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The Development of Simple Flow Injection Analysis Tandem Mass Spectrometric Methods for the Cutaneous Determination of Peptide-Modified Cationic Gemini Surfactants Used as Gene Delivery Vectors.

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Abstract

Diquaternary ammonium gemini surfactants are a class of non-viral gene delivery vectors, primarily studied for their dermal applications. However, their biological fate has rarely been investigated. In this work, we developed simple flow injection analysis tandem mass spectrometric methods, (FIA)-MS/MS, to understand the fate and biodistribution of topically applied gemini surfactant-based therapeutics in an ex-vivo skin model.

Three peptide-modified gemini surfactants with varied structures and transfection efficiencies were evaluated. For each compound, two methods were developed to quantify their presence in skin tissue and in phosphate buffered saline (PBS). The methods were developed using single-point calibration mode. Skin penetration was assessed on CD1 mice dorsal skin tissue mounted in a Franz diffusion cell after extraction. Amongst the five evaluated liquid-liquid extraction protocols, the Folch method provides the highest extraction efficiency for all compounds. Weak cationic exchange solid phase extraction was also used to further isolate gemini surfactants from endogenous skin lipids. FIA-MS/MS analysis of the skin revealed that all compounds were detected in the skin with minimal partition into the PBS compartment, which represents circulation. Interestingly, the detected amounts of gemini lipids in the skin were correlated with their transfection efficiencies.

Introduction

Recent advances in discovering the genetic basis of many dermatological disorders have found cutaneous gene therapy to be a promising therapeutic option [1]. Cutaneous delivery of genetic material offers numerous advantages over other routes of administration, such as minimizing systematic toxicity, bypassing first-pass metabolism, and avoiding rapid clearance from the systemic circulation [2]. Despite these advantages, skin is a formidable barrier to foreign materials, such as nanotechnology products [3]. Therefore, effective delivery systems capable of penetrating the cutaneous barrier and facilitating recombinant DNA uptake into the skin are needed to achieve gene expression – the ultimate goal of gene therapy.

Among topical delivery modalities currently being explored, lipid-based delivery vectors are at the forefront [4]. They have the ability to encapsulate, protect, and compact negatively charged nucleic acids, whereby forming nano-sized lipoplexes. Furthermore, the chemical composition of lipid-based nanocarriers bears some similarity to skin lipids, which enables them to fuse with the lipids in the stratum corneum, the outer layer of the skin; destabilizing the lipid matrix and enhancing drug penetration [5]. Diquaternary ammonium gemini surfactants are a class of lipid-based delivery systems that are composed of dimeric surfactants with positively charged head groups and hydrocarbon tails linked by a spacer chain (Figure 1) [6]. The structure of gemini surfactants can be tailored to overcome skin barrier functions [7]. The topical application of gemini surfactant-based nanoparticles demonstrated a promising potential in the treatment of localized cutaneous scleroderma [7, 8]. Nanoparticles of *N,N'*-bis(dimethylhexadecyl)-1,3-propanediammonium dibromide gemini surfactant complexed with pDNA encoding for interferon gamma (INF- γ) showed a significant increase in the level of INF- γ in mice [7, 8].

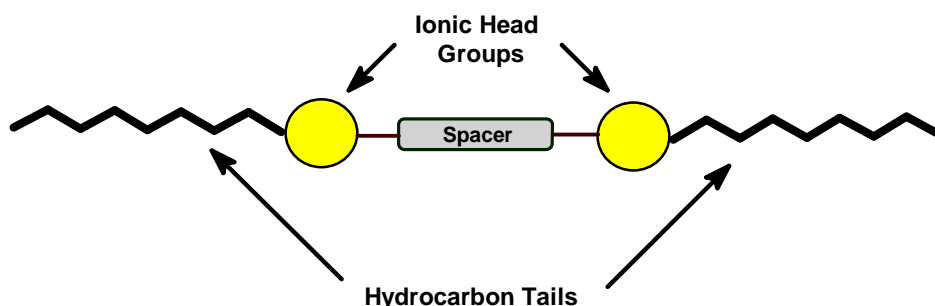


Figure 1: Schematic representation of gemini surfactants showing the two ionic head groups, hydrocarbon tails and the spacer

Despite advancements in the design of lipid-based nanocarriers, their biodistribution and biological fate have been less explored. Upon topical application, lipid-based nanoparticles distribute within various layers and cellular components. At present, the biodistribution, intracellular trafficking, and the ultimate fate of the lipid vector, after releasing its therapeutic cargo, are not fully understood. A fundamental understanding of the behavior of the lipid-based vectors in complex biological environments is essential in guiding the design of safer and more effective nanoparticles.

To track the fate and distribution of lipid-based nanoparticles, fluorescently labeled and radiolabeled carriers are the most commonly used strategies. However, labeling techniques have drawbacks, particularly their tendency to alter the physicochemical properties of the delivery system. These modifications, in turn, change the pharmacokinetic profile of the nanocarriers [9]. Furthermore, they are unable to distinguish between the localization of a labeled molecule and the

metabolites that retain the fluorescent or radioactive probes [10]. Therefore, a more robust and sensitive analytical technique should be employed to identify and quantify gene-based carriers in complex biological samples.

Mass spectrometry (MS) is an ideal technique to monitor the fate of gemini surfactants in the skin [11, 12]. It is a label-free technique with a powerful chemical identification capability and is gaining popularity in pharmaceutical sciences due to its high selectivity and sensitivity [13]. Coupling liquid chromatography to tandem mass spectrometry (LC-MS/MS) allows for reliable high throughput qualitative and quantitative analysis [14]. In fact, it is the gold standard technology for the quantification of pharmaceuticals in complex biological matrices [15].

In our laboratory, we developed two LC-MS/MS methods for the quantification of unsubstituted diquatery ammoniums gemini surfactants (*N, N*-bis(dimethylalkyl)- α,ω -alkane-diammonium), amine substituted diquatery ammoniums compounds, and heterocyclic headgroup gemini surfactants (bis(alkyl-pyridinium) in epidermal keratinocytes [16, 17]. These methods provided essential information about the rate of cellular uptake and intracellular depletion of gemini surfactants [16, 17]. Currently, these methods are being employed to determine the subcellular localization of gemini surfactants and identify any potential metabolites. Recently, a new series of peptide modified diquatery ammoniums gemini surfactants was found to exhibit superior transfection efficiency compared to previous generations of gemini surfactants [18]. Their collision-induced dissociation (CID)-MS/MS behaviour was evaluated, establishing a universal mass spectrometric fingerprint, essential for the development of targeted LC-MS qualitative and quantitative methods [11].

Herein, we resolved a significant analytical challenge, the efficient extraction of gemini surfactants from lipid rich skin tissues. Efficient analytical platforms are needed to guide the development of effective pharmaceutical formulations. Three representative compounds were selected with high, low, and moderate transfection efficiencies. Subsequently, rapid and simple flow injection analysis (FIA)-MS/MS methods were developed to detect and quantify peptide-modified gemini surfactants in skin tissues as well as in phosphate buffered saline (PBS).

Materials and methods

Materials

The evaluated peptide-modified gemini surfactants, designated as 16-7N(R)-16 where 16 is the alkyl chain length and R is the peptide-containing moiety: R= glyceryl-lysine, glyceryl-hexyl-trilysine and glyceryl-undecyl-trilysine (Figure 2), were synthesized using previously reported synthetic methods [18]. The corresponding internal standards were synthesized using the same synthetic procedure with the incorporation of deuterated lysine moiety bearing four deuterium atoms (Figure S1, Supplementary Material). The plasmid pGThCMV.IFNGFP (pDNA), encoding for murine interferon gamma (IFN- γ) and green fluorescent protein (GFP) was utilized in this work [7].

The helper lipid 1,2-dioleoyl-snglycerol-3-phosphoethanolamine (DOPE) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Sucrose, used as a stabilizing agent, and phosphate buffered saline (PBS) were purchased from Sigma Aldrich (Oakville, ON, Canada). Mass spectrometry-grade methanol, water, and acetonitrile were purchased from Fisher Scientific (Nepean, ON, Canada). Formic acid (purity 90%) was obtained from EMD Chemicals Inc. (Merck KGaA, Darmstadt, Germany). Anhydrous chloroform and methyl tert-butyl ether (MTBE) used as extraction solvents were purchased from Sigma Aldrich (Oakville, ON, Canada). Solid phase extraction cartridges, Bond Elute[®] CBA, were obtained from Agilent Technologies (Mississauga, ON, Canada).

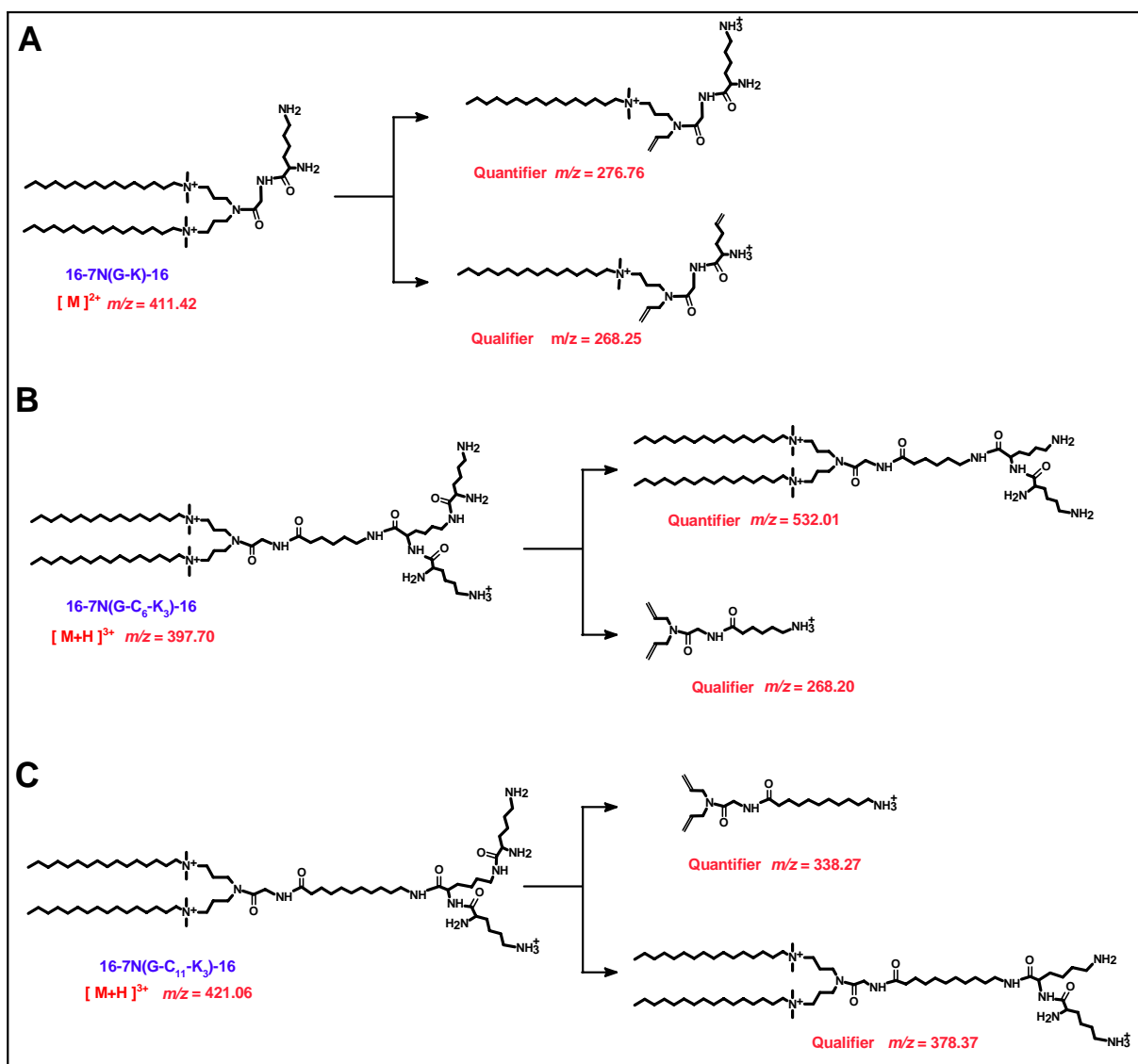


Figure 2: Chemical structure of gemini surfactants 16-7N(G-K)-16 (A), 16-7N(G-C₆-K₃)-16 (B) and 16-7N(G-C₁₁-K₃)-16 surfactants showing their precursor ion and the monitored product ions.

Preparation of topical formulations

Cationic gemini lipids were combined with 16 µg pDNA at a negative to positive charge ratio (N/P) of 1:5 in the presence of a helper lipid DOPE to create pDNA/gemini lipid/helper lipid (P/G/L) nanoparticles. The concentration of the gemini surfactants was calculated so that the amount of gemini surfactants is enough to achieve the required charge ratio. As such, an appropriate amount of 30 mM aqueous solutions of gemini surfactant was added to 2 mg/mL pDNA solution and incubated for 20 minutes at room temperature (P/G complex). 2 mM DOPE was prepared as described previously [19] then concentrated to 10mM using Eppendorf concentrator 5301(Eppendorf, Hamburg, Germany). The concentrated DOPE was added to P/G complexes at a gemini surfactant to DOPE molar ratio of 1:16 to form the final nanoparticles (P/G/L) and incubated at room temperature for 20 minutes.

***Ex vivo* skin penetration study**

Dorsal skin tissues were collected from female CD1 mice (Charles River Laboratories, Saint-Constant, QC, Canada) weighing around 22-24 g. Approval for this study was granted by the University of Saskatchewan's Animal Research Ethics Board in adherence to the Canadian Council on Animal Care guidelines for humane animal use (protocol # 20090081). The animals were shaved and the skin was collected and stored at -80 °C until use.

Skin penetration was evaluated using multi-station Franz diffusion cell system with 64 mm² surface area (PermeGear Inc., Hellertown, PA, USA). The skin tissue was mounted between the donor and receptor compartments of the Franz cell with the stratum corneum facing the donor compartment. The receiving chamber was filled with 5 mL PBS, avoiding any air bubbles between the skin and the solution. The skin tissues were allowed to equilibrate for 10 minutes before applying any formulation. A total 200 µL of peptide modified gemini surfactant-based lipoplexes containing 16 µg pDNA was placed in the donor compartment and the chamber was covered with parafilm. Throughout the experiment, the PBS in the receptor compartment was continuously stirred at 700 rpm using a magnetic stirrer bar and temperature was maintained at 32 °C using a circulating water bath (Fisher Scientific, Nepean, ON, Canada).

Aliquots of 200 µL were withdrawn from the receptor compartment at fixed intervals (2, 4, 6, 8, 12, 18 and 24 h) and replaced with an equal volume of pre-warmed PBS. After 24 hours, any remaining formulation in the donor chamber was aspirated and the skin tissue was removed from the Franz cell. The skin was rinsed thoroughly with water, blotted with tissue paper, then stripped 10 times with Scotch adhesive tape. The collected skin tissues and PBS samples were stored at -80 °C prior to analyte extraction and FIA-MS/MS analysis.

Sample preparation for FIA-MS/MS analysis

Skin tissue (40 mg) was spiked with 200 µL of methanol containing 0.03 mM of the corresponding internal standards for 16-7N(G-K_{d4})-16 as well as 16-7N(G-C₁₁-K_{d4}-K₂)-16 and 0.015 mM of 16-7N(G-C₆-K_{d4}-K₂)-16. After adding the extraction solvent, the skin was homogenized using a probe homogenizer (PRO200 Homogenizer, PRO Scientific Inc., Oxford, Connecticut, USA). During homogenization, the sample was kept on ice to avoid overheating. Five extraction solvent protocols were evaluated as explained below.

Liquid-liquid extraction protocols

Modified Folch method

The analytes were extracted as described in the Folch protocol [20] modifications by the addition of 200 µL methanol containing the internal standards, followed by the addition of 400 µL chloroform. Then, the skin sample was homogenized in the presence of ice to avoid overheating. High purity water (150 µL) was added to the homogenate to induce phase separation followed by

pulse vortexing for a few seconds. The sample was centrifuged at 14,000 rpm for 10 min at room temperature to obtain separate aqueous and organic phases. The bottom organic phase was retrieved while the upper aqueous phase and the skin pellet were re-extracted by the addition of 400 μ L chloroform as described above. The upper phase was discarded and both organic phases were combined, dried using a centrifugal evaporator, and stored at -80°C until analysis.

Modified Bligh and Dyer method (B&D)

The analytes were extracted by employing the Bligh and Dyer method with some modifications [21], by adding 80 μ L high purity water, 200 μ L methanol containing the internal standards and 100 μ L chloroform to the skin tissue. Additional 100 μ L chloroform was added, followed by 100 μ L water while the sample was homogenized. Subsequently, the homogenate was centrifuged at 14,000 rpm for 10 min at room temperature. Similar to the modified Folch protocol, the lower organic layer was collected while the upper aqueous layer and the skin pellet were re-extracted with additional 200 μ L chloroform. Finally, the organic layers were combined, dried with a centrifugal evaporator, and stored at -80°C .

Acidified/alkaline B&D method

The analytes and internal standards were extracted as described in the Bligh and Dyer protocol, except that either 2 μ L of 3M hydrochloric acid [22] or 0.3% 12 M ammonium hydroxide [23] was added to the pre-homogenization mixture.

Modified methyl-tert-butyl ether (MTBE) method

The analytes were extracted as described [24], with modifications, by adding 200 μ L methanol containing the internal standards, then 666 μ L MTBE. Subsequently, the tissue sample was homogenized in the presence of ice to avoid overheating. Afterwards, phase separation was induced by adding 166 μ L water followed by pulse vortexing for a few seconds. The homogenates were centrifuged for 10 min at 14,000 rpm then the upper organic layer was retrieved while the lower aqueous layer and the skin pellet were re-extracted by adding 666 μ L MTBE. After final centrifugation, both organic phases were combined and dried before being stored at -80°C . Figure 3 summarizes the discussed liquid-liquid extraction protocols.

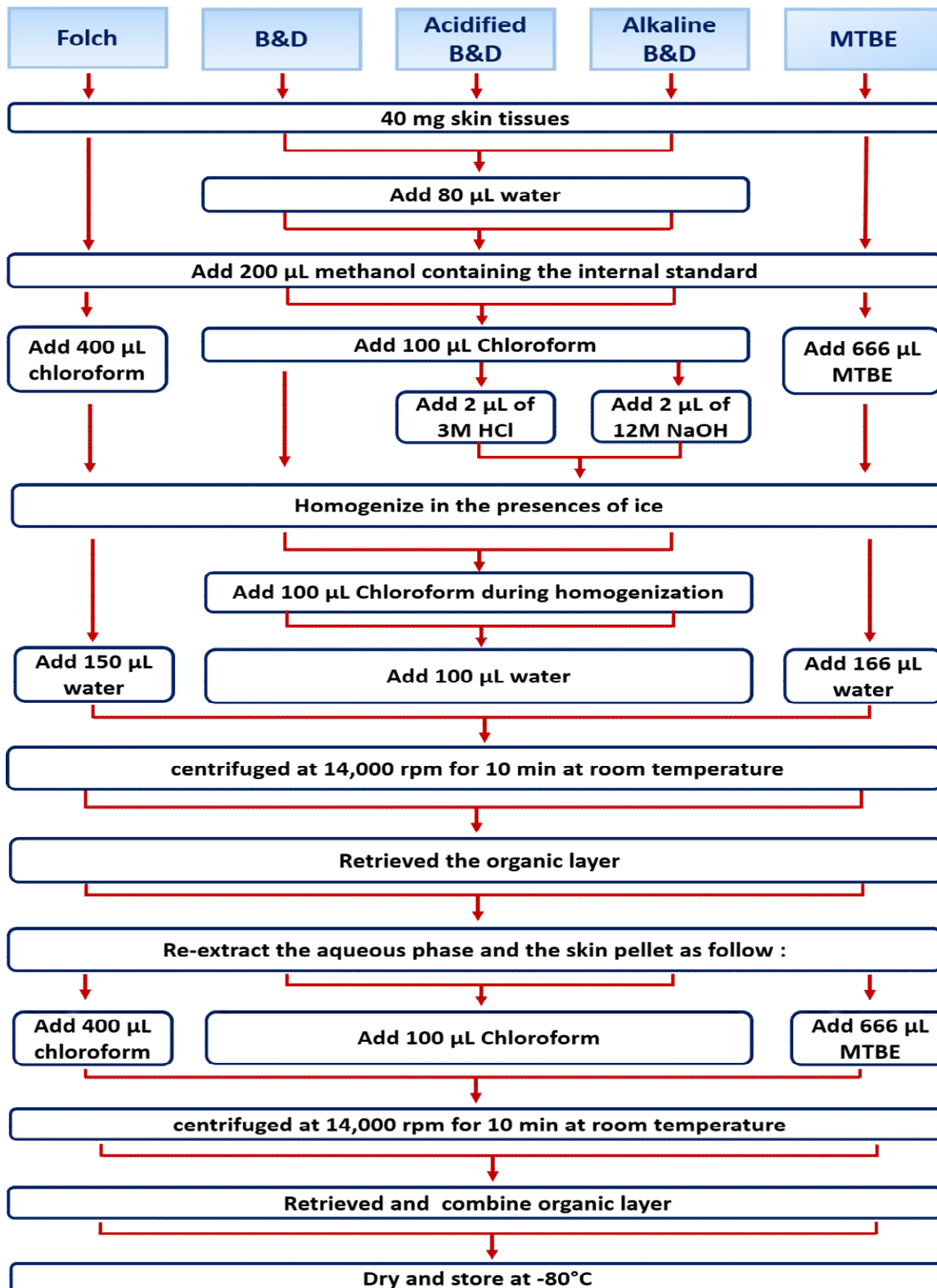


Figure 3: Flowchart summarizing the five evaluated liquid-liquid extraction methods.

Solid phase extraction (SPE) protocol

Extracts from each solvent system were solubilized in 3 mL methanol-water (50:50, v/v). The CBA cartridges were activated by successive additions of 3 mL methanol, 3 mL water, and 3 mL methanol-water mixture (50:50, v/v). Subsequently, extracts were loaded into the cartridges followed by washing steps with 3 mL pure water and 3 mL methanol. Retained analytes and internal standards were eluted with 6 mL concentrated HCL-methanol (2:98, v/v). Finally, sample elutes were dried using a centrifugal evaporator and stored at -80°C.

Prior to MS analysis, extracted analytes were reconstituted in 3 mL methanol with 0.1% formic acid. Same procedure was followed to extract the analytes of interest from the PBS used in Franz cell's receptor compartment.

FIA-MS/MS instrumentation

FIA-MS/MS was performed on an Agilent 1290 infinity UHPLC (Agilent Technologies, Mississauga, ON, Canada) interfaced to an AB SCIEX QTRAP® 6500 triple quadrupole-linear ion trap mass spectrometer (QqLIT-MS) (AB SCIEX, Concord, ON, Canada). The mobile phase was optimized for each compound to achieve better ionization and peak shape. Isocratic mobile phase composed of acetonitrile/water mixture (50:50, v/v for 16-7N(G-K)-16 and 90:10, v/v for the rest of compounds) with 0.1% formic acid was delivered at a flow rate of 0.3 mL/min for a run time of 4 min. Sample aliquots of 1 µL were injected while maintaining the auto sampler temperature at 4 °C.

The AB SCIEX QTRAP® 6500 is equipped with a “Turbo V Ion Spray” ESI source, operated in the positive ion mode and set at 5500 V ionspray voltage. Optimal detection parameters for each analyte are listed in Table 1. The MS/MS data were obtained by low energy collision-induced dissociation (CID) employing nitrogen as the collision gas. Multiple reaction monitoring (MRM) was selected as the scan mode to monitor the analytes and internal standards precursor ions to product ions transitions. Dwell time for all transitions was 150 ms at unit resolution. The monitored transitions and their MRM conditions are listed in Table 2. The structures of the monitored transitions are shown in Figure 2 as well as Figure S1 (Supplementary Material).

Table 1: Optimal detection parameters of the tested analytes on the AB SCIEX 6500 QTRAP® system.

Gemini Surfactants	Curtain gas, CUR (psi)	Nebulizer gas, GS1 (psi)	Heater gas, GS2 (psi)	Collision gas, CAD (psi)	Source temperature (°C)
16-7N(G-K)-16	45	50	80	10	700
16-7N(G-C ₆ -K ₃)-16	45	60	60	11	700
16-7N(G-C ₁₁ -K ₃)-16	45	70	90	10	600

Table 2: Conditions for MRM transitions of the gemini surfactants on AB SCIEX 6500 QTRAP® System.

Gemini surfactant	Transition (m/z)	DP (eV)	CE (eV)	CXE (eV)
16-7N(G-K)-16	411.4 \rightarrow 276.9	151	27	10
	411.4 \rightarrow 268.4	151	29	20
16-7N(G-K _{d4})-16	413.5 \rightarrow 278.9	151	27	18
16-7N(G-C ₆ -K ₃)-16	397.8 \rightarrow 532.1	101	25	26
	397.8 \rightarrow 268.2	101	33	10
16-7N(G-C ₆ -K _{d4} -K ₂)-16	399.2 \rightarrow 534.2	101	23	26
16-7N(G-C ₁₁ -K ₃)-16	421.2 \rightarrow 338.3	141	35	18
	421.2 \rightarrow 378.4	141	23	18
16-7N(G-C ₁₁ -K _{d4} -K ₂)-16	422.5 \rightarrow 379.8	116	23	18

Preparation of standard solutions

In this work, two methods were developed for each gemini surfactant: *method A* to determine their presence in the skin tissues and *method B* to monitor their cumulative amount permeated into the PBS solution.

Method A:

Aqueous stock solutions of gemini surfactants and their internal standards were prepared at a concentration of 3mM and stored at -20 °C. Working stock solutions were prepared daily by serial dilution of the stock solutions in methanol to concentrations of 0.03 mM and 0.015 mM. One point calibration standards were prepared by adding 400 μ L of 0.03 mM target analyte and 200 μ L of 0.03 mM from the corresponding internal standards to 2.4 mL pooled blank skin tissue extract. The exception was with 16-7N(G-C₆-K₃)-16 gemini surfactants where 0.015 mM of the analyte and 0.015 mM IS were used, since the concentration of 16-7N(G-C₆-K₃)-16 in the skin was less than the other two compounds. The equivalent final mass concentration of the gemini surfactants and the corresponding internal standards are listed in Table S1, Supplementary Material.

Method B:

Aqueous stock solutions of gemini surfactants and their internal standards were prepared at a concentration of 3mM and stored at -20 °C. Working stock solutions were prepared daily by serial dilution of the stock solutions in methanol to concentrations of 3 μ M and 1.5 μ M. One point calibration standards were prepared by adding 60 μ L of 3 μ M target analyte (except for 16-7N(G-C₆-K₃)-16 gemini surfactants where 1.5 μ M was used) and 40 μ L of 3 μ M from the corresponding internal standards (except for 16-7N(G-C₆-K_{d4}-K₂)-16 where 1.5 μ M was used) to 200 μ L blank

PBS extract. The equivalent final mass concentration of the gemini surfactants and the corresponding internal standards are listed in Table S1, Supplementary Material.

Method validation

The methods were partially validated with respect to selectivity, recovery, matrix effect, and process efficiency as recommended by the USFDA guidelines [25]. The performance of the methods was evaluated by statistically comparing the slope of the three-point calibration curves for three intra-run measurements.

Data analysis

Data processing for quantitative analysis was conducted using Analyst[®] software (Version 1.6.0). A sample concentration was obtained according to single point calibration mode using the following equation.

$$\text{Conc (sample)} = \frac{\text{Peak Area Ratio (sample)} \times \text{Conc(Calibration Standard)}}{\text{Peak Area Ratio (Calibration Standard)}} \quad (1)$$

The skin permeation parameters were calculated from the plot of cumulative amount of gemini surfactants permeated to the Franz diffusion cell's receptor compartment divided by 0.64 cm² to correct for the exposed skin area as a function of time. Steady-state flux (J_{ss}) was derived from the slope of the linear portion of the curve. The lag-time (t_{lag}) was estimated from the intercept of the tangent to the linear part of the absorption profile on the time axis. The permeability coefficient K_p was calculated using the following equation [26]:

$$K_p = \frac{J_{ss}}{C_{dose}} \quad (2)$$

where C_{dose} is the concentration of the applied dose.

Consequently, diffusion coefficient D_m and skin partition coefficient K_m could be calculated as follows:

$$D_m = \frac{d^2}{6 \times t_{lag}} \quad (3)$$

$$K_m = \frac{K_p \times d}{D_m} \quad (4)$$

where d is the measured skin thickness in cm.

Computational prediction

The gemini surfactants partition coefficient ($\log P$), distribution coefficient ($\log D$) at varying pH, and aqueous solubility ($\log S$) were estimated using ACD/Physchem Profiler 2016 [27] (Advanced Chemistry Development, Inc., Toronto, ON, Canada).

Statistical analysis

Statistical analyses were performed using SPSS software (Version 24.0). One way analysis of variance (ANOVA, Scheffé/Dunnett's post hoc tests) and Pearson's correlation were used for statistical analyses. Significant differences were considered at $p < 0.05$ level.

Results and discussion

Method development

The major purpose of this work is to overcome an analytical challenge by developing simple and rapid MS-based methods for the detection and relative quantification of peptide-modified gemini surfactants in skin tissues as well as in PBS. Analytical strategies should meet the need of the experiment; in our work, a fit-for-the-purpose approach was adopted as it provides the needed data to drive future formulation decisions. The developed methods aim to track the distribution of topically applied gemini surfactant-based therapeutics and investigate the impact of structural variation on the efficiency of skin penetration.

LC-MS/MS is the most widely used platform for the quantification of pharmaceuticals in complex biological matrices, offering analyte separation and matrix effect reduction capabilities [15]. Despite these advantages, LC-MS/MS significantly increases the overall analysis time and associated costs [28]. The sensitivity and selectivity of modern MS instruments and the evolution of efficient sample preparation techniques allow the development of simple analytical procedures [29, 30]. Direct introduction of the sample into the MS using FIA (i.e. loop injection) has emerged as an effective approach that offers a rapid sample possessing rate, low cost, and method simplicity [31]. It has been successfully applied in the quantitation of several analytes, including pharmaceuticals, environmental contaminants, and endogenous compounds [32, 33]. Therefore, FIA-MS/MS methods were developed removing the need for chromatographic separation while relying on the MS separation capabilities. For each gemini surfactant, two methods were developed: *method A* to determine their presence in the skin and *method B* to monitor their cumulative amount permeated into the PBS.

To ensure adequate selectivity and specificity of the developed method, we capitalized on the QTRAP capability in the MRM scan mode. The MS operational parameters such as declustering potential (DP), collision energy (CE), and collision exit potential (CXP) were optimized as shown in Table 2 to maintain ion abundance and stability. Two diagnostic MRM transitions with relatively high abundance, listed in Table 2, were selected for each compound. To improve accuracy and precision, an isotopically labelled internal standard bearing four deuterium atoms was used for each compound. The use of 4 mass unit difference between the analyte and its corresponding internal standard prevents any cross talk. The proposed structure of the monitored product ions for each gemini surfactant and their internal standards are shown in Figures 2 and S1 (Supplementary Material), respectively.

All tested compounds eluted before 0.8 min, as no column was used, with a total data acquisition time of 4 min (Figure 4A). No carryover was observed under the experimental conditions. This was significantly faster than the elution time in the recently developed HILIC-based LC-MS/MS quantification method in which second generation amine substituted gemini surfactants eluted at 7.12 min [17]. It is noteworthy that peptide-modified gemini surfactants are more polar than amine substituted gemini surfactants, hence, longer elution times are expected for peptide-modified gemini surfactants if the HILIC-MS/MS method was applied.

Selectivity

Selectivity of the developed methods was assessed by monitoring the existence of interfering peaks of the evaluated analytes and their internal standards in murine skin tissue extracts as well as in PBS solution from 6 different sources (data not shown). As shown in Figure 4 B and C, no interference from endogenous compounds against the selective bio-determination of the gemini surfactants was observed in both double blank skin tissue extracts and PBS. The ratio of the quantifier to the identifier transition ions were also monitored showing less than 9%

variations, indicating peak purity. The relative standard deviation (RSD) of the qualifier to the quantifier ratios was used as the acceptance criterion (less than 15%).

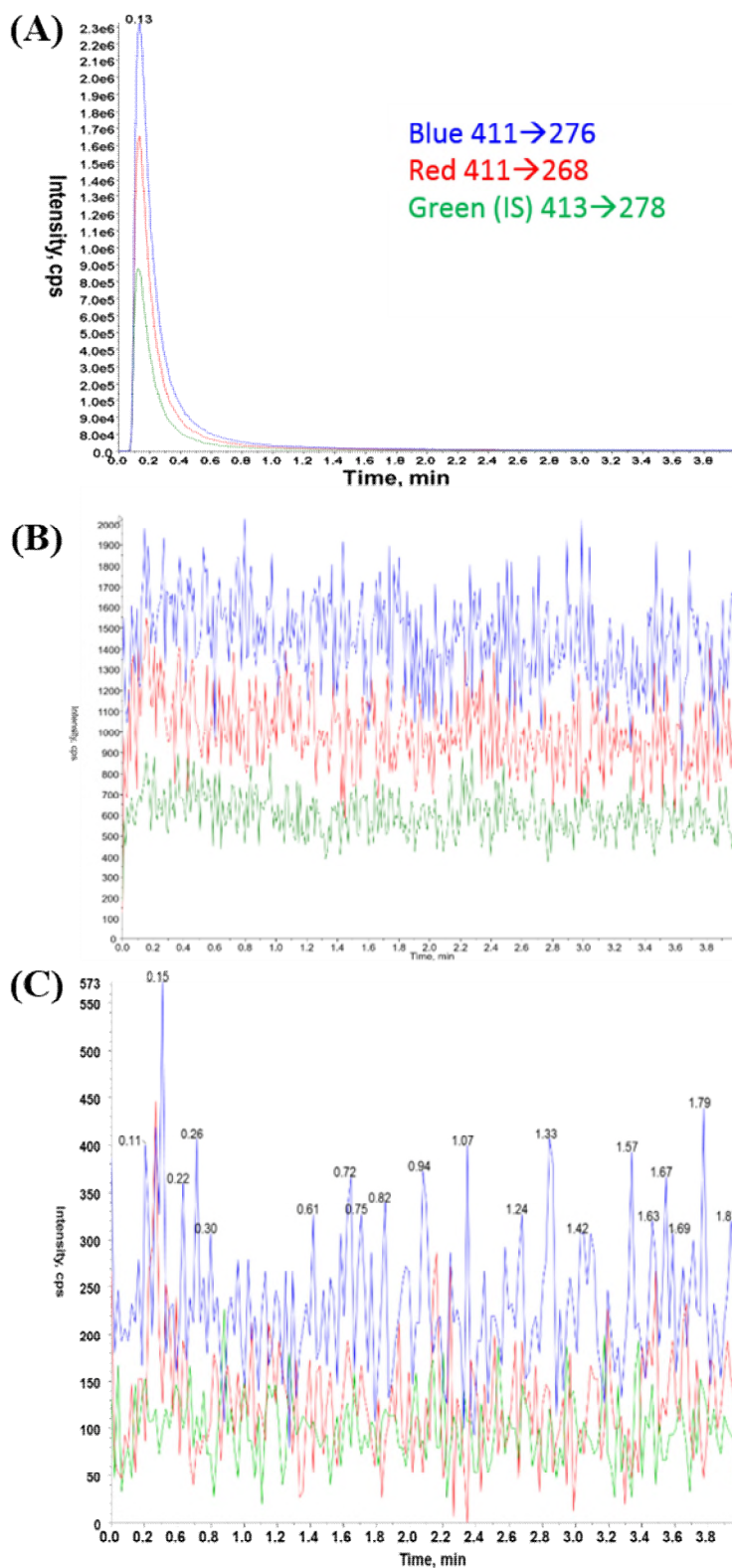


Figure 4: Representative FIA-MS/MS chromatograms of (A) skin tissue extract of 16-7N(G-K)-16 gemini surfactants, (B) double blank skin tissue extract, and (C) double blank PBS extract.

Sample preparation

Sample preparation represents one of the most critical steps for obtaining reliable and sensitive quantitative data [34]. As such, the ability to extract gemini surfactants from skin tissues was thoroughly assessed in this work using five common liquid-liquid extraction protocols, namely Folch [20], B&D [21], Acidified B&D [22], Alkaline B&D [23], and MTBE [24]. Extraction of gemini surfactants from skin tissues posed an analytical challenge due to the complexity of the skin matrix and the presence of a wide variety of interfering substances such as proteins, salts, and lipids [35]. In fact, the main challenge was the lipid-rich nature of skin tissues that caused significant matrix effects (data not shown) due to the high affinity of the endogenous lipids to the organic phase, similar to the target analyte, i.e. the gemini surfactants. Therefore, further purification was necessary to isolate gemini surfactants from the skin's endogenous lipids.

Since the skin is composed of a wide variety of lipid classes ranging from highly non-polar to polar lipids [36]; we capitalized on the gemini surfactants' unique feature, namely the two permanently charged quaternary amines to efficiently isolate them. Therefore, Bond Elute[®] CBA weak cationic exchange solid phase extraction (SPE) was used to purify extracts obtained from the liquid-liquid extractions. Bond Elute[®] CBA is a silica-based sorbent with a weak anion, carboxylic acid group, bonded to the surface. The carboxylic acid functional group has a pK_a of 4.8 that is negatively charged at pH 6.8 and higher, allowing for strong ionic interaction with the positively charged nitrogen atoms of the gemini surfactants. Washing of the cartridges with non-acidic methanol and water was used to remove non-bonded skin lipids and other interfering substances. Finally, elution of the gemini surfactants was achieved by neutralizing the carboxylic acid functional group using acidified methanol (pH 2.8 and lower).

Recovery, matrix effect, and process efficiency were evaluated across the five extraction protocol according to Matuszewski *et al.* [37] equations as follow:

$$\% \text{ Recovery} = \frac{\text{Response pre-extraction spiked sample}}{\text{Response post-extraction spiked sample}} \times 100 \quad (5)$$

$$\% \text{ Matrix effect} = \frac{\text{Response post-extraction spiked sample}}{\text{Response non-extracted neat sample}} \times 100 \quad (6)$$

$$\% \text{ Process efficiency} = \frac{\text{Response pre-extraction spiked sample}}{\text{Response non-extracted neat sample}} \times 100 \quad (7)$$

where the pre-extraction spiked sample refers to gemini surfactant standards added to the skin tissue before extraction and where samples were processed according to each liquid-liquid extraction procedure followed by purification with SPE. Response from the post-extraction spiked sample contains gemini surfactant standards added to the extracted blank tissues after passing through the SPE. The non-extracted neat sample contains the gemini surfactants added to the final reconstitution solvent (methanol with 0.1% formic acid). The determined value is the average for a set of triplicates.

Table 3 displays the effect of the extraction protocols on the recovery, matrix effect, and process efficiency. The Folch method resulted in the highest extraction efficiency in all gemini surfactants while MTBE displayed the lowest efficiency. The higher extraction efficiency of Folch compared to B&D methods was in agreement with the notion that the Folch protocol is more suited for extracting lipids from tissues, whereas B&D is more successful for biological fluids [38]. Although both Folch and B&D methods are based on the biphasic chloroform-methanol-water mixtures, the Folch method uses a higher percentage of chloroform over methanol (Folch maintains the chloroform-methanol-water mixture at 8:4:3 while the B&D ratio is 2:2:1.8) [20], [21]. Chloroform is a widely used extraction reagent for analytes with intermediate polarity such as gemini surfactants, while methanol is not an ideal extraction solvent since it is miscible with

water. In fact, methanol is incorporated into the extraction mixture to disturb the interaction of the target analyte with the cellular biopolymers such as proteins, owing to its polarity and high dielectric constant [38]. Although methanol has recently been used as a monophasic extraction system for phospholipids in blood and polar lipids in the upper layer of human skin [39], in our experiments, it showed no success in extracting gemini surfactants from the skin tissues (data not shown).

Adjustment of the pH in the B&D method is viewed as an effective way to optimize the extraction efficiency of specific classes of lipids [22, 23]. In the case of gemini surfactants, higher recovery and less ion suppression were reported with alkaline B&D compared to conventional B&D, however the differences were not statistically significant (Table 3). Alkaline medium caused an increase in logD values and a decrease in logS values of the tested gemini surfactants (Table 4), resulting in their higher partition into the organic layer. In addition, the alkaline medium increases the polarity of the phosphate moiety of the skin natural lipids, which might increase their partition into the aqueous layer, minimizing interference with gemini surfactants.

Acidified B&D was the least effective method among the B&D methods; it exhibited the highest ion suppression. This could be attributed to the higher affinity of the endogenous lipids to the organic phase under acidic conditions resulting in significant ion suppression. The addition of HCl neutralizes the negatively charged skin lipids resulting in increased hydrophobicity, hence, higher unfavorable partition into the organic phase.

Extraction with MTBE was significantly the least effective among the evaluated extraction protocols (Table 3). This could be attributed to the lower polarity index of MTBE of 2.5 compared to chloroform of 4.1. MTBE was introduced as an alternative solvent system to chloroform that offered simplified sample handling [24]. It has a lower density than water and methanol, thus, it forms the upper layer during the extraction allowing for easier analyte collection. While it was suggested that extraction with MTBE is effective for most major classes of lipids, including polar and neutral lipids [24], contradictory reports indicate that MBTE was only able to extract 10% of major polar lipids [40].

Comparison of the extraction efficiency among the three gemini surfactants revealed that 16-7N(G-C₁₁-K₃)-16 exhibited the highest recovery in all extraction methods, followed by 16-7N(G-K)-16, then 16-7N(G-C₆-K₃)-16 (Table 3). This trend was in accordance with the $\log P$ values of the gemini surfactants in which the higher the compound hydrophobicity, the higher its affinity into the organic phase, hence the higher the extraction efficiency (Table 4). However, while there is a major difference in the $\log P$ values between compound 16-7N(G-C₁₁-K₃)-16 and 16-7N(G-K)-16 (3.27 and 2.8 respectively), the differences in the extraction efficiency is not as dramatic. This could be explained by the higher number of terminal lysine moieties in compound 16-7N(G-C₁₁-K₃)-16 compared to 16-7N(G-K)-16 which exhibit higher affinity to bind to the negatively charged constituents of the skin. In fact, such speculation could explain the substantially low extraction efficiency of compound 16-7N(G-C₆-K₃)-16, which has three polar lysine residues and shorter hydrophobic spacer, conferring low lipophilicity to the molecule.

Since the modified Folch method demonstrated the highest process efficiency, it was selected as the liquid-liquid extraction method for the bioanalysis.

1 **Table 3:** Recovery, matrix effect, and process efficiency of the evaluated-peptide modified gemini surfactants using different liquid-
 2 liquid extraction protocol.
 3

Extraction Method	Gemini surfactants								
	16-7N(G-K)-16			16-7N(G-C ₆ -K ₃)-16			16-7N(G-C ₁₁ -K ₃)-16		
	% Recovery	%Matrix effects	%Process efficiency	% Recovery	%Matrix effects	%Process efficiency	% Recovery	%Matrix effects	%Process efficiency
MTBE	51.80 ± 2	89.75± 6	56.67 ± 1	21.9 ± 3	75.29 ± 7	23.1 ± 5	53.98 ± 1	83.66±4	45.86 ± 0.8
Folch	85.32 ± 7	71.48 ±6	75.06 ± 5	46.04 ± 7	71.8 ± 1	38.28 ± 0.3	93.82 ± 5	74.63 ±5	77.09 ± 3
Blight&Dyer	74.18 ± 3	77.92± 4	69.01 ± 0.5	30.7 ± 1	53.44 ± 3	30.1 ± 0.8	76.57 ± 0.8	62.71± 2	60.88 ± 4
Acidify B&D	60.17 ± 3	67.7 ± 6	60.81 ±5	28.37 ± 2	46.09 ± 0.9	29.64 ± 1	63.65 ± 5	53.91 ± 8	55.97 ± 4
Alkaline B&D	77.60 ± 2	88 ± 1	73.18 ± 2	39.9 ± 1	68.49 ± 4	31.2 ± 1	80.7 ± 6	67.24 ± 4	60.77 ± 0.5

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Table 4: Physicochemical properties of the evaluated gemini surfactants estimated using ACD/Physchem Profiler.

Physicochemical properties	Gemini surfactants		
	16-7N(G-K)-16	16-7N(G-C₆-K₃)-16	16-7N(G-C₁₁-K₃)-16
log P	2.80	1.28	3.27
log D at pH= 4	0.8	-2.7	-0.7
log D at pH= 6	1.0	-2.6	-0.6
log D at pH= 10	2.3	0.3	2.3
log S at pH= 4	-9.2	-7.6	-9.1
log S at pH= 6	-9.4	-7.7	-9.2
log S at pH= 10	-10.7	-10.6	-12.1

8 Single point calibration

9 Fully validated quantification methods are usually needed for preclinical and clinical
10 analysis, however, they require considerable time, workload, and resources [41]. In the case where
11 full validation is not required, a “fit-for-the-purpose” approach is a more suited analytical strategy
12 to obtain the needed data and is frequently used to answer predefined research questions [41, 42].
13 One common quantification strategy is single-point calibration; considered a compromise between
14 the rigor of the analytical method and the workload without sacrificing the accuracy of the results.
15 In fact, several studies compared single point to conventional multi-point calibration showing the
16 usefulness of single-point calibration in providing quantitative data with accuracy and precision
17 that meet regulatory guidelines [43, 44]. Since the scope of this work is to conduct a relative
18 comparison among three gemini surfactants with varying transfection efficiencies; a one-point
19 calibration quantitative strategy is deemed adequate to provide relative quantification data with
20 acceptable accuracy and precision, effectively reducing the required time, resources, and cost of
21 analysis.

22 In single point calibration, one reference concentration is employed for the quantification
23 of the analyte of interest. However, two conditions must be fulfilled: (i) the concentration-response
24 function is linear and (ii) the y-intercept is negligibly small [45]. In this work, a single
25 concentration of gemini surfactants was selected to serve as a calibration standard for each method:
26 *method A* and *method B* (Table S1, Supplementary Material). The calibration standard was
27 prepared in triplicate and the average response was used for quantification. The RSD values (i.e.
28 precision) in all cases did not exceed 11%. The concentrations of selected calibration standards
29 were relatively close to the concentration of the analytes in the skin tissue samples for *method A*
30 and in the mid-range of the sample concentrations in the PBS solution (Table S1, Supplementary
31 Material). The selection of each point was extrapolated from previous knowledge about the
32 penetration behaviour of similar delivery vectors, which was then adjusted experimentally to best
33 suit the method. Although some studies suggest that the linearity condition for single point
34 calibration can be avoided if the analyte amount in the evaluated samples is close to its amount in
35 the calibration standard, the linearity over the samples’ concentrations range was verified by
36 developing a three-point calibration curve in the range of 1 µg/mL to 7 µg/mL for *method A* and
37 of 50 ng/mL to 3000 ng/mL for *method B*. In order to assess the reliability and reproducibility of
38 the methods, the slope of the three-point calibration curve for each method was statistically
39 compared across at least three intra-run measurements. One way analysis of variance comparison
40 suggested that the variations between the evaluated slopes were not significantly different
41 ($p < 0.05$), indicating the reproducibility and reliability of the generated quantitative data.

42 Skin penetration study

43 The developed methods were used to assess the cutaneous deposition and penetration
44 behaviour of the three peptide-modified gemini surfactants after the topical application of P/G/L
45 nanoparticles. The selection of gemini surfactants was based on: (i) the variation in their molecular
46 structure and (ii) their differences in transfection efficiency profiles. Namely, 16-7N(G-K)-16 was
47 the lead compound with the highest transfection efficiency showing protein expression of 2.82 ± 0.2
48 ng/ 15×10^3 in PAM 212 murine keratinocytes. On the other hand, 16-7N(G-C₁₁-K₃)-16
49 demonstrated moderate transfection efficacy with protein expression of 1.73 ± 0.2 , while 16-7N(G-
50 C₆-K₃)-16 had a low transfection ability of 1.09 ± 0.1 . The choice of the three compounds was
51 based on a comprehensive evaluation of over 20 compounds (data not shown, manuscript in
52 preparation). Table 5 displays the skin disposition and penetration parameters of the gemini

53 surfactants obtained in accordance with the Organisation for Economic Co-operation and
54 Development (OECD) guideline for determining the dermal penetration of chemicals [26].

55 **Table 5:** Skin disposition and penetration parameters of the evaluated peptide-modified gemini surfactants. Results are the average of
 56 five measurements. Abbreviations: J_{ss} : the steady-state flux, t_{lag} : the lag-time, K_p : the skin permeability coefficient, D_m : diffusion
 57 coefficient and K_m : partition coefficient.

Gemini Surfactants	Applied dose ($\mu\text{g}/\text{cm}^2$)	Skin tissues		Skin penetration parameter (receptor compartment)						
		Amount in skin ($\mu\text{g}/\text{cm}^2$) \pm RSD (%)	% Deposited in skin	Total amount penetrated across skin ($\mu\text{g}/\text{cm}^2$) \pm RSD (%)	% Penetrated across skin	J_{ss} ($\text{ng}/\text{cm}^2/\text{h}$)	t_{lag} (h)	K_p (cm/h)	D_m (cm^2/h)	K_m
16-7N(G-K)-16	181.36	20.30 \pm 4	11.19	0.99 \pm 0.01	0.55	50.59	5.0	2.79 \times 10 ⁻⁴	2.70 \times 10 ⁻⁴	0.93
16-7N(G-C₆-K₃)-16	264.31	10.45 \pm 3	3.95	0.28 \pm 0.02	0.11	13.67	5.5	0.52 \times 10 ⁻⁴	2.45 \times 10 ⁻⁴	0.19
16-7N(G-C₁₁-K₃)-16	277.46	22.25 \pm 5	8.02	0.86 \pm 0.02	0.31	43.48	5.3	1.57 \times 10 ⁻⁴	2.55 \times 10 ⁻⁴	0.55

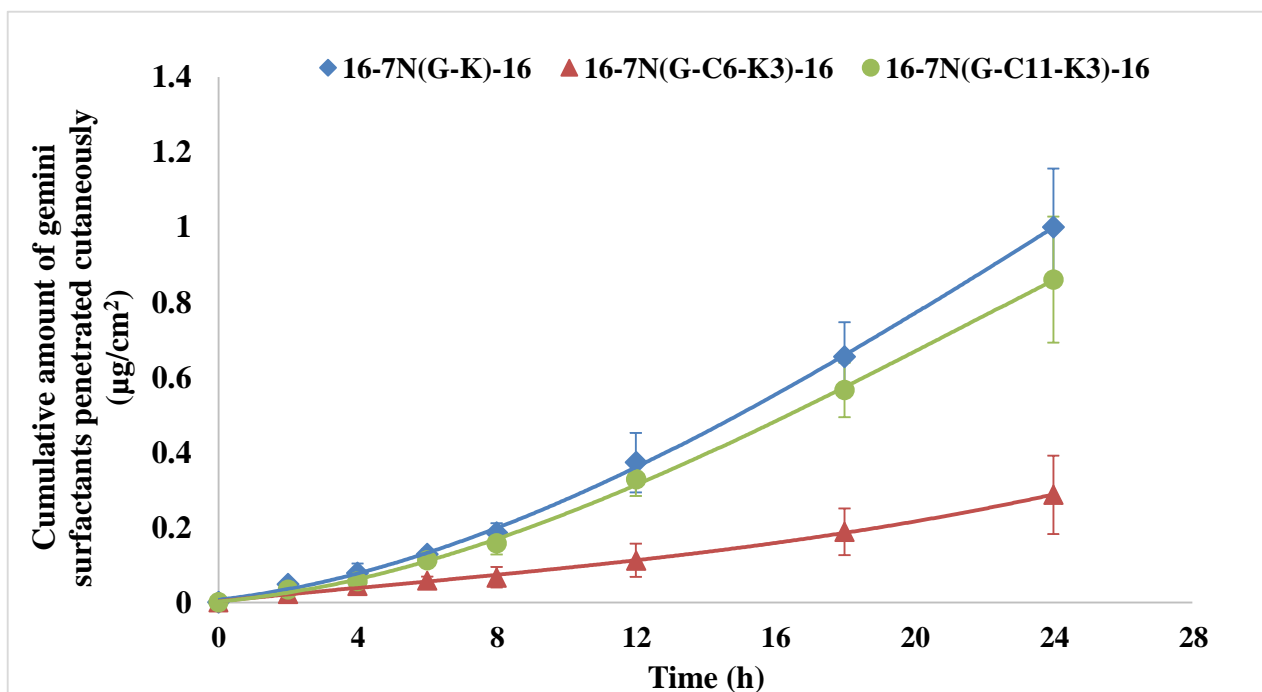
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After 24 h of topical application, FIA–MS/MS analysis of the skin tissues revealed that 3.95-11.19% of the applied dose of the three evaluated gemini surfactants was retained in the skin (Table 5). It is noteworthy that the detected amounts of gemini surfactants in the skin correlated with their transfection efficiency, where the lead compound 16-7N(G-K)-16 exhibited the highest skin deposition (11.19%) followed by 16-7N(G-C₁₁-K₃)-16 (8.02%) and the least performing compound 16-7N(G-K)-16 (3.95%). This could be explained by the gemini surfactants' physicochemical properties, particularly lipophilicity and molecular size. In fact, it has been established in the literature that molecular size and hydrophobicity are the main determinants of dermal penetration suggesting that small hydrophobic compounds have a higher tendency to pass through the different layers of the skin [46]. In the evaluated model compounds, the lead compound, 16-7N(G-K)-16, with the highest skin deposition had the smallest molecular size (M.Wt. 967.24 g/mol) among the tested compounds; in addition, it showed the highest lipophilicity ($\log D = 1.0$) at the intrinsic pH of the formulation (pH=6, Table 4). However, while compound 16-7N(G-C₆-K₃)-16 has a smaller molecular size than 16-7N(G-C₁₁-K₃)-16, it exhibited significantly less residence in the skin tissues (Table 5). This could be attributed to the significantly lower $\log D$ value of the former (-2.7) compared to the latter (-0.6) at the formulation intrinsic pH of 6 (Table 4). This is in agreement with the reported trend in the literature where lower skin penetration is expected for compounds with higher molecular weight unless they have higher lipophilicity [47].

In addition to the lipophilicity and molecular size, several other parameters such as solubility might play a role in determining skin penetration ability. In the evaluated model compounds, skin penetration correlated negatively with the compounds' aqueous solubility ($\log S$ at pH 6, Table 4). Lower aqueous solubility is usually associated with a higher ability to penetrate through the lipid-rich stratum corneum [48].

Finally, the physicochemical parameters of the P/G/L nanoparticles could also affect the dermal delivery. For example, small particle size and lower surface charge usually translate into higher skin penetration due to their superior ability to move through the complex skin matrix [49, 50]. Evaluation of the size and zeta potential of the P/G/L nanoparticles revealed that the lead compound, 16-7N(G-K)-16, exhibited the smallest particle size (85 ± 2 nm) and lowest zeta potential (34 ± 2 mV) among the tested compounds. On the other hand, compound 16-7N(G-C₁₁-K₃)-16 had a particle size of 96 ± 1 nm and a zeta potential of 44 ± 1 mV while 16-7N(G-C₆-K₃)-16 with the least skin disposition demonstrated the largest particle size and zeta potential, i.e., 107 ± 3 nm and 52 mV, respectively.

The cumulative amount of gemini surfactants that permeated across the skin into the receptor compartment increased progressively with time (Figure 5). After 24 h, only 0.11- 0.55% of the applied dose was found in the Franz cell diffusion receptor compartment. This is an indication that the gemini surfactant-based gene delivery system could be suitable for the treatment of localized skin conditions like scleroderma or melanoma with minimum passing into the systematic circulation.



99 **Figure 5:** Cumulative amount of gemini surfactants ($\mu\text{g}/\text{cm}^2$) penetrated across the skin into the
 100 Franz cell diffusion receptor compartment versus time curves. Results are the average of five
 101 measurements, error bars represent standard deviation.

102
 103 From the linear part of the curves plotted in Figure 5, the steady-state flux (J_{ss}), the lag-
 104 time (t_{lag}), the skin permeability coefficient (K_p), diffusion coefficient (D_m), and partition
 105 coefficient (K_m) were calculated (Table 5). The time required before the steady state absorption
 106 occurs denoted as t_{lag} was around 5 - 5.5 h for all compounds. The compound with the highest skin
 107 penetration 16-7N(G-K)-16 demonstrated the shortest t_{lag} of 5.0 h, followed by 16-7N(G-C₁₁-K₃)-
 108 16 (t_{lag} = 5.3 h), and 16-7N(G-C₆-K₃)-16, with t_{lag} = 5.5 h (Table 5). Lag time is a reflection of the
 109 efficiency of the compound to pass through the different layers of the skin. Therefore, it is mainly
 110 influenced by the same parameters that determine skin permeability, namely lipophilicity,
 111 solubility, and molecular size [51]. While t_{lag} inversely correlated with the $\log D$ values at the
 112 formulation intrinsic pH, it directly correlated with $\log S$ values (Table 4). The gemini surfactants'
 113 net rate of transport, once equilibrium conditions have been reached, is described by the steady-
 114 state flux (J_{ss}). Similar to t_{lag} , J_{ss} is a representation of the efficiency of the compounds to penetrate
 115 the skin. As such, J_{ss} is directly related to the total amount of gemini surfactants found in the Franz
 116 cell receptor compartment (Table 5).

117 The permeability coefficient (K_p), which depicts the rate at which gemini surfactants
 118 penetrate the skin, diffusion coefficient (D_m), and partition coefficient (K_m), are extracted from t_{lag}
 119 and J_{ss} (equations 2-4), thereby showing a direct relationship with the compounds' hydrophobicity
 120 at the intrinsic pH, lipid solubility, and skin penetration ability (Table 4 and 5). Reporting these
 121 parameters is not only valuable for drug delivery but it is also of significant interest for assessing
 122 the toxicity and occupational risk of the gemini surfactants.

123 Conclusion

124 In this work, rapid and simple FIA-MS/MS methods were developed for the relative
125 quantification of three peptide-modified gemini surfactants in skin tissues as well as PBS solution.
126 Combining the Folch liquid-liquid extraction protocol with weak cationic exchange SPE enhanced
127 the extraction efficiency of gemini surfactants from the complex lipid-rich skin tissues. As such,
128 proper sample preparation (i.e., clean extracts) eliminated the need for HPLC column, resulting in
129 fast analysis in which all compounds eluted before 0.8 min with a total acquisition time of merely
130 4 min. In addition, single-point calibration was successfully employed, resulting in a significant
131 reduction in the required time and resources for method development and sample analysis.

132 The developed methods were applied to assess the skin deposition and skin penetration
133 behavior of the evaluated gemini surfactants, generating data that could help in the design of more
134 effective cutaneous lipid-based gene delivery vectors. The skin disposition and penetration
135 behavior of gemini surfactants heavily relied on their physicochemical properties, particularly their
136 lipophilicity. Furthermore, it correlated with the transfection efficiency of the gemini surfactants.
137 A favorable deposition in the skin with minimum escape into the PBS compartment (representing
138 circulation) was observed, suggesting the feasibility of the delivery system in a topical application.
139 The developed methods will be further utilized to probe the biodistribution and fate of topically
140 applied therapeutic gemini surfactant formulations in animal models. Such knowledge is
141 fundamental before any translation from laboratory to clinical evaluation.

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