

In-vitro transfection potential of fluorinated G5 PAMAM dendrimers for miRNA delivery to MRC-5 cells

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ABSTRACT

Objectives. MicroRNAs (miRNAs) are involved in the regulation of most biological processes and also contribute to many types of disease. Fibroblast cells, such as MRC-5, are often used in biological researches utilizing cell transfection methods due to their difficult to transfect nature. Cells can be genetically engineered by using viral and non-viral methods. Poly(amidoamine) (PAMAM) dendrimers are very promising alternative as a delivery vehicle due to their well-defined characteristics. In this study, in vitro transfection potential of cystamine core generation five (G5) PAMAM dendrimers fluorinated with 2,3,4,5,6-pentafluorobenzoic acid (PFB) and pentafluoropropionic acid (PFP) for miRNA delivery to MRC-5 cells was examined. **Methods.** Spectroscopic techniques were used in the characterization of the prepared dendrimers. miRNA binding and condensation capability of dendrimers was examined by gel retardation assay. Characterization of dendriplexes was made by zeta potential, particle size measurements and transmission electron microscopy. Transfection efficiencies of the dendriplexes were determined by flow cytometry and intracytoplasmic distribution of the dendriplexes was shown by laser scanning confocal microscopy. Also, quantitative structure-activity relationship and molecular docking calculations were used to be able to discuss transfection efficiencies of the dendriplexes into the cell. **Results.** While high level of viability on MRC-5 cells was observed for dendriplexes prepared with PFB and PFP, transfection efficiency with PFP was higher than PFB. Transfection efficiency difference between these two compounds was attributed to their molecular structures. **Conclusions.** Obtained results hold promise for the usage of these compounds as a transfection reagent at MRC-5 cells. Further studies are needed to support these findings.

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Keywords: Cystamine-G5-PAMAM, pentafluorobenzoic acid, pentafluoropropionic acid, miRNA, MRC-5

Introduction

MicroRNAs (miRNAs) are one of the RNA-interference based therapeutics vehicles that will be able to be effective during the genetic engineering

process if they successfully transfected into the cell [1, 2]. However, direct administration of miRNAs in vitro or in vivo is not efficient due to their low cellular

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internalization and enzymatic degradation [3]. Researches towards to the development of efficient nucleic acid carriers, transfection reagents, are being carried out in order to bypass these drawbacks.

Poly(amidoamine) (PAMAM) dendrimers are cationic, hyper-branched, highly symmetric, nano-sized, three-dimensional macromolecules with well-defined structure [4]. Their chemical homogeneity and presence of multiple surface groups suitable for the binding of different target molecules makes them potential candidates for medical or biological applications [5, 6]. Polycationic dendrimers such as PAMAM possess primary amine groups at the surface, which participate in the DNA/small interfering RNA (siRNA) binding process and increase their cellular uptake by transforming the entire complex into nanoscale polyplexes. Also, bioreducible PAMAMs such as prepared by N,N'-dimethylcystamine (DMC) or N,N'-cystaminebisacrylamide (CBA) can provide selective intracellular release of nucleic acids [6-10]. However, these highly efficient delivery systems have been less explored for miRNA delivery [11, 12].

Despite mentioned characteristics of the dendrimers, their transfection efficiencies remain relatively low when compared to the viral vectors. For this reason, studies towards to increase transfection efficiency and biocompatibility of these polymers are being conducted. Fluorination is one of the methods used for this purpose. Fluorination improves the affinity of dendrimers to the cell membrane and also makes them able to cross the lipid bilayer of the cell membrane, as well as the endosome/lysosome membrane [13, 14].

In this study, in vitro transfection potential of fluorinated generation five (G5) PAMAM dendrimers for miRNA delivery to MRC-5 cells has been investigated. Cystamine core G5 PAMAM dendrimers were modified with 2,3,4,5,6-pentafluorobenzoic acid (PFB) and pentafluoropropionic acid (PFP) and these dendrimers were used for miRNA delivery to MRC-5 cells. In addition, the effect of structural differences of fluoro compounds over transfection efficiency was discussed by molecular docking calculations.

Methods

Materials

2-mercaptoethanol, cystamine dihydrochloride, ethylenediamine, ethyl acrylate, triethylamine,

2,3,4,5,6-pentafluorobenzoic acid, pentafluoropropionic acid and methanol were purchased from Sigma-Aldrich (Germany). The chemicals were used as received without further purification.

Synthesis and characterization of dendrimers

G5 PAMAM: Divergent method described previously was slightly modified for the synthesis of cystamine-core amine-terminated G5 PAMAM dendrimer [15]. During the synthesis, azeotropic evaporation, high vacuum or ultrafiltration with MWCO of 3000 and 10000 Da methods were used for product purifications. The products were characterized by ¹³C-NMR and FT-IR at the end of each step of the synthesis and also ¹H-NMR, ESI-MS and MALDI-TOF-MS techniques were used when required.

Fluorinated G5 PAMAM: Fluorinated G5 PAMAM dendrimers were synthesized by adding methanolic solutions of 32 equivalent-grams PFB and PFP into methanolic solution of 1 equivalent-gram G5 PAMAM dendrimer and the mixtures stirred at room temperature for 48 h. The products were lyophilized to obtain fluorinated dendrimers as pale-yellow gels. The products were characterized by ¹⁹F-NMR.

Preparation and characterization of the fluorinated dendrimer/miRNA dendriplexes

Fluorinated dendrimers and miRNA (miRIDIAN microRNA Mimic Negative Control #1, Dharmacon) were mixed in DNase/RNase free water at different w/w ratios; PFB: 3.75:1, 7.5:1, 15:1, 20:1, 40:1, 80:1, PFP: 0.7:1, 1.4:1, 3.5:1, 7:1, 14:1, 21:1. The dendriplexes were incubated for 30-60 min before characterization.

miRNA binding and condensation capability of the fluorinated dendrimers were evaluated by agarose gel retardation assay. Gel electrophoresis experiments were performed using 4.5-5% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide in a 1× Tris-boric acid-EDTA (TBE) buffer solution. The samples were run at 70 V for 60-75 min and the bands were visualized under UV illumination by UVIngenius LHR Gel Imaging System.

The zeta-potential and size analyses were performed by preparing dendriplexes at 1×, 3× and 6× ratio of dendrimer/miRNA (w/w). 1×dendrimer/miRNA (w/w) ratio was determined by examining gel retardation assay results. Dendriplexes were prepared approximately 1h before the measurement and just prior to measurement mixed in

ultrapure water, subsequently filtered through a Millex-AP Syringe Filter (EMD Millipore). The zeta-potential and size of the dendriplexes were measured by using Zetasizer Nano ZS-90 (Malvern Instruments Ltd, UK) at 25 °C in the folded capillary zeta cell (Malvern) and disposable polystyrene cuvettes, respectively.

Transmission electron microscopy (TEM) images were also obtained in order to examine morphology and size of the dendriplexes. 6×dendrimer/miRNA (w/w) dendriplexes were prepared in DNase/RNase free water at room temperature and incubated for 30-60 min. For TEM observation 10 µl of the prepared dendriplex solution was dropped on the carbon-coated copper grid and dried for 60-90 min at room temperature. TEM images of the dried specimens were obtained by using FEI Tecnai G2 220 kV transmission electron microscope at an acceleration voltage of 120 kV.

Cell culture and miRNA transfection

MRC-5 (human lung fibroblast cell line, ATCC® CCL-171™) cells were used to evaluate the transfection efficiency and cytotoxicity of the synthesized fluorinated dendrimers. The cells were cultured in 0.1% gelatin-coated plates and maintained in FibroGRO™ Complete Media Kit (EMD Millipore) at 37 °C and 5% CO₂. No antibiotics were added to the culture media. Cells were passaged with TrypLE™ Express Enzyme (Gibco).

The transfection experiments were performed with Dy547-labelled miRNA (miRIDIAN microRNA Mimic Transfection Control with Dy547, Dharmacon). MRC-5 cells were cultured in 0.1% gelatin-coated 24-well plates 24 h prior to the transfection. 10 pmol miRNA was mixed with fluorinated dendrimers at 1x, 3x and 6x dendrimer/miRNA (w/w) ratios and the dendriplexes were incubated for 30-60 min. Prepared dendriplexes were dropped to the wells and incubated for 8 h. Then, the media were replaced with fresh media and the cells were incubated for further 48 h at 37 °C and 5% CO₂.

Transfection efficiencies of the fluorinated dendrimer/miRNA dendriplexes

Transfection efficiencies were evaluated by flow cytometer analysis and also intracytoplasmic localization of the fluorinated dendrimer/miRNA dendriplexes were observed by confocal laser scanning microscopy. The transfection experiments were conducted as described above.

At the end of 8 h transfection period the media were removed. After washing with PBS twice, the cells were detached with TrypLE™ Express Enzyme and then centrifuged for 4-5 min at 200 g. The cells were resuspended in PBS and analyzed by using CytoFLEX Flow Cytometer and CytExpert 1.2 software.

For confocal imaging, at the end of incubation period the media were removed, and the cells fixed with 4% paraformaldehyde solution in PBS for 15 min at 37 °C. Then, cells were stained with 5 µg/ml WGA conjugate solution in PBS and 1:4000 Hoechst 33342 dye. Images were obtained by using LSM 780 NLO Multi Photon and Confocal Microscope (Zeiss, Germany).

Cytotoxicity of the fluorinated dendrimer/miRNA dendriplexes

Cytotoxicity of the transfections made by the fluorinated dendrimer/miRNA dendriplexes was evaluated by XTT [2,3-Bis(2-methoxy-4-nitro-5-sulfonyl)-2H-tetrazolium-5-carboxanilide inner salt] method. Cell proliferation kit, XTT based (Roche) was used according to the instructions of the manufacturer. Briefly, MRC-5 cells were cultured in 96-well plates at a density around 6×10³ cells per well overnight. The transfections were conducted as described above. At the end of 8 h transfection period, the media were removed, and the cells were incubated for further 48 h at 37 °C and 5% CO₂. Then, reaction solution containing XTT reagent and activation reagent was added to the wells and incubated for further 8 h at 37 °C and 5% CO₂. Absorbance of the each well was detected at 450 nm by a Synergy H1 Hybrid Multi-Mode Reader.

Molecular docking calculations

In order to get optimized structure of G5-PFB and G5-PFP, molecular mechanics calculations were performed by Polak-Ribiere algorithm (conjugated gradient) with root mean square (RMS) gradient of 0.010 kcal/(Åmol). Duplex miRNA was generated by Nucleic Acid Builder (<http://casegroup.rutgers.edu/>). In order to calculate atomic contact energy (ACE) values PatchDock Beta 1.3 Version program was used (<https://bioinfo3d.cs.tau.ac.il/PatchDock>). A RMS deviation tolerance for each docking was set at 8.0 Å. The remaining parameters were set as default. Quantitative structure-activity relationship (QSAR) calculations were used to obtain topological polar surface area (TPSA) values. ACE and TPSA values were evaluated for comparisons.

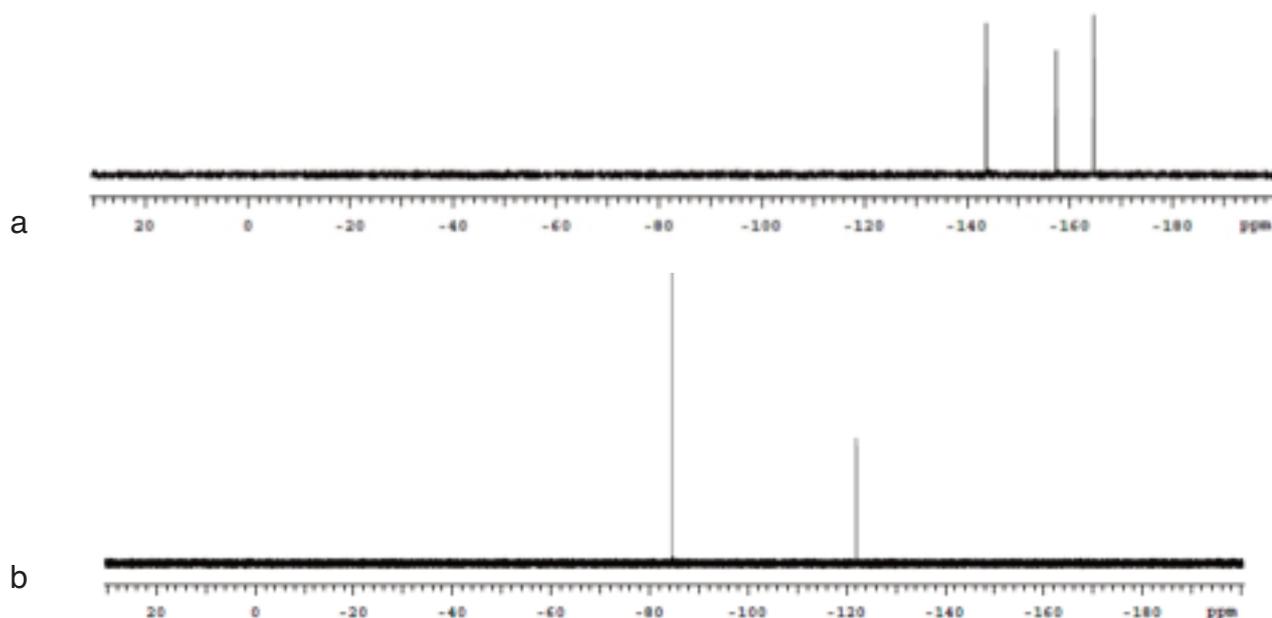


Figure 1. ^{19}F -NMR spectra of G5-PFB (a) δ ppm, CDCl_3 : -164.61, -157.22, -143.66 and G5-PFP (b) δ ppm, CDCl_3 : -121.95, -84.56.

Statistical Analysis

The data were given as mean (SD) and analyzed by One Way ANOVA Test (with Bonferroni Corrected).

Results

Synthesis and characterization of dendrimers

Synthesis of G5 PAMAM was performed by adding methanolic solutions of methylacrylate and ethylenediamine into cystamine until to get to G5 by iterative reactions of Michael addition and exhaustive amidation. Presence of the $-\text{NH}_2$ and other functional groups on the G5 PAMAM dendrimer surface and $-\text{CH}_2$ -groups on the dendrons were confirmed by FT-IR and ^{13}C -NMR analysis, respectively. FT-IR (ATR, 4000-450 cm^{-1}): 3270 (o), 3050 (o), 2925 (o),

2835 (o), 1650 (k), 1556 (k), 1460 (k), 1150 (o), 1030(o). ^{13}C -NMR (D_2O , 400 MHz, δ ppm): 32.6, 37.8, 38.7, 41.8, 42.3, 47.9, 49.1, 51.5, 177.3.

PFB and PFP were conjugated to the surface of G5 PAMAM dendrimer using an addition reaction. The number of PFB and PFP moieties modified on each G5 dendrimer was calculated using ^{19}F -NMR analysis (Figure 1). 2,2,2-trifluoroethanol was used as an internal standard. For both fluorinated dendrimer, 25% of the $-\text{NH}_2$ groups on the surface were found to be fluorinated.

Characterization of dendriplexes

The ability of fluorinated dendrimers to complex with miRNA as a function of dendrimer/miRNA weight ratio was evaluated by gel retardation assay. The amount of miRNA was kept constant at 10 pmol. As shown in Figure 2, complete complexation of

Table 1. Size and zeta potential of dendrimer/miRNA dendriplexes

Dendrimer	w/w	Z-Average, (d.nm)	Zeta potential, (mV \pm SD)
G5-PFB	1 \times	223.5	36.4 \pm 16.1
	3 \times	199.2	60.5 \pm 8.49
	6 \times	191.2	76.1 \pm 10.7
G5-PFP	1 \times	256.6	53.4 \pm 9.33
	3 \times	201.3	52.3 \pm 20.7
	6 \times	192.5	78.8 \pm 12.4

G5 = generation five, PFB = pentafluorobenzoic acid, PFP = pentafluoropropionic acid

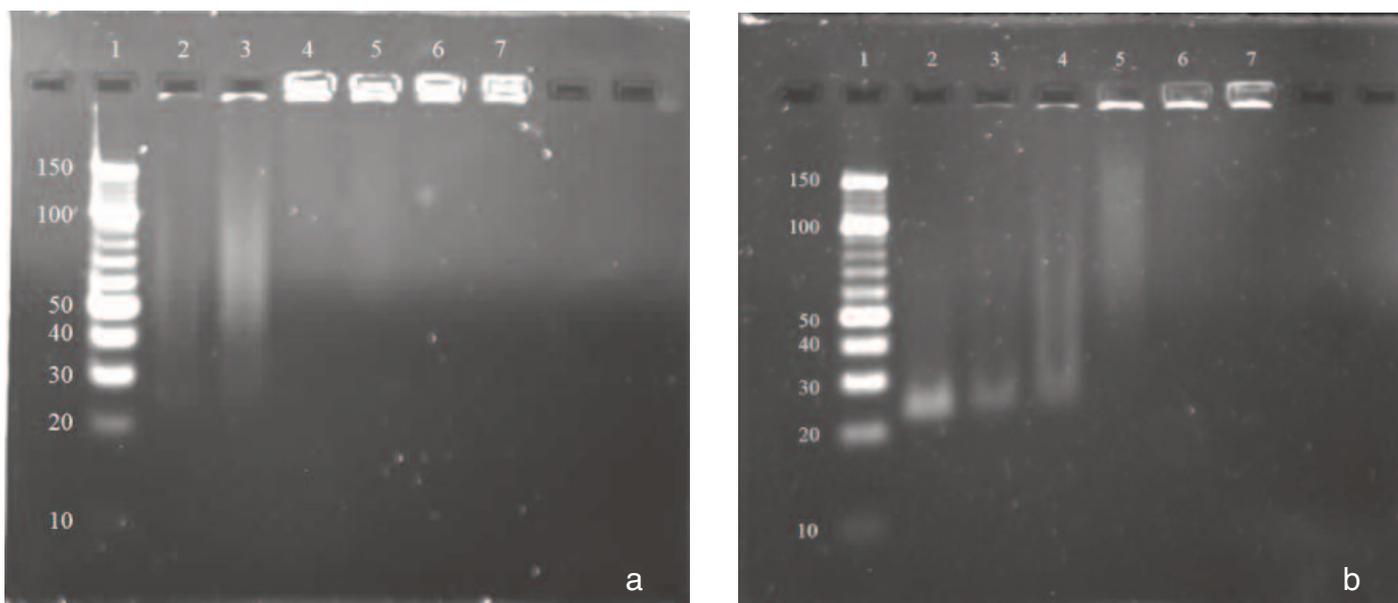


Figure 2. Agarose gel retardation assay of dendriplexes (a) G5-PFB/miRNA (w/w): Line 1- 10 bp DNA Ladder; 2- 3.75:1; 3- 7.5:1; 4- 15:1; 5- 20:1; 6- 40:1; 7-, 80:1; (b) G5-PFP/miRNA (w/w): Line 1- 10 bp DNA Ladder; 2- 0.7:1; 3- 1.4:1; 4- 3.5:1; 5- 7:1; 6- 14:1; 7-21:1.

miRNA was observed for G5-PFB and G5-PFP at a w/w ratio of 20 and 14, respectively.

After having demonstrated that fluorinated dendrimers are able to complex with miRNA, the characteristics of these dendriplexes at 1×, 3× and 6× dendrimer/miRNA (w/w) ratios were evaluated by size and zeta potential measurements. The results of size and zeta potential analysis for dendriplexes of G5-PFB and G5-PFP with miRNA are given in Table 1. Particle size analysis showed the formation of ~200 nm dendriplexes. Despite the slight changes of size with the w/w ratio, evaluation of the surface charges of the dendriplexes showed the significant increases with

increasing w/w ratios. The highest charge was observed at w/w ratio of 6× which was 76-78 mV for both G5-PFB and G5-PFP.

Since the method used for particle size measurement, which is dynamic light scattering, detects light scattering and does not measure real particle size, morphology and the particle size of dendriplexes were also evaluated by TEM. As shown in Figure 3, dendriplexes formed spherical structures with mean particle size between 120-190 nm and 80-130 nm for G5-PFB and G5-PFP, respectively.

Cytotoxicity of dendriplexes

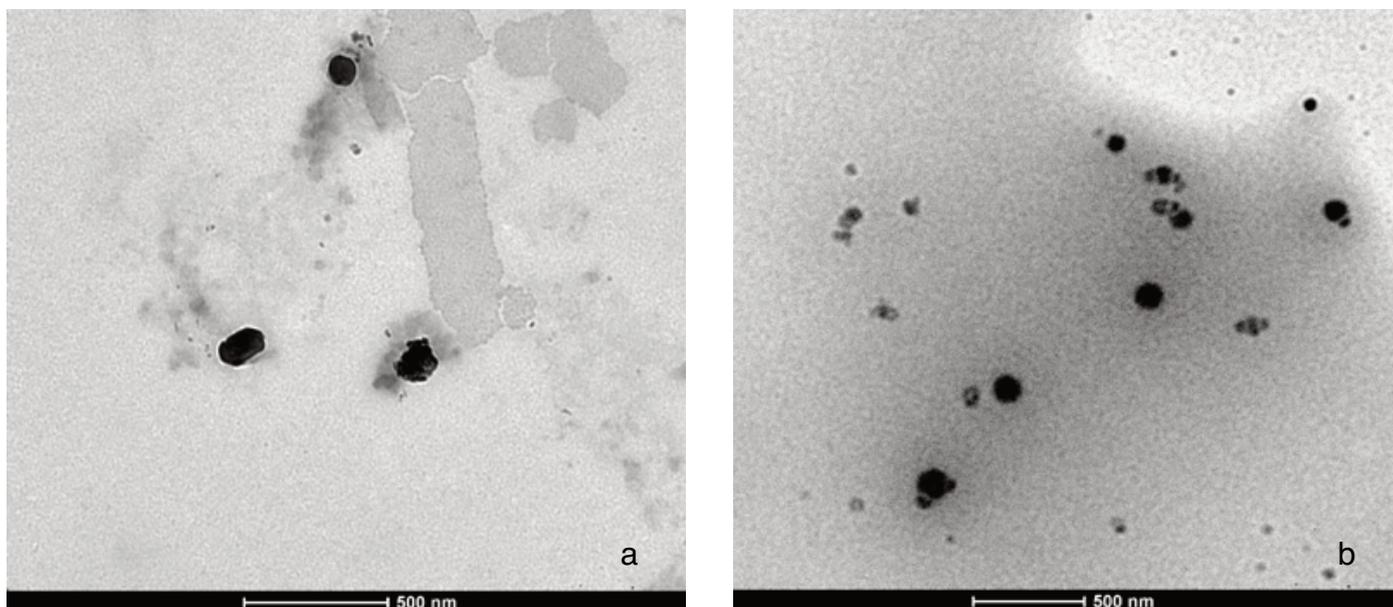


Figure 3. TEM images of G5-PFB/miRNA (a) and G5-PFP/miRNA (b) dendriplexes.

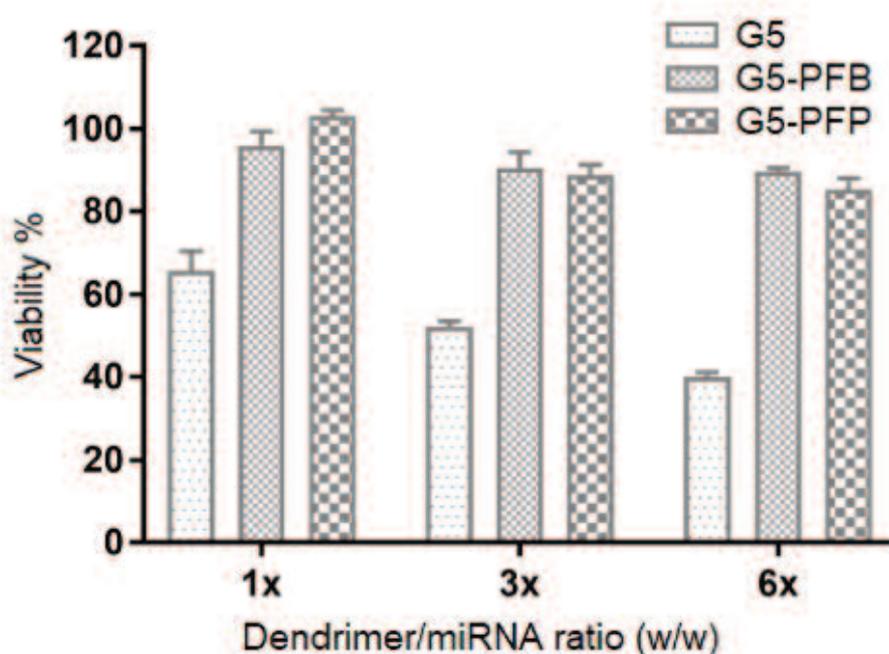


Figure 4. Cytotoxicity of dendrimer/miRNA dendriplexes.

The cytotoxicity of G5-miRNA, G5-PFB/miRNA and G5-PFP/miRNA dendriplexes in MRC-5 cells was evaluated by XTT assay. As seen in Figure 4, fluorination has increased the viability and both of the fluorinated dendrimers showed minimal cytotoxicity on MRC-5 cells. When G5-PFP and G5-PFB compared with each other, while there was not a statistical significance for 3× ratio ($p = 0.438$), there was a statistical significance for 1× ($p = 0.003$) and 6× ($p = 0.004$).

Transfection efficiencies of dendriplexes

Dy547-labeled miRNA uptake by G5-PFB and G5-PFP dendrimers was evaluated by flow cytometry. The percentage of Dy547 positive cells was used as a measure of transfection efficiency. G5-PFB, showed 4.57%, 44.5% and 68.6% efficiency for w/w ratios of 1×, 3× and 6×, respectively. For G5-PFP, transfection efficiencies were 8.77%, 75.4% and 90.7% for w/w ratios of 1×, 3× and 6×, respectively (Figure 5). G5-

PFP showed superior efficiency than G5-PFB as a transfection reagent.

In order to show cellular distribution of the miRNAs delivered by fluorinated dendrimers confocal imaging was also performed. Images were obtained for dendriplexes at 6× dendrimer/miRNA (w/w) ratio. As seen in Figure 6, bright green fluorescent signals of Dy547 were localized to cytoplasm.

Effect of structural differences over transfection efficiency

To compare two fluorinated dendrimers with each other Patchdock tool was used. Patchdock is an algorithm for molecular docking that identifies docking transformations, molecular shape complementarities and can also perform clustering and calculates the global binding energy [16, 17]. TPSA and ACE values obtained from QSAR and molecular docking calculations are given in Table 2. TPSA values of the both dendriplexes were so close to each

Table 2. The QSAR and docking results of dendriplexes

Dendriplex	Pose Score	ACE (kcal/mol)	TPSA (\AA^2)
G5-PFB/miRNA	15228	-2815.46	12041
G5-PFP/miRNA	14840	-2099.33	12054

ACE = atomic contact energy, G5 = generation five, miRNA = microRNA, QSAR = quantitative structure-activity relationship, PFB = pentafluorobenzoic acid, PFP = pentafluoropropionic acid, TPSA = topological polar surface area

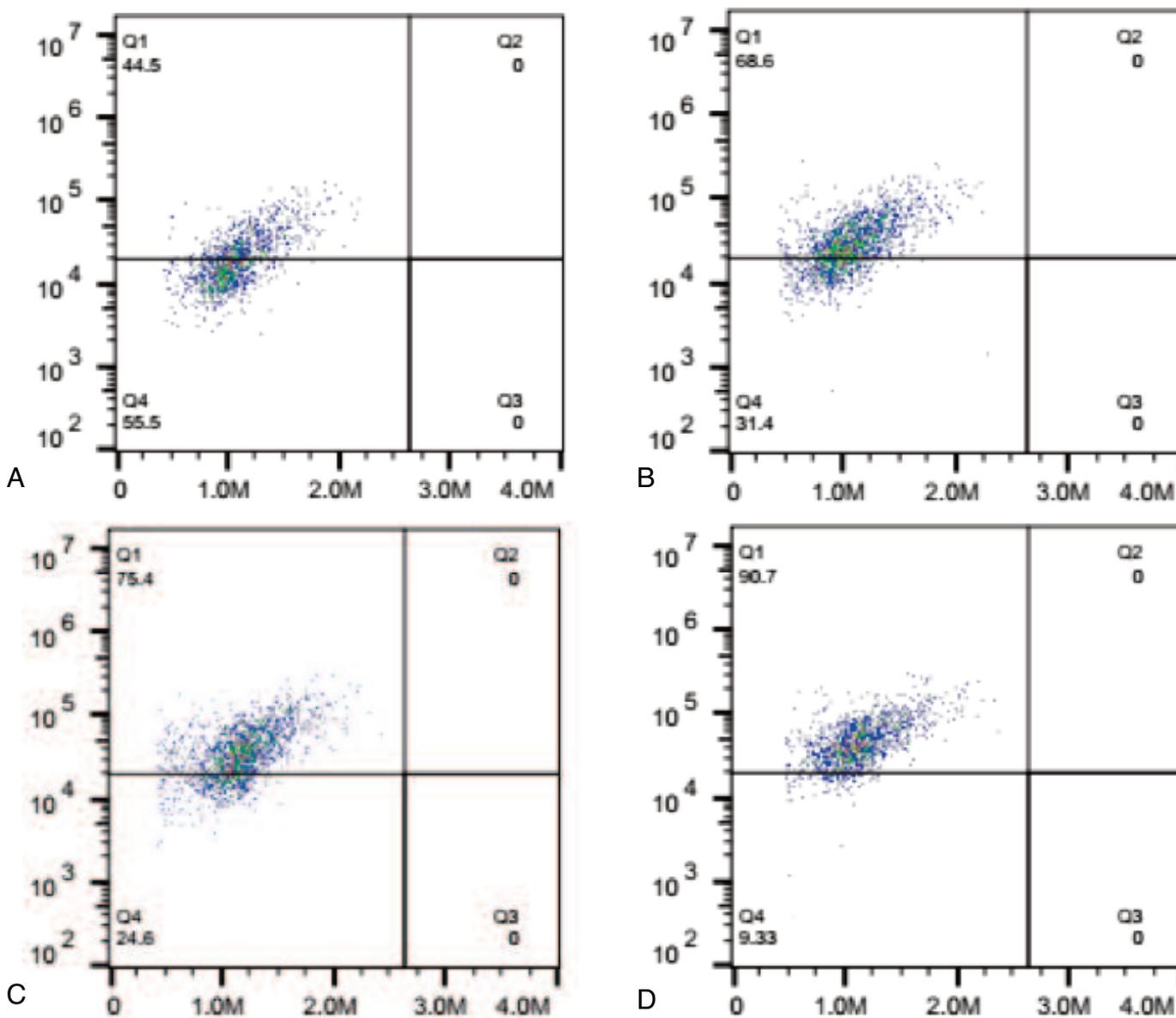


Figure 5. Transfection efficiencies of G5-PFB 3× (a), G5-PFB 6× (b), G5-PFP 3× (c) and G5-PFB 6× (d).

other. G5-PFP showed good ACE, -2099 kcal/mol, with the miRNA than G5-PFB. This means that, G5-PFP formed stronger complexation with miRNA and therefore G5-PFP/miRNA dendriplex achieved high transfection efficiency than G5-PFB/miRNA. This difference was attributed to the molecular structure of the fluoro-compounds (Figure 7).

Discussion

In this study, aliphatic (PFP) and aromatic (PFB) two fluoro compounds were used for PAMAM dendrimer modifications. When the structures given in Figure 7 taken into the account, due to chemical environment differences of the fluorines, two and three peaks are expected in the ^{19}F -NMR spectrums of the PFP and

PFB, respectively. The ^{19}F -NMR spectra of the products confirmed the addition of PFB and PFP to the surface of G5 PAMAM.

Gel retardation analysis showed the fluorinated dendrimers miRNA complexation ability. Size measurement and TEM analysis also revealed that G5-PFB and G5-PFP can condense the miRNA in to the dendriplexes below 200 nm at w/w ratios above 1×. Size of the dendriplexes is one of the factors for effective delivery of miRNA/siRNA to the cells. Studies suggest that polyplexes below or as large as 200 nm are the optimal size for the non-viral vectors because they can pass through the cell membrane by receptor-mediated endocytosis [18-20]. Zeta potential measurements of dendriplexes showed significant increases with increasing dendrimer concentration. It was 76-78 mV for G5-PFB and G5-PFP at w/w ratio

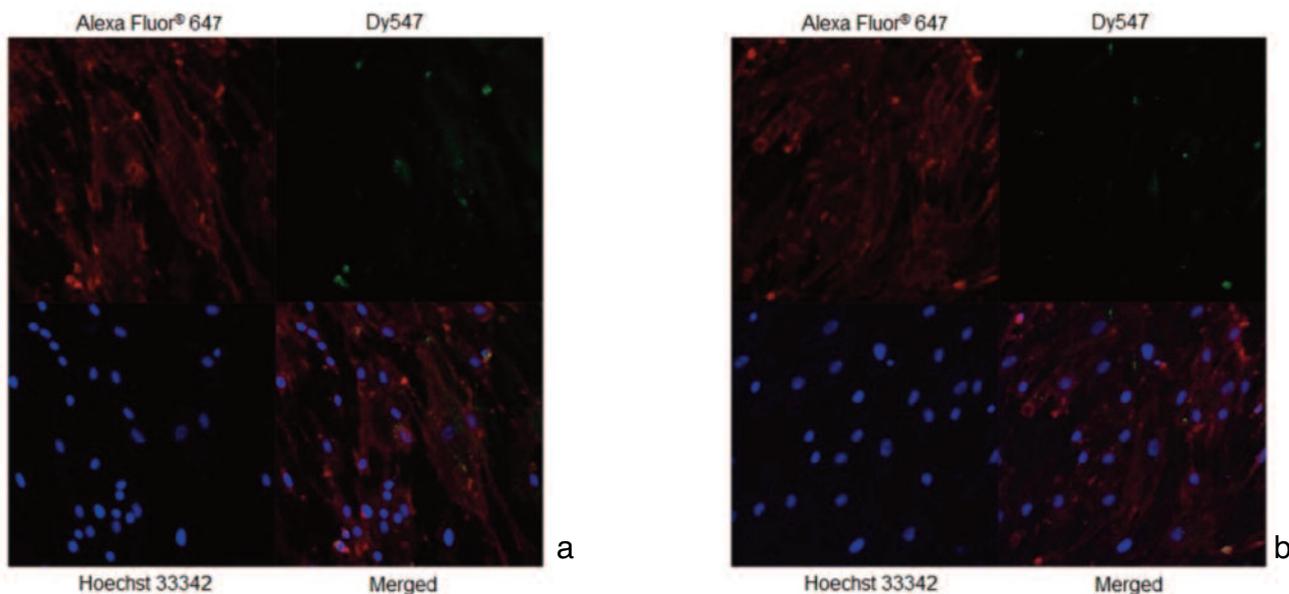


Figure 6. Confocal images of MRC-5 cells transfected with G5-PFB/miRNA (a) and G5-PFP/miRNA (b) dendriplexes. Plasma membranes were stained WGA Alexa Fluor® 647 conjugate and the nuclei were stained with Hoechst 33342. miRNA was labeled with Dy547.

of 6×. Zeta potential is a measure of the effective electric charge on the nanoparticle surface and provides information about particle stability. The higher magnitude of zeta potentials exhibits increased stability and protects them from aggregation. Positive charge of particles also facilitates the transport of target into the cell and increases their solubility in the aqueous environment [21, 22].

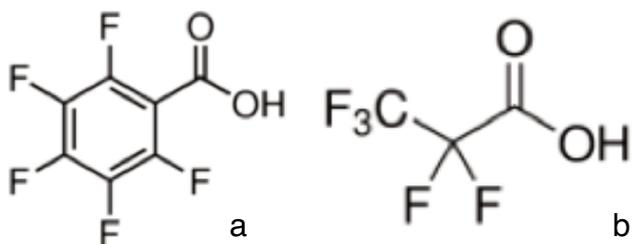


Figure 7. Structures of PFB (a) and PFP (b) molecules.

The relation between surface cationic charges and cytotoxicity is well known and has been mentioned for various cell lines in culture [23, 24]. In our results, similar relation was observed for MRC-5 cells and reduction of the surface cationic charges with fluorination has increased the cell viability. Despite dose-dependent cytotoxicity by increasing dendrimer concentrations, the cells treated with all dendriplexes showed cell viability above 85%.

To evaluate the transfection efficiencies of prepared fluorinated dendrimers, flow cytometry analysis was conducted with Dy547-labeled miRNAs. Results showed dose-dependent efficiency increase

with increasing dendrimer concentrations. It was 68.6% and 90.7% at w/w ratio of 6× for G5-PFB and G5-PFP, respectively. To explain the difference in efficiency of fluorinated dendrimers molecular docking and QSAR calculations were used. Proximity of TPSA values of dendriplexes points out their almost equality of cationic surface charges and smooth penetration capability into the cell. High pose rate and low ACE value of G5-PFP/miRNA dendriplex shows its highly stable complexation with miRNA (Table 2). On the other hand, aliphatic structure of G5-PFP leads less intramolecular interactions than aromatic structured G5-PFB. Therefore, it was concluded that higher transfection efficiency of G5-PFP was because of its structure and more stable complexation with miRNA.

Conclusions

High transfection efficiency and low cytotoxicity during transfection are the two main factors influencing cationic polymer mediated-nucleotides delivery [25]. In this study, two fluorinated dendrimers were synthesized and their miRNA delivery efficiencies were evaluated on MRC-5 cells. The fluorinated PAMAM dendrimers showed efficient miRNA complexation ability and high cell viability above 85%. In addition, PAMAM dendrimer fluorinated with PFP performed superior transfection efficiency than PFB modified. It was concluded that

fluoroaliphatic compounds such as PFP can be used for surface modifications of dendrimers. Also, the structure-function relationship seen in this study will be helpful for other dendrimer modification studies in the future.

Authorship declaration

All authors listed meet the authorship criteria according to the latest guidelines of the International Committee of Medical Journal Editors, and all authors are in agreement with the manuscript.

Author contributions

A.O. performed dendriplex characterization, cytotoxicity, transfection efficiency experiments, data analysis and also designed the study and wrote the manuscript. H.N. performed the synthesis and characterization of dendrimers, made the theoretical calculations and wrote the manuscript.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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