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The determination of gemini surfactants used as gene delivery agents in cellular matrix using validated tandem mass spectrometric method

Jin Wei [1], Ildiko Badea[1], Scot C. Leary[2], Anas El-need[1]*

[1] Drug Design & Discovery Group, College of Pharmacy and Nutrition, University of Saskatchewan, 107 Wiggins Road, Saskatoon, SK S7N 5E5, Canada.

[2] Department of Biochemistry, University of Saskatchewan, 107 Wiggins Road, Saskatoon, SK S7N 5E5, Canada.

*Corresponding author. E-mail address: anas.el-aneed@usask.ca (A. El-Aneed).

ABSTRACT:

A simple, reliable flow injection analysis (FIA)-tandem mass spectrometric (MS/MS) method was developed for the determination of gemini surfactants, designated as 16-3-16, 16(Py)-S-2-S-(Py)16 and 16-7N(GK)-16, as gene delivery agents in cellular matrix. 16-3-16 is a conventional gemini surfactant bearing two quaternary amines, linked by a 3-carbon spacer region, 16(Py)-S-2-S-(Py)16 contains two pyridinium head groups, while 16-7N(GK)-16 bears a glycine-lysine di-peptide in the space region. The method was fully validated according to USFDA guidelines. It is the first time that FIA-MS/MS method was developed for the quantification of gemini surfactants, belonging to different structural families. The method was superior to existing liquid chromatographic (LC)-MS/MS methods in terms of sensitivity and time of analysis. Positive electrospray ionization (ESI) in the multiple reaction monitoring (MRM) mode were used on a triple quadrupole-linear ion trap (4000 QTRAP®) instrument. Deuterated internal standards were used to correct for matrix effects and variations in ionization within the ESI source. Isotope dilution standard curves were established in cellular matrix, with a linear range of 10nM-1000nM for 16-3-16 and 16(Py)-S-2-S-(Py)16, and 20nM-2000nM for 16-7N(GK)-16. The precision, accuracy, recovery and stability were all within the acceptable ranges as per the USFDA guidelines. The method was successfully applied for the quantification of target gemini surfactants in the nuclear fraction of PAM 212 keratinocyte cells treated with nanoparticles, which varied significantly and may explain differences in the observed efficiency and/or toxicity of these gemini surfactants in gene delivery.

Keywords: FIA-MS/MS, quantification, gemini surfactants, lipid-based gene delivery agents, cellular matrix

1 Introduction

Gene therapy is a promising approach to treat or improve the health condition of patients by introducing therapeutic genetic materials into the patient's cells [1-4]. To date, almost 2,600 gene therapy clinical trials have been conducted worldwide, with more than half of them being in the field of cancer gene therapy [5]. The most difficult challenge in gene therapy is the issue of gene delivery. Typically, there are two main types of gene delivery methods; viral and non-viral vectors [6]. Viral vectors utilize the viruses' natural infection capability to introduce the target gene into cells [7, 8]. While viruses are efficient vectors for transfection, a limited quantity of genetic material can be delivered and they present challenges with respect to potential genotoxicity and induction of a severe immune response [9, 10]. On the other hand, non-viral vectors have relatively low toxicity profiles, are not limited to the size of genes they can encapsulate, and can be easily produced at low cost [4, 11, 12]. However, the major disadvantage of non-viral vectors is their low transfection efficiency compared to viral vectors [13]. Thus, major efforts have been made to discover and develop novel non-viral vectors that offer both high transfection efficiency and low toxicity.

A family of lipid cationic molecules, called gemini surfactants, has been investigated as gene delivery vehicles [14, 15]. They are comprised of two surfactant monomers that are chemically linked by a rigid spacer group [16]. Gemini surfactants possess dual positively charged hydrophilic head groups and hydrophobic tail regions [17] (Figure 1A). This structure enables gemini surfactants to bind and compact DNA, and subsequently facilitate their cellular entry [18, 19]. For example, the conventional bis-quaternary gemini surfactant 14-2-14 and the serine-derived bis-quaternary gemini surfactants (nSer)$_2$N5 (n=12 and 14) were shown to efficiently deliver plasmid DNA into mitochondria in HeLa cells in combination with the helper lipids 1, 2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (DOPE) and cholesterol [20]. A transfection of up to 40% of the cells was achieved, which is almost twice of that obtained with commercial transfection agent, Lipofectamine 2000.

By varying the head group, the length of the hydrophobic tails and the spacer region, a wide range of gemini surfactant compounds can be designed and synthesized. The aim is to increase efficacy while reducing toxicity. N, N-bis(dimethylhexadecyl)-1,3-propanediammonium (denoted 16-3-16, Figure 1B) is a representative of the traditional non-substituted *m-s-m* gemini surfactants, where *m* is the number of carbon atoms in the tail chain and *s* is number of carbon atoms in the spacer region [17]. Other classes of gemini surfactants with variations in the head group have also been studied, such as 1,1'-[ethane-1,2-diylbis(sulfanediylhexadecane-1,2-diyl)]dipyridinium (denoted 16(Py)-S-2-S-(Py)16, Figure 1C) [21], which displayed good DNA binding property and low cytotoxicity [21]. Most recently, bio-compatible moieties, such as amino acids, have been incorporated within the gemini surfactant structure to enhance the bio-compatibility of the delivery agent [22]. One compound, in particular, substituted with the di-peptide glycine-lysine (denoted 16-7N(GK)-16, Figure 1D) showed higher transfection efficiency with lower toxicity in comparison to earlier generations of gemini surfactants [23].

However, these gemini surfactants nonetheless vary amongst each other in terms of their gene transfer efficiency and toxicity profile. This could theoretically be attributable to their different biological fates upon transfection, such as variable cellular uptake, subcellular biodistribution and/or metabolism. Garnering such knowledge is important and may

provide a mechanistic explanation for the observed differences among these gemini surfactants in their efficiency and toxicity. However, there is a need for sensitive analytical methods that can detect, differentiate and quantify these gemini surfactants in biological matrices to determine their post-transfection fate.

Since the majority of gemini surfactants lack a chromophore or fluorophore and contain permanent positive charges, mass spectrometry (MS) is ideal for their qualitative and quantitative analysis [24-26]. Tandem mass spectrometry (MS/MS) using multiple reaction monitoring (MRM) allows for the detection and quantification of gemini surfactants using precursor ion-to-product ion diagnostic transitions, which provides specificity to target gemini surfactants in complex biological matrices. Our group has established the collision-induced dissociation (CID)-MS/MS fragmentation patterns of over 50 gemini surfactant structures belonging to various structural families and identified their diagnostic product ions [27-31]. Subsequently, we developed liquid chromatography electrospray ionization (LC-ESI)-MS/MS methods employing cyano and hydrophilic interaction liquid chromatography (HILIC) columns for the quantification of gemini surfactants within cells [25, 26]. However, these methods suffered from some drawbacks, such as ion suppression due to the addition of an ion pairing reagent, relatively long run times, and the use of an analytical column and gradient elution. Therefore, we aim herein to develop a simple and reliable method that can quantify the gemini surfactants in cellular matrix.

Flow injection analysis (FIA)-MS/MS is an analytical approach for the rapid quantitative analysis, in which no analytical column is used and both separation and detection occur simultaneously within the MS instrument [32]. The removal of the analytical column can substantially decrease the time of method development, increase the speed of analysis and simplify the acquisition of quantitative data. Furthermore, FIA-MS/MS offers high-throughput quantitative analysis without compromising the sensitivity, precision and accuracy [33]. Many studies have demonstrated the feasibility of FIA-MS/MS for the quantification of small molecules in biological samples [34-36]. For example, FIA-MS/MS has been used to quantify nifedipine in human plasma samples, with high sensitivity, selectivity and a short run time [37]. Similarly, the drug metformin was quantified in dog plasma after developing a validated FIA-MS/MS method that was superior to all existing methods in terms of speed of analysis without compromising sensitivity [38]. Most recently, a simple FIA-MS/MS method has been developed for the cutaneous determination of peptide-modifed cationic gemini surfactants used as gene delivery vectors [39].

In this work, we report for the first time the development and validation of a fast and simple FIA-MS/MS quantification method that was applied for the quantification of structurally different gemini surfactants at the subcellular level.

2 Materials and Methods

2.1 Materials

All gemini surfactants and the deuterated internal standards were previously synthesized using established protocols [21, 23, 40]. Their structures are listed in Figures 1 and S1 (supporting information). For analyte 16-3-16, the internal standard (16-3-16-D_{66}) contains 66 deuterium atoms in the alkyl tails. Analyte 16(Py)-S-2-S-16(Py) has an internal standard (16(Py)-S-2-S-16(Py)-D_{10}) with deuterated pyridinium head groups (10 deuterium atoms), while the internal standard (16-7N(GK)-16-D_4) for analyte 16-7N(GK)-16 possesses 4 deuterium atoms on the peptide group (Figure S1 in supporting information).

PAM 212 keratinocyte cells were kindly provided by Dr. S. Yuspa, National Cancer Institute, Bethesda, MD, USA. The neutral lipid, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Formic acid, chloroform, methanol and acetonitrile were purchased from Fisher Scientific (Ottawa, ON, Canada). Minimum essential media (MEM), fetal bovine serum albumin (FBS) and antibiotic-antimycotic solution were obtained from Sigma-Aldrich (Oakville, ON, Canada). Tissue culture flasks (150cm^2) and petri dishes (150cm^2) were purchased from Fisher Scientific (Ottawa, ON, Canada). The motorized tissue-grinder/pellet homogenizer (#12-141-361, 12-141-368) was purchased from Fisher Scientific (Toronto, ON, Canada).

2.2 Instrumentation

A quadrupole-linear ion trap (4000 QTRAP®) mass spectrometer (AB Sciex, Concord, ON, Canada) was coupled with an Agilent 1200 series HPLC, comprised of a quaternary pump, degasser and auto sampler (Agilent Technologies, Mississauga, ON, Canada), to perform the FIA-MS/MS analysis. 3µL of sample at 6°C was loop-injected into the turbo ion source with an isocratic mobile phase consisting of acetonitrile-water (98:2, v/v) with 0.1% formic acid at a flow rate of 0.5mL/min. The data acquisition time is 2min. No sample carryover was observed and to eliminate any chance of carryover, the injection of the highest calibration curve sample was followed by one blank sample injection during sample analysis.

The source was set at 600°C at the interface, with ion spray voltage (ISV) at 5500V, curtain gas (CUR) at 30, nebulizer gas (GS1) at 55, and heater gas (GS2) at 50. Nitrogen was used for all gas consumption. Multiple reaction monitoring (MRM) in positive electrospray ionization (PESI) mode with unit resolution was employed to monitor all analytes and internal standards. Two MRM transitions were monitored for each analyte, with one as a quantifier ion and the other as a qualifier ion (Figure 1); one MRM transition was used for each internal standard (Figure S1, supporting information). Dwell times for all transitions were set at 150ms. The monitored MRM transitions and compound-dependent parameters for analytes and internal standards are listed in Table 1. Data acquisition and analysis was performed with Applied Biosystems/MDS Sciex Analyst software (v. 1.6.0).

2.3 Standard preparation

All gemini surfactants and internal standards were prepared as aqueous stock solutions at a concentration of 3mM, and stored at -80°C under darkness. A working solution for each

analyte was prepared by serial dilution of the stock solution to achieve a concentration range of 50nM-5000nM for 16-3-16 and 16(Py)-S-2-S-(Py)16, and 100nM-10000nM for 16-7N(GK)-16. Working solutions for each internal standard were prepared at a concentration of 1000nM. For the preparation of the standard curve and quality control samples, 50µL of analyte and internal standard working solutions were added to 900µl of blank cell lysate (untreated cell lysate), the mixture then was processed in the same sample extraction process as described below. After extraction, 150µL of organic solution was transferred into an HPLC vial for FIA-MS/MS analysis.

DOPE vesicles were prepared freshly at 1mM in isotonic sucrose solution (9.25% w/v, pH=9) as per established protocol [14]. Plasmid DNA (pGT·IFN-GFP) solution at 200µg/mL was prepared in ultra-pure water and stored at -80℃. The DOPE vesicles and plasmid DNA solutions were used without further dilutions.

2.4 Cell treatment and sample collection

The plasmid DNA, gemini surfactant and lipid DOPE (P/G/L) nano-lipoplex was formulated as previously described [14]. Briefly, 190µL of gemini surfactant was added to 190µL of plasmid DNA and mixed, followed by a 15min incubation at room temperature; 4620µL of DOPE solution was then added to the binary mixture, which was incubated at room temperature for 15min to yield 5000µL of the ternary P/G/L system (nanoparticles).

PAM 212 cells were cultured inside a humidified incubator at 37°C in an atmosphere of 5% CO_2. The MEM cell culture medium was supplemented with 10% (v/v) FBS and 1 % (v/v) antibiotic-antimycotic solution. Upon reaching 80% confluence in the 150cm^2 flasks, cells were washed with phosphate buffered saline (PBS, 25ml), dissociated with a 5min incubation in a versene (5ml) and trypsin (0.5mL) mixture and collected by centrifugation (1,200rpm, 5min, 4°C). The cells were then seeded at 8×10^6 cells per dish (150cm^2) 24h prior to treatments. At 1h prior to transfection, cells were switched to serum free media. Nanoparticle formulations (500µL) were added to each dish in a dropwise manner and incubated for 5h, after which the cells were returned to supplemented media for all subsequent incubation steps. During the incubation period, triplicates of treated cell samples were trypsinized and collected along with one control (untreated cells) at 2h, 5h and 8h. The collected cells were pelleted (1,200rpm, 5min, 4°C), rinsed with PBS, reconstituted in 500µL ice-cold hypotonic homogenization buffer (10mM NaCl, 1.5mM $MgCl_2$, 10mM Tris-HCL (pH=7.5), cOmplete™ protease inhibitor cocktail) and incubated on ice for 10min.

The ice-cooled 500µL treated cell samples were gently homogenized using a motorized tissue-grinder/pellet homogenizer to release the subcellular components, and diluted in ice-cold hypertonic homogenization buffer (420mM mannitol, 140mM sucrose, 10mM Tris-HCL (pH=7.5), 2mM EDTA (pH=7.5)) to a total volume of 1000µL. Cell homogenates were then fractionated by differential centrifugation as described [41] to obtain the nuclear, mitochondrial, plasma membrane and cytosolic fractions. It is necessary to mention that the reported differential centrifugation procedure was slightly modified using 100,000 *x g*, instead of 80,000 *x g*, to separate the mitochondrial and cytosolic fractions. Finally, all fractions were suspended in an equal volume of 950µL PBS and stored at -80°C prior to sample preparation.

2.5 Sample preparation

The 950µL subcellular fractions were thawed and lysed by undergoing six freeze/thaw cycles plus 1h sonication at 25kHz on a water bath at room temperature. After that, each sample was spiked with 50µL of internal standard bringing the volume for subsequent extraction to 1mL. Liquid-liquid extraction of the analytes and internal standards from cellular matrix was carried out using the Bligh/Dyer method [42]. Briefly, each 1mL of sample was mixed with 3.75mL of methanol-chloroform (2:1, v/v), followed by mixing with 1.25mL of chloroform and finally 1.25mL of water. At each step, samples were vortexed for at least 10s to ensure that a thorough mixing was achieved. The final combined mixture was centrifuged at 4000rpm for 10min at room temperature to separate the aqueous and organic phases. The bottom organic phase (80% portion) was retrieved and dried under a N_2 gas stream, followed by reconstitution in 200µL of methanol. 150µL of methanol solution was transferred into an HPLC vial for FIA-MS/MS analysis.

2.6 Method validation

Method validation for all gemini surfactants was conducted in accordance with USFDA guidelines [43], which include matrix effects, selectivity, linearity, precision, accuracy, recovery and stability.

Matrix effects were assessed by comparing the instrument response of analytes added to the extracted cell samples to that of analytes in a methanol solution at low, mid and high concentration. Selectivity was evaluated to ensure no interference from other components of the sample matrix through the analysis of six different blank cell samples.

Linearity was explored over a wide range of analyte concentrations in the sample extract, from 10nM to 1000nM for both 16-3-16 and 16(Py)-2-S-2-(Py)-16, and from 20nM to 2000nM for 16-7N(GK)-16. Standard curves were constructed by plotting the ratio of peak areas of analytes to peak areas of internal standards versus the analyte concentrations using the least-square regression with a weighting factor of $1/x$. Each standard curve was established with the slope, intercept and coefficient of determination (r^2). The curve was accepted if the ± 15% deviation of the nominal value for each standard point other than LLOQ, which can be ± 20%, is achieved. The lower limit of detection (LLOD) was set as the lowest detectable concentration with a signal-to-noise ratio (S/N) ≥3, while the lower limit of quantification (LLOQ) was set as the lowest concentration with S/N ≥5, with precision of ± 20% coefficient of variation (CV) and accuracy of ± 20% deviation from the nominal value as per the USFDA guidelines [43].

Precision and accuracy of the method were determined by analysis of six replicates of quality control samples at four different concentrations (LLOQ; low quality control (LQC), middle quality control (MQC), and high quality control (HQC)). The LQC was within 3 fold of the LLOQ, the MQC was at the middle part of the standard curve range, and the HQC was within 80% of the upper limit of quantitation (ULOQ). Two runs per day were conducted across three consecutive days to assess the intra- and inter-day precision and accuracy. Precision was accepted if the CV is ± 15% for concentrations other than the LLOQ, which is allowed to be ± 20%; while accuracy was accepted if they were ± 20% deviation of the nominal value for the LLOQ and ± 15% deviation of nominal values for other concentrations.

Recovery experiments were conducted by preparing one set of samples with analyte pre-spiked prior to extraction and the other set with analyte post-spiked after extraction, and comparing the peak areas of analytes obtained from each set. For each analyte, three different concentrations at LQC, MQC and HQC levels in each set were used to determine the recovery.

Freeze-thaw stability, bench-top stability, auto-sampler stability and long-term stability were carried out using samples with concentrations at LQC, MQC, and HQC levels. Six replicates of samples at each concentration were prepared for the stability evaluation. Freeze/thaw stability was tested after all samples had gone through six freeze-thaw cycles, with one cycle involving taking out samples stored at -80°C for at least 24h and allowing them to thaw completely at room temperature prior to refreezing. Bench top stability was evaluated after the samples were placed on the bench at room temperature for 8h and then extracted and analyzed. For the auto-sampler stability, a set of samples was prepared and placed in the auto-sampler at 6°C for 20h prior to injection for analysis. Long-term stability was tested for samples that were stored at -80°C for 90 days. All samples were analyzed along with freshly prepared standard curves. Samples were considered stable when the USFDA criteria for precision and accuracy were met [43].

3 Results and discussion

3.1 Method development

In this work, we aimed at developing a simple and reliable method that can quantify the three target gemini surfactants in biological matrix for the assessment of their uptake and biodistribution in cells. Although gemini surfactants have been previously separated and determined using LC-MS/MS methods with various analytical columns, such as cyano [25] and HILIC columns [26], all of these methods require relatively long run time for the separation and the prior optimization of LC for the analysis. Therefore, we chose to develop a FIA-MS/MS method that relies on the mass spectrometer's separation capability, as the MRM mode in the quadrupole-linear ion trap system has the capability of selectively monitoring and accurately quantifying the analytes of interest in complex matrices.

Optimization of the FIA-MS/MS condition was the main focus in the process of method development. All source-dependent parameters, such as nebulizer gas (Gas 1) and heater gas (Gas 2), and compound-dependent parameters (i.e. DP, EP, and CE) were properly optimized so that a high sensitivity of the MRM transitions for each analyte and internal standard can be achieved. Two MRM transitions were selected for each analyte in this method; the qualifier transition was used to confirm the presence of the analyte peak, whereas the quantifier transition was used for the calculation of the concentration of analyte. Matrix effects have been identified as a challenge in ESI-MS/MS analysis due to the inconsistency in ion current response [44]. To correct for such variations, we used deuterium-labelled gemini surfactants as internal standards that have similar physicochemical properties and MS behavior to the analytes. The deuterated gemini surfactants possess various number (n=4, 10 and 66) of deuterium atoms (Figure S1, supporting information), which resulted in large mass differences between the analytes and their respective internal standards and thus eliminated the potential for cross-talk. In addition, we did not observe any interference from the endogenous compounds in the biological matrix for all standards.

For the optimization of FIA, different compositions of solvent mobile phases, including acetonitrile, methanol and water mixed with varying concentrations of formic acid, were tested. Acetonitrile was found to be superior to methanol in terms of obtaining better peak shape and reducing carry over, as gemini surfactants are amphiphilic compounds that tend to stick to the injection loop and tubing (Figure 2). Aqueous solvent and acid condition are required to achieve high ionization efficiency of gemini surfactants for better detection sensitivity. As a result, acetonitrile-water (98:2, v/v) with 0.1% formic acid was the best mobile phase system that can obtain high ionization efficiency, minimize peak tailing for all analytes and internal standards, and reduce the carry over (Figure S2 in supporting information)

The Bligh/Dyer extraction method was adapted in this study as it was reported previously to be highly efficient at extracting lipids, including gemini surfactants, from biological matrices [26, 42]. In this method, methanol-chloroform (2:1, v/v) was used as the binary extraction solvent, because it is highly compatible with the amphiphilic nature of the gemini surfactants and thus results in high extraction efficiency. To minimize variation in extraction efficiency across samples, internal standards were spiked into samples at appropriate concentrations prior to extraction.

3.2 Selectivity and matrix effects

Selectivity was assessed with the analysis of six different blank cell matrices. These blank cell samples did not contain either the analyte or the internal standard, and a typical chromatogram for the blank cell sample is shown in Figure S3 (supporting information). As illustrated, no interference peak from endogenous compound was observed in the analyte and the internal standard channels from the cell matrix. Furthermore, no cross-talk was observed between the analytes and the internal standards as they have large mass differences due to the presence of multiple (n=4, 10 and 66) deuterated atoms in the structures of internal standards. The peaks of analytes and internal standards eluted at 0.11min within the data acquisition time of 2min (Figure 3).

Matrix effects were evaluated by comparing the analyte response in the post-extracted spiked sample with non-extracted neat sample. Three different concentrations for each analyte in three replicates were used for the evaluation. The matrix effects across all concentration levels were calculated, on average, to be 41.5%±7.2% for 16-3-16, 40.2%±7.8% for 16(Py)-S-2-S-(Py)16, and 35.7%±4.4% for 16-7N(GK)-16 (Table S1, supporting information), respectively, which were caused by ion suppression. Although a column or additional preparative steps may help reduce the matrix effect, the observed ion suppression does not undermine the quantification of gemini surfactants as the sensitivity of the method was sufficient for the detection and quantification of target gemini surfactants. In addition, internal standards are used, which are considered the gold standard approach for correcting any matrix effects.

3.3 Linearity and Sensitivity

The linearity of each standard curve was established with a wide range sufficient to cover the expected concentration of analytes for quantitative analysis. The standard curves were linear at a concentration range of 10nM-1000nM for 16-3-16 and 16(Py)-S-2-S-(Py)16, and

20nM-2000nM for 16-7N(GK)-16, with r^2 ≥0.998. The LLOD was 4nM for both 16-3-16 and 16(Py)-S-2-S-(Py)16 and 8nM for 16-7N(GK)-16, whereas the LLOQ was 10nM for 16-3-16 and 16(Py)-S-2-S-(Py)16, and 20nM for 16-7N(GK)-16. Compared to the recent HILIC-MS/MS method [26], which reported an LLOQ of 67.5nM and 58.2nM for the gemini surfactants 16-3-16 and 16(Py)-S-2-S-(Py)16, respectively [26], the current FIA-MS/MS method has a lower LLOQ and thus higher sensitivity. The increase in sensitivity could be attributable to the enhanced sample preparation method and the optimized solvent mobile phase, which resulted in narrower and more symmetrical analyte peaks compared to those obtained with the HILIC-MS/MS method [26]. Such sensitivity is needed to detect the target analytes in a subcellular matrix.

3.4 Intra- and inter-day precision and accuracy

Intra- and inter-day precision and accuracy were evaluated by analyzing samples at four different concentrations LLOQ, LQC, MQC and HQC as per the USFDA guidelines [43]. Tables 2 and 3 show the precision and accuracy obtained for gemini surfactants 16-3-16 and 16(Py)-S-2-S-(Py)16 at various concentrations. The precision and accuracy of 16-7N(GK)-16 are listed in Tables 4 and 5. The precision was reported as CV% among all measurements and accuracy was expressed as a percentage of the mean of all measurements relative to the theoretical value. The intra-day precision did not exceed 7.7% for any of the gemini surfactants at the four concentration levels, while accuracy ranged between 94.4% and 108.8%. The inter-day assessment yielded a precision less than 3.3% and accuracy between from 97.2% to 108.4%.

3.5 Recovery

Recovery was determined as the ratio of the analyte peak area of the pre-spiked sample before extraction versus that of the post-spiked sample after extraction, expressed as a percentage. Three concentrations for each analyte were tested in this study. On average, the recovery was 104.9% at 30nM, 111.3% at 150nM and 94.3% at 800nM for 16-3-16, 104.0% at 30nM, 95.7% at 150nM and 102.9% at 800nM for 16(Py)-S-2-S-(Py)16; and 42.9% at 60nM, 33.1% at 400nM and 34.4% at 1600nM for 16-7N(GK)-16. Similar to previously published results [26], the Bligh/Dyer method efficiently extracted the gemini surfactants 16-3-16 and 16(Py)-S-2-S-(Py)16 from the cellular matrix. In contrast, the extraction efficiency for the gemini surfactant 16-7N(GK)-16 was significantly reduced. This finding was expected as 16-7N(GK)-16 is more hydrophilic compound due to the presence of a di-peptide in the spacer region, which decreases its partition to the organic solvent (methanol-chloroform), thereby reducing the extraction efficiency. However, with the use of deuterated internal standards spiked prior to the sample extraction, the recovery of analyte was corrected with internal standard. Therefore, the determination of 16-7N(GK)-16 is not compromised. Such low recovery explains the slightly higher LLOQ for 16-7N(GK)-16 in comparison to the other two anlaytes (Tables 2 and 4).

3.6 Stability

Freeze-thaw stability, bench top stability, auto-sampler stability and long-term stability were tested with the analysis of samples at three different concentrations as shown in Tables S2 and S3 (supporting information). Freeze-thaw stability was determined to be with

precision of ≤6.7% and accuracy between 94.7% and 104.4%. For the bench top stability, the precision and accuracy varied from 2.6% to 7.3% and from 96.7% to 100.3%, respectively. The stability of these samples in the auto-sampler resulted in a precision range of 2.8%-5.7% and accuracy range of 96.9%-102.5%. These results confirmed that the samples were stable during sample preparation and data acquisition. The stability of these samples was not compromised by long-term storage at -80°C, with the values of precision ranging from 2.0% to 7.0% and accuracy ranging from 89.1% to 101.1%.

3.7 Application

The uptake and subcellular distribution profiles of the gemini surfactants 16-3-16, 16(Py)-S-2-S-(Py)16 and 16-7N(GK)-16 in PAM 212 cells were studied using the validated FIA-MS/MS method. In this work, we show proof-of-principle application of the method to analyze these compounds in the nuclear fraction. The complete analysis of various subcellular fractions including the nucleus, mitochondria, plasma membrane and cytosol, along with other biological assessments, will be reported upon completion of the analyses. Although the standard curves and QC samples were prepared in the blank cell lysate matrix, no matrix difference was observed among the various subcellular fractions and the whole cell lysate (data not shown), which ensured the accurate quantification of the gemini surfactants in each cellular fraction.

The uptake rate of the gemini surfactants in the nuclear fraction increased rapidly over the course of the 5h treatment, reaching to approximately 800nmol for 16(Py)-S-2-S-(Py)16 and 16-7N(GK)-16 and 300nmol for 16-3-16, followed by a gradual decrease after the removal of the dosed culture media (Figure 4). The observations are consistent with the reported progressive nanoparticle uptake, which reaches a maximum before a depletion of the intracellular analyte [26]. As expected, the rate of uptake and the bio-distribution in the nuclear fraction is different among the three gemini surfactants. Such differences could explain their variable efficiency and/or toxicity. However, a definitive conclusion may only be drawn upon the completion of the analysis of all four subcellular compartments, which will be conducted in the near future.

4 Conclusion

A simple and reliable FIA-MS/MS method was successfully developed and validated for the quantification of the gemini surfactants 16-3-16, 16(Py)-S-2-S-(Py)16 and 16-7N(GK)-16 as gene delivery agents in subcellular compartments. The sensitivity of the FIA-MS/MS method was superior to the reported HILIC-MS/MS method for the determination of these gemini surfactants at the subcellular level. The specificity, precision, accuracy, recovery and stability were sufficient to quantify these gemini surfactants in a cellular matrix. Furthermore, the use of chromatographic separation, gradient elution and an ion pairing reagent was eliminated in the reported approach, thus substantially simplifying the analytical method and reducing the sample run time. The method was successfully applied to quantify the three gemini surfactants in the nuclear fraction of PAM 212 cells treated with nanoparticles, which varied significantly and may explain differences in the observed efficiency and/or toxicity of these gemini surfactants in gene delivery.

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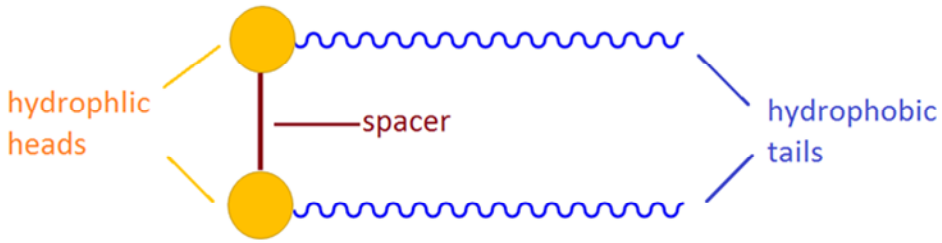
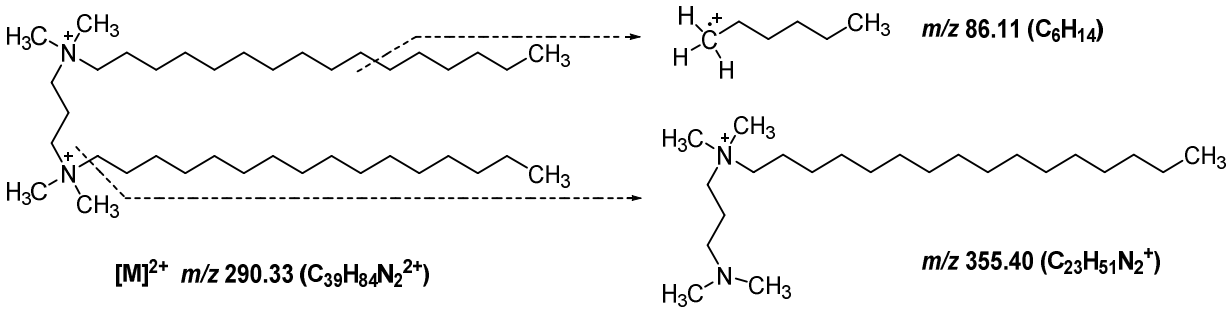
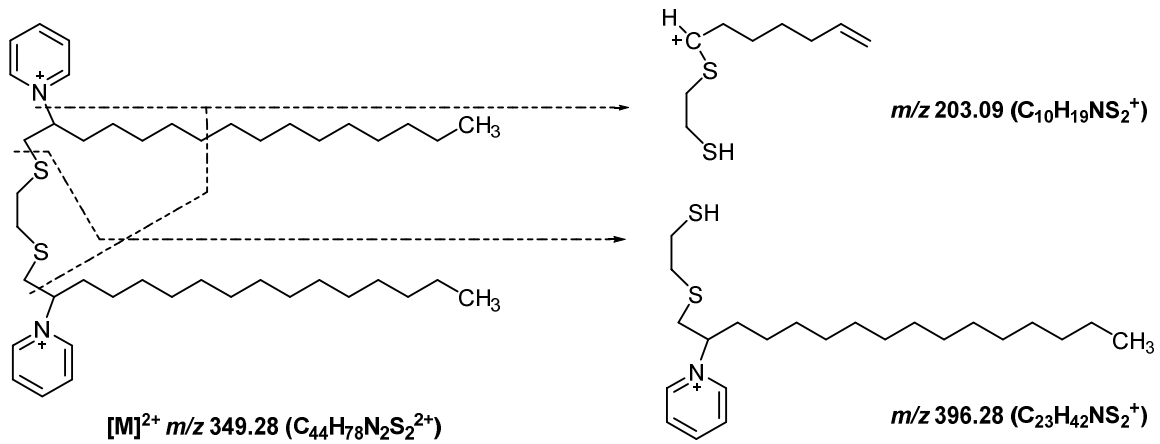


B 16-3-16



C 16(Py)-S-2-S-(Py)16



D 16-7N(GK)-16

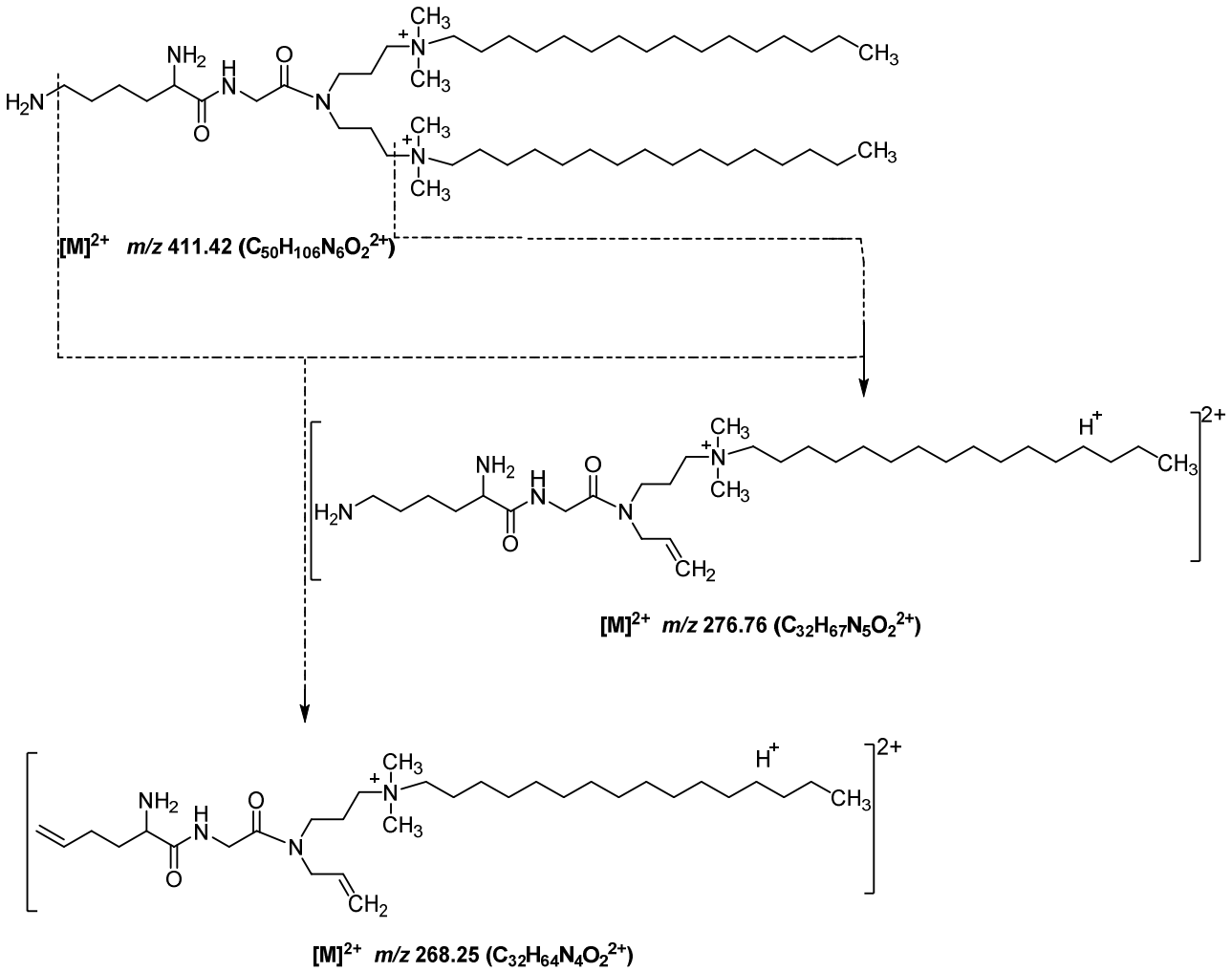


Figure 1. (A) Schematic representation of the general structure of gemini surfactant, (B) structure of 16-3-16 and the monitored product ions, (C) structure of 16(Py)-S-2-S-16(Py) and the monitored product ions, and (D) structure of 16-7N(GK)-16 and the monitored product ions.

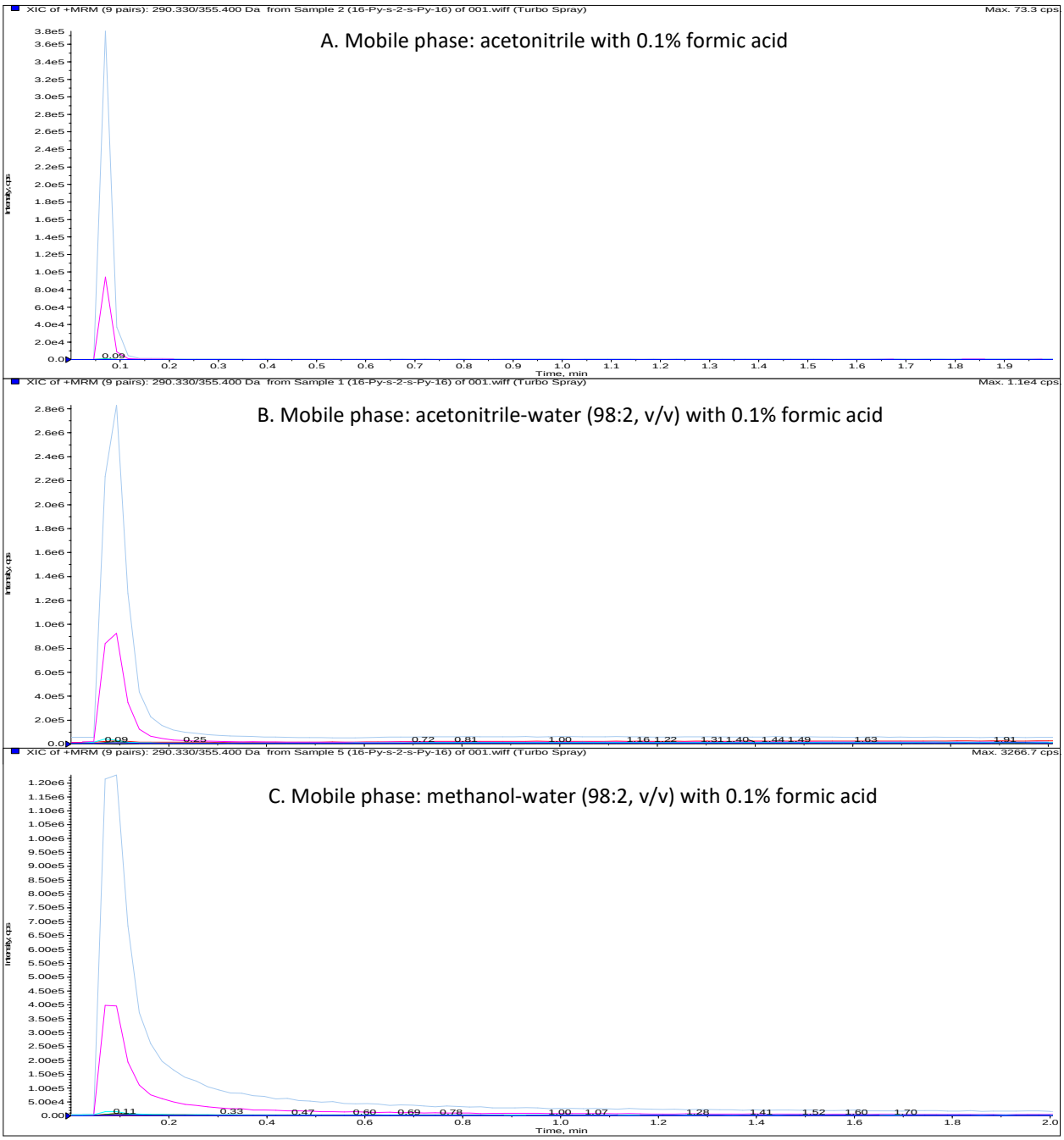


Figure 2. FIA-MS/MS chromatogram of 16(Py)-S-2-S-16(Py) using various mobile phases: (a) acetonitrile with 0.1% formic acid, (b) acetonitrile-water (98:2, v/v) with 0.1% formic acid, and (c) methanol-water (98:2, v/v) with 0.1% formic acid.

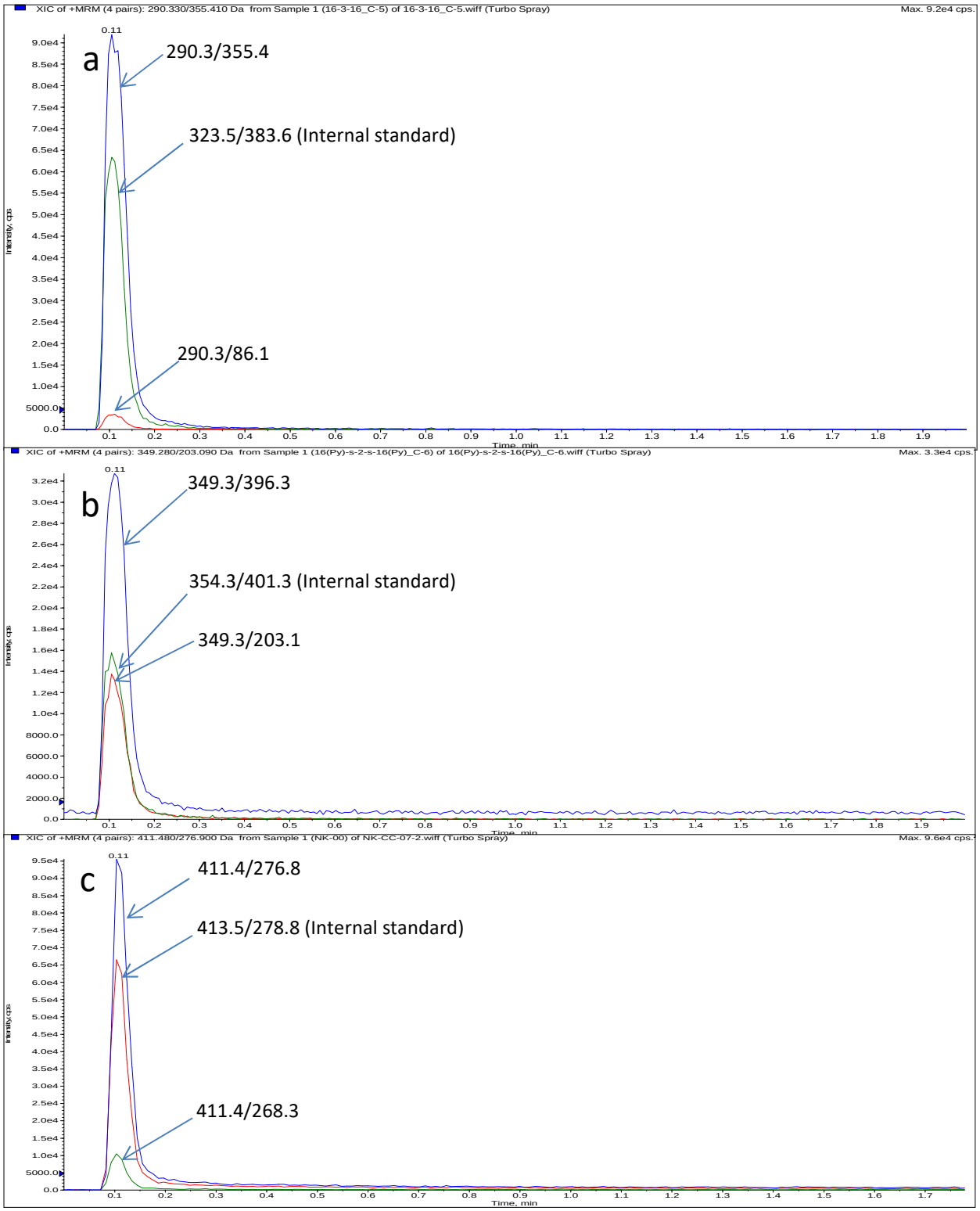


Figure. 3. FIA-MS/MS chromatograms in cellular extract. (a) 16-3-16 and internal standard, (b) 16(Py)-S-2-S-16(Py) and internal standard, and (c) 16-7N(GK)-16 and internal standard.

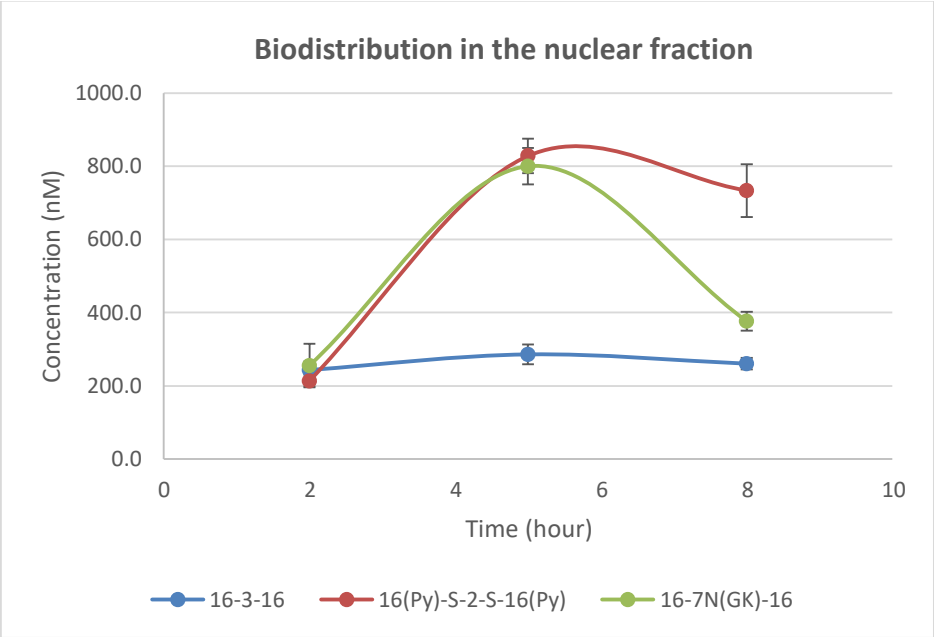


Figure 4. Distribution of gemini surfactants: 16-3-16, 16(Py)-S-2-S-16(Py) and 16-7N(GK)-16 in the nuclear fraction of PAM 212 cells treated with gemini surfactant nanoparticles.

Table 1. MRM transitions and compound-dependent parameters for analytes and internal standards

Gemini surfactants	Molecular Formula	MRM Transition (m/z)	DP	EP	CE	CXP
16-3-16	$C_{39}H_{84}N_2^{2+}$	290.3/355.4 (quantifier)	70	10	22	10
		290.3/86.1 (qualifier)	70	10	35	10
16-3-16-D_{66}	$C_{39}H_{18}D_{66}N_2^{2+}$	323.5/388.6	70	10	22	10
16(Py)-S-2-S-16(Py)	$C_{44}H_{78}N_2S_2^{2+}$	349.3/396.3 (quantifier)	50	10	22	10
		349.3/203.1 (qualifier)	50	10	22	10
16(Py)-S-2-S-16(Py)-D_{10}	$C_{44}H_{68}D_{10}N_2S_2^{2+}$	354.3/401.3	50	10	22	10
16-7N(GK)-16	$C_{50}H_{106}N_6O_2^{2+}$	411.4/276.8 (quantifier)	100	12	28	10
		411.4/268.3 (qualifier)	100	12	31	10
16-7N(GK)-16-D_4	$C_{50}H_{102}D_4N_6O_2^{2+}$	413.4/278.8	100	12	28	10

DP- declustering potential, EP-entrance potential, CE-collision energy, CXP-collision exit potential.

Table 2. Intra-day precision and accuracy of gemini surfactants 16-3-16 and 16(Py)-S-2-S-16(Py)

Samples	Day	Measured concentration (Mean± SD, nM)		Precision		Accuracy	
		16-3-16	16(Py)-S-2-S-16(Py)	16-3-16	16(Py)-S-2-S-16(Py)	16-3-16	16(Py)-S-2-S-16(Py)
LLOQ (10nM)	1	10.6±0.4	10.3±0.6	3.7%	5.5%	105.8%	102.7%
	2	10.2±0.3	10.8±0.8	3.1%	7.7%	101.9%	108.3%
	3	10.1±0.2	10.2±0.5	2.0%	5.1%	100.6%	102.0%
LQC (30nM)	1	29.7±0.5	29.0±1.8	1.6%	6.0%	98.9%	96.5%
	2	29.2±0.4	30.2±1.2	1.5%	3.9%	97.4%	100.7%
	3	28.9±0.6	28.3±1.5	1.9%	5.4%	96.3%	94.4%
MQC (150nM)	1	150.7±1.2	143.3±7.3	0.8%	5.1%	100.4%	95.6%
	2	150.2±1.5	149.8±7.2	1.0%	4.8%	100.1%	99.9%
	3	150.7±1.8	149.0±3.9	1.2%	2.6%	100.4%	99.3%
HQC (800nM)	1	805.8±8.6	761.7±17.0	1.1%	2.2%	100.7%	95.2%
	2	814.0±6.6	804.0±24.7	0.8%	3.1%	101.8%	100.5%
	3	809.5±7.9	775.7±20.2	1.0%	2.6%	101.2%	97.0%

Table 3. Inter-day precision and accuracy of gemini surfactants 16-3-16 and 16(Py)-S-2-S-16(Py)

Samples	Replicates	Measured concentration (Mean± SD, nM)		Precision		Accuracy	
		16-3-16	16(Py)-S-2-S-16(Py)	16-3-16	16(Py)-S-2-S-16(Py)	16-3-16	16(Py)-S-2-S-16(Py)
LLOQ (10nM)	18	10.3±0.3	10.4±0.4	2.6%	3.3%	102.8%	104.3%
LQC (30nM)	18	29.3±0.4	29.2±1.0	1.3%	3.3%	97.6%	97.2%
MQC (150nM)	18	150.5±0.3	147.4±3.5	0.2%	2.4%	100.3%	98.3%
HQC (800nM)	18	809.8±4.1	780.4±21.6	0.5%	2.8%	101.2%	97.6%

Table 4. Intra-day precision and accuracy of gemini surfactant 16-7N(GK)-16

Samples	Day	Measured concentration (Mean± SD, nM)	Precision	Accuracy
LLOQ (20nM)	1	21.7±0.8	3.5%	108.7%
	2	21.5±1.1	5.3%	107.7%
	3	21.8±1.0	4.5%	108.8%
LQC (60nM)	1	60.3±1.4	2.4%	100.5%
	2	60.1±0.8	1.4%	100.1%
	3	62.8±1.8	2.9%	104.6%
MQC (400nM)	1	401.2±8.4	2.1%	100.3%
	2	397.0±15.2	3.8%	99.3%
	3	389.0±10.8	2.8%	97.3%
HQC (1600nM)	1	1613.3±35.6	2.2%	100.8%
	2	1575.0±50.9	3.2%	98.4%
	3	1536.7±57.5	3.7%	96.0%

Table 5. Inter-day precision and accuracy of gemini surfactant 16-7N(GK)-16

Samples	Replicates	Measured concentration (Mean± SD, nM)	Precision	Accuracy

LLOQ (20nM)	18	21.7±0.1	0.6%	108.4%
LQC (60nM)	18	61.0±1.5	2.5%	101.7%
MQC (400nM)	18	395.7±6.2	1.6%	98.9%
HQC (1600nM)	18	1575.0±38.3	2.4%	98.4%

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