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Cellular uptake and distribution of gemini surfactant nanoparticles used as gene delivery agents

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Abstract

Gemini surfactants are promising molecules utilized as non-viral gene delivery vectors. However, little is known about their cellular uptake and distribution after they release their therapeutic cargo. Therefore, we quantitatively evaluated the cellular uptake and distribution of three gemini surfactants: unsubstituted (16-3-16), with pyridinium head groups (16(Py)-S-2-S-16(Py)) and substituted with a glycyl-lysine di-peptide (16-7N(GK)-16). We also assessed the relationship between cellular uptake and distribution of each gemini surfactant and its overall efficiency and toxicity. Epidermal keratinocytes PAM212 were treated with gemini surfactant nanoparticles formulated with plasmid DNA and harvested at various time points to collect the enriched nuclear, mitochondrial, plasma membrane and cytosolic fractions. Gemini surfactants were then extracted from each subcellular fraction and quantified using a validated flow injection analysis-tandem mass spectrometry (FIA-MS/MS) method. Mass spectrometry is superior to the use of fluorescent tags that alter the physicochemical properties and pharmacokinetics of the nanoparticles and can be cleaved from the gemini surfactant molecules within biological systems. Overall, a significantly higher cellular uptake was observed for 16-7N(GK)-16 (17.0%) compared with 16-3-6 (3.6%) and 16(Py)-S-2-S-16(Py) (1.4%), which explained the relatively higher transfection efficiency of 16-7N(GK)-16. Gemini surfactants 16-3-16 and 16(Py)-S-2-S-16(Py) displayed similar subcellular distribution patterns, with major accumulation in the nucleus, followed by the mitochondrion, cytosol and plasma membrane. In contrast, 16-7N(GK)-16 was relatively evenly distributed across all four subcellular fractions. However, accumulation within the nucleus after 5hr of treatment was the highest for 16(Py)-S-2-S-16(Py) (50.3%), followed by 16-3-16 (41.8%) and then 16-7N(GK)-16 (33.4%), possibly leading to its relatively higher toxicity.

Key words: Gemini surfactants; Gene delivery; Subcellular distribution; FIA-MS/MS; Toxicity; Transfection.

1 Introduction

Gemini surfactants are a versatile family of lipids that have a general structure of two surfactant monomers chemically linked by a spacer [1]. In particular, cationic gemini surfactants possess dual positively charged hydrophilic head groups, a spacer region and two hydrophobic tails (Figure 1) [2]. They are promising vectors for non-viral gene delivery [3-5] as their structures enable them to bind and compact DNA, facilitating cellular entry for gene transfection [6, 7]. Extensive research has been conducted to design and synthesize novel gemini surfactants with the aim of enhancing transfection efficiency while reducing toxicity. For example, the positively charged head groups were altered using various cationic moieties, such as di-quaternary amines and di-pyridines, to obtain effective compaction of DNA [2, 8]. Furthermore, amino acid moieties have been incorporated into the spacer region to enhance the biocompatibility of gemini surfactants and thus increase their transfection efficiencies [9]. In addition, the formulation of gemini surfactant-based lipoplexes and their cellular uptake mechanisms have been well studied [10, 11], and it has been found that endocytosis is the main pathway by which gemini surfactant nanoparticles are internalized by the cell [11].

Despite their promise, gemini surfactants are still limited in their gene transfection efficiency [12, 13], hindering their advancement from the experimental stage to clinical application. In addition, there are no clear explanations for the varying toxicities among different gemini surfactant structures. Therefore, a greater understanding of the mechanism of transfection and toxicity is required and will ultimately contribute to the development of more efficient and less toxic gemini surfactants. One factor that may be related to the overall efficiency and toxicity of gemini surfactants is their intracellular biological fate post-transfection; that is, how they are distributed at the subcellular and tissue levels. However, as of yet, their biological fate is poorly understood, and little is known about their cellular uptake, distribution and metabolite formation upon transfection. Garnering such knowledge will contribute to the design and development of more effective gemini surfactants. In addition, an understanding of the cellular distribution of the delivery agents is crucial to achieve targeted delivery at the subcellular level [14]. Our research hypothesis is that the structure of a gemini surfactant significantly influences its cellular uptake and subsequent partitioning which in turn has profound consequences with respect to efficiency and toxicity. In fact, we have recently demonstrated the role of the molecular structure of gemini surfactants in determining their skin penetration efficiency [15].

Gemini surfactants designated as 16-3-16, 16(Py)-S-2-S-16(Py) and 16-7N(GK)-16) have been studied as non-viral gene delivery agents [16-18]. Structurally, 16-3-16 is a conventional *m-s-m* type gemini surfactant bearing two quaternary amines, linked by a 3-carbon spacer region (*m* is the number of carbon atoms in the tail and *s* is the number of carbon atoms in the spacer); while 16(Py)-S-2-S-(Py)16 is a pyrdinium derived gemini surfactant containing two pyridines in the head groups, and 16-7N(GK)-16 bears a glycine-

lysine di-peptide within the spacer region (Figure 1), allowing for a better biocompatibility. In fact, these gemini surfactants have been successfully used for *in vitro* and *in vivo* gene delivery [16-20]. For instance, the 16-3-16 nanoparticles have shown great promise in the treatment of localized scleroderma, as transgene expression in animal models was significantly increased with treatment of the nanoparticles compared with naked DNA, showing the effectiveness of gemini surfactant-based gene delivery systems [6, 20].

The selection of the three gemini surfactants (Figure 1) was based on the variations in their molecular structures, transfection efficiency and toxicity profiles [6, 8, 21]. Although these gemini surfactants belong to three different structural families that possess different head groups and spacer regions, they have the same number of carbon atoms in the tails (Figure 1). We are currently conducting a wider assessment of the gemini surfactants in various families with respect to the relationship between cellular uptake, subcellular distribution, efficiency and toxicity. As such, we chose these three structures as model compounds. Previously, we determined that the trend of cellular uptake and clearance of 16-3-16 and 16(Py)-S-2-S-(Py)16 nanoparticles were comparable in PAM 212 epidermal keratinocytes, which provides no explanation of the differential toxicities between the two compounds [22]. We, therefore, are testing the hypothesis that the relative efficiency and toxicity of these compounds is explained by a difference in their cellular uptake and subcellular distribution.

To assess the cellular uptake and distribution of lipid-based nanoparticles, fluorescent tags have often been incorporated into their structures [14, 23]. However, the use of fluorescent tags suffers from two main drawbacks. First, the addition of such structure-modified moieties alters the physicochemical properties and pharmacokinetics of the nanoparticles [24, 25]. Second, the fluorescent tags can be cleaved from the gemini surfactant molecules within biological systems, confounding subsequent data interpretation. Such limitations motivated us to develop mass spectrometry (MS)-based methods to monitor the fate of gemini surfactant nanoparticles in cells [26, 27]. The main advantage of MS is its capability to measure the original intact molecule with high selectivity and sensitivity [26]. Most recently, we developed and validated a simple flow injection analysis-tandem mass spectrometry (FIA-MS/MS) method that allows for the tracking of gemini surfactants at the subcellular level [28].

In the present study, the validated FIA-MS/MS method is applied to provide for the first time a quantitative assessment of the cellular uptake and subcellular distribution of three gemini surfactants gene delivery nanoparticles (Figure 1) within PAM 212 cells. We found that variable cellular uptake of the three gemini surfactants explained the differences in transfection efficiency, and that accumulation of gemini surfactant in the nucleus may provide insights into the observed increased toxicity.

2 Materials and Methods

2.1 Materials

Gemini surfactants 16-3-16, 16(Py)-S-2-S-16(Py) and 6-7N(GK)-16 (Figure 1) and their deuterated internal standards 16-3-16-D_{66}, 16(Py)-S-2-S-16(Py)-D_{10} and 16-7N(GK)-16-D_4 (Figure S1, supporting information) were synthesized according to established protocols [16, 19, 29]. The neutral lipid 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Chloroform, methanol, acetonitrile, formic acid, tissue culture flasks (75cm^2, 150cm^2) and petri dishes (150cm^2) were purchased from Fisher Scientific (Ottawa, ON, Canada). 96-well tissue culture plates were obtained from Falcon (BD Mississauga, ON, Canada). PAM 212 keratinocyte cells were kindly provided by Dr. S. Yuspa, National Cancer Institute, Bethesda, MD, USA. Fetal bovine serum albumin (FBS), antibiotic-antimycotic solution and minimum essential media (MEM) were obtained from Sigma-Aldrich (Oakville, ON, Canada). The protease inhibitor cocktail was purchased from Invitrogen (Burlington, ON, Canada). The plasmid DNA (pGTmCMV.IFN-GFP) was constructed in-house as previously described [6]. The motorized homogenizer was purchased from Fisher Scientific (Toronto, ON, Canada).

2.2 Formulation

Gemini surfactants and internal standards were prepared at a concentration of 3mM in aqueous solutions and stored at -80°C under darkness. DOPE vesicles were prepared freshly at a concentration of 1mM in isotonic sucrose solution (9.25% w/v, pH=9) -based on an established protocol [5]. The plasmid DNA solution was prepared at 200µg/mL in ultra-pure water and stored at -80°C.

The nano-lipoplex formulation (P/G/L) was prepared with plasmid DNA, gemini surfactant and lipid DOPE as previously described [6] with a nitrogen (cationic) to phosphate (anionic) charge ratio (N/P) at 10 for 16-3-16 and 16(Py)-S-2-S-16(Py) and at 2.5 for 16-7N(GK)-16. Briefly, to prepare 1mL of the P/G/L for 16-3-16 and 16(Py)-S-2-S-16(Py), 38µL of 3mM gemini surfactants was added to 38µL of 200µg/mL plasmid DNA, gently mixed by pipetting up and down several times, and incubated for 15min at room temperature. Subsequently, 924µL of 1mM DOPE solution was added to the binary mixture, gently mixed with a pipette and incubated for 15min at room temperature to produce the ternary P/G/L system (nanoparticles). To prepare 1mL of the P/G/L for 16-7N(GK)-16, 9.5µL of 3mM gemini surfactant was added to 38µL of 200µg/mL plasmid DNA, mixed and incubated for 15min at room temperature, 952.5µL of 1mM DOPE solution was then added, mixed and incubated to generate the nanoparticles.

2.3 *In vitro* transfection

PAM 212 cells were cultured in MEM media supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution in 75cm^2 tissue culture flasks in a humidified incubator at 37°C at an atmosphere of 5% CO_2. Upon reaching 80% confluence, cells were washed with phosphate buffered saline (PBS, 8mL), dissociated with a 5min incubation in a Versene (10x, 3mL) and Trypsin (10x, 0.3mL) mixture and collected by centrifugation (250 *x g*, 5min, 4°C). 24h prior to transfection, three 96-well tissue culture plates were seeded with PAM 212 cells at a density of 2×10^4 cells/well. MEM was replaced with serum-free media 1h prior to transfection. Cells were treated with 20µL of the P/G/L nanoparticles per well and incubated for 5h. The cells were then returned to supplemented MEM for further incubation and the culture media was collected at 48h for interferon-gamma (IFN-Y) measurement.

An enzyme-linked immunosorbent assay (ELISA) was carried out to measure secreted IFN-Y using flat bottom 96-well plates (Immulon 2, Greiner Labortechnik, Germany) as per the BD Pharmingen protocol. An IFN-Y standard curve was established using recombinant mouse IFN-Y (BD Biosciences, Missisauga, ON, Canada) to allow for the concentration of secreted IFN-Y in the cell media to be quantified. The experiments were conducted in three plates of quadruplicate wells.

2.4 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

A 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed to determine the cytotoxicity of the gemini surfactants. PAM 212 cells were seeded in three 96-well cell culture plates at a density of 2×10^4 cells/well and treated with the P/G/L nanoparticles. Plates were incubated for 5h at 37°C with 5% CO_2 in a humidified incubator and then the cell media was switched to supplemented MEM media. After 48h of incubation, the cell media was removed and cell toxicity was evaluated by the determination of cell viability. Briefly, 100µL of 0.5mg/mL sterile MTT (Invitrogen, USA) in the supplemented media was added to each well and the plates were incubated for 3h at 37°C. The media was then removed and 200µL of dimethyl sulfoxide (DMSO) (spectroscopy grade, Sigma-Aldrich, ON, Canada) was added to each well to dissolve the formed purple formazan crystal. Subsequently, the plates were incubated at 37°C for 10min. Absorbance was measured at 550nm using a microplate reader (Bio-Tek® Microplate Synergy, HT, VT, USA). The experiments were conducted in three plates of quadruplicate wells, and the cytotoxicity of gemini surfactants reflects cell viability expressed as a percentage of the non-transfected cells (control).

2.5 Size and zeta-potential measurements

Size and zeta-potential of the gemini surfactants-based nanopartilces were measured using a Zeta-sizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK). The

nanoparticles were prepared as described in section 2.2. Three measurements were conducted for each sample. The reported results are the mean of triplicate measurements ± standard deviation.

2.6 Cell treatment and sample collection

PAM 212 cells were cultured in 150cm^2 flasks until they reached 80% confluence. Cells were then washed with PBS (25mL), dissociated with Versene (10x, 5mL) and Trypsin (10x, 0.5mL) and collected by centrifugation (250 *x g*, 5min, 4°C). 24h prior to treatment, 8×10^6 cells were seeded in each petri dish (150cm^2). Cells were switched to serum-free media 1h prior to transfection. 500µL of freshly prepared P/G/L nanoparticles were added to each dish in a drop-wise manner. Following a 5h of incubation, the cells were returned to supplemented MEM media for subsequent incubation steps. Triplicates of treated cell samples and one control (untreated cell) were trypsinized and collected at 2h, 5h and 8h, respectively. The collected cells were pelleted by centrifugation (250 *x g*, 5min, 4°C), rinsed with PBS three times, resuspended in 500µL of ice-cold hypotonic homogenization buffer (10mM NaCl, 1.5mM $MgCl_2$, 10mM Tris-HCl [PH 7.5], and cOmplete™ protease inhibitor) and incubated on ice for 10min.

2.7 Subcellular fractionation using differential centrifugation

Cells were gently homogenized on ice to break the plasma membrane and release subcellular organelles. The cell homogenates were then diluted with ice-cold hypertonic buffer (420mM mannitol, 140mM sucrose, 10mM Tris-HCl [pH 7.5], and 2mM EDTA [pH 7.5]) to a final volume of 1mL and enriched nuclear, mitochondrial, plasma membrane and cytosolic fractions were isolated by differential centrifugation using an established protocol [30], with slight modifications (Figure 2). Briefly, homogenates were first centrifuged at 1,000 *x g* for 10min at 4˚C, and the S_1 supernatant was transferred to a clean ice-cooled microcentrifuge tube while the P_1 pellet was collected as the nuclear fraction (nuclei, unbroken cells and cell debris). The S_1 supernatant was then subjected to further centrifugation at 15,000 *x g* for 15min at 4˚C, yielding the S_2 supernatant and the P_2 pellet which contained the mitochondrial fraction. The S_2 supernatant was then centrifuged at 100,000 *x g* for 60min at 4˚C. The resultant S_3 supernatant contained the cytosol and the P_3 pellet (the plasma membrane along with microsomes, ER and Golgi). All collected fractions were kept on ice prior to being diluted to equal 950µL volume with PBS and stored at -80°C. To verify the successful isolation and relative purity of enriched fractions, Western blot analysis was performed. Relevant experimental details and results are shown in the supporting information (see Appendix B).

2.8 Sample preparation

As we previously described [28], subcellular fractions (950µL) were lysed and spiked with 50µL of internal standard and sample extractions were conducted using the Bligh/Dyer

method [31]. Briefly, 3.75mL of methanol-chloroform (2:1, v/v) was added per 1mL of sample, followed by the addition of 1.25mL of chloroform and 1.25mL of water. At each step, samples and the extraction solvent were vortexed thoroughly. The final mixture was centrifuged at 2,800 *x g* for 10min at room temperature to separate the aqueous and organic phases. The bottom organic phase (80% portion) was collected and dried under a N_2 gas stream, followed by reconstitution in 200µL of methanol. Finally, 150µL of methanol solution was transferred into an HPLC vial for analysis.

2.9 FIA-MS/MS analysis

The FIA-MS/MS analysis was performed on a quadrupole-linear ion trap (4000 QTRAP®) mass spectrometer with an electrospray ionization (ESI) source (AB Sciex, Concord, ON, Canada), coupled with an Agilent 1200 series HPLC (a quaternary pump, degasser and auto sampler) (Agilent Technologies, Mississauga, ON, Canada). As recently described [28], 3µL of sample was injected into the ESI source at a flow rate of 0.5mL/min with an acetonitrile-water mixture (98:2, v/v) containing 0.1% formic acid as the mobile phase. The instrument source temperature was set at 600°C and the ion spray voltage was at 5,500V. Nitrogen gas was used for curtain gas at 30, nebulizer gas at 55, and heater gas at 50. Multiple reaction monitoring (MRM) in positive ion mode was used to monitor all analytes and internal standards. The monitored MRM transitions were as follows: 16-3-16 $[M]^{2+}$ *m/z* 290.3→355.4, 86.1; 16-3-16-D_{66} $[M]^{2+}$ *m/z* 323.5→388.6; 16(Py)-S-2-S-(Py)16 $[M]^{2+}$ *m/z* 349.3→396.3, 203.1; 16(Py)-S-2-S-(Py)16-D_{10} $[M]^{2+}$ *m/z* 354.3→401.3; and 16-7N(GK)-16 $[M]^{2+}$ *m/z* 411.4→276.8, 268.3; 16-7N(GK)-16-D_4 $[M]^{2+}$ *m/z* 413.4→278.8 (Figure 1 and Figure S1). The compound-dependent parameters for analytes and internal standards were optimized as previously described [28]. A stable isotope dilution standard curve and three quality control samples (low, medium and high) were run along with the samples in each batch. The data acquisition time per sample was 2min. Data acquisition and analysis was performed using AB Sciex Analyst software (version 1.6.0).

2.10 Ethidium bromide dye exclusion assay

The plasmid DNA (200µg/mL) was complexed with the three gemini surfactants at various charge ratios in the presence or absence of DOPE on 96-well plates. Ethidium bromide was added to all samples at a final concentration of 1µg/mL. The samples were then incubated for 10min at room temperature. After that, fluorescence excitation was carried out at 530nm and emission was measured at 590nm using a microplate reader (Biotek Microplate Synergy HT, VT, USA). The relative fluorescence of the P/G/L and P/G complexes was expressed as a percentage of fluorescence of the pure plasmid DNA solution. Measurements were conducted in triplicate.

2.11 Langmuir studies

Langmuir trough was used to measure the monolayer surface area of the gemini surfactant head group. Surface pressure-mean molecular area isotherms were obtained using a KSV 2,000 Langmuir trough instrument (KSV Instruments Ltd, Helsinki, Finland).. A stock solution of each gemini surfactant was prepared at 1mM in chloroform and 40µL of each stock solution was added dropwise on the sub-phase using a Hamilton syringe. The monolayer was left for a minimum of 10min to allow chloroform to evaporate, a constant rate compression of 20mm/min was then applied on the monolayer molecules until collapse of the monolayer lipid. The ultra-pure water (Millipore, resistivity 18 MΩ· cm) was used as a sub-phase in the trough and the sub-phase temperature was set at 22°C. Triplicate measurements were collected for each gemini surfactant and data collection was performed using the KSV software (KSV Instruments Ltd, Helsinki, Finland).

2.12 Statistical analysis

Statistical analyses were performed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests using SPSS 25 software. Significant difference was established at the $p < 0.05$ level of significance. Results are expressed as the mean of triplicates ± standard deviation.

3 Results and discussion

3.1 *In vitro* transfection activity

These gemini surfactant nanoparticles have shown great promise in treating the localized scleroderma, a rare skin disease [6, 20], we are currently tuning the gemini surfactant nanoparticles to develop an effective, non-invasive topical gene delivery system for the treatment of the fibrotic skin conditions. As such, the epidermal keratinocytes, PAM 212, was used as the cell model in this study. To evaluate the efficiency of the gemini surfactants to mediate transfection in PAM 212 cells, the amount of secreted IFN-Ɣ was quantified 48h post-transfection. As determined in previous work [6, 16], the optimal N/P to obtain the best transfection efficiency for 16-3-16 is 10 and for 16-7N(GK)-16 is 2.5. We also determined that the optimal N/P for 16(Py)-S-2-S-16(Py) is 10 based on the assessment of various N/P ratios at 0.5, 1, 2.5, 5, 10 and 20 for transfection (data not shown). For the best comparison of transfection capability as well as for proper toxicity assessment, the *in vitro* transfection study was conducted at the optimal N/P of each gemini surfactant. It reflects the real conditions in which these compounds are used for in vitro gene transfer. At the optimal N/P of 10, the P/G/L of 16(Py)-S-2-S-(Py)16 resulted in a significantly lower level of IFN-Ɣ (1.19±0.08 ng/2x10^4 cells) when compared with that of 16-3-16 (2.77±0.13 ng/2x10^4 cells) (p <0.05) (Figure 3). In comparison, the P/G/L of 16-7N(GK)-16 at its optimal N/P of 2.5 yielded a IFN-Ɣ level (3.76±0.27 ng/2x10^4 cells) [21] that is significantly higher than that of 16-3-16 (p<0.05). In sum, the relative transfection efficiency of the three gemini surfactant

nanoparticles was determined, with 16-7N(GK)-16 being the most effective and 16(Py)-S-2-S-(Py)16 the least effective.

3.2 Cytotoxicity

In the present study, the cytotoxicity of the gemini surfactants 16-3-16 and 16(Py)-S-2-S-(Py)16 was evaluated in PAM 212 cells. It was observed that cell viability was significantly higher upon treatment with the P/G/L of 16-3-16 (76%) compared with that of 16(Py)-S-2-S-(Py)16 (61%, p <0.05) (Figure 4), indicating that the former has significantly lower cytotoxicity than the latter for the tested PAM 212 cell line. However, 16-7N(GK)-16 was previously reported to have an even lower toxicity (89% cell viability) than 16-3-16 [21]. Hence, 16-7N(GK)-16 possesses the lowest cytotoxicity and 16(Py)-S-2-S-(Py)16 has the highest cytotoxicity among the three gemini surfactants.

3.3 Determination of size and zeta-potential

Size and zeta potential of the P/G/L nanoparticles were measured as they are important characteristics of the delivery systems, which can have an influence on their stability, cellular uptake, and cytotoxicity [32, 33]. At the optimal N/P of 10, the P/G/L of 16-3-16 displayed a size of 131.9±1.48 nm and positive zeta potential at 17.3±1.38 mV, and the P/G/L of 16(Py)-S-2-S-(Py)16) showed a comparable size of 123.1±0.76 nm and zeta potential at 23.3±0.32 mV. Compared with the P/G/Ls of 16-3-16 and 16(Py)-S-2-S-(Py)16, the P/G/L of 16-7N(GK)-16 at its optimal N/P of 2.5 showed a similar zeta potential at 24±2.00 mV but a bit smaller size of 80±1.00 nm as reported in previous work [21]. Endocytosis has shown to be the main mechanism for the internalization of gemini surfactant-based nanoparticles [11, 34]. In particular, clathrin and caeolae-mediated endocytosis are most common pathways for the cellular uptake of gemini surfactant nanoparticles. In fact, the internalization of amino acid-substituted gemini surfactant nanoparticles involves equally both clathrin and caeolae-mediated endocytosis [11]. While particles with smaller sizes of 60-80nm typically undergo caveolae-mediated endocytosis for their internalization, particles with relatively larger size in the range of 120-200nm often enter the cells via clathrin-mediated endocytosis [35, 36]. As such, all three gemini surfactant nanoparticles have the particle sizes that are appropriate for their cellular uptake. However, the smaller size of the P/G/L of 16-7N(GK)-16 could be a contributing factor for its high efficiency in gene transfection, as nanoparticles with small size at 70nm have been reported to display significantly higher transfection efficiency than large-sized nanoparticles at 200nm [32].

3.4 Cellular uptake and distribution of gemini surfactants

To determine the cellular uptake and distribution of the gemini surfactants in PAM 212 cells, fractions enriched for nuclei, mitochondria, plasma membrane and cytosol were isolated by differential centrifugation, extracted and analyzed by the validated FIA-MS/MS method [28]. Differential centrifugation is an isolation technique that uses stepwise increases in

centrifugal force to precipitate subcellular components based on their distinct density, size and shape [37]. During the FIA-MS/MS analysis, the standard curve achieved a less than 15% deviation of the nominal value for each standard point other than the lower limit of quantification (LLOQ), which was 20%, and the quality control (QC) samples were accepted with a less than 15% deviation of the nominal values, as per FDA guidelines [38].

Cellular uptake of the three gemini surfactants, expressed as percentage of dose, was observed to increase rapidly over the course of a 5hr treatment in PAM 212 cells, reaching a maximum of 17.0% for 16-7N(GK)-16, 1.4% for 16-3-6 and 3.6% for 16(Py)-S-2-S-16(Py), followed by a gradual depletion after the removal of the nanoparticles from the media (Figure 5a). A significantly higher cellular uptake was observed for the gemini surfactant 16-7N(GK)-16 compared with 16-3-16 and 16(Py)-S-2-S-16(Py) (Figure 5a) (p <0.05). It should be noted that the optimal N/P for 16-3-16 and 16(Py)-S-2-S-16(Py) was 10, whereas for the 16-7N(GK)-16 was 2.5 as previously determined in our lab [6, 16], and all transfections were conducted with equal amounts of plasmid DNA in the P/G/L nanoparticles. Therefore, the higher cellular uptake of 16-7N(GK)-16 implies that there is a higher transfection efficiency with this gemini surfactant relative to 16-3-16 and 16(Py)-S-2-S-16(Py) and offers a mechanistic explanation as to why PAM 212 cells exposed to 16-7N(GK)-16 secrete greater amounts of IFN-Ɣ (Figure 3).

While the gemini surfactant 16(Py)-S-2-S-16(Py) accumulated more significantly than 16-3-16 in PAM 212 after a 5hr treatment (p <0.05), less IFN-Ɣ was produced, arguing that transfection efficiency is lower with 16(Py)-S-2-S-16(Py) than 16-3-16. The relatively higher toxicity of 16 (Py)-S-2-S-16 (Py) (Figure 4), which caused greater cell death, might have contributed to reducing the overall gene transfection efficiency.

In terms of subcellular distribution, normalized for each surfactant to the total cellular uptake, the gemini surfactants 16-3-16 and 16(Py)-S-2-S-16(Py) exhibited comparable partitioning with significant accumulation in the nucleus, followed by mitochondria, cytosol and plasma membrane (Figure 5b and c) (p <0.05). In contrast, the distribution of 16-7N(GK)-16 was relatively even across the four subcellular compartments (Figure 5d). No significant difference was observed among the three gemini surfactants with respect to their relative distribution in the mitochondrial and cytosolic fractions. However, accumulation in the nucleus was the highest for 16(Py)-S-2-S-16(Py) (50.3%), followed by 16-3-16 (41.8%) and then 16-7N(GK)-16 (33.4%) (p <0.05) at the 5h duration of treatment (Figure 5e), which correlates with the relative toxicity observed for the three gemini surfactants (Pearson's correlation r =0.9993). Even at the 2h and 8h time points post treatment, 16-7N(GK)-16 still displayed significantly lower accumulation in the nucleus relative to 16(Py)-S-2-S-16(Py) and 16-3-16 (p <0.05). Although there is a difference in the accumulation among the three gemini surfactants at 2h time point, this accumulation might not be informative as the cellular uptake are still in rapid progress at this stage. Surprisingly, 16(Py)-S-2-S-16(Py) showed a similar distribution percentage in the nucleus as 16-3-16 at the 8h time point;

however, the distribution quantity (in terms of absolute amount) of 16-3-16 is significantly less compared with that of 16(Py)-S-2-S-16(Py) (Table S1 in supporting information) as 16-3-16 has much lower cellular uptake in comparison with 16(Py)-S-2-S-16(Py) (Figure 5a), resulting in less toxicity of 16-3-16.

Nuclear accumulation may result form either the entry of the gemini surfactants into the compartment or their association with the nuclear envelope, as it has been reported that lipoplexes can fuse with the membrane and release their DNA cargo into the nucleus [39]. However, it is currently not known that the entry of gemini surfactants into the nucleus is in the form of lipid molecules or lipoplexes. Although the entry of lipoplexes is unlikely due to the small pore size of nuclear membrane, which can take place during cell mitosis. As such, it could be in either form or both.

The nucleus has crucial functions within a cell, ensuring the faithful storage and expression of genetic material essential to regulating cellular metabolism and growth [40]. Therefore, nuclear association or accumulation of gemini surfactants could impact outer membrane integrity and normal organelle function. As such, the higher cellular uptake and preferential accumulation of 16(Py)-S-2-S-16(Py) within the nucleus could provide a basis for its higher toxicity relative to 16-3-16 and 16-7N(GK)-16. Thus, to date, our findings provide the only quantitative distinction in the subcellular profiles of these three gemini surfactants, without the use of a florescent tag. The results may offer the first mechanistic insight into the various efficiencies and toxicities observed for the three promising gene delivery agents.

In addition, it was observed that 16-7N(GK)-16 has a significantly higher distribution within the plasma membrane compared with 16(Py)-S-2-S-16(Py) and 16-3-16 (Figure 5b-d) (p <0.05). However, this did not result in higher toxicity, as evidenced by the viability of PAM 212 cells treated with the 16-7N(GK)-16 nanoparticles (Figure 4). In fact, 16-7N(GK)-16 displayed the lowest cytotoxicity among the three gemini surfactants in gene transfection. Stefanutti *et al.* [41] reported that the internalization of lipoplexes of DMPC and a cationic gemini surfactant traversing cell membrane did not cause a significant biological damage to the cells. In addition, Marjan *et al*. [42] reported that although nanoparticle treatment disturbed membrane integrity, the cells were still alive and metabolically active during the transfection process. Therefore, accumulation in the plasma membrane does not appear to cause toxicity.

3.5 Ethidium bromide dye exclusion assay

To explore why unique gemini surfactants differentially accumulate within distinct subcellular compartments, an ethidium bromide dye exclusion assay was conducted to investigate their DNA binding and compaction capabilities. Gemini surfactants bind and compact plasmid DNA via electrostatic interactions to form nanosized particles, which hinder the penetration of ethidium bromide into the complexes. As a result, fluorescence is

quenched due to the lack of intercalation between ethidium bromide and the base-pairs of DNA. The stronger the compaction of DNA by gemini surfactants, the more intense the fluorescence quenching in the complex. As shown in Figure 6, the lowest fluorescence emission was observed at the N/P of 5, with 13.0% for 16-7N(GK)-16, 8.1% for 16-3-16 and 8.9% for 16(Py)-S-2-S-16(Py) in the absence of the helper lipid DOPE. The data indicate that 16-7N(GK)-16 has a significantly lower DNA compaction capability compared with the other two compounds (p <0.05). In the presence of a helper lipid, the fluorescence values were increased to 45.7% for 16-7N(GK)-16, 23.6% for 16-3-16 and 28.3% for 16(Py)-S-2-S-16(Py), again showing 16-7N(GK)-16 is significantly less efficient at binding and compacting DNA relative to 16-3-16 and 16 (Py)-S-2-S-16(Py) (p <0.05).

However, the relatively lower DNA binding capability of 16-7N(GK)-16 does not undermine its gene transfection capability, as evidenced in the transfection study, since gene transfection requires not only effective compaction of DNA for their protection and cellular entry, but also efficient release from the complex into the nucleus for transgene expression . It is believed that the presence of glycyl-lysine moiety in the spacer offers conformational flexibility of the structure, which bestows 16-7N(GK)-16 with softened DNA binding properties [9, 43]. Although 16-7N(GK)-16 has a relatively weaker DNA binding ability than the other two compounds as indicated by its higher fluorescence emission of 13% compared with 8.1% for 16-3-16 and 8.9% for 16(Py)-S-2-S-16(Py), such binding provides the DNA with much needed protection against enzymatic degradation while also facilitating its intracellular release, thereby enhancing overall transfection efficiency [9, 43]. This special binding capability may be caused by the overall interaction of multiple bonding forces, including hydrogen bonding and electrostatic interactions [44]. In addition, the various amine groups in the amino acids can allow for additional buffering capacity, which helps in the disruption of the endosomal membrane for the intracellular release of DNA and their nuclear transport [9].

Due to the weaker DNA binding of 16-7N(GK)-16, the encapsulated DNA could be released more efficiently from the lipoplexes into the cytoplasm to translocate into the nucleus for gene expression. As such, this led to a lesser accumulation of 16-7N(GK)-16 in the nucleus. Conversely, the lipoplexes formed with 16-3-16 and 16(Py)-S-2-S-16(Py) release the DNA less efficiently and potentially at a later stage due to their stronger association with DNA (Figure 6), which could be one of the reasons for their lower transfection efficiency relative to 16-7N(GK)-16, as it has been reported that slow vector unpacking is linked to a decreased transfection efficiency [45, 46]. In this case, the lipoplexes rather than free DNAs are more likely to be translocated into the nucleus. Consistent with this idea, we observed elevated accumulation of both 16-3-16 and 16(Py)-S-2-S-16(Py) in the nucleus (Figure 5b-c).

3.6 Molecular packing parameter

In addition to their DNA compaction and binding properties, the molecular shape of gemini surfactants also have an great impact on the performance of the gene delivery system [16, 47]. Therefore, to further understand the behavioral differences among the three gemini surfactants, structural differences in the formed aggregates was evaluated using the molecular packing parameter (*P*) [47]. The *P* was estimated based on the structures of gemini surfactants and the behavior of gemini surfactants at the air-water interface [48], and is defined as:

$$P = v/a_o l$$

Where *v* is the volume of the hydrophobic tails, *l* is the length of the hydrocarbon tails and a_o is the head group area per molecule in aqueous solution. The *v* and *l* are the geometrical properties of gemini surfactants, which can be calculated from the chemical structures [49, 50]. The a_o is an equilibrium parameter dependent upon both the attractive forces of the hydrophobic chains and the repulsive forces of the head groups, which can be determined by the Langmuir studies. A specific *P* value can be linked to a particular geometrical shape [48]. Spherical micelles typically have a *P* value of less than 0.33; cylindrical micelles possess a *P* value between 0.33 and 0.50, whereas flexible bilayers (vesicles) usually have a *P* value between 0.5 and 1.0 [48].

As the three gemini surfactants have the same *l* and *v* of hydrophobic tails, the a_o is the main determinant of the *P* value. The *l* was calculated to be 21.74 Å using the Avogardro software [51], and the *v* was calculated to be 918 $Å^3$ with the Gaussian 09 software (revision B. 01) [52] (Table 1). Based on the Langmuir studies, 16-7N(GK)-16 showed the smallest a_o of 84$Å^2$ and thus the largest *P* of 0.51, indicating the aggregates formed by 16-7N(GK)-16 are typically flexible bilayers. 16-3-16 displayed an a_o of 116$Å^2$ and a *P* value of 0.36, which suggested the formation of aggregates shaped as cylindrical micelles (Table 1). This is in agreement with the literature [50, 53] reporting that aggregates formed by *m-3-m* gemini surfactants tend to form cylindrical micelles. Similarly, 16(Py)-S-2-S-16(Py) had an a_o of 110$Å^2$ and a *P* value of 0.38, which argues it too forms cylindrical micelles in aqueous solution.

Although 16-3-16 and 16(Py)-S-2-S-16(Py) have unique structural head groups, their areas are comparable and enable them to have similar packing parameters and a preference towards forming cylindrical micelle aggregates (Table 1). The same aggregates would allow for a similar internalization process of the two gemini surfactant nanoparticles for gene delivery, which explained the similar trends observed for their uptake and subcellular distribution (Figure 5b-c). Conversely, the substitution of a di-peptide in the spacer region provides 16-7N(GK)-16 with conformational flexibility [9]. It has a much smaller head group area and thus a flexible bilayer structure (Table 1), which allows for the formation of the inverted hexagonal phase of the lipoplex [47]. Such a conformation facilitates not only the

destabilization of endosomal membrane to promote the cytoplasmic release of DNA but also the dissociation of DNA from the lipoplexes, thus resulting in enhanced gene transfection [54].

4 Conclusions

The cellular uptake and distribution of the gemini surfactants 16-3-16, 16(Py)-S-2-S-16(Py) and 16-7N(GK)-16 as gene delivery agents in PAM 212 cells was evaluated by analyzing subcellular fractions collected by differential centrifugation using a validated FIA-MS/MS method. The three gemini surfactants varied with respect to their uptake and subcellular distribution profiles, with 16-7N(GK)-16 exhibiting greater uptake and a higher transfection efficiency. Preferential nuclear accumulation or association of 16(Py)-S-2-S-16(Py) may explain its relatively higher toxicity. DNA binding and molecular packing experiments provided explanations to the different cellular behaviors of the gemini surfactants. Overall, the results presented herein demonstrate the general applicability of the combined differential centrifugation and MS approach for assessing the uptake and subcellular distribution of gemini surfactants and emphasize that it is superior to a fluorescence-labelling method as it does not require any structural modifications. We are currently investigating the metabolite formation of the three structures, which may provide additional insight into their relative efficiencies and toxicities. In the future, it may be worthwhile isolating more cellular organelles, such as the endosomes and lysosomes to better understand the cellular trafficking of lipid-based gene delivery agents.

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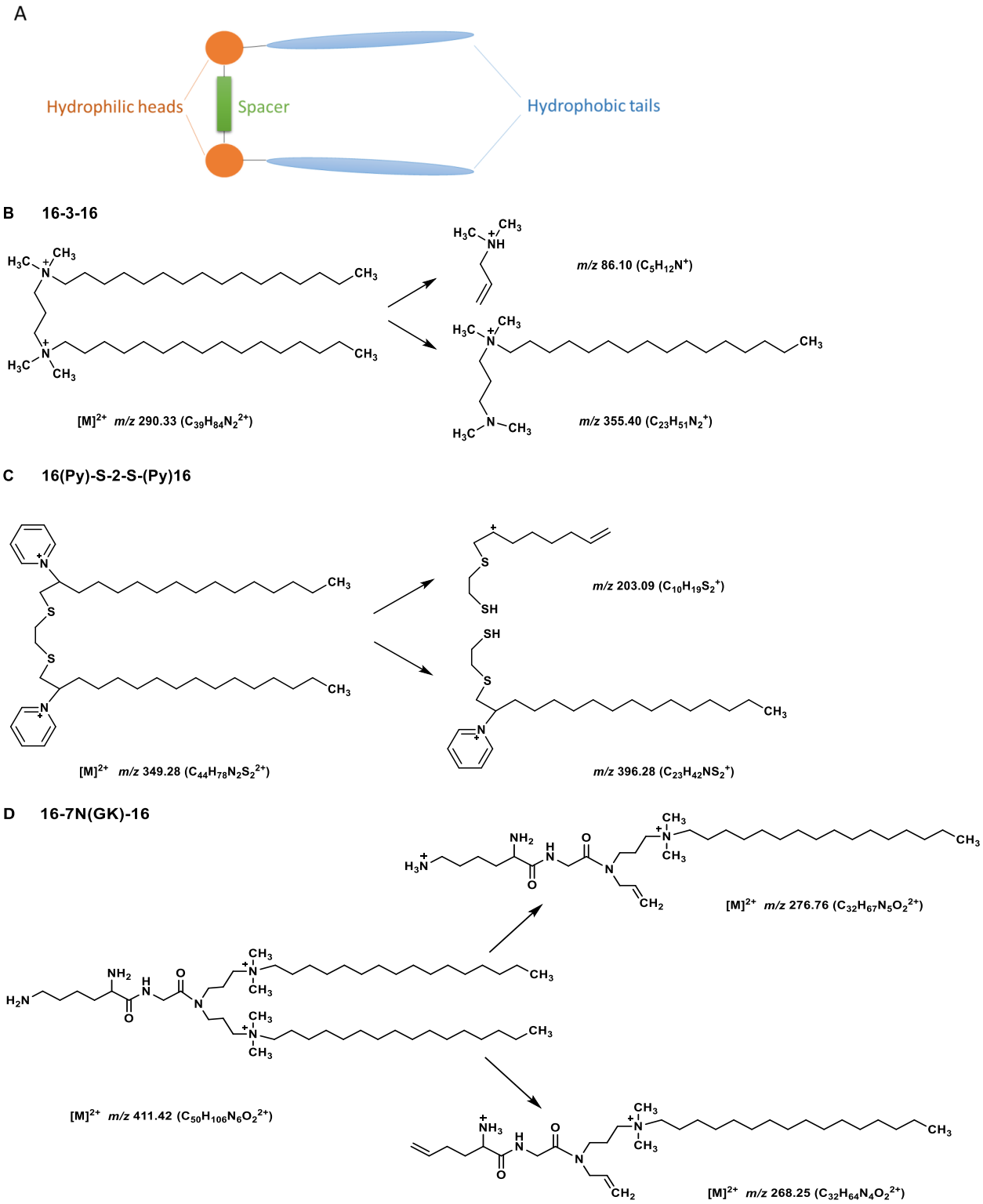

Figure 1. Schematic representation of the general structure of a gemini surfactant (A). The structures of gemini surfactants 16-3-16 (B), 16(Py)-S-2-S-16(Py) (C) and 16-7N(GK)-16 (D), showing their *m/z* values as well as the ions monitored during the FIA-MS/MS analysis.

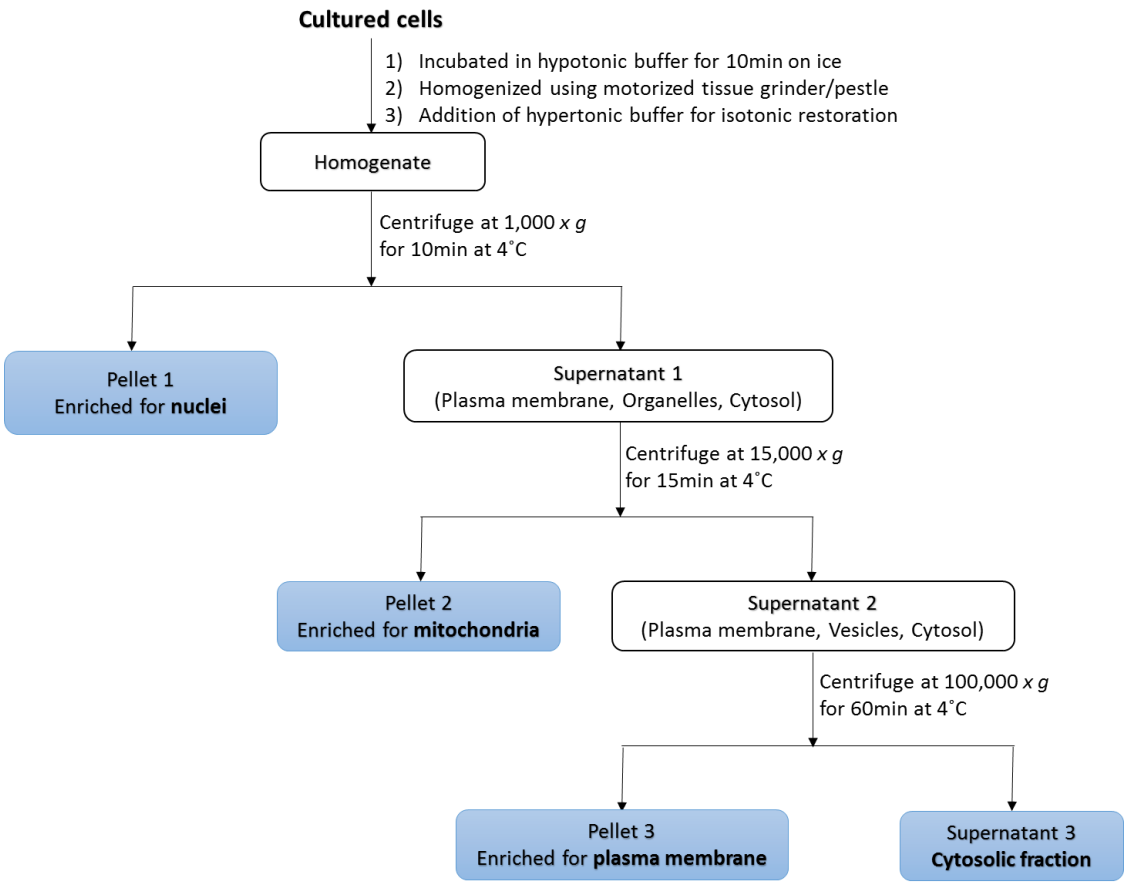

Figure 2. Schematic illustration of the homogenization and subcellular fractionation protocol. Differential centrifugation was used to isolate several subcellular fractions, including those enriched for nuclei, mitochondria, plasma membrane and cytosol.

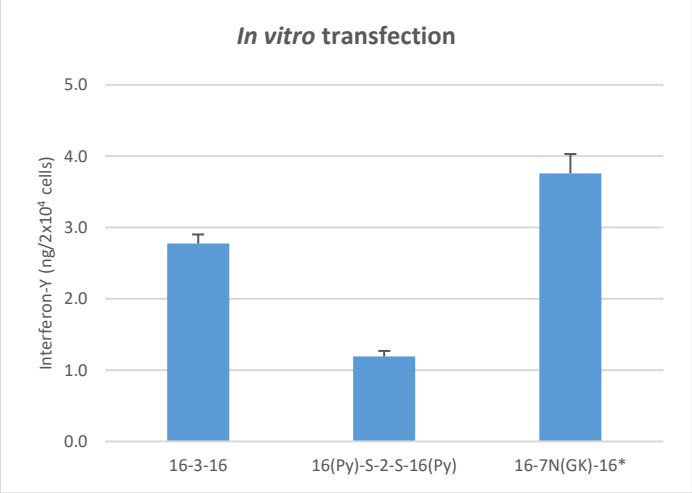

Figure 3. Transfection efficiencies of the P/G/Ls of 16-3-16, 16(Py)-S-2-S-(Py)16 and 16-7N(GK)-16 in PAM 212 cells. *16-7N(GK)-16 transfection was recently reported by our group, extracted from ref. [21].

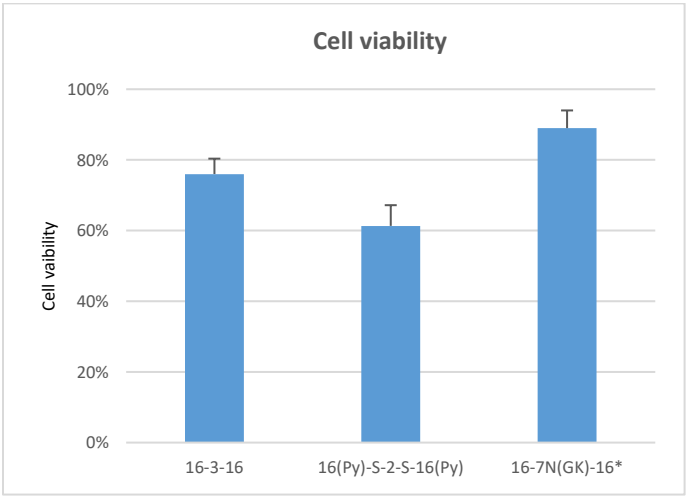

Figure 4. Cytotoxicity of the P/G/L of 16-3-16, 16(Py)-S-2-S-(Py)16 and 16-7N(GK)-16 in PAM 212 cells. *16-7N(GK)-16 cytotoxicity was recently reported by our group, extracted from ref. [16, 21].

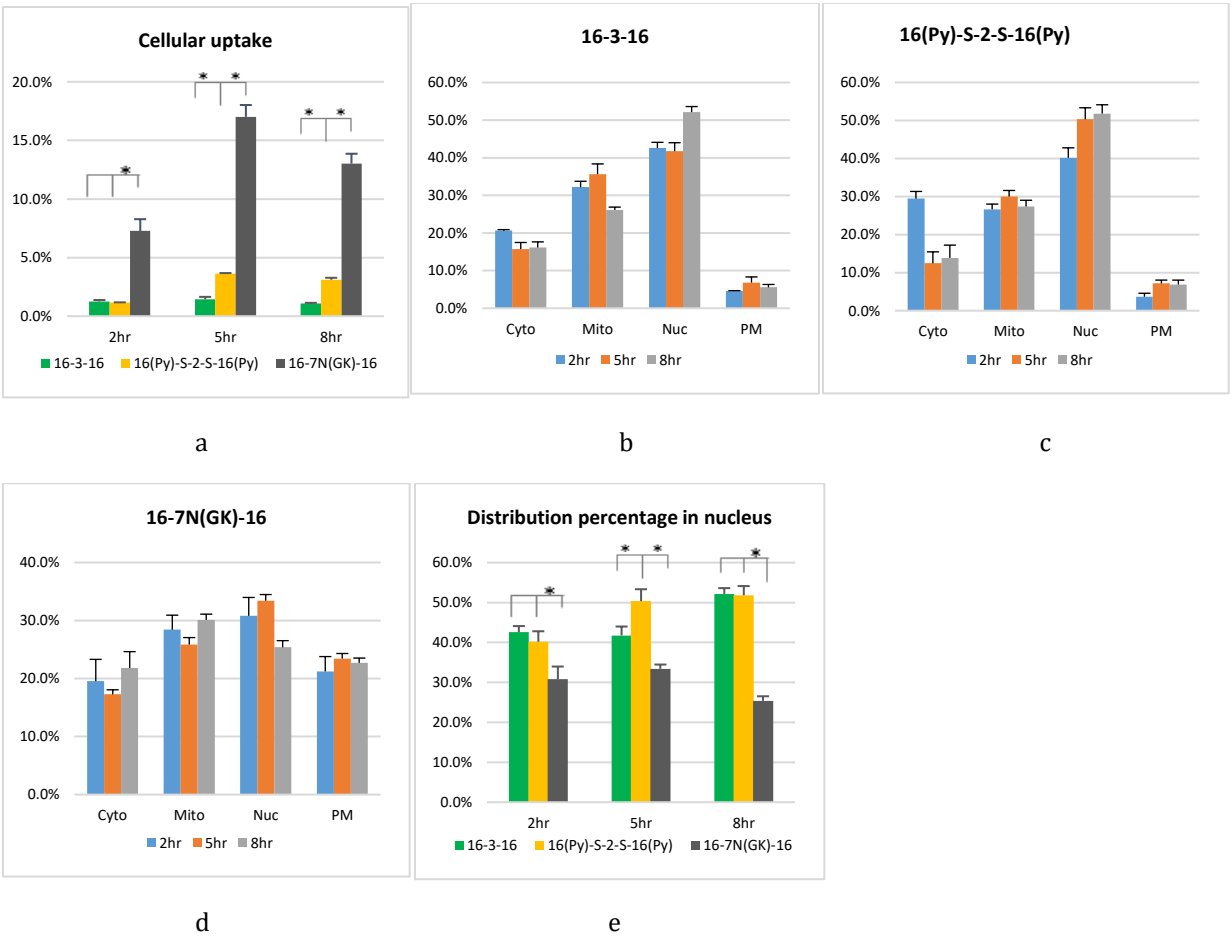

Figure 5. The cellular uptake and distribution of gemini surfactants in PAM 212 cells. a) Cellular uptake, normalized based on the dose, of three gemini surfactants; b-d) Subcellular distribution, normalized based on the total cellular uptake, of 16-3-16, 16(Py)-S-2-S-(Py)16 and 16-7N(GK)-16; and e) Distribution percentage in nucleus. (Cyto-cytosol, Mito-mitochondria, Nuc-nucleus and PM-plasma membrane), * indicates p <0.05.

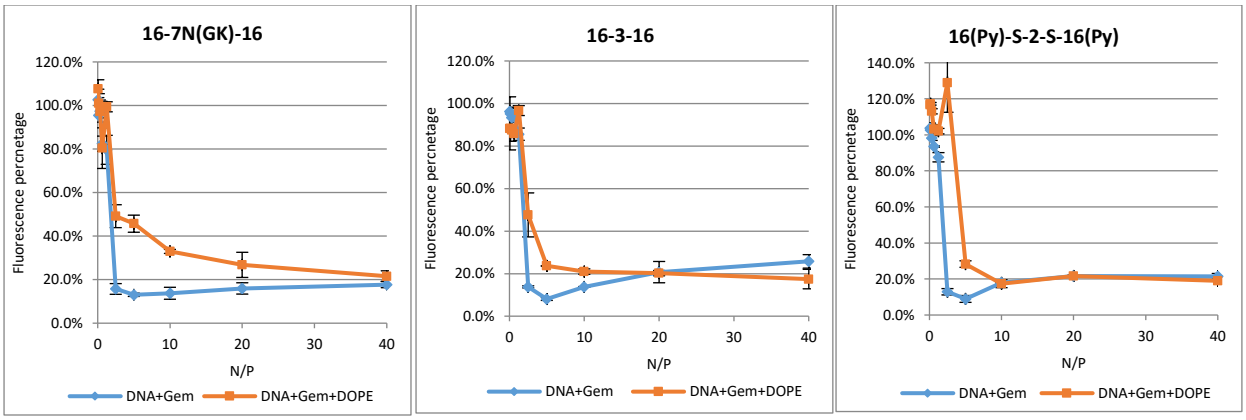

Figure 6. Ethidium bromide dye exclusion assay to evaluate the DNA binding and compaction capability of the gemini surfactants. A lower fluorescence indicates stronger DNA binding and compaction.

Table 1. Estimated molecular packing parameter (p) and shapes of aggregates of the gemini surfactants

Gemini surfactant	a_0(Å^2)	l(Å)	v(Å^3)	P	Shape of aggregate
16-3-16	116	21.74	918	0.36	Cylindrical micelle
16(Py)-S-2-S-16(Py)	110	21.74	918	0.38	Cylindrical micelle
16-7N(GK)-16	84	21.74	918	0.51	Flexible bilayer, vesicle