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Published title
Hydrophilic interaction liquid chromatography–tandem mass spectrometry quantitative method for the cellular analysis of varying structures of gemini surfactants designed as nanomaterial drug carriers

Alternate title
HILIC-LC-MS/MS quantitative method for the cellular analysis of varying structures of gemini surfactants designed as nanomaterial drug carriers.

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SHORT TITLE: HILIC-LC-MS/MS METHOD FOR THE BIO-ANALYSIS OF GEMINI SURFACTANT

ABSTRACT
Diquaternary gemini surfactants have successfully been used to form lipid-based nanoparticles that are able to compact, protect, and deliver genetic materials into cells. However, what happens to the gemini surfactants after they have released their therapeutic cargo is unknown. Such knowledge is critical to assess the quality, safety, and efficacy of gemini surfactant nanoparticles. We have developed a simple and rapid liquid chromatography electrospray ionization-tandem mass spectrometry (LC-ESI–MS/MS) method for the quantitative determination of various structures of gemini surfactants in cells. Hydrophilic interaction liquid chromatography (HILIC) was employed allowing for a short simple isocratic run of only 4 minutes. The lower limit of detection (LLOD) was 3 ng/mL. The method was valid to 18 structures of gemini surfactants belonging to two different structural families. A full method validation was performed for two lead compounds according to USFDA guidelines. The HILIC-MS/MS was compatible with the physicochemical properties of gemini surfactants that bear a permanent positive charge with both hydrophilic and hydrophobic elements within their molecular structure. In addition, an effective liquid-liquid extraction method (98% recovery) was employed surpassing previously used extraction methods. The analysis of nanoparticle-treated cells showed an initial rise in the analyte intracellular concentration followed by a maximum and a somewhat more gradual decrease of the intracellular concentration. The observed intracellular depletion of the gemini surfactants may be attributable to the bio-transformation into metabolites and exocytosis from the host cells. Obtained cellular data showed a pattern that grants additional investigations , evaluating metabolite formation and assessing the subcellular distribution of tested compounds.

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ABBREVIATIONS

16-3-16: N,N-bis(dimethylhexadecyl)-1,3-propane-diammonium
16-3-16-$\boldsymbol{d_{66}}$: N,N-bis(dimethylhexadecyl-d_{16})-1,3-propane-diammonium
16(Py)-S-2-S-(Py)16: 1,1'-[ethane-1,2-diylbis(sulfanediylhexadecane-1,2-diyl)]dipyridinium
16(Py)-S-2-S-(Py)16-$\boldsymbol{d_{10}}$: 1,1'-[ethane-1,2-diylbis(sulfanediylhexadecane-1,2-diyl)]dipyridinium-d_{10}
CID: Collision-induced dissociation
DOPE: 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine
DESI: Desorption electrospray ionization
DNA: Deoxyribonucleic acid
ESI: Electrospray ionization
FBS: Fetal bovine serum
FC: Fast chromatography
GS1: Nebulizer gas
GS2: Heater gas
HILIC: Hydrophilic interaction liquid chromatography
HQC: High quality control
LC-ESI-MS/MS: Liquid chromatography-Electrospray Ionization- tandem mass spectrometry
LLE: Liquid-liquid extraction
LLOD: Lower limit of detection
LLOQ: Lower limit of quantification
LQC: Low quality control
MALDI: Matrix assisted laser desorption
MEM: Minimum essential media (Eagle's formulation)
MRM: Multiple reaction monitoring
MQC: Medium quality control
PBS: Phosphate-buffered saline
P/G/L: Plasmid, gemini surfactant and lipid as components
pGT·IFN-GFP: DNA in plasmid form, coding for IFN-γ protein
RSD: Relative standard deviation
R_t: Retention time
S/N: Signal-to-noise ratio
TEA: Triethyl amine
USFDA: United States Food and Drugs Administration
v/v: Volume-to-volume ratio
ZIC: Zwitterionic

1. INTRODUCTION

Dicationic gemini surfactants are a promising class of lipids for nanoparticle-mediated gene delivery [1,2,3] as they bind to DNA and facilitate its entry into cells. However, the fate of gemini surfactants after they have released their therapeutic cargo is unknown. Effective

analytical methods are needed to assess the quality, safety, and efficacy of gemini surfactant nanoparticles.

Gemini surfactants consist of two monomer surfactants with polar or ionic head-groups connected via a spacer moiety (Figure 1A). In dicationic gemini surfactants (frequently used as the bromide salts), the head-groups are positively charged to allow DNA complexation. The structural modification in gemini surfactants increases transfection efficiency and reduces toxicity. In fact, a variety of gemini surfactant structures demonstrate effectiveness as non-viral DNA delivery agents.[4,5,6,7]

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) offers the combined capability of analyte separation alongside sensitive, high-throughput, and selective analysis.[8,9,10] Gemini surfactants are not compatible with fluorescent/UV-detection due to the lack of fluorescent or UV-active moieties. Therefore, LC-MS/MS is an ideal platform for the quantitative determination of the nanoparticles' amphiphilic constituents within complex biological mixtures. In addition, LC-MS/MS analysis using multiple reaction monitoring (MRM) allows gemini surfactants to be monitored using precursor ion-to-production diagnostic transitions, which provides specificity to target gemini surfactant analytes.

To allow for the assessment of the cellular fate of gemini surfactants, our laboratory initiated studies to establish the collision-induced dissociation (CID)-MS/MS fragmentation patterns of several gemini surfactant structures.[11,12,13,14] These qualitative studies allowed for the subsequent development of an LC- (electrospray ionization) ESI-MS/MS method employing a *cyano* chromatographic column for the quantification of gemini surfactants in epidermal keratinocytes.[15] However, this method suffered from drawbacks, such as ion suppression due to the addition of an ion pairing reagent, long run times, and the use of gradient elution. Other MS-based quantification methods, such as fast chromatography (FC)-MS, desorption electrospray ionization (DESI-MS/MS), and matrix-assisted laser desorption (MALDI)-MS were also developed in our lab and their analytical capabilities were compared;[16] however, all the developed methods were only applicable to one structural family of gemini surfactants with full validation possible for the *N,N*-bis(dimethylhexadecyl)-1,3-propanediammonium compound, denoted as 16-3-16 (Figure 1B). In fact, the fastest MS quantification method,[16] MALDI-MS, was not applicable to gemini surfactant structures that have more than three carbons within their spacer region due to the incomplete dissociation of the gemini surfactants within the MALDI source; preventing the possibility of developing quantification methods. Hence, there is need for a universal method that can produce quantitative data for varying gemini surfactant structures through the adoption of suitable chromatographic platform such as hydrophilic interaction liquid chromatography (HILIC). HILIC is an ideal stationary phase that can combine the benefits of both the normal and reversed-phase of separation.[17] It has been widely used for the analysis of polar compounds including those bearing quaternary amines.[18]

In addition to traditional, non-substituted alkanediyl-α,ω-bis(dimethyl alkyl-ammonium) gemini surfactants (designated as *m-s-m*), other classes of gemini surfactants, especially bis(alkyl-pyridinum) compounds, have emerged.[19,20] The latter are reported to be less toxic when compared to *m-s-m*.[19] The representative *m-s-m* compound is *N,N*-bis(dimethylhexadecyl)-1,3-propanediammonium (denoted 16-3-16; Figure 1B), while its *bis(alkyl-pyridinum)* counterpart is 1,1'-[ethane-1,2-diylbis(sulfanediylhexadecane-1,2-

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diyl)]dipyridinium (denoted 16(Py)-S-2-S-(Py)16; Figure 1C). The 16-3-16 compound imposed higher toxicity than 16(Py)-S-2-S-(Py)16 [19,21] irrespective of its molecular structure. It is hypothesized that possible differences in how gemini surfactants enter the cells, or in bio interactions, bio-distribution, bio-transformation, or bio-persistence, could explain differences in toxicity. Therefore, we developed a simple and fast LC-ESI-MS/MS method that can effectively quantify varying structures of gemini surfactants within treated cells.

Herein, we report the utilization of HILIC-LC-MS/MS to allow for the quantitative determination of variable structures of gemini surfactants within cells. The method is fast (4 min), simple, and requires an isocratic mobile phase. As we discuss below, its analytical capability surpasses previously developed methods. In fact, for the first time, we were able to quantify gemini surfactants that belong to two different structural families, namely 16-3-16 and 16(Py)-S-2-S-(Py)16 (Figure 1).

2. MATERIALS AND METHODS

2.1 *Materials*

Diquaternary ammonium and dipyridinium gemini surfactants and their deuterated internal standards were synthesized based on established methods.[19,22,23] For the 16-13-6 analyte, the internal standard (16-13-6-$\boldsymbol{d_{66}}$) incorporated deuterated alkyl tails, resulting in a mass difference of +66 Da; while for 16(Py)-S-2-S-(Py)16, deuteration was within the pyridinium heads, accounting for a +10 Da mass difference with the internal standard, 16(Py)-S-2-S-(Py)16-$\boldsymbol{d_{10}}$ (Figure S1 in the supplementary information).

The neutral lipid 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Chloroform, methanol, and 1-Octanol (99%) were purchased from Fisher Scientific (Ottawa, ON, Canada). Ammonium formate, formic acid, mass spectrometric-grade water, and acetonitrile were purchased from Fisher Scientific (Ottawa, ON, Canada). PAM 212 cells were kindly provided by Dr. S. Yuspa, National Cancer Institute, Bethesda, MD, USA. The cell growth medium contained minimum essential media (MEM), fetal bovine serum albumin (FBS), and antibiotic-antimycotic obtained from Sigma-Aldrich (Oakville, ON, Canada). Tissue culture flasks (with capacities: 150-cm^3, 75-cm^3, 25-cm^3) as well as 6-well plates, were purchased from Fisher Scientific (Ottawa, ON, Canada).

2.2 *LC-MS/MS instrumentation*

The hybrid LC-ESI-MS/MS analytical system comprised an Agilent 1200 Series HPLC with a quaternary pump, degasser, and auto sampler (Agilent Technologies, Mississauga, ON, Canada) coupled to an AB Sciex API 4000 QTRAP mass spectrometer (AB Sciex, Concord, ON, Canada). The chromatography step used a ZIC®-HILIC column (150 × 2.1 mm, 5 µm, 200 Å; Merck SeQuant AB) held at 50 ˚C and an isocratic mobile phase flowing at500 µL/min to analyze 2 µL injected aliquots. The mobile phase was 80:20 (v/v) acetonitrile/buffer, with the buffer containing 2.5 mM ammonium formate and 25 mM formic acid. No sample carryover was detected and to eliminate any chances of carryover, double blank injections were run after the injection of the highest calibration curve concentration.

The AB Sciex QTRAP 4000 mass spectrometer is equipped with an ESI source set at 5500 V ionspray voltage and 600 ºC at the ion source interface, with 30 psi being set as the curtain gas pressure and 40 psi as the pressures for both GS1 (nebulizer gas) and GS2 (heater gas). The mass

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spectrometry data were obtained using low energy collision tandem mass spectrometry CID-MS/MS operated in the multiple reaction monitoring (MRM) mode to monitor the analytes and the deuterated internal standards as $[M]^{2+}$ species, including the following transitions: 16-3-16 $[M]^{2+}$ *m/z* 290.33 → 355.40, 86.11; 16-3-16-$\boldsymbol{d_{66}}$ $[M]^{2+}$ *m/z* 323.54 → 388.61; and, 16(Py)-S-2-S-(Py)16 $[M]^{2+}$ *m/z* 349.28 → 396.28, 203.09; 16(Py)-S-2-S-(Py)16-$\boldsymbol{d_{10}}$ $[M]^{2+}$ *m/z* 354.31 → 401.31. The MRM conditions and fingerprint structures for the monitored transitions are presented below (Table 1, Figure 1, and Figure S1 in the supplementary information).

2.3 *Preparation of standard solutions*

Aqueous stock solutions of all gemini surfactants (3 mM) were used with or without dilutions and stored at -20 ˚C under darkness. The mass concentrations (equivalent to 3 mM) for analytical quantitation are detailed on Table S1. An isotonic sucrose solution (9.25% w/v, pH 9) containing DOPE vesicles (1 mM) was prepared fresh. Plasmid DNA (pGT·IFN-GFP) solution, 40 µg/mL, was prepared in ultrapure, organic-free water and stored at -20 ˚C. The DOPE and plasmid DNA solutions were used without further dilutions.

2.4 *Preparation of nanoparticle formulations*

Plasmid DNA/gemini surfactant/lipid DOPE (P/G/L) nano-lipoplex formulations were prepared in a 1:10:100 molar ratio (combined in the order given) as previously described.[22] The mole of DNA is calculated per DNA base-pair with an average molecular weight of 660 Da.[24] Briefly, the required transfection dose of plasmid DNA was measured, to which the appropriate amount of gemini surfactant was added with an allowed 30-minute incubation at room temperature. DOPE was then added to the binary mixture, followed by a further 15-minute incubation at room temperature to obtain a ternary (final) P/G/L system. The transfection dose of plasmid DNA was 0.3 µg for 1×10^6 cells/treatment.

2.5 *PAM 212 cell treatment and sample collection*

PAM 212 cells were routinely cultured inside humidified 95% air/5% CO_2 incubators at 37 ˚C. The MEM culture medium was supplemented with 10% FBS and 1 unit/mL antibiotic-antimycotic. Cells cultured to 80% confluence were harvested by trypsinization and either subcultured or prepared for treatment. At 24 h before treatment, cells were seeded at 1×10^6 cells/mL/well within 6-well plate format. At 1 h to transfection, the cell supernatant media was replaced with FBS-free media. Nanoparticle formulations, freshly prepared (*Section 2.3*), were added and allowed to interact with cells by placing them in the CO_2 incubator (37 ˚C). This marked the reference point for recording any post-treatment incubation time-points when further steps are taken.

After 5 hours, the nanoparticle-dosed cell media was replaced with supplemented media (MEM with 10% FBS, 1 unit/mL), followed by a continued incubation of cells. All treatments were carried out in replicates (n ≥ 3) along with appropriate controls on at least three separate days. The controls included cells that underwent no treatment as well as cells that received formulation prepared without the inclusion of the gemini surfactant component. During the incubation period, replicates of treated cell samples were, at different time-points, trypsinized and collected into 1.5 mL volumes. The collected cells were pelleted (14,000 rpm, 5 min, 4 ˚C), rinsed with phosphate-buffered saline (PBS), reconstituted into smaller sample volumes of 200 µL in PBS, and stored at -80 ˚C prior to analyte extraction and LC-ESI-MS/MS analysis.

To better conduct sample collection and reduce tediousness in the process, an alternative approach was also used. During the post-treatment incubation period, replicates of treated cells were, at different time-points, retrieved from the CO_2 incubator, rinsed (3×) with PBS and placed at -80 ˚C. All cells kept at -80 ˚C were then thawed, trypsinized, and collected into 1.5-mL volumes. The 1.5-mL samples were reduced into smaller sample volumes of 200 µL using centrifugal evaporation (speed vacuum concentration). Results were identical using both approaches.

2.6 *Sample Preparation for LC-ESI-MS/MS analysis*

The 200-µL treated cell samples kept at -80 ˚C were lysed by six freeze/thaw cycles along with 1-h sonication at 25 kHz. The samples were spiked with constant amounts of the internal standards by addition of 50-µL solutions obtained from a dilution of $^1/_{250}$ parts of the pure stock internal standard in water; the resultant concentration was 1,938 ng/mL for 16-3-16-$\boldsymbol{d}_{66}$ and 2,086 ng/mL for 16(Py)-2-S-2-(Py)16-$\boldsymbol{d}_{10}$. For the construction of calibration curves, 200 µL cell lysates (equivalent to 1×10^6 cells each), which had not received any nanoparticle treatment, were used. The untreated samples were spiked with 50-µL solutions containing the analyte and the corresponding internal standard. The resultant volume was 250 µL for all samples prior to subsequent steps.

Liquid-liquid extraction (LLE) of the analytes and internal standards from the cellular matrix were achieved using the Bligh/Dyer method.[25] Briefly, the 250-µL samples were mixed with 950 µL (3.8 vols) of 2:1 (v/v) methanol/chloroform, followed by mixing with 310 µL (1.24 vols) of both chloroform and water, in that order. Mixing was achieved by vortexing in all cases. The combined mixtures were centrifuged at 14,000 rpm for 10 min at room temperature to obtain separate aqueous and organic phases. The bottom organic phase (80% portion) was retrieved and dried using a gentle N_2 gas stream, followed by reconstitution of the extracted analytes in 200 µL of n-octanol prior to LC-ESI-MS/MS analysis.

For comparison, a recently reported alternative method, *octanol* extraction, was also used.[15] For the *octanol* extraction, which involves extracting samples with equal volumes of n-octanol, the 250-µL samples were mixed with 250 µL of n-octanol. Separation of the aqueous and organic phases was achieved by centrifugation as above. The organic phase (200-µL, i.e., 80% portion) was retrieved for LC-ESI-MS/MS analysis.

2.7 *LC-ESI-MS/MS method validation*

The LC-ESI-MS/MS method was validated for the 16-3-16 and 16(Py)-2-S-2-(Py) gemini surfactants in accordance with USFDA guidelines.[26,27] Linearity was probed over a wide range of analyte concentrations, including 50 – 5,000 ng/mL. Using least-square analysis along with $1/\chi$ as the weighting factor, data was processed by plotting the ratio of summed peak areas for 16-3-16 and 16(Py)-2-S-2-(Py)16 over the peak areas for their respective internal standards, 16-3-16-$\boldsymbol{d}_{66}$ and 16(Py)-2-S-2-(Py)16-$\boldsymbol{d}_{10}$. Linearity was established with the slope, intercept, and coefficient of determination (r^2). The limit of detection (LLOD) was set as the lowest detectable concentration with a signal-to-noise (S/N) ratio of 3, while the lowest limit of quantification (LLOQ) was set at the lowest concentration having S/N ≥ 5, with precision and accuracy within ±20% of the nominal value as per the USFDA guidelines.[27]

The intra- and inter-day precision and accuracy of the method was established through the analysis of six sample replicates at four different concentrations (lower limit of quantification,

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LLOQ; lower quality control, LQC; middle quality control, MQC; and high quality control, HQC) on three different days. Single assay runs were accepted only when the relative standard deviation (RSD) was found to be less than ±15% at concentrations other than the LLOQ, which is allowed at ±20%. The criterion for accuracy was set at ±15% of the nominal concentration of the QC samples and ±20% for LLOQ.

Studies involving freeze/thaw stability, bench-top stability, and long-term stability were undertaken at LQC, MQC, and HQC. Freeze/thaw stability was tested after three freeze/thaw cycles spaced at least 24 h apart with sample storage at -20 ± 5 ˚C between sample thawing. The twenty-four hour stability of 16-3-16 and 16(Py)-2-S-2-(Py)16 in PAM212 cell lysate under bench-top conditions was evaluated. Predicted concentrations were calculated using newly prepared calibration curves. Samples were stored at -20 ± 5 ˚C for 105 days prior to analysis of long-term stability. Samples were considered stable when the USFDA criteria for precision and accuracy were met.[27]

3. RESULTS -

3.1 *HILIC-LC-MS/MS specificity for gemini surfactant bio-analysis*

The HILIC-LC-MS/MS method provided selectivity through the observed analyte-specific retention times and specificity through the MRM mode, which monitors the analytes using precursor → product ion transitions. The specific retention times established for each analyte and the elimination of carryover effects made the HILIC chromatographic separation an ideal choice for the analysis of gemini surfactants. In addition, the HILIC-LC-MS/MS method was applicable to various gemini surfactant structures as shown in Table 2.

A characteristic chemical structure-dependent elution order was observed, establishing predictable HILIC retention time trends important for distinguishing the gemini surfactants. For instance, the alkyl tail length of three gemini surfactants with the same spacer moiety, increased in the order 12(Py)-S-2-S-(Py)12 (tail: *dodecyl*), 14(Py)-S-2-S-(Py)14 (tail: *tetradecyl*) and 16(Py)-S-2-S-(Py)16 (tail: *hexadecyl*); the corresponding retention times (R_t) decreased in the reverse order, 2.88 min, 2.80 min, and 2.62 min, respectively (Table 2). Such a trend was also notable in the case of the alkyl spacer portions. The increase in the alkyl spacer chains led to shorter retention times as observed for gemini surfactants: 12-3-12 (spacer: *propyl*) R_t = 2.96, 12-8-12 (spacer: *octyl*) R_t = 2.56, 12-12-12 (spacer: *dodecyl*) R_t = 2.43 min (Table 2). Thus, gemini surfactants with longer hydrophobic alkyl tails or longer alkyl spacer chains showed shorter retention times on the HILIC column.

All tested compounds eluted before 4 minutes making the analysis a very fast approach for varying gemini surfactant structures. The only exception is 12-7NH-12 eluting at 7.12 min (Table 2); this compound contains a secondary amine within the spacer region. Therefore, it bears increased polarity within its structure leading to an overall stronger interaction and retention on the zwitterionic sulfoalkylbetaine stationary phase (ZIC®-HILIC column).

In addition, the HILIC-LC-MS/MS platform demonstrated a capability for allowing the analysis and differentiation of isobaric gemini surfactants, namely 16-7-16 and 18-3-18 (identical composition $C_{43}H_{92}N_2^{2+}$, $[M]^{2+}$ m/z 318). The differentiation of the isobaric gemini surfactants relied on the established characteristic chromatographic retention behaviour in combination with the analyte's unique mass spectrometric fingerprints (Table 2). Finally, the developed method

was not only applicable to the two classes of gemini surfactants, but it was also capable of separating the gemini surfactant Py-3-12, which contains two asymmetric tails, dodecyl chain and fluorescent pyrenyl-hexyl tail. The gemini surfactant Py-3-12 is generally used as a fluorescence probe for studying DNA complexation with gemini surfactants in addition to its gene transfection potential.[28,29]

3.2 *Method validation for bio-analysis of 16(Py)-S-2-S-(Py)16 and 16-3-16*

The method validation of the HILIC-LC-MS/MS method for the bio-analysis of two specific gemini surfactants, 16(Py)-S-2-S-(Py)16 and 16-3-16, was conducted in accordance with USFDA guidelines.[27] These two promising molecules are currently being evaluated at the pre-clinical stage, assessing their toxicity alongside gene transfer capabilities. All method validation parameters are summarized in Table S2 (supplementary materials).

3.3 *Selectivity and matrix effects*

Selectivity was achieved as illustrated for both 16(Py)-S-2-S-(Py)16 and 16-3-16 gemini surfactants per the MRM chromatograms in Figure S2 (supplementary materials). The absence of peaks in Figure S2A confirms that "double blank" samples contain no trace of either the analyte or the internal standard. Hence, the blank matrix has no interference or co-eluting peaks against the selective bio-determination of 16(Py)-S-2-S-(Py)16. Further, no cross-interference occurred between the analytes and internal standards. As an illustration, Figure S2B and Figure S2C for samples containing only the analyte 16(Py)-S-2-S-(Py)16 and internal standard 16(Py)-S-2-S-(Py)16-$\boldsymbol{d_{10}}$, respectively, show peaks for only the respective compound. Thus as expected, no peaks of the analyte were detected in samples containing only the internal standard and vice versa. Similar data for 16-3-16 is shown in the supplementary materials (Figure S2D-F).

The cellular matrix did not have an effect on the ionization efficiency, that is, neither ionization enhancement nor suppression was observed. The matrix effect was calculated to be approximately 101% for both 16(Py)-S-2-S-(Py)16 and 16-3-16 (criteria: >100% indicates enhancement, <100% indicates suppression). Determination of the matrix effect followed the method of Matuszewski *et al*,[30] as given below:

$$\text{Matrix effect}(\%) = \frac{\text{Response post-extraction spiked sample}}{\text{Response non-extracted neat sample}} \times 100 \qquad (1);$$

where the post-extraction spiked sample refers to standards spiked after extraction and contains the standard analyte added to extracted blank cell lysate. The non-extracted neat sample contains the analyte added to octanol (pure, cellular matrix-free). The determined value is the average for a set of 6 replicates.

3.4 *Calibration curve linearity and sensitivity*

During validation, the calibration curves for the 16(Py)-S-2-S-(Py)16 and 16-3-16 gemini surfactant analytes were linear at a concentration range of 50 – 5000 ng/mL, with an r^2 value ≥ 0.997 as shown in Figure S3 (supplementary materials). Figure 2 shows a representative chromatograms for 16(Py)-S-2-S-(Py)16 at 6, 50 and 5,000 ng/mL. The concentration determined as the lower limit of detection (LLOD) was based on a set limit of S/N ≥ 3; this led to an LLOD of 6 ng/mL for 16(Py)-S-2-S-(Py)16, (Figure 2C). The LLOD for 16-3-16 was 3 ng/mL, with corresponding chromatograms given in the supplementary information (Figure S4).

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HILIC-LC-MS/MS showed a substantially higher sensitivity in terms of ability to detect the target analytes in comparison with a recent cyano-based stationary phase LC-ESI-MS/MS method for the cellular analysis of 16-316 gemini surfactant, with reported LLOD of 180 ng/mL.[15] With the reported cyano-LC-ESI-MS/MS method, triethylamine (TEA) along with gradient elution were needed and the analyte extraction from aqueous medium utilized *n*-octanol, which had a 71% recovery.[15] In the current work, the increased limit of detection up to 60-fold over previous work can be attributed to the change to the utilization of HILIC chromatography, coupled with the exclusion of the ion-pairing reagent, TEA, and better analyte recovery using the Bligh/Dyer lipid extraction method.[25]

3.5 *Recovery*

Liquid-liquid extraction of the 16(Py)-S-2-S-(Py)16 gemini surfactant analytes gave more than 96% recovery using the Bligh/Dyer lipid extraction method while the recovery was 67.5-73.9% for octanol extraction (Table 3). The corresponding data for 16-3-16 is available in the supplementary materials (Table S3) showing similar trends. The recovery values were computed as proposed by Matuszewski *et al*,[30]:

$$\text{Recovery}(\%) = \frac{\text{Response from extracted cells}}{\text{Response from spiked-extracted cells}} \times 100 \qquad (2);$$

where the response from extracted cells refers to standards spiked before extraction where samples were processed as per the extraction procedure. Response from spiked extracted cells refers to known standards spiked after the extraction procedure was employed on cells.

For the separate extraction methods, the average recoveries were consistent for replicate determinations and were equal for the analyte (at three concentrations: lower, middle, and upper limit of quantitation). With such improved recovery (Figure 3 and Figure S5 in supplementary materials), our routine analyte extractions adopted the Bligh/Dyer extraction for its better efficiency, increasing the sensitivity of the LC-ESI-MS/MS method.

3.6 *Accuracy and precision*

In general, the validated method satisfied the USFDA-recommended accuracy and precision limits for all standard curve and quality control samples. Table 4 shows the accuracy and precision obtained for 16(Py)-S-2-S-(Py)16 gemini surfactant at four concentrations chosen per USFDA guidelines: LLOQ, LQC, MQC and HQC (corresponding data for 16-3-16 are available in the supplementary information, Table S4). For both the intra- and inter-day experiments, the accuracy (computed by expressing the observed concentrations as percentage of the theoretical values) varied between ~92 – ~110%. For precision, the values were within ~3 – ~11% relative standard deviation for both intra- and inter-day cases.

3.7 *Stability*

Both the 16(Py)-S-2-S-(Py)16 and 16-3-16 analytes were stable beyond 24 h when stored at room temperature (bench top stability) and long-term stability when stored at -20 ˚C (Table 5, Table S5). The analytes also showed very good freeze/thaw stability after three freeze/thaw cycles. The autosampler stability was within USFDA guidelines for accuracy/precision and was determined for samples re-injected for analysis for up to two consecutive days. Overall, the

stabilities were within ~96 – ~105% accuracy and ~2 – ~9% precision for all the determinations (Table 5, Table S5).

3.8 *Bio-analysis of P/G/L-nanoparticle-treated cells*

Using the validated HILIC-LC-MS/MS methods, the uptake and intracellular deposition profile of the 16(Py)-S-2-S-(Py)16 and 16-3-16 gemini surfactants was studied within PAM 212 cells treated with the gemini surfactant-containing P/G/L-nanoparticles. Figure 4 shows the results of an experiment as the intracellular concentration vs. time profile for 16(Py)-S-2-S-(Py)16 and 16-3-16. The intracellular concentration of the analyte increased rapidly throughout the 5-h duration when the administered nanoparticles remain in contact with cells, followed by a noticeable decrease within the next 5 h after removal of the nanoparticle-dosed supernatant culture media. The intracellular amount showed a decreased by ~60% after the initial rise. A similar trend was observed in the case of 16-3-16; however, its intracellular decreawas ~40% of the maximum value and occurred less rapidly than that observed for 16(Py)-S-2-S-(Py)16. It is important to note that we ran many batches for the analysis of the cellular fate of gemini surfactants. It was noted on occasions that during the post-validation application of the HILIC-LC-MS/MS method, the LLOQ quality control sample within the standard curve did not pass in terms of accuracy, despite performing the full method validation according to the USFDA as discussed above. Therefore, for scientific accuracy, the cellular data in Figure 4 was only reported for data with concentration above 200 ng/ml which is the concertation of the second data point within the standard curve. This did not affect the results or the observed trend shown in Figure 4.

The observations within the cellular data are consistent with a progressive nanoparticle uptake, which reached a maximum before a seeming depletion of the intracellular analyte. For both analytes, an observed intracellular remnant proportion did not undergo further depletion within the intracellular investigation lasting 54-h. For the 16-3-16 gemini surfactant, its partial depletion from the host cells is in disagreement with our recent reports[15,16] showing no depletion of this compound. The apparent discrepancy is attributed to differences in the extraction method and the extensive washing of treated cell cultures in the present work. Adequate washing steps were not conduced in the past work. Gemini surfactants tend to be adherent to bio-membranes and to plastics including walls of plastic cell culture containers. The washing was thus critical for eliminating potential gemini surfactants that is adherent to plastic walls or loosely adsorbed to exterior cell surfaces. To verify the results, the cell transfection experiments and the subsequent LC-ESI-MS/MS quantification procedures were repeated. The results reported attests to the consistency of the quantitative results on the uptake and subsequent intracellular profile of the investigated gemini surfactants.

4. DISCUSSIONS

The present study describes the development of fast and simple HILIC-MRM-MS/MS methods that were applicable to 18 gemini surfactants with varying molecular structures (Table 2). To-date, only one LC-ESI-MS/MS method has been reported for the quantification of gemini surfactants[15] but was only applicable to one class of gemini surfactants, the m-s-m family. This gives importance to the demonstrated applicability of the HILIC-LC-MS/MS platform to different structures of gemini surfactants.

The choice of HILIC chromatography (with sulfoalkyl betaine-based zwitterionic stationary phase, ZIC-HILIC column[31,32]) contributed to a high LLOD, which is 60-fold higher relative to the the method reported for the analysis of 16-3-16.[15] Our choice of the ZIC-HILIC column, based on its compatibility with the diquaternary ammonium gemini surfactant analytes through $^{+}N/^{-}SO_3$ HILIC mode interaction, led to more efficient chromatographic separation. Unlike the previous method,[15] no ion pairing reagent (i.e. TEA) was needed resulting in better ionization efficiency within the ESI source. TEA can compete for ionization within the ESI source resulting in undesirable ion suppression. Another major advantage of the HILIC-MS/MS is the use of isocratic elution rather than gradient system. In fact, the isocratic option was not possible when the *cyano* column was utilized.[15]

The mobile phase was optimized to ensure that retention times are established to correlate with the gemini surfactant structural properties; a low buffer concentration (2.5 mM formate) was used. This buffer concentration (lower than concentrations of up to 50 mM in some cases) allows the chance for dominant interaction between the stationary phase and the analyte so that the gemini surfactants can display specific retention times as a result of their different structural features. Here, an added potential benefit is that low buffer concentrations are associated with better sensitivity as opposed to high buffer concentrations. In addition, the buffered mobile phase was optimized to pH 6.2; deviation from this pH resulted in weak and inconsistent response as well as unstable retention times. The HILIC system is very sensitive to variations in analytical conditions including pH and temperature. In fact, deviation from the optimized column temperature resulted in increased pressure within the column thus preventing chromatographic separation.

In addition to adopting HILIC chromatography, a high efficiency analyte recovery was achieved through the use of Bligh/Dyer lipid extraction. Bligh/Dyer method uses 2:1 (v/v) methanol/chloroform binary extractant-solvent allowing for high compatibility with the gemini surfactants' amphiphilic nature[25] (as defined by having both hydrophobic and hydrophilic moieties, Figure 1). In fact, we have previously employed liquid-liquid extraction using either methanol or octanol with 63% and 70% recoveries[16], respectively. While methanol may favor the solubilisation of the hydrophilic component within gemini surfactants, octanol is more compatible with the hydrophobic elements. The methanol/chloroform system of the Bligh/Dyer method can, therefore, solubilize both components within gemini surfactants resulting in approximately 98% extraction efficiency. The high recovery is clearly illustrated in Figures 6 and S6 in which a direct comparison with octanol extraction is shown. Overall, the superior sensitivity achieved for the reported HILIC-LC-MS/MS method underscores a strategic use of HILIC chromatography in conjunction with high analyte extraction efficiency and a suitable mobile phase.

Data from the analysis of nanoparticle-treated cells showed an initial rise in the analyte intracellular concentration followed by a maximum and a somewhat more gradual decrease of the intracellular analyte. Herein, the findings are consistent with a progressive nanoparticle uptake, which reached a steady state before a seeming depletion of the intracellular analyte. Two events that may account for the intracellular depletion of the gemini surfactant are its bio-transformation into metabolites and its exocytosis from the host cells. An advantage with LC-ESI-MS/MS includes its versatility that allows adapting the described method, with major or minor modifications, for the investigation of both bio-transformation and exocytosis. The

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knowledge regarding the bio-fate of gemini surfactants can impact decisions regarding the safe use of biomedical nanoparticles as well as provide insights into engineering new nanoparticles.

CONCLUSION

We have developed simple and fast HILIC-LC-MS/MS methods for the determination of the 16(Py)-S-2-S-(Py)16 and 16-3-16 gemini surfactants in cells. The need for gradient elution as well as ion pairing reagent was eliminated, substantially simplifying the analytical methods. A high-efficiency liquid-liquid extraction was also adopted along with a ZIC-HILIC column to attain linear response and a run time of merely 4 minutes. The method is suitable for monitoring the fate of therapeutic nano-lipoplexes within cells. Obtained cellular data showed a pattern that grants additional investigations evaluating metabolite formation and assessing the subcellular distribution of tested compounds.

We are currently investigating the intracellular deposition of gemini surfactants as well as metabolite formation. Such knowledge can shed light into the varying toxicities reported for gemini surfactants.[19,33] Finally, it should be noted that gemini surfactants have many other industrial applications[34] such as detergents, cosmetics, and solubilisation agents. The newly developed versatile HILIC-LC-MS/MS can serve as a starting point for the analysis of gemini surfactants regardless of the application. The developed method was applicable to various structures of gemini surfactants making it a universal method that can be adopted by others who wish to quantify gemini surfactants, regardless of the application.

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FIGURES & FIGURE CAPTIONS:

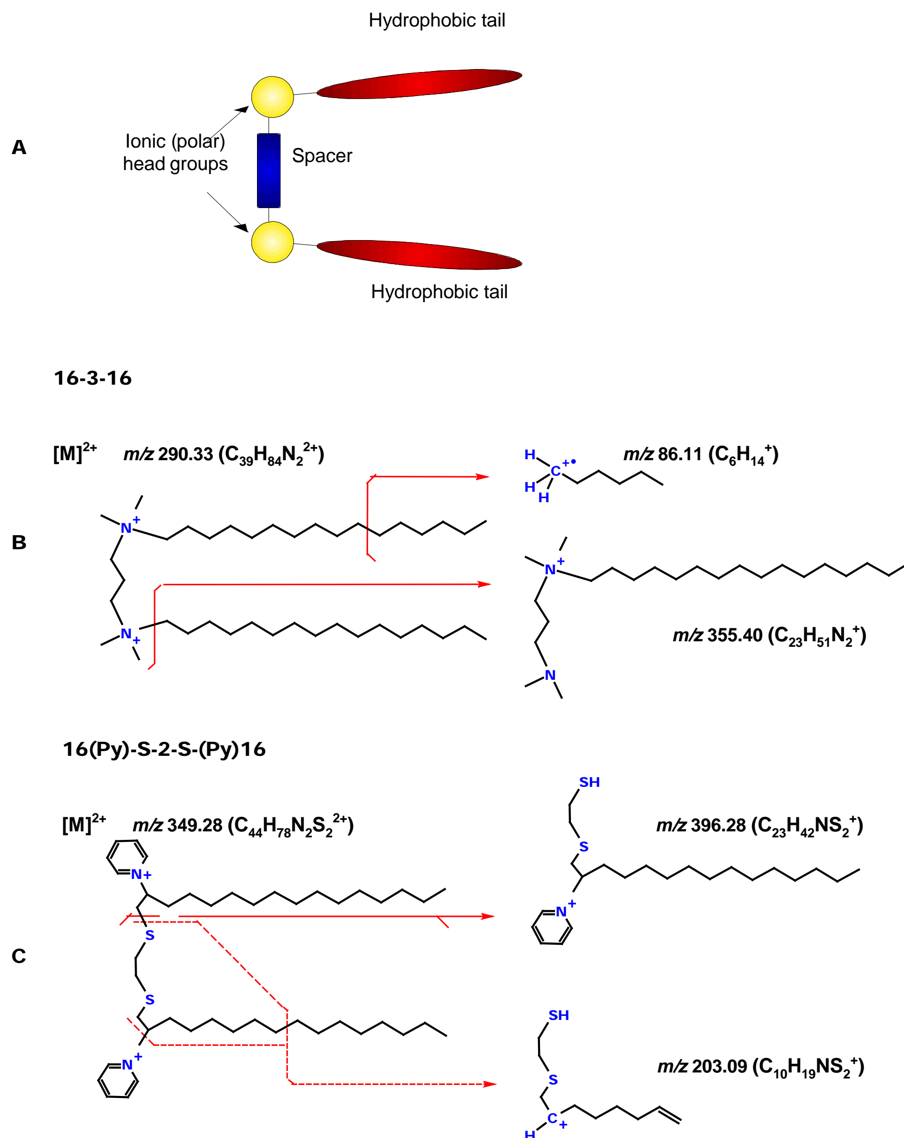

FIGURE 1: Schematic representation of gemini surfactant general structure (A). The exact molecular structures of intact compounds and monitored product ions of 16-3-16: *N,N*-bis(dimethylhexadecyl)-1,3-propanediammonium (B) and16(Py)-S-2-S-(Py)16: 1,1'-[ethane-1,2-diylbis(sulfanediylhexadecane-1,2-diyl)]dipyridinium (C)

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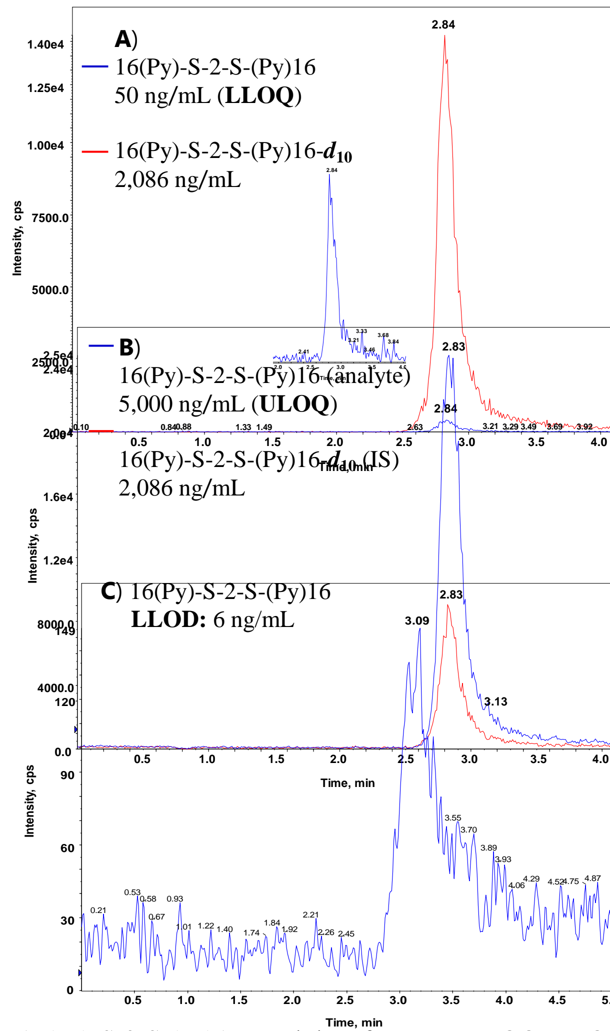

FIGURE 2: Chromatograms of the 16(Py)-S-2-S-(Py)16 gemini surfactant at LLOQ, ULOQ and LLOD. The relative response signal is shown for the analyte at: **A**) LLOQ – insert is a zoomed in spectrum of the analyte and **B**) ULOQ in relation to the internal standard, 16(Py)-S-2-S-(Py)16-d_{10}, which was present at a constant concentration. **C**) Extracted ion chromatogram for 16(Py)-S-2-S-(Py)16 at LLOD.

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Figure 3:

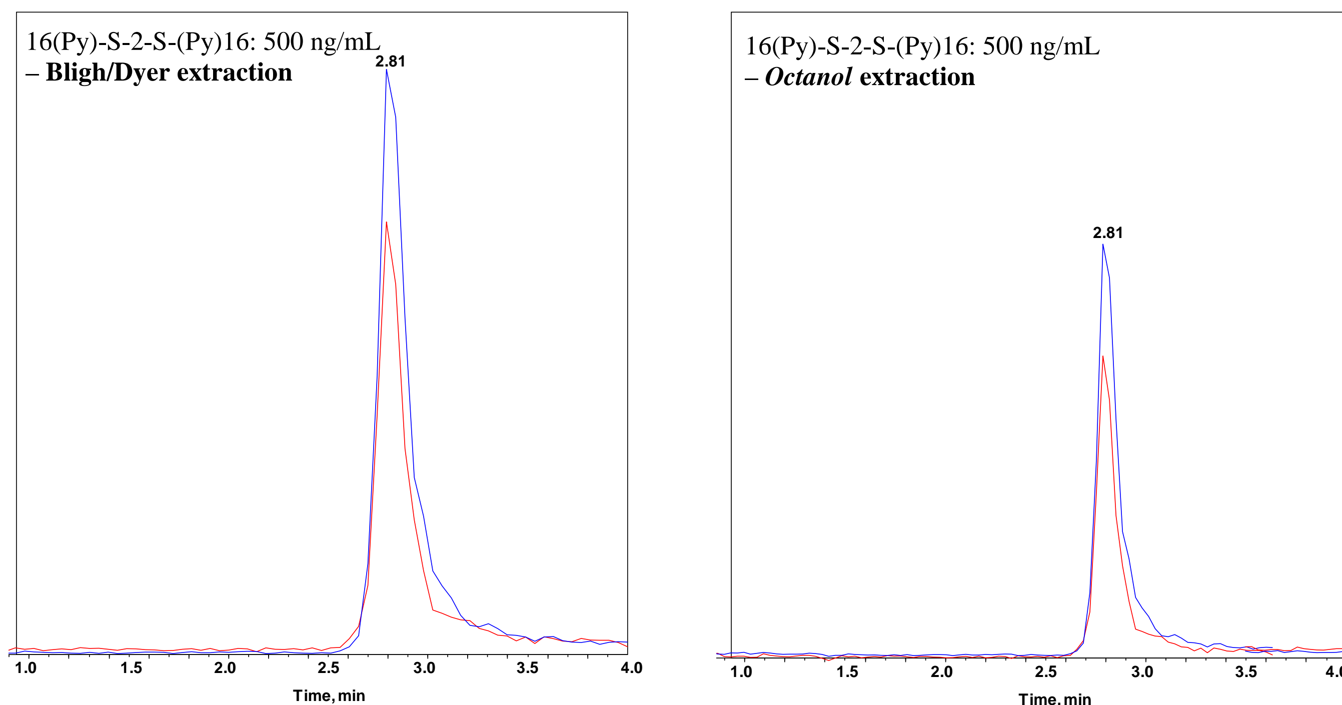

Figure 3. Analyte recovery efficiencies for Bligh/Dyer lipid extraction vs octanol extraction. Bligh/Dyer lipid extraction gave a better recovery (typically 98%) of the analyte and was the chosen liquid-liquid extraction method, departing from a recent report in which octanol extraction (70% efficiency) was used. Unsurprisingly, the new HILIC-LC-MS/MS methods reported herein show better sensitivity (60-fold increase).

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Figure 4:

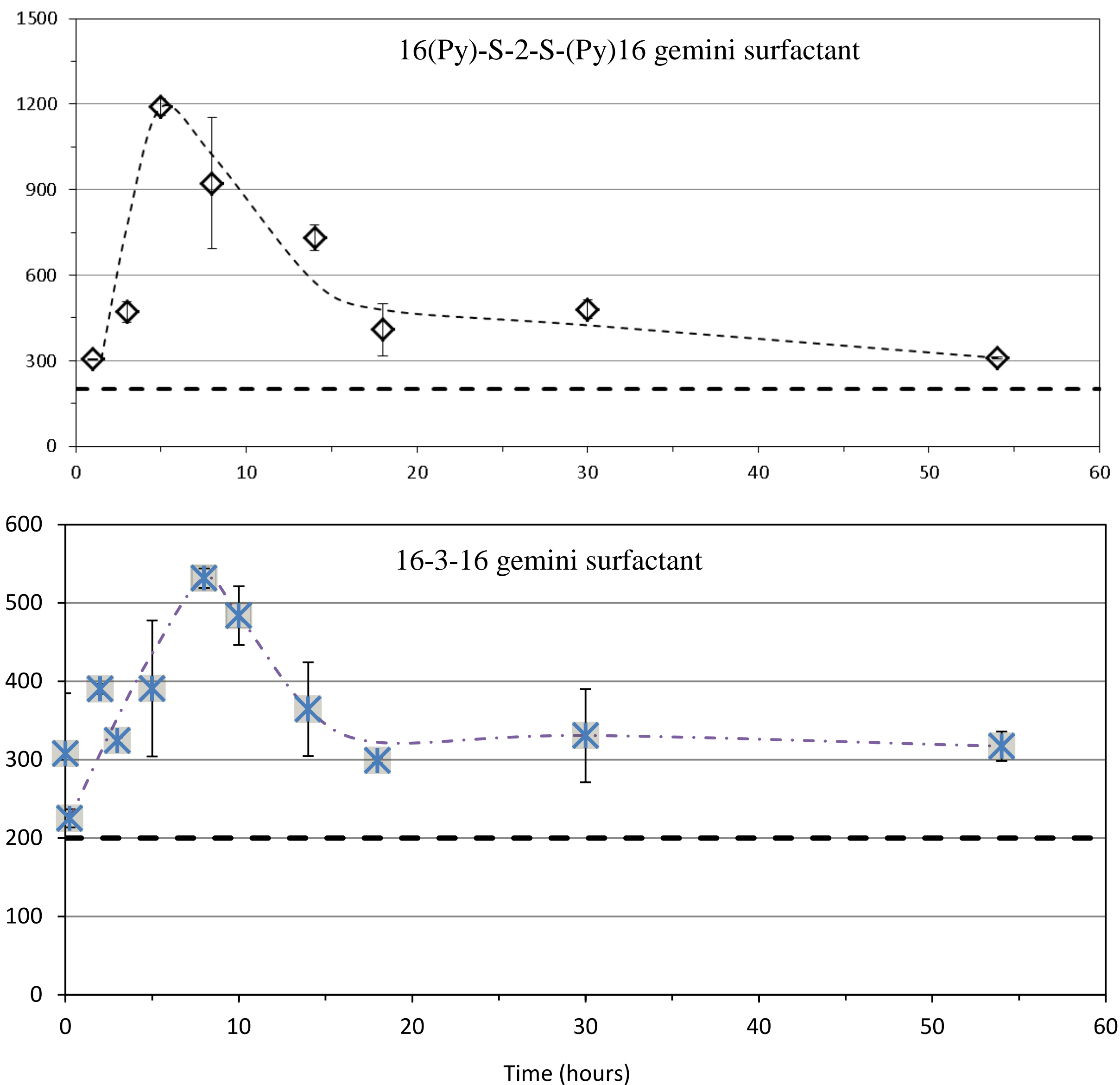

Figure 4. Representative experiments showing the intracellular concentration vs. time profile of the gemini surfactants, 16(Py)-S-2-S-(Py)16 (top panel) and 16-3-16 (bottom panel). The intracellular concentration increased progressively throughout the duration of nanoparticle administration to cells, followed by a gradual decrease after removal of the nanoparticle-dosed supernatant culture media. Each plotted data point represents mean ± SD, n = 3.

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TABLES & TABLE CAPTIONS:

Table 1. Conditions for MRM transitions of the analytes on AB Sciex 4000 QTRAP® System

Analyte		Transition		DP*	CE*	CXE*
Gemini surfactant	Molecular Formula	$[M]^{2+} \rightarrow [M - X]^{+}$	*m/z* → *m/z*	(eV)	(eV)	(eV)
16(Py)-S-2-S-(Py)16	$C_{44}H_{78}N_2S_2^{2+}$	$[M]^{2+} \rightarrow [M - C_{21}H_{36}N]^{+}$	*m/z* 349 → 396	30	22	10
16(Py)-S-2-S-(Py)16	$C_{44}H_{78}N_2S_2^{2+}$	$[M]^{2+} \rightarrow [M - C_{34}H_{59}N_2]^{+}$	*m/z* 349 → 203	30	22	10
16(Py)-S-2-S-(Py)16-$\boldsymbol{d_{10}}$	$C_{44}H_{68}\mathbf{D_{10}}N_2S_2^{2+}$	$[M]^{2+} \rightarrow [M - C_{21}H_{31}\mathbf{D_5}N]^{+}$	*m/z* 354 → 401	30	25	10
16-3-16	$C_{39}H_{84}N_2^{2+}$	$[M]^{2+} \rightarrow [M - C_{16}H_{33}]^{+}$	*m/z* 290 → 355	40	21	10
16-3-16	$C_{39}H_{84}N_2^{2+}$	$[M]^{2+} \rightarrow [M - C_{33}H_{70}N_2]^{+}$	*m/z* 290 → 86	40	35	6
16-3-16-$\boldsymbol{d_{66}}$	$C_{39}H_{18}\mathbf{D_{66}}N_2^{2+}$	$[M]^{2+} \rightarrow [M - C_{16}\mathbf{D_{33}}]^{+}$	*m/z* 323 → 388	35	25	10

***Abbreviation:**
DP: declustering potential, CE: collision energy, CXE: collision cell exit potential; X: neutral loss

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Table 2. HILIC-LC-MS/MS bio-analysis of 17 gemini surfactants with varying molecular structures

Gemini surfactant Symbol	Spacer region		Tail region		MRM transitions	Retention time
	Name	Molecular formula	Name	Molecular formula	*m/z* → *m/z*	(min)
12-3-12	Propyl	C_3H_6	Dodecyl	$C_{12}H_{25}$	234 → 299	2.96
12-4-12	Butyl	C_4H_8	Dodecyl	$C_{12}H_{25}$	241 → 313	2.91
12-8-12	Octyl	C_8H_{16}	Dodecyl	$C_{12}H_{25}$	269 → 369	2.57
12-12-12	Dodecyl	$C_{12}H_{24}$	Dodecyl	$C_{12}H_{25}$	297 → 425	2.43
12-16-12	Hexadecyl	$C_{16}H_{32}$	Dodecyl	$C_{12}H_{25}$	325 → 481	2.42
16-3-16	Propyl	C_3H_6	Hexadecyl	$C_{16}H_{33}$	290 → 355	2.86
16-7-16	Heptyl	C_7H_{14}	Hexadecyl	$C_{16}H_{33}$	318 → 411	2.52
18-3-18	Propyl	C_3H_6	Octadecyl	$C_{18}H_{37}$	318 → 383	2.44
18-7-18	Heptyl	C_7H_{14}	Octadecyl	$C_{18}H_{37}$	346 → 439	2.37
18:1-3-18:1	Propyl	C_3H_6	Octadec-9-ene	$C_{18}H_{35}$	316 → 381	2.57
18:1-6-18:1	Hexyl	C_6H_{12}	Octadec-9-ene	$C_{18}H_{35}$	337 → 423	2.50
12(Py)-S-2-S-(Py)12	1,2-ethanedithiol	$C_2H_4S_2$	Dodecyl	$C_{12}H_{25}$	293 → 340	2.86
14(Py)-S-2-S-(Py)14	1,2-ethanedithiol	$C_2H_4S_2$	Tetradecyl	$C_{14}H_{29}$	321 → 368	2.79
16(Py)-S-2-S-(Py)16	1,2-ethanedithiol	$C_2H_4S_2$	Hexadecyl	$C_{16}H_{33}$	349 → 396	2.78
18(Py)-S-2-S-(Py)18	1,2-ethanedithiol	$C_2H_4S_2$	Octadecyl	$C_{18}H_{37}$	377 → 424	2.63
12-7NH-12	Dipropylamine	$C_6H_{13}N$	Dodecyl	$C_{12}H_{25}$	263 → 356	7.12
Py-3-12	Propyl	C_3H_6	Dodecyl, Hexapyrenyl,	$C_{12}H_{25}$, $C_{22}H_{23}$	293 → 417	2.96

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Table 3. Recovery of 16(Py)-S-2-S-(Py)16 from the aqueous cellular matrix

Theoretical concentration	Bligh/Dyer extraction			*Octanol* extraction		
	Extracted cells (mean ± SD, ng/mL)	**Spiked extracted cells** (mean ± SD, ng/mL)	Recovery (%)	**Extracted cells** (mean ± SD, ng/mL)	**Spiked extracted cells** (mean ± SD, ng/mL)	Recovery (%)
16(Py)-S-2-S-(Py)16						
50 ng/mL (LLOQ)	47.44 ± 4.73	48.31 ± 3.74	96.2	32.88 ± 4.42	48.73 ± 4.74	67.5
150 ng/mL (LQC)	151.16 ± 10.61	155.36 ± 9.31	97.3	104.51 ± 11.02	145.36 ± 8.98	71.9
375 ng/mL (MQC)	369.75 ± 41.33	377.68 ± 37.93	97.9	270.87 ± 19.16	375.68 ± 47.92	72.1
4375 ng/mL (HQC)	4309.51 ± 359.41	4344.25 ± 222.53	99.2	3209.64 ± 179.11	4343.22 ± 182.33	73.9

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Table 4. Intra- and inter-day accuracy and precision in the analysis of 16(Py)-S-2-S-(Py)16

Quality Control		Observed concentration (mean ± SD, ng/mL)	Accuracy (%)	Precision (%RSD)
INTRA-DAY:				
	Analysis Day (#)			
LLOQ: 50 ng/mL	Day 1	46.44 ± 4.73	92.9	10.2
	Day 2	51.26 ± 5.08	102.5	9.9
	Day 3	55.06 ± 3.33	110.1	6.0
LQC: 150 ng/mL	Day 1	145.96 ± 8.61	97.3	9.0
	Day 2	154.64 ± 13.9	103.1	8.4
	Day 3	161.14 ± 9.99	107.4	6.2
MQC: 375 ng/mL	Day 1	363.75 ± 11.33	97.0	3.1
	Day 2	393.36 ± 21.73	104.9	5.5
	Day 3	369.03 ± 19.77	98.4	5.4
HQC: 4375 ng/mL	Day 1	4032.50 ± 359.41	92.2	8.9
	Day 2	4411.38 ± 272.55	100.8	6.2
	Day 3	4321.99 ± 323.09	98.8	7.5
INTER-DAY:				
	Concentration			
LLQC	50 ng/mL	51.15 ± 4.84	102.3	9.7
LQC	150 ng/mL	157.25 ± 10.84	104.8	7.7
MQC	375 ng/mL	375.38 ± 17.61	100.1	4.7
HQC	4375 ng/mL	4255.29 ± 318.53	97.3	7.5

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Table 5. Stability of 16(Py)-S-2-S-(Py)16 analyte within the sample matrix

Quality Control	Storage condition/ period	Observed concentrations (mean ± SD, ng/mL)	Accuracy (%)	Precision (%RSD)
LQC: 150 ng/mL	0 h	150.33 ± 11.14	102.3	9.7
	24 h on bench top	148.83 ± 10.04	99.3	7.7
	Three freeze/thaw cycles	145.87 ± 13.13	94.3	8.5
	–20 ˚C for 105 days (LT)	146.16 ± 9.91	96.7	10.1
	48 h extract in autosampler	150.15 ± 8.43	96.6	9.2
MQC: 375 ng/mL	0 h	365.03 ± 18.18	102.3	9.7
	24 h on bench top	381.73 ± 17.98	99.3	7.7
	Three freeze/thaw cycles	355.05 ± 18.17	94.3	8.5
	–20 ˚C for 105 days (LT)	355.22 ± 18.68	96.7	10.1
	48 h extract in autosampler	375.88 ± 15.76	96.6	9.2
HQC: 4375 ng/mL	0 h	4358.23 ± 198.08	102.3	9.7
	24 h on bench top	4398.23 ± 201.27	99.3	7.7
	Three freeze/thaw cycles	4151.53 ± 229.38	94.3	8.5
	–20 ˚C for 105 days (LT)	4324.56 ± 186.83	96.7	10.1
	48 h extract in autosampler	4308.93 ± 191.77	96.6	9.2

LT (long term): –20 ˚C for 105 days

…|…