Advances in Chemical Engineering and Science, 2017, 7, 191-205
http://www.scirp.org/journal/aces
ISSN Online: 2160-0406
ISSN Print: 2160-0392

New Amphiphilic Amino Acid Derivatives for Efficient DNA Transfection *in Vitro*

Lucía C. Peña[1], María F. Argarañá[2], María M. De Zan[3], Antonella Giorello[4], Sebastián Antuña[5], Claudio C. Prieto[5], Carolina M. I. Veaute[6], Diana M. Müller[1*]

[1]LAQUIMAP, Dto. Química Orgánica, Universidad Nacional del Litoral, Santa Fe, Argentina
[2]Cát. Microbiología General, Universidad Nacional del Litoral, Santa Fe, Argentina
[3]Laoratorio de Control de Medicamentos, Universidad Nacional del Litoral, Santa Fe, Argentina
[4]Instituto de Investigaciones en Catálisis y Petroquímica, Universidad Nacional del Litoral, Santa Fe, Argentina
[5]Laboratorio de Cultivos Celulares, Universidad Nacional del Litoral, Santa Fe, Argentina
[6]Laboratorio de Inmunología Básica, Universidad Nacional del Litoral, Santa Fe, Argentina

Email: *dmuller@fbcb.unl.edu.ar

How to cite this paper: Peña, L.C., Argarañá, M.F., De Zan, M.M., Giorello, A., Antuña, S., Prieto, C.C., Veaute, C.M.I. and Müller, D.M. (2017) New Amphiphilic Amino Acid Derivatives for Efficient DNA Transfection *in Vitro*. *Advances in Chemical Engineering and Science*, **7**, 191-205.
https://doi.org/10.4236/aces.2017.72014

Received: February 16, 2017
Accepted: April 8, 2017
Published: April 11, 2017

Abstract

Nucleic acids-based therapies have recently developed as next-generation agents for treating and preventing viral infection, cancer, and genetic disorders, but their use is still limited due to its relatively poor delivery into targeted cells. We designed and synthesized new amphiphilic amino acid derivatives (cysteine-based) of low molecular weight, formed by the same pentapeptide (AG2: WWCOO) N-acylated, with different hydrophobic chains containing from 12 to 18 carbons, named AG2-C_n (N), which dimerize by oxidation in the presence of pLenti-CMV-GFP Puro plasmid (P) in the respective *gemini*. We determined transfection efficiency, critical micelle concentration, particle size, ζ-potential and cytotoxicity for the derivatives obtained. We found that all the synthesized compounds were active for DNA delivery and had greater ability to transfect CHO-K1 cells. In particular, AG2-C_{18} is a promising carrier for gene delivery because it showed no cytotoxicity and its activity was greater than or equal to the commercial actives currently used.

Keywords

Amphiphile, N-Acylated, Cysteine, Gemini, Ornithine, Transfection

1. Introduction

Gene therapy is a promising approach, with a potential to improve human health [1]. A successful gene therapy depends on efficient, safe and stable gene delivery systems. Chemically mediated non-viral vectors, such as cationic lipids, exhibit low immunogenicity, compared to viral vectors [2] [3]. Amphiphilic

gemini, a specific group of cationic lipids, has shown efficient transfection activity [4] [5]. These are dimeric amphiphiles with two hydrophilic heads and two hydrophobic groups per molecule, separated by a covalently bound spacer chain at the head groups, and are primarily used in material sciences because of their characteristic low surface tension [6] [7] [8] [9]. In recent years, there has been extensive research on *gemini* amphiphiles as non-viral gene delivery carriers for both *in vitro* and *in vivo* applications. These agents have a versatile chemical structure, can be easily produced on a laboratory scale, can compact DNA to nano-sized lipoplexes and show relatively low toxicity, compared to monomeric surfactants [4]. The transfection activity of *gemini* is influenced by the chemical nature of the head groups, length and saturation of the hydrophobic chains and by the chemical composition and length of the spacer [10] [11] [12].

Several classes of natural amino acid-based *gemini* have been synthesized and characterized for the purpose of gene delivery [13] [14] [15] [16]. One of the methods most commonly used for the synthesis of amphiphilic amino acid derivatives is the peptide *N*-terminal acylation [16]. If the peptide has cysteine in its sequence, it may dimerize by oxidative coupling obtaining the corresponding *gemini* [17] [18] [19] [20].

We recently described a new *gemini 3b* derived from a tetrapeptide consisting of tryptophan and ornithine ($COCH_3$-WWOO-$CONH_2$), designed with structural requirements similar to those for AMPs (antimicrobial peptides) and CPPs (cell penetrating peptides) [21]. This *gemini* is active towards bacteria causing foodborne diseases and has a potential longer biological half-life, as ornithine gives *gemini* enzymatic resistance. Structure activity relationship studies (SARS) determined that *gemini* 3b shows greater activity when the sequence has tryptophan residues and the ornithine residues are adjacent [22] [23].

The current study presents the development of new amphiphilic carriers with simple structure (molecular mass < 1 KDa), aimed to achieve high DNA *in vitro* transfection efficiency. We describe the synthesis of a series of new *N*-acyl and cysteine-based amphiphilic amino acid derivatives named AG2-C_n(N) (**Table 1** and **Figure 1**), where the head group derives from the sequence of the *gemini* 3b (WWCOO) and the tail group, acylated to the *N*-terminus of peptide, has been systematically varied.

Table 1. Sequences, characterization and CMC of the synthetic monomeric amphiphiles.

Identification	Sequence	Molecular mass[a] (Da)		Net Charge[b]	RT by RP-HPLC[c]	CMC (μM)[d]
AG2-C_{12}	CH_3-$(CH_2)_{10}$-CO-WWCOO-$CONH_2$	902.574	903.3	+2	22.049	54,5
AG2-C_{14}	CH_3-$(CH_2)_{12}$-CO-WWCOO-$CONH_2$	930.605	931.1	+2	22.071	18,54
AG2-C_{16}	CH_3-$(CH_2)_{14}$-CO-WWCOO-$CONH_2$	930.605	936.5	+2	22.085	64,2
AG2-C_{18}	CH_3-$(CH_2)_{16}$-CO-WWCOO-$CONH_2$	986.668	985,6	+2	22.093	21,6

[a]Calculated (left) and determined by ESI-MS (right). [b]Calculated at *pH* 7.4. [c]A C4 column was used, and peptides were eluted using a gradient elution of 5% - 95% of ACN in water containing 0.1% TFA. [d]Determined in Hepes 15 m*MpH* 7.4, DTT 10 mM.

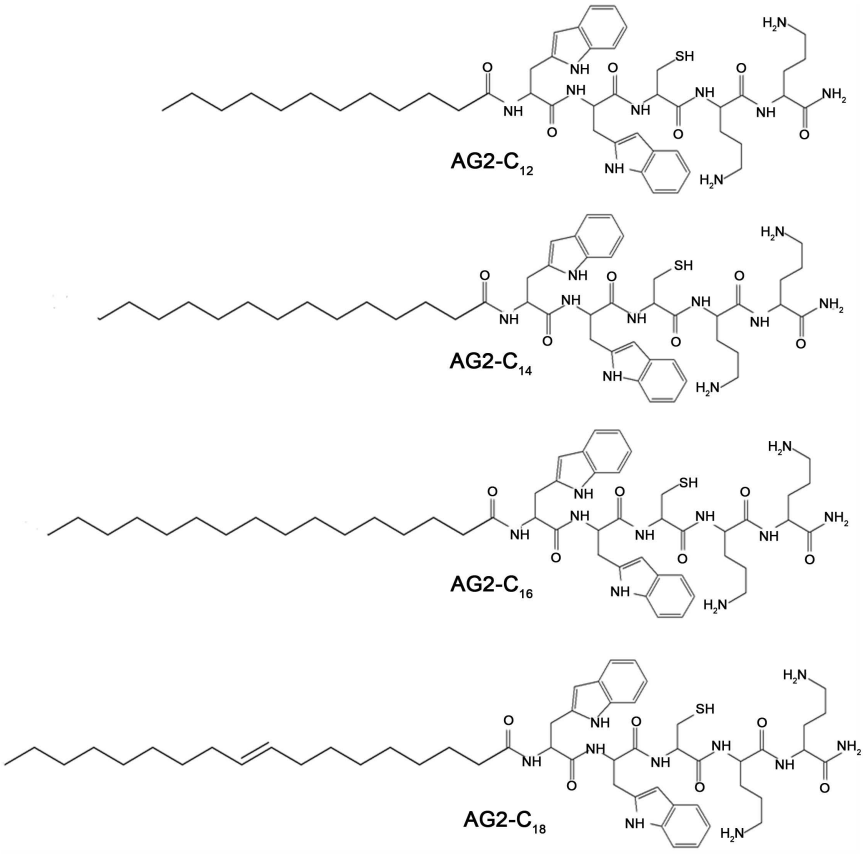


Figure 1. Structure of the synthetic monomeric amphiphiles.

The design of the structure of these compounds is potentially better than that of *gemini* 3b for two reasons, firstly the technique used in the hydrophobization of the peptide sequence has greater efficiency and yield and, secondly, the presence of the cysteine residue, gives AG2-C_n better interfacial properties and therefore greater biological activity.

AG2-C_n(monomer) dimerizes by oxidation in the presence of pLenti-CMV-GFP Puroplasmid (P) in the respective *gemini* during the formation of the lipoplex.

For the derivatives obtained, we determined critical micelle concentration (CMC), particle size, ζ-potential, cytotoxicity, antimicrobial activity, and gene transfection efficiency for HEK293 T and CHO-K1 cells using pEGFP as reporter gen.

2. Materials and Methods

2.1. Synthesis and Characterization of Monomeric Amphiphiles

Firstly, AG2 peptide (NH_2-WWCOO-$CONH_2$) was synthesized using Fmoc-solid phase peptide synthesis method [24]. Secondly, the lipophilic acid (lauric, myristic, palmitic and oleic) was attached to the *N*-terminus of a resin-bound peptide using the same synthesis method as for AG2 to obtain AG2-C_{12}; AG2-C_{14}; AG2-C_{16} and AG2-C_{18}, respectively (Table 1). Rink amide 4-Methylbenzy-

drylamine resin (MBHA) was used to prepare the C-terminal peptide amide. Couplings were performed by PyBOP ((Benzotriazol-1-yloxy)tripyrrolidi-nophosphonium hexafluorophosphate) and NMM (4-methylmorpholine) was used as catalyst; Fmoc-deblockings were done with 20% piperidine in DMF (Dimethylformamide) (v/v). The final cleavage from the resin was achieved by a mixture of TFA (trifluoracetic acid) /H2O/EDT (Ethanedithiol)/TIS (Triisopropylsilane) (94.5: 2.5: 2.5: 0.5) (v/v). After 3 h, the resin was filtered off and the crude peptide was precipitated in dry cold diethyl ether, centrifuged and washed several times with cold diethyl ether until scavengers were removed. The product was then dissolved in water and lyophilized twice.

The amphiphiles were analyzed by analytical RP-HPLC (Reverse Phase High Pressure Liquid Chromatography) using a Jupiter (Phenomenex, Torrence, CA, USA) C4 column (5 μm, 300 Å, 150 × 4.60 mm). The amphiphiles were eluted with a linear gradient of 5% - 95% acetonitrile with 0.1% TFA at flow rate of 0.8 mL per min for 33 min at 30˚C. The parameters corresponding to the chromatographic analysis were those obtained after an optimization process. The broad gradient range of acetonitrile (5% - 95%) was applied to detect the presence of impurities of different degrees of hydrophobicity. The application of higher temperature to room temperature is to facilitate the elution of hydrophobic compounds of interest and to take care of the useful half life of the column used. The absorbance was measured at 220 and 240 nm. All the amphiphiles synthetically prepared were analyzed by ESI-MS (electrospray ionization mass spectrometry)using UPLC-MS SQD 2 (Waters) and the peptide amino acid sequence was confirmed by automatic Edman Degradation, performed on a Shimadzu PPSQ-23-A sequencer.

2.2. Determination of Critical Micellar Concentration

Critical micellar concentrations of monomeric amphiphiles were obtained by measuring surface tension using a CSC Scientific Du Nouytensiometer. Serial dilutions were prepared from a concentrated stock solution of the amphiphiles in Hepes buffer (10 mM, *pH* 7.4) containing DTT (Dithiothreitol) (15 mM) to avoid detergent oxidation. A plot of the tension (mN/m) fluorescence versus the logarithm of the surfactant concentration displayed a sharp break and the corresponding concentration was considered to be the CMC.

2.3. Preparation and Characterization of Complexes

The plasmid pLenti CMV GFP Puro (658-5) was a gift from Eric Campeau (Addgene plasmid # 17448) [25]. Lipoplexes were prepared according to the technique described by Wang *et al.* [26]. DNA complexes at N/P ratios of 1.2, 2.4, 4.8, 9.6, 15.0, 19.2, 28.0, and 38.4 were prepared by adding AG2-C_n from a 5 mM methanol stock solution to a 20 μg pLenti-CMV-GFP Puro (Addgen plasmid DNA into 1 mL Hepes buffer (15 mM, *pH* 7.4) under constant stirring. The mixture was incubated for 30 minutes at room temperature. DNA concentration was checked by measuring its absorbance at 260 nm. Complexes were kept at

room temperature to allow cross-linking to occur prior to further characterization. The particle size and ζ-potential of the complexes were further determined by dynamic light scattering using a ZetaSizer Nano ZS90 (Malvern, Worcestershire, U.K) with the following specifications: refractive index, 1.45 (typical liposome RI); medium viscosity, 1.054 cP; medium dielectric constant, 80; scattering angle, 90°; temperature, 25˚C. Data were analyzed using the multimodal number distribution software included in the instrument. ζ-potentials were measured according to the following specifications: refractive index, 1.45 (typical liposome RI); medium viscosity, 1.054 cP; medium dielectric constant, 80; scattering angle, 90˚; temperature, 25˚C.

2.4. Agarose Gel Electrophoresis

The ability of the amphiphiles to condense the DNA was determined by agarose gel electrophoresis. The AG-2-C_n/pLenti-CMV-GFP Puro plasmid complex was prepared at N/P ratios ranging from 0.6 to 9.6. After 30-minute incubation, the complex was electrophoresed at 100 V for 15 minutes on agarose gels (1.0%, w/v).The location of plasmid bands was visualized under ultraviolet light by Gelred™ fluorescent dye.

2.5. Gene Transfection Assay

Gene transfection efficiency of AG2-C_n/pLenti-CMV-GFP Puro plasmid complex was evaluated in HEK293 T and CHO-K1 cells, using GFP (green fluorescent protein) as reporter gene. Briefly, HEK293 T and CHO-K1 cells were seeded into 24-well plates at a density of 3×10^5 cells per well, respectively. After 24 h of incubation, the culture medium was removed. Cells were added with fresh culture medium containing AG2-C_n/pLenti-CMV-GFP Puro complexes, with different N/P ratios, at 37˚C, and incubated for another 3 h. The culture medium was then replaced and the cell culture was expanded. After additional 21 h incubation, GFP expression in the transfected cells was observed by fluorescent microscopy and quantitated by flow cytometry. Commercial transfection reagents: Lipofectamine 2000 and PEI (Polyethylenimine) were used as controls. The experiment was repeated three times.

2.6. Detection of Antimicrobial Activity

The agar-well-diffusion assay was used to investigate antimicrobial activity of amphiphiles [27]. For this purpose, 1 mL overnight culture of each indicator strain was added to 19 mL of molten Nutrient Agar Mueller Hinton (Difco) and poured into a sterile Petri dish. After cooling, wells of 5 mm diameter were cut out in the agar plates and filled with 50 µL of amphiphile aqueous solution, at two concentrations, one below and one above CMC. The plates were incubated at 37˚C for 24 h and the diameters of the clear inhibition zones were subsequently measured. Each assay was repeated three times and the results were expressed as an average of the values obtained. The following bacterial strains from ATCC (American Type Culture Collection) were used as indicators of lipopep-

tideantimicrobial activity: *Listeria monocytogenes* ATCC 15313; *Bacillus subtilis* ATCC 6633; *Staphylococcus aureus* ATCC 25923; *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27857.

2.7. Hemolytic Assay

For the determination of hemolytic activity, Triton X-100 (1%, w/v), free AG2-C_n, and AG-2-C_n/pLenti-CMV-GFP Puro plasmid complexes were dissolved in PBS (phosphate buffered saline) with *pH* adjusted to 7.4. Serial dilutions were performed for amphiphile-containing solutions to obtain concentrations ranging from 1000 μg/mL to 0.5 μg/mL. Aliquots (200 μL) for each sample were transferred into 0.6 mL microcentrifuge tubes. RBCs (Rat red blood cells) were suspended in PBS (0.4 % v/v) at *pH* 7.4, and 200 μL of RBCs solution was mixed with the sample solution and incubated at 37˚C for 1 h. The mixture was then centrifuged at 1500 rpm for 10 min. 100 μL of supernatant was collected from each sample, and the absorbance was measured at 540 nm using a microplate reader to determine the hemoglobin concentration released. The relative hemolytic capacity was calculated by normalizing the absorbance of samples to that treated with Triton X-100 [28].

2.8. Compound Toxicity Assay by Crystal Violet Dye

The toxicity of each free amphiphile was determined by crystal violet staining. This method is useful for the rapid detection of highly toxic compounds at 48 h. Since the dye stains viable cells, the less intensively coloured cells indicate compound toxicity. Cells were seeded in 1×10^5 cell/well into 0.2 ml growing medium and incubated at 37˚C and 5% CO_2 for 24 h. Supernatants were discarded and two-fold serial dilutions of each free amphipile were evaluated in triplicate. The amphiphile concentrations ranged from 1000 μg/ml to 0.1 μg/ml. Serial dilutions were performed for surfactant-containing solutions to achieve the surfactant concentrations. Between 1000 μg/ml and 0.5 μg/ml cells were incubated for 48 h at 37˚C and 5% CO_2. The supernatants from each well were discarded and 50 μl/well of crystal violet dye was added and incubated at 37˚C and 5% CO_2 for 30 min. The dye was then removed and the plates were generously washed with water. 100 μl/well of acetic acid was added and colour intensity was measured by spectrophotometer or by a 96-well plate reader capable of measuring absorbance at 540 nm. Untreated cells were considered to be the negative control. The non-toxic limit concentration was calculated as the highest concentration of compound which produced the same colour intensity than that of the negative control.

3. Results and Discussion

3.1. Synthesis and Characterization of AG2-C_n

The monomeric amphipiles AG2-C_n were synthesized with high purity (>95%), the mass-average molecular weights are summarized in **Table 1**. Amphiphiles have a net positive charge of +2 at *pH* 7.4, due to the presence of two residues of

ornithine. The RT (retention times) of the different amphiphiles determined by RP-HPLC and the experimental molecular mass determined by ESI-MS are also shown in Table 1.

3.2. DNA Binding Assay

Gel retardation is a technique widely used for assessing complex formation between plasmid DNA and gene delivery vectors. Amphiphiles condense DNA into large particles that remain in the loading well. Figure 2 shows the results of agarose gel electrophoresis for the binding affinity for pLenti-CMV-GFP Puro plasmid to AG2-C_n. All the compounds tested formed lipoplexes but at different N/P ratios. The number of cationic nitrogen of *gemini* required per phosphorous residue of pDNA (*i.e.*, N/P ratio) for complete complexation was found to be close to 4.8 and 9.6 for AG2-C_{12} and AG2-C_{14}, respectively, and 2.4 for AG2-C_{16} and AG2-C_{18}, which is comparable to other cationic amphiphiles with excellent gene transfection ability [15]. During gel electrophoresis, fluorescent dye bands were present with equal intensity within the N/P ratios 0 - 0.6 for all the AG2-C_n, which indicates no prominent binding with pDNA. In contrast, the fluorescent dye band disappeared at N/P ratio of 4.8 for AG2-C_{12}, 9.6 for AG2-C_{14}, and 2.4 for AG2-C_{16} and AG2-C_{18}. During the formation of DNA-AG2-C_n complexes the migration of DNA is retarded and the fluorescent dye is displaced by *gemini*, which may explain the disappearance of the DNA bands [29].

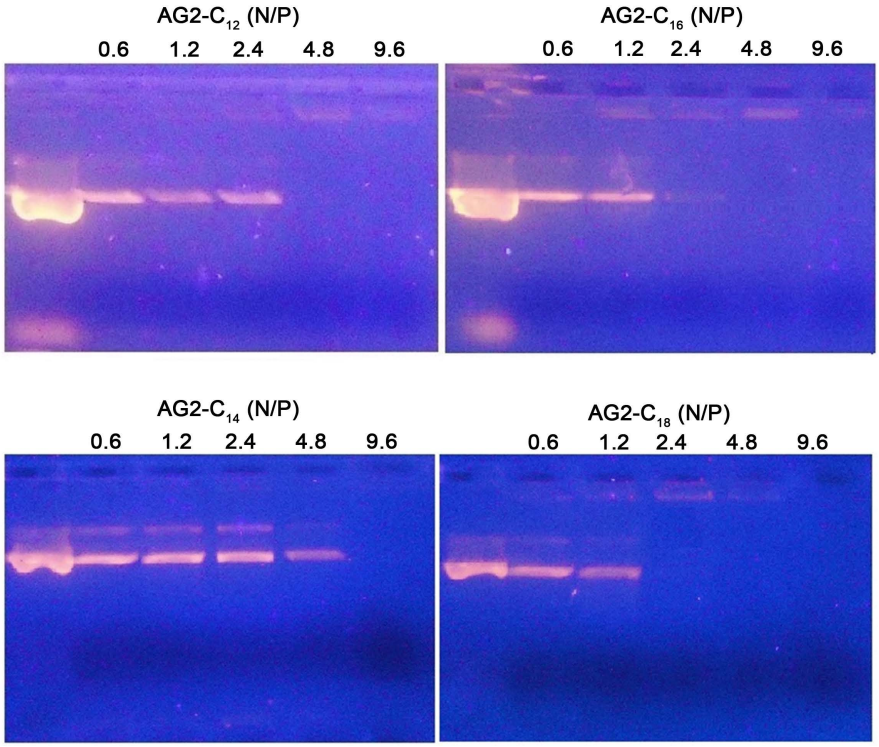


Figure 2. Agarose gel electrophoresis shift assay of AG2-C_n/pLenti-CMV-GFP Puro complexes at indicated N/P ratios.

3.3. Characterization of AG2-C_n/pLenti-CMV-GFP Puro Complexes

Appropriate size and ζ-potential are important for efficient gene transfection [12]. In this study, the particle size and ζ-potential of AG2-C_n/pLenti-CMV-GFP Puro complexes were studied by light-scattering technique at different N/P ratios (Table 2 and Table 3). According to Dauty *et al.* [12], lipoplexes size increases with the increase of N/P ratio or the chain length (C_{12} to C_{14}) of the amphiphile, as it leads to a growth in the particle size (Table 2). The average size is reproducible at low charge ratios. However, size values fluctuate considerably above N/P ratio of 19.6 due to the onset of precipitation that reduces the accuracy of the light scattering measurement. Lipoplex size was reported to have a close relationship with transfection efficiency [30]. However, a specific correlation between lipoplex size and transfection efficiency using the AG2-C_n compounds as vectors were not discernible as all the lipoplexes at optimum charge ratio showed a similar size (around 110 - 350 nm diameter) and lipoplex sizes were variable to N/P ratio, while the compounds showed maximum activity.

ζ-potentials are negative at low N/P ratio due to excess DNA and become less negative as N/P ratio increases to the estimated isoelectric point at N/P ratios ranging from 2.4 to 4.8 (Table 3) and remain positive up to an N/P ratio of 28.0. The formation of AG2-C_n/pLenti-CMV-GFP Puro complexes appears to occur well above the N/P ratio of 1:1 that has been reported for traditional single tail, single head surfactants with DNA, or for single head, double tail surfactants with DNA [31].

Table 2. Nanoparticle diameter (nm) of AG2-C_n/pLenti-CMV-GFP Puro complexes as measured by dinamic light scattering.

(N/P)[a]	AG2-C_{12}[b]	AG2-C_{14}[b]	AG2-C_{16}[b]	AG2-C_{18}[b]
9.6	146 ± 45	343 ± 175	155 ± 22	107 ± 4
15	169 ± 15	242 ± 28	215 ± 16	146 ± 32
19.2	203 ± 81	222 ± 41	195 ± 31	173 ± 47
28	210 ± 8	331 ± 72	350 ± 128	327± 31

[a]Ratio of amphiphile amine functions to DNA phosphates. [b]Mean diameter from the multimodal distribution analysis; average and standard deviation of $n = 3$ determinations.

Table 3. Zeta-potential (mV) of AG2-C_n/pLenti-CMV-GFP Puro complexes at *pH* 7.4 as measured by dinamic light scattering.

N/P[a]	AG2-C_{12}	AG2-C_{14}	AG2-C_{16}	AG2-C_{18}
1.2	−42 ± 0.833	−40.1	−34.4 ± 0.153	−23.7 ± 0.814
2.4	−30 ± 2.29	−7.74 ± 3.1	−10.2 ± 0.482	−28 ± 0.751
4.8	30.1 ± 2.46	33	32.3 ± 2.25	14.9 ± 0.643
9.6	33.4 ± 5.02	38.5 ± 0.611	38.2 ± 0.351	42.9 ± 0.805
15	37.8 ± 1.16	38 ± 0.889	37.9 ± 0.513	39.3 ± 2.43
19.2	37.9 ± 0.551	37.4 ± 0.265	37.5 ± 0.416	37.8 ± 1.79
28	35.2 ± 1.82	34.4 ± 0.569	34.8 ± 0.493	37.1 ± 0.2

[a]Ratio of amphiphile amine functions to DNA phosphates.

The ζ-potential of the lipoplexes might be considered to be a good indicator of the importance of the first step in the overall transfection process, which is the adhesion of the lipoplex to the negatively charged cell membrane. Nevertheless, as it has been previously shown [25], a higher positive ζ-potential does not appear to correlate to higher transfection efficiency, since although lipoplexes acquire a positive charge between +33 and +38 up to an N/P ratio of 4.8, the minimum and a maximum activity are observed at N/P ratios of 15.0 and 28.0, respectively.

3.4. Critical Micellar Concentrations

Knowledge on CMC is of upmost importance for transfection: the presence of excess cationic micelles of the amphiphiles during the complex formation step may trigger the aggregation of anionic condensed DNA particles; a high CMC is thus preferable. Onthe other hand, once oxidized, the resulting dimeric amphiphile (*gemini*) should have a very low CMC to avoid early extraction from the amphiphile/DNA complexes during the gene delivery process [32] [33]. The CMCs of the monomeric amphiphiles were determined at neutral *pH* in 10 mM DTT using a Du Nouytensiometer. The results are presented in **Table 1**. In this paper, we assume that the differences observed for the CMCs of the synthesized compounds are due only to the different hydrophobic chains formed. Similarly to the trend exhibited by conventional amphiphiles, the CMC of AG2-C_n decreased as alkyl chain length increased from C_{12} to C_{14} or from C_{12} to C_{18}. We attribute the unexpected value for C_{16} to the presence of AG2 pentapentide not hydrophobised, because the quality of palmitic acid used was not optimal, compared to the other fatty acids employed. Finally, AG2-C_{14} and AG2-C_{18} were the molecules that showed the lowest CMC value, with a surface tension at concentration higher than the rest, conditions which allow the efficient formation of lipoplexes.

3.5. Gene Transfection Efficiency

Gene transfection efficiency in HEK293 T and CHO-K1 cells was evaluated by expression assays, using GFP as the reporter gen. PEI and Lipofectamine 2000 were used as controls. It was determined that all the compounds tested were active for both cell lines tested, significantly higher against CHO-K1 cells, except AG2-C_{12}. In particular, AG2-C_{18} was able to transfect 30% - 40% more CHO-K1 cells. In comparison, AG2-C_{18} transfected 67% CHO-K1 cells, obtaining a value similar to the one obtained for PEI (67%) and greater than the one obtained for Lipofectamine 2000 (55%). It seemed that gene transfection efficiency was dependent on N/P ratio, which could be observed for both HEK293 T and CHO-K1 cells (**Figure 3(a)** and **Figure 3(b)**). Except for the least active amphiphile, AG2-C_{16}, all compounds showed two peaks of activity at N/P ratio of 15 and 28, respectively. Normally, most compounds of this type have a rising activity curve with a single maximum [11] [28] [34]. Although the reasons are not clear yet, we can assume that the low activity observed for all compounds atan N/P ratio of 15

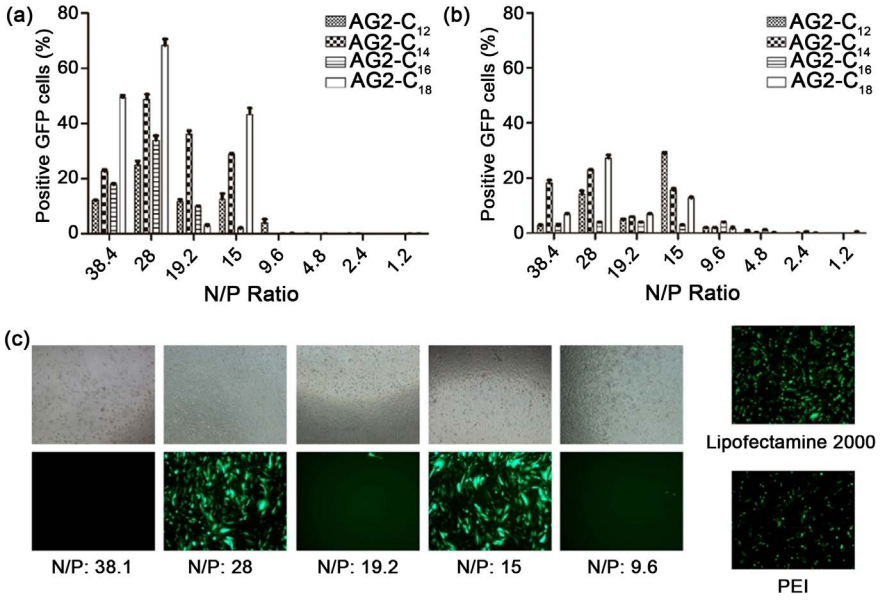


Figure 3. *In vitro* transfection efficacy of AG2-C_n/pLenti-CMV-GFP Puro complexes. Notes: Quantitative measurement of transfection efficiency forAG2-C_n/pLenti-CMV-GFP Puro complexes at diferent N/P ratios in CHO-K1 cells (a) and HEK293 T17 cells (b) using flow citometry. Fluorescent images of transfection efficiency for AG2-C_n/pLenti-CMV-GFP Puro complexes at diferent N/P ratios in CHO-K1 cells (c).

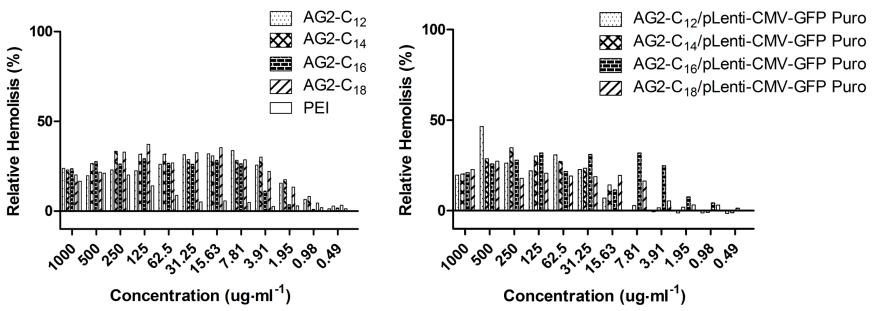
can be because lipoplexesare still not fully formed. As measured by DLS sizes are smaller (average 192 nm) than those observed at an N/P ratio of 28 (average 305 nm) (**Table 2**). The fluorescence intensity of AG2-C18/pLenti-CMV-GFP Puro complexes at N/P ratio of 28 was comparable to that of PEI/ pLenti-CMV-GFP Puro and Lipofectamine 2000/pLenti-CMV-GFP Puro complexes (**Figure 3(c)**). Given that for all amphiphiles the peptide sequence (polar head) is the same, we can attribute the increased activity for AG2-C_{18} to the presence in the molecule of the hydrophobic tail of 18 carbon unsaturated oleic acid. This agrees with Fielden *et al.* [11] and Castro *et al.* [35], who reported that the introduction of unsaturation to C_{18} tails (85% cis) transfects CHO-K1 cells with efficiency comparable to Lipofectamine Plus/2000.

The amphiphiles designed for this paper are based on the amino acid sequence of a *gemini* compound tested by our group as antimicrobials [22] [23]. These *gemini* were designed considering the structural requirements reported for antimicrobial peptides (AMPs) and cell penetrating peptides (CPPs) regarding the need for positive charges (basic peptides: Lys, Arg, Orn) and the presence of hydrophobic residues (Trp, Tyr, Phe) involved in the membrane destabilization processes [21] [36] [37]. The original sequence of these antimicrobials amphiphiles was modified by the addition of the cysteine residue in order to be tested as DNA transfection agents. The thiol group of cysteine from AG2-C_n undergoes oxidative coupling to yield cystine and produces *gemini* in presence of DNA. These *gemini* surfactants self-assembled at a much lower concentration than their monomeric counterparts and they also showed a lower surface tension

at the CMC. This improvement in the interfacial properties makes them less suitable to act individually at cell membrane level, and more suitable to cross without damaging it. The residues of ornithine are positively charged at neutral *pH*, which helps particles to interact with plasmid DNA and cellular membrane. Ornithine also helps overcome one of the biological barriers associated with the process of lipofection, which is DNA escape from the endosome (to avoid the formation of lysosomes and the destruction of plasmid by nucleases). Meanwhile, the interaction of tryptophane residues with GAG (glycosaminoglycan) cell membranes promote the endocytosis of DNA [38] [39]. The design of these amphiphiles is new. The literature describes the importance of the coexistence of tryptophane and cysteine in the sequence of some CPP for cellular internalization [40], but there are no reports on amphiphilic amino acid derivatives which combine tryptophan, cysteine, and ornithine residues in the same structure. We believe that this combination could be the explanation for the fact that the lipoplexes formed from these amphiphiles are much more easily internalized in CHO-K1 cells than in other cell lines, which should be further researched.

3.6. Hemolytic Activity Study

The membrane disruption of AG2-C_n was evaluated by hemolytic assay. **Figure 4(a)** shows the hemolysis of the free amphiphiles in a concentration range from 1000 μg/mL to 0.5 μg/mL. All amphiphiles showed concentration-dependent hemolysis at *Ph* 7.4. However, the hemolytic activity observed is markedly lower than the activity observed for similar molecules [40]. In fact, for a concentration of about 6 μM Xu *et al.* [41] detected between 10% and 60% hemolysis while, at the same concentration, our amphiphiles reached between 7% and 20% hemolysis. The AG2-C_n/pLenti-CMV-GFP Puro complexes also show concentration-dependent hemolytic activity (**Figure 4(b)**). However, except for AG2-C_{16}, for the entire range of concentrations tested, hemolytic activity decreases significantly when amphiphileis as part of the complex. Only 15% hemolysisis observed when the concentration of amphiphile corresponds to the maximum activity (N/P of 28, approximately 20.5 μg/mL).



(a) (b)

Figure 4. Hemolytic activities of AG2-C_n (Free carriers) (a) and AG2-C_n/pLenti-CMV-GFP Puro complexes (b) at variable concentrations at *pH* 7.4. TritonX-100 (1%, w/v), PBS and its complexes with pLKV1-EGFP plasmid were used as controls with 100% and <10% of hemolytic activity respectively.

3.7. Cytotoxicity Assay

The cytotoxicity for AG2-C_n in CHO-K1 cells is shown in Figure 5. As it can be seen, all the amphiphilic amino acid derivatives showed low cytotoxicity. No cytotoxicity was observed over the 24-h period for AG2-C_{12} and AG2-C_{16} at 0.48 - 62.5 μg/mL concentrations and for AG2-C_{14} and AG2-C_{18} at 0.48 - 32.25 μg/mL concentrations, which indicates favourable biocompatibility with CHO-K1 cells. The concentrations at which maximum activity can be observed for these compounds (N/P ratio 28) are within those ranges The low cytotoxicity observed is consistent with the lack of antimicrobial activity observed for these compounds. In fact, all compounds were tested in their activity towards pathogenic bacteria at two concentrations, one below and one above CMC. Activity was observed only for AG2-C_{12}, which was poorly active against *Bacillus subtilis*.

4. Conclusion

Four biocompatible and dimerizable amphiphilic aminoacid-based derivatives were designed and synthesized for delivering nucleic acids. The carriers had low critical micelle concentrations and formed nanoparticles with plasmid DNA. The nucleic acid nanoparticles with all the carriers showed low cytotoxicity and high activity at physiological *pH*. The amphiphilic carriers were more effective to transfect CHO-K1 cell, mainly AG2-C_{18}, with efficiency 12% higher than that of Lipofectamine 2000. Further studies on a greater number of cell lines are required to establish specificity of action, to correlate the physicochemical and structural properties for AG2-C_n/pLenti-CMV-GFP Puro complexes with *in vitro* transfection of CHO-K1 cells, and to contribute to a better understanding of the gene delivery process.

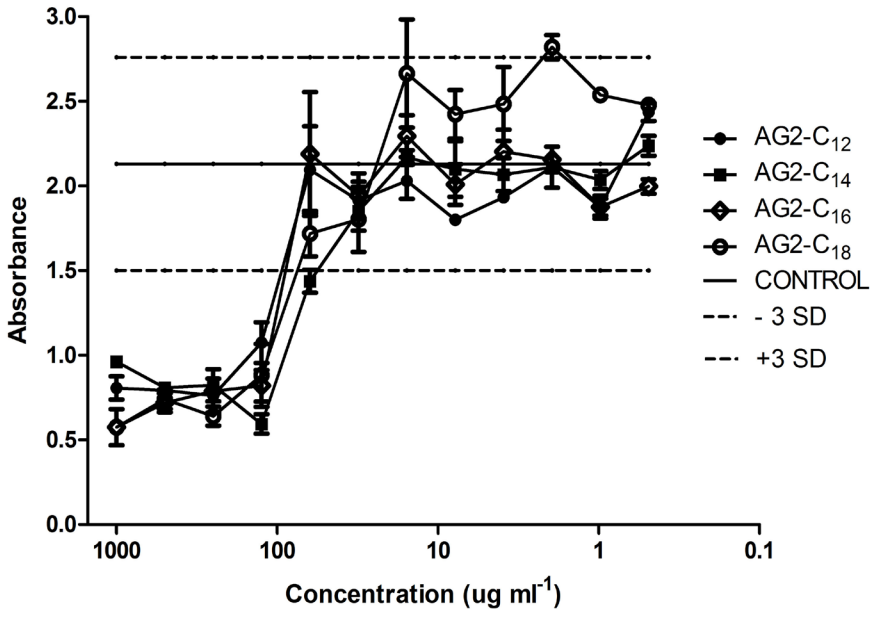


Figure 5. Cytotoxicity of AG2-C_n on CHO-K1 cells. Data are shown as the mean ± standard deviation (n = 3).

Acknowledgements

This work was supported by grants from Universidad Nacional del Litoral (U.N.L) (CAI+D, 2011), República Argentina.

References

[1] Friedmann, T. (1999) The Development of Human Gene Therapy. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

[2] Thomas, C.E., Ehrhardt, A. and Kay, M.A. (2003) Progress and Problems with the Use of Viral Vectors for Gene Therapy. *Nature Reviews Genetics*, **4**, 346-358. https://doi.org/10.1038/nrg1066

[3] Ilies, M., Seitz, W.A. and Balaban, A.T. (2002) Cationic Lipids in Gene Delivery: Principles, Vector Design and Therapeutical Applications. *Current Pharmaceutical Design*, **8**, 2441-2473. https://doi.org/10.2174/1381612023392748

[4] Badea, I., Verrall, R. and Baca-Estrada, M. (2005) *In Vivo* Cutaneous Interferon-γ Gene Delivery Using Novel Dicationic (Gemini) Surfactant-Plasmid Complexes. *The Journal of Gene Medicine*, **7**, 1200-1214. https://doi.org/10.1002/jgm.763

[5] Singh, J., Yang, P., Michel, D., Verrall, R., Foldvari, M. and Badea, I. (2011) Amino Acid-Substituted Gemini Surfactant-Based Nanoparticles as Safe and Versatile Gene Delivery Agents. *Current Drug Delivery*, **8**, 299-306. https://doi.org/10.2174/156720111795256200

[6] Menger, F.M. and Littau, C. (1991) Gemini-Surfactants: Synthesis and Properties. *Journal of the American Chemical Society*, **113**, 1451-1452. https://doi.org/10.1021/ja00004a077

[7] Menger, F. and Littau, C. (1993) Gemini Surfactants: A New Class of Self-Assembling Molecules. *Journal of the American Chemical Society*, **115**, 10083-10090. https://doi.org/10.1021/ja00075a025

[8] Donkuru, M.D., Wettig, S.D., Verrall, R.E., Badea, I. and Foldvari, M. (2012) Designing pH-Sensitive Gemini Nanoparticles for Non-Viral Gene Delivery into Keratinocytes. *Journal of Materials Chemistry*, **22**, 6232-6244. https://doi.org/10.1039/c2jm15719e

[9] Yang, P., Singh, J., Wettig, S., Foldvari, M., Verrall, R.E. and Badea, I. (2010) Enhanced Gene Expression in Epitelial Cells Transfected with Amino Acid-Substituted Gemini Nanoparticles. *European Journal of Pharmaceutics and Biopharmaceutics*, **75**, 311-320.

[10] Kirby, A.J., Camilleri, P., Engberts, J.B.F.N., Feiters, M.C., Nolte, R.J.M., *et al.* (2003) Gemini Surfactants: New Synthetic Vectors for Gene Transfection. *Angewandte Chemie International Edition*, **42**, 1448-1457. https://doi.org/10.1002/anie.200201597

[11] Wettig, S.D., Verrall, R.E. and Foldvari, M. (2008) Gemini Surfactants: A New Family of Building Blocks for Non-Viral Gene Delivery Systems. *Current Gene Therapy*, **8**, 9-23. https://doi.org/10.2174/156652308783688491

[12] Dauty, E., Remy, J.S., Blessing, T. and Behr, J.P. (2001) Gemini Surfactants Structurally Related to 1, Derived from the Oxidative Dimerization of Cysteine-Based Monomers, Have Been Reported by the Strasbourg Group to Show Interesting Transfection Capabilities. *Journal of the American Chemical Society*, **123**, 9227-9234. https://doi.org/10.1021/ja015867r

[13] Ronsin, G., Perrin, C., Guédat, P., Kremer, A., Camilleri, P. and Kirby, A.J. (2001) Novel Spermine-Based Cationic Geminisurfactants for Gene Delivery. *Chemical Communications*, No. 21, 2234-2235. https://doi.org/10.1039/b105936j

[14] Fielden, M.L., Perrin, C., Kremer, A., Bergsma, M., Stuart, M.C. and Camilleri, P. (2001) Sugar-Based Tertiary Amino Gemini Surfactants with a Vesicle-to-Micelle-transition in the Endosomal pH Range Mediate Efficient Transfection *in Vitro*. *The FEBS Journal*, **268**, 1269-1279. https://doi.org/10.1046/j.1432-1327.2001.01995.x

[15] Candiani, G., Frigerio, M., Viani, F., Verpelli, C., Sala, C. and Chiamenti, L. (2007) Dimerizable Redox-Sensitive Triazine-Based Cationic Lipids for *in Vitro* Gene Delivery. *ChemMedChem*, **2**, 292-296.

[16] Pérez, L., Pinazo, A., Pons, R. and Infante, M.R. (2014) Gemini Surfactants from Natural Amino Acids. *Advances in Colloid and Interface Science*, **205**, 134-155.

[17] Yoshimura, T., Sakato, A., Tsuchiya, K., Ohkubo, T., Sakai, H. and Abe, M. (2007) Adsorption and Aggregation Properties of Amino Acid-Based N-Alkyl Cysteine Monomeric and N,N'-Dialkyl Cystine Gemini Surfactants. *Journal of Colloid and Interface Science*, **308**, 466-473.

[18] Faustino, C.M.C., Calado, A.R.T. and Garcia-Rio, L. (2010) Dimeric and Monomeric Surfactants Derived from Sulfur-Containing Amino Acids. *Journal of Colloid and Interface Science*, **351**, 472-477. https://doi.org/10.1016/j.jcis.2010.08.007

[19] Jennings, K., Marshall, I., Birrell, H., Edwards, A., Haskins, N., Sodermann, O. and Kirby, A.J. (1998) The Synthesis and Aggregation Properties of a Novel Anionic Gemini Surfactant. *Chemical Communications*, **18**, 1951-1952.

[20] Miller, A.J. (1998) Cationic Liposomes for Gene Therapy. *Angewandte Chemie International Edition*, **37**, 1768-1785. https://doi.org/10.1002/(SICI)1521-3773(19980803)37:13/14<1768::AID-ANIE1768>3.0.CO;2-4

[21] Bechara, C. and Sagan, S. (2014) Cell-Penetrating Peptides: 20 Years Later, Where Do We Stand? Historical Perspective. *Advances in Colloid and Interface Science*, **205**, 134-155.

[22] Müller, D.M., Ingaramo, M.C., Argañará, M.F. and Murguía, M.C. (2011) Síntesis y actividad biológica de nuevos surfactantes peptídicos tipo gemini. *CIT*, **22**, 11-20. https://doi.org/10.4067/S0718-07642011000500003

[23] Garello, C.P., Ingaramo, M.C., Argarañá, M.F., Murguía, M.C. and Müller, D.M. (2012) Estudios de relación estructura—Actividad sobre surfactantes gemini con actividad antimicrobiana. http://www.grupomontevideo.edu.uy/index.php/programas/jovenes-investigadores http://issuu.com/ufprdigital/docs/xx_jornadas_completo

[24] Chang, W.C. (2004) Fmoc Solid Phase Peptide Synthesis: A Practical Approach. Oxford University Press, Oxford.

[25] Campeau, E., Ruhl, V.E., Rodier, F., *et al.* (2009) A Versatile Viral System for Expression and Depletion of Proteins in Mammalian Cells. *PLoS ONE*, **4**, e6529. https://doi.org/10.1371/journal.pone.0006529

[26] Wang, C., Li, X., Wettig, S.D., Badea, I., Foldvarid, M. and Verral, R.E. (2007) Investigation of Complexes Formed by Interaction of Cationic Gemini Surfactants with Deoxyribonucleic Acid. *Physical Chemistry Chemical Physics*, **9**, 1616-1628. https://doi.org/10.1039/b618579g

[27] Tagg, J.R. and Mcgiven, A.R. (1971) Assay Systems for Bacteriocins. *Applied and Environmental Microbiology*, **21**, 943-947.

[28] Yao, C., Tai, Z., Wang, X., Liu, J., Zhu, Q., Wu, X., Zhang, L., Zhang, W., Tian, J., Gao, Y. and Gao, S. (2015) Reduction-Responsive Cross-Linked Stearyl Peptide for Effective Delivery of Plasmid DNA. *International Journal of Nanomedicine*, **10**, 3403-3416.

[29] Kumar, V., Chatterjee, A., Kumar, N., Ganguly, A., Chakraborty, I. and Banerjee, M. (2014) D-Glucose Derived Novel Gemini Surfactants: Synthesis and Study of Their Surface Properties, Interaction with DNA, and Cytotoxicity. *Carbohydrate Research*, **397**, 37-45.

[30] Mahato, R.I., Anwer, K., Tagliaferri, F., Meaney, C., Leonard, P., Wadhwa, M., Logan, S., French, M. and Rolland, A. (2008) Biodistribution and Gene Expression of Lipid/Plasmid Complexes after Systemic Administration. *Human Gene Therapy*, **9**, 2083-2099. https://doi.org/10.1089/hum.1998.9.14-2083

[31] Lobo, B.A., Rogers, S.A., Wiethoff, C.M., Choosakoonkriang, S., Bogdanowich-Knipp, S. and Middaugh, C.R. (2001) Characterization of Cationic Vector-Based Gene Delivery Vehicles Using Isothermal Titration and Differential Scanning Calorimetry. *Meth. Mol. Med*, **65**, 319-348.

[32] Marsh, D. and King, M.D. (1986) Prediction of the Critical Micelle Concentrations of Mono- and Di-Acyl Phospholipids. *Chemistry and Physics of Lipids*, **42**, 271-277.

[33] Yoshimura, T., Sakato, A. and Esumi, K. (2013) Solution Properties and Emulsification Properties of Amino Acid-Based Gemini Surfactants Derived from Cysteine. *Journal of Oleo Science*, **62**, 579-586. https://doi.org/10.5650/jos.62.579

[34] Koloskova, O.O., Nikonova, A.A., Budanova, U.A., Shilovskiy, I.P., Kofiadi, I.A., Ivanov, A.V., Smirnova, O.A., Zverev, V.V., Sebaykin, Yu.L., Andreev, S.M. and Khaitov, M.R. (2016) Synthesis and Evaluation of Novel Lipopeptide as a Vehicle for Efficient Gene Delivery and Gene Silencing. *European Journal of Pharmaceutics and Biopharmaceutics*, **102**, 159-167.

[35] Castro, M., Griffiths, D., Patel, A., Pattrick, N., Kitson, C. and Ladlow, M. (2004) Effect of Chain Length on Transfection Properties of Spermine-Based Gemini Surfactants. *Organic & Biomolecular Chemistry*, **2**, 2814-2820. https://doi.org/10.1039/b410240a

[36] Takayama, K., Nakase, I., Michiue, H., Takeuchi, T., Tomizawa, K., Matsui, H. and Futaki, S. (2009) Enhanced Intracellular Delivery Using Arginine-Rich Peptides by the Addition of Penetration Accelerating Sequences (Pas). *Journal of Controlled Release*, **138**, 128-133.

[37] Chan, D.I., Prenner, E.J. and Vogel, H.J. (2006) Tryptophan- and Arginine-Rich Antimicrobial Peptides: Structures and Mechanisms of Action. *Biochimica et Biophysica Acta*, **1758**, 1184-1202.

[38] Bechara, C., Pallerla, M., Zaltsman, Y., Burlina, F., Alves, D.I., Lequin, O. and Sagan, S. (2013) Tryptophan within Basic Peptide Sequences Triggers Glycosaminoglycan-Dependent Endocytosis *The FASE Journal*, **27**, 738-749.

[39] Bechara, C., Pallerla, M., Burlina, F., Illien, F., Cribier, S. and Sagan, S. (2015) Massive Glycosaminoglycan-Dependent Entry of Trp-Containing Cell-Penetrating Peptides Induced by Exogenous Sphingomyelinase or Cholesterol Depletion. *Cellular and Molecular Life Sciences*, **72**, 809-820. https://doi.org/10.1007/s00018-014-1696-y

[40] Fang, S.L., Fan, T.C., Fu, H.W., Chen, C.J., Hwang, C.S., Hung, T.J., Lin, L.Y. and Chang, M.D. (2013) A Novel Cell-Penetrating Peptide Derived from Human Eosi-nophil Cationic Protein. *PLoS ONE*, **8**, e57318. https://doi.org/10.1371/journal.pone.0057318

[41] Xu, R., Wang, X.L. and Lu, Z.R. (2010) New Amphiphilic Carriers Forming pH-Sensitive Nanoparticles for Nucleic Acid Delivery. *Langmuir*, **26**, 13874-13882. https://doi.org/10.1021/la1024185