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In situ assembly of transmembrane proteins from expressed and synthetic components in giant unilamellar vesicles

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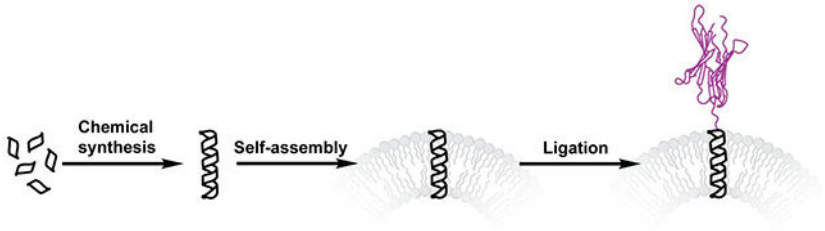
Abstract

Reconstituting functional transmembrane (TM) proteins into model membranes is challenging due to the difficulty of expressing hydrophobic TM domains, which often require stabilizing detergents that can perturb protein structure and function. Recent model systems solve this problem by linking the soluble domains of membrane proteins to lipids, using non-covalent conjugation. Herein, we test an alternative solution involving the *in vitro* assembly of TM proteins from synthetic TM domains and expressed soluble domains using chemoselective peptide ligation. We developed an intein-mediated ligation strategy to semi-synthesize single-pass TM proteins in synthetic giant unilamellar vesicle (GUV) membranes by covalently attaching soluble protein domains to a synthetic TM polypeptide, avoiding the requirement for detergent. We show that the extracellular domain of programmed cell death protein 1, a mammalian immune checkpoint receptor, retains its ligand-binding function at a membrane interface after ligation to a synthetic TM peptide in GUVs, facilitating the study of receptor-ligand interactions.

Graphical Abstract



INTRODUCTION

Transmembrane (TM) proteins comprise ~23% of the human proteome and conduct essential cellular functions such as solute transport, cell-to-cell communication, signaling,

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Supporting Information

Materials, instrumentation, experimental methods, supplemental sequences and structures, supplemental tables and figures, and supplemental spectra are listed in the Supporting Information. This material is available free of charge *via* the Internet.

The authors declare no competing financial interest.

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and lipid synthesis.[1,2] As such, TM proteins have high therapeutic relevance, and are targeted by ~60% of currently available pharmaceuticals.[3] Reconstituting TM proteins into model membranes (*e.g.* unilamellar vesicles, supported lipid bilayers, and nanodiscs) enables functional and structural studies of these proteins in controlled lipid environments.[4–7] Expressing and purifying TM proteins is often challenging due to their hydrophobicity, leading to aggregation.[8,9] Protein reconstitution into model membranes such as giant unilamellar vesicles (GUVs) relies on expressing and purifying full-length TM proteins in the presence of stabilizing detergents, which can perturb protein function and structure.[7,10–16] Furthermore, TM domains pose a problem for protein overexpression and purification due to their toxicity in cell membranes at high concentrations and insolubility in aqueous environments.[17,18] Solutions involve chemically lipidating proteins or expressing truncated soluble domains of TM proteins, followed by conjugation to synthetic lipids in model membranes to circumvent expressing the hydrophobic TM region.[19–25] While eliminating the TM domain helps faciliate hydrophobic protein expression, this strategy is not ideal, as TM domains in integral membrane proteins are highly conserved and play a variety of roles such as lipid environment sensing, controlling lateral diffusion, participating in dimerization, and facilitating transport and signaling across the bilayer.[26–36] Thus, there is a need to improve the synthesis and reconstitution of TM proteins, ideally without the use of detergents.

To address this problem, we developed a simplified synthesis method for bitopic (single-pass) TM proteins, which represent about half the TM proteins in the human genome,[37,38] involving the *in situ* assembly of a TM protein in vesicles. To circumvent TM-related reconstitution/expression problems, we chemically synthesized a TM domain, embedding the TM domains during self-assembly of model lipid membranes, and used peptide bond forming reactions to ligate the soluble domains to the membrane-anchored TM domain (Figure S1A).[39] We adapted a split intein-mediated expressed protein ligation strategy to build a semisynthetic TM protein into GUVs,[40–43] which, unlike other protein semi-synthesis strategies, such as native chemical ligation, does not require additional chemical modifications after protein and peptide production.[43–46]

We used our method in GUVs to ligate the extracellular domain of fully glycosylated mammalian programmed cell death protein 1 (PD-1), a bitopic TM receptor on T-cells, to a synthestic TM. PD-1 is activated by its ligand PD-L1, suppressing T-cell activation,[47,48] and defining an “immune checkpoint” which is subverted in certain cancers to evade immune survailance. While immune checkpoint inhibitor antibodies have been developed as cancer immunotherapies, little is known about the biophysical mechanisms that govern checkpoint protein interactions.[48] Minimal T-cell models have been developed using PD-1 extracellular domains non-covalently conjugated to lipids in GUVs,[49] however, the lack of the TM domain and absense of covalent anchoring in the phospholipid membrane cannot recapitulate biological PD-1 activity (Figure S1B).[50] As a reconstitution system for modeling T-cell extracellular interactions, we have used split intein-mediated ligation to covalently link PD-1 to a TM peptide in GUVs (Figure S1B) and show that PD-1 retains its surveillance activity in a commonly used GUV-based functional assay.

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RESULTS AND DISCUSSION

Expression and synthesis of split intein constructs

For protein ligation we chose the engineered Cfa_{GEP} split intein system, derived from the ultrafast Cfa_{WT}, for its improved extein tolerance, enabling versatility in protein semisynthesis.[40] Cfa_{GEP} is reportedly robust for semisynthesis, contains a small C intein (38 amino acids) ideal for peptide synthesis, and results in minimal amino acid scarring (incorporation of non-native amino acids) between exteins.[40] To ask whether Cfa_{GEP} split intein ligation could be used for building TM proteins in an artificial membrane, we designed a proof-of-concept semisynthetic pair capable of ligating in phospholipid membranes (Figure 1A). Green fluorescent protein (GFP) was chosen as the protein extein for downstream imaging experiments. GFP fused to Cfa^N with a C-terminal polyhistidine tag (GFP-Cfa^N-His_6) was expressed in *E. coli* and purified by Ni-NTA column (Figure 2B, Figure S3). The well-characterized, single-pass TM peptide, WALP, provides a model synthetic TM peptide extein.[51,52] WALPs classically contain leucine and alanine (LA) repeats flanked by two tryptophans (WW) on each terminus. A Cfa^C-WALP peptide was produced via solid phase peptide synthesis (SPPS) on a peptide synthesizer (CEM Liberty Blue; Figure 1B). A fluorescent derivative of Cfa^C-WALP containing a lysine side chain conjugated to carboxyfluorescein (Cfa^C-WALP-CF) was also synthesized. After purification of the TM peptides on a reverse phase C-18 column, liquid chromatography electrospray ionization time of flight mass spectrometry (LC-ESI-TOFMS) confirmed their purity and mass (Supplementary spectra 1 & 2).

We verified the reconstitution of TM peptide into phospholipid bilayers using confocal fluorescence microscopy, confirming localization of Cfa^C-WALP-CF to hydrated DOPC vesicles (Figure 2A). Cryo-transmission electron microscopy (cryo-TEM) showed that the vesicles were intact after peptide incorporation and remained intact after the split intein ligation (Figure S4). Circular dichroism (CD) spectra verified that the peptide folds into a secondary alpha-helix structure once reconstituted into unilamellar vesicles (Figure 2B, Figure S5).[51–54] These results show that the synthetic Cfa^C-WALPs are reconstituting into DOPC bilayers as alpha helical TM peptides.

Split intein-mediated ligation in phospholipid vesicle membranes

We carried out the split intein-mediated GFP-WALP ligation in multilamellar large vesicles (MLVs) formed by hydrating a lipid-peptide film in splicing buffer (150 mM sodium phosphates, 100 mM NaCl, 5 mM EDTA, 1 mM TCEP, pH 7.2). Mimicking previously established conditions for Cfa_{GEP} splicing, we reacted soluble GFP-Cfa^N-His_6 to liposome-reconstituted Cfa^C-WALP (1:4) in splicing buffer.[40] After 1 and 24 h, LC-ESI-TOFMS analysis confirmed the presence and expected mass of the predicted GFP-WALP product, **F**, which contains an eight amino acid linker (GGCFNGGG) between the GFP and WALP (Figure 3A & B, Supplementary spectra 3 and 4). SDS-PAGE corroborated these results, where even at 0 min, there is a rapid conversion of all reacting GFP-Cfa^N-His_6, **E**, into an intermediate, **H** (Figure 3C). LC-ESI-TOFMS suggests that **H** is a covalently-linked, branched intermediate of the intein ligation (GFP-Cfa^C-WALP). The irregular m/z distribution in the mass spectrum of peak B indicates unknown molecules which most

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likely correspond to protein and synthetic peptide breakdown (Supplementary spectra 3). All observed masses are within the acceptable error (0.01%) of ESI-MS.[55] Branched-chain polypeptides have faster than expected migration times compared to their linear counterparts.[56–58] Although **H** has a higher calculated and validated molecular weight than **F**, its covalently-linked, branched structure leads to a faster migration in SDS-PAGE. The gel bands of **H** and **F** show the conversion of **H** to **F** over time.

To reconstitute Cfa^{C}-WALP into GUVs in splice buffer under more physiologically relevant conditions, we adapted previously established detergent-free electroformation procedures, using "high salt" (~300 mOsm), thereby eliminating buffer exchange and chemical workup after synthesis (Table S1).[59–65] Ligation of GFP-Cfa^{N}-His_6 to TM peptide in GUVs was tracked using confocal fluorescence microscopy and observing fluorescence signal at the lipid membrane (Figure 3D and Figure S6). The GFP construct alone does not nonspecifically bind to vesicles (Figure 3D). Taken together, the LC-ESI-TOFMS, SDS-PAGE, and confocal fluorescence microscopy data indicate that the semisynthetic split intein-mediated ligation of protein and TM peptide takes place on liposomes.

Semisynthesis of PD-1-WALP in GUV membranes

Wondering whether we could reconstitute PD-1 into GUVs via a TM domain using our semisynthesis method, we expressed and purified a fully glycosylated extracellular domain of PD-1 fused to an N-terminal SNAP-tag, used for fluorescent labeling, and C-terminal Cfa^{N}, in mammalian (hamster) HEK293F cells. The recombinant protein was labeled with Janelia Fluor 646 (JF) for fluorescence microscopy experiments and purified using standard procedures (Figure S7). Glycosylation of PD-1 resulted in smeared starting material and product bands which are difficult to interpret by SDS-PAGE (Figure 4A, Figure 4B, Figure S7, Figure S8) but revealed an intermediate, **K**, similar to that observed in GFP-WALP semisynthesis. The yield of GUVs liberated from the elctroformation chambers is low, which limits analysis of multiple GUVs in the same image. However, multiple fluorescence microscopy images of GUVs are shown to validate the reproducibility of the procedure (Figure 4C, Figure S8, Figure S9, Figure S10).

PD-1 retains its function after split intein-mediated ligation in GUV membranes

To test whether the extracellular domain of PD-1 retains its PD-L1 binding function after split intein-mediated semi-synthesis, we used total internal reflection fluorescence (TIRF) microscopy to visualize trans PD-1:PD-L1 interaction at a membrane interface between a PD-1 GUV and a PD-L1-attached planar lipid bilayer. To produce PD-1 GUVs, we reacted JF-PD-1-Cfa^{N} with electroformed GUVs containing semi-sythetic Cfa^{C}-WALP-CF in splice buffer. To construct a ligand (PD-L1) presenting membrane, we attached polyhistidine-tagged PD-L1 to a supported lipid bilayer (SLB) doped with 5% NTA-DGS lipids.[19] Upon incubating the semi-sythetic PD-1 GUVs with PD-L1 SLBs, we visualized PD-1 distribution at the GUV-SLB contact area by TIRF fluorescence microscopy (Figure 4D). The PD-1 ectodomain and TM peptide fluorescent signals were co-enriched at the SLB-GUV interface, as previously observed for the PL-1:PD-L1 interaction,[49] consistent with ligation and the ability of the semisythetic PD-1 to engage PD-L1 in trans (Figure 4E). As a control, a PD-1 antibody blockade, pembrolizumab, was used to inhibit the binding of PD-1 to PD-L1 and

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the sunken GUV was unable to bind but remained in close proximity to the SLB, indicated by the bright field image of the bottom of the GUV and the minor PD-1 fluorescent signal (Figure 4E, Figure S11). Thus, semisynthetic PD-1 retains its binding function after ligation to TM peptides within GUV membranes.

Most bitopic TM proteins, such as PD-1, have soluble extracellular and intracellular domains connected by a membrane-spanning, hydrophobic TM domain. Here, we have constructed a single-pass TM protein from the soluble extracellular domain of PD-1 and a synthetic WALP TM domain, using our split intein-mediated ligation method in the presence of artificial phospholipid membranes, including GUVs, without the need of detergents. As there is no additional chemical work-up after protein and peptide purification, this method is more accessible than other conjugation techniques, requiring few chemical tools and therefore allowing the facile creation of minimal cell models with bitopic membrane proteins.[45,46]

For applications in synthetic biology and therapeutic development, the faithful recapitulation of structure and function after protein reconstitution is essential. Having been successfully targeted for cancer immunotherapy in a fraction of patients, the PD-1/PD-L1 axis is an urgent focus for translational research.[48] The extracellular domain of PD-1 has previously been non-covalently conjugated to lipids within artificial membranes in an effort to reconstitute the protein at the phospholipid membrane and bind PD-L1.[19] We showed that the fully-glycosylated, mammalian cell-expressed extracellular domain of PD-1 could be covalently ligated to a TM peptide and the semisynthetic TM protein retained the trans-PD-1:PD-L1 interaction at the GUV-SLB interface after ligation.

In previous studies using intein-mediated ligation within living cell membranes, cellularly expressed TM domains were coupled with an exogenously added expressed or synthetic soluble protein usually containing additional tags to colocalize the split inteins or facilitate correct protein folding.[66,67] Although useful for labeling a living cell surface, these methods do not enable the reconstitution of isolated TM proteins for biophysical studies. Encouraged by previous strategies conjugating proteins to tagged membrane lipids, we set out to ligate proteins to a peptide anchor in artificial vesicles.[19–25] This strategy bypasses the necessity of using cellular machinery to build and insert TM domains into an artificial vesicle or a crowded living cell membrane before reconstitution.[66,68,69] In our work, the inherent challenge of building proteoliposomal models with GUVs has been overcome by using synthetic TM domains. By covalently linking a glycosylated mammalian protein to a TM domain anchored in the phospholipid membrane while retaining its extracellular binding functions, we have created a reconstitution system for cell biologists to model extracellular interactions.

Given the versatility of the Cfa_{GEP} and other split intein ligations, it should be possible to expand the range of semisynthetic protein products.[40] While this initial study focused on ligating only the extracellular domain of a bitopic membrane protein, we plan to leverage the orthogonality of split inteins to semisynthesize full-length, single-pass TM proteins by incorporating an additional intein-mediated ligation site on the C-terminus of the TM peptide that will ligate to an expressed intracellular domain.[70] We envision that this method

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will improve biochemical, biophysical, and pharmacological research on TM proteins as it will expedite the production of reconstituted proteins anchored by synthetic or native TM domains in GUVs.

METHODS

Methods are provided in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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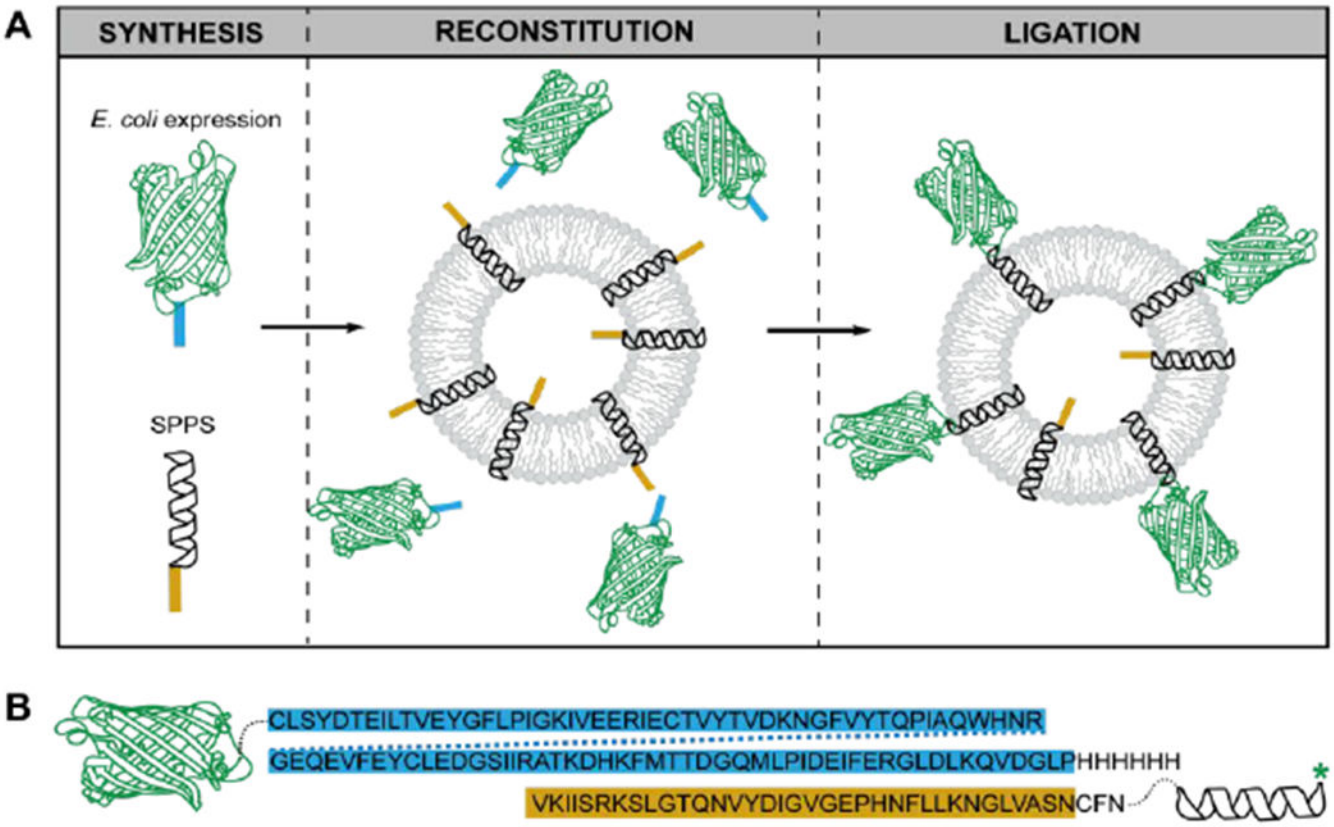

Figure 1.

Semisynthetic split intein-mediated ligation. **(A)** GFP (green), fused to the Cfa^N split intein domain (blue) is expressed in *E. coli*, while the WALP (black) fused to the Cfa^C split intein domain (yellow) is fabricated via SPPS. Cfa^C-WALP is reconstituted into GUVs in randomly distributed orientations within the membrane. Upon addition of the soluble GFP-Cfa^N construct to peptide-loaded GUVs, the split intein-mediated ligation produces GFP-WALP embedded GUVs. **(B)** Amino acid sequences of Cfa^N (blue) and Cfa^C (yellow) inteins and their respective protein and peptide fusions. Dashed lines represent glycine linkers. Green asterisk denotes the position of the CF conjugated to the peptide.

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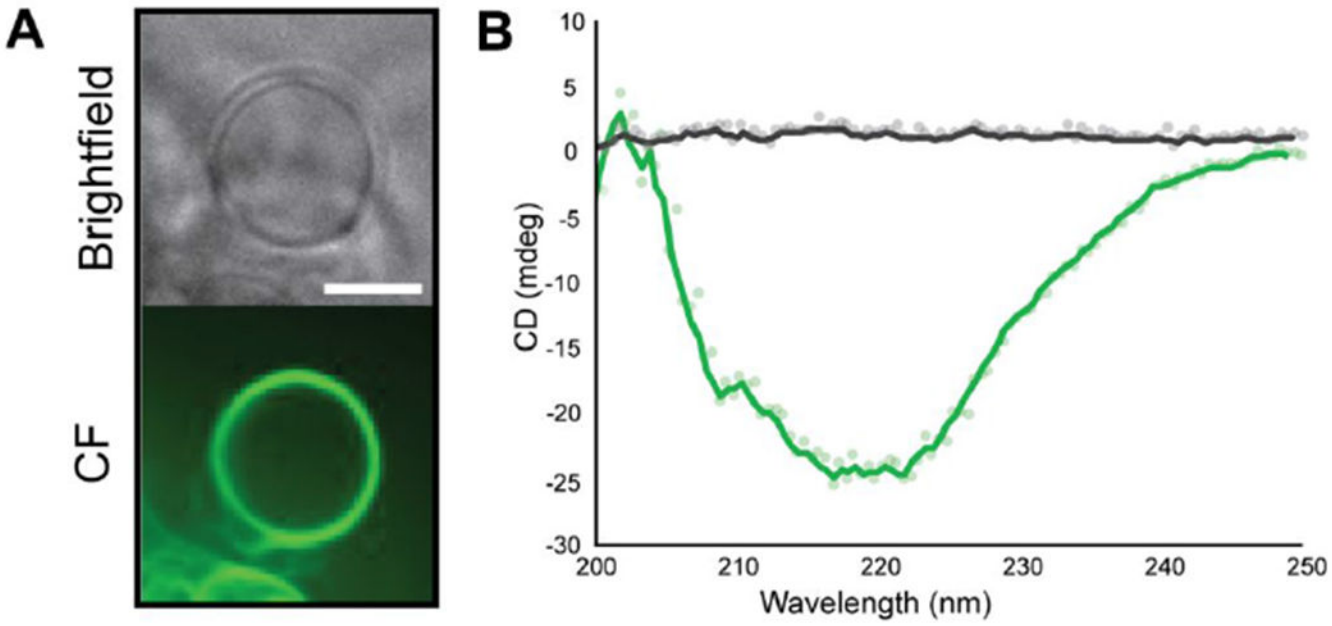

Figure 2.

Transmembrane peptide reconstitution. **(A)** Brightfield and fluorescence (488 nm excitation) microscopy images of a hydrated DOPC vesicle containing Cfa^{C}-WALP-CF. Scale bar 10 μm. **(B)** CD spectra of 20 μM Cfa^{C}-WALP before (black) and after (green) reconstitution into small unilamellar vesicles. CF = carboxyfluorescein.

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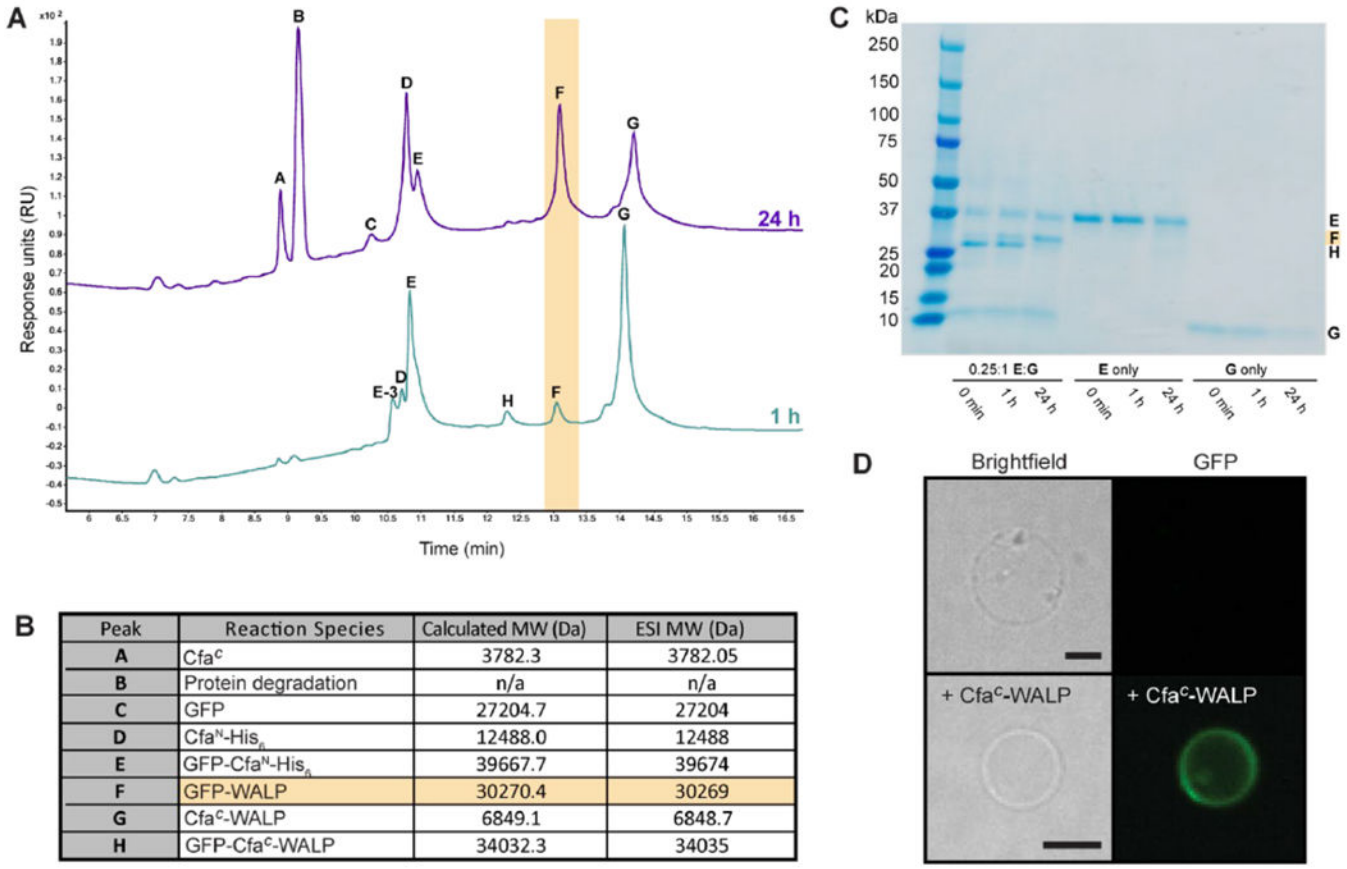

B

Peak	Reaction Species	Calculated MW (Da)	ESI MW (Da)
A	Cfa^{C}	3782.3	3782.05
B	Protein degradation	n/a	n/a
C	GFP	27204.7	27204
D	Cfa^{N}-His_6	12488.0	12488
E	GFP-Cfa^{N}-His_6	39667.7	39674
F	GFP-WALP	30270.4	30269
G	Cfa^{C}-WALP	6849.1	6848.7
H	GFP-Cfa^{C}-WALP	34032.3	34035

Figure 3.

Semisynthetic split intein-mediated ligation to form a transmembrane protein in GUV membranes. **(A)** Chromatogram of an LC-ESI-TOFMS run of the reaction between GFP-Cfa^{N}-His_6, **E**, and Cfa^{C}-WALP, **G**, in vesicles. Each peak corresponds to a reactant, intermediate, or product that is listed in **(B)** with their corresponding calculated molecular weight (MW) and experimental ESI MW. **(C)** SDS-PAGE gel of the reaction in (A). Lanes 2-4 are the reaction between **E** and **G**, lanes 5-7 is **E** only, and lanes 8-10 are **G** only. The GFP-WALP product, **F**, is highlighted in yellow boxes throughout the figure. **H** is the branched intermediate. **(D)** Confocal fluorescence micrographs show that GFP (488 nm excitation) does not bind to GUVs alone (upper row), but does bind to GUVs containing Cfa^{C}-WALP after 24 h (lower row). Scale bar, 5 μm.

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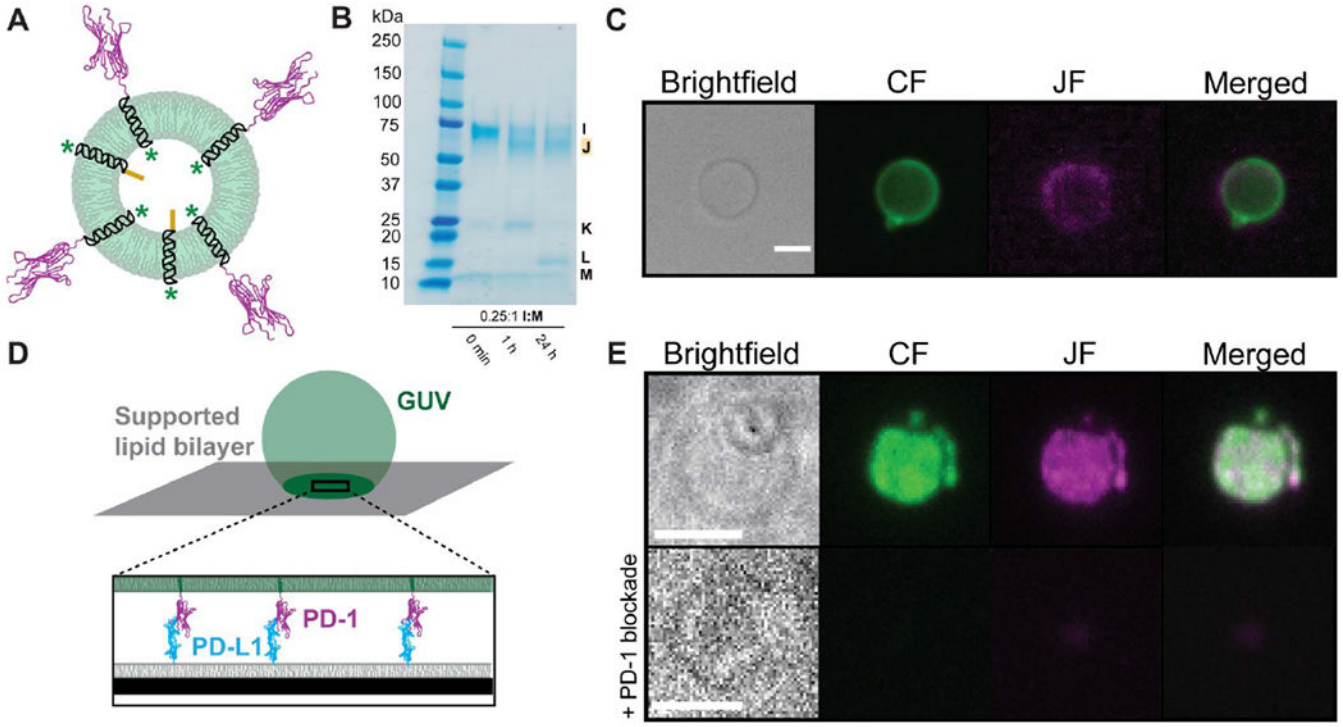

Figure 4.

Building a functional semisynthetic transmembrane protein in GUVs. **(A)** A cartoon representation depicts the CF-labeled (green asterisks) TM peptide (black) fused to the extracellular domain of PD-1-JF (magenta). **(B)** SDS-PAGE gel of the reaction between JF-PD-1-Cfa^N, **I**, and Cfa^C-WALP-CF, **M** (lanes 2-4). The JF-PD-1-WALP-CF product, **J**, is highlighted in a yellow box. **K** is an intermediate and **L** is the Cfa^N split intein. **(C)** Brightfield and fluorescent micrographs of the semisynthetic JF-PD-1-WALP-CF TM product in a GUV. CF fluorescence does not bleed into the JF channel, as indicated by Cfa^C-WALP-CF controls (Figure S9). CF= 488 nm excitation. JF 646 = 638 nm excitation. **(D)** Cartoon schematic of the PD-1 activity experiment where a GUV (green) contacts a SLB leading to the enrichment of PD-1 at the GUV-SLB interface due to trans-PD-1:PD-L1 interaction. **(E)** TIRF brightfield and fluorescence micrographs of the GUV-SLB interface showing enrichment of CF and JF signals at the interface. In the presence of PD-1 blockade (bottom row), there is no enrichment of either signal although a GUV remains at the SLB surface. Because the GUV is unbound to the SLB surface, the GUV is not clearly seen on this focal plane. A mid-GUV cross section better depicts this unbound GUV (Figure S11). The merged images in **(B)** and **(E)** reveal the colocalization of CF and JF to the GUVs, indicating the presence of the product, JF-PD-1-WALP-CF, at the phospholipid membrane. CF = carboxyfluorescein. JF = Janelia Fluor 646. Scale bars, 10 μm.