Bioconjugate Chemistry

Article

pubs.acs.org/bc

Synthesis of Multifunctional PAMAM–Aminoglycoside Conjugates with Enhanced Transfection Efficiency

Alessandra Ghilardi,[†] Daniele Pezzoli,[‡] Maria Cristina Bellucci,[∥] Chiara Malloggi,[†] Armando Negri,[§] Aurora Sganappa,[†] Gabriella Tedeschi,[§] Gabriele Candiani,*[,†,‡] and Alessandro Volonterio*[,†]

[†]Department of Chemistry, Materials, and Chemical Engineering "G. Natta", Politecnico di Milano, via Mancinelli 7, 20131 Milano, Italy

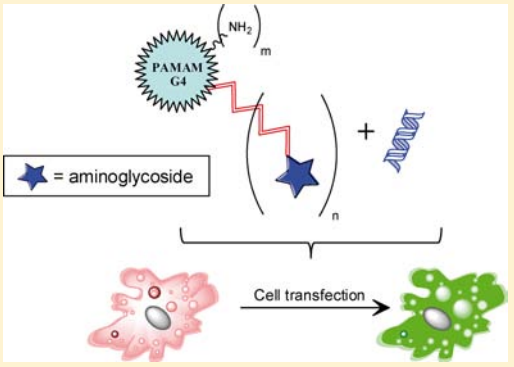
[‡]Politecnico di Milano Research Unit, National Interuniversity Consortium of Materials Science and Technology - INSTM, via Mancinelli 7, 20131 Milano, Italy

[∥]Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, via Celoria 2, 20133 Milano, Italy

[§]Department of Veterinary Science and Public Health, Università degli Studi di Milano, via Celoria 10, 20133 Milano, Italy

Supporting Information

ABSTRACT: The development of multifunctional vectors for efficient and safe gene delivery is one of the major challenges for scientists working in the gene therapy field. In this context, we have designed a novel type of aminoglycoside-rich dendrimers with a defined structure based on polyamidoamine (PAMAM) in order to develop efficient, nontoxic gene delivery vehicles. Three different conjugates, i.e., PAMAM G4–neamine, –paromomycin, and –neomycin, were synthesized and characterized by nuclear magnetic resonance (NMR) and MALDI analysis. The conjugates were found to self-assemble electrostatically with plasmid DNA, and unlike neamine conjugate, each at its optimum showed increased gene delivery potency compared to PAMAM G4 dendrimer in three different cell lines, along with negligible cytotoxicity. These results all disclosed aminoglycosides as suitable functionalities for tailoring safe and efficient multifunctional gene delivery vectors.



INTRODUCTION

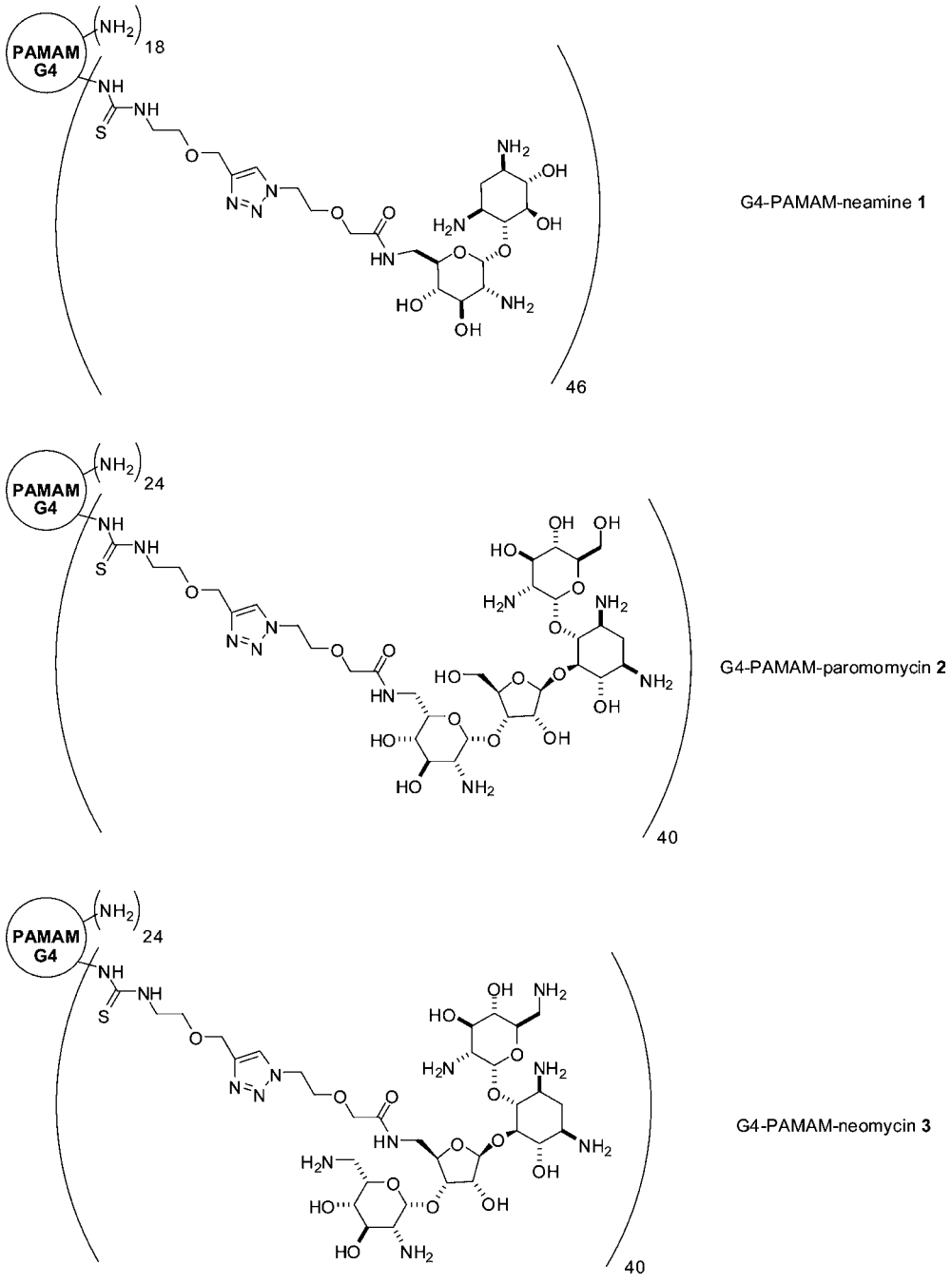
One of the most challenging tasks to reach efficient delivery of genes into cells is the development of safe and (multi)functional gene carriers. In this sense, different viral and nonviral vectors have been developed in the past decades, each with its pros and cons. Although viral vectors are the most widely used in ongoing gene therapy trials due to their superior delivery properties,[1,2] safety issues arising from insertional mutagenesis and possible recombination with wild-type viruses, intrinsic immunogenicity, and toxicity still are major concerns.[3,4] For these reasons, much effort has been devoted recently to the development of nonviral vehicles[5,6] such as cationic polymers (both natural or synthetic)[7,8] and lipids[9] able to "self-assemble" with negatively charged nucleic acids to form polyplexes and lipoplexes, respectively. Although substantial progress has been made in the development of nonviral vectors, their efficiency is still lower than that of viral counterparts, and cytotoxicity is a major concern, thus restricting their widespread clinical application. Indeed, although poly(ethylenimine) (PEI) is considered a gold standard among polymeric transfection agents, it is certainly far from being ideal because of its non-negligible toxicity and sharp reduction in efficacy when administered *in vivo*.[10,11] In this scenario, starburst poly(amidoamine) (PAMAM) dendrimers represent a very attractive alternative to PEIs because of their low cytotoxicity and well-defined geometry and chemistry that allow ease of modification to increase their efficiency.[12,13] In this regard, the functionalization of the outer primary amines of PAMAM dendrimers with, for instance, lipophilic[14] or hydrophilic moieties[15,16] has been extensively reported.

Aminoglycosides, due to their natural affinity for nucleic acids and their structural variety and functionality, have been used as polar headgroups in the synthesis of cationic lipids with different spacers and lipophilic moieties, displaying remarkably high gene delivery efficiencies both *in vitro* and *in vivo*.[17,18] Furthermore, the groups of Prof. Esko and Prof. Tor have recently shown a related family of aminoglycosides[19] that, depending on the valency of the transporter,[20−23] are able to mediate the uptake of high molecular weight (M_w) cargos into cells. Aside from these interesting features, aminoglycosides are a group of well-known antibiotics.[24] Collectively, this experimental evidence supports the idea that aminoglycosides, while not extensively studied for this purpose, are suitable and versatile scaffolds for the synthesis of new gene delivery vectors with interesting antibacterial properties. In this scenario, very recently gentamicin and

Received: August 1, 2013
Revised: October 19, 2013
Published: October 22, 2013

dx.doi.org/10.1021/bc4003635 | *Bioconjugate Chem.* 2013, 24, 1928−1936

Scheme 1. PAMAM G4−Aminoglycosides 1−3



kanamycin were used as monomers for the construction, by Michael addition with *N*,*N*′-methylenebisacrylamide, of hyperbranched cationic polymers which displayed low cytoxicity, high buffering capacity, strong pDNA condensation ability, and good antitumor and antibacterial activity.[25,26]

Herein, we introduce for the first time a class of oligomeric aminoglycosides conjugated to PAMAM generation four (PAMAM G4), i.e., PAMAM−aminoglycoside conjugates, as novel nonviral delivery vectors (Scheme 1). The surface of dendritic PAMAM G4 was grafted through an isothiocyanate linker with three different aminoglycosides, namely, neamine, paromomycin, and neomycin, and the degree of grafting determined by ^{1}H nuclear magnetic resonance (^{1}H NMR) and matrix-assisted laser desorption/ionization (MALDI) analysis. Their *in vitro* cytotoxicity and transfection efficiency in three different eukaryotic cell lines were evaluated. PAMAM G4−paromomycin and −neomycin conjugates displayed enhanced gene delivery activities compared to the parent dendrimer PAMAM G4, even greater than or equal to that of the gold standard 25 kDa branched PEI (bPEI), along with low cytotoxicity. These results all disclosed aminoglycosides as suitable functionalities for tailoring safe and efficient multifunctional gene vectors.

MATERIALS AND METHODS

Materials. Neomycin sulfate, paromomycin sulfate, PAMAM G4 dendrimer (ethylenediamine core, 64 surface groups), 25 kDa bPEI (M_w ~25 kDa by LS, average M_n ~10 kDa by GPC),

dx.doi.org/10.1021/bc4003635 | *Bioconjugate Chem.* 2013, 24, 1928−1936

and all other organic reactants, solvents, and culture reagents were purchased from Sigma-Aldrich (Milan, Italy) if not specified differently, and used as received. Use of a cationic polymer based on PEI for transfection is covered by US Patent 6,013,240, European Patent 0,770,140, and foreign equivalents, for which Polyplus-transfection is the worldwide exclusive licensee. Neamine was synthesized from neomycin as described in literature.[27] Spectra/Por dialysis bags (MWCO = 1 and 8 kDa) were from Spectrum Laboratories (Compton, CA, USA). The luciferase expression plasmid (pGL3-Control vector) encoding for the modified firefly luciferase and the luciferase expression kit were from Promega (Milan, Italy). Plasmid DNA (pDNA) was prepared as previously reported.[8] AlamarBlue cell viability assay was from Life Technologies (Monza, Italy) while BCA Protein Assay Kit was from Thermo Fisher Scientific (Rockford, IL, USA). HeLa (human cervix carcinoma), U87-MG (human glioblastoma-astrocytoma epithelial-like), and COS-7 (African green monkey kidney fibroblast-like) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). ^{1}H NMR spectra were recorded on 400 MHz spectrometers. Chemical shifts are expressed in ppm (δ), using tetramethylsilane (TMS) as internal standard for ^{1}H and ^{13}C nuclei (δ_H and δ_C = 0.00). MALDI−TOF analysis was carried out by a Bruker Daltonics Reflex IV instrument (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm) and operated in linear mode using the dry droplet technique and 2,3-dihydroxybenzoic acid 10 mg/mL in CH_3CN:TFA/1:1 as a matrix. External standards were used for calibration (Bruker protein calibration standard 1 and 2). Each spectrum was accumulated for at least 600 laser shots.

Synthesis of PAMAM G4−aminoglycoside Conjugates 1−3: General Procedure. The organic solvent of commercial 10% w/w methanolic solution of PAMAM G4 was evaporated *in vacuo* and coevaporated twice with dichloromethane. Neat PAMAM G4 was dissolved in DMSO and a solution of aminoglycoside-isothiocyanate linker (1.2 equiv per NH_2 group) in a minimal volume of DMSO was added. The solution was stirred at 60 °C for 24 h and then dialyzed against MeOH (8 h, the solvent reservoir was renewed thrice, MWCO 1 kDa for the neamine derivative and 8 kDa for the paromomycin and neomycin derivatives). The solution was evaporated under reduced pressure to give *N*-Boc-protected PAMAM G4-aminoglycosides. After ^{1}H NMR characterization, the resulting conjugates were dissolved in trifluoroacetic acid (TFA) and stirred for 30 min at room temperature (r.t.). The excess of TFA was evaporated under reduced pressure, the crude dissolved in water, and the solution dialyzed against water (overnight, MWCO 1 kDa). Lyophilization of the water solution led to recovery of a fluffy, white solid. Complete *N*-Boc deprotection occurred in all cases as evidenced by the spectra recorded which clearly show the absence of the Boc signals. For the complete description of the synthesis of PAMAM G4-aminoglycoside conjugates **1**−**3** see Supporting Information.

Cell Cultures. HeLa, MG63, and U87-MG cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C, in Dulbecco's Modified Eagle Medium (DMEM) containing 1 mM sodium pyruvate, 10 mM HEPES buffer, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM glutamine and supplemented with 10% (v/v) fetal bovine serum (FBS), hereafter referred to as 10% FBS.

Preparation of Polyplexes and Evaluation of DNA Complexation Ability. Polyplexes were prepared by adding pGL3 plasmid DNA to PAMAM G4 and PAMAM G4-aminoglycoside conjugates in 10 mM HEPES pH 7.0 at the desired polymer concentration to yield different nitrogen to phosphate (N/P) ratios, and incubated for 20 min at r.t. before use. The final DNA concentration in the transfection solution was 20 ng/μL. The DNA complexation ability of PAMAM G4 and of its derivatives was determined by fluorophore-exclusion titration assay. Briefly, polyplexes were prepared as described above using 0.2 μg of pGL3 and were diluted to 100 μL in 10 mM HEPES pH 7.0 containing 2× SYBR Green I (λ_{ex} = 497 nm; λ_{em} = 520 nm). The fluorescence of the resulting solutions was measured using the microplate reader GENios Plus (Tecan, Segrate, Italy) and normalized over the fluorescence of noncomplexed plasmid DNA.

Measurement of Mean Hydrodynamic Diameter and ζ-Potential of Polyplexes. The mean hydrodynamic diameter (D_H) of polyplexes was evaluated by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS instrument (Rome, Italy), fitted with a 633 nm laser at a fixed scattering angle of 173°. The ζ-potential of the same complexes was measured by Laser Doppler Velocimetry using the same apparatus. Polyplexes (1 μg of pGL3) were diluted 1:9 in 10 mM HEPES pH 7, and equilibrated at 25 °C prior to measurements.

***In Vitro* Transfection Experiments.** Cells were plated at a density of 2 × 10^4 cells/cm^2 in 96-well culture plates. Twenty-four hours after seeding, cells were washed in PBS and culture medium was replaced with 100 μL/well of polyplex-containing medium (160 ng/cm^2 of pGL3). Three different transfection media were tested: serum-free DMEM, 10% FBS, and DMEM supplemented with 50% FBS (hereafter referred to as 50% FBS). After transfection, cells were incubated for 48 h at 37 °C. Cytotoxicity was tested using AlamarBlue cell viability assay and expressed in percent relative to the untreated controls. Luciferase activity was measured by Luciferase Assay System using a Mithras luminometer (Berthold Technologies, Brugherio, Italy) and normalized to the total protein content, evaluated by BCA assay, in cell lysates.

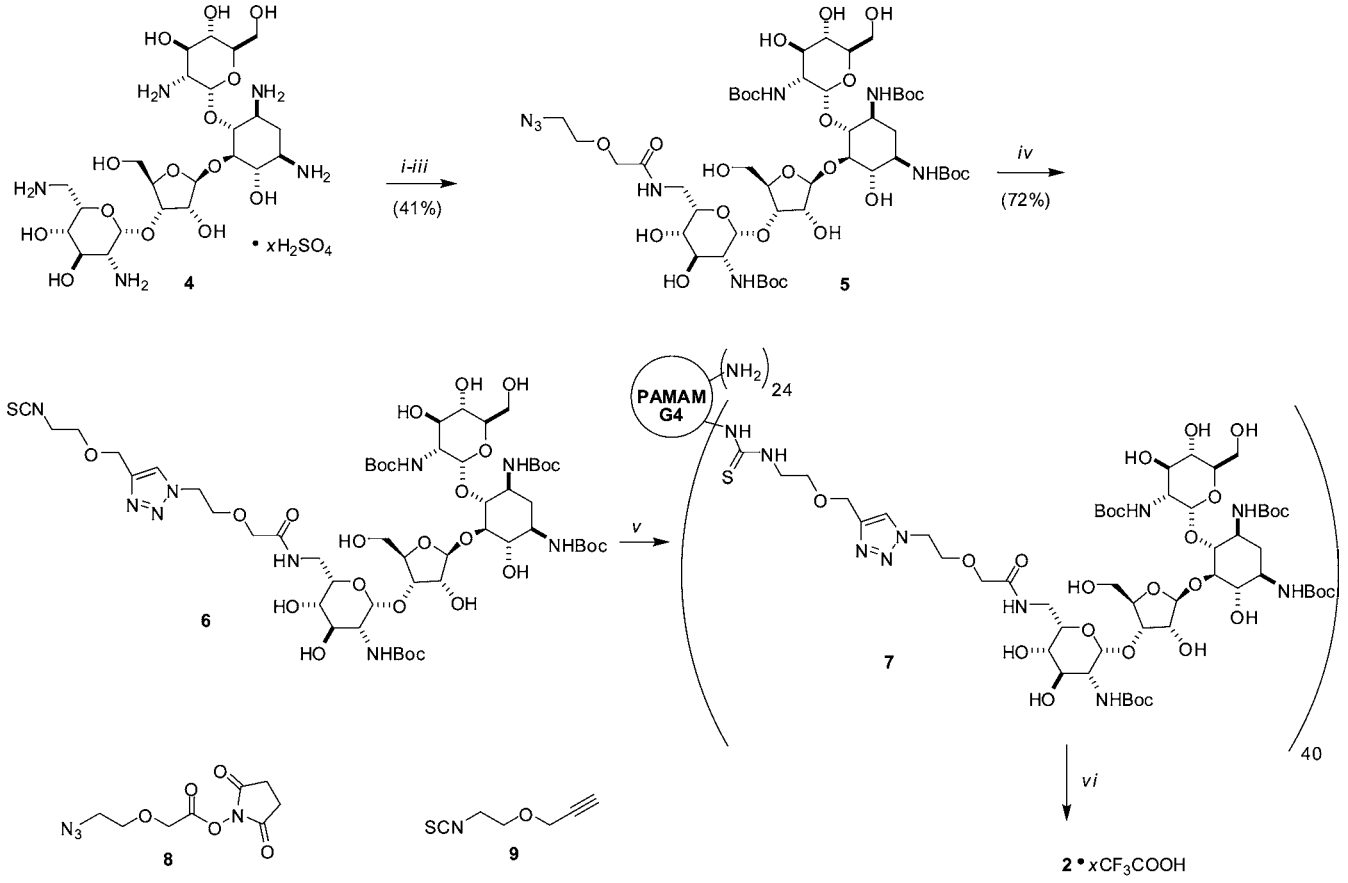
Atomic Force Microscopy. The shape of polyplexes was evaluated by atomic force microscopy (AFM; NTEGRA-Spectra, NT-MDT, Moscow, Russia). Polyplexes were prepared at N/P 15 (except for 25 kDa bPEI, N/P 10) as described above and a drop of 2 μL of polyplex solution was placed on a silicon wafer and dried at r.t.. AFM images were collected in tapping mode (semicontact) at a scanning speed of 0.6 Hz and images were analyzed using NT-MDT *Nova* software (v1.0.26).

Statistical Analysis. Experiments were performed at least in quadruplicate. Results were expressed as mean ± SD and statistical analyses were carried out by GraphPad *Prism* v 5.04 (GraphPad software, La Jolla, CA, USA). Comparisons among groups were performed by one-way ANOVA with posthoc Dunnett test. Significance was retained when $p < 0.05$.

RESULTS AND DISCUSSION

Synthesis and Characterization of PAMAM G4−Aminoglycoside Conjugates. Among the known strategies to graft the dendrimer termini with biomolecules, we have used the well-established reaction between isothiocyanate group-containing linkers and amino-terminated PAMAM dendrimers. This approach has the great advantage that neither additional coupling reagents are required for the reaction nor byproducts are formed, thus facilitating the purification step. PAMAM G4 was selected as the dendritic platform because relatively small in size (i.e., M_w) but displaying a reasonable number of tertiary amino groups responsible for the so-called "proton sponge effect".[28] On the

Scheme 2. Synthesis of PAMAM G4−Paramomycin Conjugate 2



i. Amberlist IRA 400 OH⁻; *ii*. **8**, CH_3CN/H_2O; *iii*. Boc_2O, dioxane/H_2O; *iv*. **9**, $Cu(OAc)_2$, Na-ascorbate, MeOH/THF/H_2O; *v*. PAMAM-G4, dmso 40°C; *vi*. TFA

other hand, aminoglycosides are multifunctional molecules having different amino and hydroxyl functional groups in their skeleton. In order to obtain selective functionalization, we exploited the presence of a singular, less hindered amino methylene moiety in neamine (position 6′) and paromomycine (position 6‴) and the presence of a singular primary alcohol in neomycin (position 5″). Accordingly, we adopted the synthetic strategy depicted in Scheme 2 to graft the formers, while it was followed by a slightly different approach to prepare PAMAM G4-neomycin conjugate (Scheme S4 in Supporting Information). Commercially available paromomycin sulfate **4** was first neutralized with Amberlist resin and then treated with *N*-hydroxysuccinimide ester **8**, which reacted selectively with the less sterically hindered amine affording, after protection of the remaining free amino groups with Boc_2O, derivative **5** in reasonable yield. "Click" reaction of **5** with propargyl derivative **9** led to the formation of **6** which possesses the suitable isothiocyanate function to be used to anchor the aminoglycoside to the PAMAM dendrimer. The latter reaction was carried out in DMSO at 60 °C for 24 h in such a way as to maximize the degree of grafting. However, probably due to the steric hindrance of the aminoglycoside, we did not reach a complete conversion of the outer PAMAM amino groups, as it has been previously reported to occur with small amino acids,[15,16] but *ca.* 40 out of 64 terminal primary amines were functionalized affording, after Boc-deprotection with TFA, PAMAM G4-paromomycin conjugate **2** with a slightly greater than 60% degree of grafting.

The structure of synthesized PAMAM G4-aminoglycoside conjugates **1**−**3** as well as the degree of grafting were confirmed by ^{1}H NMR spectroscopy. In Figure 1, the ^{1}H NMR spectra of PAMAM G4 (red spectrum), free paromomycin (green spectrum), and PAMAM G4−paromomycin conjugate **2** (blue spectrum), all recorded in D_2O, are represented as an example. In the spectrum of PAMAM G4−paromomycin conjugate **2**, characteristic peaks belonging to the PAMAM G4 dendrimer and to the paromomycin aminoglycoside are well observable: (1) at 2.76 ppm the signals of the protons in α position of the carbonyl group in PAMAM dendrimer (H_c protons, Figure 1); (2) the two characteristic protons H' belonging to the 2-desoxystreptamine ring of the aminoglycoside which resonate at 2.43 and 1.81 ppm, respectively; (3) the three anomeric protons H'' belonging to the paromomycin molecule which appear as three small doublets between 5.71 and 5.15 ppm; and (4) the proton resonating at *ca.* 8.00 ppm which belongs to the triazole heterocycle of the linker. The integration of these protons was used to calculate the degree of grafting (see Figure S10 in Supporting Information). Indeed, the integration ratio between the PAMAM H_c protons and the two H' protons was found to be 248:41:41 (the same ratio was found between H_c protons and the anomeric protons H'') confirming that slightly more than 40 amino groups over 64 were functionalized (degree of grafting ∼63%). The same result was observed for conjugate **3** where neomycin was grafted to PAMAM G4 (Figure S11, Supporting Information), while a slightly higher degree of grafting (∼72%,

dx.doi.org/10.1021/bc4003635 | *Bioconjugate Chem.* 2013, 24, 1928−1936

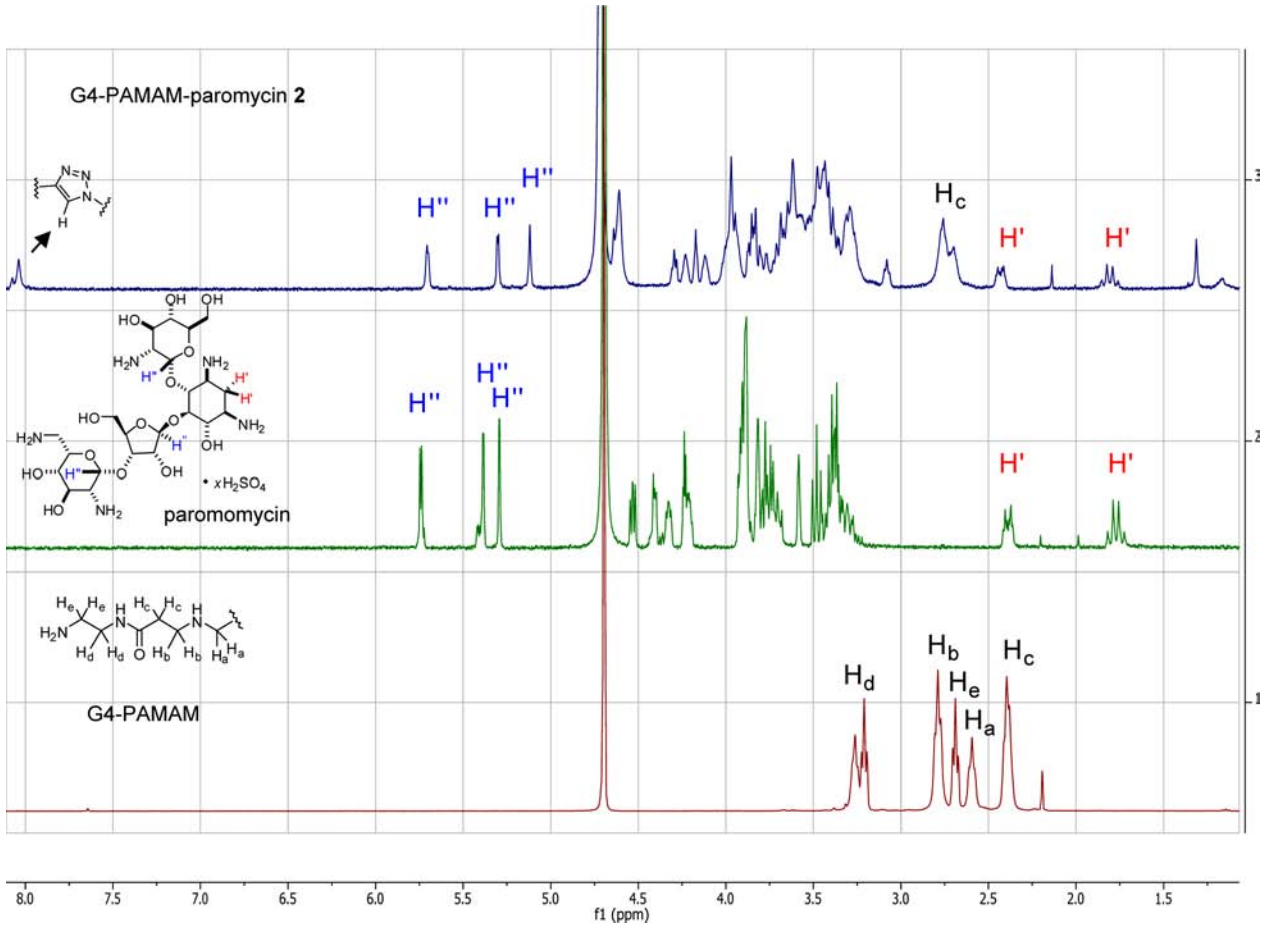


Figure 1. ^{1}H NMR spectra recorded in D_2O of PAMAM G4 dendrimer (red spectrum), paramomycin (green spectrum), and PAMAM G4–paromomycin conjugate (blue spectrum).

Figure S9 in Supporting Information) was found for the smaller aminoglycoside neamine.

The calculated degree of grafting was confirmed by MALDI spectroscopy with the only exception of the PAMAM–neamine conjugate **1** which could not be detected by MALDI analysis. Table 1 reports the molecular mass determined by mass spectroscopy and the corresponding values calculated for the single- and the double-charged parent ions. As shown in Supporting Information (Figure S12), the MALDI peaks detected correspond to the double-charged ions of the PAMAM G4-paramomycin and -neomycin conjugates analyzed, while PAMAM G4 presents the single-charged ion.

Table 1. Molecular Mass Determined by MALDI Mass Analysis and the Corresponding Values Calculated for the Single and the Double Charged Parent Ions

	experimental value (m/z)	calculated value $[M+H]^+$ (m/z)	calculated value $[M+2H]^{2+}$ (m/z)
PAMAM G4	13226	13226	6613
PAMAM G4–paramomycin conjugate **2**	24600	49186	24593
PAMAM G4–neomycin conjugate **3**	24580	49146	24573

Biophysical Behavior of pDNA/PAMAM G4–Aminoglycosides. One of the main requirements of gene delivery vectors is that they do have to be able to effectively complex and condense nucleic acids.[29] We thus evaluated first by fluorophore-exclusion titration assay the ability of each PAMAM G4-derivative to complex DNA as a function of the nitrogen to phosphate (N/P) ratio (Figure 2A). Due to their globular shape and the positioning of amino groups at the surface, PAMAM dendrimers, and possibly PAMAM G4-aminoglycosides, are well suited to engage in multivalent interactions with nucleic acids, thus allowing strong binding of the phosphodiester groups. In fluorophore-exclusion titration assay (Figure 2A), using SYBR Green I as DNA dye, maximal complexation of pDNA was found for N/P $\geq$ 2 for PAMAM G4 and derivative **3** and for N/P $\geq$ 3 for derivatives **1** and **2** that showed similar complexation behavior and did displace slightly less SYBR Green I than the former, indicating that they have a lower affinity for nucleic acids. These results are not surprising for two reasons: (1) it is well-known that neomycin is a better DNA-binder than neamine and paromomycin, and (2) in conjugates **1** and **2**, the amino methylene groups (position 6′ of neamine and 6‴ of paromomycin) are converted into an amide removing the most basic amine from contributing to strong electrostatic interactions with negatively charged DNA.[30]

Recent evidence disclosed surface charge and size (hydrodynamic diameter) of polyplexes as prominent factors affecting their biological behavior.[31] We have thus evaluated the mean diameter and the ζ-potential of polyplexes as a function of the N/P and results are shown in Figure 2B–F. Typical ζ-potential curves had a marked sigmoidal shape as a function of N/P, with

dx.doi.org/10.1021/bc4003635 | *Bioconjugate Chem.* 2013, 24, 1928–1936

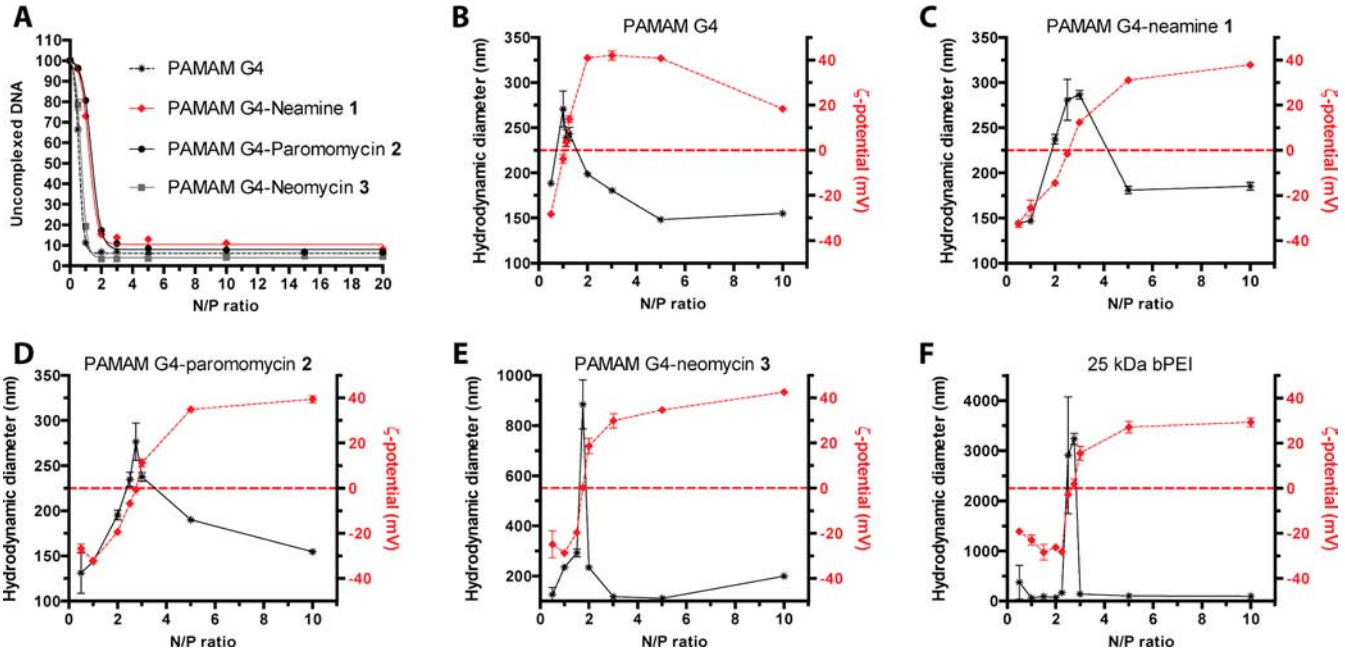


Figure 2. DNA complexation abilities of PAMAM G4 dendrimer and PAMAM G4-derivatives **1**–**3**. (A) Comparative DNA complexation ability of PAMAM G4 (black stars and dotted line) and of PAMAM G4-neamine **1** (red rhombi and solid line), PAMAM G4-paromomycin **2** (black circles and solid line) and PAMAM G4-neomycin **3** (gray squares and solid line) evaluated by monitoring the fluorochrome exclusion from polyplexes as a function of nitrogen (N) to phosphate (P) ratio (N/P). Average hydrodynamic diameters (black stars) and ζ-potentials (empty red rhombi) of (B) PAMAM G4, (C) derivative **1**, (D) **2**, (E) **3**, and (F) 25 kDa bPEI, measured over a wide range of N/P ratios. Results are expressed as mean ± standard deviation ($n \geq 3$).

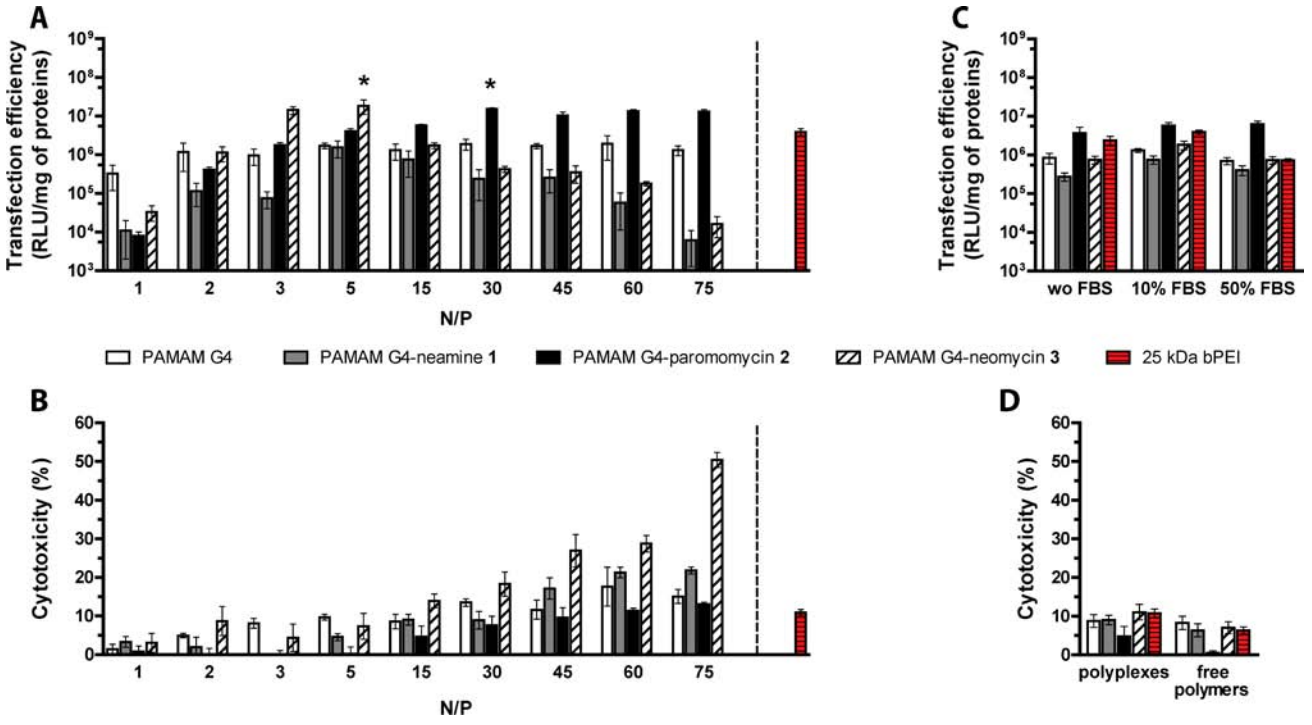


Figure 3. Transfection efficiency and cytotoxicity of PAMAM G4 and PAMAM G4-derivatives in HeLa cells. (A) Transfection efficiency and (B) cytotoxicity of polyplexes prepared with pGL3 at different N/P were evaluated after incubation for 48 h in 10% FBS. (C) Influence of FBS content on the transfection efficiency of polyplexes prepared at N/P 15. (D) Comparative cytotoxicity assay of PAMAM G4 dendrimer and PAMAM G4-derivatives **1**–**3** delivered as polyplexes at N/P 15 and free in solution at equivalent concentration. 25 kDa bPEI was utilized at N/P 10, according to the existing literature. Transfection efficiency was expressed as relative luminescence normalized against the total protein content in cell lysates while cytotoxicity was expressed as toxicity percent relative to untreated controls. Results are expressed as mean ± standard deviation ($n \geq 4$) (*, $p < 0.05$ vs 25 kDa bPEI).

the inversion point (~0 mV) corresponding to a spike in size distribution profiles principally due to polyplex aggregation.[8] Noteworthy, the aforementioned differences in DNA complexation ability between the couple of derivatives **1** and **2** (Figure 2C

dx.doi.org/10.1021/bc4003635 | *Bioconjugate Chem.* 2013, 24, 1928–1936

and D, respectively) and that of unconjugated PAMAM G4 and the PAMAM G4−neomycin conjugate **3** (Figure 2B and E, respectively) was confirmed by the displacement of the inversion points toward higher N/P. However, PAMAM G4 and all the derivatives at N/P > 5 showed similar characteristics ($D_H \sim 150-200$ nm; ζ-potential ~30−40 mV), comparable to those of the gold standard polymeric transfectant 25 kDa bPEI (Figure 2F). Polyplexes (N/P 15 for PAMAM G4 and PAMAM G4 derivatives; N/P 10 for 25 kDa bPEI) were also characterized by AFM. Submicrometric structures with globular shape together with some bigger aggregates were observed for all the transfectants (Figure S13, Supporting Information), in accordance with the results obtained by DLS.

Nevertheless, as the effectiveness of shielding the DNA is likewise recognized as a poor indicator of optimal transfection effectiveness,[8] we investigated the transfection efficiency and the cytotoxicity of each PAMAM G4 derivative/pGL3 polyplex at varying N/P.

***In Vitro* Transfection and Cytotoxicity of pDNA/PAMAM G4−Aminoglycosides.** It is now widely accepted that nanoparticles, and among them polyplexes and lipoplexes, when dispersed in biological fluids (plasma or otherwise) do associate with a range of biopolymers, especially proteins, that organize into a dynamic structure called protein corona that deeply influence their behavior.[32,33] Although serum has often been reported to adversely impact transgene expression,[26] mammalian cells do require serum for their maintenance in culture. Initial transfection experiments were thus carried out in complete medium, on HeLa, Cos-7, and U87-MG cell lines. The transfection efficiency was assessed with a luciferase reporter system, as this is a more sensitive method than GFP evaluation allowing us to distinguish between less and more active transfectants,[34] while eventual cytotoxicity was assayed with AlamarBlue cell viability assay. As expected, the extent of transfection for each PAMAM G4 derivative was dramatically affected by the N/P ratio, as shown in Figure 3 for HeLa cells and in Figure S14 and S15 (Supporting Information) for Cos-7 and U87-MG cells, respectively. Interestingly, the transfection profiles of the PAMAM derivatives **1**−**3** as a function of the N/P were different and cell-specific, making it impossible to find a common optimal N/P. Instead, their *in vitro* toxicity always increased proportionally to the N/P used. Although for PAMAM G4-derivatives the increase in a certain range of concentrations (i.e., N/P) enhanced transfection, this was counterbalanced without exception by a parallel rise in cytotoxicity. Generally speaking, transfection levels were the highest for Cos-7 cells but the lowest for HeLa cell line. Of note, the conjugation of paromomycin (conjugate **2**) and neomycin (conjugate **3**) to PAMAM G4 led to a dramatic increase in transfection efficiency (more than 1 order of magnitude at their optimal N/P when compared to the parent polymeric dendrimer; $p < 0.05$) in all cell lines, while the neamine derivative (conjugate **1**) displayed a significative increase in efficiency only in U87-MG cells ($p < 0.05$). Noteworthy, derivatives **2** and **3** at their optimal N/P exhibited greater transfection efficiency than the gold standard polymeric transfectant 25 kDa bPEI in two out of three cell types (HeLa and U87-MG; $p < 0.05$). Furthermore, in these conditions and in all cell lines tested, PAMAM derivatives were found to be as toxic as or even less toxic than 25 kDa bPEI.

In agreement with previous studies,[34] we found no strict association between transfection efficiencies and DNA binding abilities of transfectants. In fact, PAMAM G4−neomycin **3** derivative was generally as efficient as PAMAM G4−paromomycin **2** in transfecting cells but displayed stronger DNA binding behavior. As a mostly identical hierarchical order of transfection was observed for PAMAM G4-derivatives among different cell types, these results allowed unveiling of general structure−activity relationships (SAR).

Tremendous efforts have been undertaken to develop serum-resistant, efficient gene delivery vectors. In this scenario, several modifications to possibly counteract the inhibitory effect of serum on transfection have been reported,[15,35] such as conjugating PAMAM with biocompatible poly(ethylene glycol), poly[*N*-(2-hydroxypropyl) methacrylamide], dextran, and others, but limited success has been achieved so far. In line with this, we performed additional experiments questioning whether or not PAMAM G4-derivatives **1**−**3** were sensitive to the presence of serum in cell culture medium, in other words if they may be well-suited for *in vivo* use. We have thus evaluated the influence of serum content in culture medium on the activity of PAMAM G4-conjugates **1**−**3** and unconjugated PAMAM at a fix N/P of 15, assuming this as a good compromise between high transfection efficiency and low cytotoxicity in all cell lines tested. Therefore, the diverse cell types were challenged with polyplexes in the absence (without, wo FBS) and in the presence of increasingly high concentrations of serum in cell culture medium (10% and 50% FBS). It is noteworthy that in HeLa cells (Figure 3C), PAMAM G4-derivatives **1**−**3** gave, on average, a 2.3-fold higher transfection efficiency in complete medium (10% FBS) when compared to culture medium devoid of FBS (wo FBS) while unconjugated PAMAM G4 and 25 kDa bPEI displayed a more modest enhancement of luciferase activity (1.5- and 1.6-fold, respectively), as previously shown also by others.[15] Most interestingly, in the presence of high serum content (i.e., 50% FBS) all PAMAM G4-conjugates elicited no inhibition, but instead a significant increase, on average, by a factor 1.4 compared to serum-free conditions. In sharp contrast, unconjugated PAMAM G4 dendrimers and 25 kDa bPEI displayed a mild-to-severe decrease in transfection efficiency in 50% FBS (by 0.8- and 0.3-fold, respectively) when compared to transfections carried out in plain DMEM. In Cos-7 and U87-MG cell lines the situation was roughly the same as for HeLa cells (Figure S14 and S15 in Supporting Information), although the extent of variation was cell-dependent. Importantly, PAMAM G4-paromomycin **2** was always the best transfectant in 50% FBS ($p < 0.05$ vs all). Taken together, these data indicate that, unlike PAMAM G4 and 25 kDa bPEI, the positive sensitivity to serum is a very general feature of polyplexes based on PAMAM G4-derivatives **1**−**3** which make them particularly attractive for future *in vivo* therapeutic applications.

Although a high cationic charge density allows polymers to more avidly and productively associate with DNA into nanoscaled aggregates amenable to cellular internalization via endocytosis,[36,37] such characteristic are also known to adversely impact cell viability.[38] As the overall cytotoxicity of a given nonviral gene delivery vector depends on its inherent toxicity, namely, on its chemical structure and composition, as well as on the biophysical properties of the resulting polyplexes (i.e., their size, surface charge, and shape), we have challenged the three different cell lines with both polyplexes at N/P 15 (25 kDa bPEI at N/P 10, its optimum in transfection) and with the equivalent concentration of PAMAM G4 and PAMAM G4-derivatives **1**−**3** (Figure 3, S14 and S15). Nontreated cells were used as a positive control. The cytotoxicity of polyplexes at such N/P basically mirrored that of free polymers, probably because they were mostly not engaged in interactions with pDNA.[39] Most

important, these results highlight the inherently low cytotoxicity of free PAMAM G4-conjugates that, depending on the cell line and the derivative considered, was comparable to or even lower than that of unconjugated PAMAM G4 and/or 25 kDa bPEI.

CONCLUSION

The construction of multifunctional gene vehicles from natural functional small molecules is a promising strategy in the modern gene delivery field. In this contest, aminoglycosides provide a fascinating source and have been exploited for the synthesis of lipids[17,18] and hyperbranched polymers[25,26] with interesting results either *in vitro* and *in vivo*. Herein, aminoglycosides such as neamine, paromomycin, and neomycin have been used to functionalize the periphery of PAMAM G4 dendrimer, providing the formation of a novel class of oligomeric aminoglycosides. The degree of grafting, which depends on the steric hindrance of the aminoglycoside, has been determined by ^{1}H NMR and MALDI spectroscopy. The resulting multifunctional gene vectors possess enhanced transfection efficiency compared to PAMAM G4 dendrimers and low cytotoxicity. Moreover, unlike PAMAM G4 and gold standard 25 kDa bPEI, the transfection efficiency of these conjugates is not affected by the presence of serum which makes them particularly attractive for future *in vivo* therapeutic applications. These results all disclosed aminoglycosides as suitable functionalities for tailoring safe and efficient multifunctional gene delivery vectors. Moreover, since guanidinoglycosides, a family of synthetic carriers made by replacing the amino moieties of aminoglycosides with guanidino groups, are able to selectively mediate the cellular uptake of macromolecules in a heparan sulfate-dependent manner,[19−23] it might be very interesting to study the properties of related oligomeric PAMAM G4−guanidino conjugates either in terms of cellular uptake and gene delivery properties. These issues, as well as the antibiotic activity of PAMAM−aminoglycoside conjugates, are currently being investigated in our laboratories.

ASSOCIATED CONTENT

Supporting Information

Scheme of the synthesis of PAMAM G4-neamine conjugate **1** and -neomycin conjugate **3**. Copies of the ^{1}H and ^{13}C NMR spectra for all new compounds and MALDI spectra of PAMAM-G4 and the PAPAM G4-paromomycin and PAMAM G4-neomycin conjugates. AFM images of polyplexes prepared with PAMAM G4, PAMAM G4-derivatives and 25 kDa bPEI. Transfection efficiency and cytotoxicity of PAMAM G4 and PAMAM G4-derivatives in Cos-7 and U87-MG cells. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: gabriele.candiani@polimi.it.

*E-mail: alessandro.volonterio@polimi.it. Phone: +39-02-23993139. Fax: +39-02-23993180.

Author Contributions

Alessandra Ghilardi and Daniele Pezzoli equally contributed to the presented work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Politecnico di Milano (FARB-Finanziamento di Ateneo per la Ricerca di Base, to G.C. and A.V.) and by the Italian Ministry of Education, University and Research (MIUR-FIRB, grant number: RBFR08XH0H, to G.C.). We would like to thank Mr. Pietro Suraci and Mr. Carlo Enrico Scaietta for technical support and Dr. Luca Magagnin for providing AFM images.

REFERENCES

(1) Rainov, N. G., and Ren, H. (2003) Clinical trials with retrovirus mediated gene therapy–what have we learned? *J. Neurooncol. 65*, 227−36.

(2) Bartel, M. A., Weinstein, J. R., and Schaffer, D. V. (2012) Directed evolution of novel adeno-associated viruses for therapeutic gene delivery. *Gene Ther. 19*, 694−700.

(3) Thomas, C. E., Ehrhardt, A., and Kay, M. A. (2003) Progress and problems with the use of viral vectors for gene therapy. *Nat. Rev. Genet. 4*, 346−358.

(4) Cavazzana-Calvo, M., Thrasher, A., and Mavilio, F. (2004) The future of gene therapy. *Nature 427*, 779−781.

(5) Mintzer, M. A., and Simanek, E. E. (2009) Nonviral vectors for gene delivery. *Chem. Rev. 109*, 259−302.

(6) Pezzoli, D., and 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*, 1−27.

(7) Samal, S. K., Dash, M., Van Vlierberghe, S., Kaplan, D. L., Chiellini, E., van Blitterswijk, C., Moroni, L., and Dubruel, P. (2012) Cationic polymers and their therapeutic potential. *Chem. Soc. Rev. 41*, 7147−7194.

(8) Pezzoli, D., Olimpieri, F., Malloggi, C., Bertini, S., Volonterio, A., and Candiani, G. (2012) Chitosan-graft-branched polyethylenimine copolymers: influence of degree of grafting on transfection behavior. *PLoS One 7*, e34711.

(9) Labas, R., Beilvert, F., Barteau, B., David, S., Chevre, R., and Pitard, B. (2010) Nature as a source of inspiration for cationic lipid synthesis. *Genetica 138*, 153−168.

(10) Gharwan, H., Wightman, L., Kircheis, R., Wagner, E., and Zatloukal, K. (2003) Nonviral gene transfer into fetal mouse livers (a comparison between the cationic polymer PEI and naked DNA). *Gene Ther. 10*, 810−817.

(11) Park, T. G., Jeong, J. H., and Kim, S. W. (2006) Current status of polymeric gene delivery systems. *Adv. Drug Delivery Rev. 58*, 467−486.

(12) Jager, M., Schubert, S., Ochrimenko, S., Fischer, D., and Schubert, U. S. (2012) Branched and linear poly(ethylene imine)-based conjugates: synthetic modification, characterization, and application. *Chem. Soc. Rev. 41*, 4755−4767.

(13) Medina, S. H., and El-Sayed, M. E. H. (2009) Dendrimers as carriers for delivery of chemotherapeutic agents. *Chem. Rev. (Washington, DC, U. S.) 109*, 3141−3157.

(14) Kono, K., Akiyama, H., Takahashi, T., Takagishi, T., and Harada, A. (2005) Transfection activity of polyamidoamine dendrimers having hydrophobic amino acid residues in the periphery. *Bioconjugate Chem. 16*, 208−214.

(15) Choi, J. S., Nam, K., Park, J., Kim, J. B., Lee, J. K., and Park, J. (2004) Enhanced transfection efficiency of PAMAM dendrimer by surface modification with L-arginine. *J. Controlled Release 99*, 445−456.

(16) Yu, G. S., Bae, Y. M., Choi, H., Kong, B., Choi, I. S., and Choi, J. S. (2011) Synthesis of PAMAM dendrimer derivatives with enhanced buffering capacity and remarkable gene transfection efficiency. *Bioconjugate Chem. 22*, 1046−1055.

(17) Sainlos, M., Hauchecorne, M., Oudrhiri, N., Zertal-Zidani, S., Aissaoui, A., Vigneron, J. P., Lehn, J. M., and Lehn, P. (2005) Kanamycin A-derived cationic lipids as vectors for gene transfection. *ChemBioChem 6*, 1023−1033.

(18) Mevel, M., Sainlos, M., Chatin, B., Oudrhiri, N., Hauchecorne, M., Lambert, O., Vigneron, J. P., Lehn, P., Pitard, B., and Lehn, J. M. (2012)

Paromomycin and neomycin B derived cationic lipids: Synthesis and transfection studies. *J. Controlled Release 158*, 461−469.
(19) Luedtke, N. W., Baker, T. J., Goodman, M., and Tor, Y. (2000) Guanidinoglycosides: A novel family of RNA ligands. *J. Am. Chem. Soc. 122*, 12035−12036.
(20) Luedtke, N. W., Carmichael, P., and Tor, Y. (2003) Cellular uptake of aminoglycosides, guanidinoglycosides, and poly-arginine. *J. Am. Chem. Soc. 125*, 12374−12375.
(21) Elson-Schwab, L., Garner, O. B., Schuksz, M., Crawford, B. E., Esko, J. D., and Tor, Y. (2007) Guanidinylated neomycin delivers large, bioactive cargo into cells through a heparan sulfate-dependent pathway. *J. Biol. Chem. 282*, 13585−13591.
(22) Sarrazin, S., Wilson, B., Sly, W. S., Tor, Y., and Esko, J. D. (2010) Guanidinylated neomycin mediates heparan sulfate-dependent transport of active enzymes to lysosomes. *Mol. Ther. 18*, 1268−1274.
(23) Dix, A. V., Fischer, L., Sarrazin, S., Redgate, C. P. H., Esko, J. D., and Tor, Y. (2010) Cooperative, heparan sulfate-dependent cellular uptake of dimeric guanidinoglycosides. *ChemBioChem 11*, 2302−2310.
(24) Zhou, J., Wang, G., Zhang, L. H., and Ye, X. S. (2007) Modifications of aminoglycoside antibiotics targeting RNA. *Med. Res. Rev. 27*, 279−316.
(25) Chen, M. S., Wu, J. L., Zhou, L. Z., Jin, C. Y., Tu, C. L., Zhu, B. S., Wu, F. A., Zhu, Q., Zhu, X. Y., and Yan, D. Y. (2011) Hyperbranched glycoconjugated polymer from natural small molecule kanamycin as a safe and efficient gene vector. *Polym. Chem. 2*, 2674−2682.
(26) Chen, M. S., Hu, M., Wang, D. L., Wang, G. J., Zhu, X. Y., Yan, D. Y., and Sun, J. (2012) Multifunctional hyperbranched glycoconjugated polymers based on natural aminoglycosides. *Bioconjugate Chem. 23*, 1189−1199.
(27) Park, W. K. C., Auer, M., Jaksche, H., and 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.
(28) Behr, J. P. (1997) The proton sponge: A trick to enter cells the viruses did not exploit. *Chimia 51*, 34−36.
(29) Pezzoli, D., Chiesa, R., De Nardo, L., and Candiani, G. (2012) We still have a long way to go to effectively deliver genes! *J. Appl. Biomater. Funct. Mater. 10*, 82−91.
(30) Arya, D. P. (2011) New approaches toward recognition of nucleic acid triple helices. *Acc. Chem. Res. 44*, 134−146.
(31) van Gaal, E. V. B., van Eijk, R., Oosting, R. S., Kok, R. J., Hennink, W. E., Crommelin, D. J. A., and Mastrobattista, E. (2011) How to screen non-viral gene delivery systems in vitro? *J. Controlled Release 154*, 218−232.
(32) Ganta, S., Devalapally, H., Shahiwala, A., and Amiji, M. (2008) A review of stimuli-responsive nanocarriers for drug and gene delivery. *J. Controlled Release 126*, 187−204.
(33) Pezzoli, D., Zanda, M., Chiesa, R., and Candiani, G. (2013) The yin of exofacial protein sulfhydryls and the yang of intracellular glutathione in in vitro transfection with SS14 bioreducible lipoplexes. *J. Controlled Release 165*, 44−53.
(34) Le Gall, T., Baussanne, I., Halder, S., Carmoy, N., Montier, T., Lehn, P., and Decout, J. L. (2009) Synthesis and transfection properties of a series of lipidic neamine derivatives. *Bioconjugate Chem. 20*, 2032−2046.
(35) Wu, H. M., Pan, S. R., Chen, M. W., Wu, Y., Wang, C., Wen, Y. T., Zeng, X., and Wu, C. B. (2011) A serum-resistant polyamidoamine-based polypeptide dendrimer for gene transfection. *Biomaterials 32*, 1619−1634.
(36) Rejman, J., Bragonzi, A., and Conese, M. (2005) Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. *Mol. Ther. 12*, 468−474.
(37) Rejman, J., Conese, M., and Hoekstra, D. (2006) Gene transfer by means of lipo- and polyplexes: Role of clathrin and caveolae-mediated endocytosis. *J. Liposome Res. 16*, 237−247.
(38) Putnam, D., Gentry, C. A., Pack, D. W., and Langer, R. (2001) Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. *Proc. Natl. Acad. Sci. U. S. A. 98*, 1200−1205.
(39) van de Wetering, P., Moret, E. E., Schuurmans-Nieuwenbroek, N. M. E., van Steenbergen, M. J., and Hennink, W. E. (1999) Structure-activity relationships of water-soluble cationic methacrylate/methacrylamide polymers for nonviral gene delivery. *Bioconjugate Chem. 10*, 589−597.