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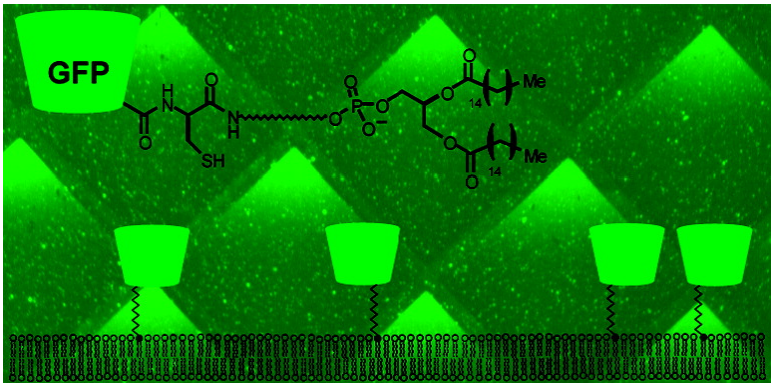

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Synthesis of Lipidated Green Fluorescent Protein and Its Incorporation in Supported Lipid Bilayers

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Abstract: Herein we report a semisynthetic method of producing membrane-anchored proteins. Ligation of synthetic lipids with designed anchor structures to proteins was performed using native chemical ligation (NCL) of a C-terminal peptide thioester and an N-terminal cysteine lipid. This strategy mimics the natural glycosylphosphatidylinositol (GPI) linkage found in many natural membrane-associated proteins; however, the synthetic method utilizes simple lipid anchors without glycans. Synthetically lipidated recombinant green fluorescent protein (GFP) was shown to be stably anchored to the membrane, and its lateral fluidity was quantitatively characterized by direct fluorescence imaging in supported membranes. Circumventing the steps of purification from native cell membranes, this methodology facilitates the reconstitution of membrane-associated proteins.

Introduction

The separation of cellular phenomena into compartments by membranes is characteristic of all living cells, and the dynamics and maintenance of these membranes is central to cellular homeostasis. A substantial fraction of cellular proteins are membrane-associated, and the concentration of cell factors on membranes and within subdomains of membranes contributes to the high activity and selectivity of proteins which are otherwise dilute or promiscuous. A significant subset of these proteins is associated with cell membranes by virtue of lipids covalently attached to the polypeptide side chains or backbone. These modifications represent a level of control over protein localization separate from peptide transmembrane domains, for timed posttranslational lipidation allows changes in the localization of cell factors independent of regulated protein expression and transport. Furthermore, the unique chemical properties of the fatty acid, prenyl, and phosphatidyl lipids expand the possibilities of membrane protein localization and physical behavior.

Highlighting the potential complexity of these modifications, glycosylphosphatidylinositol (GPI) proteins are cell-surface proteins with a lipo−pentasaccharide membrane anchor amide-linked to the protein C-terminus.[1] The GPI anchor occurs on a wide range of proteins, and some GPIs appear to be sequestered by the lipid anchor in membrane subdomains, termed lipid rafts, which are enriched in cholesterol, sphingomyelin, and other factors.[2,3] The structural basis for this phenomenon and the general role for the elaborate GPI structural modification are not well-described, for the effects of lipid structures on protein behavior and function are difficult to address directly with traditional genetic methods. To gain chemical control over protein lipidation, we herein report the semisynthesis of lipidated proteins with defined anchor structures, and we demonstrate the incorporation of a synthetic lipoprotein into supported lipid bilayers for physical studies. The feasibility of such an approach has recently been demonstrated for Ras by the semisynthesis and evalution of several protein lipoforms.[4,5] Our methods are in principle general for many proteins and lipids and produce homogeneous materials previously inaccessible for the physical and biological investigation of lipid modifications.

Results and Discussion

Synthesis of Lipidated-GFPs. We envisioned ligating synthetic lipids to proteins using native chemical ligation (NCL) of a C-terminal peptide thioester and a lipid-modified cysteinyl peptide (Figure 1).

NCL has emerged as a robust technology for assembling large peptides and proteins, under mild aqueous reaction conditions, forming a stable amide bond and remaining tolerant of most functional groups and native protein structures.[6,7] We have previously utilized NCL to prepare glycoproteins and glyco-

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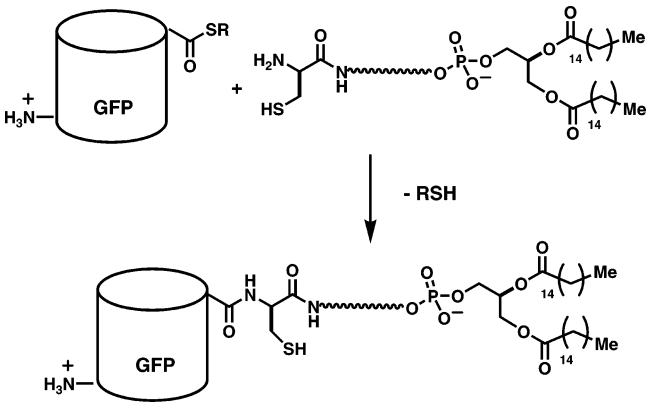
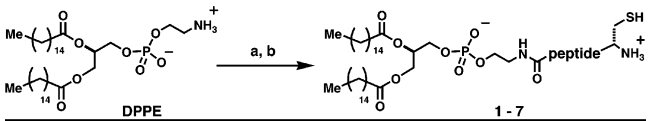


Figure 1. Native chemical ligation of green fluorescent protein (GFP) C-terminal thioester to an N-terminal cysteine−phosphatide lipid to form a covalent amide-bonded lipoprotein.



Compound	*Product Lipopeptide*	*% yield for coupling*	*% yield for deprotection*
1	DPPE-Cys-NH_2	80	82
2	DPPE-Ala-Cys-NH_2	68	77
3	DPPE-Ala-Arg-Ser-Tyr-Arg-Gly-Cys-NH_2	55	89
4	DPPE-Ala-Lys-Ser-Tyr-Lys-Gly-Cys-NH_2	61	98
5	DPPE-Ala-Ser-Pro-Ala-Ser-Pro-Cys-NH_2	62	86
6	DPPE-Ala-Glu-Ser-Tyr-Glu-Gly-Cys-NH_2	87	55
7	DPPE-Ala-Asp-Ser-Tyr-Asp-Gly-Cys-NH_2	73	54

Figure 2. Synthesis of lipopeptides for NCL. (a) Protected peptide, EDCI and NHS (1 equiv each), $CHCl_3$/MeOH (4:1), 23 °C, 1 h; *i*Pr_2EtN (3.6 equiv), DPPE (1.2 equiv), 23 °C, 12 h, 50−75% yield. For **1**, EDCI and HOBt (1 equiv each) with $NaHCO_3$ (2 equiv) in $CHCl_3/H_2O$ (3:1), 23 °C for 12 h efficiently gave DPPE-Cys(Tr)Boc. (b) 1% Et_3SiH, 2.5% EDT, 2.5% H_2O in TFA, 23 °C, 1 h.

peptides bearing homogeneous patterns of glycosylation.[8,9] Other small molecules have also been attached to native protein structures by NCL.[10] The dissection of lipoprotein synthesis into two components, cysteine-bearing lipids and protein thioesters, represents a flexible route for preparing a wide range of C-terminally lipidated proteins. Initially, we focused on coupling lipid structures to GFP as model protein due to its ease of optical imaging.

For lipoprotein synthesis by NCL, the phospholipids **1**−**7** of varied length and charge were assembled by the coupling of peptides to dipalmitoylphosphatidylethanolamine (DPPE) (Figure 2). Fmoc-based solid-phase peptide synthesis (SPPS) followed by mild acid cleavage from 2-chlorotrityl resin produced *N*-*t*Boc-, *O*-*t*Bu-, and *S*-triphenylmethyl (Tr)-protected heptapeptides for coupling to DPPE. Crucial to the success of the amide forming reaction was both the preactivation of the peptides with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide·HCl (EDCI) and *N*-hydroxysuccinimide (NHS) and the use of chloroform/methanol to dissolve the phospholipid. However, coupling of the amino acid Cys(Tr)Boc to produce **1** was most effective with EDCI and hydroxybenzotriazole (HOBt) in an aqueous chloroform mixture. Removal of the peptide protecting groups with trifluoroacetic acid (TFA) containing 1% triethylsilane, 2.5% ethanedithiol (EDT), and 2.5% water cleanly afforded lipopeptides **1**−**7**. To construct simpler phosphatides, dipalmitoyl glycerol and a protected cysteinyl-oligoethylene glycol were joined by phosphoramidite methodology to produce lipids **8** and **9** (Figure 3).

In addition to phospholipids **1**−**9**, farnesol ester **10** and cholesterol ester **11** were prepared from Fmoc- and *t*Boc-protected cysteines, respectively. Lipids **1**−**11** were designed to vary with respect to charge, length, and lipid properties.

C-Terminal peptide thioesters for the lipidation strategy outlined in Figure 1 have been prepared by either Boc- or Fmoc-based SPPS followed by thiol cleavage from solid support.[11,12] Alternatively, the technique of expressed protein ligation (EPL) has been developed to generate protein thioesters for NCL and is not constrained by the size limits of SPPS.[13] Applying EPL, we recombinantly expressed, from *Escherichia coli*, GFP fused by its C-terminus to an intein- and chitin-binding domain (CBD). This triprotein fusion was purified on chitin resin and treated with mercaptoethanesulfonic acid (MESNA) to release pure GFP−thioester by action of the intein, as indicated by SDS−PAGE.

To produce lipo−GFP adducts by NCL with the lipids such as **1**−**11**, 1% (w/v) cysteine-bearing lipid solubilized with 1% β-octyl glucoside (β-OG) was mixed as a slurry with the GFP−intein fusion bound on chitin resin. For farnesol and cholesterol esters **10** and **11**, addition of 1% Triton X-100 was necessary to maintain lipid solubility in the mixture. After shaking for 48 h at 23 °C, filtration of the mixture and analysis by SDS−PAGE revealed complete conversion of GFP−thioester to lipo−GFPs for **1**−**11**. As shown in Figure 4 for a representative set of lipo−GFPs, the soluble GFP is resolved from farnesol−GFP (GFP·**10**) and the gel shifts correlate to the size of the appended lipids, reliably indicating the progression of ligations. Of the cysteine-lipids examined to date, only those not entirely soluble in the ligation mixture were slow to react, and milligram quantities of lipidated GFP adducts were readily obtained.

After ligation, the lipo−GFP adducts become distinctly hydrophobic, adsorbing to hydrophobic supports and requiring detergent to remain in solution. Extraction of the ligation products with Triton X-114[14] revealed that the lipidated proteins partition into a separate 11% detergent phase, while soluble GFP remains entirely in the aqueous phase. The phospholipids, with partition coefficients >30:1, showed the highest propensity for extracting, while cholesterol− and farnesol−GFP were decreasingly hydrophobic (16:1 and 1.3:1, respectively). As an exception, GFP·**9** with an ∼1 kDa poly(ethylene glycol) tether was extracted poorly into the detergent phase.

Incorporation of Lipo−GFP in Supported Membranes. Taking advantage of the hydrophobicity of the attached lipid, lipo−GFPs were incorporated by dialytic removal of β-OG into small unilaminar vesicles (SUVs), composed largely of phosphatidylcholine and doped with phosphatidylserine and phosphatidylethanoleamine. Characteristics of GFP·**8** in lipid bilayers were quantitatively analyzed as a test case. These vesicles were

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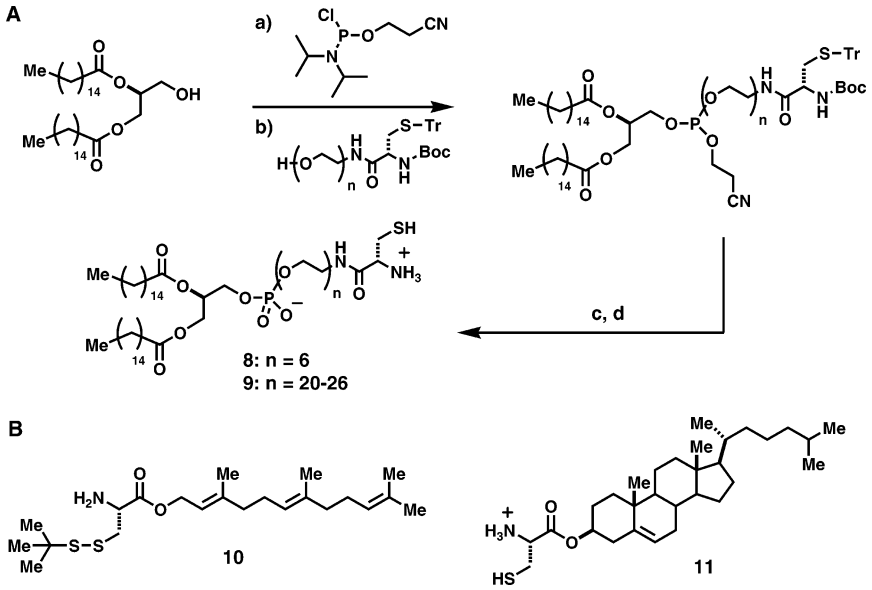

Figure 3. (A) Synthesis of cysteine-bearing lipids for NCL. (a) iPr$_2$EtN (2.2 equiv), CH_2Cl_2, 23 °C, 1 h. (b) tetrazole (1 equiv), CH_2Cl_2, 23 °C, 1 h, 83%. (c) tBuOOH (3 equiv), Et_3N (5 equiv), CH_2Cl_2, 23 °C, 2 h; tBuNH$_2$/CH_2Cl_2 (1:1), 23 °C, 2 h, 95% for **8**. (d) 1% Et_3SiH, 2.5% EDT, TFA, 23 °C, 2 h, 85% yield of **8**. (B) Farnesol and cholesterol esters to cysteine **10** and **11** for NCL.

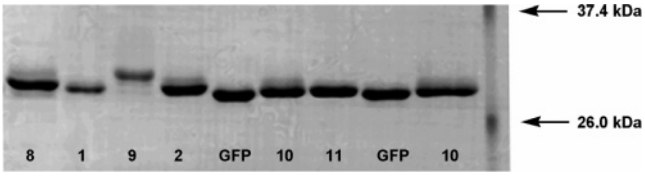

Figure 4. Coomassie blue-stained 12% SDS−PAGE of representative lipo−GFP adducts from ligations to GFP. The lipids used for the ligation are listed by compound number at the bottom of lanes 1−9.

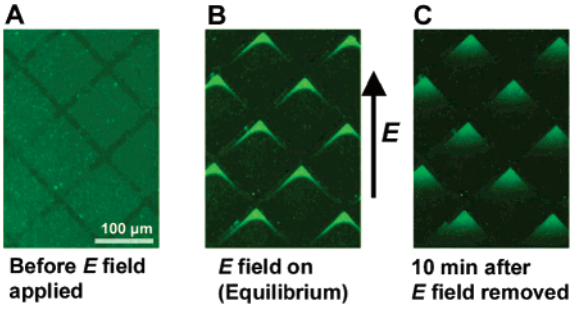

Figure 5. Fluorescence microscopy of GFP·**8** in lipid bilayer membranes supported on glass patterned with a grid of bovine serum albumen (BSA) barriers, forming isolated 100 μm square regions of membranes. (A) Stationary bilayer after proteoliposomes deposition. (B) Six minutes after application of a 20V/cm field. (C) Ten minutes after releasing the field.

deposited on a glass surface patterned with a grid of bovine serum albumin (BSA) barriers, forming isolated 100 μm square regions of supported lipid bilayers.[15] Lipo−GFP incorporated into outer leaflets of SUVs faced the bulk water phase in supported membranes,[16] and therefore the effect of substrate on the diffusion of protein was negligible. Observed by fluorescence microscopy, the bilayers contained a clear GFP signal (Figure 5A) that was evenly dispersed across the glass surface. Unlike GFP·**8**, soluble GFP was not competent for incorporation into SUVs and bilayers, producing no GFP fluorescence above background.

Application of a 20 V/cm electric field parallel to the plane of the supported bilayers rapidly induced lateral concentration of GFP·**8** into the corner of the membrane corrals by electro-osmotic flow (Figure 5B).[17,18] Only a small fraction of incorporated GFPs was immobile in electrophoresis (Figure 5B), indicating that the amount of proteins adsorbed to substrate during the deposition of proteoliposomes was negligible. Based on the 4 min time scale of this process, we estimate the electrophoretic drift velocity, v, of the membrane-tethered GFP to be ~0.6 μm/s. Quantitative analysis of fluorescence intensity images of the concentration gradient at equilibrium indicated approximately 3.3×10^3 GFP/μm^2 density of protein in the fluid membrane prior to field-induced rearrangement. Furthermore, the shape of the equilibrium profile, $\varphi(r)$, results from a competition between diffusion, D, and field-induced drift;[17]

$$\varphi(r) = \frac{1}{\exp[-v(r - r_0)/D] + 1} \qquad (1)$$

A quantitative fit of the observed GFP concentration profile to this functional form indicates $v/D = 0.37 \pm 0.03$, which, combined with the previous estimate of v, implies $D = 1.6\ \mu$m^2/s (Figure 6).

This value of D is consistent with GFP associated in the membrane as monomers or small clusters; large clusters would not be expected to exhibit such rapid diffusion. After release of the field, the concentrated protein relaxed back to uniformity by diffusion processes, the time scale of which was consistent with the value of D determined above. These long-range field-induced redistributions of GFP in the supported membrane confirm that GFP·**8** is stably linked to the membrane by lipid **8** and that it is subject to the same constraints as other membrane components.

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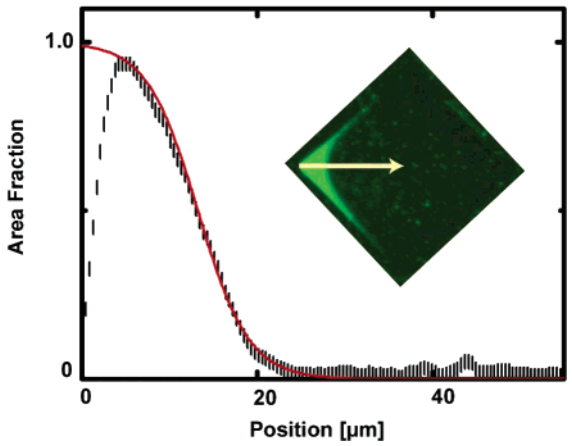

Figure 6. Field-induced equilibrium concentration profile of fluorescence intensity in one of the 100 μm square regions of membrane. The profile corresponds to the arrow in an inset image. The solid line is the best fit of eq 1 to the data.

Conclusion

In summary, proteins linked to phospholipids **1**−**9**, though lacking glycans, are simple surrogates for the GPI anchor structure. The synthesis and characterization of such model lipoproteins in model membranes will permit the design of biomolecule analogs with known physical behaviors.

Experimental Section

Protein Expression. GFP was cloned from pcDNA3.1/ctGFP (Invitrogen) into the expression vector pTYB1 (New England Biolabs) with the restriction enzymes Not1 and EcoR1, and the GFP−intein−CBD fusion from this plasmid was expressed in *E. coli* strain ER2566. The protein fusion from the lysed pellet of a 1 L culture was applied on two batches of chitin resin (2 mL each), washed with column buffer (5 × 10 mL; 20 mM HEPES, 250 mM NaCl, 0.5 mM EDTA at pH 7.0) and then ligation buffer (5 × 10 mL; 200 mM NaCl and phosphate, pH 7.2). A slurry of this resin bed was used for lipid ligations.

Solid-Phase Peptide Synthesis. Fmoc-based synthesis of heptapeptides for the preparation of lipids **3**−**7** was performed on 2-chorotrityl polystyrene resin charged with Fmoc-Alanine. Fmoc-L-amino acids (10 equiv) were coupled with sequential rounds of HBTU/HOBt and (iPr)$_2$EtN (10 equiv each) in DMF followed by Fmoc deprotection with 20% piperidine in DMF. Cleavage of the peptide from resin with 0.5% TFA in CH_2Cl_2 and purification by C18 reversed-phase HPLC with 0.1% TFA in MeCN/H_2O produced pure protected peptide C-terminal acids for amide coupling to DPPE.

HO-Ala-Arg(N^G-Pmc)-Ser(O-tBu)-Tyr(O-tBu)-Arg(N^G-Pmc)-Gly-Cys(S-Tr)Boc: MS (ESI$^+$): 1799.8 [M + H]$^+$.

HO-Ala-Lys(N^α-Boc)Ser(O-tBu)-Tyr(O-tBu)-Lys(N^α-Boc)-Gly-Cys(S-Tr)Boc: MS (ESI$^+$): 1434.8 [M + H]$^+$.

HO-Ala-Ser(O-tBu)-Pro-Ala-Ser(O-tBu)-Pro-Cys(S-StBu)Boc: MS (ESI$^+$): 932.2 [M + H]$^+$.

HO-Ala-Glu(O-tBu)-Ser(O-tBu)-Tyr(O-tBu)-Glu(O-tBu)-Gly-Cys-(S-Tr)Boc: MS (ESI$^+$): 1346.6 [M + Na]$^+$.

HO-Ala-Asp(O-tBu)-Ser(O-tBu)-Tyr(O-tBu)-Asp(O-tBu)-Gly-Cys-(S-Tr)Boc: MS (ESI$^+$): 1318.7 [M + Na]$^+$.

Peptide Coupling to DPPE. A 2 mL 3:1 $CHCl_3$/MeOH solution of protected peptide (0.05 mmol) was stirred for 1 h at 23 °C with NHS (0.05 mmol) and EDCI (0.05 mmol). Dipalmitoyl-phosphatidylethanolamine (DPPE, 0.06 mmol) and (iPr)$_2$EtN (0.06 mmol) were added, and the mixture was stirred vigorously for 12 h at 23 °C. The mixture was diluted with CH_2Cl_2 (10 mL) and washed with 10% aqueous NH_4Cl (3 mL). The aqueous layer was extracted with CH_2Cl_2 (2 × 10 mL), and the combined organic layers were concentrated. Purification by silica gel chromatography (1% NH_4OH, 5−10% MeOH in CH_2Cl_2) gave pure protected lipopeptides.

DPPE-Ala-Cys(S-Tr)Boc: 68% yield. R_f 0.25 (1% NH_4OH, 10% MeOH/CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39 (d, 8 H, J = 8.0 Hz), 7.28 (m, 12 H), 6.91 (bs, 1H), 5.17 (bs, 1 H), 4.33 (m, 2 H), 4.10 (m, 1 H), 3.87 (bs, 2 H), 3.78 (bs, 2 H), 3.45 (bs, 1 H), 3.17 (bs, 1 H), 2.65 (m, 1 H), 2.50 (m, 1 H), 2.23 (m, 4 H), 1.55 (m, 4 H), 1.55 (bs, 4 H), 1.38 (s, 9 H), 1.24 (s, 59 H), 0.86 (t, 6 H, J = 6.4 Hz) ppm. MS (ESI$^+$): 1209.5 [M − H]$^+$.

DPPE-Ala-Arg(N^G-Pmc)-Ser(O-tBu)-Tyr(O-tBu)-Arg(N^G-Pmc)-Gly-Cys(S-Tr)Boc: 55% yield. R_f 0.30 (1% NH_4OH, 10% MeOH/CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40 (d, 8 H, J = 8.0 Hz), 7.27 (bs, 10 H), 7.19 (bs, 3 H), 5.15 (bs, 1 H), 4.50 (bs, 1 H), 4.35 (bs, 3 H), 4.20 (bs, 1 H), 4.08 (bs, 3 H), 3.91 (bs, 6 H), 3.73 (bs, 3 H), 3.08 (bs, 8 H), 2.64 (bs, 6 H), 2.55 (bs, 11 H), 2.25 (m, 4H), 2.08 (s, 15 H), 1.78 (bs, 6 H), 1.55 (bs, 6 H), 1.41 (m, 30 H), 1.29 (s, 25 H), 1.24 (s, 44 H), 1.09 (bs, 8 H), 0.86 (t, 6 H, J = 6.4 Hz) ppm. MS (ESI$^-$): 2471.7 [M − H]$^-$.

DPPE-Ala-Lys(N^α-Boc)Ser(O-tBu)-Tyr(O-tBu)-Lys(N^α-Boc)-Gly-Cys(S-Tr)Boc: 61% yield. R_f 0.25 (1% NH_4OH, 10% MeOH/CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40 (d, 8 H, J = 8.0 Hz), 7.27 (bs, 12 H), 7.19 (d, 1 H, J = 11.2 Hz), 6.86 (d, 1 H, J = 11.2 Hz), 5.15 (bs, 1 H), 5.10 (s, 1 H), 4.40 (m, 3 H), 4.15 (m, 2 H), 3.88 (bs, 5 H), 3.74 (bm, 2 H), 3.45 (bs, 1 H), 3.19 (m, 1 H), 3.01 (bs, 4 H), 2.65 (d, 1 H, J = 8.0 Hz), 1.26 (bs, 11 H), 1.56 (bs, 29 H), 1.29 (s, 9 H), 1.24 (bs, 65 H), 0.86 (t, 6 H, J = 6.4 Hz) ppm. MS (ESI): 2085.3 [M + H]$^+$.

DPPE-Ala-Ser(O-tBu)-Pro-Ala-Ser(O-tBu)-Pro-Cys(S-StBu)Boc: 62% yield. R_f 0.35 (1% NH_4OH, 10% MeOH/CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$/MeOD-d_4): δ 5.15 (bs, 1 H), 4.60 (m, 1 H), 4.45 (m, 2 H), 4.21 (m, 7 H), 3.73 (m, 2 H), 3.65 (m, 5 H), 3.35 (m, 1 H), 3.28 (m, 2 H), 2.72 (m, 1H), 2.65 (m, 1 H), 2.00 (bm, 3 H), 1.81 (bm, 6 H), 1.22 (bs, 11 H), 1.14 (bs, 13 H), 1.03 (s, 11 H), 0.95 (bs, 20 H), 0.63 (t, 6 H, J = 6.4 Hz) ppm. MS (ESI): 1607.6 [M + H]$^+$.

DPPE-Ala-Glu(O-tBu)-Ser(O-tBu)-Tyr(O-tBu)-Glu(O-tBu)-Gly-Cys(S-Tr)Boc: 87% yield. R_f 0.25 (1% NH_4OH, 10% MeOH/CH_2Cl_2). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.22 (d, 8 H, J = 8.0 Hz), 7.09 (bs, 12 H), 6.92 (d, 1 H, J = 8.4 Hz), 6.86 (d, 1 H, J = 8.4 Hz), 5.17 (bs, 1 H), 4.55 (bs, 1 H), 4.19 (m, 3 H), 3.89 (m, 5 H), 3.70 (m, 1 H), 3.37 (m, 1 H), 3.15 (m, 1 H), 2.98 (m, 1 H), 2.64 (m, 2 H), 2.35 (m, 2 H), 2.25 (bm, 4 H), 2.12 (bm, 3 H), 1.85 (vbs, 11 H), 1.55 (bs, 4 H), 1.40 (bs, 18 H), 1.29 (bs, 9 H), 1.25 (bs, 58 H), 0.87 (t, 6 H, J = 6.6 Hz) ppm. MS (ESI): 1999.2 [M + H]$^+$.

DPPE-Ala-Asp(O-tBu)-Ser(O-tBu)-Tyr(O-tBu)-Asp(O-tBu)-Gly-Cys(S-Tr)Boc: 73% yield. R_f 0.35 (1% NH_4OH, 10% MeOH/CH_2Cl_2). ^{1}H NMR (300 MHz, $CDCl_3$/MeOD-d_4): δ 7.12 (d, 6 H, J = 8.7 Hz), 7.01 (m, 12 H), 6.87 (d, 1 H, J = 8.7 Hz), 6.62 (d, 1 H, J = 8.4 Hz), 4.96 (bs, 1 H), 4.37 (m, 2 H), 4.12 (m, 2 H), 3.89 (m, 1 H), 3.73 (m, 1 H), 3.60 (m, 3 H), 3.36 (m, 3 H), 3.18 (m, 1 H), 2.51 (d, 1 H, J = 4.8 Hz), 2.36 (m, 1 H), 2.04 (m, 4 H), 1.32 (bs, 5 H), 1.18 (s, 27 H), 1.12 (bs, 28 H), 1.01 (s, 9 H), 0.99 (bs, 65 H), 0.61 (t, 6 H, J = 6.5 Hz) ppm. MS (ESI): 1993.1 [M + Na]$^+$.

DPPE-Cys(S-Tr)Boc. A 1.5 mL $CHCl_3$ solution of Cys(S-Tr)Boc (0.068 mmol) with HOBt (0.068 mmol) and EDCI (0.068 mmol) was stirred for 1 h at 23 °C. DPPE (0.05 mmol), $NaHCO_3$ (0.09 mmol), and water (0.5 mL) were added, and the mixture was vigorously stirred for 12 h at 23 °C. The mixture was diluted with CH_2Cl_2 (10 mL) and washed with 10% aqueous NH_4Cl (3 mL). The aqueous layer was extracted with CH_2Cl_2 (2 × 10 mL), and the combined organic layers were concentrated. Purification by silica gel chromatography (1% NH_4OH, 5−7% MeOH in CH_2Cl_2) gave pure DPPE-Cys(S-Tr)Boc in 80% yield. R_f 0.5 (1% NH_4OH, 10% MeOH/CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40 (d, 8 H, J = 8.0 Hz), 7.28 (m, 12 H), 7.18 (d, 2 H, J = 8.6 Hz), 5.17 (bs, 1 H), 5.01 (bs, 1 H), 4.30 (dd, 1 H, J = 4.5, 8.5 Hz), 4.08 (m, 1 H), 3.90 (bd, 4 H), 3.55 (bs, 1 H), 3.26 (bs, 1 H), 2.55 (m, 1 H), 2.45 (m, 1 H), 2.21 (t, 4 H, J = 6.0 Hz), 1.55 (m, 4 H), 1.42

(d, 1 H, $J = 4.0$ Hz), 1.35 (s, 9 H), 1.22 (s, 56 H), 0.86 (t, 6 H, $J = 6.4$ Hz) ppm. MS (ESI^+): 1160.5 $[M + Na]^+$.

Deprotection of Lipids 1−8, 10. Lipopeptide (0.026 mmol) was stirred in a 1% Et_3SiH, 2.5% ethanedithiol (EDT), 2.5% H_2O TFA solution for 0.5−2 h at 23 °C. The mixture was concentrated, and the product was purified by silica gel chromatography or trituration with *t*BuOMe, as indicated.

DPPE-Cys (1): Purified by silica gel chromatography (1% NH_4-OH, 10% MeOH, CH_2Cl_2) to give **2** in 82% yield. R_f 0.20 (1% NH_4-OH, 12% MeOH/CH_2Cl_2). ^1H NMR (400 MHz, $CDCl_3$): δ 5.07 (bs, 1 H), 4.22 (dd, 1 H, $J = 8.5, 3.5$ Hz), 3.79 (m, 4 H), 3.31 (m, 1 H), 3.05 (m, 1 H), 2.17 (m, 4 H), 1.45 (m, 4 H), 1.11 (m, 56 H), 0.74 (t, 6 H, $J = 6.4$ Hz) ppm. MS (ESI): 795.9 $[M + H]^+$.

DPPE-Ala-Cys (2): Purified by silica gel chromatography (1% NH_4-OH, 10−20% MeOH, CH_2Cl_2) to give **2** in 77% yield. R_f 0.50 (1% NH_4OH, 20% MeOH/CH_2Cl_2). ^1H NMR (300 MHz, $CDCl_3$): δ 5.15 (bs, 1 H), 4.18 (m, 1 H), 4.08 (m, 1 H), 3.94 (m, 1 H), 3.85 (m, 3 H), 3.40 (bs, 10 H), 2.85 (m, 1 H), 2.24 (m, 4 H), 1.52 (m, 4 H), 1.33 (m, 3 H), 1.21 (s, 56 H), 0.80 (t, 6 H, $J = 6.9$ Hz) ppm. MS (ESI): 866.2 $[M + H]^+$.

DPPE-Ala-Arg-Ser-Tyr-Arg-Gly-Cys (3): 89% yield after trituration with *t*BuOMe. ^1H NMR (300 MHz, $CDCl_3$/pyridine-d_5/MeOD-d_4): δ 7.55 (bs, 2H), 6.98 (bs, 2 H), 5.50 (bs, 1 H), 4.80 (bm, 2 H), 4.45 (m, 1 H), 4.24 (m, 5 H), 3.85 (m, 1 H), 3.45 (m, 3 H), 2.50 (m, 4 H), 1.78 (bs, 2H), 1.60 (m, 3 H), 1.45 (s, 52 H), 1.03 (bs, 6 H) ppm. MS (ESI^+): 1486.8 $[M + H]^+$.

DPPE-Ala-Lys-Ser-Tyr-Lys-Gly-Cys (4): 98% yield after trituration with *t*BuOMe. ^1H NMR (300 MHz, $CDCl_3$/pyridine-d_5/MeOD-d_4): δ 6.75 (d, 2H, $J = 8.4$ Hz), 6.35 (d, 2 H, $J = 8.4$ Hz), 4.90 (bs, 1 H), 3.80 (m, 1 H), 3.60 (m, 2 H), 2.81 (m, 1 H), 2.50 (m, 4 H), 1.96 (m, 4 H), 1.19 (m, 11 H), 1.04 (d, 3 H, $J = 9.3$), 0.90 (s, 52 H), 0.52 (t, 6 H, $J = 6.3$ Hz) ppm. MS (ESI^+): 1430.1 $[M + H]^+$.

DPPE-Ala-Ser-Pro-Ala-Ser-Pro-Cys (5): 86% yield after trituration with *t*BuOMe. ^1H NMR (300 MHz, $CDCl_3$/pyrdine-d_5/MeOD-d_4): δ 4.65 (m, 1 H), 4.45 (m, 4 H), 4.16 (m, 1 H), 4.03 (bs, 3 H), 3.80 (m, 2 H), 3.51 (m, 1 H), 3.40 (m, 1 H), 2.23 (m, 4 H), 2.00 (bs, 3 H), 1.80 (m, 2 H), 1.53 (bs, 3 H), 1.32 (bs, 5 H), 1.16 (s, 52 H), 0.77 (t, 6 H, $J = 6.9$ Hz) ppm. MS (ESI^+): 1305.9 $[M + H]^+$.

DPPE-Ala-Glu-Ser-Tyr-Glu-Gly-Cys (6): 55% yield after trituration with *t*BuOMe. In a range of solvents, clear ^1H NMR spectra could not be acquired. MS (ESI^-): 1430.2 $[M]^-$.

DPPE-Ala-Asp-Ser-Tyr-Asp-Gly-Cys (7): 54% yield after trituration with *t*BuOMe. ^1H NMR (300 MHz, $CDCl_3$/pyrdine-d_5/MeOD-d_4): δ 6.62 (d, 2 H, $J = 8.4$ Hz), 6.32 (d, 2 H, $J = 8.4$ Hz), 4.83 (bs, 1 H), 3.97 (bs, 1 H), 3.76 (bs, 2 H), 2.69 (bs, 1 H), 2.54 (bs, 1 H), 1.89 (bs, 4 H), 1.18 (m, 4 H), 0.95 (m, 8 H), 0.85 (bs, 48 H), 0.77 (t, 6 H, $J = 6.9$ Hz) ppm. MS (ESI^+): 1425.9 $[M + Na]^+$.

***N*-Fmoc-*S*-*t*Buthio-cysteine Farnesol Ester.** Farnesol (0.275 mmol), Cys(*S*-StBu)Fmoc (0.25 mmol), DCC (0.25 mmol), and DMAP (0.025 mmol) were stirred in THF (2.5 mL) for 5 h at 23 °C. Filtration of the white precipitate and concentration in vacuo gave a residue that was purified by silica gel chromatography (5% ethyl acetate in hexanes) to afford the farnesol ester in 88% yield. ^1H NMR (400 MHz, $CDCl_3$): δ 7.78 (d, 2 H, $J = 7.6$ Hz), 7.63 (d, 2 H, $J = 7.6$ Hz), 7.41 (t, 1 H, $J = 7.2$ Hz), 7.39 (t, 1 H, $J = 7.2$ Hz), 5.77 (d, 1 H, $J = 8.0$ Hz), 5.36 (t, 1 H, $J = 6.4$ Hz), 5.10 (t, 2 H, $J = 5.6$ Hz), 4.71 (m, 3 H), 4.39 (m, 2 H), 4.26 (t, 1 H, $J = 7.2$ Hz), 3.22 (m, 2 H), 2.05 (m, 6 H), 1.97 (m, 2 H), 1.73 (s, 3 H), 1.68 (s, 6 H), 1.61 (s, 6 H), 1.33 (s, 9 H), 1.26 (m, 1 H) ppm. MS (ESI^+): 636.3 $[M + Na]^+$.

***S*-*t*Buthio-cysteine Farnesol Ester (10).** *N*-Fmoc-*S*-S*t*Bu-cysteine farnesol ester (0.11 mmol) dissolved in CH_2Cl_2 (5 mL) was treated with piperidine (0.5 mL) for 4 h at 23 °C. The solution was diluted with water (5 mL) and extracted with ethyl acetate (2 × 10 mL). The organic layers were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. C-18 Reversed-phase HPLC (40−95% MeCN/H_2O) gave pure 10 in 40% yield. ^1H NMR (400 MHz, $CDCl_3$): δ ppm. MS (ESI^+): 414.2 $[M + H]^+$, 436.2 $[M + Na]^+$.

***N*-Boc-*S*-Tr-cysteine Cholesterol Ester.** Cholesterol (1.0 mmol), Cys(*S*-StBu)Fmoc (1.0 mmol), DCC (1.0 mmol), and DMAP (0.1 mmol) were stirred in THF (10 mL) for 8 h at 23 °C. Filtration of the white precipitate and concentration in vacuo gave a residue that was purified by silica gel chromatography (5% ethyl acetate in hexanes) to afford the cholesterol ester in 65% yield. ^1H NMR (400 MHz, $CDCl_3$): δ 7.42 (d, 6 H, $J = 8.0$ Hz), 7.28 (t, 6 H, $J = 8.0$ Hz), 7.23 (m, 3 H), 5.39 (d, 1 H, $J = 4.4$), 5.15 (d, 1 H, $J = 8.0$ Hz), 4.63 (m, 1 H), 4.27 (m, 1 H), 3.22 (m, 1 H), 2.57 (m, 2 H), 2.31 (m, 2 H), 2.02−2.81 (m, 8 H), 1.75 (m, 2 H), 1.65−1.23 (m, 7 H), 1.45 (s, 3 H), 1.19 (m, 4 H), 1.07 (s, 3 H), 0.95 (d, 3 H, $J = 6.2$ Hz), 0.90 (s, 3 H), 0.89 (s, 3 H), 0.71 (s, 3 H) ppm. MS (ESI^+): $[M + H]^+$.

Standard Conditions for Lipidation of GFP. For a typical ligation, cysteine-bearing lipid and β-octylglucoside (5 mg each) were dissolved in dichloromethane and dried in a 1.5 mL Eppendorf tube to a film under a stream of N_2 and then in vacuo. The mixture was resuspended in ligation buffer (250 μL) and mixed for 48 h at 23 °C with sodium mercaptoethanesulfonate (15 mg) and a slurry of GFP−intein fusion on chitin resin (250 μL). Filtration of the mixture yielded a 0.1−0.7 mg/mL solution of lipo−GFP, as indicated by Coomassie blue-stained 12% SDS−PAGE. For incorporation into SUVs, the lipo−GFP solution was cooled to 4 °C and any white precipitate was pelleted by centrifugation. The clarified solution was concentrated 10-fold by Micron-10 centrifugal dialysis (Millipore) and used for proteoliposome formation.

Incorporation of GFP·8 into Planar Supported Bilayers. SUVs composed of egg phosphatidylcholine with 5% dioleolylphosphatidylserine and 0.6% Texas red-dihexadecanoylphosphatidylethanolamine (Texas red-DPPE, Molecular Probes, Eugene, OR) were prepared in water, by modification of the Barenholz procedure.[17,19] The SUVs were changed into Dulbecco's phosphate-buffered saline (PBS, pH 7.4) to a total lipid concentration of 1 mM, and these vesicles were dialyzed with 2 mM GFP·**8** at 23 °C overnight against four buffer changes of PBS.[20] Glass coverslips were etched in 3:1 H_2SO_4/30% aqueous H_2O_2 and patterned with BSA by microcontact printing.[15] Proteoliposomes were deposited on these coverslips for form fluid bilayers and were exposed to lateral electric fields as previously described, with a 20 V/cm electric field in PBS diluted by 1/5.[17] Supported membranes were viewed with a Nikon TE300 inverted fluorescence microscope (Nikon, Tokyo, Japan) equipped with a mercury arc lamp. Images were recorded with a cooled charge-coupled device camera (Hamamatsu C4742-98 ORCAII, Hamamatsu, Japan) through a Nikon 20× long working distance objective.

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