estimated by subtracting the bilayer thickness (see below) from L.

We also performed X-ray diffraction measurements on a silicon substrate to determine the thickness of lipid bilayers formed from compound **1**. The X-ray diffraction measurements were performed using an in-house X-ray spectrometer. Compound **1**, like many other amphiphilic lipids, can be induced to self-assemble as a lamellar phase when deposited on a smooth solid substrate (such as a Silicon wafer) by slow evaporation of the solvent, and annealing in a high humidity atmosphere for several hours. The final multilamellar structure can be studied and identified by carrying out X-ray reflectivity measurements from the deposited film.[33] We observed that up to five lamellar equally spaced Bragg peaks were present (Figure S8A). The lamellar repeat distance of compound **1** at room temperature and 98% relative humidity was 3.0 nm, which included both the lipid bilayer and the water channel thicknesses. In order to determine the lipid bilayer thickness, we determined its Electron Density Profile (EDP). When a sample owns large-positional order, like a multistack of lipid bilayers, the one-dimensional EDP can be extracted by the magnitude of the structure factor ($|F_h|$) showed below:

$$F_h = |F_h| \cdot e^{i\Phi h} \quad \text{(eq. 3)}$$

where F_h is the structure factor and $e^{i\Phi h}$ is its phase term. $|F_h|$ is related to the integrated intensities of Bragg peaks I_h as:

$$|F_h| = \pm \sqrt{(I_h)} \quad \text{(eq. 4)}$$

We solved the phase problem ($+|F_h|$ or $-|F_h|$) using the well-established swelling method (see a detailed explanation in the Supporting information).[34,35] The EDP from a multistack of lipid bilayers has a characteristic shape (Figure S8B), where the minimum represents the methyl groups of the lipid hydrocarbon chains and the two maxima represent the lipid headgroup of each lipid layer. Therefore, the lipid bilayer thickness can be established as the distance between these two maxima.

The EDP of **1** displayed the characteristic shape for lipid bilayers, with a minimum representing the center of the bilayer (z = 0 Å, for convenience) and the two maxima representing the two lipid headgroups of the bilayer. However, the EDP presents small contrast between the headgroup and water region compared to natural diacylphospholipids, which could be explained by the

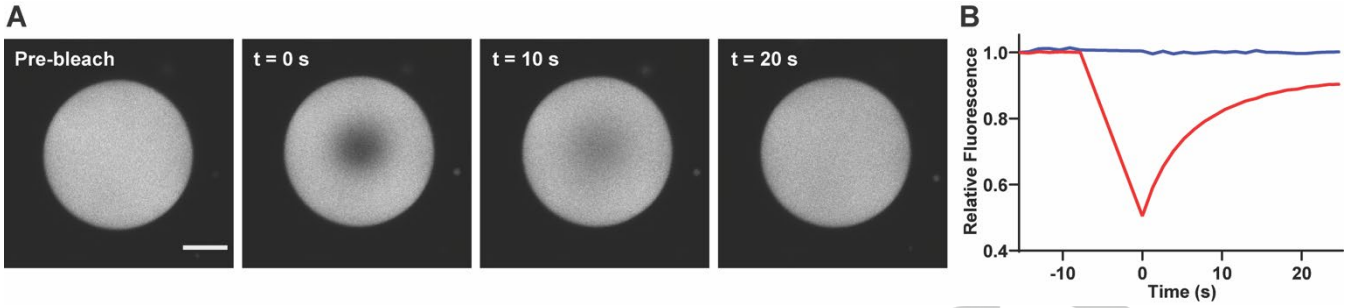

Figure 2. FRAP experiments in lipid sponge phase droplets. (A) Fluorescence microscopy images of lipid sponge phase droplets containing 0.1 mol% of Rhodamine-DHPE, showing fluorescence recovery after photobleaching (Scale bar: 20 μm). A circular region of interest (ROI) was delineated at the center of the droplet and bleached for 5 s with 561 nm laser. Fluorescence recovery of the bleached region was monitored for 20 s after laser treatment. ROIs delineated in the background were also analyzed as a control. (B) Relative fluorescence recovery of Rhodamine-DHPE in lipid sponge phase droplets of the ROI (red) and control (blue), demonstrating almost complete fluorescence recovery.

absence of the ester groups in compound **1**. The distance between headgroups was measured as 2.2 nm, which correlates well with the predicted molecular structure of **1**. Because the lipid sponge is a swollen phase, the lamellar repeat distance determined by X-ray diffraction (3.0 nm) is smaller than the sponge characteristic repeat distance (3.5 nm) obtained by SAXS analysis, with a ratio of about 1.2 between these two phases. A similar ratio was previously found for a cetylpyridinium/hexanol/dextrose/brine system.[36] By subtracting the thickness of the bilayer (2.2 nm) from the sponge phase repeat distance (3.5 nm), the average diameter size of the water channels in the sponge phase was estimated to be 1.3 nm.

Cellular organelles regulate complex networks of biochemical reactions by governing the spatial and temporal organization of enzymes and other biomolecules involved in such processes. To achieve spatial regulation, it is important that molecules associated with the lipid bilayer can still move freely within the compartment. To study the lateral diffusion of molecules embedded in the bilayer of lipid sponge phase droplets, we carried out fluorescence recovery after photobleaching (FRAP) measurements on lipid sponge phase droplets enriched with the membrane-anchored dye (*N*-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Rhodamine-DHPE). After bleaching the center of a droplet, we recorded the fluorescence recovery over time (Figure 2A), observing a half-time recovery of 4.0 s (Figure 2B) and a coefficient diffusion (D) of 6.5 $\mu m^2/s$. The FRAP results show that membrane-anchored molecules are able to freely diffuse throughout the entire sponge structure, supporting that the environment in the droplet is one in which sequestered molecules can dynamically interact.

To carry out biochemical processes in artificial lipid compartments, mechanisms must exist to ensure that the required biomolecules can selectively partition into the compartments at an adequate concentration. In particular, while nonpolar molecules can spontaneously localize to the hydrophobic lipid bilayer, the enrichment of lipid compartments with polar hydrophilic molecules such as water-soluble proteins is more challenging. Ideally, the incorporation of hydrophilic proteins into lipid membranes should be achieved using robust bioconjugation methods that enable the stable attachment of proteins to the bilayer, and ensure adequate exposure of the protein to the aqueous bulk.[37–42]

Along these lines, we explored the feasibility of using SNAP-tag conjugation for controlling protein localization into our lipid sponge phase droplets (Figure 3A).[43,44] SNAP-tag bioconjugation offers several advantages compared to other protein anchoring methods. First, the reaction occurs at physiological conditions, which is ideal for retaining protein structural integrity, and does not require the use of

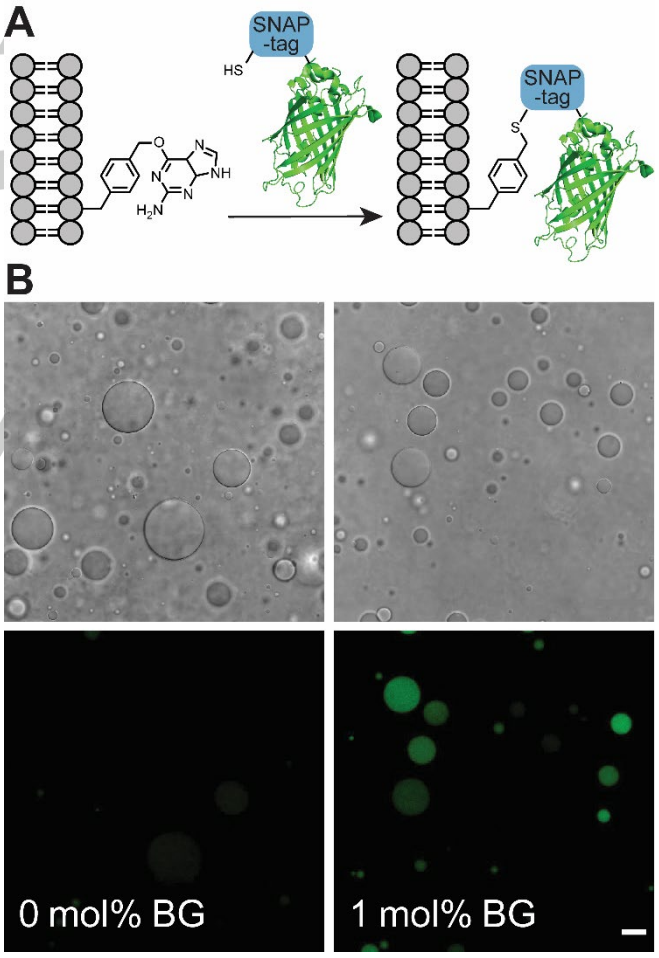

Figure 3. SNAP-sfGFP enrichment in lipid sponge phase droplets. (A) Illustration of the SNAP-sfGFP conjugation with BG-modified phospholipids. (B) Phase contrast *(top)* and fluorescence *(bottom)* microscopy images of sfGFP enrichment in lipid sponge phase droplets. Experiments were performed in the absence *(left)* and presence *(right)* of 1 mol% **18:1 PE-PEG2000-BG** phospholipids.

catalysts or coupling reagents. Second, the protein of interest is genetically fused to the SNAP-tag protein, and does not require any further chemical modification prior to the functionalization reaction. The SNAP-tag can often be appended onto the N- or C-terminus of proteins without affecting the function of the fusion proteins. The reaction between BG derivatives and the reactive cysteine residue in the SNAP-tag results in an irreversible covalent thioether bond (Figure 3A).[43] We previously described a straightforward SNAP-tag bioconjugation approach for tethering proteins to vesicle phospholipid membranes in a controlled fashion.[40] Considering this, we hypothesized that the SNAP-tag bioconjugation could be applied to program protein enrichment in lipid sponge phase droplets. We first performed SAXS measurements to confirm that the presence of BG-modified phospholipids in the droplets did not affect the stability of the sponge phase. For this purpose, we prepared lipid sponge phase droplets containing 1 mol% of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[benzylguanine(polyethylene glycol)-2000] (**18:1 PE-PEG2000-BG**). The SAXS data indicated that lipid sponge phase droplets, with or without **18:1 PE-PEG2000-BG** present, have similar structural characteristics (Figure S7). We observed periodic repeat distance values of 3.5 nm for the pure gemini **1** samples and 3.4 nm for the samples containing **18:1 PE-PEG2000-BG**, indicating a similar lipid packing. This periodic repeat distance is also similar to other previously reported sponge phases. For instance, the sponge phase composed from glycerol monooleate has a repeat distance of 3.5 nm.[45] Finally, we noticed that the presence of **18:1 PE-PEG2000-BG** leads to an increase in the correlation length (ξ), suggesting an increase of the lipid order in the assembled structure (Figure S7). This shows that the architecture of lipid sponge phase droplets can undergo some structural modification in the presence of other lipid components. Increasing lipid order could determine changes at the lipid-water interface, with more water molecules that are excluded from the lipid bilayer, which may affect the local environment of the protein attached.

To test if SNAP-tag bioconjugation can sequester proteins in lipid sponge phase droplets, we first expressed a SNAP-tag superfolded green fluorescent protein (SNAP-sfGFP) fusion in *E. coli*. This protein was purified and subsequently used to evaluate sfGFP enrichment in lipid sponge phase droplets doped with **18:1 PE-PEG2000-BG** (Figure 3A). After incubating the SNAP-sfGFP with lipid sponge phase droplets bearing 1 mol% of **18:1 PE-PEG2000-BG** for 15 min at room temperature, the reaction mixture was examined by fluorescence confocal microscopy, showing a clear accumulation of sfGFP into the droplets (Figure 3B). As a control, the reaction performed on lipid sponge phase droplets without **18:1 PE-PEG2000-BG** showed no sfGFP enrichment (Figure 3B). SNAP-tag bioconjugation product formation was confirmed by electrospray ionization mass spectrometry (ESI-MS) analysis of the reaction mixture (Figures S10, S11). These results demonstrated that the non-specific binding of the SNAP-sfGFP to the lipid sponge phase droplets was negligible, and support that the observed GFP enrichment was due to covalent bond formation with **18:1 PE-PEG2000-BG**. Our findings showed that the SNAP-tag methodology is an effective method to specifically recruit hydrophilic proteins into lipid sponge phase droplets.

Finally, to evaluate the compatibility of our lipid sponge phase droplets with complex biochemical reactions, we performed *in vitro* protein expression in the presence of lipid sponge phase droplets using a recombinant cell-free transcription-translation (TX-TL) system (PURExpress) (Figure 4). Sequestration occurs in the presence of TX-TL machinery, simulating the ability of lipid organelles to sequester synthesized proteins. The PURE TX-TL system is an optimized system containing all the components necessary for transcription and translation to occur.[46] First, we investigated the ability of lipid sponge phase droplets to spontaneously sequester transmembrane proteins expressed *in vitro,* similar to our previously described approach with GOA/IGEPAl droplets.[24] We thus carried out a PURE TX-TL reaction in the presence of lipid sponge phase droplets, using a plasmid with a gene encoding an sfGFP-fused bacterial diacylglycerol kinase (DAGK), a 13.2-kDa protein containing three transmembrane domains.[47] The green fluorescence signal observed by fluorescence microscopy analysis confirmed efficient sfGFP-DAGK partitioning into lipid sponge phase droplets (Figure 4A). Our results indicate that the biochemical machinery required for protein synthesis is still functional in the presence of gemini **1**. Moreover, these results demonstrate that *in vitro* produced transmembrane proteins can be promptly sequestered into the droplets, presumably due to protein hydrophobicity, which could favor, at least in part, the integration of sfGFP-DAGK into the lipid bilayers of the sponge phase droplets.

Next, we investigated if we could gain control over water-soluble protein enrichment into lipid sponge phase droplets during TX-TL using SNAP-tags. We conducted cell-free protein expression of SNAP-sfGFP in the presence of lipid sponge phase droplets doped with the **18:1 PE-PEG2000-BG** phospholipid. We carried out a PURE TX-TL reaction using a DNA plasmid encoding for the SNAP-sfGFP both in presence of lipid sponge phase droplets doped with 1 mol% **18:1 PE-PEG2000-BG**, and in presence of gemini **1** only as a control (Figure 4B). After 3 h, we

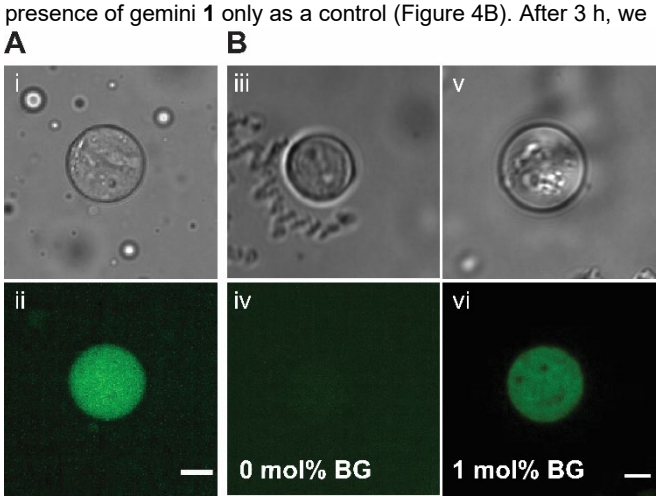

Figure 4. Gemini droplets enrichment of PURE TX-TL expressed proteins. (A) Localization of the protein sfGFP-DAGK into gemini **1** droplets (i, ii) (Scale bar: 20 µm). (B) Selective partitioning of SNAP-GFP into lipid **1** droplets doped without (iii, iv) and with (v, vi) 1 mol% of **18:1 PE-PEG2000-BG** (Scale bar: 5 µm).

observed that the SNAP-sfGFP expressed in the presence of **18:1 PE-PEG2000-BG** was localized into the lipid sponge phase droplets. In contrast, no partitioning was obtained for the control sample. These results demonstrate that the SNAP-tag technology is an efficient method to sequester proteins into lipid compartments even spontaneously when droplets are incubated during *in vitro* protein synthesis.

Conclusion

In summary, we have explored the suitability of SNAP-tag bioconjugation to drive the enrichment of hydrophilic proteins into lipid sponge phase droplets. This one-step conjugation method enables control over the partitioning of proteins into a lipid sponge L_3 phase, providing a stable covalent bond between a protein of interest and the bilayer of the lipid membrane, under aqueous conditions and neutral pH. Additionally, we have demonstrated that the SNAP-tag bioconjugation strategy can be used to direct protein sequestration in the presence of the complex biochemical machinery required for cell-free protein expression. We plan on using the SNAP-tag technology to program enzymatic activity into lipid sponge phase droplets, enabling the development of lipid-based structures that mimic the functions of cellular organelles.

Experimental Section

General information. All the reagents and solvents were purchased from Sigma-Aldrich Co. LLC (Merck KGaA, Darmstadt, Germany) unless differently described. Water was purified by Millipore purification system (Merck KGaA). NMR spectra were recorded on a Jeol ECA-500 (Jeol Ltd., Tokyo, Japan). Solvent mixtures for chromatography are reported as volume/volume (v/v) ratios. HPLC analyses were carried out on an Agilent 1260 Infinity with an Eclipse Plus C8 analytical column with Phase A/Phase B gradients [Phase A: H_2O with 0.1% formic acid; Phase B: MeOH with 0.1% formic acid]. The HPLC was equipped with a Varian 380-LC evaporative light scattering detector (ELSD), and an Agilent 6120 single quadrupole Electrospray Ionization-Mass spectrometer (ESI-MS). Microscopy images were acquired either with a Yokagawa spinning-disk system (Yokagawa, Japan) inverted microscope (Carl Zeiss Microscopy GmbH, Germany) or Leica sp8 with lighting deconvolution (Leica GmbH, Wetzlar, Germany). The samples were prepared by depositing 2 µL of sample on a microscope slide (Fisher brand) and topping with a coverslip. ZEN Blue (Carl Zeiss Microscopy GmbH, Germany), and LAS x Life Science imaging software were used respectively. Images were further analyzed with ImageJ software (version 2.1.0). Thermogravimetric analysis were performed using a SDT650 TGA instrument to measure the weight loss of the coalesced phase as a function of temperature and time. Measurements were performed at a rate of 10 °C per minute from 30 to 700 °C, under a constant flow of N_2 gas. Thermal decomposition was observed at temperatures higher than 300 °C. The fluorescence spectra were recorded using a Tecan infinite F200 plate reader instrument.

Preparation of lipid sponge phase droplets. The desired amount of compound **1** was weighed and dissolved in $CHCl_3$ in a glass vial. A thin film of gemini **1** was deposited on a glass vial by carefully evaporating the solvent under a flow of Ar. The desired volume of aqueous solvent (e.g. PBS) was added and vortexed until compound **1** was completely dispersed to form a turbid suspension. Droplets bearing 1% of **18:1 PE-PEG2000-BG** (Avanti polar lipids, Inc.) were prepared following the same procedure but adding 1 mol% of **18:1 PE-PEG2000-BG** to the initial solution of compound **1** in $CHCl_3$.

Small angle X-ray analysis (SAXS). SAXS measurements were performed at the 12-ID-B beamline from the Argonne Photon Source synchrotron. The samples were contained in glass capillaries with a diameter of 1 mm and a wall thickness of 0.01 mm (Hampton Research®).

X-ray diffraction analysis. X-ray diffraction measurements were carried out using an in-house Cu Kα tube spectrometer with wavelength 1.54 Å operating in the horizontal plane. Experiments were performed on multistacks of oriented lipid bilayers deposited on freshly cleaned hydrophilic silicon [100] wafers.

SNAP-tag bioconjugation on lipid sponge phase droplets. A SNAP-sfGFP bearing a 6xHis-tag was expressed by BL21 competent *E. coli* cells (Agilent Technologies, Santa Clara, CA) and purified by affinity chromatography using a Ni-NTA resin (Thermo Fisher Scientific, Inc.) (see Supporting Information for the details). For the SNAP-tag bioconjugation on lipid sponge phase droplets, 2 µL of a 330 µM PBS pH 7.4 solution of the expressed SNAP-sfGFP, containing dithiothreitol (5 mM) was added in a microcentrifuge tube containing 20 µL of compound **1** (5 mM equivalent concentration) doped with 1 mol% **18:1 PE-PEG2000-BG** (1:1 SNAP-sfGFP:**18:1 PE-PEG2000-BG**). As a control, the same procedure was carried out using lipid sponge phase droplets without **18:1 PE-PEG2000-BG**. Samples were carefully mixed and incubated at room temperature for 15 min. After the incubation period, mixtures were vortexed and 2 µL of each sample were loaded on the microscope slide for microscopy analyses. Microscopy images of SNAP-tag-GFP bioconjugation and control samples were acquired under the same gain and laser power.

PURE TX-TL in the presence of lipid sponge phase droplets. *In vitro* cell-free transcription-translation reactions were performed using the PURExpress *in vitro* protein synthesis kit (New England Biolabs-E6800S). A master mix was prepared for the reactions by combining PURExpress Solutions A and B (10 µL/reaction and 7.5 µL/reaction respectively), 20 units/reaction of murine RNAse inhibitor (NEB-M0314S), the linear DNA template for the superfolded-GFP-diacylglycerol kinase (sfGFP-DAGK)[24] (13.4 nM/reaction), and water to make the final volume of the master mix 20 µL/reaction. To prepare the lipid sponge phase droplets, a thin lipid film of compound **1** was rehydrated in water to an equivalent concentration of 30 mM by gently vortexing until a cloudy dispersion was visible. Each reaction was then created by combining 20 µL of the master mix with either 5 µL of water (control reaction) or 5 µL of the prepared dispersion of compound **1** to get a final equivalent concentration of 5 mM. After mixing the reactions, the samples were incubated for 3 h at 37 °C while shaking. After incubation, 2 µL of each reaction mixture was loaded onto a microscope slide for visualization by confocal microscopy.

To test the SNAP-tag functionalization, the reactions were set up as described above except for the DNA used: the master mix was prepared such that each reaction would contain 222 ng of plasmid for the SNAP-sfGFP expression.[40] A dispersion of lipid sponge phase droplets (25 mM equivalent concentration) with 1 mol% of **18:1 PE-PEG2000-BG** was prepared. 5 µL of the dispersion was added to each reaction for a final equivalent concentration of 5 mM for compound **1** in each 25 µL reaction. After mixing, the samples were incubated for 3 h at 37 °C while shaking. After incubation, 2 µL of each reaction mixture was loaded onto a microscope slide for visualization by confocal microscopy.

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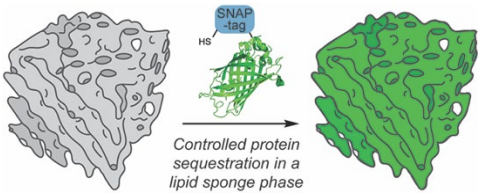

We describe a one-step SNAP-tag bioconjugation strategy for controlling protein capture in lipid sponge phase droplets. The approach provides a stable covalent bond between the protein of interest and the lipid bilayer of sponge droplets, enabling protein sequestration even in the presence of the complex biochemical machinery required for cell-free protein expression.

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