Exploring the Efficiency of Gallic Acid-based Dendrimers and their Block Copolymers with PEG as Gene Carriers

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Abstract

The synthesis of a new family of amino-functionalized gallic acid-triethylene glycol (GATG) dendrimers and their block copolymers with poly (ethylene glycol) (PEG) has recently being disclosed. Additionally these dendrimers have shown potential for gene delivery applications, as they efficiently complex nucleic acids and form small and homogeneous dendriplexes. On this basis, the present study was aimed to explore the interaction of the engineered dendriplexes with blood components, as well as their stability, cytotoxicity, and ability to enter and transfect mammalian cells. Results show that GATG dendrimers can form stable dendriplexes, protect the associated pDNA from degradation, and are biocompatible with HEK293T cells and erythrocytes. More importantly, dendriplexes are effectively internalized by HEK293T cells, which are successfully transfected. Besides, the presence of PEG residues strongly affects these properties. While PEGylated GATG dendrimers have improved biocompatibility, the long PEG chains limit their uptake by HEK293T cells and thus their ability to transfect them. As a consequence, the degree of PEGylation in dendriplexes containing dendrimer/block copolymer mixtures emerges as an important parameter to be conveniently modulated in order to obtain an optimized stealth formulation able to effectively induce the expression of the encoded protein.

Key Words: Dendrimers, PEG, Gene delivery, in vitro, HEK293

INTRODUCTION

Relevant scientific breakthroughs in the genomics field and the understanding of the role of genes in disease explain the importance of gene therapy as a promising strategy for treating inherited and acquired diseases [1]. The most efficient gene vectors to date are viral carriers. However concerns such as host immune responses, residual pathogenicity, and potential induction of neoplasic growth following insertional mutagenesis, have led to the exploration of alternative non-viral transfer systems. Consequently, the design of new and safer synthetic delivery systems has been identified as a critical step to move gene therapy forward into the clinic [2].

In the last decades important advances have been made towards the rational design of optimized non-viral vectors for gene delivery. It is well known that the success of gene nanomedicines is strongly limited by a number of barriers that need to be conveniently overcome: nanocarriers must be stable in the systemic circulation, escape the mononuclear phagocytic system, leave the capillary vessels, reach the tissues, overpass the extracellular matrix and enter the target cells to deliver the associated nucleic acids to the cell nuclei in order to express the protein of interest [3].

Among the different materials employed for the engineering of nanocarriers, dendrimers have unique molecular architectures and properties that make them attractive candidates in gene therapy [4, 5]. Dendrimers are synthetic tree-like macromolecules constituted of repetitive layers of branching units that are prepared in a controlled iterative fashion, through generations with discrete properties. They are characterized by well-defined structures of nil dispersity. Additionally, their inherent multivalency allows for the controlled display of specific ligands, drugs, targeting and imaging agents at the periphery and internal groups, providing a high versatility for drug and gene delivery applications, and other biomedical applications [4-8]. Two main families of commercially available dendrimers,

namely poly (amido amine) (PAMAM) and poly (propylene imine) (PPI) dendrimers, currently dominate the field of gene therapy [4, 5]. Despite of this, the toxicity of cationic compounds is still a concern. This is particularly true for cationic dendrimers as they typically display toxicity in a generation-dependent manner, as a consequence of a larger number of amino groups [9, 10]. A common strategy for increasing the biocompatibility of cationic carriers has traditionally relied on the shielding of the positive charge with hydrophilic polymers such as poly (ethylene glycol) (PEG), which has lead to the formation of stealth vectors. Stealth vectors are characterized by lower toxicities, enhanced solubility, and longer circulation times in the blood stream [11-13].

The preparation of gallic acid-triethylene glycol (GATG) dendrimers, a dendritic family comprising a gallic acid core and triethylene glycol spacer arms, and their block copolymers with PEG (PEG-GATG), has recently being disclosed [14-16]. The presence of peripheral azides on GATG dendrimers has allowed their straightforward functionalization by means of the Cu (I) catalyzed azide alkyne cycloaddition (CuAAC). The resulting dendrimers have emerged as interesting tools in the study of the multivalent carbohydrate-receptor interaction, the dynamics of dendrimers, and the preparation of polyion complex (PIC) micelles [17-19]. More recently, GATG-based contrast agents for magnetic resonance imaging (MRI) and as inhibitors of the dimerization of the capsid protein (CA) of HIV-1 have been also reported [20, 21].

In a recent study, the ability of amino terminated GATG and PEG-GATG dendrimers (PEG5000) (Figure 1) to complex plasmid DNA (pDNA) has been explored [22]. As a result of this work, it was shown that the GATG dendritic architecture (dendrimer generation and PEGylation) determines the physicochemical properties of the resulting dendriplexes, offering a great opportunity for fine-tuning the requirements for specific gene therapy applications. In addition, these dendriplexes were reported as core-shell nanostructures with sterically induced stoichiometry (SIS) [23], where the relative size

between the condensed pDNA at the core and the shell of dendrimers limits the core-shell stoichiometry by steric reasons. With these premises in mind, the objective of this work has been to investigate the potential of GATG and PEG-GATG dendrimers of generation 2 and 3 (G2 and G3) as synthetic gene carriers. To this end, we have systematically explored their behaviour and that of the dendriplexes resulting upon pDNA complexation, under simulated physiological conditions. Results concerning their biocompatibility, stability, and ability to transfect cells from human origin, are herein reported.

[*Insert Figure 1*]

MATERIALS AND METHODS

1. Chemicals

Plasmid DNA (pDNA) encoding green fluorescent protein (pEGFP-C1) and driven by a CMV promoter, was purchased from Elim Biopharmaceuticals (USA). pDNA encoding secreted alkaline phosphatase (pSEAP) based on the gWiz™ high-expression vector system, was purchased from Aldevron (USA). HEPES, PBS, ethidium monoazide (EMA), ethidium bromide, DNase I, dextran sulphate, and heparin were all supplied by Sigma-Aldrich (Spain). Sodium hyaluronate (170KDa) was a gift from Bioibérica (Spain). Phalloidin Bodipy® 650/665 was purchased to Molecular Probes, Invitrogen (USA). One KBp DNA ladder was obtained from Life Technologies (Spain). All other solvents and chemicals were of the highest commercial grade available. Amino-terminated GATG and PEG-GATG dendrimers of generation G2 and G3 were prepared and characterized as previously reported [15, 22].

2. Preparation and characterization of dendriplexes

The precise conditions for efficient complexation of pDNA with the GATG dendrimers and the PEGylated GATG dendrimers of generation 2 and 3 (G2, PEG-G2, G3 and PEG-G3), as well as an exhaustive physicochemical characterization of the resulting dendriplexes, were recently reported by Raviña et al [22]. In this work, we prepared dendriplexes at N/P charge ratios from 1/1 to 40/1, upon addition of 240 µL of the GATG dendrimer aqueous solution to 40 µL of the pDNA solution (175 µg/mL in HEPES Buffer Saline 50 mM, pH 7.4 (HBS)). The final pDNA concentration in the dendriplex was 25 µg/mL. Dendrimer concentration was varied in order to achieve the desired charge ratio (N/P). The formation of dendriplexes was verified by measuring their size by Photon Correlation Spectroscopy (Zetasizer® 3000HS Malvern Instruments, UK) and their superficial charge by Lasser Dopper

Anemometry (Zetasizer Nanoseries Nano-ZS, Malvern Instruments, England). Three independent experiments were performed.

3. Cytotoxicity

Human embryogenic kidney cells (HEK293T) were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively) (Sigma-Aldrich, Spain), and maintained at 37 ºC in a 5% CO_2 humidified atmosphere. Cells were seeded at a density of 6 x 10^4 cells per well into 96 multiwell culture plates (Costar, Cambridge, UK) 24 h before starting the experiment. The culture medium was then removed, and replaced with 100 µL of HBSS per well (Sigma Aldrich, Spain). Increasing concentrations of a suspension of GATG or PEG-GATG dendrimer, or the corresponding dendriplexes (N/P 40/1), were incubated with the cells for 5 h at 37 ºC. The compounds were then removed, and the cell viability determined by the colorimetric MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymetoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Promega Corp., USA). Six replicates per concentration were assayed in each of four independent experiments performed.

4. Interaction of the dendriplexes with red blood cells

4.1. Isolation of erythrocytes

Fresh blood from rat was collected and immediately mixed with sodium citrate to a final concentration of 25 mM. Erythrocytes were obtained after multiple rounds of centrifugation in cold phosphate buffered saline (PBS), until the supernatant became clear (1000 g at 4 ^{o}C for 10 min). Eventually, a 1 % or 3 % (w/v) suspension of erythrocytes was prepared.

4.2. Haemolysis assay

200 µL of the suspension of erythrocytes (3% w/v) were mixed with increasing volumes of dendriplexes (from 25 to 200 µL; GATG /pDNA, N/P 40/1) and the final volumes adjusted to 400 µL to maintain the dilution factor constant. To ensure iso-osmolarity, PBS 10x was added to reach a final concentration of PBS 1x. After 1 h of incubation at 37 °C, the cells were removed by centrifugation and the supernatant analysed spectrophotometrically for haemoglobin release (λ=540 nm). As controls, PBS 1x (negative haemolysis, i.e. 0% haemoglobin release) or 2% Triton X-100 solution (positive haemolysis, i.e. 100% haemoglobin release) were used. Three independent experiments were performed in triplicate.

4.3. Erythrocyte aggregation assay

200 µL of the suspension of erythrocytes (1% w/v) were mixed with increasing volumes of dendriplexes (200 µL, 100 µL, or 25 µL; GATG /pDNA N/P 40/1) and the final volumes were adjusted to 400 µL to maintain the dilution factor constant. To ensure iso-osmolarity, PBS 10x was added to reach a final concentration of PBS 1x. Dendriplexes were incubated with the cells during 1 h at 37 °C in 24-well plates (Nunc, Rochester, NY, USA). To determine the degree of aggregation, images of each well were taken in triplicate (40x magnification) using a Nikon video camera attached to an Eclipse TE-2000 inverted microscope (Nikon). Three independent experiments were performed.

5. Stability of the complexes under mimicked physiological conditions.

5.1. Interaction of the dendriplexes with highly charged polyanions

Condensation and relaxation experiments were carried out to determine 1) the ability of GATG dendrimers to efficiently condense the pDNA and 2) the strength of the interaction in the presence of highly charged polyanions, such as glycosaminoglycans (GAGs). Condensation assays were performed in 96 well plates (0.6 µg of pDNA per well). The ability of the dendrimers to condense the pDNA was

calculated as relative to the maximum fluorescent signal, obtained when the ethidium bromide (EtBr, 5 μg/mL) was incubated with naked pDNA (100 % EtBr accessibility) (Perkin-Elmer LS 50 B, USA; λem=300 nm; λex=600 nm). For the relaxation experiments, a solution of the negatively charged polymers dextran sulfate, heparin or hyaluronan, was added to the complexes. The number of negative groups of the anionic polymers was in all cases fixed as 3-fold in excess to the negative groups of the complexed pDNA [24]. The fluorescent signal corresponding to the EtBr bounded to the displaced pDNA was measured after an incubation period of 2 h. Three independent experiments were performed. Statistical analyses were performed using the software Origin®. One-way ANOVA was followed by Bonferroni post hoc test, and values of $P < 0.05$ were considered statistically significant.

5.2. Incubation with endonucleases.

DNase I was incubated with the dendriplexes GATG/pDNA N/P 40/1 (1 unit of DNase per 1 μg of pDNA) during 5, 30, and 60 min at 37 ºC, under horizontal shaking. After incubation, the samples were rapidly placed on ice and the enzymatic reaction terminated by addition of 5 μL of 0.5 M EDTA. The complexed pDNA was partially dissociated from the dendriplexes by incubation with a far excess of dextran sulphate (50 mg/mL) at 37 ºC for 20 min. The topology of the displaced pDNA was eventually examined by agarose gel electrophoresis (1 % agarose gel with EtBr, 90 min at 60 V in tris-acetate-EDTA (TAE) buffer; Sub-Cell GT 96/192, Bio-Rad Laboratories Ltd., England). Unprotected naked pDNA was used as a control. Three independent experiments were performed.

6. Uptake and intracellular distribution of the dendriplexes

The pDNA was labelled with ethidium monoazide (EMA) according to the procedure described by Ruponen et al [25]. In order to determine the cellular uptake of the dendriplexes by confocal laser

scanning microscopy (CLSM), HEK293T cells were seeded at a density of 3 x 10^5 cells per well in 24 multiwell culture plates (Costar, Cambridge, UK), over sterile glass covers previously coated with Poly-L-Lysine (Sigma, Spain). Cells were grown during 24 h, and the growing medium replaced by HBSS at the beginning of the experiment. GATG/EMA-pDNA dendriplexes (N/P 40/1) were added to each well (1 μg of pDNA per well, equivalent to a dendrimer concentration of 0.13 mg/mL for G2 and G3 dendriplexes, 0.40 mg/mL for PEG-G2 dendriplexes and 0.20 mg/mL for PEG-G3 dendriplexes). After 5 h of incubation, the cells were washed with PBS and 1) fixed in freshly made paraformaldehide (4% in PBS), or 2) alternatively allowed to grow in normal conditions for 24 h more, prior fixation. The cell nuclei and the actin filaments were counterstained with DAPI and Phalloidin Bodipy® 650/665, respectively. Finally, the coverslips were mounted, and the cellular uptake and localization of the EMA-pDNA explored by confocal microscopy (Leica TCS SP2, Leica Microsystems). Three independent experiments were performed.

7. Transfection efficiency

HEK293T cells were seeded at a density of 3 x 10^5 cells per well into 24 multiwell culture plates (Costar, Cambridge, UK). Cells were grown during 24 h, and the culture medium replaced with 300 μL of HBSS before the transfection. GATG/pDNA dendriplexes were added over the cells and incubated for 5 h (1 μg of complexed pDNA/well, equivalent to a dendrimer concentration of 0.13 mg/mL for G2 and G3 dendriplexes, 0.40 mg/mL for PEG-G2 dendriplexes and 0.20 mg/mL for PEG-G3 dendriplexes for dendriplexes prepared at a charge ratio N/P 40/1, and proportionally lower for dendriplexes prepared at charge ratios < N/P 40/1). The cells were subsequently washed, and allowed to grow under normal conditions. When cells were transfected with pEGFP, the gene expression was evaluated with a fluorescence microscope (Eclipse TE 2000-S, Nikon Ltd., UK) 48h post-transfection. Additionally, MTS assay (as described in section 3) was used to assess the viability of the cells at this time point. In

the case of transfection with gWiz™-pSEAP, samples of the culture medium were collected after 2, 4, or 6 days post-transfection and SEAP expression was quantified using the Great EscAPe^{TM} SEAP Reporter System Kit protocol (BD Biosciences Clontech, USA) and a fluorescence plate reader (Perkin-Elmer LS 50 B, USA). The cells were continuously imaged under the optical microscope for assessing that the cell density and morphology were comparable to the untreated control cells. Three replicates per condition were assayed in each of three independent experiments performed. Statistical analyses were performed using the software Origin®. One-way ANOVA was followed by Bonferroni post hoc test, and values of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

1. Association of pDNA to GATG dendrimers and characterization of the resulting dendriplexes

Dendriplexes within a nanometric size, a narrow distribution and a positive zeta potential were obtained at the assayed N/P charge ratios, as previously reported by our research groups [22]. In agreement with those results, G2 and G3 dendriplexes could be obtained from charge ratios N/P 10/1 and N/P 2/1, respectively, having a small size (< 100nm) that was not influenced by either the charge ratio or the dendrimer generation, in agreement with their SIS nature. Bigger dendriplexes (> 100nm) were obtained in the case of PEGylated dendriplexes, due to the known interference of PEG with the formation of compact condensates [26, 27] and the PEG creating a palisade around the dendriplex core [28] (Figure 2A). With respect to the zeta potential of the dendriplexes, an inversion from negative to positive values was observed as the charge ratio N/P increased. The negative values of zeta potential are attributed to the presence of uncondensed pDNA. After complete condensation, dendriplexes had a positive zeta potential, with larger N/P having little effect on charge. Non-PEGylated dendriplexes showed a higher surface charge compared to PEGylated dendriplexes. Generation-dependent charge neutralization was only observed in the case of the PEGylated dendrimers, a result that has been interpreted again as resulting from the SIS nature of these structures (Figure 2B).

[*Insert Figure 2*]

In general, the properties of the resulting dendriplexes are adequate for gene therapy applications intended to reach distal organs or solid tumours. It is known that a small size exerts a positive effect on their ability to travel through capillaries and extravasate [29]. With respect to the surface properties, a

slightly positive charge will favour their interaction with negative charged cell surfaces, while the presence of PEG is meant to increase their circulation time and minimize their interaction with erythrocytes [12, 13].

2. Cellular toxicity of GATG dendrimers

Toxicity can be a major limitation for the use of new cationic dendrimers in gene delivery [9]. For this reason the cellular toxicity of the amino-terminated GATG dendrimers was evaluated in a human derived cell line, HEK293T cells, as a crucial step before exploring their efficacy as synthetic pDNA nanocarriers. The cells were incubated with the GATG and the PEGylated block copolymers up to a concentration of 5mg/mL, being that their solubility limit in aqueous-based media. As seen in Figure 3A, the toxicity attributed to the dendrimers is concentration dependent. For the non-PEGylated G2 and G3 dendrimers a decrease on cell viability below 50% of cell survival was only appreciated for concentrations above 2.5mg/mL, which is particularly good in relation to toxicities reported for other well-known families of cationic dendrimers [9, 10, 30]. PEGylated GATG dendrimers were found to be even less cytotoxic than the non-PEGylated dendrimers, as expected by considering the partial shielding of the cationic amino groups provided by the PEG moieties and bearing in mind previous reports in the field [11, 13]. With regards to the generation, the results were comparable for both G2/G3 and PEG-G2/PEG-G3 dendrimers.

The toxicity of the GATG/pDNA and PEG-GATG/pDNA dendriplexes was further explored following the same methodology. For that specific purpose, we decided to evaluate the toxicity of the dendriplexes formulated at the highest charge ratio among those prepared in the present work (i.e. containing the highest amount of dendrimers, which could then be presumable more toxic to the cells). As seen in Figure 3B, the cells were completely viable at the end of the study even in the case of dendriplexes prepared by non-PEGylated GATG dendrimers at a charge ratio N/P 40/1. While

dendriplexes were non-toxic, free-dendrimers at the same concentration (i.e. 1mg/mL) caused a decrease on cell viability (Figure 3A). The improved compatibility of the dendriplexes can be attributed to the fact that part of the cationic groups of the dendrimers, related to the appearance of toxic effects, are partially neutralized as they are occupied in the interaction with the negatively charged phosphate groups of the pDNA, in agreement with previous works [31].

[*Insert Figure 3*]

3. Interaction with erythrocytes: Haemolysis and hemagglutination assays

In most cases, for the practical application of in vivo gene therapies, vector systems need to be directly injected into the bloodstream. To minimize the interaction of dendriplexes with erythrocytes is particularly important, as this interaction would decrease the circulation half-life of the dendriplexes and may even cause vascular obliteration and embolism due to the formation of big aggregates [32]. These phenomena are typically related to the presence of cationic groups at the surface of nanostructures/dendriplexes that bind to the membranes of the erythrocytes. Consequently, the blood-compatibility of the dendriplexes was evaluated. For that purpose, dendriplexes were prepared at a charge ratio N/P 40/1, using the G3 GATG dendrimer as it holds the highest density of positive groups. Considering that PEGylation has been reported to diminish the interaction of DNA complexes with plasma components as a consequence of the shielding effect [33], PEG-G3/pDNA was also evaluated for comparative purposes.

The membrane-damaging capacity of the dendriplexes was evaluated by determining the percentage of haemoglobin that is released upon incubation of increasing concentrations of dendriplexes, (expressed in Table 1 as concentration/dose of pDNA) with freshly extracted red blood cells. According

to the results presented here, it is possible to state that GATG dendriplexes are highly compatible with erythrocytes. In all cases the percentages of haemoglobin released were lower than 10 %, which is regarded as safe for an intravenous administration [34, 35]. As expected, this percentage was even lower in the case of PEG-G3 dendrimers, due to the shielding of the cationic groups provided by the PEG and to a minor interaction with the red blood cell membranes.

Table 1. Percentage of haemoglobin released upon incubation of G3/pDNA or PEG-G3/pDNA dendriplexes (N/P 40/1) with red blood cells, and taking as a positive control erythrocytes incubated with 2% Triton X-100 solution (100% haemoglobin release). Different concentrations of dendriplexes were incubated with an erythrocyte suspension (200 µL) for 1 h, and the amount of haemoglobin released was quantified spectrophotometrically. Data are presented as means ± SD (*n*=3).

Final pDNA concentration (µg/mL)	% Haemoglobin released	
	G3 dendriplex	PEG-G3 dendriplex
1.6	8.1 ± 3.1	3.6 ± 1.3
3.1	9.5 ± 3.6	4.0 ± 1.8
6.5	7.2 ± 5.1	2.1 ± 3.6
9.4	7.2 ± 3.3	2.0 ± 2.6
12.5	7.6 ± 3.2	4.1 ± 2.8

In a parallel study, the degree of erythrocyte aggregation was evaluated by means of optical microscopy. Dendriplexes were incubated with the erythrocytes and, as seen in the images shown in Figure 4, small aggregates were only detected in samples incubated with G3/pDNA dendriplexes. The

degree of aggregation was dependent on the dendriplex concentration, and a higher degree of erythrocyte aggregation was observed when the pDNA concentration increased up to 6.5-12.5 μg/mL (equivalent to a final G3 dendrimer concentration of 0.23-0.45 mg/mL). As observed, even for those concentrations the PEG chains were effective in preventing the aggregation of the erythrocytes. Similar results have already been reported for other polycations such as polyethyleneimine [36]. This points out the importance of preparing stealth formulations for an intravenous mode of delivery.

[*Insert Figure 4*]

4. Stability of the dendriplexes upon interaction with polyanions

Extracellular matrix penetration is another critical process that can severely compromise the success of gene carriers. It is widely known that cationic vectors are frequently destabilized due to non-specific interactions with negatively charged components such as glycosaminoglycans (GAGs), leading to the unpacking and releasing of the pDNA before reaching the target site and consequently diminishing the efficacy of the carriers for gene transfection [25]. As GATG/pDNA and PEG-GATG/pDNA dendriplexes are held together by electrostatic interactions, it was thought that their incubation with competing highly charged polyanions would shed light on the ability of these dendrimers to overcome the extracellular matrix and successfully deliver their payload to the target.

With this aim, G3 and PEG-G3 dendriplexes were incubated with solutions of negatively charged polymers (dextran sulfate, heparin, and hyaluronan) and the accessibility to the pDNA measured by monitoring the fluorescent signal that results from the binding of EtBr to the DNA. When the pDNA is condensed into dendriplexes its accessibility is severely hampered, and the binding of EtBr and fluorescent signal consequently reduced. Conversely, if the extracellular soluble GAGs were to displace

the pDNA, a subsequent increase in the fluorescence signal would be observed [24]. Naked pDNA (pDNA fully accessible, giving the highest fluorescent signal when incubated with EtBr) was used as a positive control.

In agreement with previous experiments, we first explored the stability of dendriplexes (G3/pDNA and PEG-G3/pDNA) prepared at a charge ratio N/P 40/1. These dendriplexes remained completely stable. Subsequently, complexes prepared at lower charge ratios were also analysed. Figure 5 shows that larger N/P charge ratios than those required for complete pDNA condensation (black columns: 2/1 for G3/pDNA and 5/1 for PEG-G3/pDNA dendriplexes) are necessary in order to prevent the displacement of the pDNA upon incubation with a variety of polyanions. Overall it can be concluded that, irrespective of the PEGylation, no important pDNA release occurs after interaction of GATG/pDNA dendriplexes with competing polyanions when the dendriplexes are prepared at charge ratios above those required for pDNA condensation.

[*Insert Figure 5*]

5. Endonucleases protection assay

The protection of nucleic acids from endogenous nucleases is a crucial parameter in gene delivery as pDNA is rapidly degraded in the presence of serum (within 5 min after i.v. injection) [37]. In order to evaluate the ability of GATG and PEG-GATG dendrimers to protect the associated pDNA, G3 or PEG-G3 dendriplexes (N/P 40/1) were incubated during 5, 30, or 60 min with the enzyme DNase I. Subsequently, to analyze the pDNA topology and integrity, the complexed pDNA was displaced from the union to the dendrimers and samples were analysed in an agarose electrophoresis gel (Figure 6).

[*Insert Figure 6*]

Naked pDNA, fully accessible to DNases, was completely degraded after 5 min of incubation with DNase I. In the case of the pDNA associated to the G3 dendrimers it was protected from DNase I degradation and most of it remained in the form of dendriplex, as it can be detected from the dendriplex signal in the wells of the gel. Moreover, the pDNA was only moderately converted to the open circular form, which indeed is considered to be an active form able to effectively transfect cells [38]. Some smear only appears at the bottom of the gel after long incubation times, 60min. In contrast, PEGylated dendrimers were not able to protect the associated pDNA from degradation during long periods of time. Thus, although an important amount of the pDNA remained in the active form after 5 min incubation, pDNA was completely degraded after 30 min. This fact may be related to the interference of the PEG chains with the formation of compact condensates being permissive for the DNases accessing the pDNA [26, 27]. Similar results were previously observed for other PEGylated polycations, such as PEGylated poly-L-lysine (PEG-PLL) [39]. Interestingly in this particular example, and besides the limited capacity of the PEG-PLL to protect the pDNA, the transfection efficiencies after systemic administration to mice were superior to those reported for the non-PEGylated carriers, due to a facilitated release of the associated pDNA upon entering the cells [39].

6. Cellular uptake of the GATG dendriplexes

The interaction of dendrimers with lipid bilayers is known to be mediated by both electrostatic interactions and hydrophobic forces [40]. Within this premise cationic dendrimers have extensively been functionalized with lipid residues to further enhance their transit across cell membranes [41, 42]. In the particular case of cationic GATG dendrimers, they naturally present hydrophobic gallic acid groups in their structure, and are therefore expected to easily enter mammalian cells.

To track the complexed pDNA, it was fluorescently labelled with ethidium monoazide bromide (EMA-pDNA). Upon incubation of the dendriplexes with HEK293T cells for 5 h, the cells were visualized with the confocal microscope. As it can be seen in the following images (Figure 7A), green fluorescence corresponding to the labelled EMA-pDNA complexed with G3 dendrimers was observed all over the cells. In contrast, the EMA-pDNA was not able to enter the cells (undetectable green fluorescence) upon complexation with PEG-G3 dendrimers (Figure 7B). The reason for this lack of internalization could be the steric hindrance caused by the long and abundant PEG chains hampering the interaction of the PEGylated dendriplexes with the cell surface. This fact also explains the high biocompatibility reported for PEGylated dendriplexes (section 2). As described in our previous paper [22] the PEG is creating a palisade around the dendriplex core. The negative effects of PEGylation for PEGylated nanostructures to enter the cells have been previously described by Mishra et al [43]. In our case, this negative effect was efficiently counterbalanced by tuning the molar ratio in GATG/PEG-GATG mixtures, as will be described in the following section.

With respect to the intracellular distribution of the labelled EMA-pDNA, it was mainly located on the cell cytoplasm after short incubation times (5h) (Figure 7A). Encouragingly, after longer incubation times (24 h) the green signal corresponding to the EMA-pDNA was already distributed around and into the cell nuclei (Figure 7C). Thus, it looks apparent that the G3 dendrimers not only facilitate the uptake of the pDNA but also promotes its access to the cell nuclei for transcription.

[*Insert Figure 7*]

7. Transfection efficiency of GATG dendriplexes

To evaluate the transfection efficiency, dendriplexes were prepared with a model plasmid that encodes the green fluorescent protein (pEGFP). G3/pEGFP and PEG-G3/pEGFP dendriplexes (N/P 40/1)

were incubated with HEK293T cells for 5h, removed, and the cells were allowed to grow in normal conditions for 48 h. EGFP expression was then determined by fluorescence microscopy and complete cell viability was confirmed with the MTS assay. While G3/pEGFP dendriplexes rendered detectable levels of gene expression (Figure 8A), PEG-G3/pEGFP dendriplexes were unable to transfect the cells, a fact that could nevertheless be predicted based on the results of cellular uptake. In an attempt to explore if the charge ratio could have an influence on the process, dendriplexes were prepared at increased charges ratios, from N/P 1/1 to N/P 40/1. As depicted in Figure 8A, the transfection efficiency of the G3/pEGFP dendriplexes increased as the N/P did. Thus, while detectable gene expression was achieved at G3/pEGFP N/P 2/1, an efficient expression of the encoded protein was only observed for the largest N/P ratios. This pattern has already been observed for other dendrimers, and can be due to an improved pDNA complexation and/or to the presence of free GATG dendrimers [44]. For peptide and PAMAM dendrimers (and other cationic carriers) an excess of free dendrimer helps dendriplexes to enter the cells by improving their interaction and even favouring the formation of small reversible holes in the cell membranes [40, 45]. With respect to the PEG-G3 dendrimers, they failed to transfect the cells irrespective of the PEG-G3/pEGFP charge ratio (N/P). G2 and PEG-G2 dendrimers were similarly evaluated for transfection, and similar results were observed with regards to the effect of the charge ratio and PEGylation in gene expression (data not shown).

Additional experiments were performed to quantify the amount of encoded protein expressed as a function of the dose of pDNA and time of evaluation. In this particular case, the selected reporter gene was gWiz™-pSEAP (G3/pSEAP N/P 40/1). It was found that the efficacy of the transfection was proportional to the dose of pSEAP, suggesting that the process of internalization and subsequent transfection was not saturated. As seen in Figure 8B, the levels of secreted alkaline phosphatase increased over the time during the period of evaluation, showing a long-lasting transfection for up to 6 days. This effect was more remarkable for the highest dose of pDNA (4 μg).

[*Insert Figure 8*]

As reported above (section 6), uptake experiments showed the inability of PEGylated GATG/pDNA dendriplexes to enter HEK293T cells due to the interference of the PEG chains. Indeed, the density of the PEG groups on the surface of nanostructures is known to be critical for the interaction of nanostructures with biological surfaces and severely affects the transfection process [13, 42, 46-49]. Consequently, and bearing in mind the benefits of stealth formulations, we attempted to obtain dendriplexes with a variable proportion of PEG (mixtures GATG/PEG-GATG) so that their capacity to interact with mammalian cells and subsequently transfect them would be preserved. As seen in Figure 9, increasing the molar ratio of the PEGylated G3 dendrimer in the dendriplexes has a profound effect on protein expression in agreement with transfection efficiency being significantly influenced by the PEGylation degree. Indeed, the transfection efficacy of the dendriplexes severely decreased as the amount of PEG increased, with transfection efficacies comparable to the non-PEGylated dendriplexes being observed up to molar ratios PEG-G3/G3 of 1%. Further in vivo studies will be required to determine if this PEG substitution is, in the particular case of the GATG dendriplexes, enough to increase the circulation times of the dendriplexes and prevent uptake by the reticuloendothelial system after intravenous injection, since reported data for other nanostructures are very variable [34, 50, 51].

[*Insert Figure 9*]

8. Conclusions

The results presented here show the potential of amino terminated GATG dendrimers as gene carriers. GATG dendrimers can associate pDNA in a very effective manner and protect it against

nuclease degradation. Moreover, GATG dendrimers exhibit a good biocompatibility with erythrocytes and cells, especially in the case of PEGylated dendrimers. Several critical factors that affect the ability of GATG dendriplexes to transfect cells have been analysed in the present work. While dendrimer generation does not seem to affect the biological properties of GATG/pDNA dendriplexes, the density of PEGylation at the dendriplex surface has been revealed as a critical factor that needs to be carefully adjusted in order to obtain delivery systems benefiting from the stabilization and biocompatibility provided by PEG, but without compromising the transfection efficiency. The systems reported here have been shown to effectively transfect cells. Further in vivo studies are aimed to demonstrate the efficacy of this stealth formulation in animal models.

9. SUMMARY POINTS

Association of pDNA and characterization of the resulting dendriplexes

1. GATG dendrimers and the PEG block copolymers associate pDNA in a very efficient manner.
2. The capacity of complexation is dependent on the dendrimer generation and PEGylation: G3 dendrimers can complex pDNA at lower charge ratios than G2 dendrimers, while higher amounts of PEGylated dendrimers are required for complete pDNA complexation.
3. As PEG is creating a palisade around the core of the nanostructures, PEGylated dendriplexes are bigger and present lower surface charge values than non-PEGylated dendriplexes.

Biocompatibility of the dendriplexes

4. Overall, GATG dendrimers, and especially the PEGylated block copolymers, are highly biocompatible with mammalian cells irrespective of the generation (HEK293T).

5. The positive effect on biocompatibility attributed to the presence of PEG can also be observed when looking at the interaction of the dendriplexes with blood components. PEGylated dendriplexes do not induce haemolysis or hemagglutination upon incubation with erythrocytes.

Stability of the dendriplexes

6. Non-PEGylated dendriplexes resulted to be stable upon interaction with highly charged negative polymers and glycosaminoglycans, and can efficiently protect pDNA from degradation. In contrast, PEGylated dendrimers are stable but fail to protect the associated pDNA from endonuclease degradation, as pDNA remains accessible to the enzyme.

Ability of the dendriplexes to be taken up and subsequently transfect mammalian cells

7. Besides stealth dendriplexes present adequate physicochemical properties for gene delivery and are highly biocompatible, they cannot enter the cells as manifested in the uptake studies, most probably due to the long and abundant PEG chains that hamper their interaction with the cell membranes. Consequently, dendriplexes formed from the PEG block copolymers cannot transfect HEK293 cells.
8. In the case of dendriplexes prepared with non-PEGylated dendrimers, HEK293 cells successfully internalize them, and the transport of pDNA to the cell nuclei is favoured. The transfection experiments show as these dendriplexes can transfect mammalian cells in a dose dependent manner. Moreover, the transfection efficiency increases accordingly to the N/P charge ratio, in agreement with previous reports in the field.
9. Most encouragingly is the fact that by modulating the proportion of PEG block copolymers that are included in the formulations, i.e. the PEGyaltion degree, density of PEG groups, it is possible

to obtain stealth dendriplexes that render levels of protein expression similarly to non-PEGylated dendriplexes.

10. ACKNOWLEDGEMENTS

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11. REFERENCES

1. Cotrim AP, Baum BJ. Gene therapy: Some history, applications, problems, and prospects. *Toxicol. Pathol.* 36(1), 97-103 (2008).
2. Jin S, Ye K. Nanoparticle-mediated drug delivery and gene therapy. *Biotechnol. Progr.* 23(1), 32-41 (2007).
3. Rettig GR, Rice KG. Non-viral gene delivery: From the needle to the nucleus. *Expert Opin. Biol. Ther.* 7(6), 799-808 (2007).
4. **Dufes C, Uchegbu IF, Schaltzlein AG. Dendrimers in gene delivery. Adv. Drug Deliv. Rev. 57(15), 2177-2202 (2005).**

*** Recommended review on the field**

5. Raviña M, Paolicelli P, Seijo B, Sanchez A. Knocking down gene expression with dendritic vectors. *Mini Rev.Med. Chem.* 10(1), 73-86 (2010).
6. **Gillies ER, Fréchet JMJ. Dendrimers and dendritic polymers in drug delivery. *Drug Discov. Today* 10(1), 35-43 (2005).**

*** Recommended review on the field**

7. Gitsov I. Hybrid linear dendritic macromolecules: From synthesis to applications. *J. Polym. Sci. A Polym. Chem.* 46(16), 5295-5314 (2008).
8. Menjoge AR, Kannan RM, Tomalia DA. Dendrimer-based drug and imaging conjugates: design considerations for nanomedical applications. *Drug Discov. Today* 15(5-6), 171-185 (2010).
9. Duncan R, Izzo L. Dendrimer biocompatibility and toxicity. *Adv. Drug Deliv. Rev.* 57(15), 2215-2237 (2005).
10. Jain K, Kesharwani P, Gupta U, Jain NK. Dendrimer toxicity: Let's meet the challenge. *Int. J. Pharm.* 394(1-2), 122-142 (2010).
11. Jevprasesphant R, Penny J, Jalal R, Attwood D, Mckeown NB, D'Emanuele A. The influence of surface modification on the cytotoxicity of PAMAM dendrimers. *Int. J. Pharm.* 252(1-2), 263-266 (2003).
12. Ryan SM, Mantovani G, Wang X, Haddleton DM, Brayden DJ. Advances in PEGylation of important biotech molecules: Delivery aspects. *Exp. Opin. Drug Deliv.* 5(4), 371-383 (2008).
13. Yang H, Lopina ST, Dipersio LP, Schmidt SP. Stealth dendrimers for drug delivery: Correlation between PEGylation, cytocompatibility, and drug payload. *J. Mater. Sci. Med.* 19(5), 1991-1997 (2008).
14. **Amaral SP, Fernandez-Villamarin M, Correa J, Riguera R, Fernandez-Megia E. Efficient multigram synthesis of the repeating unit of gallic acid-triethylene glycol dendrimers. *Org. Lett.* 13(17), 4522-4525 (2011).**

*** Improved procedure for the multigram synthesis of the repeating unit of GATG dendrimers through an efficient and cost-effective route.**

15. Fernandez-Megia E, Correa J, Riguera R. "Clickable" PEG - Dendritic block copolymers. *Biomacromolecules* 7(11), 3104-3111 (2006).
16. **Fernandez-Megia E, Correa J, Rodriguez-Meizoso I, Riguera R. A click approach to unprotected glycodendrimers. Macromolecules 39(6), 2113-2120 (2006).**

**** Synthetic procedure and characterization of GATG dendrimers.**

17. Munoz EM, Correa J, Fernandez-Megia E, Riguera R. Probing the relevance of lectin clustering for the reliable evaluation of multivalent carbohydrate recognition. *J. Am. Chem. Soc.* 131(49), 17765-17767 (2009).

18. Novoa-Carballal R, Säwén E, Fernandez-Megia E, Correa J, Riguera R, Widmalm G. The dynamics of GATG glycodendrimers by NMR diffusion and quantitative 13C relaxation. *Phys. Chem. Chem. Phys.* 12(25), 6587-6589 (2010).
19. Sousa-Herves A, Fernandez-Megia E, Riguera R. Synthesis and supramolecular assembly of clicked anionic dendritic polymers into polyion complex micelles. *Chem. Commun.* (27), 3136-3138 (2008).
20. Doménech R, Abian O, Bocanegra R et al. Dendrimers as potential inhibitors of the dimerization of the capsid protein of HIV-1. *Biomacromolecules* 11(8), 2069-2078 (2010).
21. Fernández-Trillo F, Pacheco-Torres J, Correa J et al. Dendritic MRI contrast agents: An efficient prelabeling approach based on CuAAC. *Biomacromolecules* 12(8), 2902-2907 (2011).
22. Raviña M, de la Fuente M, Correa J et al. Core-shell dendriplexes with sterically induced stoichiometry for gene delivery. *Macromolecules* 43(17), 6953-6961 (2010).
**** Development and physicochemical characterization of GATG/pDNA complexes.**
23. Tomalia DA. Dendrons/dendrimers: Quantized, nano-element like building blocks for soft-soft and soft-hard nano-compound synthesis. *Soft Matter* 6(3), 456-474 (2010).
24. Ruponen M, Ylä-Herttuala S, Urtti A. Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: Physicochemical and transfection studies. *Biochim. Biophys. Acta* 1415(2), 331-341 (1999).
25. Ruponen M, Rönkkö S, Honkakoski P, Pelkonen J, Tammi M, Urtti A. Extracellular glycosaminoglycans modify cellular trafficking of lipoplexes and polyplexes. *J. Biol. Chem.* 276(36), 33875-33880 (2001).
*** Non-specific interactions of extracellular glycosaminoglycans with gene carriers may alter both their cellular uptake and intracellular behavior.**
26. Männistö M, Vanderkerken S, Toncheva V et al. Structure-activity relationships of poly(L-lysines): Effects of pegylation and molecular shape on physicochemical and biological properties in gene delivery. *J. Control. Release* 83(1), 169-182 (2002).
27. Rackstraw BJ, Stolnik S, Davis SS, Bignotti F, Garnett MC. Development of multicomponent DNA delivery systems based upon poly(amidoamine)-PEG co-polymers. *Biochim. Biophys. Acta* 1576(3), 269-286 (2002).
28. Putnam D, Zelikin AN, Izumrudov VA, Langer R. Polyhistidine-PEG:DNA nanocomposites for gene delivery. *Biomaterials* 24(24), 4425-4433 (2003).
29. Alexis F, Pridgen E, Molnar LK, Farokhzad OC. Factors affecting the clearance and biodistribution of polymeric nanoparticles. *Mol. Pharm.* 5(4), 505-515 (2008).
30. Shcharbin D, Pedziwiatr E, Blasiak J, Bryszewska M. How to study dendriplexes II: Transfection and cytotoxicity. *J. Control. Release* 141(2), 110-127 (2010).
31. Cherng JY, Van De Wetering P, Talsma H, Crommelin DJA, Hennink WE. Effect of size and serum proteins on transfection efficiency of poly ((2-dimethylamino)ethyl methacrylate)-plasmid nanoparticles. *Pharm. Res.* 13(7), 1038-1042 (1996).
32. Chollet P, Favrot MC, Hurbin A, Coll JL. Side-effects of a systemic injection of linear polyethylenimine-DNA complexes. *J. Gene Med.* 4(1), 84-91 (2002).
33. Ogris M, Brunner S, Schüller S, Kircheis R, Wagner E. PEGylated DNA/transferrin-PEI complexes: Reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther.* 6(4), 595-605 (1999).
34. Neu M, Germershaus O, Behe M, Kissel T. Bioreversibly crosslinked polyplexes of PEI and high molecular weight PEG show extended circulation times in vivo. *J. Control. Release* 124(1-2), 69-80 (2007).

35. Richardson SCW, Kolbe HVJ, Duncan R. Potential of low molecular mass chitosan as a DNA delivery system: Biocompatibility, body distribution and ability to complex and protect DNA. *Int. J. Pharm.* 178(2), 231-243 (1999).
36. Petersen H, Fechner PM, Martin Al et al. Polyethylenimine-graft-poly(ethylene glycol) copolymers: Influence of copolymer block structure on DNA complexation and biological activities as gene delivery system. *Bioconjug. Chem.* 13(4), 845-854 (2002).
37. Kawabata K, Takakura Y, Hashida M. The fate of plasmid DNA after intravenous injection in mice: Involvement of scavenger receptors in its hepatic uptake. *Pharm. Res.* 12(6), 825-830 (1995).
38. Adami RC, Collard WT, Gupta SA, Kwok KY, Bonadio J, Rice KG. Stability of peptide-condensed plasmid DNA formulations. *J. Pharm. Sci.* 87(6), 678-683 (1998).
39. Kwoh DY, Coffin CC, Lollo CP et al. Stabilization of poly-L-lysine/DNA polyplexes for in vivo gene delivery to the liver. *Biochim. Biophys. Acta* 1444(2), 171-190 (1999).
40. Klajnert B, Janiszewska J, Urbanczyk-Lipkowska Z, Bryszewska M, Epand RM. DSC studies on interactions between low molecular mass peptide dendrimers and model lipid membranes. *Int. J. Pharm.* 327(1-2), 145-152 (2006).
41. Bayele HK, Sakthivel T, O'donell M et al. Versatile peptide dendrimers for nucleic acid delivery. *J. Pharm. Sci.* 94(2), 446-457 (2005).
42. Takahashi T, Kono K, Itoh T, Emi N, Takagishi T. Synthesis of novel cationic lipids having polyamidoamine dendrons and their transfection activity. *Bioconjug. Chem.* 14(4), 764-773 (2003).
43. Mishra S, Webster P, Davis ME. PEGylation significantly affects cellular uptake and intracellular trafficking of non-viral gene delivery particles. *Eur. J. Cell Biol.* 83(3), 97-111 (2004).
*** PEGylation led to significantly reduced uptake and gene expression of the reporter genes with polycations.**
44. Zinselmeyer BH, Mackay SP, Schatzlein AG, Uchegbu IF. The lower-generation polypropylenimine dendrimers are effective gene-transfer agents. *Pharm. Res.* 19(7), 960-967 (2002).
45. Hong S, Leroueil PR, Janus EK et al. Interaction of polycationic polymers with supported lipid bilayers and cells: Nanoscale hole formation and enhanced membrane permeability. *Bioconjug. Chem.* 17(3), 728-734 (2006).
46. Csaba N, Köping-Höggard M, Fernandez-Megia E, Novoa-Carballal R, Riguera R, Alonso MJ. Ionically crosslinked chitosan nanoparticles as gene delivery systems: Effect of PEGylation degree on in vitro and in vivo gene transfer. *J. Biomed. Nanotechnol.* 5(2), 162-171 (2009).
*** Exploring the role of a PEG coating on the transfection properties of synthetic vectors in vitro and in vivo.**
47. Fant K, Esbjörner EK, Jenkins A, Grossel MC, Lincoln P, Nordén B. Effects of PEGylation and acetylation of PAMAM dendrimers on DNA binding, cytotoxicity and in vitro transfection efficiency. *Mol. Pharm.* 7(5), 1734-1746 (2010).
48. Singh M, Borain J, Noor-Mahomed N, Ariatti M. The effect of pegylation on the transfection activity of two homologous cationic cholesteryl cytofectins. *Af. J.Biotechnol.* 10(8), 1400-1407 (2011).
49. Zhang X, Pan SR, Hu Hm et al. Poly(ethylene glycol)-block-polyethylenimine copolymers as carriers for gene delivery: Effects of PEG molecular weight and PEGylation degree. *J. Biomed. Mat. Res. A* 84(3), 795-804 (2008).

50. Kunath K, Von Harpe A, Petersen H et al. The structure of PEG-modified poly(ethylene imines) influences biodistribution and pharmacokinetics of their complexes with NF-κB decoy in mice. *Pharm. Res.* 19(6), 810-817 (2002).
51. Sato A, Choi SW, Hirai M et al. Polymer brush-stabilized polyplex for a siRNA carrier with long circulatory half-life. *J. Control. Release* 122(3), 209-216 (2007).

Figure 1. Structure of GATG dendrimers (G2 and G3) and the block copolymers with PEG (PEG-G2 and PEG-G3)

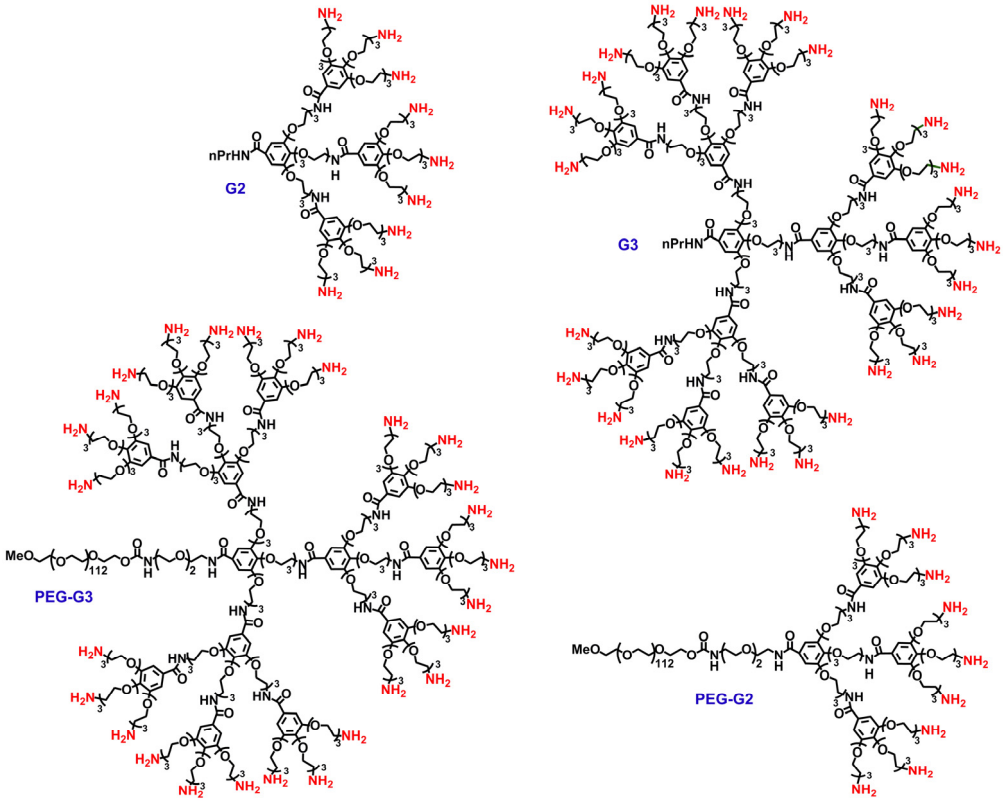
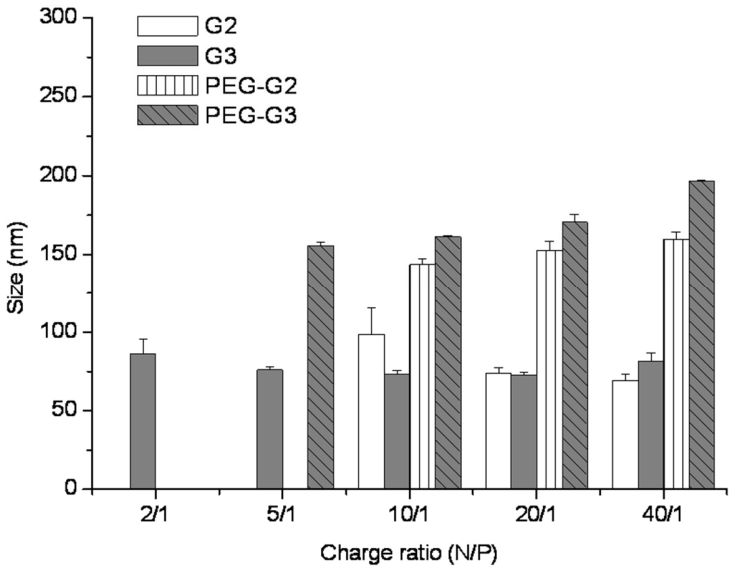


Figure 2. Size (A) and zeta potential (ζ) (B) of dendriplexes obtained upon complexation of pDNA (25μg/mL) with GATG dendrimers (G2 and G3) and the PEGylated block copolymers (PEG-G2 and PEG-G3) at increasing N/P charge ratios (means ± SD, *n*=3) ([22]). No data is given for charge ratios lower than required for complete pDNA complexation

A) Size



B) Zeta potential

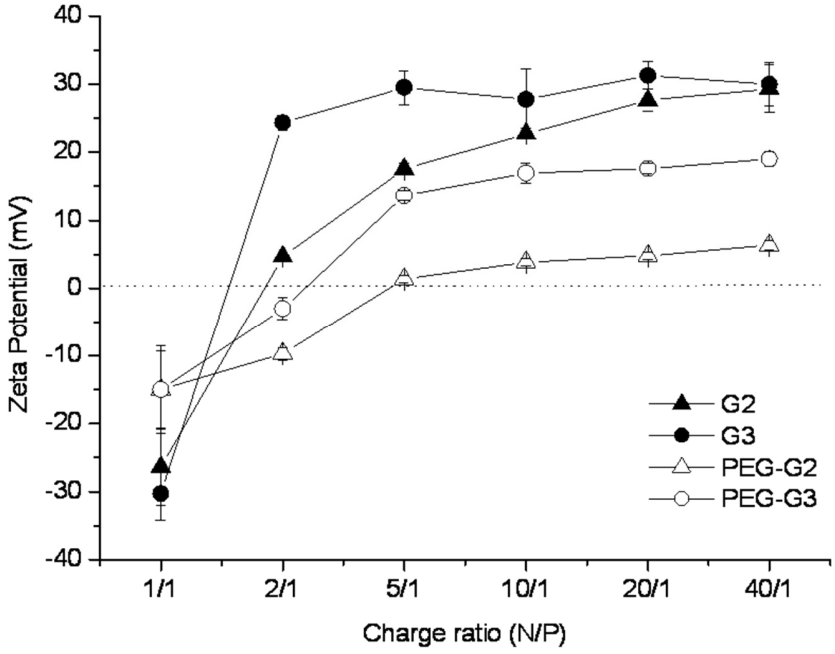


Figure 3. Percentage of cell survival (HEK293T cells) determined upon 5 h incubation of the cells with GATG and PEG-GATG dendrimers in solution (A) or dendriplexes GATG/pDNA and PEG-GATG/pDNA prepared at a charge ratio (N/P) 40/1 (B) (MTS assay, means ± SD, n=4).

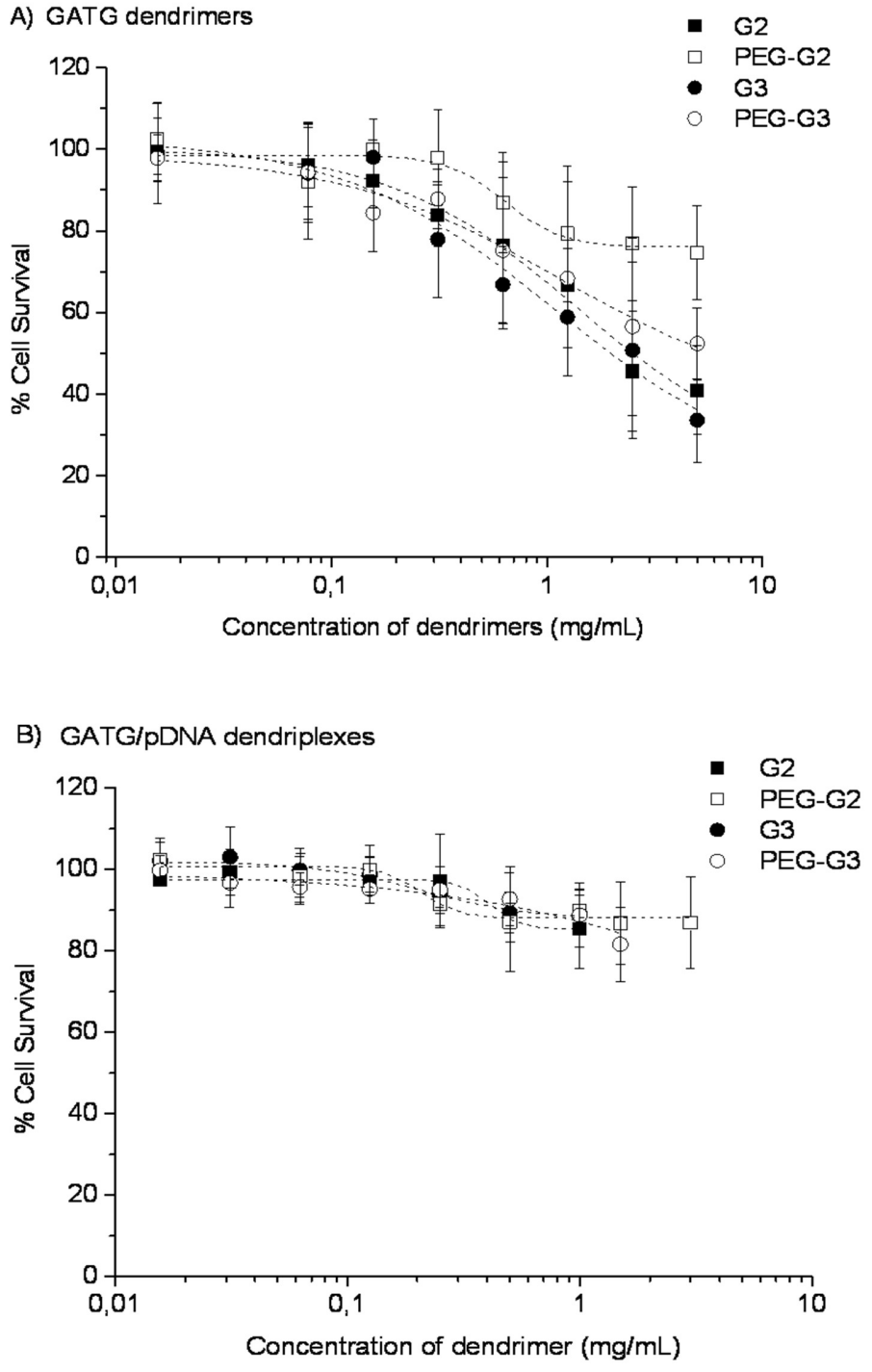


Figure 4. Erythrocyte aggregation assay for G3/pDNA (A, C, E) and PEG-G3/pDNA (B, D, F) dendriplexes prepared at a charge ratio (N/P) 40/1. Different concentrations of dendriplex suspensions were incubated with a 1% red blood cell suspension in order to reach a final pDNA concentration of 1.5 μg/mL (A, B), 6.5 μg/mL (C,D), and 12.5 μg/mL (E, F). Untreated erythrocytes are shown as control (G). Images representative of three independent experiments are shown.

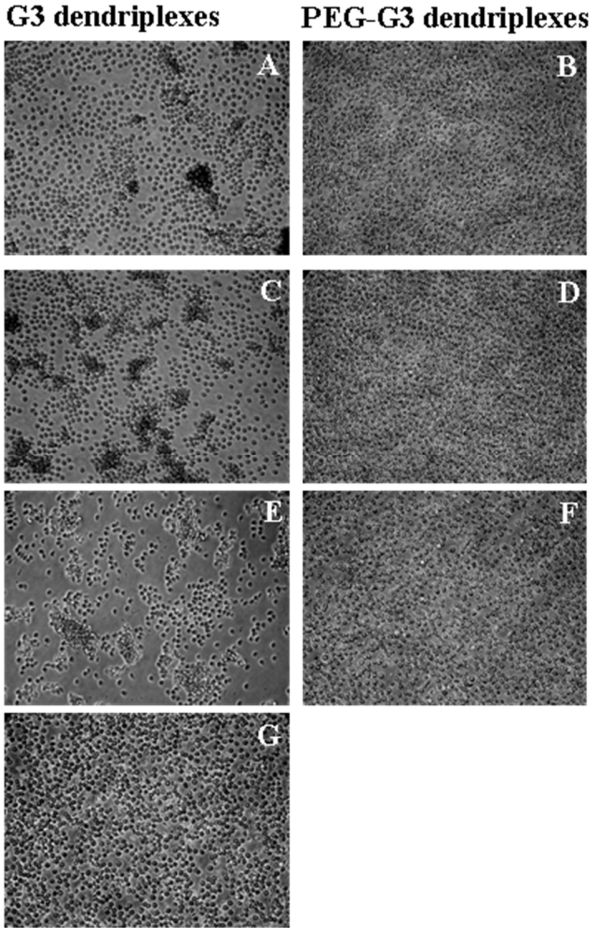


Figure 5. Relative accessibility of EtBr to the pDNA complexed G3/pDNA (A) or PEG-G3/pDNA (B) dendriplexes prepared at several charge ratios (N/P). The complexes were incubated for 2h with several polyanions (3-fold charge excess with respect to the dendrimer), namely heparin (white bars), dextran sulphate (stripped bars) and hyaluronan (grey bars) (means ± SD, n=3). For reference, the accessibility of EtBr to freshly prepared complexes is reported (black bars). Statistical significance between the values of reference (freshly prepared G3 or PEG-G3 dendriplexes) and the values upon incubation of dendriplexes with polyanions are denoted as * (P < 0.05)

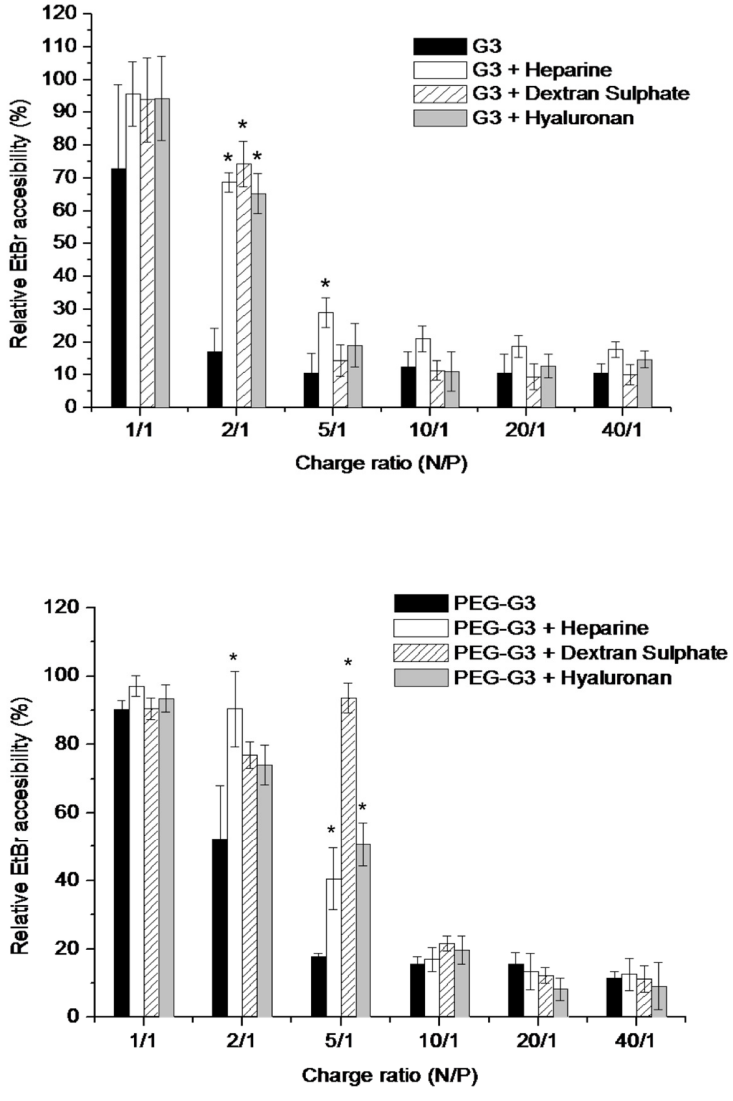


Figure 6. Topology of the pDNA determined by agarose gel electrophoresis. DNase I (1 unit) was added over naked pDNA, or G3/pDNA and PEG-G3/pDNA dendriplexes prepared at a charge ratio (N/P) 40/1 (1 μg of pDNA per sample). After 5 (a), 30 (b) or 60 (c) min incubation, the DNases were inactivated and the samples incubated with a far excess of dextran sulphate (50 mg/mL) for pDNA displacement and loaded in an agarose gel. 1 Kb ladder and pDNA were used as controls for the experiment. A gel representative of three independent experiments is shown.

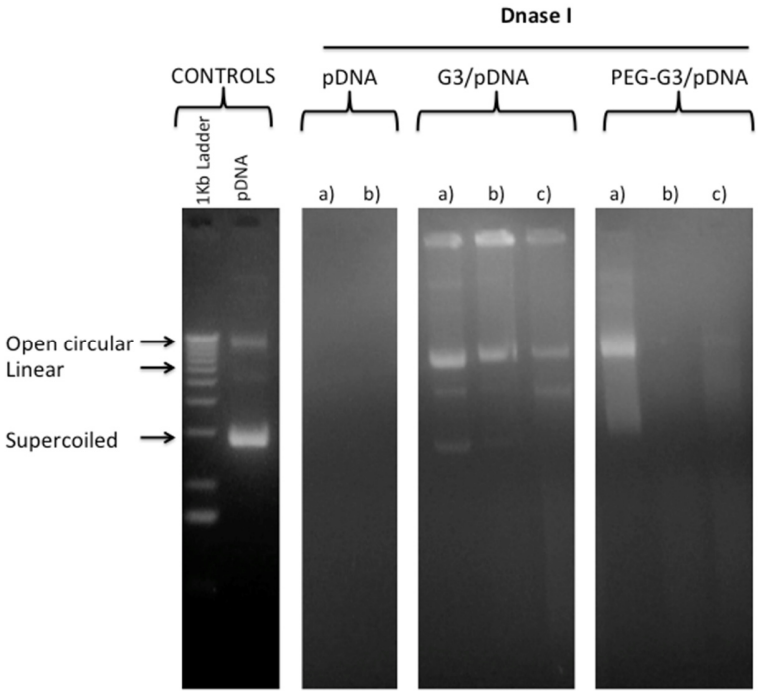
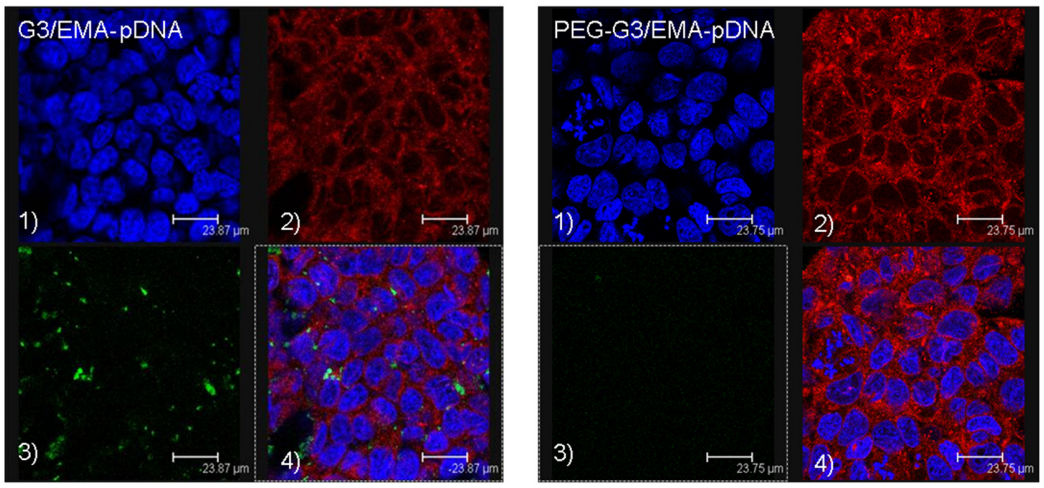


Figure 7. Confocal images of HEK 293T cells incubated during 5 h (A, B) or 24 h (C) with G3/EMA-pDNA (A, C) or PEG-G3/EMA-pDNA (B) dendriplexes prepared at a charge ratio N/P 40/1 (1 µg EMA-pDNA/well). Confocal images show: 1) blue signal corresponding to the cell nuclei stained with DAPI, 2) red signal corresponding to the actin filaments of the cytosketelon stained with Phalloidin-Bodipy® 650/665, 3) green signal attributed to the internalized EMA-pDNA and 4) the overlapping image of the three channels. Image C is a cross-section to facilitate the visualization of the green signal (EMA-pDNA) overlapping with the blue signal (cell nuclei). Images representative of three independent experiments are shown.

A) B)



C)

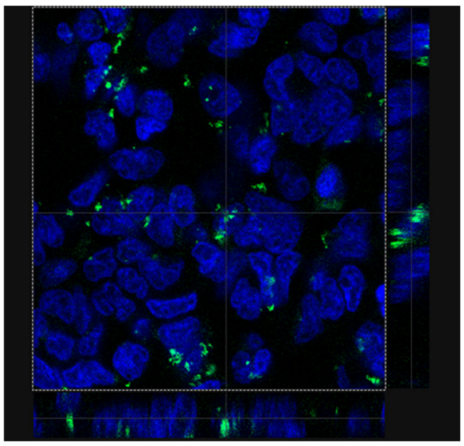
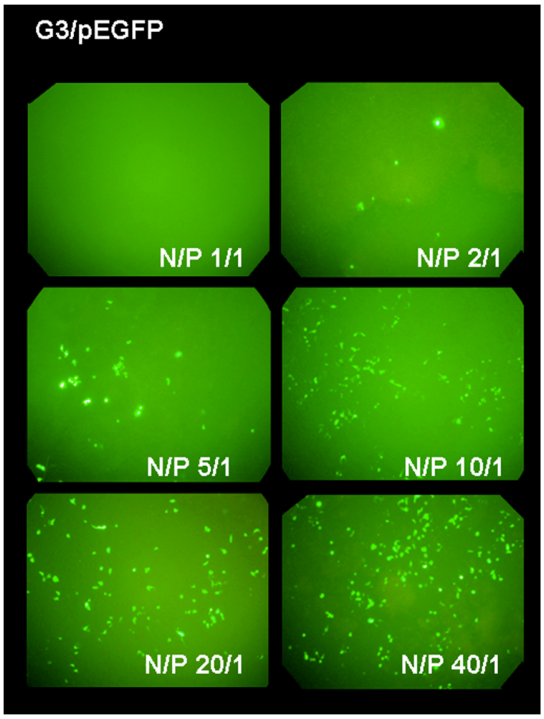


Figure 8. Expression of green fluorescent protein evaluated 48h after transfection of HEK293T cells (1µg pEGFP per well) with G3/pEGFP dendriplexes prepared at the indicated charge ratio (N/P) (A) and expression of the secreted protein SEAP, quantified at 2, 4 or 6 days after transfection of HEK293T cells (1, 2 or 4µg pSEAP per sample) with G3/pSEAP dendriplexes prepared at a charge ratio (N/P) 40/1 (means ± SD, n=3; * Significant differences, $P < 0.05$) (B).

A)



B)

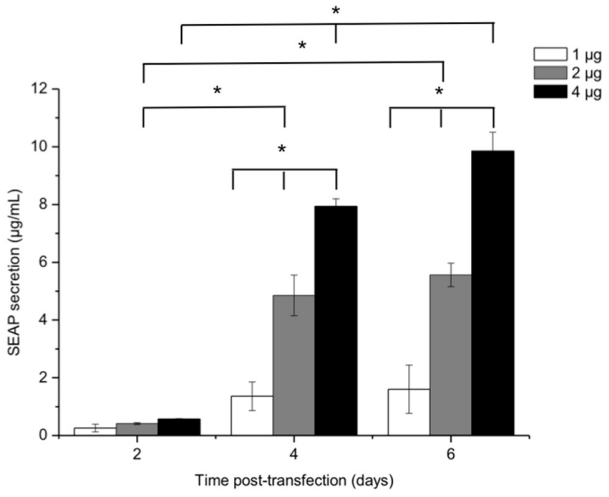


Figure 9. Expression of EGFP in HEK293T cells transfected with dendriplexes prepared at a charge ratio (N/P) 40/1 and with increasing molar ratios of PEG-G3/G3 (1μg pEGFP per well). The following images were acquired with the fluorescent microscope and correspond to cells transfected with dendriplexes prepared at increasing molar ratios (%) of PEG-G3/G3: a) 0% (i.e. solely G3), b) 0.5% c) 1% d) 5% e) 10% f) 20% g) 50% h) 100% (i.e. solely PEG-G3). EGFP expression was evaluated 48 h post-transfection. Images representative of three independent experiments are shown.