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Efficient Cell Transfection with Melamine-Based Gemini Surfactants

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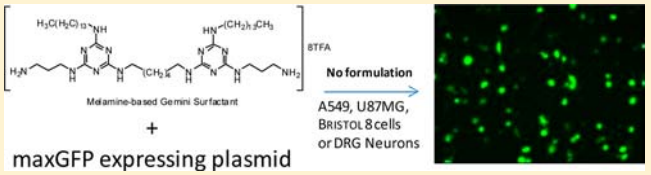
Supporting Information

ABSTRACT: Gemini surfactants consisting of two melamine scaffolds connected by a *n*-hexyl linker and functionalized with a 1-propylammonium polar head and a lipophilic chain having variable carbon length (from C8 to C16) were synthesized. These were then used successfully for the transfection of A549, U87 MG, and Bristol 8 cell lines with maxGFP expressing plasmid. The transfection protocol was optimized appropriately (confluence, reagent/pcDNA ratio, compaction time, and transfection time) for each cell line. Under optimized conditions, the C12 and C14 melamine gemini surfactants showed little toxicity and remarkable transfection efficiency, superior to the gold-standard Lipofectamine 2000. These reagents were also able to efficiently transfect primary DRG neurons, which are notoriously difficult to transfect. The presence of serum completely inhibited the transfection capacity of these reagents. Owing to their ready availability, straightforward synthesis, high chemical stability (even in solution), ease of use (no formulation is required), improved transfection ability, and low toxicity, melamine-based gemini surfactants are very promising reagents for cellular DNA transfection.



INTRODUCTION

Gene transfection is a powerful tool to study protein expression and gene function in cells. The development of efficient and reliable gene delivery systems remains a very active area of research. The currently available systems are affected by a number of drawbacks, most notably the prohibitive costs of most transfectants and the need to rely on complex formulations, which affect the long-term reagents' stability and require careful storage.[1]

As a result of size and charge, and several cellular barriers, the spontaneous entry of naked DNA and its subsequent expression by the replicative machinery of the cell is a very inefficient process. For this reason, the success of gene transfection is largely dependent on the development of a suitable carrier molecule ("vector" or "vehicle") that can deliver DNA selectively and efficiently to target cells, leading to the expression of the therapeutic gene.

Over the years, a range of techniques and vehicles for the transfer of nucleic acids into cultured eukaryotic cells (transfection) have been developed. Early transfection methods include calcium phosphate coprecipitation[2] and physical methods, such as direct microinjection of nucleic acids into cells or nuclei[3] and electroporation,[4] where cell membranes become permeable to DNA by exposure to electrical fields.

More recent approaches involve the use of a carrier molecule to deliver genetic material to the cells. Vehicles for gene delivery are usually divided into two major groups: viral and nonviral.

Although viral vectors (mainly recombinant retrovirus and adenovirus) have been found to be effective in gene transfer, they have several intrinsic drawbacks. For adenoviral vectors, packaging capacity is low, and production is labor-intensive.[5] With retroviral vectors, there is the potential for activation of latent disease and, if there are replication-competent viruses present, activation of endogenous retrovirus and limited transgene expression.[6] Furthermore, viral capsids have the potential to generate a severe immune response *in vivo*, as has been demonstrated in a number of animal experiment and clinical trials.[7−10] As a result, nonviral gene delivery is currently the subject of increasing attention because of its relative safety and ease of use.

Among nonviral-based approaches, cationic lipid-mediated gene transfer or lipofection represents the most extensively investigated method.[11−15] Since first being used as novel transfection vectors in 1987 by Felgner et al.,[16] numerous cationic lipids (also called cytofectins or lipofection reagents) have been synthesized and used for delivery to cell culture, animals, and patients enrolled in phase I, II, and III of clinical trials (http://www.wiley.co.uk/genmed/clinical/).

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These reagents contain amphiphilic molecules with a hydrophobic hydrocarbon domain connected to a cationic polar headgroup via a linker structure. The positive charge on the headgroup facilitates spontaneous electrostatic attraction to the phosphate backbone of DNA, leading to compaction of DNA and the formation of cationic lipid/DNA complexes (lipoplexes). In addition, the positive charge facilitates binding of the resulting lipoplexes to the negatively charged components of the cell membrane prior to cellular uptake.[17−19]

Cationic lipids possess many features of an "ideal" gene delivery vector. Although the gene expression levels obtained using these systems are lower than with viral vectors, they present several advantages including safety, large-scale production, and capacity to deliver large gene fragments. Moreover, since they are easy to produce and to modulate chemically for improved transfection efficiency, research efforts in this particular area have increased dramatically in recent years.[18]

Since the initial description, many cationic lipids, generally bearing either a single tertiary or quaternary ammonium headgroup or containing protonatable polyamines linked to dialkyl or cholesterol anchors, have been designed for transfection of a variety of cell types in culture.[18−22]

These lipids are usually formulated as an aqueous liposome suspension containing two lipid species, a cationic amphiphile and a neutral phospholipid, typically dioleoyl phosphatidyl ethanolamine (DOPE). DOPE is often used in conjunction with cationic lipids to achieve high transfection efficiency because of its membrane destabilizing effects which promote endolysosomal escape.[23]

Cationic liposomes with the polycationic lipid 2,3-dioleyloxy-*N*-[2-spermine carboxamide] ethyl-*N*,*N*-dimethyl-1-propanammonium trifluoroacetate (DOSPA) and DOPE at 3:1 w/w are available commercially as Lipofectamine Reagent; Lipofectin Reagent is a 1:1 w/w liposome formulation of the cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium (DOTMA) and DOPE. Other important commercially available reagents for lipofection include 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP), *N*-[1-(2,3-dimyristyloxy)propyl]-*N*,*N*-dimethyl-*N*-(2-hydroxyethyl) ammonium bromide (DMRIE), 3*β*-[*N*-(*N*′,*N*′-dimethylaminoethane) carbamoyl]cholesterol (DC-Chol), and dioctadecyl amidoglyceryl spermine (DOGS).

In the panorama of new positively charged amphiphiles, gemini surfactants (GS),[24,25] featuring two hydrophobic tails and two polar head groups connected by a linker, seem promising compounds. They typically show greatly enhanced physical−chemical properties compared to their corresponding monomeric (single chain, single headgroup) counterparts.[24−26] This makes them of special interest for biomedical applications, such as gene delivery, where they are generally much more efficient transfection reagents than the corresponding monomeric structures.[26,27]

Although a large number of cationic lipids have been synthesized, most of the present liposomal formulations are not useful due to their poor performance, including cytotoxicity and low transfection efficiency,[28] especially compared to viral vectors. Furthermore, the prices associated with most commercially available transfection tools are very high. For these reasons, there is still a need to develop cationic lipid-based gene transfer systems with improved transfection properties and low cytotoxicity that are readily accessible, easy to use, cheap, and cost-effective.

In this study, we report new nonviral gene transfer vectors belonging to the GS class. Five novel triaminotriazine (melamine)-based GS, that differ only in the hydrophobic tail length, have been synthesized; and their ability to transfect plasmid DNA containing maxGFP reporter gene into adherent and suspension cell lines, as well as primary cells, has been examined.

In vitro transfection experiments demonstrated a remarkable correlation between the length of the lipophilic tail and the transfection efficiency, and a structure−activity relationship was identified.

Unlike many cationic lipids used in gene transfection studies, these new melamine-based GS are synthesized from relatively inexpensive starting materials, are easy to handle, and are readily soluble at room temperature in aqueous medium. Moreover, some of these compounds are very effective in cell transfection using a simple experimental protocol, in which no formulation or liposome preparation is required. Last but not least, these reagents are stable indefinitely in aqueous solution at room temperature (they can be stored for months on the bench, without any apparent deterioration or loss of performance). This makes these molecules efficient, cost-effective, and therefore very attractive and competitive reagents for gene delivery applications.

EXPERIMENTAL SECTION

Materials. pMaxGFP vector (3.4 kb), encoding the green fluorescent protein from *Potellina sp.*, was purchased from Lonza. Lipofectamine 2000 was obtained from Invitrogen Life Technologies. All chemicals were of reagent-grade or higher quality and were purchased from Sigma unless otherwise specified.

Methods. *Chemistry.* Experimental procedures for the synthesis of gemini surfactants **GS C8−C16** and spectroscopic/analytical characterization of all the compounds described herein are provided in the Supporting Information section.

Cell Culture. Transfection studies were performed using two adherent cell lines, A549 (human lung adenocarcinoma epithelial) and U-87 MG (human glioblastoma-astrocytoma, epithelial-like) cell lines, and a suspension cell line, Bristol 8 (normal human HLA-A2 positive B lymphoblastoid) cell line. These cell lines were maintained in culture according to ECACC guidelines.

Primary dorsal root ganglion (DRG) neurons were also used for *in vitro* transfection experiments and were prepared as previously described (Murray et al., 2009).

In preparation for transfection experiments of adherent cell lines, cells were seeded at a density of 7.5×10^4 cells/cm^2 in 35 mm culture dishes the day prior to transfection. The cells were incubated for 24 h at 37 °C to reach ∼70% confluence on the day of transfection.

For the Bristol 8 suspension cell line, on the day of transfection the cell pellet was suspended in serum-free medium and 500 μL of cell suspension was seeded at a density of 7.5×10^4 cells/cm^2 in 35 mm culture dishes.

For the primary DRG neurons, cells were removed from the animal and dissociated into culture dishes coated with polylysine (1 μg/mL) and laminin (10 μg/mL) to promote adherence to the culture surface.

In Vitro Transfection. Transfection Protocol for Adherent Cells. The A549 and U-87 MG transfection protocol using **GS**

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Table 1. A549 Cells and GS C8-C16[a]

parameters optimized	GS C8	GS C10	GS C12	GS C14	GS C16
seeding density	7.5×10^4 cells/cm^2	7.5×10^4 cells/cm^2	7.5×10^4 cells/cm^2	7.5×10^4 cells/cm^2	7.5×10^4 cells/cm^2
concentration of GS used in lipoplex preparation	---	6 μM	6 μM	6 μM	6 μM
GS-pcDNA complex formation time (compaction time)	---	60 min	60 min	60 min	30 min
exposure time	---	4 h	4 h	4 h	4 h

[a]Optimal transfection conditions. Transfection efficiency for each **GS** was evaluated by visualization of maxGFP positive cells under fluorescent microscope and confirmed quantitatively using flow cytometry.

Table 2. U-87 MG Cells and GS C8-C16[a]

parameters optimized	GS C8	GS C10	GS C12	GS C14	GS C16
seeding density	7.5×10^4 cells/cm^2	7.5×10^4 cells/cm^2	7.5×10^4 cells/cm^2	7.5×10^4 cells/cm^2	7.5×10^4 cells/cm^2
concentration of GS used in lipoplex preparation	---	6 μM	6 μM	3 μM	6 μM
GS-pcDNA complex formation time (compaction time)	---	60 min	30 min	45 min	30 min
exposure time	---	4 h	4 h	4 h	4 h

[a]Optimal transfection conditions. Transfection efficiency for each **GS** was evaluated by visualization of maxGFP positive cells under fluorescent microscope and confirmed quantitatively using flow cytometry.

C8-C16 was developed and optimized throughout the passage of this work.

Briefly, **GS** 20× stock solutions were prepared by dissolving cationic lipids in distilled water. Preparations of **GS** molecules in distilled water are stable for at least 6 months at 4 °C.

GS-pmaxGFP lipoplexes were obtained by simple mixing 1 μg of pmaxGFP vector (diluted in 500 μL of serum free medium) with an equal volume of the cationic lipid solution in serum free medium. At this stage, pcDNA concentration was 1 μg/mL and **GS** lipid was at the desired concentration.

The **GS**-pcDNA mixture was incubated at room temperature for the indicated compaction time to allow the transfection complexes to form and these were added to dishes containing cells. Before adding lipoplexes, the cells were rinsed in serum free medium to remove traces of serum.

Following the indicated exposure time at 37 °C (typically 4 h), the transfection medium was replaced by fresh serum-containing culture medium. A similar protocol was used to assess the capacity of **GS C12** and **C14** to transfect primary DRG neurons. Control transfections were carried out using optimized Lipofectamine 2000, according to the manufacturer's protocols.

Transfection Protocol for Suspension Cells. Bristol 8 cells were transfected with **GS C12** or **C14** surfactants using the optimal lipofection conditions found for both A549 (Table 1) and U-87 MG (Table 2) cells. Briefly, **GS C12** or **GS C14** 20× stock solutions were prepared by dissolving cationic lipids in distilled water.

GS-pmaxGFP lipoplexes were prepared by simple mixing 1 μg of pmaxGFP vector (diluted in 250 μL of serum free medium) with an equal volume of the cationic lipid solution in serum free medium. At this stage, pcDNA concentration was 2 μg/mL and **GS** lipid was at 2× concentration.

The **GS**-pcDNA mixture was incubated at room temperature for the indicated compaction time to allow the transfection complex to form, and these were added to dishes containing 500 μL of cells. At this stage, plasmid DNA concentration was 1 μg/mL and **GS** lipid was at the desired concentration. Following the indicated exposure time at 37 °C (typically 4 h), 2 mL of serum-containing culture medium was added to the dish.

GFP Detection. Cellular expression of maxGFP in A549, U-87 MG, Bristol 8, and DRG cells was evaluated 24 h post-transfection using fluorescence microscopy. The cells were imaged and photographed using Nikon Eclipse E400 microscope equipped with Q-Imaging QICAM Fast 1394 Color 12 bit digital camera. Images were captured using *ImageJ* software.

FACS Analysis. A549 and U-87 MG cells were transfected using **GS C8-C16** surfactants, according to the optimized protocols, and using optimized Lipofectamine 2000 (positive control), according to the manufacturer's protocols. Non-transfected samples (negative control) were cultured at the same time and had the same period of time in serum-free medium as transfected samples.

One day post-transfection, the cells were dissociated from their culture dishes with trypsin-EDTA solution, and transfection efficiency, evaluating the percentage of cells expressing maxGFP in each sample, was measured by means of fluorescence-activated cell sorting (FACS) analysis.

Gates and instrument settings were set according to forward and scatter characteristics, and a total of 10 000 events were collected for each sample. Data, based on triplicate measurements, were expressed as means as mean ± SEM.

RESULTS AND DISCUSSION

The novel synthetic transfection reagents **GS C8**, **C10**, **C12**, **C14**, and **C16** are melamine-based gemini surfactants with an overall net positive charge determined by the presence of 8 equiv of trifluoroacetic acid. Their structures are shown in Figure 1.

These molecules are composed of two units, each consisting of a triaminotriazine (melamine) scaffold supporting three different side chains: (1) a linear lipophilic tail with increasing

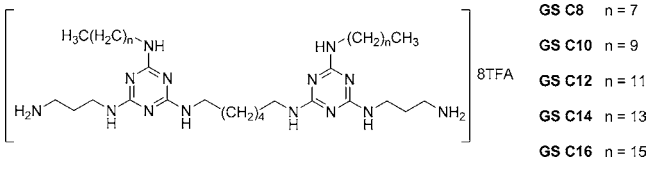

Figure 1. Chemical structure of the five cationic amphiphiles used in this study.

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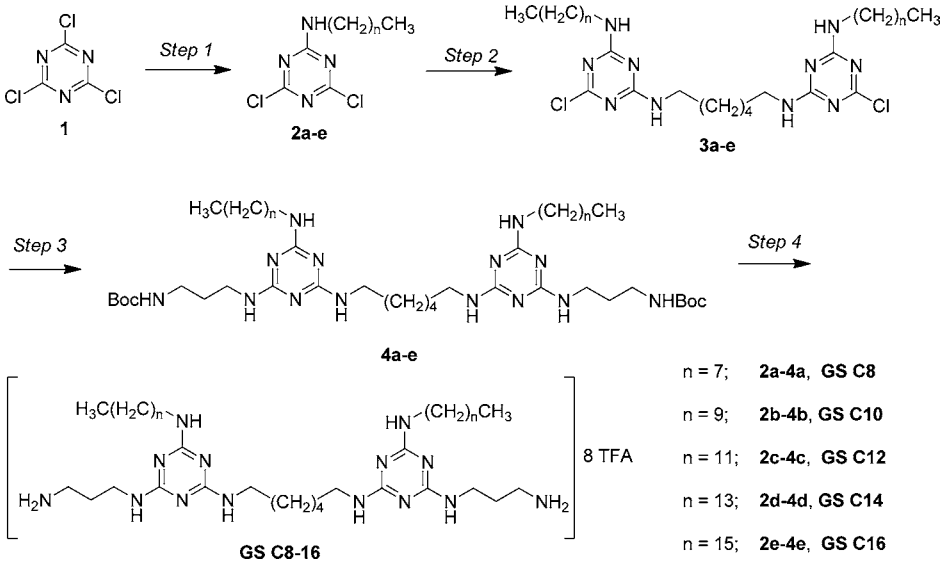
Scheme 1. Synthesis Scheme for GS C8-C16[a]



[a]Reagents and conditions: Step 1: $CH_3(CH_2)_nNH_2$, DIPEA, acetonitrile, room temperature (RT), 2 h, 45−60%; Step 2: $NH_2(CH_2)_6NH_2$, DIPEA, acetonitrile, RT, 3 h, 32−35%; Step 3: $NH_2(CH_2)_3NHBoc$, benzene (anhydrous), 125 °C, sealed tubes, 24 h, 52−83%; Step 4: TFA, DCM (anhydrous), RT, 3 h, 90−95%. Key: DIPEA = *N*,*N*-diisopropylethylamine, TFA = trifluoroacetic acid, DCM = dichloromethane.

alkyl chain length of 8 (**GS C8**), 10 (**GS C10**), 12 (**GS C12**), 14 (**GS C14**), and 16 (**GS C16**) carbon atoms, (2) a 3-propylammonium group which acts as cationic polar head, and (3) a *n*-hexyl spacer connected to the melamine headgroup of the other unit (Figure 1).

These amphiphilic **GS** molecules are highly stable, with a considerably improved chemical stability compared to the triazine-based redox-sensitive agents previously described in the literature.[29−32] The presence of a saturated alkyl spacer makes **GS C8-C18** compounds resistant to air oxidation and to the intracellular reducing environment with respect to the cationic lipids based on the thiol/disulfide (-SH/-SS-) equilibrium.[29−32]

The preparation of the new cationic lipids is relatively quick, produces gram amounts of the target **GS** molecules, and uses relatively inexpensive materials, which certainly increases the value of these melamine-based **GS** as transfection reagents. The synthesis of **GS 8−16** proceeded via a simple multistep strategy making use of cyanuric chloride **1** as starting material, as shown in Scheme 1. The activated triazine ring of **1** allows sequential dechloroamination substitution reactions with nitrogen nucleophiles. The hydrophobic hydrocarbon moiety was introduced first on the triazine core (Scheme 1). The single tail dichlorotriazine **2** was then reacted with 1,6-diaminohexane to provide the dimeric compounds **3a−e** featuring two symmetric single tail-monochlorotriazine units connected via the *n*-hexyl spacer (step 2, Scheme 1). The subsequent dechloroamination reaction (step 3), followed by cleavage of the *N*-protecting group (step 4) produced the amphiphilic **GS 8−16** as trifluoroacetic salts, which were purified by flash chromatography on silica gel (Scheme 1).

The number of equivalents of CF_3CO_2H (TFA) incorporated by each **GS** was assessed by ^{19}F NMR titration experiments, using trifluoromethylbenzene as an internal probe. Each **GS** was found to incorporate 8 equiv of TFA; therefore, the basic primary amine functions and some nitrogen atoms of each melamine unit are actually protonated in these molecules. The pK_b of melamine is 9.00 vs a pK_b = 3.29 of a basic dialkylamine such as dimethylamine.[33a] Recently, melamine ($C_3H_6N_6$) has been shown to crystallize with two molecules of trifluoroacetic acid, each protonating a ring nitrogen.[33b] It is therefore likely that the samples of **GS 8−16** used in this study contained 2 to 4 equiv of TFA in excess, as a result of the specific workup, isolation, and purification procedures used herein (see Supporting Information for details). However, no significant cell toxicity that could be ascribed to TFA was ever observed at the concentrations of **GS 8−16** used in the transfection experiments. It is worth noting that the serum free medium used in the transfection experiments contained a bicarbonate buffering system that was supplemented by storing cells in an incubator supplying 5% CO_2. Additionally, the medium contained the pH indicator phenol red. Throughout all of the experiments described, no change in the color of the indicator was observed, and therefore, no significant changes in the pH of the cell cultures were evident.

Optimization of the Transfection Protocol for Each GS Using A549 and U87mg Cells. The cationic compounds used in this study maintain common features in the head groups and spacer, but vary the length of the lipophilic tails (Figure 1). It is well-known that the hydrophobic moiety of amphiphiles is a key determinant of lipoplex structure and lipofection activity. Therefore, we looked for a structure−activity relationship to determine whether differences in the alkyl side chain length resulted in altered biological activity.

The gene transfer properties of the surfactants **GS C8-C16** were studied using a maxGFP expressing plasmid in two different adherent cell lines, A549 (human lung adenocarcinoma epithelial) and U-87 MG (human glioblastoma-astrocytoma, epithelial-like) cells. Since **GS C8-C16** are novel compounds, before making a comparison across all **GS**, the optimal conditions for transfection of both cell lines were determined. A successful transfection requires several variables to be optimized. In fact, a number of parameters can greatly influence transfection efficiency, such as: (a) degree of cell confluence, (b) ratio of transfection reagent to DNA, (c) length of incubation time between DNA and cationic lipid

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(compaction time), (d) length of the time cells are exposed to DNA-cationic lipid complex (exposure time), and (e) presence or absence of serum. The optimization results of each **GS** and A549 and U-87 MG cells are summarized in Table 1 and 2, respectively, and discussed below.

Seeding Density (Confluence). Usually it is necessary to transfect cells at 60−80% confluence. Too few cells will cause the culture to grow poorly as a result of lack of cell-to-cell contact. Too many cells results in contact inhibition, making cells resistant to uptake of foreign DNA. Ideally, cells should be in an exponential phase of growth. For the *in vitro* experiments, A549 and U-87 MG cells were seeded on 35 mm dishes the day prior to transfection at 7.5×10^4 cells/cm^2 density and allowed to adhere overnight (Tables 1−2). For both cell lines, this seeding density was found to produce ∼70% confluent cells on the day of transfection.

Concentration of Cationic Transfection Reagent and Compaction Time. The key step in the lipofection process is the formation of the complex between cationic lipids and DNA (lipoplex) and the consequent compaction of the extended, high molecular-mass, negatively charged DNA into a dense, positively charged particle small enough to be taken up by the cell.[26] Electrostatic interactions between the positively charged cationic lipid head-groups and the negative phosphate DNA backbones are the main driving forces for lipoplex formation and DNA compaction.[19] Hydrophobic interactions between the apolar hydrocarbon tails enhance compaction further.[34] This step determines the characteristics and thus the transfection potential of lipoplexes. Consequently, both the cationic transfection reagent-to-DNA ratio and the compaction time are factors that should be considered carefully in any protocol of lipoplex preparation.

Concentration of GS Reagent (Ratio of Cationic Transfection Reagent to pcDNA). In the lipoplex, the amount of positive charge contributed by cationic lipids should equal or exceed the amount of negative charge contributed by the phosphates on the DNA backbone, resulting in neutral or positively charged cationic lipid/nucleic acid complex. It is widely accepted that preparation of lipoplexes with an overall net positive charge confers a higher transfer efficiency, presumably because this allows a more efficient interaction of the complexes with the negatively charged cell surface.[16,35,36]

For each **GS** molecule, by varying the concentration of the gemini surfactant (3 μM, 6 μM, 12 μM, 25 μM) and keeping constant the concentration of pmaxGFP (1 μg/mL), different **GS**-pcDNA lipoplexes, at increasing charge ratio (CR±), were prepared. Transfection ability of each **GS**-pcDNA preparation was then evaluated qualitatively, by visualization of the cells under fluorescence microscope. No formulation or liposome preparation was required for our transfection protocol and all the **GS** were readily dissolved in distilled water. **GS**-pcDNA complexes were obtained by simple mixing of the **GS** solution (at the desired concentration) and the pcDNA solution in serum free medium. The **GS**-pcDNA mixture was incubated for a compaction time of 15 min at room temperature and then the cells were exposed to **GS**-pcDNA complexes for 4 h at 37 °C (see Experimental Section). By observation of maxGFP positive cells, it was found that **GS C8** based lipoplexes were not able to transfer DNA into A549 or U-87 MG cells, whereas transfection was observed with all the other gemini amphiphile-based lipoplexes (**GS C10**−**C16**).

A **GS C10** and **C16** concentration of 6 μM (lipoplexes at CR ± = 16), among the different concentrations tested, led to the maximum GFP expression in both cell lines (Tables 1−2). Despite the use of this optimal lipid concentration, the transfection efficiency of **GS C10** or **C16** based complexes remained modest (approximately 10%). A higher transfer activity was observed with **GS C12** and **C14**. For **GS C12** surfactant, 6 μM worked most effectively with U-87 MG cells (Figure 2) and with A549 cells (Tables 1−2). For the **GS C14** molecule, a concentration of 6 μM (CR± = 16) and 3 μM (CR ± = 8) led to an effective transfection with A549 and U-87 MG cell line, respectively (Tables 1−2). A visible decrease in the number of U-87 MG fluorescent cells was observed using **GS C14** concentrations lower than 3 μM, confirming 3 μM as the optimal concentration for this cell line. As shown in Figure 2 for **GS C12** and U-87 MG cells, an increase in lipid concentration improved transfection. However, high levels of **GS C12** (above the optimal concentration) resulted in decreased fluorescence (Figure 2C). The decline in transfection activity at high concentrations of **GS C12** correlated with a

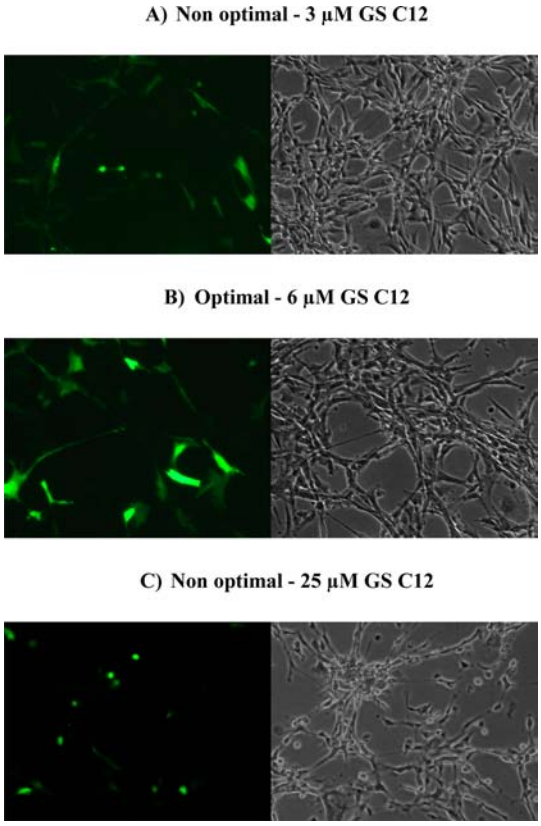

Figure 2. U-87 MG cells transfected with different concentrations of **GS C12** amphiphile. Images collected 24 h post-transfection by fluorescence microscopy (10× magnification); images are representative of at least three other independent experimental repeats. *In vitro* transfection experiments were performed using lipoplexes prepared with increasing **GS C12** concentration (3 μM, 6 μM, or 25 μM) and a pmaxGFP concentration of 1 μg/mL. The **GS C12**-pcDNA mixture was incubated for 15 min (compaction time) at room temperature. The cells were then exposed to **GS C12**-pcDNA lipoplexes for 4 h. (A) **GS C12** concentration of 3 μM led to a nonoptimal GFP expression in U-87 MG cells. (B) A very effective U-87 MG transfection and a low toxicity was obtained using **GS C12** concentration of 6 μM. (C) **GS C12** concentration of 25 μM resulted in a decrease in the number of fluorescent cells and an increase in cell death.

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visible reduction in the number of viable cells per dish (Figure 2C). This increase in cell death may be due to the incorporation of the cationic chemical reagents into cellular membranes. At high concentrations, the cationic lipid content of cellular membrane may be sufficient to significantly alter the net charge of the membranes and adversely affect the activity of a number of enzymes, membrane receptors, and ion channels. Alternatively, the cationic lipids could interfere with the attachment of the cells to the extracellular matrix, leading to the detachment of cells from the dish.[37,38] It should be noted though that, throughout, toxicity was only observed at high surfactant concentrations (>12 μM), and the effective transfection concentrations were well tolerated as determined by MTT assay. At the concentrations of **GS C12** and **GS C14** used in optimized transfections, the cell populations remained at an equivalent (or higher) level of viability than those cell populations transfected with Lipofectamine 2000 (data not shown).

GS-pcDNA Complex Formation Time (Compaction Time). A key aspect of the transfection protocol optimization is the time required for complex formation between the pcDNA and **GS** lipids (compaction time). The compaction of DNA by cationic surfactants presumably involves an initial interaction of a small cluster of surfactant molecules with DNA. Cationic amphiphiles can compact and stabilize polyanionic nucleic acids by a combination of attractive electrostatic interactions and hydrophobic interactions between the apolar hydrocarbon tails.[34] In order to evaluate the best pcDNA-**GS** incubation time, the appropriate amount of pmaxGFP vector (diluted in serum free medium) was mixed with diluted **GS** used at optimal concentration established by the previous set of transfection experiments (**GS C14** concentration was 3 μM for U-87 MG cells; all the other **GS** concentrations were 6 μM for both cell lines). The **GS**-pcDNA mixture was incubated for different times (0 min, 15 min, 30 min, 45 min, 60 min, 90 min) at room temperature and then added to dishes containing cells.

The results of the *in vitro* transfection experiments, analyzed by fluorescence microscopy, show for each **GS** molecule an increase of maxGFP expression levels with compaction time, until an optimum is reached (Figure 3A,B). Exceeding the optimal time decreased maxGFP positive cell numbers (Figure 3C). This decrease in fluorescence may be related to a loss of energy or degradation of the complexes with time. Therefore, if left too long the complexes became progressively less effective at transfecting cells.

For **GS C10** and **C16**, the optimal **GS**-pcDNA compaction time was observed to be cell-type independent. In particular, for **GS C10**, fluorescence microscopy revealed a pcDNA-**GS** incubation time of 60 min as optimal for successful transfection of both cell lines (Tables 1−2). For **GS C16**-pcDNA lipoplexes, an optimal transfection of both cell lines was obtained after a 30 min compaction time (Tables 1−2). For **GS C12** and **C14**, the optimal time of compaction changed depending on the cell line being studied. A **GS C12**-pcDNA compaction time of 60 and 30 min corresponded to the most effective transfection of A549 and U-87 MG cells, respectively (Tables 1−2). By comparing the images of the A549 transfection experiments (Figure 3), it is clear that 60 min is the best **GS C14**-pcDNA compaction time. When U-87 MG cells were used, an incubation time of 45 min led to a very effective transfection (Table 2).

The results obtained with **GS C12** or **GS C14** amphiphiles show a clear cell-type dependence for compaction time.

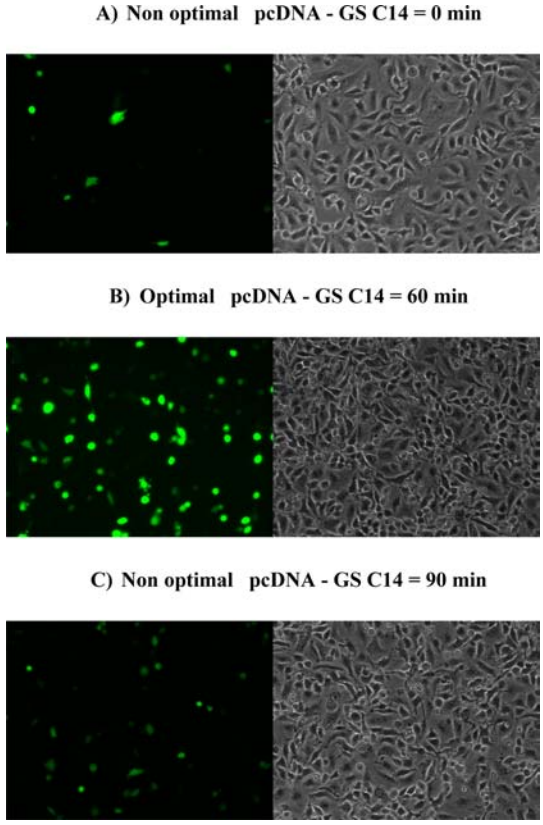

Figure 3. A549 cells and **GS C14**-pcDNA compaction time. Images collected 24 h post-transfection by fluorescence microscopy (10× magnification); images are representative of at least three other independent experimental repeats. The **GS C14**-pcDNA mixture, prepared using a surfactant concentration of 6 μM and pmaxGFP concentration of 1 μg/mL, was incubated at room temperature for increasing compaction times (0, 60, or 90 min). The cells were then exposed to **GS C14**-pcDNA lipoplexes for 4 h. (A) A nonoptimal maxGFP expression in A549 cells was achieved with a **GS C14**-pcDNA compaction time of 0 min. (B) A very effective transfection was obtained using a **GS C14**-pcDNA compaction time of 60 min. (C) A **GS C14**-pcDNA compaction time of 90 min led to a decrease in A549 fluorescent cells.

Different cell lines, having different membrane properties, composition, and charge respond differently to **GS**-pcDNA complexes. The compaction time will determine the nature and "maturity" of these complexes, and their most optimal form is slightly different between the two cell types.

Exposure Time (or Transfection Time). In the previous transfection experiments, both A549 and U-87 MG cells were incubated with **GS**-pcDNA mixture for 4 h. The growth medium containing lipoplexes was then replaced with fresh medium. To help minimize toxic effects of the **GS**-pcDNA complexes and find the best exposure time, the initial incubation time was varied between 30 min and 24 h (30 min, 1 h, 2 h, 4 h, 6 h, 24 h). As shown in Figure 4 for **GS C12** based lipoplexes and U-87 MG cells, replacement of the medium after 30 min of cell incubation with **GS**-pcDNA mixture led to few fluorescent cells per dish (Figure 4A). As the treatment time with **GS C12**-pcDNA complexes increased, higher transfection efficiency was observed. A 4 h transfection time was confirmed to be optimal for U-87 MG cells, achieving the best compromise between the number of cells transfected and alive at the end of the process (Figure 4B). Exposure times

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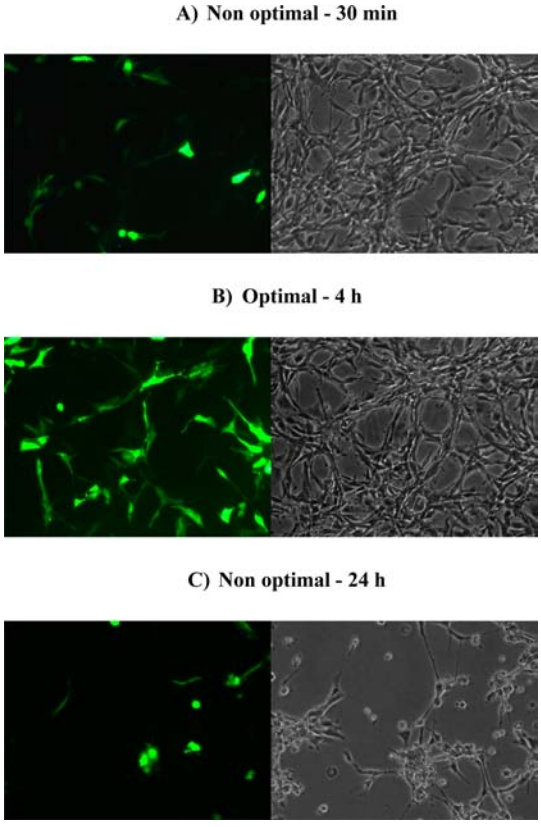

Figure 4. U-87 MG cells and different exposure times to **GS C12**-pcDNA lipoplexes. Images collected 24 h day post-transfection by fluorescence microscopy (10× magnification); images are representative of at least three other independent experimental repeats. The cells were incubated with **GS C12** based lipoplexes (**GS C12** concentration = 6 μM, pmaxGFP concentration = 1 μg/mL, compaction time **GS C12**-pmaxGFP = 30 min) for 30 min, 4 h, or 24 h. (A) A low U-87 MG transfection efficiency was obtained with 30 min transfection time. (B) U-87 MG cells incubated with **GS C12**-based lipoplexes for 4 h showed high levels of maxGFP expression and the morphology of the nontransfected cells. (C) Exposure time of 24 h resulted in an inhibition of cell growth.

longer than 4 h resulted in mild inhibition of cell growth (Figure 4C).

Removal of the medium containing the **GS C12**-pcDNA complexes after 4 h also resulted in the highest transfection efficiency for A549 cells (Table 1). Similar results were obtained from the *in vitro* transfection experiments with all the other **GS** based lipoplexes showing an optimal exposure time of 4 h for both cell lines (Tables 1−2). In each case, light microscopy analysis of transfected cells showed that the morphology of the nontransfected cells was preserved (data not shown).

Effect of Serum. Transfection protocols often require serum-free conditions for optimal performance because serum can interfere with many commercially available transfection reagents. It is thought that serum components, such as negatively charged proteins, may destabilize the positively charged lipid/DNA complex, leading to lipid vector disintegration, with DNA release and degradation.[39,40] The experiments performed with **GS** molecules, in both A549 and U-87 MG cell lines, showed that these cationic lipids also need serum free conditions for successful transfection. In fact, the presence of serum completely inhibited the ability of these compounds to transfect, even over extended periods of exposure (data not shown).

FACS Analysis. Once the optimal transfection conditions were found, a quantitative comparison of transfection properties between all the **GS** molecules was carried out. Furthermore, the capacities of compounds **GS C8-C16** to mediate the transfer of maxGFP gene reporter across A549 and U-87 MG cell membranes were compared to that of Lipofectamine 2000 (Life Technologies), regarded as the gold standard of commercially available transfection tools. Fluorescence-activated cell sorting (FACS) analysis was performed in order to get some quantitation for each of the optimized surfactant protocols, and consistently these confirmed the qualitative judgements made using the fluorescence microscope. Figures 5 and 6 show FACS analysis for selected experiments, specifically maxGFP expression levels, presented as the fluorescence intensity per cell number (fluorescent intensity, *x*-axis; cell number, *y*-axis) in A549 and U-87 MG cells using Lipofectamine 2000, **GS C10**, **C14**, and Lipofectamine 2000, **GS C12**, **C16** vectors, respectively. The results indicate that A549 transfection with **GS C14** (Figure 5D) yielded a higher fluorescence intensity per population than Lipofectamine 2000 (Figure 5B) (i.e., maxGFP expression in population **D** is shifted further to the right than population **B**). On the contrary, the level of maxGFP expression obtained in **GS C10** transfected cells (Figure 5C) was lower than the Lipofectamine 2000 transfected sample.

Compared to the positive control (Figure 6B), U-87 MG cells transfected with **GS C12** vector exhibited a significantly higher fluorescence intensity (Figure 6C), while lower levels of maxGFP expression were obtained with **GS C16** surfactant (Figure 6D). FACS analysis also showed that cell populations were consistent, healthy, and comparable across all experiments (data not shown).

FACS results are summarized and population fluorescence is quantified in Figures 7 and 8, where the percentage of the sample population that has shifted outside the M1 regions indicated in Figures 5 and 6 is plotted. This gives a measure of the comparative distribution and level of fluorescence within the population. In both cell types, the transfection efficiency of surfactants **GS C8-C16** exhibited a Gaussian bell-shaped distribution with the highest transfection performance corresponding to the **GS C12** amphiphile. Particularly remarkable is that its transfection properties are significantly better (3−4 times higher) than that of Lipofectamine 2000. As shown in Figures 7 and 8, 44% and 74% of cells were fluorescent using **GS C12**, whereas only 11% and 23% were fluorescent following Lipofectamine 2000 transfection in A549 and U-87 MG cells, respectively. The percentage of A549 reporter gene-expressing cells transfected with the compound **GS C14** was also higher than for the Lipofectamine 2000-transfected cells (Figure 7). **GS C14** was at least as effective as Lipofectamine 2000 in U-87 MG cells (Figure 8). The compounds with a lipophilic tail shorter than C12 (**GS C10**) or longer than C14 (**GS C16**) demonstrated a low transfection capacity. The **GS C8** molecule was completely ineffective.

These results allowed us to identify a consistent structure−activity relationship. In particular, the activity of the cationic surfactants is affected significantly by changes in the composition of the aliphatic tail. As the aliphatic chain length was increased from 12, 14, and 16 carbon atoms, the transfection capacity of the resulting compounds **GS C12**, **C14**, and **C16** progressively declined (Figures 7 and 8). Our

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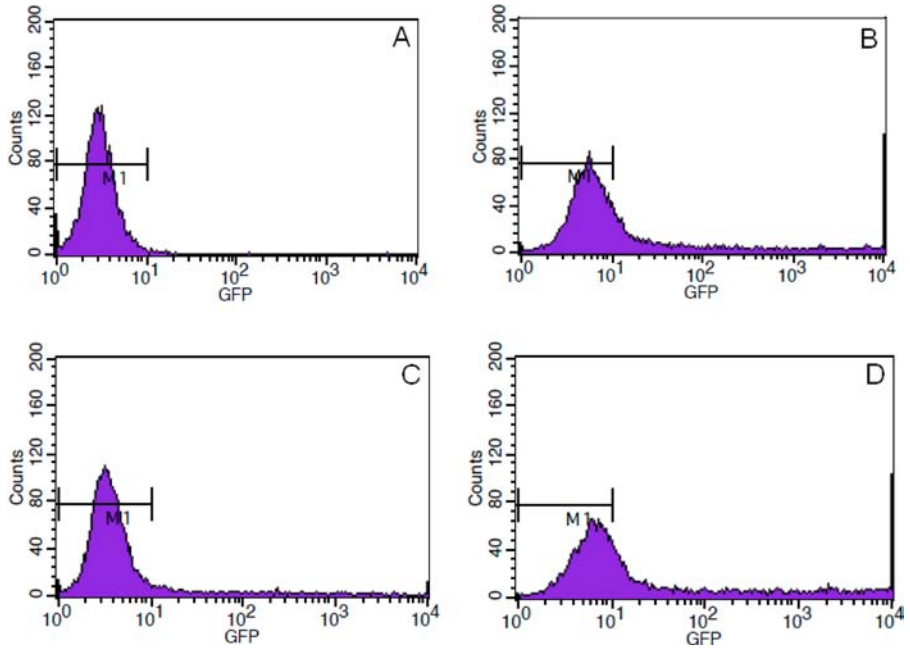

Figure 5. Expression of maxGFP in A549 cells evaluated by FACS analysis 24 h post-transfection. Optimized Lipofectamine 2000 was used as positive control according to the manufacturer's procedures (B). Transfections of A549 cells with **GS C10** (C) and **C14** (D) were performed in serum free conditions according to the optimized parameters reported in Table 1. (A) A549 cells in the absence of transfectants (negative control). (B) A549 cells transfected with Lipofectamine 2000 (positive control). (C) A549 cells transfected with **GS C10**. (D) A549 cells transfected with **GS C14**.

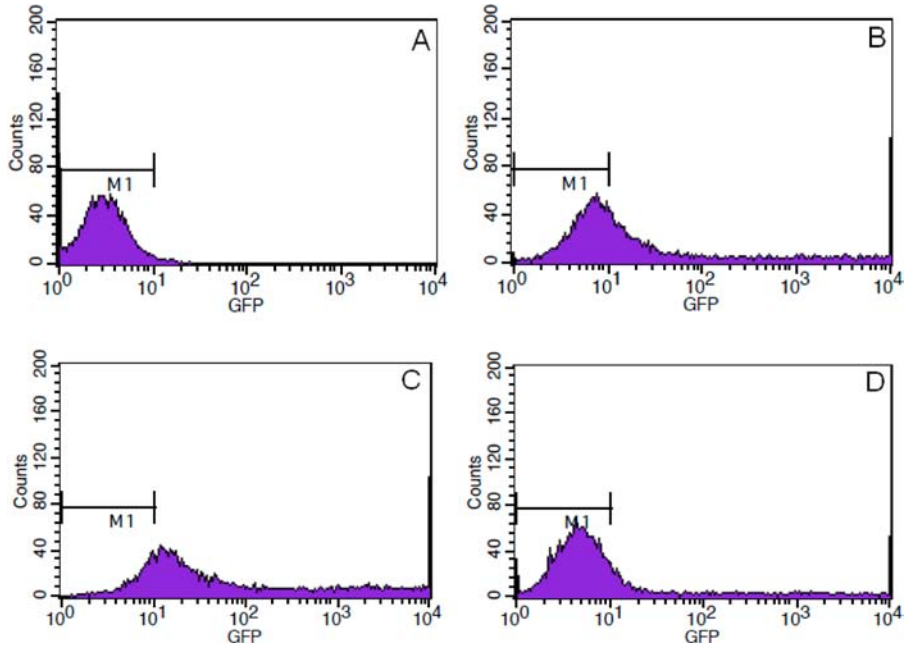

Figure 6. Expression of maxGFP in U-87 MG cells evaluated by FACS analysis 24 h post-transfection. Optimized Lipofectamine 2000 was used as positive control according to the manufacturer's guidelines (B). Transfections of U-87 MG cells with **GS C12** (C) and **C16** (D) were performed in serum free conditions according to the optimized parameters reported in Table 2. (A) U-87 MG cells in the absence of transfectants (negative control). (B) U-87 MG cells transfected with Lipofectamine 2000 (positive control). (C) U-87 MG cells transfected with **GS C12**. (D) U-87 MG cells transfected with **GS C16**.

results were consistent with previous studies.[41−43] Surfactant-mediated gene delivery requires a membrane destabilization of endosomal compartments, which allows translocation of the gene into the cytosol via an as yet poorly defined mechanism.[19,44] An exchange between cationic lipid molecules in the lipoplex and endosomal membrane has been suggested to weaken the integrity of the endosome leading to pcDNA cytoplasmic delivery.[19,44] The shorter alkyl tails, favoring a higher intermembrane transfer rate and faster rate of lipid mixing,[45,46] may enhance the endosome escape and thus the transfection capacity of **GS** molecules. The low transfection efficiency or the complete ineffectiveness of the compounds

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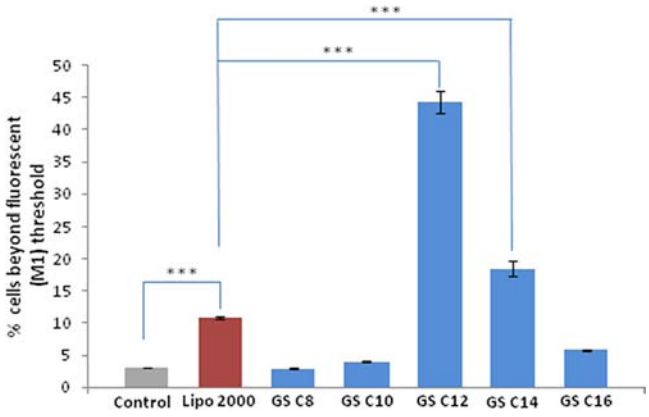

Figure 7. Transfection efficiency of compounds **GS C8-C16** in A549 cells 24 h post-transfection. *In vitro* transfection experiments, for each **GS**, were performed according to the optimal conditions reported in Table 1. Transfection capacity of **GS C8-C16** was compared with that of optimized Lipofectamine 2000 (used according to the manufacturer's guidelines). Fluorescent values are derived from Figure 5 and quantify the % of each cell population that elicits fluorescent signal beyond the M1 threshold indicated in Figure 5. Data are expressed as mean ± SEM, and are compared using one-way ANOVA with a Bonferroni post hoc test. *** = $p < 0.005$.

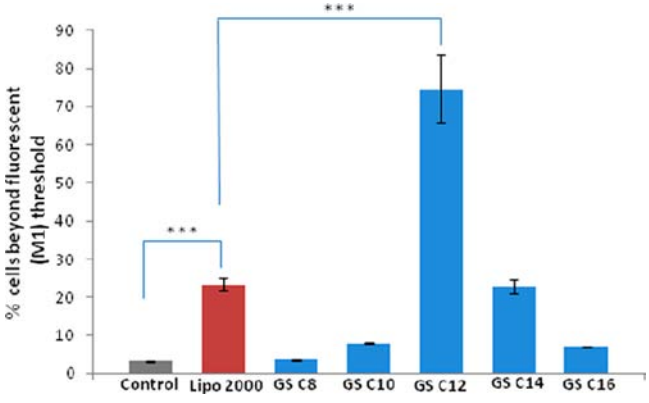

Figure 8. Transfection efficiency of compounds **GS C8-C16** in U-87 MG cells 24 h post-transfection. *In vitro* transfection experiments, for each **GS**, were performed according to the optimal conditions reported in Table 2. Transfection capacity of **GS C8-C16** was compared with that of optimized Lipofectamine 2000 (used according to the manufacturer's guidelines). Fluorescent values are derived from Figure 6 and quantify the % of each cell population that elicits fluorescent signal beyond the M1 threshold indicated in Figure 6. Data are expressed as mean ± SEM, and are compared using one-way ANOVA with a Bonferroni post hoc test. *** = $p < 0.005$.

with a hydrophobic moiety shorter than 12 carbon atoms, **GS C10** and **GS C8**, could be explained by a significant decrease or loss of surfactant properties of the compounds, respectively.

Transfection of Suspension Cells Using GS C12 and GS C14. In the previous experiments, the gene transfer properties of the lipids **GS C8-C16** were studied in two adherent cell types, A549 and U-87 MG cells. The **GS C12** and **C14** surfactants, showing the best transfection efficiency in A549 and U-87 MG cells, were also assessed for their capacity to transfer pcDNA into suspension cell lines such as Bristol 8 (normal human HLA-A2 positive B lymphoblastoid) cells. Bristol 8 cells were transfected with **GS C12** or **C14** using the optimal lipofection conditions found for both A549 (Table 1) and U-87 MG (Table 2) cells. As shown in Figures 9 and 10, for both surfactants, A549 optimal transfection conditions worked very effectively with Bristol 8 cells.

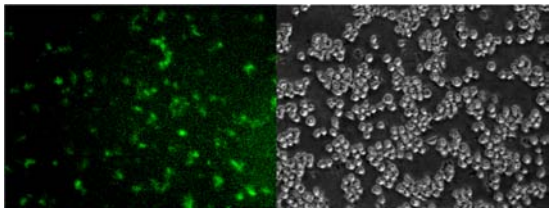

Figure 9. Bristol 8 cells transfected with **GS C12**.

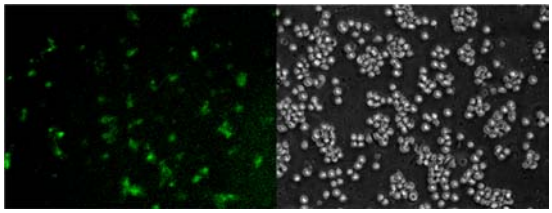

Figure 10. Bristol 8 cells transfected with **GS C14**.

Images collected 24 h post-transfection by fluorescence microscopy (10× magnification); images are representative of at least three other independent experimental repeats. Transfection experiments were performed in serum free conditions according to A549 optimized parameters reported in Table 1.

Images collected 24 h post-transfection by fluorescence microscopy (10× magnification); images are representative of at least three other independent experimental repeats. Transfection experiments were performed in serum free conditions according to A549 optimized parameters reported in Table 1.

Transfection of Primary DRG Neurons Using GS C12 and GS C14. The surfactants **GS C12** and **C14** were also tested for their ability to transfect primary cell cultures, such as primary DRG (dorsal root ganglion) neurons, which are notoriously difficult to transfect. These DRG neurons, obtained from a rat one day prior to transfection, were plated in 35 mm culture dishes with the growth area coated with polylysine (1 μg/mL) and laminin (10 μg/mL) to aid adherence of the cells to the substratum as previously described.[47,48] DRG neurons were transfected in the absence of serum with increasing concentrations of **GS C12** or **C14** (3 μM, 6 μM, 12 μM). Lipofectamine 2000 was used as positive control in the transfection experiments, although it lacks efficacy with cells of this type. Images in Figure 11 clearly show 6 μM of **GS C14** not only transfected these cells, but also did so with a greater potency than Lipofectamine 2000. Interestingly, the neurons visible in the bright field channel of Figure 11B show clear evidence of neurite outgrowth, and this is more pronounced in **GS**-transfected than Lipofectamine-transfected cells (compare Figure 11A,B). This provides evidence that these cells have tolerated this procedure particularly well, as such outgrowth only occurs if cells are physiologically functional and "settled". Indeed, there is a further suggestion here that a greater level of outgrowth is occurring post transfection with **GS C14** and this transfection method is therefore at least as well tolerated as Lipofectamine. For the **GS C12** surfactant, a 6 μM concentration of surfactant also led to transfection substantially more effective than with Lipofectamine 2000 (data not shown).

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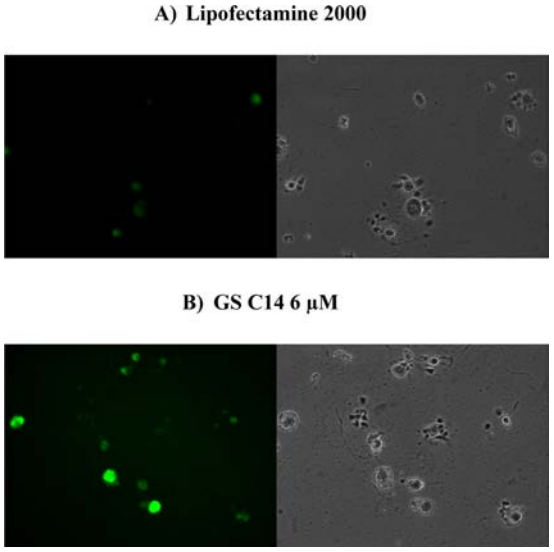

Figure 11. Comparative transfection efficiency of Lipofectamine 2000 and **GS C14** in DRG neurons. Expression of maxGFP evaluated by fluorescence microscopy. Images collected 24 h post-transfection (10× magnification). Images are representative of at least three other independent experimental repeats. (A) DRG neurons transfected with Lipofectamine 2000 (according to the manufacturer's procedures). (B) DRG neurons transfected with **GS C14**. **GS C14**-based lipoplexes were prepared using 6 μM of **GS C14** (pmaxGFP concentration = 1 μg/mL, compaction time **GS C14**-pmaxGFP = 1 h, transfection time = 4 h, serum-free conditions).

CONCLUSIONS

We have described the synthesis of novel gemini surfactants **GS C8-C16**, characterized by two symmetric melamine-based units connected by a six-carbon linker. Compounds **GS C12** and **GS C14** were then used as vectors to efficiently transfect two different adherent cell lines (A549 and U-87 MG), normal suspension cells (Bristol 8), and primary neuronal cells (DRG neurons). The ability of these **GS** molecules to deliver nucleic acid, such as DNA, into eukaryotic cells was found to be clearly dependent on the lipophilic tail length. The surfactant **GS C14** showed A549 and U-87 MG cells transfection properties comparable to or higher than those of Lipofectamine 2000, whereas **GS C12** capacity to deliver pcDNA in both cell types was significantly better (3−4-fold more effective) than that of the gold-standard transfection reagent. In addition, **GS C12** and **C14** surfactants effectively transfected suspension cells, such as Bristol 8 cells. Since these cell lines are very different with respect to each other, it is safe to state that **GS C12** and **GS C14** reagents can be efficiently used to transfect a broad range of cell lines.

The possibility to transfect primary cell cultures, which are notoriously difficult to transfect, was also investigated, and DRG neurons were transfected using **GS C12** or **C14** based lipoplexes to a greater degree than Lipofectamine 2000 based lipoplexes, showing low levels of toxicity.

Compared to the commonly used lipofection methods, melamine-based **GS** reagents offer a number of advantages such as synthesis from relatively inexpensive starting materials, a user-friendly protocol in which no formulation or liposome preparation is required and high chemical stability and remarkable effectiveness are achieved even using minimal pcDNA quantity. These features render these gemini surfactants **GS** a very attractive and effective alternative to conventional lipofecting reagents.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures and spectroscopic characterization of compounds **1**−**4** and **GS C8**−**C16**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): M.U. is current employee of KemoTech s.r.l., whereas P.L. and M.Z. are cofounders of KemoTech s.r.l. (www.kemotech.it).

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