VOLUME 123, NUMBER 26
JULY 4, 2001

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Rational Approaches to the Design of Cationic Gemini Surfactants for Gene Delivery

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Received October 10, 2000

Abstract: We report a new class of amphiphilic gemini surfactants as vehicles for gene delivery into cells, and the beginnings of a systematic structure−activity study. Preliminary results suggest that combining gemini surfactants with dioleoylphosphatidylethanolamine (DOPE) should allow the preparation of liposomes of various sizes and lipid compositions. Control of such colloidal changes could be as significant as the changes in the molecular composition of the gemini surfactants in delivering optimum gene expression in animal models.

Introduction

A range of techniques and vehicles have been developed for the transfer of genes into cells to induce protein expression. Earlier methods included the direct injection of DNA in the presence of calcium phosphate,[1] and electroporation, where DNA is driven across cell membranes by the application of an electric current.[2] More recent approaches involve the use of a carrier molecule (the vehicle, or "vector") to deliver the engineered or foreign gene into target cells. Vehicles for the delivery of genetic material are usually divided into two groups: those that use viruses (e.g., retrovirus, adenovirus)[3] and those using cationic lipids, either alone or in formulation with a "helper" lipid, such as dioleoylphosphatidylethanolamine (DOPE).[4]

Although entry into a targeted cell using a viral vector is generally very efficient, the use of such vectors is not without the risk of adverse or immunogenic reaction, or replication, depending on the virus being used. As a result, delivery of DNA mediated by cationic lipids has in many cases become a preferred alternative to viral gene delivery into eukaryotic cells, although levels of gene expression are usually lower than those achieved by the use of viral vectors.[5]

Cationic lipids possess many features of an "ideal" vector. In contrast to most viral systems, the size of the plasmid that can be delivered is unrestricted. Moreover, the physicochemical properties of cationic lipids can be varied to facilitate formulation and adaptation to Good Manufacturing Practice. Commercially attractive cationic lipids can in principle be designed to combine good transfection efficiency with other desirable features such as lower levels of toxicity and immunogenicity as well as ease of manufacture.

Although a large number of cationic lipids have been synthesized as potential gene delivery vehicles, we are not aware of published attempts to generate quantitative structure−activity

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10.1021/ja005681c CCC: $20.00
Published on Web 06/07/2001

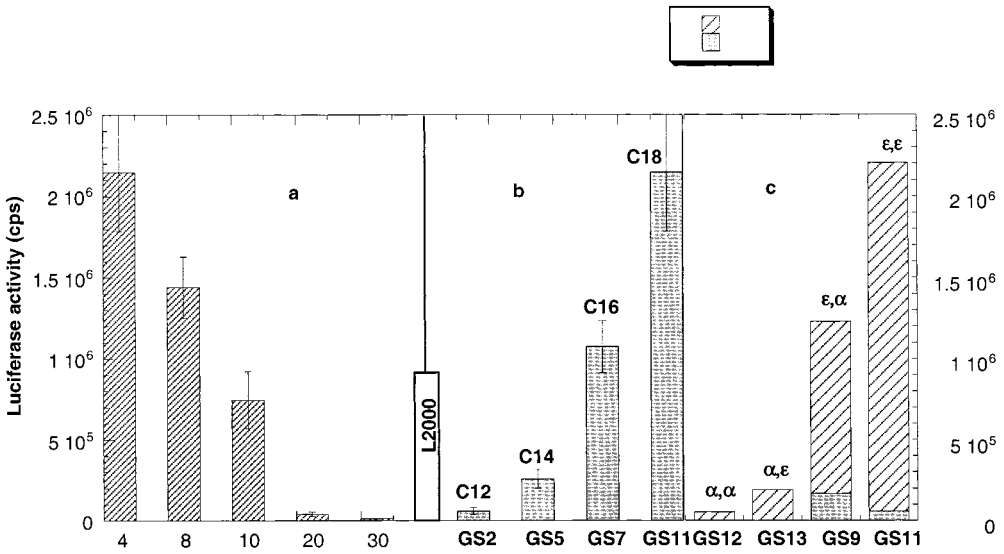


Figure 1. Structure−activity dependence on hydrocarbon tail length and peptide head section (see Table 1 for compound structures). CHO-DG44 cells were incubated with DNA and five different concentrations (4, 8, 10, 20, and 30 μM) of gemini surfactant, and luciferase activity (in counts per second) averaged over four measurements. (a) The full set of data is shown for compound **GS11** as a function of increasing gemini concentration; highest activity was generally observed at 4 μM, and data for other compounds (panels b and c) represent measurements in the presence of 4 μM surfactant. Data for L2000 (used under the recomended conditions) are included for comparison. (b) Effect of varying chain length (group R) for the series with ϵ, ϵ-linked trilysine. (c) Effect of varying trilysine linkage; data are shown for compounds with the oleyl (C_{18}) side-chain and (inset) for the corresponding C_{12} derivatives (**GS14**, **4**, **3**, and **2**).

Table 1. Structures of Selected Peptide-Based Gemini Surfactants[a]

GS	$(AA)_n$	R	GS	$(AA)_n$	R
1	$(Lys)_2Lys-$	C_{12}	**8**	Lys-α-Lys-α-Lys-	C_{16}
2	Lys-ϵ-Lys-ϵ-Lys-	C_{12}	**9**	Lys-α-Lys-ϵ-Lys-	$C_{18:1}^{\Delta 9}$
3	Lys-ϵ-Lys-α-Lys-	C_{12}	**10**	$(Lys)_2Lys-$	$C_{18:1}^{\Delta 9}$
4	Lys-α-Lys-ϵ-Lys-	C_{12}	**11**	Lys-ϵ-Lys-ϵ-Lys-	$C_{18:1}^{\Delta 9}$
5	Lys-ϵ-Lys-ϵ-Lys-	C_{14}	**12**	Lys-α-Lys-α-Lys-	$C_{18:1}^{\Delta 9}$
6	Lys-α-Lys-α-Lys-	C_{14}	**13**	Lys-ϵ-Lys-α-Lys-	$C_{18:1}^{\Delta 9}$
7	Lys-ϵ-Lys-ϵ-Lys-	C_{16}	**14**	Lys-α-Lys-α-Lys-	C_{12}

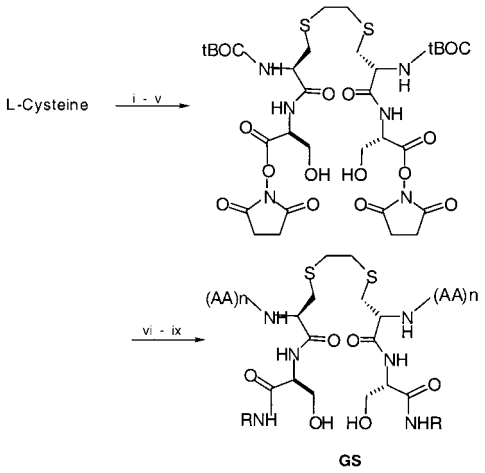
[a] Lys-α-Lys indicates a normal peptide linkage: Lys-ϵ-Lys-ϵ-Lys− is linked through the side-chain ϵ-amino-groups (see Scheme 2), and $(Lys)_2Lys-$ has lysines linked by amide bonds to both α- and ϵ-amino groups of the first lysine.

relationships (QSAR), essential for the optimization of drug activity.[6] We described recently the synthesis of several peptide-based gemini surfactants which showed significant activity for gene transfection.[7] We now report the synthesis of a series of novel multivalent gemini surfactants **GS** (Table 1), where the nature of both the "head" peptide $(AA)_n$ and the "tail" group (R, unbranched) has been systematically varied. The generic structure and the general synthesis of these surfactants are shown in Scheme 1.

Results and Discussion

We observe levels of transfection at least an order of magnitude higher than for the original **GS** systems.[7] Furthermore, these levels can be raised severalfold by appropriate formulation. Control experiments (data not shown) showed that single-chain analogues based on *S*-methyl cysteine were inactive.

Scheme 1. General Route for the Synthesis of Gemini Surfactants **GS**[8]



The capabilities of compounds **GS 1**−**14** to mediate the transfer of a luciferase reporter gene across Chinese hamster ovary (CHO-DG44) cell membranes were compared to that of LipofectAMINE 2000 (L2000), a potent nonviral vehicle recently commercialized by Life Technologies. Transfection activity was determined by assay for luciferase activity (Figure 1a).

Increasing the length of the hydrocarbon "tail" leads to a substantial increase in transfection activity (Figure 1b). Gene expression was also found to depend on the nature of the amide linkages between the three lysine residues in the headgroup. Thus, three lysines linked through their ϵ-amino groups rather than through partial or total α-linkage appear to provide optimal interaction of these dimeric cationic lipids with DNA (Figure 1c). The interaction must be at least partially electrostatic and dependent on the spacing of the ammonium groups of the

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(8) Conditions. (i) $BrCH_2CH_2Br$, $NaHCO_3$, 88% yield. (ii) Di-*tert*-butyldicarbonate, NaOH, 66% yield. (iii) *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, quantitative. (iv) L-Serine, K_2CO_3, 90% yield. (v) *N*-Hydroxysuccinimide, dicyclohexylcarbodiimide, quantitative. (vi) RNH_2 (dodecylamine, tetradecylamine, hexadecylamine, natural oleylamine (85/15 *cis*/*trans*, oleylamine, and elaidylamine), triethylamine, 50−90% yield. (vii) concentrated HCl, $CHCl_3$, 70−90% yield. (viii) *N*-hydroxysuccinimide ester of BOC-protected peptide (N.B. all peptides were prepared by traditional methods of peptide bond formation, NaOH, 50−70% yield. (ix) concentrated HCl, CH_3OH, 50−70% yield.

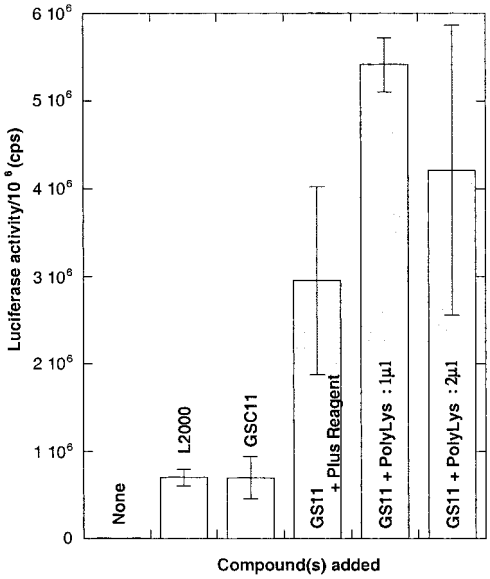


Figure 2. Effects of basic polypeptides on the gene expression efficiency of **GS11** in C2C12 mouse muscle cells. The luciferase reporter gene (0.5 μg) was complexed with either PLUS reagent (1.5 μL) or poly-D,L-lysine (molecular weight range 1000 to 4000, 1 and 2 μL of stock solution) prior to the addition of **GS11**. The L2000:DNA ratio was 1:3 (w:w), as recommended by Life Technologies. Stock solutions contained 1 mg/mL of PLUS reagent or polylysine.

vehicle; thus, the two $(Lys)_2Lys$ derivatives (**GS1** and **10**) also showed low transfection activities. High transfection activity requires both the right cationic headgroup and the optimal chain length; although the activities of the α,α-linked trilysyl systems (**GS14**, **6**, **8**, and **12**) do increase with increasing chain length, the most effective (oleyl) derivative **GS12**, is still less efficient than the least effective compound with an ϵ,ϵ-linkage.

It is well-known that the gene expression efficiency of cationic lipids can be improved in the presence of basic peptides,[9] which are thought to act as DNA "packaging" agents. This effect is also observed for our gemini surfactants, notwithstanding the basic tripeptide units built in. Thus, the gene expression efficiencies of all of the gemini surfactants in Table 1 are at least doubled in the presence of the PLUS reagent, a basic polypeptide available commercially from Life Technologies (proprietary composition). Replacement of the PLUS reagent by the considerably less expensive polylysine had the same enhancing effect, as shown in Figure 2, which compares the transfection efficiencies of **GS11** in C2C12 mouse muscle cells in the presence of these additives. The addition of either the pure enantiomeric form of polylysine (molecular weight range 1000−4000) to the plasmid gave the same result as the racemic mixture; the addition of polylysine of a higher-molecular weight range produced a much smaller effect (data not shown).

The good gene expression efficiency found for cationic peptide-based lipids of the type shown in Table 1 is found in a variety of eukaryotic cells other than CHO-DG44 and C2C12.

We are studying cells of various types (human cell lines of kidney or neuronal origin, mouse cell lines, etc.) and measuring, as described above, the activity of luciferase following transfection with plasmid DNA. We have obtained satisfactory levels of gene expression in such cell lines as SHSY-5Y or 1321N1, of neuronal origin, normally considered difficult to transfect. Similar or higher levels of gene-transfer activity were observed using **GS11** in combination with polylysine, compared with results using LipofectAMINE 2000; increases of 2−5-fold were observed in human neuronal SHSY-5Y and mouse MOPC-315 cells.

Nonviral approaches to in vitro gene transfer and expression mediated by cationic lipids have used helper lipids to improve efficiency, giving rise to so-called lipoplex systems. (This formulation approach has led to the commercialization of transfection reagents such as Lipofectamine). An appropriate lipoplex formulation can have substantial advantages for a chosen route of administration: lipoplexes can preserve the structural integrity of plasmid DNA and have the potential to achieve targeted delivery. As part of a planned series of in vivo studies of the use of gemini surfactants, we have investigated a similar formulation approach, combining a gemini surfactant, **GS11**, with DOPE. We have carried out preliminary structural characterizations of these formulations and tested their ability to effect gene transfer and expression of luciferase in a CHO

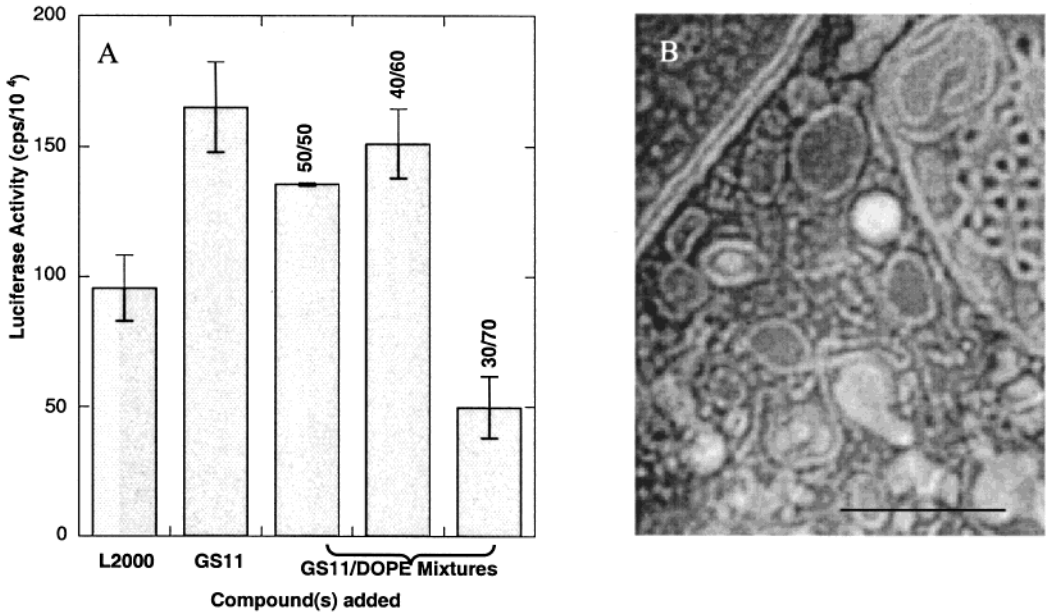


Figure 3. (A) Transfection experiment in CHO-DG44. The cells were incubated with the transfection mixtures overnight. **GS11** was used alone or in combination with DOPE. (B) Negative stained TEM micrograph of 70 mol % DOPE and 30 mol % **GS11** in water. Bar = 0.10 μm.

cell line in vitro. Suspensions of DOPE/**GS11** mixtures in water give rise to a mixture of lipid vesicles and more complex structures corresponding to particles ≤500 nm in diameter (see Figure 3b). DOPE/**GS11** formulations in molar ratios of 50/50, 60/40, and 70/30 were found to effect luciferase expression in CHO cells at levels comparable to those attained using **GS11** alone (see Figure 3a) and to Lipofectamine. Finally, we note that the cytotoxicity of these systems is low. Cell death after overnight incubations with **GS11**, either alone or mixed with DOPE, was no more than that observed after 3 h incubations with lipofectAMINE 2000.

Conclusions

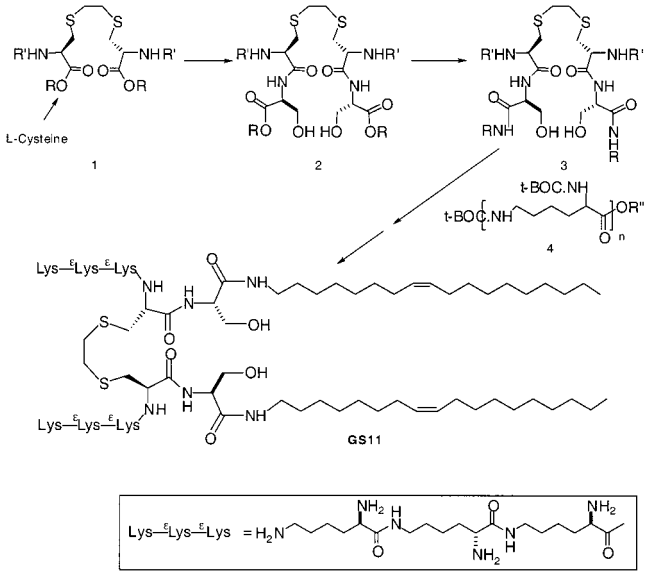
We have systematically varied the structure of peptide-based gemini surfactants **GS** (Scheme 1), leading to an increase in levels of gene expression in vitro compared to well-established nonviral reagents. These cationic lipids are relatively simple to synthesize using standard peptide chemistry, and their chemical structures can readily be tailored for effective interaction with DNA. We have shown also that they are incorporated into liposome formulations, providing further dimension in the optimization of these molecules for in vivo studies.

We are currently introducing peptide and carbohydrate cell-recognition sequences into the "head" groups of these systems. These structural changes are designed to confer a degree of selectivity in transfection by targeting specific tissues, perhaps via cell surface receptors.

Experimental Section

Synthesis of Gemini Surfactants. Synthesis (Scheme 2) involved the construction of the protected dimeric central unit (**1**), activation of the cysteine carboxyl groups, followed by coupling with serine to give **2**, and then similar activation of the serine carboxyl groups to allow acylation of the long chain primary amine tail. Deprotection and

Scheme 2



acylation of the cysteine amino groups with the protected, activated peptide gave the protected target compound. The procedure is described for the preparation of **GS11**. The other surfactants were prepared by straightforward modification of this route, which allows for independent variation of all of the component parts of the surfactant.

^{1}H NMR spectra were recorded at ambient probe temperature on Bruker AC250F (250 MHz), Bruker WM250 (250 MHz), or Bruker DX400 (400 MHz) instruments, using the solvent as an internal deuterium lock. ^{13}C NMR spectra were recorded on Bruker AC200 (50 MHz), Bruker AC250F (62.9 MHz), or Bruker AM400 (100 MHz) instruments using internal lock and proton decoupling. Moisture-sensitive reactions were performed under an inert argon atmosphere, and all glassware was either flame- or oven-dried before use. Yields are approximate because assessing purity is a problem; the compounds are difficult to separate by TLC, while the hydrophobic tails make for strong affinity with reverse phase HPLC columns. FAB (LSIMS) mass spectra were obtained on a Micromass Autospec high-resolution double-focusing instrument. Accurate masses were performed using poly-(ethylene glycol) as a mass reference. Electrospray analysis was performed on a Micromass Quattro II low-resolution triple quadrupole mass spectrometer or Finnigan MAT 900 XLT high-resolution double focusing instrument. Nebulization was pneumatically assisted by a flow of nitrogen through a sheath around the capillary. Mass spectroscopic analysis was performed by the EPSRC National Mass Spectrometry Service Centre, University of Wales, Swansea.

The Central Unit. *S*,*S*′-Ethanediyl-bis-L-cysteine (**1, R = R′ = H**)[10] was protected as the *N*,*N*′-bis-*tert*-butoxycarbonyl derivative and converted to the bis-*N*-hydroxysuccinimide (NHS) ester, as follows.

1, R = R′ = H (2.68 g, 0.01 mol) was suspended in 30 mL of H_2O and 0.96 g (0.024 mol) of NaOH was added. After 5 min a clear solution had formed, and the mixture was cooled to <10 °C. A solution of 4.36 g (0.02 mol) of $(BOC)_2O$ in 30 mL of THF was added dropwise over 30 min and the mixture stirred at room temperature overnight. After further addition of a solution of 0.25 g (0.006 mol) of NaOH in 2.5 mL of H_2O and 1.5 g (0.007 mol) of $(BOC)_2O$ in 7.5 mL of THF, the mixture was stirred for a further 18 h.

The mixture was then acidified to pH 2 with 2 M HCl, 30 mL of brine was added, and the solution was extracted with 3 × 40 mL of THF and then 2 × 30 mL of ethyl acetate. The combined organic layers were dried over $MgSO_4$, and the solvent was removed under reduced pressure. The resulting solid was recrystallized from MEK/pentane (stirred at room temperature for 2 h and then kept at −20 °C for 2 h) to give 3.1 g (65.9%) of **1 (R = H, R′ = COOBu-t)** as white crystals, mp 124−126 °C. ^{1}H NMR (d_6-DMSO, 400 MHz) δ 1.35 (s, 18H, *t*-BOC group), 2.70 (s, 4H, $(CH_2)_2$, ethylene linker), 2.74 and 2.88 (Two dd, 4H, J = 4.5, 15.05 Hz and 7.14, 15.05 Hz, CH_2 groups from cysteine fragment), 4.05 (dt, 2H, J = 4.5, 10 Hz, CHNH-*t* BOC group), 7.00 (d, 2H, J = 8.41 Hz, NH group) and 12.35 (s, 2H, carboxyl group). *m*/*z* 491.6432 $[M + Na]^+$. $C_{18}H_{32}O_8N_2S_2$ requires 468.5588.

To a solution of 2.96 g (6.33 mmol) of **1 (R = H, R′ = COOBu-t)** in 100 mL of THF (still-dried over lithium aluminum hydride) were added 1.46 g (12.67 mmol) of *N*-hydroxysuccinimide and 2.61 g (12.67 mmol) of dicyclohexyl carbodiimide (DCC). The mixture was stirred at room temperature for 18 h and then filtered through 1 cm of Celite, the residue was washed with 100 mL of THF, and the filtrate was evaporated to yield **1 (R = N-succinimido, R′ = COOBu-t)** as an off-white solid (4.09 g, 97.69), mp 140 °C (dec). ^{1}H NMR (d_6-DMSO, 400 MHz) 1.35 (s, 18H, *t*-BOC group), 2.85 (s, 4H, CH_2 on linker), 2.74 (s, 4H, succinimide CH_2). 3.00 (two dd, 4H, J = 4.5, 15.1 Hz and 7.14, 15.1 Hz, CH_2 groups from cysteine fragment), 4.5 (dt, 2H, J = 4.5, 10 Hz, CHNH-*t* BOC group), and 7.7 (d, 2H, J = 8.21 Hz, NH group). *m*/*z* 685.6914 $[M + Na]^+$. $C_{26}H_{38}O_{12}N_4S_2$ requires 662.7342.

Coupling of the Central Unit to L-Serine. L-Serine (2.10 g, 20 mmol) and K_2CO_3 (2.76 g, 20 mmol) were dissolved in 150 mL of H_2O. A suspension of 6.29 g (9.5 mmol) of **1 (R = N-succinimido, R′ = COOBu-t)** in 150 mL of THF was added. The cloudy mixture was stirred at room temperature for 72 h. Most of the THF was removed from the yellowish solution by evaporation, and the aqueous solution was acidified to pH 1 with 1 M HCl. The mixture was extracted with CH_2Cl_2 + 15% methanol (125, 50, and 50 mL) and the combined organic layers were dried over $MgSO_4$, filtered, and then evaporated. **2** (5.5 g, 90.22%) **(R = H, R′ = COOBu-t)** was collected as a colorless solid foam. ^{1}H NMR (d_6-DMSO, 400 MHz) 1.35 (s, 18H, *t*-BOC group), 2.50 (s, 4H, linker CH_2), 3.00 (two dd, 4H, J = 4, 15 Hz and 7, 15 Hz, CH_2 group from cysteine fragment), 3.70 (dd, 4H, J = 4, 12 Hz, serine CH_2), 4.15 (dt, 2H, J = 4, 10 Hz, CH-NH-*t*-BOC), 4.25 (m, 2H, serine

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CH-NH), 6.9 (d, 2H, $J = 9$ Hz, serine NH), 8.0 (d, 2H, $J = 8$ Hz, *t*-BOC protected NH) and 12.25 (s, 2H, serine carboxyl group). *m/z* 665.1879 $[M + Na]^+$. $C_{26}H_{38}O_{12}N_4S_2$ requires 642.7438.

Attachment of the Long-Chain Amine Tail. Compound **2** (2.02 g,3.15 mmol) **(R = H, R′ = COOBu-t)** prepared above was dissolved in 47.5 mL of THF (still-dried over lithium aluminum hydride) under argon, 0.82 g (7.13 mmol) of *N*-hydroxysuccinimide and 1.47 g (7.13 mmol) of DCC were added, and the mixture was stirred at room temperature for 24 h. The reaction mixture was then filtered through 1 cm Celite, the filter was rinsed with 12 mL of THF, and the filtrate was evaporated to give 2.75 g (>100%) of off-white solid. This solid was dissolved in refluxing diisopropyl ether/THF 50/50 v/v and precipitated by adding diisopropyl ether to yield **2 (R = N-succinimido, R′ = COOBu-t)** as an off-white powder (1.49 g, 56.6%), mp 52 °C (dec). ^{1}H NMR (d_6-DMSO, 400 MHz) 1.5 (s, 18H, *t*-BOC group), 2.70 (s, 4H, linker CH_2), 2.72 (s, 4H, succinimide CH_2),3.02 (m, 4H, cysteine CH_2), 3.90 (t, 4H, $J = 6$ Hz, serine CH_2), 4.30 (m, 2H, CH-NH-*t*-BOC), 4.81 (dd, 2H, $J = 6$, 8 Hz, serine CH-NH), 5.35 (t, 2H, $J = 6$ Hz, serine OH), 7.01 (d, 2H, $J = 8$ Hz, *t*-BOC protected NH), 8.70 (d, 2H, $J = 8$ Hz, serine NH), and 7.70 (d, 2H, $J = 8.21$ Hz, NH group). *m/z* 859.9234 $[M + Na]^+$ $C_{32}H_{48}O_{16}N_6S_2$ requires 836.8892.

2 (3.59 g, 4.29 mmol) **(R = N-succinimido, R′ = COOBu-t)** prepared above was dissolved in 65 mL of THF and stirred with oleylamine (Aldrich, natural mixture: 2.35 g, 8.77 mmol) and 0.89 g (1.22 mL, 8.77 mmol) of NEt_3 at room temperature for 48 h. The THF was removed by evaporation, and the off-white residue was redissolved in 86 mL of chloroform ($CHCl_3$) and extracted with 2 × 49 mL of brine. The combined brine layers were extracted with 17 mL of $CHCl_3$ The combined $CHCl_3$ layers were then dried over $MgSO_4$, and the solvent was removed by evaporation. The residue was purified on a silica column by flash chromatography using dichloromethane/ethyl acetate/methanol 4/5/1 v/v/v. This produced 3.1 g (63.4%) of **3 (R = oleyl, R′ = COOBu-t)** as an off-white solid. ^{1}H NMR ($CDCl_3$, 400 MHz) 0.9 (t, 6H, $J = 6$ Hz, terminal CH_3 of oleyl chain), 1.2 (bs, 44H, CH_2 on long chain), 1.4 (s, 18H, *t*-BOC group), 1.95 (s, 8H, CH_2CH═CH), 2.73 (s, 4H, linker CH_2), 3.01 (m, 8H, cysteine CH_2 and tail NCH_2 groups), 3.60 (m, 4H, serine CH_2), 4.15 (m, 4H, CH-NH-*t*-BOC and serine α-CH-NH), 4.90 (m, 2H, serine OH) and 5.21 (m, 4H, alkene CH). In d_6-DMSO amide NH peaks were observed at 7.01 (d, 2H, $J = 9$ Hz, *t*-BOC-protected NH), 7.70 (t, 2H, $J = 5$ Hz, on oleylamine NH) and 7.95 (d, 2H, $J = 8$ Hz, serine NH). *m/z*: 1164.6111 $[M + Na]^+$. $C_{60}H_{112}O_{10}N_6S_2$ requires 1141.7088.

Assembly of the Protected Peptide Unit. BOC_2LysOSuc (1.64 g, 3.69 mmol) was dissolved in 18 mL of THF, and a solution of 1 g (4.1 mmol) of *N*-α-*t*-BOC-L-lysine and 0.57 g (4.1 mmol) of K_2CO_3 in 18 mL of H_2O was added immediately. The mixture was stirred at room temperature for 72 h and then most of the THF was removed by evaporation and the remaining slurry acidified to pH 2 with 1 M HCl. This mixture was extracted with CH_2Cl_2 (2 × 54 mL), and the combined organic layers were dried over Na_2SO_4 and evaporated to yield tri-*t*-BOC-ϵ-linked lysyl-lysine **4 (*n* = 2, R″ = H)** as a white solid foam (1.99 g, 93.91%). ^{1}H NMR (d_6-DMSO, 400 MHz) 1.20 (s, 27H, *t*-BOC group), 1.4−1.6 (m, 12H, lysine CH_2), 2.85 (m, 2H, lysine CH_2NH-*t*-BOC), 2.90 (m, 2H, lysine CH_2NH (peptide)), 3.78 (m, 2H, CH-NH-*t*-BOC), 6.6 (d, 1H, $J = 8$ Hz, *t*-BOC-protected NH-CH), 6.73 (t, 1H, $J = 6$ Hz, *t*-BOC protected NH-CH_2), 7.00 (d, 1H, $J = 8$ Hz, *t*-BOC-protected NH attached to α-CH), 7.75 (t, 1H, $J = 6$ Hz, peptide bond N*H* attached to CH_2) and 12.15 (s, 1H, COOH). *m/z* 597.1729 $[M + Na]^+$. $C_{27}H_{50}O_9N_4$ requires 574.7110.

4 (1.99 g, 3.47 mmol) **(*n* = 2, R″ = H)** prepared above was dissolved under argon in 60 mL of THF (still-dried over lithium aluminum hydride) containing *N*-hydroxysuccinimide (0.40 g, 3.47 mmol) and DCC (0.72 g, 3.47 mmol). The mixture was stirred at room temperature for 24 h and then filtered through 1 cm Celite, and the residue was washed with THF. The filtrate was evaporated to yield **4 (*n* = 2, R″ = N-succinimido**, 2.40 g) as a white solid foam (>100% yield). ^{1}H NMR (d_6-DMSO, 400 MHz) 1.30 (s, 27H, *t*-BOC groups), 1.41−1.60 (m, 12H, lysine CH_2, 2.75 (s, 4H, *N*-hydroxysuccinimide CH_2), 2.85 (m, 2H, lysine CH_2NH-*t*-BOC), 2.91 (m, 2H, lysine CH_2NH (peptide)), 3.78 (m, 1H, CH-NH-*t*-BOC), 4.24 (m, 1H, CH-NH-*t*-BOC), 6.81 (m, 2H, *t*-BOC-protected NH-CH and *t*-BOC-protected NH-CH_2), 7.52 (d, 1H, $J = 8$ Hz, *t*-BOC-protected NH attached to α-CH), 7.75 (t, 1H, $J = 6$ Hz, peptide bond NH attached to CH_2). *m/z* 694.8951 $[M + Na]^+$. $C_{31}H_{53}O_{11}N_5$ requires 671.7837.

4 (2.40 g, max. 3.47 mmol) **(*n* = 2, R″ = N-succinimido)** prepared above was dissolved in 17 mL of THF, and a solution of 0.94 g (3.82 m mol) *N*-α-*t*-BOC-L-lysine and 0.53 g (3.82 mmol) K_2CO_3 in 17 mL of H_2O was added immediately. The mixture was stirred at room temperature for 72 h, and then most of the THF was removed by evaporation and the remaining slurry acidified to pH 2 with 1 M HCl. This mixture was then extracted with CH_2Cl_2 (2 × 50 mL), and the combined organic layers were dried over Na_2SO_4 and evaporated to yield **4 (*n* = 3, R″ = H)** as a white solid foam (2.74 g, 98.56%).^{1}H NMR (d_6-DMSO, 400 MHz) 1.25 (s, 36H, *t*-BOC group), 1.42−1.62 (m, 18H, lysine CH_2), 2.85 (m, 2H, lysine CH_2NH-*t*-BOC), 2.91 (m, 4H, lysine CH_2NH (peptide)), 3.79 (m, 3H, CH-NH-*t*-BOC), 6.61 (d, 2H, $J = 8$ Hz, *t*-BOC-protected NH-CH), 6.72 (t, 1H, $J = 6$ Hz, *t*-BOC-protected NH-CH_2), 7.02 (d, 1H, $J = 8$ Hz, *t*-BOC-protected NH-CH), 7.75 (t, 2H, $J = 6$ Hz, peptide bond NH attached to CH_2) and 12.15 (s, 1H, carboxyl group). *m/z* 826.4496 $[M + Na]^+$. $C_{38}H_{70}O_{12}N_6$ requires 803.0010.

Final Coupling Reaction. Activation of the Peptide Fragment. 4 (2.74 g, 3.42 mmol) **(*n* = 3, R″ = H)**, prepared above, was dissolved under argon in 57 mL of THF (still-dried over lithium aluminum hydride) containing 0.39 g (3.42 mmol) of *N*-hydroxysuccinimide and 0.71 g (3.42 m mol) of DCC. The mixture was stirred at room temperature for 24 h and then filtered through 1 cm Celite, and the residue was washed with THF. The filtrate was evaporated to yield 3.11 g (>100%) of **4 (*n* = 3, R″ = N-succinimido)** as a white solid foam. ^{1}H NMR (d_6-DMSO, 400 MHz) 1.30 (s, 36H, *t*-BOC), 1.41−1.63 (m, 18H, lysine CH_2), 2.78 (s, 4H, *N*-hydroxysuccinimide CH_2), 2.90 (m, 2H, lysine CH_2NH-*t*-BOC), 3.01 (m, 4H, lysine CH_2NH (peptide)), 3.79 (m, 2H, CH-NH-*t*-BOC), 4.27 (m, 1H, CH-NH-*t*-BOC), 6.6 (d, 2H, $J = 8$ Hz, *t*-BOC-protected NH-CH), 6.70 (t, 1H, $J = 6$ Hz, *t*-BOC-protected NH-CH_2), 7.55 (d, 1H, $J = 8$ Hz, *t*-BOC-protected NH-CH), 7.71 (t, 2H, $J = 6$ Hz, peptide bond NH attached to CH_2). *m/z* 922.4095 $[M + Na]^+$. $C_{42}H_{73}O_{14}N_7$ requires 899.5215.

Deprotection of the central unit. 3.1 g (2.72 mmol) of compound **3 (R = oleyl, R′ = COOBu-t)** was dissolved in 35 mL of CH_2Cl_2 and cooled in ice while 35 mL of HCl was added. The solution was stirred until the reaction mixture reached room temperature. The CH_2Cl_2 was then removed by evaporation, ethanol was added to form an azeotrope, and the remaining solvent was removed slowly under vacuum. The yellow solid was washed with several portions of diethyl ether to give 2.6 g (92.9%) of **3 (R = oleyl, R′ = H)**, mp 59−61 °C (dec). ^{1}H NMR (CD_3OD, 400 MHz) 0.90 (t, 6H, $J = 6$ Hz, terminal CH_3 of oleyl chains), 1.21 (s, 44H, CH_2 of oleyl chains), 1.95 (s, 8H, CH_2CH═CH), 2.71 (s, 4H, linker CH_2), 3.00 (m, 8H, cysteine CH_2 and tail NCH_2 groups), 3.61 (m, 4H, serine CH_2), 4.15 (m, CH-NH-*t*-BOC and serine CH-NH) and 5.21 (s, 4H, alkene CH). In d_6-DMSO additional (NH) peaks were observed at 7.65 (t, 2H, $J = 6$ Hz, oleylamine NH on tail), 7.64 (m, 4H, cysteine CH−NH_2) and 8.75 (d, 2H, $J = 8$ Hz, serine NH). *m/z* 941.8432 $[M]^+$: $C_{50}H_{96}O_6N_6S_2$ requires 941.6832.

Final Coupling. Compound **3 (R = oleyl, R′ = H)** 425 mg (0.42 mmol) prepared above was dissolved in 13 mL of H_2O, and 37 mg (0.93 mmol) of NaOH was added, followed by enough THF (25 mL) to give a clear solution. A solution of activated protected peptide **4 (*n* = 3, R″ = N-succinimido**, 875 mg, ≥0.97 mmol) dissolved in 13 mL of THF was added immediately, and the reaction mixture was stirred at room temperature for 48 h. Most of the THF was removed by evaporation, and another 13 mL of H_2O was added; stirring continued for 1 h. The solid precipitate was collected by filtration, rinsed with water, and dried to yield the BOC-protected intermediate (1.03 g, 98%). ^{1}H NMR (CD_3OD, 400 MHz) 0.90 (t, 6H, $J = 6$ Hz, terminal CH_3 on oleyl chains), 1.30 (s, 44H, oleyl chain CH_2), 1.43 (s, 72H, *t*-BOC−CH_3),1.49−1.63 (m, 16H, lysine and oleyl CH_2), 1.65−1.74 and 1.8−1.87 (m, 24H, lysine CH_2), 2.00 (m, 8H, CH_2−CH═CH), 2.95 and 3.1−3.25 (m, 24H, CH_2 groups from cysteine fragment, hydrocarbon chain, lysine and linker CH_2), 3.79−3.89 (s, 4H, serine CH_2), 3.91−3.99 (s, 6H, lysine α-CH-NH_2), 4.39−4.44 (s, 4H, serine and cysteine α-CH-NH), 5.33 (*cis*) and 5.38 (*trans*) (t, 4H, $J = 6$ Hz, alkene CH).

Final Deprotection. The solid BOC-protected intermediate (1.03 g, 0.49 mmol) prepared above was dissolved in 20 mL of CH_3OH, and the solution was cooled in ice while 20 mL of HCl was added. This mixture was stirred (ca.1 h) until it reached room temperature. Ethanol was added to form an azeotrope, and the solvent was removed slowly under reduced pressure. The solid residue was redissolved in CH_3OH; addition of diethyl ether precipitated **GS11** (0.56 g) as an off-white powder (68%). ^{1}H NMR (CD_3OD, 400 MHz) 0.87 (t, 6H, $J = 6$ Hz, terminal CH_3 on oleyl chains), 1.30 (s, 44H, oleyl chain CH_2), 1.4−1.55 (m, 16H, lysine and oleyl CH_2), 1.56−1.67 and 1.7−1.8 (m, 24H, lysine CH_2), 1.93−1.99 (m, 8H, $C\underline{H}_2$−CH═CH), 2.91−3.00 and 3.15−3.28 (m, 24H, CH_2 groups of cysteine fragment, hydrocarbon chain, lysine, and linker group), 3.72−3.86 (m, 4H, serine CH_2), 3.89−4.09 (m, 6H, lysine α-C$\underline{H}$-NH_2), 4.4−4.55 (m, 4H, serine and cysteine α-C$\underline{H}$-NH), 5.33 (*cis*) and 5.38 (*trans*) (t, 4H, $J = 6$ Hz, alkene CH). *m*/*z* 1710.2579 $[M]^+$. $C_{86}H_{168}O_{12}N_{18}S_2$ requires 1710.5192.

Preparation of DOPE/GS11 Lipid Mixtures. DOPE and **GS11** were prepared individually as ethanol stock solutions. The stock solutions were warmed briefly to approximately 50 °C, and aliquots of each were taken to prepare 5 mg (total lipid) quantities of DOPE/**GS11** mixtures at 50/50, 60/40, and 70/30 molar ratios. The ethanol was evaporated under a stream of nitrogen, leaving a thin lipid film from which trace ethanol was removed under high vacuum overnight.

The DOPE/**GS11** dried films were resuspended in Nanopure-filtered water using one cycle of vortex mixing, bath sonication, and vortex mixing. Samples were stored at approximately 4 °C.

TEM Negative Staining. Specimens (2 μL) were adsorbed on carbon/Formvar-coated grids (Agar Scientific) and stained with 2.5% w/v ammonium molybdate, containing 0.5% w/v trehalose, pH 7.0, for 15 s and blotted dry. Samples were examined in a Hitachi H7100 microscope fitted with a Gatan MSC791 digital camera controlled by Gatan Digital micrograph V.2.5 software (Gatan). The microscope and camera system were calibrated using a MAG*I*CAL Si Ge lattice (Norrox Scientific).

Cell Culture and DNA Transfection. Transfection studies were performed using the adherent Chinese hamster ovary cell line, CHO-DG44, and mouse muscle cell line, C2C12. Complete medium for CHO-DG44 consisted of MEM alpha medium supplemented with 10% v/v fetal bovine serum and 1× L-glutamine. C2C12 cells were grown in DMEM medium supplemented with 10% v/v fetal bovine serum. All media and supplements were obtained from Life Technologies.

In Vitro Gene Transfection. Cells were seeded into Biocat poly-D-lysine 96 well/plates (Becton Dickinson) 16−18 h prior to transfection at an approximate density of $2-4 \times 10^4$ cells per well. For transfection, 0.5 μg of the luciferase reporter gene plasmid, pGL3-Control Vector (Promega), was incubated with various concentrations of the gemini surfactants and complexing agents in a final volume of 100 μL. After 30 min incubation at rt, 0.4 mL of Opti-MEM medium (Life Technologies) was added to the transfection mixture and the solution placed on the cells (100 μL/well). Following 3 h or overnight incubation at 37 °C, the transfection solution was replaced with complete medium, and the cells were incubated at 37 °C. Control transfections were carried out using lipofectAMINE 2000 (Life Technologies) according to the manufacturer's guidelines. Reporter gene assays were performed according to the manufacturer's guidelines (Roche Diagnostics) approximately 48 h post-transfection. Luminescence was measured in a Packard TopCount NXT Microplate Scintillation and Luminescence Counter.

Acknowledgment. This work is a contribution from the European Network on Gemini Surfactants, supported by the TMR Program of the European Commission.

JA005681C