# Design and synthesis of biologically active cationic amphiphiles built on the calix[4]arene scaffold

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A promising strategy to design safer and more effective cationic lipids for gene delivery with inherent anti-bacterial properties is to covalently tether a lipophilic moiety with oligomeric aminoglycosides (AGs), a large family of Gram-negative-active antibiotics. Herein, we reported the development of a new class of multicationic-head AG-based amphiphiles built on the tetramino-tetrahexyloxycalix[4]arene (4A4Hex-calix-calix[4]) scaffold. Three different conjugates, namely 4A4Hex-calix-calix[4]-neomycin, -neamine, and -paromomycin, were syn-thesized and characterized. Due to the inherent multivalency of AGs and the amphiphilic behaviour, every 4A4Hex-calix-calix[4]-AG exhibited greater DNA binding ability than the gold standard transfectant 25 kDa *b*PEI and striking DNA packing ability. DNA/4A4Hex-calix-calix[4]-AG complexes at charge ratios (CRs, +/−) used for transfections displayed good colloidal stability, with a hydrodynamic diameters of ≈150 nm and an overall surface charges of ≈ +30 mV. DNA/4A4Hex-calix[4]-AGs nanoassemblies, everyone tested at the optimal CR, invariably showed good transfection efficiency in two cell lines, along with low-to-negligible cytotoxicity. Besides, DNA/4A4Hex-calix-calix[4]-AG complexes exhibited appreciable antimicrobial activity against Gram-negative bacteria, even greater than uncomplexed 4A4Hex-calix-calix[4]-AGs. Altogether, these results disclose 4A4Hex-calix[4]-AGs as promising gene delivery tools with unique antibacterial properties.

*Keywords:*
Calix[4]arenes Aminoglycosides
Non-viral gene delivery vectors
Antibacterial activity
Transfection
Lipoplexes

## 1. Introduction

Since the first attempts to deliver exogenous nucleic acids (NAs), i.e. DNA or RNA, to mammalian cells by means of cationic lipids (CLs) (Fraley et al., 1980) and polymers (Boussif et al., 1995; Wu and Wu, 1987), gene delivery vectors have made significant strides forward. Although nowadays viral vectors are still the most employed vehicles in gene therapy applications, their safety and other issues related to costs of large scale production and quality control have steered the research towards alternative technologies that are synthetic carriers (vectors). In this context, non-viral gene delivery systems are extremely attractive and promising due to their ability to electrostatically bind, condense and protect the genetic material within a few tens to hundreds nan-ometer-large particles, called lipoplexes and polyplexes for CLs and cationic polymers respectively, that allow for their cell internalization and NAs activity (Candiani et al., 2010; Pezzoli et al., 2012).

Recent advances in nanotechnology and molecular synthesis have inspired much research on new chemical agents for NAs delivery to intended targets. One of the most successful approaches lies in the use of pre-constituted building blocks, such as dendrimers (Lee et al., 2005) and macrocyclic molecules, to design highly organized micro- and nano-assemblies (Nimse and Kim, 2013; Rodik et al., 2014). In this scenario, calix[n]arenes are very promising due to their well-defined three-dimensional (3D) architecture and their capacity to bear multiple moieties to interact with NAs (Mochizuki et al., 2015; Ortiz Mellet et al., 2010; Rodik et al., 2015; Sansone et al., 2006). One of the most striking examples of this point is the work done by Ungaro's group, who has for long time been working on calix[4]arenes. By way of

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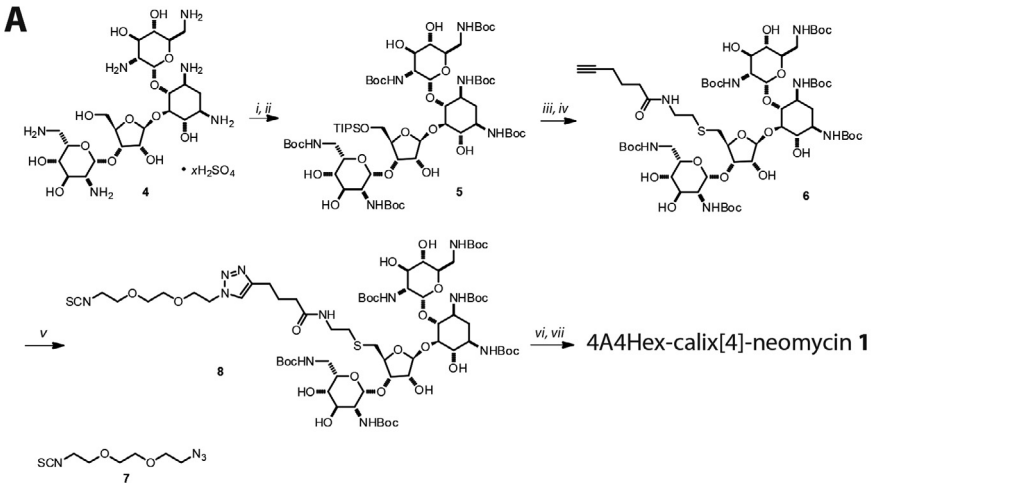


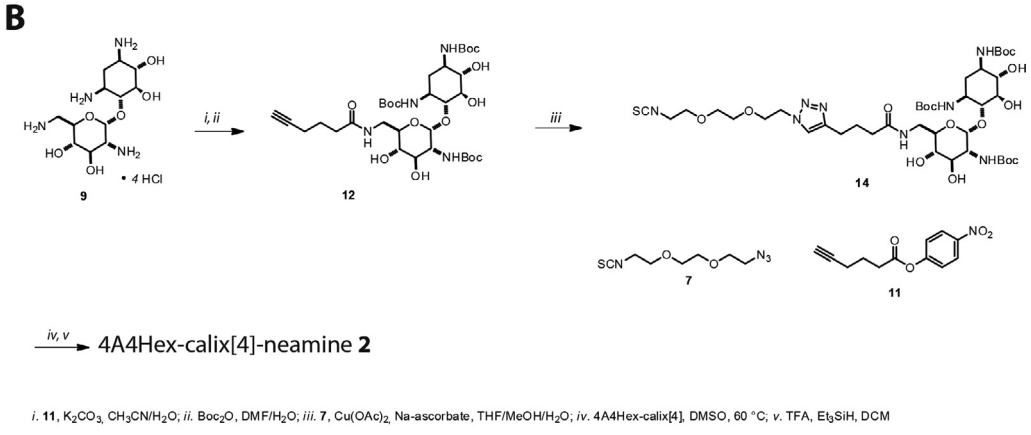


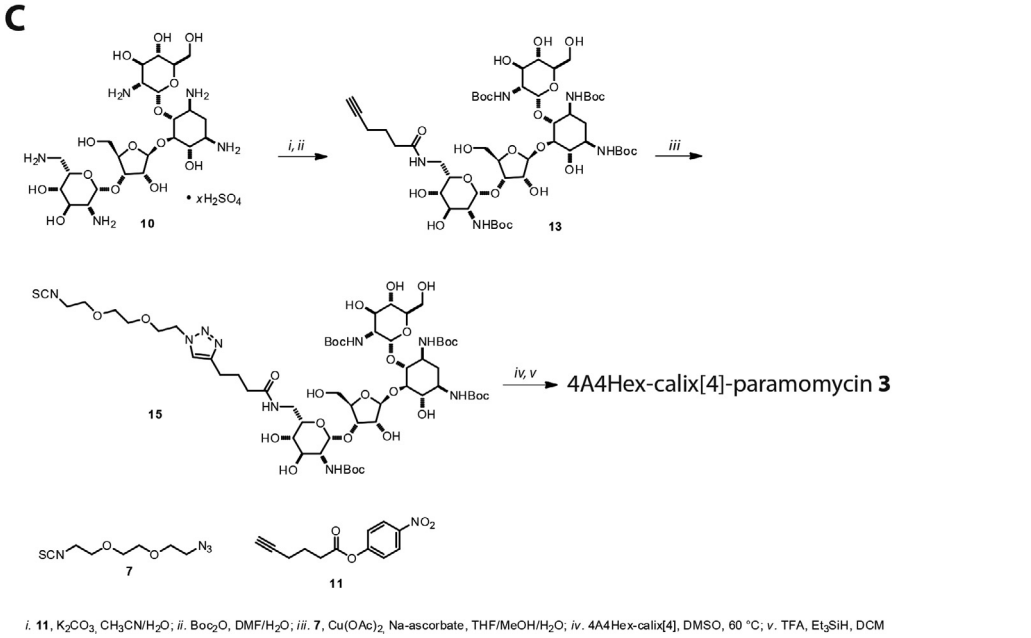


**Fig. 1.** Synthesis of 4A4Hex-calix[4]arene-AG conjugates. Every AG was first functionalized with an isothiocyanate-teminated linker exploiting the less hindered position. Then the isothiocyanate portion was reacted with the amino group of the upper rim of the 4A4Hex-calix[4]arene.

illustration, the conjugation of such macrocyclic molecules at their lower and upper rim with hydrophobic moieties, arginine clusters, and guanidino groups allowed a significant improvement of NAs condensation and delivery abilities of the resulting complexes (Bagnacani et al., 2013, 2012, 2008).

Besides, aminoglycosides (AGs), such as neomycin, kanamycin, paromomycin, are so attractive because of their inherent affinity for NAs, including bacterial and eukaryotic RNA and plasmids (pDNAs) (Tor, 2003), and their structural variety and valency, that make them very interesting cationic moieties for gene vectors. Indeed, AGs are multifunctional compounds containing up to six amine groups and several hydroxyl groups, capable of electrostatically bind NAs. For this reason, oligomeric AGs have been already used to synthesize polymeric vectors (Chen et al., 2012; Huang et al., 2016; Miryala et al., 2015, 2016) and in particular CLs with remarkably high gene delivery efficiencies both *in vitro* than *in vivo* (Belmont et al., 2002; Chatin et al., 2015; Desigaux et al., 2007; Le Gall et al., 2009; Mevel et al., 2012; Napoli et al., 2005; Sainlos et al., 2005; Zhang et al., 2013). It is worthy of note that AGs are a large family of antibiotics used in the treatment of Gram-negative infections (Fosso et al., 2014; Houghton et al., 2010). Taken together, these evidences support the idea that AGs can be proficiently used as a cationic head for the design of gene delivery vectors with antibacterial properties.

Drawing inspiration from our recent advances in the development of efficient vectors for gene delivery (Ghilardi et al., 2013; Sganappa et al., 2017), we herein report the synthesis and characterization of a novel class of multicationic-head amphiphiles built on tetramino-tetrahexyloxycalix[4]arene (hereafter referred to as 4A4Hex-calix[4]), selected on the basis of previous observations (Sansone et al., 2006), through the grafting of four units of three different AGs, namely neomycin, neamine, and paromomycin, by means of isothiocyanate linkers at the upper rim of the calix[4]arene. The efficiency of AGs grafting on 4A4Hex-calix[4] scaffolds was determined by $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR). Besides, the DNA binding capacity together with the cytotoxicity and the transfection efficiency of such amphiphilic 4A4Hex-calix[4]-AG derivatives were evaluated on two different cell lines and their antibacterial activity was evaluated as well.

## 2. Materials and methods

### 2.1. Materials and reagents

Neomycin sulfate was purchased from Fluorochem (Hadfield, UK), paromomycin sulfate was from Apollo Scientific Ltd. (Bredbury, UK), neamine was synthesized from neomycin as described in literature (Park et al., 1996). Spectra/Por dialysis bags (MWCO = 1 kDa) were from Spectrum Laboratories (Compton, CA, USA). 25 kDa branched poly(ethylenimine) (*b*PEI) was from Sigma-Aldrich (Milan, Italy).

HeLa (human cervix carcinoma) and U87-MG (human glioblastoma-astrocytoma epithelial-like) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). AlamarBlue Cell Viability Assay was purchased from Life Technologies Italia (Monza, Italy), while BCA Protein Assay Kit was from ThermoFisher (Monza, Italy). pDNA encoding the modified firefly luciferase (pGL3-Control Vector, 5.2 kbp; hereafter referred to as pGL3) and Luciferase Assay System were obtained from Promega (Milan, Italy).

*Escherichia coli* DSM 3423 (*E. coli* JM109) were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), while Sarcina lutea (*S. lutea*) ATCC 9341 were from ATCC.

All the other chemicals were from Sigma-Aldrich, if not differently specified.

### 2.2. Synthesis of 4A4Hex-calix[4]-aminoglycosides **1**–**3**: general procedure

Neat 4A4Hex-calix[4] was dissolved in DMSO and a solution of AG-isothiocyanate linker (1.2 equivalent per $NH_2$) in a minimal volume of DMSO was added (Fig. 1). The solution was stirred at 60 °C for 24 hrs, then dialyzed for 8 hrs against MeOH (the solvent reservoir was renewed 3 times, MWCO 1 KDa). The solution was evaporated under reduced pressure to give N-Boc-protected 4A4Hex-calix[4]-AG. After $^{1}H$ NMR characterization, the resulting conjugates were dissolved in a 1:1 mixture of trifluoroacetic acid (TFA)/dichloromethane (DCM) and stirred for 30 min at room temperature (r.t). The excess of TFA was stripped off under reduced pressure, the crude dissolved in deionized water ($dH_2O$) and the solution dialyzed against $dH_2O$. Lyophilization led to fluffy, white solid products, i.e. the 4AHex-calix[4]-AGs **1**–**3**. Complete N-Boc deprotection occurred in all cases as evidenced by the spectra recorded. For the complete synthetic pathway refer to Fig. 1.

### 2.3. Preparation of transfectant solutions

Amphiphilic 4A4Hex-calix[4]-neomycin **1**, 4A4Hex-calix[4]-neamine **2**, and 4A4Hex-calix[4]-paromomycin **3** were diluted in $dH_2O$ to a final concentration of 4.06 mg/mL, 5.2 mg/mL, and 5.2 mg/mL, respectively, corresponding invariably to a final nitrogen concentration ([N]) of 13 mM. 25 kDa *b*PEI was diluted in 10 mM HEPES to a final concentration of 0.86 mg/mL and a [N] = 20 mM, considering that there is one nitrogen per repeat PEI unit (-$NHCH_2CH_2$-, $M_w$ = 43 Da)(Zhang et al., 2004).

### 2.4. Complexes preparation and evaluation of DNA complexation ability

pDNA was amplified, isolated, purified and diluted in 0.1 × TE buffer (1 mM Tris, pH 8; 0.1 mM EDTA) as previously described (Malloggi et al., 2015).

Polyplexes were prepared at r.t. by mixing the aqueous solutions of pGL3 to amphiphilic 4A4Hex-calix[4]-AGs at the desired lipid concentration, yielding different charge ratios (CRs, +/−) and a final DNA concentration of 20 ng/μL. CR is defined as the number of amines (N, cationic) of the AG which is used to complex the phosphate groups (P, anionic) of a given quantity of DNA (i.e. cationic *vs.* anionic charge ratio). pDNA/4A4Hex-calix[4]-AG complexes were prepared in $dH_2O$ and incubated for 20 min at r.t. prior to use.

The DNA complexation ability of every 4A4Hex-calix[4]-AG conjugate was monitored by a fluorophore-exclusion titration assay. For each condition, 0.12 μg of pDNA in 2.4 μL of 20 × SYBR Green I ($\lambda_{ex}$ = 497 nm, $\lambda_{em}$ = 520 nm) were added to 3.6 μL of CL solutions at different concentrations. Afterwards, lipoplexes were incubated for 20 min at r.t., then diluted 1:5 (v/v) in $dH_2O$. Fluorescence measure-ments (n = 3 per condition) were performed with a GENios Plus Reader (Tecan, Segrate, Italy) in 384-well black plates. Data are expressed as relative fluorescence normalized to the fluorescence of uncomplexed pDNA.

### 2.5. Measurement of size and zeta-potential of complexes

The hydrodynamic diameter ($D_H$) and the zeta potential ($\zeta_P$) of the lipoplexes were measured at 25 °C by Dynamic Light Scattering (DLS) and Laser Doppler micro-electrophoresis using a Malvern Zetasizer Nano ZS instruments (Malvern, Italy), fitted with a 5 mV HeNe laser ($\lambda$ = 633 nm) and a scattering angle of 173°. Fifty μL of complexes containing 1 μg of pDNA were prepared as described above, incubated for 20 min at r.t. then diluted 1:9 in $dH_2O$. Samples were equilibrated for 5 min at 25 °C prior the measurement.

### 2.6. In vitro cells transfection experiments

#### 2.6.1. Cell cultures

Mycoplasma-free HeLa and U87-MG cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1 mM sodium pyruvate, 10 mM HEPES buffer, 100 U/mL penicillin, 0.1 mg/mL

streptomycin, 2 mM glutamine and supplemented with 10% (v/v) fetal bovine serum (FBS) (hereafter referred to as complete culture medium, cDMEM) in a humidified atmosphere under constant supply of 5% $CO_2$ and at 37 °C (hereafter referred to as standard culture conditions).

*2.6.2. In vitro cells transfection*

Cells were passaged 24 h before plating in 96-well plates at a density of $2 \times 10^4$ cells/cm$^2$ and maintained in standard culture conditions. Twenty-four hrs after seeding, 160 ng/well of pGL3 were complexed in $dH_2O$ with 4A4Hex-calix[4]-AG solutions to yield different CRs, as described herein above, and cells were incubated with complexes in 100 μL/well of cDMEM under standard culture conditions for further 24 hrs. Cells transfected with 25 kDa *b*PEI/DNA complexes at varying CRs were used as the internal reference.

Twenty-four hr-post transfection, cytotoxicity was evaluated by means of AlamarBlue assay according to manufacturer's instructions. Briefly, medium was removed and each well was loaded with 100 μL of cDMEM containing 1 × resazurin dye. Cells were next incubated in standard culture conditions for 2 hrs, then the fluorescence of the medium was read with a GENios Plus reader (Tecan, Italy) ($\lambda_{ex}$ = 540 nm; $\lambda_{em}$ = 595 nm). Viability of untransfected cells (CTRL) was assigned to as 100% and cytotoxicity was determined as follows:

$$\text{Cytotoxicity}[\%] = 100\% - \text{Viability}[\%]$$

Transfection efficiency was evaluated measuring the luciferase activity by means of the Luciferase Assay System, according to manufacturer's instructions. Briefly, cells were washed with phosphate buffered saline (PBS) and lysed with 110 μL/well of Cell Culture Lysis Reagent (Promega, Italy). Following a freeze-thawing cycle to improve cell disruption, 20 μL of cell lysates were mixed with 50 μL of Luciferase Assay Reagent and luminescence was measured by means of a GENios Plus reader. The luminescence signal (expressed as relative light units, RLU) of each sample was normalized to its protein content, determined by BCA assay and data are expressed as RLU/mg of proteins.

*2.7. Antimicrobial activity of calix[4]-AGs*

*E. coli* JM109 and *S. lutea* bacterial strains were pre-cultured in 5 mL of Luria-Bertani broth at 37 °C under shaking at 130 rpm for 20 hrs, until reach an optical density at λ = 600 nm ($OD_{600nm}$) ≈ 1, corresponding to ≈$10^9$ bacteria/mL. Bacterial suspensions were then diluted to obtain a final concentration of ≈$10^6$ bacteria/mL, hereafter used as the test inoculum. Afterwards, bacterial suspension (50 μL/well) were inoculated in 96-well plates at a density of $1.5 \times 10^5$ bacteria/cm$^2$ in 50 μL/well of LB containing pDNA/4A4Hex-calix[4]-AGs complexes prepared as described hereinabove or uncomplexed (i.e. DNA-free) 4A4Hex-calix[4]-AGs solutions (prepared at the same lipid concentration used to complex pDNA), and incubated at 37 °C for 24 hrs. Bacteria inoculated in 50 μL/well of LB were used as positive controls (CTRL$^+$) for bacterial growth (Yadav et al., 2014), while bacteria inoculated in 50 μL/well of free AGs solution (i.e. neomycin, neamine, paramomycin at different AG concentrations) and 4A4Hex-calix[4] were used as in-ternal references. The antibacterial efficacy of every compound was evaluated by means of both indirect (i.e. turbidity – $OD_{600nm}$ mea-surements) (Chen et al., 2012; Huang et al., 2016; Zimmermann et al., 2013) and direct (i.e. plate count) methods, according to the ISO 10932:2010 (E) norm and Taylor et al. (1983). The $MIC_{90}$ was as the lowest concentration (or CR) of every compound that reduced the OD of the inoculum by 90% within a 24 hr-incubation with respect to the CTRL$^+$ (Hu et al., 2017). Briefly, 24 hrs after inoculation, the $OD_{600\ nm}$ of each well (n ≥ 3 per compound) was read by means of a Sunrise microplate reader (Tecan, Italy). The number of viable bacteria was next counted on LB-agar Petri dishes after serial 10-fold dilutions of the bacterial suspensions and plating. Briefly, for every compound, wells displaying an $OD_{600\ nm}$ across the minimum were plated. The bacterial reduction was calculated according to the following equation:

$$\text{antibacterial reduction}\ [\%] = [1-(N_{compound}/N_{CTRL+})] \times 100$$

where N is the number of Colony Forming Units (CFU) specific to every compound.

*2.8. Statistical analysis*

Statistical analysis was carried out by GraphPad version 6 (GraphPad software, La Jolla, CA, USA). All data were initially analyzed using D'Agostino & Pearson omnibus normality test. Comparisons among groups were performed by multiple *t*-test. Significance was retained when $p < 0.05$. Data are expressed as mean ± standard deviation (SD). Experiments were performed at least in triplicate.

## 3. Results and discussion

CL-based gene delivery (lipofection) was one of the earliest ways used to introduce exogenous genetic materials into mammalian cells (Felgner et al., 1987). Basically, CLs are attractive delivery vehicles because they can be easily synthesized, functionalized and formulated with co-lipids to increase their transfection efficiency and decrease their cytotoxicity. In general, CLs comprise three basic domains that are the cationic head(s), the hydrophobic group(s), and the linker moiety tethering polar and hydrophobic moieties (Pezzoli and Candiani, 2013). In this context, a number of lipid vectors have been developed so far through the proper design and the combination of such structural domains (Martin et al., 2005).

Herein, we propose the synthesis of a class of CL vectors relying on the use of a suitably decorated calix[4]arene macrocycle scaffold. A previous study by Bagnacani and coworkers (Bagnacani et al., 2013) showed that calix[4]arenes tethered with a six-carbon lipophilic chain to the lower rim were extremely effective in transfecting a variety of cell lines. Taking a clue from this study, we propose the synthesis of a novel class of lipid vectors displaying a (lipophilic) hydrophobic do-main consisting of 4A4Hex-calix[4] scaffold. Besides, multivalent CLs do generally display enhanced NAs binding and delivery abilities as compared to the monocationic counterparts (Bhattacharya and Bajaj, 2009). As we have proficiently used AGs as polar heads of polycationic vectors (Ghilardi et al., 2013), we did tether the calix[4] macrocyclic scaffold with AG headgroups, namely neomycin, neamine and paromomycin.

*3.1. Synthesis and characterization of 4A4Hex-calix[4]-AG derivatives*

The synthetic strategy used to graft 4A4Hex-calix[4] is well established in our laboratory (Ghilardi et al., 2013), and relies on the reaction between the AG modified with the isothiocyanate-terminated linker and the amine at the upper rim of 4A4Hex-calix[4]. In order to selectively functionalize neomycin, we exploited the presence of only one, less hindered primary hydroxyl group in the molecular skeleton. Accordingly, neomycin B **4** was treated with $Boc_2O$ and the resulting Boc-protected neomycin reacted with triisopropyl sulfonyl chloride producing the selective formation of intermediate **5** in good yields (Fig. 1A). Substitution with thioethanolamine followed by coupling with hexynoic acid lead to the formation of propargylic derivative **6**. Finally, click reaction between **6** and azido-linker **7** (for the synthesis, see Supporting Information) bearing the needed isothiocyanate func-tional group produced in good yields aminoglycoside **8**, which was reacted with 4A4Hex-calix[4] in DMSO at 60 °C in order to maximize the degree of grafting, yielding 4A4Hex-calix[4]-AG **1**. Instead, for the functionalization of neamine **9** and paromomycin **10** we exploited the presence in their skeleton of only one, less hindered aminomethylene moiety which reacts smoothly with activated *p*-nitrophenyl ester **11** (for the synthesis, see Supporting Information) giving rise to the formation of intermediates **12**, **13**, respectively, followed by protection of the remaining amines with $Boc_2O$ (Fig. 1B for neamine and Fig. 1C for

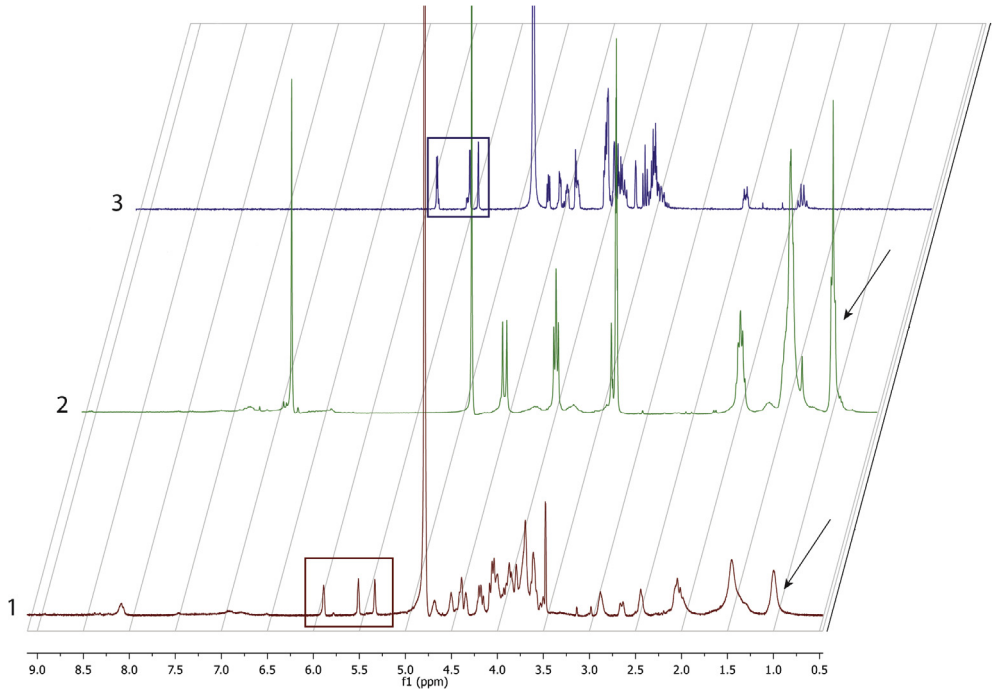


**Fig. 2.** Representative $^{1}$H NMR spectra. $^{1}$H NMR spectra of: 1) 4A4Hex-calix[4]arene-paramomycin conjugate 3 (recorded in $D_2O$), 2) 4A4Hex-calix[4]arene (recorded in MeOD) and 3) free paromomycin (recorded in $D_2O$). The spectrum of the 4A4Hex-calix[4]arene-paramomycin 3 displays the three anomeric protons (spectrum 1, red rectangle) belonging to paromomycin (spectrum 3, blue rectangle) and the terminal methyl groups of the 4A4Hex-calix[4] (spectra 1 and 2, arrows) confirmed that the functionalization occurred. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

paromomycin). Following the same synthetic strategy, propargylic-functionalized aminoglycosides **12**, **13** were submitted to click reaction with azido-linker **7** producing the corresponding isothiocyanate-functionalized aminoglycosides **14**, **15**, which were reacted with 4A4Hex-calix[4] giving rise to the formation of 4A4Hex-calix[4]-AG **2** and **3**, respectively.

The conjugates were next characterized through $^{1}$H and $^{13}$C NMR (see Supplementary Information, Figs. S1–S6). Specifically, the efficiency of AG grafting on the 4A4Hex-calix[4] scaffold was determined by $^{1}$H NMR. In the spectra of 4A4Hex-calix[4]-AG **3**, reported as typical example in Fig. 2, it is evident the characteristic protons of the triazole at 8.1 ppm, the signals of the three anomeric protons of the AG between 5.3 and 5.9 ppm and the broad singlet of the methyl groups of the terminal hexyl moiety of calix around 1.0 ppm. Their integrations are respectively 4H for each anomeric proton and 12H. This confirms that each amino group of the 4A4Hex-calix[4] was covalently functionalized with an AG. Besides, the aromatic protons of the calix[4]arene result in broad resonances, most likely due to the formation of high molecular weight aggregates and/or for the resonance between the aromatic rings and the thiourea groups. The fact that the ratio between the integrations of the signals belonging to the protons of the AGs and the calix is 4:1, together with the fact that the signals of the anomeric protons do not present any side peaks belonging to the free AG indicates that the conjugates **1–3** are pure and that the excess of the free aminoglycosides used in the "click" isothiocyanate/amine reaction was completely removed by dialysis.

### *3.2. Biophysical properties of pDNA/4A4Hex-calix[4]-AGs*

The formation of lipoplexes has known to be driven by electrostatic interactions between the cationic moieties of the transfectant and the anionic phosphates of NAs, leading to the charge neutralization and the compaction of polynucleotides (Lucotti et al., 2014; Mintzer and Simanek, 2009). Accordingly, one of the key requirements of an efficient gene delivery vector lies on its ability to effectively bind and condense NAs. We thus evaluated the ability of every 4A4Hex-calix[4]-AG derivative to complex pDNA as a function of N-to-P (+/−) ratio (CR). In fluorophore exclusion titration assay (Fig. 3A), 4A4Hex-calix[4]-AGs invariably exhibited a maximal complexation ability at CR $\geq$ 1.5. This means that they have higher affinity for NAs if compared with the gold standard 25 kDa *b*PEI, which exhibited a maximal complexation at CR $\geq$ 3. A reason for such strong interactions with NAs can be ascribed to multivalent binding sites, due to the presence of four AGs (Wang and Tor, 1997), and to the clustering of the binders on the rigid scaffold calix[4]arene.

We have recently pointed out a strict relationship between the dimensions of gene delivery complexes and their transfection efficiency (Pezzoli et al., 2017). Besides, the surface charge of complexes is considered as an essential factor affecting their biological fate as well (Mintzer and Simanek, 2009). In this context, we evaluated the physico-chemical properties of 4A4Hex-calix[4]-AG lipoplexes, i.e. their average $D_H$ and $\zeta_P$, at varying CRs. Since physico-chemical properties of complexes are highly dependent on the CR (i.e. the transfectant-to-pDNA ratio), in this study a wide range of CR was considered, in order to find out the lowest ratio at which DNA was condensed into small and weakly cationic particles. As reported in Fig. 3B–E, every $\zeta_P$ curve displayed a sigmoidal shape as a function of CR. Very interestingly, the charge-inversion point (0 mV) did correspond to the CR at which maximal complexation occurred for all the 4A4Hex-calix[4] derivatives. Conversely, the $D_H$ profile of every 4A4Hex-calix[4]-AG derivative was fairly constant at CR $\geq$ 5. It is worthy of note that all pDNA/4A4Hex-calix[4] lipoplexes displayed similar $D_H$ ($\approx$150 nm) and $\zeta_P$ ($\approx$25 ÷ 40 mV) at 5 $\leq$ CR $\leq$ 80 ($p > 0.05$ for every derivative series). Nevertheless, derivatives **2**- and **3**-based assemblies displayed high $D_H$ at CR 80, although not significantly greater than those at other CRs (CR 80 *vs.* CR 10-20-40; $p > 0.05$ for all). The $D_H$ and $\zeta_P$ profiles of *b*PEI/DNA complexes were similar to those of 4A4Hex-calix[4]-AGs **1–3**.

The ability of 4A4Hex-calix[4]-AG derivatives to effectively bind the DNA, together with their biophysical behavior similar to those of the gold standard *b*PEI, prompted us to challenge cells *in vitro* with such vectors.

### *3.3. In vitro transfection of pDNA/4A4Hex-calix[4]-AGs*

To investigate the influence of biophysical properties of pDNA/4A4Hex-calix[4]-AG lipoplexes on the gene delivery behavior, we evaluated their transfection effectiveness at varying CRs. Besides physico-chemical properties, biological activity of complexes is highly

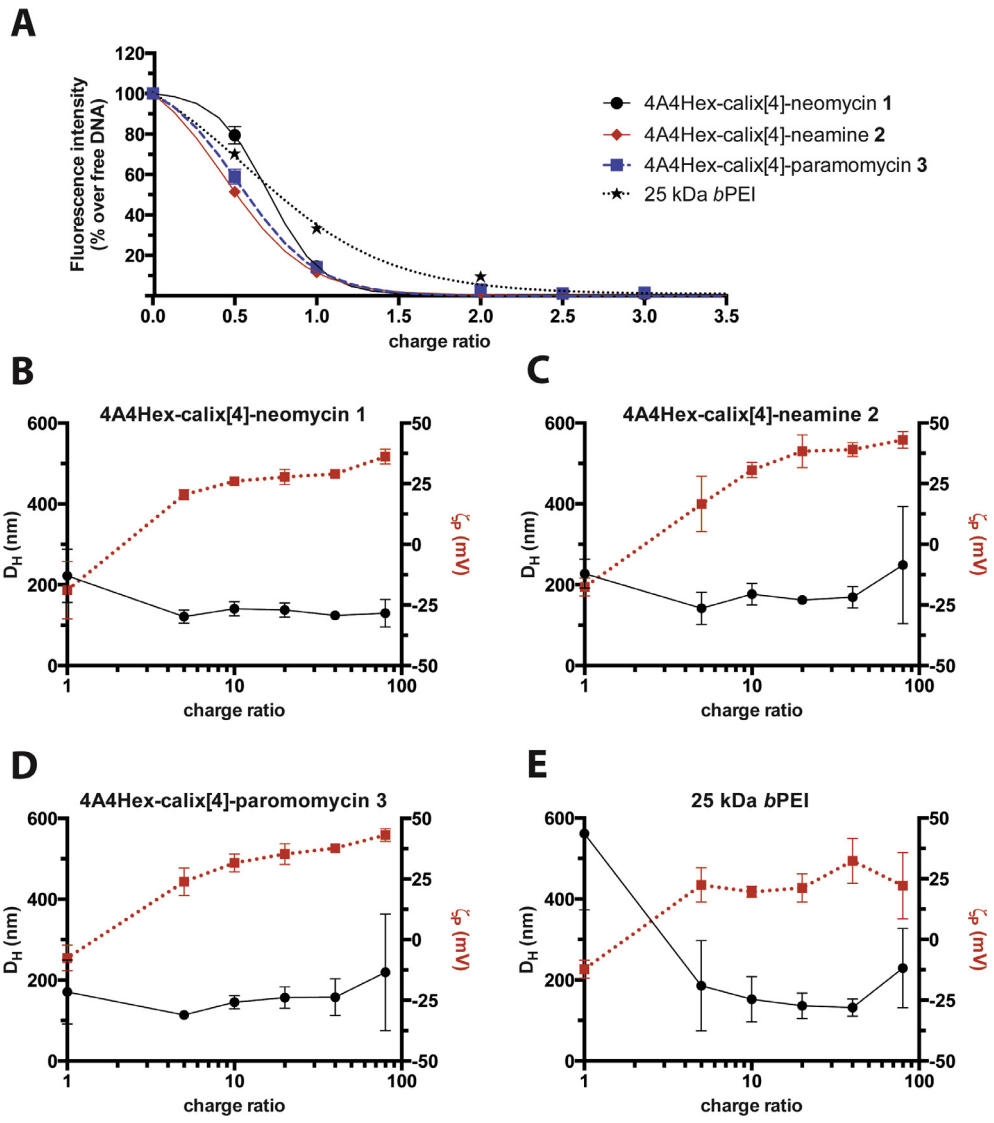


**Fig. 3.** DNA complexation ability and physico-chemical characterization of 4A4Hex-calix[4]arene-AG conjugates **1**–**3** *vs.* 25 kDa *b*PEI. A) Comparative DNA complexation ability of 4A4Hex-calix[4]arene-neomycin **1** (black circle and solid line), 4A4Hex-calix[4]arene-neamine **2** (red circle and solid line), 4A4Hex-calix[4]arene-paromomycin **3** (blue square and dotted line), evaluated by monitoring the fluorochrome exclusion from complexes as a function of charge ratio (CR, +/-). Mean hydrodynamic diameter ($D_H$; black circle and solid line) and overall charge ($\zeta_P$; red square and dotted line) of B) 4A4Hex-calix[4]arene-neomycin **1**, C) 4A4Hex-calix[4]arene-neamine **2**, D) 4A4Hex-calix[4]arene-paromomycin **3** and E) 25 kDa *b*PEI, measured by Dynamic Light Scattering (DLS) and Laser Doppler microelectrophoresis. Results are expressed as mean ± SD (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dependent on the CR as well. Accordingly, we tested lipoplexes prepared over a wide range of CR to find out the minimal ratio to attain high gene expression and low cytotoxicity. Since *b*PEI is a gold standard transfectant (Boussif et al., 1995; Malloggi et al., 2015; Mintzer and Simanek, 2009; van Gaal et al., 2011), for the sake of comparison, *b*PEI/DNA complexes were herein used as the reference (Fig. S7). pGL3 encoding the firefly luciferase was used to check the transfection efficiency in two extensively used cell lines, namely HeLa and U87-MG cells (van Gaal et al., 2011). As expected, the transfection profiles of 4A4Hex-calix[4]-AG derivatives **1**–**3** were strongly dependent on the CR (Fig. 4A and B). More into detail, amphiphilic derivative **1** exhibited the highest transfection efficiency in both cell lines at CR 20, while 4A4Hex-calix[4]-neamine conjugate **2** and -paromomycin conjugate **3** did display a different CR-dependent and cell-dependent transfection behavior. Indeed, derivative **2** was more effective at CR 20 and 40 when used to transfect HeLa and U87-MG cells, respectively. Derivative **3**, instead, did show higher transfection levels at CR 80 and 20, in HeLa and U87-MG cells, respectively. Most important, it is worth noting that every derivative, when tested at each respective optimal CR, invariably exhibited similar or even greater transfection efficiency than the gold standard 25 kDa *b*PEI used in the most effective conditions, that were CR 10 and CR 40 for HeLa and U87-MG cells, respectively (Fig. 4). Yet, without exception, pDNA/4A4Hex-calix[4]-AG lipoplexes exhibited very low toxicity on HeLa cells, close to that of *b*PEI-based complexes ($p > 0.05$) (Fig. 4C). Similar results were found with U87-MG cells as well, even though slightly greater cytotoxicity levels were found as compared to HeLa cells (Fig. 4D).

From a general point of view, we found no strict relationship between the physico-chemical features (i.e. $D_H$ and $\zeta_P$) and the transfection efficiency of pDNA/4A4Hex-calix[4]-AG derivatives. In fact, even though different lipoplex types displayed similar size and surface charge, the different surface chemistry of each specific assembly might account for some differences in transfection.

Overall, gene delivery studies highlight the pretty good transfection properties and low cytotoxicity of amphiphilic 4A4Hex-calix[4]-AG derivatives. Interestingly, such remarkable results were obtained with pure 4A4Hex-calix[4]-AG, therefore without the inclusion of any helper lipids in lipoplex formulations. Indeed, a lipid adjuvant, such as the neutral phospholipid dioleoylphosphatidylethanolamine (DOPE), is sometimes used to substantially improve the transfection efficiency of CLs (Bagnacani et al., 2013; Candiani et al., 2010; Zuhorn et al., 2002). This underlines the inherent gene delivery efficacy of this novel class of cationic amphiphiles.

### *3.4. Antibacterial properties of 4A4Hex-calix[4]-AG derivatives and pDNA/4A4Hex-calix[4]-AGs*

An important feature sometimes undervalued or ignored by

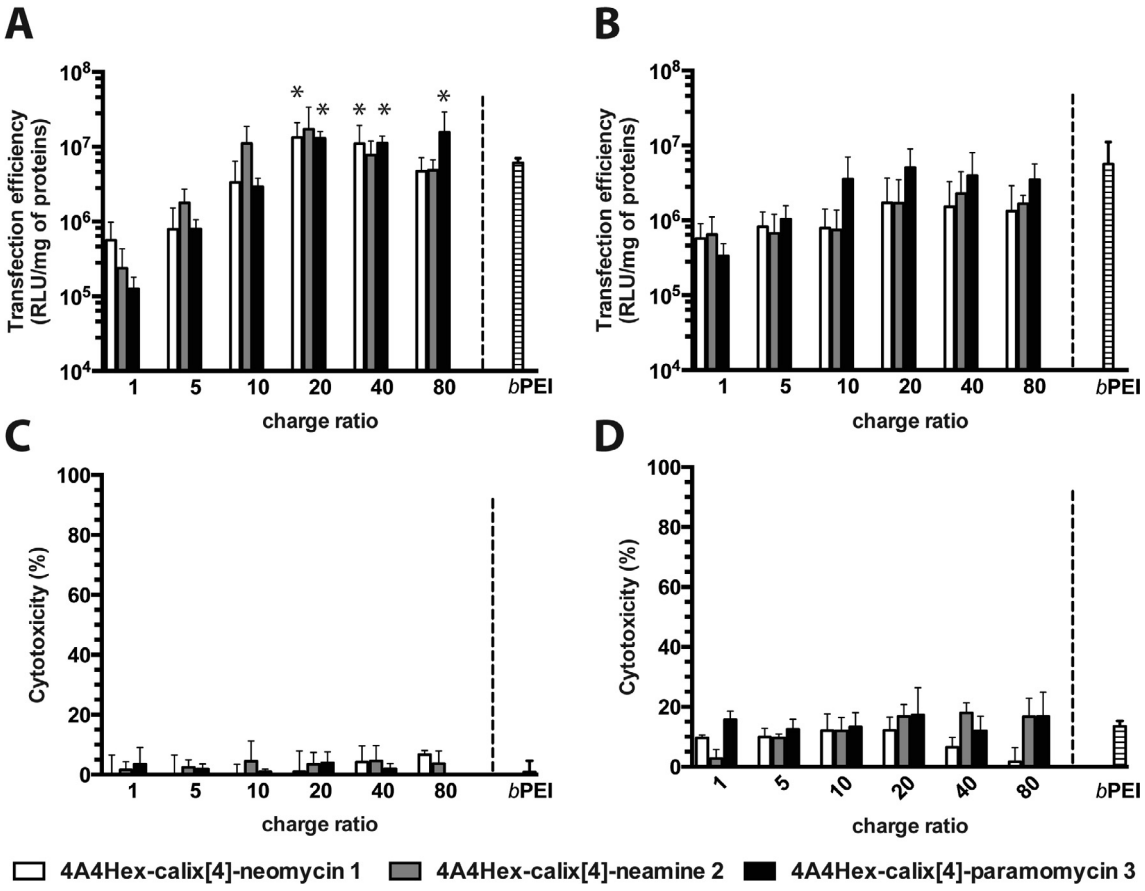


**Fig. 4.** Transfection efficiency and cytotoxicity and of 4A4Hex-calix[4]arene derivatives in HeLa and U87-MG cells. Comparative transfection efficiency of complexes prepared by mixing pGL3 with 4A4Hex-calix[4]arene-AG derivatives **1–3** at different charge ratios (CRs, +/−) and 25 kDa *b*PEI, expressed as luminescence signal (RLU) normalized to the total protein content in each cell lysate: A) HeLa cells; B) U87-MG cells. Cytotoxicity of the aforementioned complexes on C) HeLa and D) U87-MG cells. Results are expressed as mean ± SD (n ≥ 3) ($^{*}p < 0.05$ *vs.* 25 kDa *b*PEI).

fundamental scientists but receiving considerable attention from clinical investigators is the desired side antibacterial effect of medicinal drugs, especially when diseases associated with immunosuppression, such as certain types of cancer, are treated. We hypothesized that 4A4Hex-calix[4]-AGs, found to be effective as gene delivery agents, would also be suited to this purpose. It is worthy of note that, AGs are a group of clinically relevant antibiotics, which function through the binding to the 30S subunit of ribosomes. In turn, this perturbs the elongation of the nascent protein chain by impairing the proofreading process controlling translational accuracy. The use of AGs as potential antiviral (HIV) agents has also been reported (Zhou et al., 2007). On the other hand, calix[n]arenes have been extensively used in the last few years as molecular platforms to attach binding moieties for the selective recognition of molecular species (Nimse and Kim, 2013). Due to their three-dimensional architecture and the possibility to be specially tethered with different groups at their lower and upper rim, calix[n]arenes are well suited to engage in multivalent interactions, allowing these compounds to possibly interfere with the function of critical bacterial virulence determinants. In these context, several pharmacological properties, including antibacterial (Casnati et al., 1996), antifungal, and antiviral activities of calix[n]arene derivatives have been already reported in literature (Colston et al., 2004).

We therefore tested and compared the potential antimicrobial activity of 4A4Hex-calix[4]-AG conjugates **1–3**, used as aqueous (lipid) solutions and in the form of suspensions of pDNA/4A4Hex-calix[4]-AG complexes, and compared their performances to those of free AGs and the 4A4Hex-calix[4] lipophilic scaffold.

The antibacterial activity of such free molecules and particles were tested against Gram-negative *E. coli* and Gram-positive *S. Lutea* bacteria, which are part of the human flora (*E. coli* are found in gut microbiota, while *S. lutea* may be found in the skin and large intestine). In solution, 4A4Hex-calix[4]-neomycin derivative **1** displayed the strongest antibacterial activity as it was largely more effective in inhibiting Gram-negative *E. coli* growth with respect to the 4A4Hex-calix[4]-neamine **2** and the 4A4Hex-calix[4]-paromomycin molecule **3** (Fig. 5). It is worth noting that the antibacterial activity of 4A4Hex-calix[4]-AGs was not affected by their association with pDNA, as previously pointed out by Kichler and colleagues for other transfectants (Kichler et al., 2003). In our hands, the antibacterial efficiencies of 4A4Hex-calix[4]-AGs complexed with pDNA were even greater than the same derivatives in solution (DNA-free lipids), but were slightly less active against *E. coli* than the corresponding free AGs (Fig. 5). Indeed, for derivative **1**-based lipoplexes, the $MIC_{90}$ was found at CR 40 (corresponding to 9.4 μg of Neomycin per mL) (Fig. 5A), with a bacterial reduction of ≈100%(plate count method, Table 1), while its free lipid counterpart displayed a bacterial reduction of 76% at the same lipid concentration. For derivative **3**, lipoplexes and free lipid showed a 100% bacterial reduction at its $MIC_{90}$, that is at CR 80 (corresponding to an AG concentration of 28.2 μg/mL). On the other hand, Neomycin and Paromomycin, tested as free AGs, displayed the $MIC_{90}$ at ≈4 μg/mL, a slightly lower concentration with respect to their conjugated counterparts (Figs. 5A and C and Fig. S8A and C), while 4A4Hex-calix[4] lipophilic scaffold alone was roughly ineffective (Fig. 5D). Of note, it is evident that 4A4Hex-calix[4]-AG conjugates hide in a sort of way the antimicrobial potential of free AGs. Specifically, derivatives **1** and **3** exerted a strong antibacterial effects at CR ≥ 20 (Table 1) while, irrespective of the

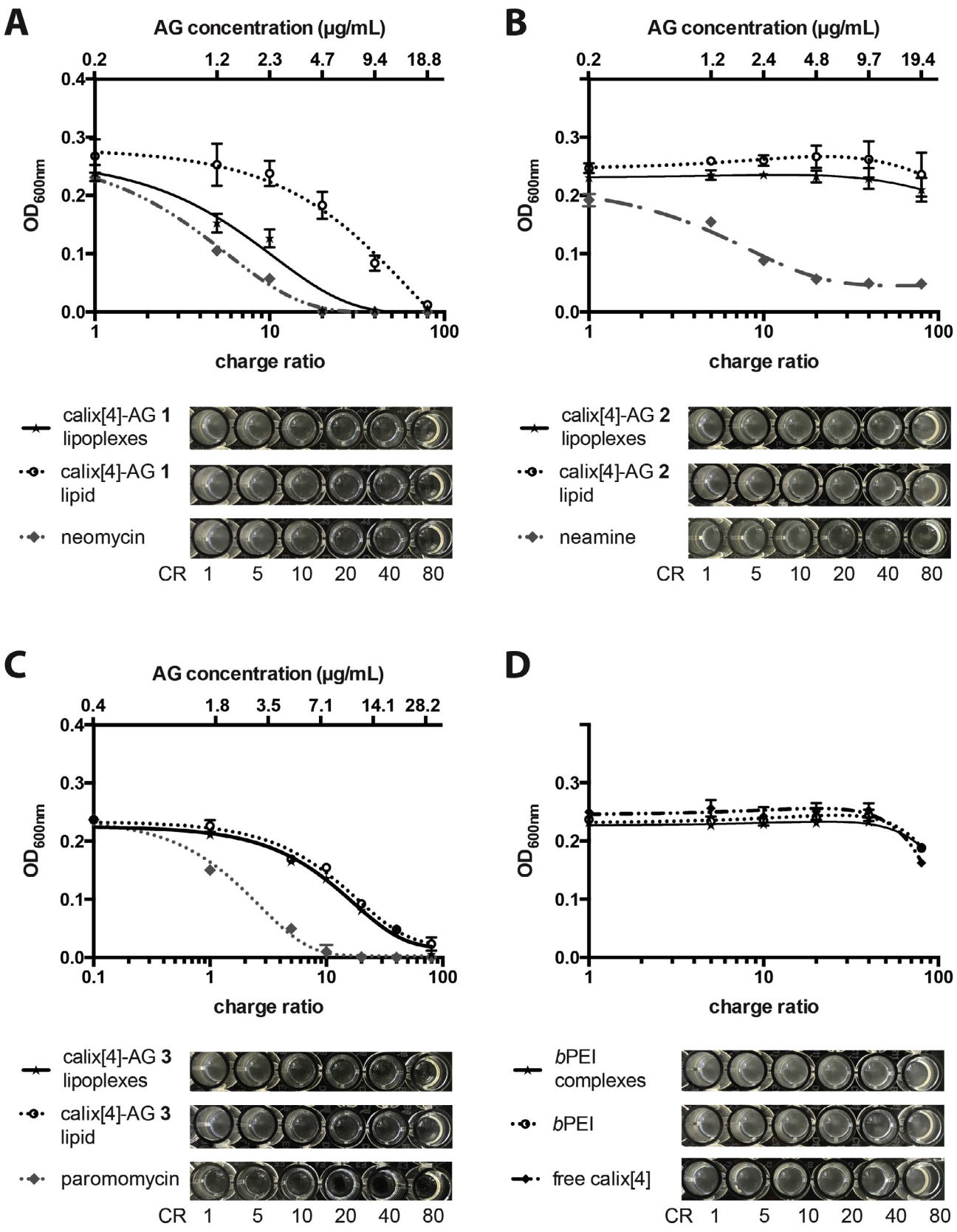


**Fig. 5.** Antibacterial activity of 4A4Hex-calix[4]arene derivatives in solution and pDNA/4A4Hex-calix[4]-AG assemblies against Gram-negative *E. coli*. Complexes were prepared by mixing pDNA with 4A4Hex-calix[4]arene-neomycin **1** (A), -neamine **2** (B), and -paromomycin **3** (C) at different charge ratios (CRs, +/−), and 25 kDa *b*PEI (D). Data are expressed as mean absorbance ($OD_{600\ nm}$) ± SD (n ≥ 3). Free AGs, namely neomycin (A), neamine (B), paromomycin (C), and 4A4Hex-calix[4]arene lipophilic scaffold (D) were used as internal references.

**Table 1**
Antibacterial efficiency of 4A4Hex-calix[4]-neomycin **1**, 4A4Hex-calix[4]-neamine **2**, 4A4Hex-calix[4]-paromomycin **3** lipids and lipoplexes, their parent AGs, and the 4A4Hex-calix[4] scaffold, as determined by means of the (direct) plate count method.

| compound | antibacterial efficiency (%) | | |
|---|---|---|---|
| | CR 20 | CR 40 | CR 80 |
| 4A4Hex-calix[4]-Neomycin **1** | 73% | 76% | 100% |
| 4A4Hex-calix[4]-Neomyin **1**/pDNA | 73% | 95% | 100% |
| 4A4Hex-calix[4]-Neamine **2** | n.a. | n.a. | n.a. |
| 4A4Hex-calix[4]-Neamine **2**/pDNA | n.a. | n.a. | n.a. |
| 4A4Hex-calix[4]-Paramomycin **3** | 65% | 90% | 100% |
| 4A4Hex-calix[4]-Paramomycin **3**/pDNA | 75% | 90% | 100% |
| *b*PEI | n.a. | n.a. | n.a. |
| *b*PEI/pDNA | n.a. | n.a. | n.a. |
| Neomycin | 100% | 100% | 100% |
| Neamine | n.a. | n.a. | n.a. |
| Paromomycin | 98% | 100% | 100% |

complexation with DNA, derivative **2** (and PEI as well) was found to be ineffective against *E. coli* (Fig. 5B and D). Of note, the $MIC_{90}$ of Neamine was found at 32 μg/mL (Supplementary Information, Fig. S8B), therefore the lack of antibacterial properties cannot be ascribed to the ineffectiveness of the AG *per se*, but may relay on the conjugation of the antibiotic and the 4A4Hex-calix[4] scaffold. These data about the MIC of free AGs are in good agreement with the literature (Hu et al., 2017; Zimmermann et al., 2013).

We evaluated the antibacterial activity of 4A4Hex-calix[4]-AG derivatives also against Gram-positive bacteria. We found a mild effect of such compounds against *S. lutea* (see Supplementary Information, Fig. S9), and it was not particularly surprising because parent AGs are known to be mostly effective against Gram-negative bacteria (Dworkin, 1999; Mingeot-Leclercq et al., 1999; Vakulenko and Mobashery, 2003).

Altogether these results disclosed 4A4Hex-calix[4]-AG derivatives **1** and **3** as potential antimicrobials against Gram-negative bacteria, especially when complexed with DNA. Besides, since in every conjugate the AGs were found to be covalently bound to the 4A4Hex-calix[4] as shown by NMR analysis (which means that no free AG is detectable), the antibacterial activity displayed by 4A4Hex-calix[4]-AG as free lipids and their relative lipoplexes can be ascribed to the compounds themselves rather than to the presence of some free AGs. Indeed, due to their cationic nature, 4A4Hex-calix[4]-AG derivatives **1** and **3** did display antimicrobial properties, probably because of the interactions happening with the bacterial wall, as reported for other bioactive chemicals (Carmona-Ribeiro and de Melo Carrasco, 2013).

Taken together, these data disclose 4A4Hex-calix[4]-AGs as effective gene delivery tools with inherent antibacterial properties.

## 4. Conclusion

We have herein reported the synthesis and characterization of a novel class of multivalent CLs through the tethering of the upper rim of the 4A4Hex-calix[4] scaffold with three different AGs, namely neamine, neomycin, and paromomycin. 4A4Hex-calix[4]-AG derivatives **1–3** did induce very effective DNA complexation, as demonstrated by the fluorophore exclusion assay, thus giving rise to nano-assemblies ($D_H \approx 150$ nm) with superior transfection efficiencies *in vitro* and weak cytotoxicity on HeLa and U87-MG cells. Besides, these nano-assemblies displayed inherent antimicrobial activity against Gram-negative (*E. coli*) bacteria.

In order to provide more insight into the structure–activity relationship, further studies will aim to elucidate the mechanisms involved in cellular uptake and intracellular trafficking of this class of multivalent and multifunctional gene delivery vectors and to shed light on their mode of actions against Gram-negative bacteria.

Altogether, these findings highlight the potential of 4A4Hex-calix[4]-AGs assemblies as efficient multifunctional carries capable of delivering NAs and blunting Gram-negative bacterial infections at once.


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## Conflicts of interest

There are no conflicts to declare.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijpharm.2018.08.020.

## References


Bagnacani, V., Franceschi, V., Bassi, M., Lomazzi, M., Donofrio, G., Sansone, F., Casnati, A., Ungaro, R., 2013. Arginine clustering on calix[4]arene macrocycles for improved cell penetration and DNA delivery. Nat. Commun. 4, 1721.

Bagnacani, V., Franceschi, V., Fantuzzi, L., Casnati, A., Donofrio, G., Sansone, F., Ungaro, R., 2012. Lower rim Guanidinocalix[4]arenes: macrocyclic nonviral vectors for cell transfection. Bioconjug. Chem. 23, 993–1002.

Bagnacani, V., Sansone, F., Donofrio, G., Baldini, L., Casnati, A., Ungaro, R., 2008. Macrocyclic nonviral vectors: high cell transfection efficiency and low toxicity in a lower rim guanidinium calix[4]arene. Org. Lett. 10, 3953–3956.

Belmont, P., Aissaoui, A., Hauchecorne, M., Oudrhiri, N., Petit, L., Vigneron, J.P., Lehn, J.M., Lehn, P., 2002. Aminoglycoside-derived cationic lipids as efficient vectors for gene transfection in vitro and in vivo. J. Gene Med. 4, 517–526.

Bhattacharya, S., Bajaj, A., 2009. Advances in gene delivery through molecular design of cationic lipids. Chem. Commun. (Camb) 4632–4656.

Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P., 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc. Natl. Acad. Sci. U.S.A. 92, 7297–7301.

Candiani, G., Pezzoli, D., Ciani, L., Chiesa, R., Ristori, S., 2010. Bioreducible liposomes for gene delivery: from the formulation to the mechanism of action. PLoS One 5, e13430.

Carmona-Ribeiro, A.M., de Melo Carrasco, L.D., 2013. Cationic antimicrobial polymers and their assemblies. Int. J. Mol. Sci. 14, 9906–9946.

Casnati, A., Fabbi, M., Pelizzi, N., Pochini, A., Sansone, F., Ungaro, R., DiModugno, E., Tarzia, G., 1996. Synthesis, antimicrobial activity and binding properties of calix[4] arene based vancomycin mimics. Bioorg. Med. Chem. Lett. 6, 2699–2704.

Chatin, B., Mevel, M., Devalliere, J., Dallet, L., Haudebourg, T., Peuziat, P., Colombani, T., Berchel, M., Lambert, O., Edelman, A., Pitard, B., 2015. Liposome-based formulation for intracellular delivery of functional proteins. Mol. Therapy 4, e244. Chen, M., Hu, M., Wang, D., Wang, G., Zhu, X., Yan, D., Sun, J., 2012. Multifunctional hyperbranched glycoconjugated polymers based on natural aminoglycosides. Bioconjug. Chem. 23, 1189–1199.

Colston, M.J., Hailes, H.C., Stavropoulos, E., Herve, A.C., Herve, G., Goodworth, K.J., Hill, A.M., Jenner, P., Hart, P.D., Tascon, R.E., 2004. Antimycobacterial calixarenes enhance innate defense mechanisms in murine macrophages and induce control of Mycobacterium tuberculosis infection in mice. Infect. Immun. 72, 6318–6323.

Desigaux, L., Sainlos, M., Lambert, O., Chevre, R., Letrou-Bonneval, E., Vigneron, J.P., Lehn, P., Lehn, J.M., Pitard, B., 2007. Self-assembled lamellar complexes of siRNA with lipidic aminoglycoside derivatives promote efficient siRNA delivery and interference. Proc. Natl. Acad. Sci. U.S.A. 104, 16534–16539.

Dworkin, R.J., 1999. Aminoglycosides for the treatment of gram-negative infections: therapeutic use, resistance and future outlook. Drug Resist. Updates 2, 173–179.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., Danielsen, M., 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. U.S.A. 84, 7413–7417.

Fosso, M.Y., Li, Y., Garneau-Tsodikova, S., 2014. New trends in aminoglycosides use. Medchemcomm 5, 1075–1091.

Fraley, R., Subramani, S., Berg, P., Papahadjopoulos, D., 1980. Introduction of liposome-encapsulated SV40 DNA into cells. J. Biol. Chem. 255, 10431–10435.

Ghilardi, A., Pezzoli, D., Bellucci, M.C., Malloggi, C., Negri, A., Sganappa, A., Tedeschi, G., Candiani, G., Volonterio, A., 2013. Synthesis of multifunctional PAMAM-aminoglycoside conjugates with enhanced transfection efficiency. Bioconjug. Chem. 24, 1928–1936.

Houghton, J.L., Green, K.D., Chen, W., Garneau-Tsodikova, S., 2010. The future of aminoglycosides: the end or renaissance? Chembiochem 11, 880–902.

Hu, Y., Liu, L., Zhang, X., Feng, Y., Zong, Z., 2017. In vitro activity of neomycin, streptomycin, paromomycin and apramycin against carbapenem-resistant enterobacteriaceae clinical strains. Front. Microbiol. 8, 2275.

Huang, Y., Ding, X., Qi, Y., Yu, B., Xu, F.J., 2016. Reduction-responsive multifunctional

hyperbranched polyaminoglycosides with excellent antibacterial activity, biocompatibility and gene transfection capability. Biomaterials 106, 134–143.
Kichler, A., Leborgne, C., Marz, J., Danos, O., Bechinger, B., 2003. Histidine-rich amphipathic peptide antibiotics promote efficient delivery of DNA into mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 100, 1564–1568.
Le Gall, T., Baussanne, I., Halder, S., Carmoy, N., Montier, T., Lehn, P., Decout, J.L., 2009. Synthesis and transfection properties of a series of lipidic neamine derivatives. Bioconjug. Chem. 20, 2032–2046.
Lee, C.C., MacKay, J.A., Frechet, J.M., Szoka, F.C., 2005. Designing dendrimers for biological applications. Nat. Biotechnol. 23, 1517–1526.
Lucotti, A., Tommasini, M., Pezzoli, D., Candiani, G., 2014. Molecular interactions of DNA with transfectants: a study based on infrared spectroscopy and quantum chemistry as aids to fluorescence spectroscopy and dynamic light scattering analyses. RSC Adv. 4, 49620–49627.
Malloggi, C., Pezzoli, D., Magagnin, L., De Nardo, L., Mantovani, D., Tallarita, E., Candiani, G., 2015. Comparative evaluation and optimization of off-the-shelf cationic polymers for gene delivery purposes. Polym. Chem.-Uk 6, 6325–6339.
Martin, B., Sainlos, M., Aissaoui, A., Oudrhiri, N., Hauchecorne, M., Vigneron, J.P., Lehn, J.M., Lehn, P., 2005. The design of cationic lipids for gene delivery. Curr. Pharm. Des. 11, 375–394.
Mevel, M., Sainlos, M., Chatin, B., Oudrhiri, N., Hauchecorne, M., Lambert, O., Vigneron, J.P., Lehn, P., Pitard, B., Lehn, J.M., 2012. Paromomycin and neomycin B derived cationic lipids: synthesis and transfection studies. J. Control. Release 158, 461–469.
Mingeot-Leclercq, M.P., Glupczynski, Y., Tulkens, P.M., 1999. Aminoglycosides: activity and resistance. Antimicrob. Agents Chemother. 43, 727–737.
Mintzer, M.A., Simanek, E.E., 2009. Nonviral vectors for gene delivery. Chem. Rev. 109, 259–302.
Miryala, B., Feng, Y., Omer, A., Potta, T., Rege, K., 2015. Quaternization enhances the transgene expression efficacy of aminoglycoside-derived polymers. Int. J. Pharm. 489, 18–29.
Miryala, B., Godeshala, S., Grandhi, T.S., Christensen, M.D., Tian, Y., Rege, K., 2016. Aminoglycoside-derived amphiphilic nanoparticles for molecular delivery. Colloids Surf., B 146, 924–937.
Mochizuki, S., Nishina, K., Fujii, S., Sakurai, K., 2015. The transfection efficiency of calix [4]arene-based lipids: the role of the alkyl chain length. Biomater. Sci. 3, 317–322.
Napoli, S., Carbone, G.M., Catapano, C.V., Shaw, N., Arya, D.P., 2005. Neomycin improves cationic lipid-mediated transfection of DNA in human cells. Bioorg. Med. Chem. Lett. 15, 3467–3469.
Nimse, S.B., Kim, T., 2013. Biological applications of functionalized calixarenes. Chem. Soc. Rev. 42, 366–386.
Ortiz Mellet, C., Benito, J.M., Garcia Fernandez, J.M., 2010. Preorganized, macromolecular, gene-delivery systems. Chemistry 16, 6728–6742.
Park, W.K.C., Auer, M., Jaksche, H., Wong, C.H., 1996. Rapid combinatorial synthesis of aminoglycoside antibiotic mimetics: use of a polyethylene glycol-linked amine and a neamine-derived aldehyde in multiple component condensation as a strategy for the discovery of new inhibitors of the HIV RNA Rev responsive element. J. Am. Chem. Soc. 118, 10150–10155.
Pezzoli, D., Candiani, G., 2013. Non-viral gene delivery strategies for gene therapy: a “menage a trois” among nucleic acids, materials, and the biological environment Stimuli-responsive gene delivery vectors. J. Nanopart. Res. 15.
Pezzoli, D., Chiesa, R., De Nardo, L., Candiani, G., 2012. We still have a long way to go to effectively deliver genes!. J. Appl. Biomater. Funct. Mater. 10, 82–91.
Pezzoli, D., Giupponi, E., Mantovani, D., Candiani, G., 2017. Size matters for in vitro gene delivery: investigating the relationships among complexation protocol, transfection medium, size and sedimentation. Sci. Rep. 7, 44134.
Rodik, R.V., Anthony, A.S., Kalchenko, V.I., Mely, Y., Klymchenko, A.S., 2015. Cationic amphiphilic calixarenes to compact DNA into small nanoparticles for gene delivery. New J. Chem. 39, 1654–1664.
Rodik, R.V., Klymchenko, A.S., Mely, Y., Kalchenko, V.I., 2014. Calixarenes and related macrocycles as gene delivery vehicles. J. Incl. Phenom. Macro 80, 189–200.
Sainlos, M., Hauchecorne, M., Oudrhiri, N., Zertal-Zidani, S., Aissaoui, A., Vigneron, J.P., Lehn, J.M., Lehn, P., 2005. Kanamycin A-derived cationic lipids as vectors for gene transfection. Chembiochem 6, 1023–1033.
Sansone, F., Dudic, M., Donofrio, G., Rivetti, C., Baldini, L., Casnati, A., Cellai, S., Ungaro, R., 2006. DNA condensation and cell transfection properties of guanidinium calixarenes: dependence on macrocycle lipophilicity, size, and conformation. J. Am. Chem. Soc. 128, 14528–14536.
Sganappa, A., Wexselblatt, E., Bellucci, M.C., Esko, J.D., Tedeschi, G., Tor, Y., Volonterio, A., 2017. Dendrimeric guanidinoneomycin for cellular delivery of bio-macromolecules. Chembiochem 18, 119–125.
Taylor, P.C., Schoenknecht, F.D., Sherris, J.C., Linner, E.C., 1983. Determination of minimum bactericidal concentrations of oxacillin for Staphylococcus aureus: influence and significance of technical factors. Antimicrob. Agents Chemother. 23, 142–150.
Tor, Y., 2003. Targeting RNA with small molecules. Chembiochem 4, 998–1007.
Vakulenko, S.B., Mobashery, S., 2003. Versatility of aminoglycosides and prospects for their future. Clin. Microbiol. Rev. 16, 430–450.
van Gaal, E.V., van Eijk, R., Oosting, R.S., Kok, R.J., Hennink, W.E., Crommelin, D.J., Mastrobattista, E., 2011. How to screen non-viral gene delivery systems in vitro? J. Control. Release 154, 218–232.
Wang, H., Tor, Y., 1997. Dimeric aminoglycosides: design, synthesis and RNA binding. Bioorg. Med. Chem. Lett. 7, 1951–1956.
Wu, G.Y., Wu, C.H., 1987. Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. J. Biol. Chem. 262, 4429–4432.
Yadav, S., Mahato, M., Pathak, R., Jha, D., Kumar, B., Deka, S.R., Gautam, H.K., Sharma, A.K., 2014. Multifunctional self-assembled cationic peptide nanostructures efficiently carry plasmid DNA in vitro and exhibit antimicrobial activity with minimal toxicity. J. Mater. Chem. B 2, 4848–4861.
Zhang, C., Yadava, P., Hughes, J., 2004. Polyethylenimine strategies for plasmid delivery to brain-derived cells. Methods 33, 144–150.
Zhang, Y., Pelet, J.M., Heller, D.A., Dong, Y., Chen, D., Gu, Z., Joseph, B.J., Wallas, J., Anderson, D.G., 2013. Lipid-modified aminoglycoside derivatives for in vivo siRNA delivery. Adv. Mater. 25, 4641–4645.
Zhou, J., Wang, G., Zhang, L.H., Ye, X.S., 2007. Modifications of aminoglycoside antibiotics targeting RNA. Med. Res. Rev. 27, 279–316.
Zimmermann, L., Bussiere, A., Ouberai, M., Baussanne, I., Jolivalt, C., Mingeot-Leclercq, M.P., Decout, J.L., 2013. Tuning the antibacterial activity of amphiphilic neamine derivatives and comparison to paromamine homologues. J. Med. Chem. 56, 7691–7705.
Zuhorn, I.S., Oberle, V., Visser, W.H., Engberts, J.B., Bakowsky, U., Polushkin, E., Hoekstra, D., 2002. Phase behavior of cationic amphiphiles and their mixtures with helper lipid influences lipoplex shape, DNA translocation, and transfection efficiency. Biophys. J. 83, 2096–2108.