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Synthesis and evaluation of functional hyperbranched polyether polyols as prospected gene carriers

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Abstract

Hyperbranched polyether polyols have been partially functionalized with quaternary or tertiary ammonium groups. Five derivatives have been prepared bearing 4, 8 and 12 quaternary or 4 and 21 tertiary ammonium groups. The resulting dendritic polymers interact with plasmid DNA affording the corresponding polyplexes. The complexes were physicochemically characterized while their transfection ability was assessed by gel retardation assay, ethidium bromide exclusion assay and cell culture transfection. All the investigated polymers were shown to have marginal to low cytotoxicity in mammalian cells. Transfection efficiency comparable to that of polyethylenimine was exhibited by selected quaternized polymers. However, the introduction of tertiary amino groups on polyglycerol did not improve the transfection of the ineffective parent polymer, despite the fact that the derivatives obtained exhibited additional buffering capacity (sponge effect). The observed transfection efficiency for the quaternized polymers has been attributed to the destabilization of the lysosomal membrane originating from the interaction between the cationic polymers and the anionic moieties located at the membrane. These results are encouraging for the prospective application of these polyols as gene delivery vectors.

Keywords: Gene delivery; Hyperbranched polyether polyols; Polyplex; Quaternary ammonium group; Tertiary ammonium group; Transfection

1. Introduction

Non-viral gene delivery systems (Tomlinson and Rolland, 1996; Brown et al., 2001; Liu and Huang, 2002) including complexes of DNA with cationic liposomes (Oku et al., 2001; Guo and Szoka, 2003; El-Aneed, 2004), cationic polymers (De Smedt et al., 2000; Gebhart and Kabanov, 2001; Merdan et al., 2002; Krämer et al., 2004; Park et al., 2006) or dendrimeric derivatives (Eichman et al., 2000; Cloninger, 2002; Dennig and Duncan, 2002; Boas and Heegaard, 2004) are of significant interest, primarily due to the safety advantages offered by these systems as compared to viruses (Robbins et al., 1998; Cavazzana-Calvo et al., 2004). The application of non-viral systems has, however, disadvantages associated with their lower efficiency as compared to viruses and the inability to target gene expression to the area of pathology. For efficient gene expression, genes must be transported to the interior of cell nucleus, having to overcome a number of extracellular and intracellular barriers. These include targeting barriers, need for endosomal escape following endocytosis and transferring of genes to nucleus.

Hyperbranched polymers (Sunder et al., 2000; Frey and Haag, 2002; Stiriba et al., 2002) which are easily prepared compared to dendrimers (Tully and Fréchet, 2001; Lee et al., 2005; Svenson and Tomalia, 2005) are highly branched polymers exhibiting compact, globular structures in combination with a great number of functional groups. Low toxicity and biocompatibility of hyperbranched polyether polyols (Kainthan et al., 2006a,b) are promising incentives for undertaking investigations to develop novel functional derivatives based on these polymers that could be employed as gene delivery systems following proper functionalization of their surface groups. In principle, functionalization (Smith and Diederich, 1998; Vögtle et al., 2000; Sideratou et al., 2006; Paleos et al., 2006) with positively charged groups can induce complex formation of

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the polymer with DNA by electrostatic interactions. Alternatively, functionalization with tertiary amino groups can both render the functional hyperbranched polymer positively charged at physiological pH and also attribute to the carrier the proton sponge capacity (Haensler and Szoka, 1993; Boussif et al., 1995; Godbey et al., 1999; Akinc et al., 2005). According to the proton sponge hypothesis, the buffering capacity of tertiary amino groups leads to osmotic swelling and rupture of endosomes, resulting in the release of the vector into the cytoplasm.

Polycations, originating primarily from polyamines that contain primary, secondary, tertiary or quaternary amines (Thomas and Klibanov, 2002; Lee et al., 2003; Schatzlein et al., 2005; Yudovin-Farber et al., 2005; Swami et al., 2007; Tziveleka et al., 2007), form complexes with DNA and are widely used as gene delivery systems. Under physiological conditions, polyamines are cationic and condense with DNA forming compact structures. Polycations reduce the electrostatic repulsion between DNA and the cell membrane by neutralizing the DNA negative charge and also protect it from enzymatic digestion by nucleases in serum and extracellular fluids.

Within the context of developing new gene delivery systems, functional hyperbranched polymers were prepared based on polyether polyols. Thus, partial functionalization of the hyperbranched polyether polyols with either 4, 8 and 12 quaternary (6, 11 and 17% molar coverage) or 4 and 21 tertiary ammonium groups (6 and 31% molar coverage) was achieved through the interaction with glycidyltrimethylammonium chloride, or 2,3-epoxypropyldiethylamine, respectively (Scheme 1). The resulting dendritic derivatives were interacted with DNA and the complexes obtained, following their physicochemical characterization, were investigated in vitro as far as their transfection properties were concerned on human embryonic kidney (HEK) 293 cell line, which is widely employed for transfection studies, and also on monkey kidney fibroplast COS-7 cell line. These complexes showed marginal toxicity when tested on HEK 293 cells.

2. Materials and methods

2.1. Materials

Hyperbranched polyether polyol (polyglycerol $M_n = 5000$, $M_w/M_n = 1.5$, PG), Scheme 1, bearing 68 hydroxy groups (Felekis et al., 2005) and branched polyethylenimine (PEI, MW 25,000) were purchased from Hyperpolymers GmbH, Germany, and used as received. The molecular weights of all other synthesized polyglycerol derivatives reported in this study are based on this M_n taking into account the degree of functionalization of each derivative. Glycidyltrimethylammonium chloride and epichlorohydrin (ECH) were purchased from Fluka while diethylamine (DEA) was purchased from BDH. Diethylamine was dried by distillation over sodium hydroxide while epichlorohydrin was purified by drying over calcium oxide followed by distillation. Heat inactivated foetal bovine serum (FBS), high glucose Dulbecco's modified Eagle Medium (DMEM), OPTIMEM, Trypsin-EDTA, L-glutamine and penicillin–streptomycin solution were purchased from GIBCO. Luciferase and MTT assay kits were purchased from Promega and Sigma, respectively.

2.2. Characterization

^{1}H and ^{13}C NMR spectra were recorded in D_2O or $CDCl_3$ employing a Bruker 500 spectrometer operating at 500 and 125.1 MHz, respectively. For UV–vis absorption spectra a Perkin Elmer Lambda-16 spectrophotometer was employed. Fluorescence spectra were recorded employing a Perkin Elmer LS-5B spectrophotometer.

2.3. Synthesis of functionalized polyglycerol

2.3.1. Synthesis of quaternized polyglycerol [PG-Q-n]

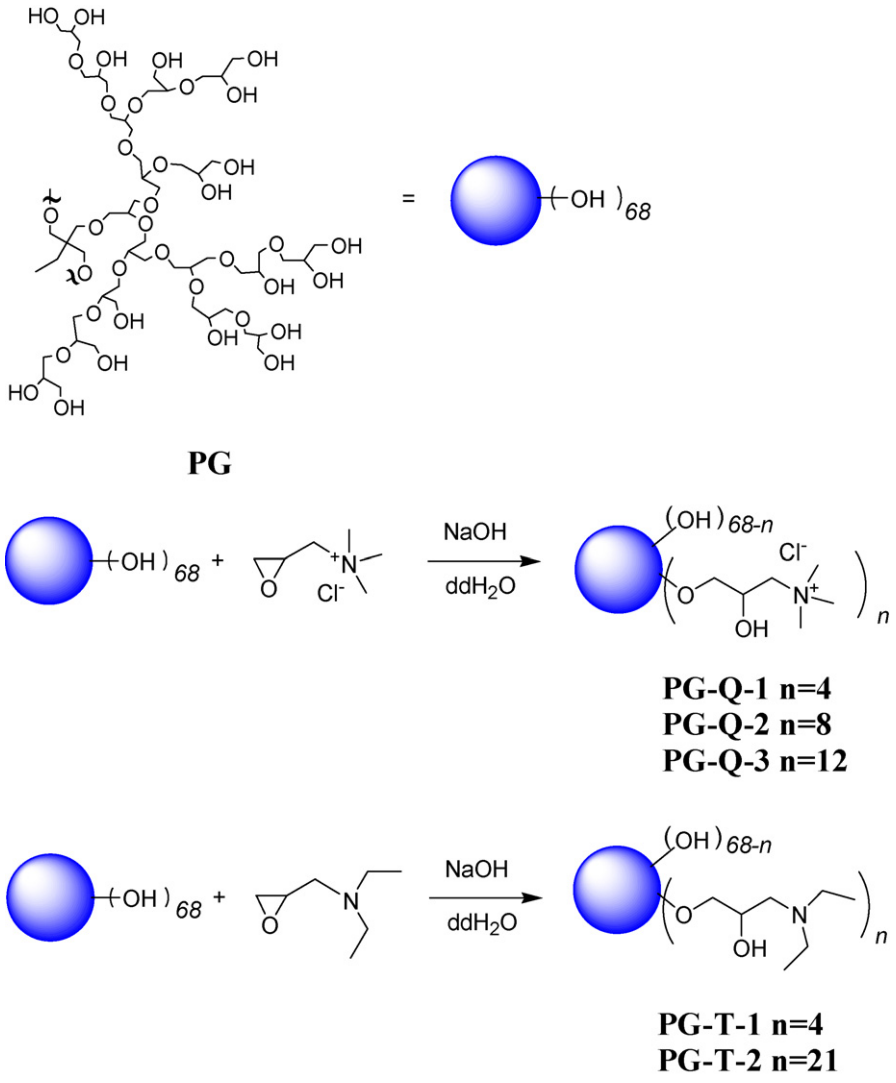
The introduction of the quaternary ammonium group on polyglycerol was conducted according to a slightly modified, previously reported method (Wilke and Mischnick, 1995). Thus, to a concentrated aqueous solution of PG (1eq), 2.4 equiv. of NaOH (13.5 N) were added. The mixture was cooled at about 0 °C and reacted with 1.2 equiv. of glycidyl trimethylammonium chloride, which were added dropwise. The reaction mixture was allowed to react overnight and subsequently neutralized with HCl (6 M). The solvent was removed under vacuum and methanol was added for precipitating NaCl, which was removed by centrifugation. The supernatant solvent was distilled off and the remaining product was redissolved in water and subjected to dialysis with a 1200 MW cut-off membrane and finally lyophilized. The partial introduction of the quaternary moiety was established by ^{1}H, ^{13}C and 2D NMR experiments recorded in D_2O, while the degree of substitution was determined by integration of the signals of interest in the ^{1}H NMR spectrum. The degree of substitution was also estimated by elemental analysis.

^{1}H NMR (500 MHz, D_2O) δ: 4.35 (br s, 1H, C*H*OHCH$_2$N$^+$(CH$_3$)$_3$), 4.03–3.27 (br m, CH_2, C*H* of PG, OCH_2CHOHCH_2N$^+$(CH$_3$)$_3$), 3.16 (s, 3H, N$^+$(CH_3)$_3$), 1.31 (br s, 2H, CCH_2CH$_3$), 0.81 (br s, 3H, CCH$_2$CH_3). ^{13}C NMR (125.1 MHz, D_2O) δ: 80.5–79.6 (L$_{13}$), 79.0–78.0 (D), 73.9–73.2 (O*C*H$_2$CHOHCH$_2$N$^+$(CH$_3$)$_3$), 72.5 (2L$_{14}$, T), 72.1 (C(*C*H$_2$)$_3$O), 71.7–70.4 (2D, 2T), 70.0–69.0 (L$_{13}$, D, L$_{14}$), 68.6 (*C*H$_2$N$^+$(CH$_3$)$_3$), 65.3 (*C*HOH*C*H$_2$N$^+$(CH$_3$)$_3$), 63.0 (T), 61.2 (L$_{13}$), 54.6 (N$^+$(*C*H$_3$)$_3$), 43.6 (CH$_3$CH$_2$*C*(CH$_2$)$_3$O), 22.4 (C*C*H$_2$CH$_3$), 7.5 (CCH$_2$*C*H$_3$).

2.3.2. Synthesis of 2,3-epoxypropyldiethylamine

2,3-Epoxypropyldiethyl-amine was prepared as previously described (Gilman and Fullhart, 1949; Burness and Bayer, 1963). The so-prepared compound was immediately used after distillation. A $n_D^{22} = 1.4338$ (Lit, $n_D^{25} = 1.4362$ (Gilman and Fullhart, 1949), $n_D^{20} = 1.4306$ (Burness and Bayer, 1963)) was determined. The structure of the product was established with ^{1}H and ^{13}C NMR.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.92–2.83 (broad m, 1H, C*H*), 2.63–2.20 (br m, 8H, CH_2(O)CHCH_2N(CH_2CH$_3$)$_2$), 0.94–0.80 (br m, 6H, N(CH$_2$CH_3)$_2$). ^{13}C NMR (125.1 MHz, $CDCl_3$) δ: 55.5 (*C*H$_2$N(CH$_2$CH$_3$)$_2$), 50.5 (*C*H), 47.2 (N(*C*H$_2$CH$_3$)$_2$), 44.9 (*C*H$_2$(O)CHCH$_2$N(CH$_2$CH$_3$)$_2$), 11.4 (N(CH$_2$*C*H$_3$)$_2$).



Scheme 1. Functionalization of hyperbranched polyether polyglycerol.

2.3.3. Introduction of tertiary amino groups to polyglycerol [PG-T-n]

The introduction of the tertiary amino group on polyglycerol surface was conducted according to the above described reaction conditions for epoxide interaction with hydroxyl groups (Wilke and Mischnick, 1995). The partial introduction of the tertiary moiety was established by ^{1}H, ^{13}C and 2D NMR recorded in D_2O, while the degree of substitution was determined by integration of the signals of interest of the ^{1}H NMR spectrum. As mentioned above the degree of substitution was also estimated by elemental analysis.

^{1}H NMR (500 MHz, D_2O) δ: 4.00–3.27 (br m, CH_2, CH of PG, $OCH_2CH(OH)CH_2N(CH_2CH_3)_2$), 2.70–2.48 (br, m, 6H, $CH_2N(CH_2CH_3)_2$), 1.30 (br s, 2H, CCH_2CH_3), 1.00 (t, 6H, $CH_2N(CH_2CH_3)_2$), 0.81 (br s, 3H, CCH_2CH_3). ^{13}C NMR (125.1 MHz, D_2O) δ: 80.4–79.5 (L_{13}), 79.3–78.0 (D), 74.1 ($OCH_2CH(OH)CH_2N(CH_2CH_3)_2$), 72.6 ($2L_{14}$, T), 72.1–70.2 (2D, 2T), 70.1–68.8 (L_{13}, D, L_{14}), 68.4–67.3 ($OCH_2CH(OH)CH_2N(CH_2CH_3)_2$), 63.0 (T), 61.2 ($L_{13}$), 55.1 ($CH_2N(CH_2CH_3)_2$), 47.8 ($N(CH_2CH_3)_2$), 43.6 ($CH_3CH_2C(CH_2)_3O$), 22.3 ($CCH_2CH_3$), 10.2 ($N(CH_2CH_3)_2$), 7.2 ($CCH_2CH_3$).

2.4. Plasmid DNA

Plasmids (pEGFP-C2, 4700 bp; and pGL3 luciferase reporter vector, 4818 bp) were grown in DH5α *Escherichia coli* and their purification was performed using a QIAGEN Maxi Plasmid Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA concentration was calculated according to its absorbance at 260 nm and DNA purity was determined by the ratio of absorbance at 260 and 280 nm. All samples showed an A_{260}/A_{280} ratio of 1.7–1.9, which indicates that DNA of high degree of purity was obtained. DNA purity was also confirmed by agarose gel electrophoresis.

2.5. Gel retardation assay

Complexes for this assay were formed at polymer:plasmid DNA ratios of 0, 0.5, 1, 2.5, 5, 10 and 20 (w/w). In each case,

an appropriate amount of the PG-Q-n or PG-T-n derivative was dissolved in distilled water and mixed with 2 μg of the plasmid DNA (pEGFP-C2) to a final volume of 50 μl, in OPTIMEM. The solutions were incubated at room temperature for 30 min, mixed with a dye solution (0.25% Bromophenol blue, 30% glycerol in water, six times concentrated), and loaded into agarose gel wells (0.8% agarose in Tris-acetate EDTA buffer containing 0.5 μg ethidium bromide/ml). Electrophoresis was carried out at 100 V for 40 min, and DNA was visualized by UV illumination.

2.6. Ethidium bromide exclusion assay

The ability of the functional hyperbranched polyether polyols to effectively condense with DNA was monitored by observing the reduction in fluorescence intensity of ethidium bromide (EtBr) due to its exclusion from DNA. A solution containing 10 μg/ml of plasmid DNA (pEGFP-C2) and 1 μg/ml ethidium bromide in 5% dextrose or 10 mM Hepes, pH 7.4, was at first incubated at room temperature for 15 min to ensure interaction between DNA and ethidium bromide. The fluorescence of plasmid DNA solution with EtBr was set at 100%. Consequently, an appropriate quantity of the PG-Q-n or PG-T-n derivatives was added in order to reach the desired w/w ratio followed by further incubation for 15 min after each addition. Controlled experiments were also conducted which only contained ethidium bromide and the hyperbranched polyether polyols treated in the same manner. Results were normalized against the controlled experiments and expressed as % reduction of the relative fluorescence intensity. The spectrofluorometer was operated with an excitation wavelength at 526 nm and emission wavelength at 592 nm.

2.7. DNA-polymer size and ζ-potential

Dynamic light scattering (DLS) was employed for determining the size of the polymer–pDNA complexes. A light scattering apparatus (AXIOS-150/EX, Triton Hellas) with a 30 mW laser source and an Avalanche photodiode detector at an angle of 90° was employed. The viscosity (0.89 mPa s) and refractive index (1.332) of distilled water at 25 °C were used for data analysis. Each experiment was performed in triplicate. Ten measurements were acquired for each dispersion and the correlation functions were analyzed using the CONTIN algorithm to obtain the apparent hydrodynamic radii. Samples were prepared in the same manner and at the same concentrations as the ones used in transfection experiments. Generally, particles were first formed in distilled water, and then added either to OPTIMEM, or to 10% serum containing OPTIMEM media. The final concentration of the pDNA was 2 μg/ml.

For ζ-potential measurements of polymer–pDNA polyplexes a ZetaPlus apparatus of Brookhaven Instruments Corporation was employed. Ten ζ-potential measurements were collected for each dispersion, and the results were averaged. Also for the ζ-potential measurements the final concentration of DNA was 2 μg/ml.

2.8. Atomic force microscopy

Atomic Force Microscopy was used for imaging the shape of polyplexes at a weight ratio of 20 and 40 employing a Nanoscope III System (Digital Instruments, Inc.). Polyplexes were formed at a total of 2 μg/ml of pDNA (pEGFP-C2) concentration in OPTIMEM. After incubation for 30 min at room temperature, dispersions containing 0.1 μg of pDNA were applied to freshly cleaved mica and the excess fluid was removed using filter paper. The solution was allowed to dry for 10 min at room temperature prior to imaging. All imaging was conducted in tapping mode, with 512 × 512 data acquisition, at a scan speed of 1 Hz at ambient conditions. The experiments were repeated twice, and the observed structures were found to be reproducible. All post-imaging analysis was carried out on Nanoscope software. Background slope was removed using a first or second order polynomial function.

2.9. Cell culture

The human embryonic kidney (HEK 293) and the monkey kidney fibroplast (COS-7) cells were maintained in high glucose DMEM, supplemented with 10% heat inactivated FBS, 2 mM L-glutamine and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2.

2.10. Transfection studies

Polyplexes of pDNA and hyperbranched polymers were prepared at various weight ratios in 20 μl of distilled water, and the mixtures were incubated for 30 min at room temperature. An equivalent volume of OPTIMEM was added, and the mixture was further incubated for another 30 min. For all the transfection studies, cells were seeded at a density of 1×10^5 cells per well, in 12-well plates, 48 h before the transfection. Before the initiation of transfection experiments, the growth medium was removed from each well and the cells were washed once with serum- and antibiotics-free medium. The polyplexes were then diluted to a final volume of 1 ml OPTIMEM per well and the transfection was carried out for 4 h at 37 °C. The level of pDNA dosed with the polymers was 2 μg per well. The transfection medium was then removed and the cells were further incubated, under the same conditions, in a complete growth medium for 24 h. The cells were then washed with phosphate-buffered saline (PBS) and harvested, lysed with 400 μl per well of reporter lysis buffer (Promega), following the manufacturer's protocol and assayed for the expression of luciferase. Luciferase activity was determined by measuring the light emission from 20 μl of cell lysate incubated with 2.35×10^{-2} μmol of luciferin substrate. The light emission was measured in relative light units (RLU) using a chemiluminometer LB 9507 (Berthold, Germany) and adjusted to the protein concentration of the sample. The protein concentration in the cell lysates was determined according to Bradford (1976). Luciferase expression was normalized by total cellular protein. PEI was used as a positive control. Transfection efficiency is expressed relatively to PEI. Control experiments

were also performed in order to check the background levels of DNA and polymers. Transfection experiments were performed twice and the results are their mean value ± S.D. ($n = 6$).

2.11. In vitro cytotoxicity studies

Cytotoxicity assay was performed by the modified MTT assay (Mosmann, 1983). Thus, HEK 293 cells were seeded at a density of 5×10^4 cells per well in a 96 well plate and grown in 200 μl of growth medium 24 h prior to the incubation with polymers. Following the treatment of the cells with PEI and PG derivatives for 4 h under the same conditions used for the serum free transfection experiments, the medium was replaced with completed growth medium and incubated for 24 h. After this period the medium was replaced with 200 μl per well of MTT solution (0.5 mg/ml diluted in growth medium). The cells were further incubated for 3 h at 37 °C and 5% CO_2. Subsequently, the medium was removed and 200 μl of isopropanol was added per well, so as to dissolve the formazan crystals. The absorbance was measured at 570 nm (reference filter, 690 nm) using a microplate reader (Spectra MAX 190, Molecular Devices). Measurements were converted to percent viability by comparison to control experiments in which no polymers had been added.

3. Results and discussion

3.1. Functionalization of polyether polyol

Three quaternized derivatives of polyglycerol were prepared bearing 4 (PG-Q-1), 8 (PG-Q-2) or 12 (PG-Q-3) quaternary ammonium groups, as shown in Scheme 1. The introduction of the quaternary group renders polyglycerol cationic for interacting with negatively charged DNA for the formation of complexes.

Ether bond formation with the hydroxy groups of PG was confirmed by the appearance in the ^{1}H NMR spectra of a characteristic broad signal at 4.35 ppm corresponding to the hydroxylated methine proton due to ring opening. The new signals at 3.43 and 3.16 ppm were attributed to the α-methylene and methyl protons adjacent to the quaternary group, respectively. Furthermore, the appearance of four signals in the ^{13}C NMR spectrum at 54.6, 65.3, 68.6 and 73.4 ppm established the introduction of the quaternary group. The α-methylene and methyl groups attached at the quaternary center were observed at 68.6 and 54.6 ppm, respectively.

The degree of substitution was estimated by integration of the appropriate signals in the ^{1}H NMR spectrum. By calculating the integration ratio of the total area corresponding to all protons of the polymer to the characteristic methyl protons next to the quaternary amino group, it was found that approximately 6, 11 and 17% of the total hydroxy groups have been interacted leading to the derivatives PG-Q-1, PG-Q-2 and PG-Q-3, respectively. Taking into consideration that each polymer bears 68 hydroxy groups, the average number of attached quaternary moieties per polymer is 4, 7.6 and 11.5, respectively. An inverse gated (IG) ^{13}C NMR experiment was also conducted for PG-Q-2 in order to confirm the ^{1}H NMR-estimated substitution. By calculating the integration ratio of the total area corresponding to all carbons of the polymer to the characteristic methyl carbons next to the quaternary group, the previous results were confirmed. The degree of substitution was also estimated by elemental analysis. The average number of attached quaternary moieties per polymer as calculated by elemental analysis is 5.5, 8.9 and 13.5, respectively. The C/N ratios are also given in Table 1.

Additionally, two derivatives of polyglycerol were prepared bearing 4 (PG-T-1) or 21 (PG-T-2) tertiary amino groups as shown in Scheme 1. The introduction of the tertiary amino group renders polyglycerol positively charged at physiological pH and also capable of exhibiting buffering capacity.

Ether bond formation with the hydroxy groups of PG was established by the appearance in the ^{1}H NMR spectra of a characteristic broad signal at 2.70–2.48 ppm corresponding to the α-methylenes adjacent to the tertiary amino group. Furthermore, the triplet at 1.00 ppm corresponds to the methyl protons of the introduced moiety. The appearance of four signals in the ^{13}C NMR spectrum at 10.2, 47.8, 55.1, 67.7 and 74.1 ppm confirmed the introduction of the tertiary amino group. The α-methylenes adjacent to the tertiary amino group and the β-methyl groups attached at the tertiary center were observed at 55.1, 47.8 and 10.2 ppm, respectively.

The percentage substitution was estimated by ^{1}H NMR experiments. By calculating the integration ratio of the total area corresponding to all protons of the polymer to the characteristic methyl carbons next to the tertiary amino group, it was found that approximately 6 and 31% of the total hydroxy groups have been modified (4.2 and 20.9 tertiary amino moieties per polymer

Table 1
Chemical characterization of the PG derivatives

	PG	PG-Q-1	PG-Q-2	PG-Q-3	PG-T-1	PG-T-2
Mn[a]	5000[b]	5602[c]	6145[c]	6732[c]	5543[c]	7700[c]
Number of *N* per molecule[d]	0.0	4.0	7.6	11.5	4.2	20.9
C/N[e]	–	35.9	24.2	17.6	38.7	13.2
Number of *N* per molecule[f]	0.0	5.5	8.9	13.5	5.0	21.7

[a] Average molecular weight.
[b] Average molecular weight as provided by the manufacturers.
[c] Molecular weight as determined by ^{1}H NMR analysis of each polymer.
[d] Number of nitrogen atoms per molecule of each polymer as determined by ^{1}H NMR analysis.
[e] Found carbon to nitrogen ratio (elemental analysis).
[f] Number of nitrogen atoms per molecule of each polymer as determined by elemental analysis.

for PG-T-1 and PG-T-2, respectively). The degree of substitution was also estimated by elemental analysis. The average number of attached tertiary amino moieties per polymer as calculated by elemental analysis is 5.0, and 21.7, respectively. The C/N ratios are also given at Table 1. Molecular weights of the functionalized PG derivatives as determined by ^{1}H NMR analysis, along with the degree of substitution for each derivative, estimated by both ^{1}H NMR and elemental analysis, are shown in Table 1.

3.2. Determining complex formation by agarose gel electrophoresis and ethidium bromide exclusion assay

The PG-Q-n or PG-T-n derivatives of varying degree of substitution were mixed with plasmid DNA. The mixtures were subjected to electrophoresis in agarose gel to verify polyplex formation (Fig. 1). Polyplexes were prepared at various ratios ranging from 0.5 to 20 (w/w). The complexes were not precipitated and retained their water solubility at all ratios examined. As expected, no indication of polyplex formation between PG and DNA was found (data not shown). Complete retardation of DNA was observed in all three different PG-Q-n derivatives. As the degree of quaternization increased, complete complexation was obtained at lower polymer to DNA ratios (w/w). Highly quaternized polymers were more efficient in polyplex formation. Actually, derivative PG-Q-3 could retard the migration of DNA completely even at a ratio of 5 (w/w) (N/P = 3.0), while PG-Q-1 and PG-Q-2 could not retard polyplex until the w/w ratio of the polyplex reached the value of 20 (N/P = 5.0) and 10 (N/P = 4.4), respectively. Complete retardation of DNA was also observed for PG-T-n derivatives. PG-T-1 derivative retards the migration of DNA completely at a ratio of 5 (w/w) (N/P = 1.3), while PG-T-2 at a ratio of 1 (1.0 N/P = 1.0).

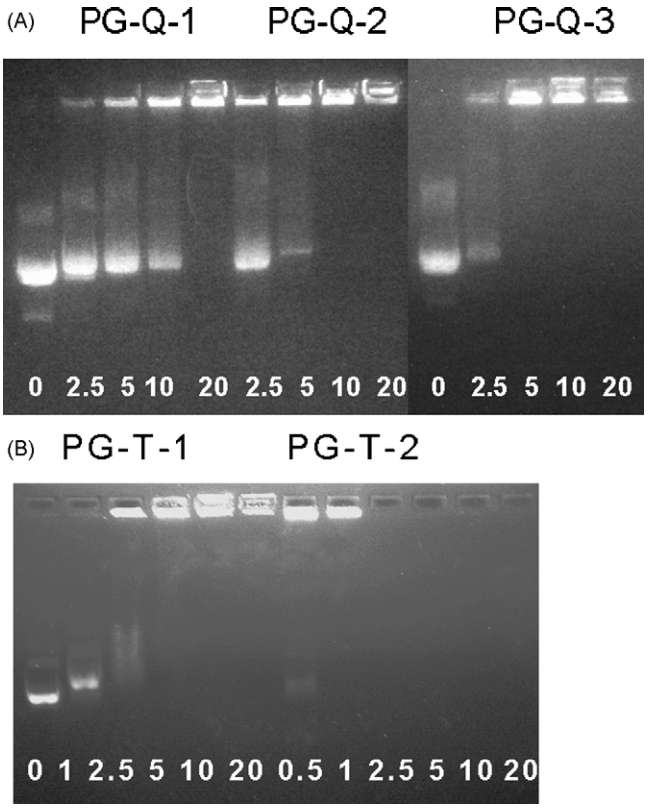


Fig. 1. Agarose gel electrophoretic analysis of PG-Q-n/pDNA (A) or PG-T-n/pDNA (B) polyplexes in OPTIMEM. Hyperbranched polymers and plasmid DNA were mixed at varying w/w ratios and incubated for 15 min at room temperature. Weight/weight ratios are indicated beneath each lane. Samples were electrophoresed in 0.8% agarose gel at 100 V for 40 min in Tris-acetate, EDTA buffer.

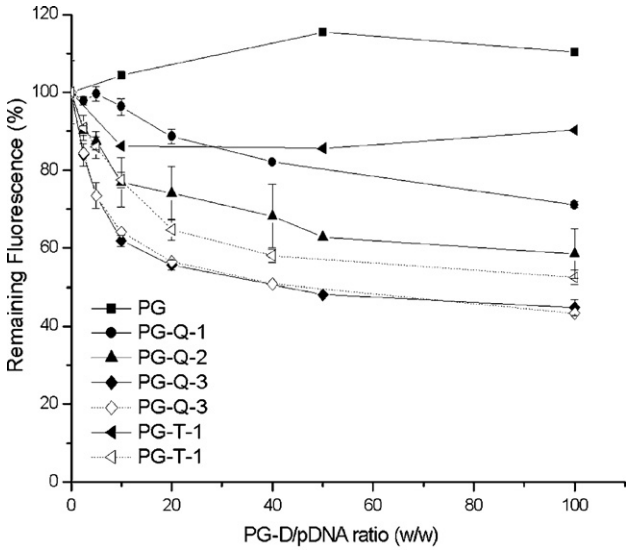


Fig. 2. Ethidium bromide exclusion assay comparing parent polymer, PG, and polyglycerol derivatives, PG-Q-n and PG-T-n. Polyplexes were developed in both 5% dextrose (closed symbols) and 10 mM Hepes, pH 7.4 (open symbols).

Comparing the various derivatives, differing in the degree of substitution, it is obvious that it is the N/P ratio that affects the interaction with DNA. The N/P ratio necessary for the complete complexation of the polymers with the pDNA is about 4 for the PG-Q-n and 1 for the PG-N-n derivatives.

Complex formation was also studied by the ethidium bromide exclusion assay (Fig. 2). Once again, no indication of polyplex formation between the parent polymer, PG, and DNA was found. In all of the weight ratios studied, complete exclusion of the intercalating agent could not be observed, indicating a partial condensation of the plasmid and formation of loose conjugates. The extent of complexation increased parallel to the increase of the degree of quaternization, reaching a plateau at almost 50% dye exclusion. When the quaternized PG-Q-3 derivative was employed, the nature of the medium did not alter the extent of complexation. On the contrary, when the PG-T-1 derivative was tested, its complexating ability depended on the buffer used. Protonation of the tertiary amines occurred at pH 7.4 rendering the derivative positively charged and therefore, able to form complexes with the pDNA. When dextrose was used for complexation, only minor complexation was observed.

3.3. Characterization of PG-Q-n/DNA polyplexes

The effect of the degree of functionalization and the charge ratio increase on the size of polyplexes was investigated

Table 2
Size (radius, nm) of PG-Q-n and PG-T-n/DNA polyplexes at various time intervals as determined by dynamic light scattering

Polyplexes	Ratio (w/w)	0 h	1 h	2 h	3 h	4 h
PG-Q-2/pDNA	20	814 ± 175	735 ± 231	796 ± 262	1001 ± 448	877 ± 294
	40	670 ± 224	629 ± 157	793 ± 165	810 ± 235	785 ± 213
	100	449 ± 250	484 ± 177	459 ± 200	566 ± 211	432 ± 160
PG-Q-3/pDNA	20	332 ± 79	386 ± 116	452 ± 143	404 ± 122	523 ± 122
	40	204 ± 37	304 ± 60	328 ± 60	377 ± 54	433 ± 77
PG-T-2/pDNA	20	146 ± 27	221 ± 32	267 ± 62	293 ± 34	356 ± 39
	40	120 ± 19	140 ± 17	130 ± 21	193 ± 18	154 ± 17

Data are the mean radii of each polyplex observed in OPTIMEM and at 2 μg/ml of plasmid DNA concentration.

(Table 2). In order to elucidate the potential relationship between size and transfection efficiency, polyplexes were prepared at the same concentrations as they were used for typical *in vitro* bioassays.

Table 2 shows the effect of the weight ratio on the size of polyplexes and whether their size remains unchanged over time (up to 4 hs). It is clear that by increasing the weight ratio used and also the charge ratio, i.e. the degree of functionalization, the size of the polyplexes is reduced. For the PG-Q-2/pDNA polyplex the size of the particles is in the μm range. Even at the high weight ratio of 100 (N/P = 43.6), the particles formed are still large. For the PG-Q-3/pDNA polyplex the particles formed are smaller compared to PG-Q-2 and almost independent on the weight ratio used. Finally, for the PG-T-2/pDNA polyplex the particles formed are even smaller and the weight ratio does not affect their size. The size of the particles increases slightly in parallel with the incubation time, the bigger ones reaching a plateau value faster.

As far as the effect of serum on the particle size is concerned, both PG-Q-n and PG-T-n/pDNA particles delivered in serum supplemented OPTIMEM are slightly smaller, though their size is still large, than those formed in the non serum supplemented culture media (data not shown). Serum proteins have been shown (Ogris et al., 1998; Green et al., 2006) to reduce particle size and increase stability, presumably, by preventing particle/particle contact through the adsorption of serum proteins to the complex surface.

The surface charge of the polyplexes was determined by ζ-potential measurements (Fig. 3). It was found that the surface charge of the PG-Q-2 derivative was slightly positive and increased in parallel to the weight ratio (from 20 to 40, w/w). On the other hand, the PG-Q-3 derivative was slightly negative at the weight ratio of 20 and as previously, its charge increased as the weight ratio increased. Incubation of the polyplexes at room temperature resulted in a slight decrease of the surface charge. In the case of the PG-T-2 derivative, and at the same weight ratios, the surface charge was positive, independent on the weight ratio used and constant relative to incubation. It is evident that under the experimental conditions employed, all the derivatives are positively charged, a requirement for efficient transfection (Takeuchi et al., 1996; Zelphati and Szoka, 1996a; Labhasetwar, 2005).

The morphology of the polyplexes, as a function of the degree of substitution, was investigated by AFM (Fig. 4). Polyplexes were formed at a weight ratio of 20 or 40. PG-Q-2/pDNA polyplexes at the weight ratio of 20 formed mainly very large and loose condensates with sizes up to 1 μm (Fig. 4A). At the higher ratio of 40 only compact spheres could be observed (Fig. 4B). Analogous compact spheres have also been observed for PG-Q-3/pDNA polyplexes when formed at a weight ratio of 20.

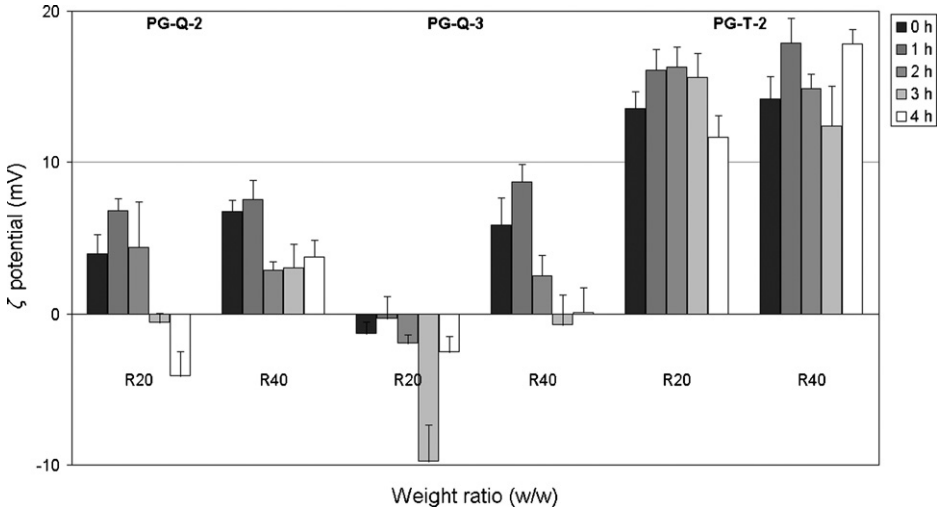


Fig. 3. ζ-potential values of polyplexes obtained after the interaction of PG-Q-2, PG-Q-3 or PG-T-2, with pDNA at various weight ratios (w/w), in OPTIMEM. Results are shown as mean ± S.D. ($n = 10$).

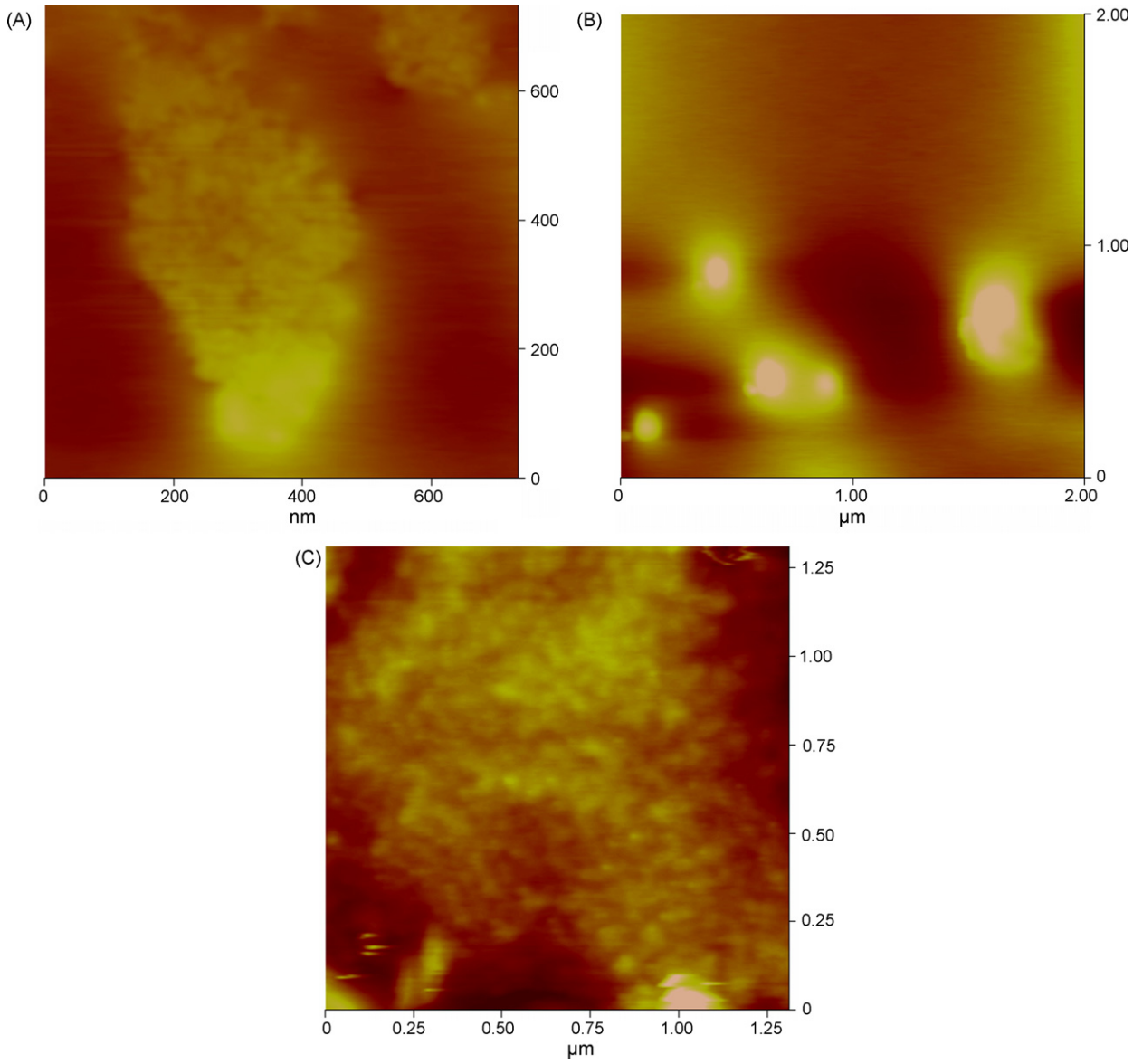


Fig. 4. AFM images of particles obtained after the interaction of pDNA with PG-Q-2 (A) at a weight ratio (w/w) of 20, and PG-Q-2 (B) and PG-Q-3 (C) both at a weight ratio (w/w) of 40 in OPTIMEM.

Furthermore, PG-Q-3/pDNA polyplexes formed at the weight of 40 are imaged as loose aggregates (Fig. 4C). The presence of loose or compact polyplexes is directly correlated with transfection efficiency (*vide infra*). On the other hand, if we consider the size of the polyplexes alone, there appears to be a trend and only a loose correlation between particle size and transfection ability.

3.4. Transfection efficiency of functional PGs

The transfection of the polymeric derivatives was performed in HEK 293 transformed human kidney cells and COS-7 transformed monkey kidney fibroplast cells. In the second cell line neither of the polymers tested did exhibit any transfection efficiency, therefore, only the results obtained with HEK 293 are presented. The transfection efficiency was quantitatively evaluated by luciferase reporter gene assay using pGL3 plasmid DNA at various weight ratios in the absence and in the presence of serum (Fig. 5).

After an initial screening using the green fluorescent protein (GFP) as the reporter gene (data not shown), the most promising functional dendritic polymers were selected for studying the transfection efficiency of their polyplexes with pGL3. It was found that only the quaternized polymers exhibited transfection efficiency, while the introduction of the tertiary amino group on polyglycerol did not improve the transfection of the ineffective parent polymer (Fig. 5). The transfection efficiency (almost 120% relative to PEI) of the most efficient quaternized derivative, PG-Q-2, reached its maximum when a weight ratio of 40 (N/P = 17.4) was used. Nevertheless, the transfection efficiency remained satisfactory (almost 90% relative to PEI) even at the higher ratio of 100 (N/P = 43.6). The highly quaternized derivative, PG-Q-3 exhibited its maximum activity (almost 80% relative to PEI) at a weight ratio equal to 20 (N/P = 12.0).

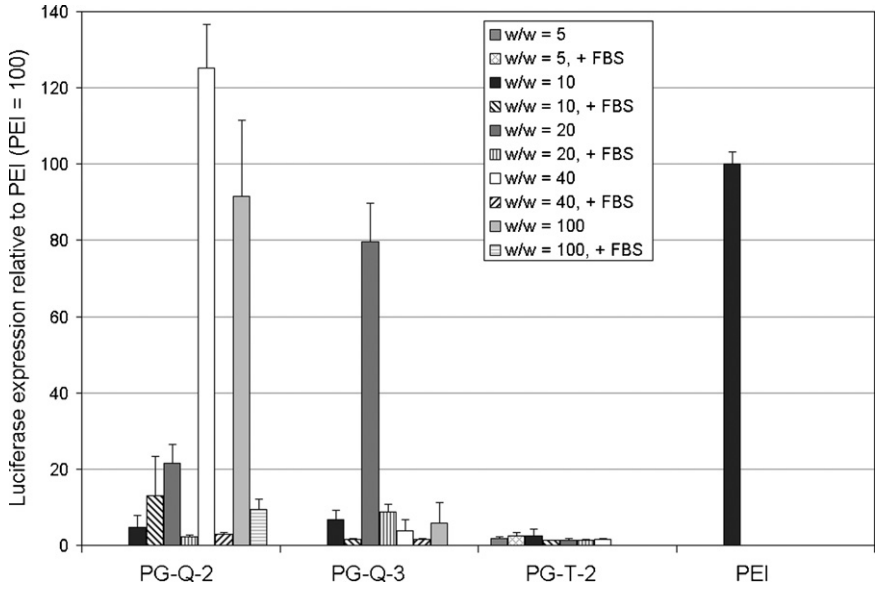


Fig. 5. Transfection efficiency of hyperbranched polyether polyols against the HEK 293 cell lines, in the absence (filled bars) and in the presence (hatched bars) of FBS at various w/w ratios. Luciferase expression was normalized by total cellular protein and a DNA dose of 2 μg/well was used. Data are represented as the mean ± S.D. ($n = 6$). Results are presented relative to PEI.

On the other hand, PG-T-2 exhibited marginal transfection efficiency at all the ratios tested (Fig. 5). It seems that the increase of the buffering capacity of these polymers does not affect their transfection efficiency (Funhoff et al., 2004). This fact indicates the contribution of the lysosomolytic effect of the functional cationic polymers to their transfection efficiency, as previously has been shown for cationic lipids and liposomes (Zelphati and Szoka, 1996b; Wattiaux et al., 1997). This destabilization effect on the lysosomal membrane apparently stems from the interaction between the cationic polymers and the anionic molecules present in the membrane (Fuchs and Raines, 2006). Our results are in line with previous reports (Ogris et al., 1998; Green et al., 2006), according to which large, aggregated particles are more efficient transfection agents. In an attempt to correlate the results obtained from the physicochemical characterization of the PG-Q-n and PG-T-n/pDNA polyplexes, we could conclude that large compact polyplexes with a slightly positive charge act as efficient transfection agents. On the other hand, polyplexes with low transfection efficiency form loose aggregates, as imaged by AFM (Fig. 4).

For studying the serum effect on the transfection efficiency of the polyplexes, experiments were conducted employing the HEK 293 cell line. It was found (Fig. 5) that the addition of FBS to the transfection medium resulted in a major decrease in transfection efficiency for all the polymers tested. A decrease in the transfection efficiency in the presence of serum has also been reported for the majority of the investigated non-viral vectors (Boussif et al., 1996; Yang and Huang, 1997; Escriou et al., 1998; Guo and Lee, 2001; Green et al., 2006). The obstructive effect of serum may be partly attributed to the non-specific interaction of the vector complexes with proteinous components, which induces the decrease in the cellular uptake and gene expression (Johnstone et al., 2001).

3.5. Cytotoxicity of derivatized PG-Q-n and PG-T-n

The cytotoxicity of the PG-Q-n and PG-T-n derivatives was examined by MTT assay. HEK 293 transformed human kidney cells were used in these experiments. The concentrations employed for the cytotoxicity evaluation were within the range used for the transfection efficiency experiments. As shown in Fig. 6, the viability of the HEK 293 cells decreased abruptly with increasing concentration of PEI, which was used as control. Cytotoxicity was marginal for the PG-Q-n derivatives even at high concentrations (90% cell viability was registered for PG-Q-2 and PG-Q-3 at 200 μg/ml which corresponds to the highest weight ratio of 100 employed in the transfection experiments). It is noticeable that the toxicity of the PG-Q-n derivatives remained constant independent on the increase in the degree of functionalization. On the other hand, PG-T-n polymers exhibited a higher cytotoxicity. Specifically, for the PG-T-1 derivative the cell viability was 80%, while for the PG-T-2 derivative it reached 40% at the maximium concentration tested (200 μg/ml). These results demonstrate the enhanced safety and biocompatibility of the quaternized polymers (Kainthan et al., 2006a,b) and suggest their potential as gene delivery carriers for further *in vivo* applications.

In conclusion, a series of quaternary or tertiary amino group functional polyether polyol derivatives were synthesized and their complexes with pDNA were characterized and investigated for their potential application as gene delivery vectors. Quaternized derivatives yield large polyplexes that exhibit similar transfection efficiency compared to that of PEI, while limited cytotoxicity in mammalian cells rendered these dendritic derivatives more attractive gene delivery carriers. Variation in the degree of quaternization of the parent dendritic polymer affects the transfection efficiency and cytotoxicity of the derivatives obtained.

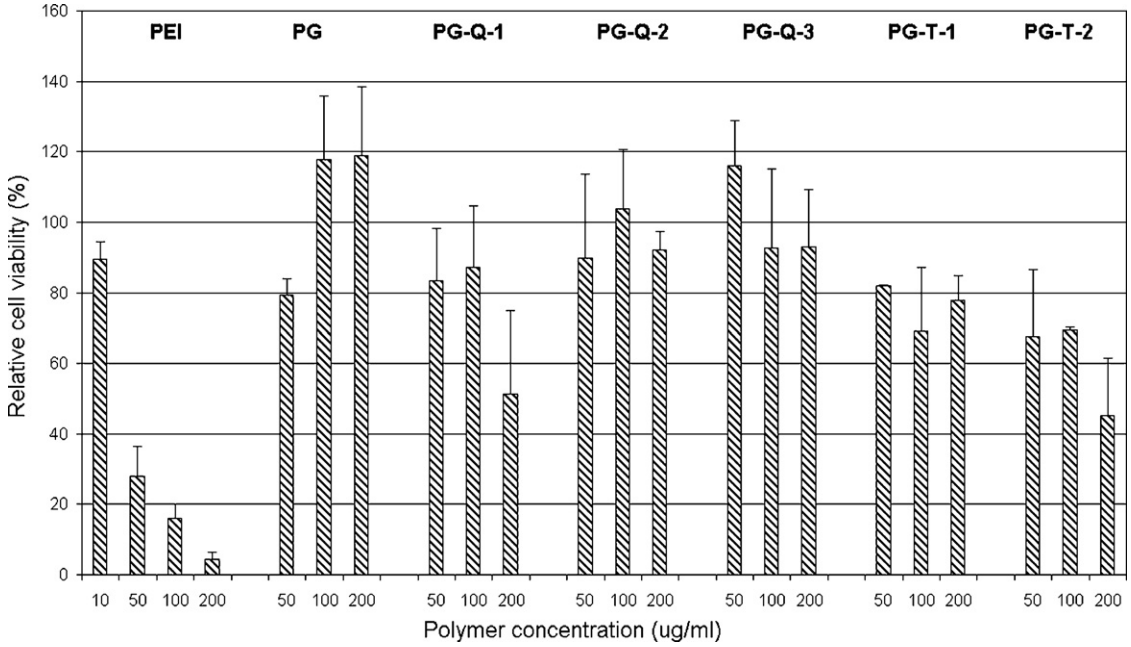


Fig. 6. Cytotoxicity assay in HEK 293 cells by MTT assay. Relative cell viability (%) was calculated according to the following equation: relative cell viability (%) = [(A_{570} of polymer-treated cells − A_{570} of blank)/(A_{570} of non-treated cells − A_{570} of blank)] × 100. Data are represented as the mean ± S.D. (n = 3).

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