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Pentafluoropropionic Anhydride Functionalized PAMAM Dendrimer as miRNA Delivery Reagent

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Abstract: Poly(amidoamine) (PAMAM) dendrimers are good candidates for nucleic acid delivery with their well-defined characteristics. MicroRNA mediated regulation of biological process is also active an area of investigation. Fibroblast cells, such as MRC-5, are one of the cell lines used in biological researches due to their hard to transfect nature. In this two-staged study, cystamine core generation 5 PAMAM dendrimers were synthesized and fluorinated with pentafluoropropionic anhydride and subsequently tested as miRNA delivery reagent on MRC-5 cells. Effect of fluorination against to naked generation 5 dendrimer on transfection efficiency was also investigated by molecular docking and quantitative structure-activity relationship calculations. Structural characterization of the synthesized dendrimers was verified by spectroscopic techniques. Gel retardation assay, particle size and transmission electron microscopy results demonstrated polyplex formation of fluorinated dendrimers with miRNA at nanoscale level. Zeta potential values indicated non-aggregation and increased stability of the polyplexes. Prepared polyplexes with fluorinated dendrimer showed over 90% cell viability and transfection efficiency. In silico calculations confirmed the stable complexation with miRNA and good penetration capability into the cell.

Keywords: Cystamine core, PAMAM dendrimer, pentafluoropropionic anhydride, microRNA, MRC-5.

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INTRODUCTION

PAMAM [poly(amidoamine)] dendrimers are well defined nano-sized architectural macromolecules and they are potential candidates for nanoscale nucleic acid delivery vehicles (1, 2). PAMAM dendrimers transform nucleic acid-dendrimer complex into nanoscale dendriplexes with its primary amine terminal groups at the periphery. Dimethyl- or bisacrylamide-cystamine as a core initiator provides bioreducibility of PAMAM dendrimers and selective intracellular release of nucleic acids (2-4). Since the cytotoxicity of naked dendrimers is increased with its higher generations, despite increase in their transfection efficiency, studies are being conducted in order to increase biocompatibility and transfection efficiency of PAMAM dendrimers. One of the strategies used in this context is fluorination and it increases dendrimer affinity to the lipid bilayer and enables them to get across cell and endosome/lysosome membrane (5, 6).

MicroRNAs (miRNAs) are remarkable candidates for the RNA-interference based therapeutic approach if they are successfully delivered to the target (7, 8). However, the major drawbacks for direct miRNA administration in vitro or in vivo are low cellular internalization and enzymatic degradation (9). Researches to overcome these obstacles and to develop efficient nucleic acid delivery agents are being conducted.

In literature, pentafluoropropionic anhydride (PA) and heptafluorobutyric anhydride modified PAMAM dendrimers have been tested in HEK293, HeLa, NIH3T3, COS-7, CHO and primary mouse mesenchymal stem cells for DNA and siRNA delivery and promising results have been reported (5, 10, 11). In this study, cystamine

core generation 5 (G5) PAMAM dendrimers were synthesized and fluorinated with PA and subsequently cytotoxicity and transfection efficiency of the prepared polyplexes were examined on MRC-5 cells which are known with their hard-to-transfect nature. Also, the effect of fluorination on polyplex formation was evaluated by molecular docking and quantitative structure-activity relationship (QSAR) calculations.

EXPERIMENTAL

Preparation of G5 PAMAM dendrimer

Ethyl acrylate, cystamine dihydrochloride, ethylenediamine, pentafluoropropionic anhydride, trimethylamine, methanol, 2-mercaptoethanol and toluene were purchased from Sigma-Aldrich (Germany) and they were used as received. Cystamine core G5 PAMAM dendrimer was synthesized by using divergent method. Methanolic solutions of methylacrylate and ethylenediamine were added into cystamine according to iterative Michael addition and exhaustive amidation reactions until to get a G5. During the synthesis process azeotropic evaporations were made by using 10:1 (v/v) toluene:methanol mixture and product purification of the half generations were performed by column chromatography on silica gel [Kieselgel 60 (230-400 mesh ASTM); Merck, Germany] and Sephadex LH-20 (GE Healthcare, Sweden) (12, 13).

The products were characterized by $^{13}C\{H\}$-NMR (Varian Mercury 400 MHz NMR Spectrometer, Agilent) and FT-IR (IRAffinity-1 FT-IR Spectrometer, Shimadzu) at the end of each step. Also ^{1}H-NMR (Varian Mercury 400 MHz Spectrometer NMR, Agilent), ESI-MS (Micromass ZQ Mass Spectrometer with 2695 HPLC Separations Module, Waters) and MALDI-TOF-MS (MALDI Synapt G2-Si High Definition Mass Spectrometry, Waters) were used when required. PAMAM G5: FT-IR (ATR, 4000-450 cm^{-1}): 3338 (m), 3275 (m), 3190 (m), 3050 (w), 2924 (m), 2834 (m), 1650 (s), 1556 (s), 1460 (s), 1150 (m), 1030 (m); ^{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.

Preparation of fluorinated dendrimers

Fluorinated dendrimers were prepared by adding methanolic solutions (1 mL) of PA (696 µL, 3.52 mmol) into a methanolic solution (1 mL) of G5 PAMAM dendrimer (100 mg, 3.46 µmol) and following the 48 h stirring at room temperature the mixture was dialyzed against distilled water. The products were lyophilized and examined by ^{19}F-NMR. ^{19}F-NMR (CD_3OD, 400 MHz, δ ppm): -84.577, 84.722, 84.898 ($-CF_3$), -122.010, 123.184, 124.468 ($-CF_2$-CO-). Fluorination ratios of the dendrimers were calculated by using integrals of the internal standard (2,2,2-trifluoroethanol; Sigma-Aldrich, Germany). About 25% of the $-NH_2$ groups on the surface of the G5 PAMAM dendrimers was fluorinated with PA.

Polyplexes' preparation and characterization

Different amount of G5-PA and 10 pmol (w/w) cel-miR-67 (Dharmacon, Germany) were mixed in nuclease-free water and incubated at room temperature for 60 min. The polyplexes were subjected to electrophoresis on 4.5-5% (w/v) agarose gels and run at 70 V for 60-75 min to find minimum required dendrimer amount. Polyplexes at 1× (determined from gel retardation results), 3× and 6× ratio of dendrimer/miRNA (w/w) were prepared for zeta-potential and size analyses. Size and zeta-potential of the polyplexes were measured by using Zetasizer Nano ZS-90 (Malvern, UK) at 25 °C in disposable polystyrene cuvettes and folded capillary zeta cell, respectively. Morphology and size of the polyplexes at 6× ratio were also examined by using Tecnai G2 220 kV transmission electron microscope (FEI, USA) at an acceleration voltage of 120 kV.

Cell culture and transfections

MRC-5 (human lung fibroblast cell line, ATCC® CCL-171™) cells were maintained in 0.1% gelatin-coated plates containing FibroGRO™ Complete Media Kit (EMD Millipore, Germany) and no antibiotics at 37 °C and 5% CO_2. TrypLE™ Express Enzyme solution (Gibco, USA) was used for passaging the cells. 10 pmol Dy547-labelled cel-miR-67 (Dharmacon, Germany) was used for transfection. 24 h prior to the transfection MRC-5 cells were cultured and polyplexes prepared at 1×, 3× and 6× ratios were dropped on to the cells. Subsequent to 8 h incubation the media were changed and cells were incubated for additional 48 h.

Cytotoxicity and transfection efficiency of fluorinated dendrimer/miRNA polyplexes

Cell Proliferation Kit, XTT based (Biological Industries, Israel) was used to examine the cytotoxicity of the G5-PA/miRNA polyplexes. Briefly, MRC-5 cells were seeded at a density of 6×10^3 cells/well on 96-well plates and incubated overnight. Transfections were made and following 48 h post-transfection period, the reaction solution containing XTT [2,3-bis(2-methoxy-4-nitro-5-sulfonyl)-2H-tetrazolium-5-carboxanilide inner salt] reagent and PMS (N-methyl dibenzopyrazine methyl sulfate) was added to the wells and incubated for further 8 h at 37 °C and 5% CO_2. Five repeats were conducted for each sample and untransfected cells were used as controls. Absorbance of the each well was measured at 450 nm by a microplate reader (Synergy H1, BioTek, USA). The data were given as mean (SD) and analyzed by One Way ANOVA Test (with Bonferroni Corrected).

Cells were transfected as described above and following 8 h post-transfection period cells were detached with TrypLE™ and centrifuged for 4-5 min at 200 g. Resuspended cells in PBS were analyzed by flow cytometer on a Beckman

Coulter's CytoFLEX in order to evaluate transfection efficiencies. To examine intracytoplasmic localization of the polyplexes confocal imaging was also performed (LSM 780 NLO Multi Photon and Confocal Microscope, Zeiss, Germany). Following to transfections and 48 h post-transfection, cells were fixed with 4% paraformaldehyde solution in PBS (Affymetrix, Germany) for 15 min at 37 °C and then stained with 5 µg/mL wheat germ agglutinin (WGA), Alexa Fluor® 647 conjugate solution (Thermo Fisher, USA) in PBS and 1:4000 Hoechst 33342 (Thermo Fisher, USA) dye.

Molecular docking and QSAR calculations

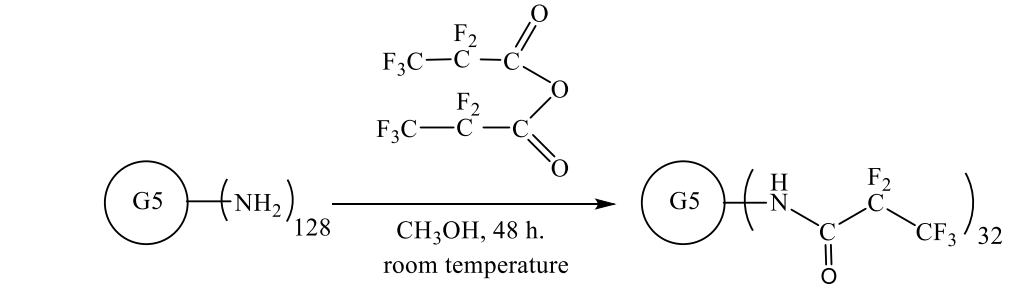
In order to discuss the effect of fluorination on polyplex formation, global energy (binding energy), atomic contact energy (ACE) and topological polar surface area (TPSA) values of the naked and PA modified G5 PAMAM dendrimers were calculated. Molecular mechanics calculations of the fluorinated dendrimers were performed by Polak-Ribiere algorithm (conjugated gradient) with root mean square (RMS) gradient of 0.010 kcal/(Å mol). Duplex miRNA was generated by the Nucleic Acid Builder (http://casegroup.rutgers.edu/). Docking analysis was carried out using PatchDock Beta 1.3 Version program and RMS deviation tolerance for each docking was set at 4.0 Å (14, 15). The refinement of the first 10 docked complexes determined by PatchDock was carried out using FireDock and global energy and ACE values were obtained (16, 17). TPSA values were obtained by QSAR calculations (18).

RESULTS AND DISCUSSION

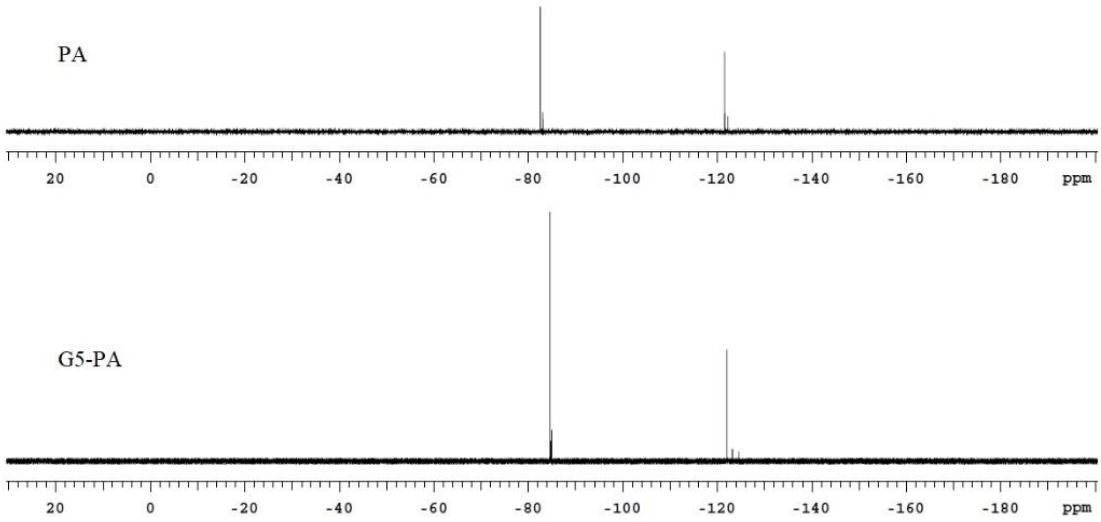
Synthesis and characterization of dendrimers

Due to the symmetric structure of the G5 PAMAM dendrimer, obtained by iterative addition reactions of methyl acrylate and ethylene diamine, FT-IR and ^{13}C-NMR spectra give specific peaks. When the FT-IR values given in the preparation of G5 PAMAM dendrimer section has examined, stretching bands at 3338 cm^{-1} and 3275 cm^{-1}, 3190 cm^{-1} are belong to the -NH and -NH_2, respectively. -CH- and -CH_2- stretching bands forming the skeletal structure of G5 are observed at 2924 cm^{-1} and 2854 cm^{-1}, respectively. Stretching bands of the -CO- group distributed throughout the structure and S-S bending vibrations of the cystamine constituting the dendrimer core appear at 1642 cm^{-1} and 815 cm^{-1}, respectively. -C=O- and -S-CH_2-CH_2-NH_2 specific peaks of the G5 are located at 177.3 ppm and 37.8 ppm in ^{13}C-NMR spectrum.

Fluorination reaction of G5 PAMAM dendrimer and ^{19}F-NMR spectra of the PA and G5-PA are given in Figure 1a and Figure 1b, respectively. When ^{19}F-NMR spectrum of the G5-PA at the Figure 1b is examined, two singlet peaks belonging to the -CF_3- and -CF_2- of the PA are seen at 84.58 ppm and 122.01 ppm with 1.5 ppm and 0.5 ppm shifts, respectively.



a)



b)

Figure 1. a) Fluorination reaction of G5 PAMAM, b) ^{19}F-NMR spectra of PA and G5-PA.

Characterization of polyplexes

Complete miRNA complexation ability was observed for G5-PA dendrimers by gel retardation assay (Figure 2). Size and zeta potentials for three different G5-PA/miRNA (w/w) ratios are given in Table 1. Zeta potential and particle size measurements showed the formation of positively charged and ~200 nm polyplexes. Size is one of the factors effecting polyplex transfection and studies suggest that the optimal size for the non-viral vectors are below 200 nm (19-21). Nanoparticles with zeta potentials of greater than +30 mV are considered strongly cationic and exhibits increased stability. The positive charge of particles also facilitates their permeation from the membranes and increases their solubility in the aqueous environment (22, 23). Dynamic Light Scattering (DLS), is a popular technique and allows particle sizing down to 1 nm diameter but it detects light scattering rather than real particle size. Therefore, information about particle size and morphology of the polyplexes were also obtained by transmission electron microscopy. As seen in Figure 3, spherical polyplexes below 200 nm were formed with G5-PA.

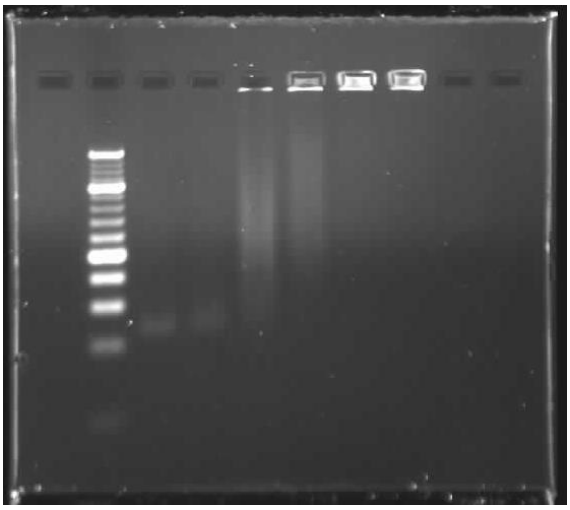


Figure 2. G5-PA/miRNA (w/w) gel retardation assay: Line 1- 10 bp DNA Ladder; 2- 0.7:1; 3- 1.4:1; 4- 3.5:1; 5- 7:1; 6- 14:1; 7- 21:1.

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

Dendrimer	w/w	Z-Average, (d.nm)	Zeta potential, (mV ± SD)
G5	1×	223.0	18.91 ± 3.93
	3×	220.7	28.9 ± 5.89
	6×	190.1	56.1 ± 8.70
G5-PA	1×	237.4	55 ± 8.03
	3×	216.2	80.3 ± 11.2
	6×	207.7	74.6 ± 10.6

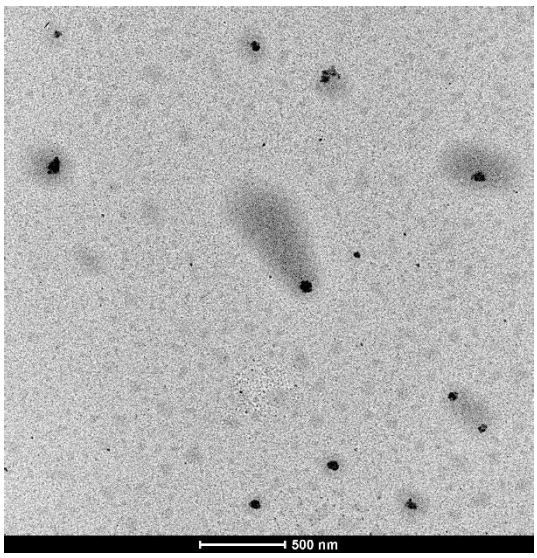


Figure 3: TEM image of G5-PA/miRNA polyplexes.

Cytotoxicity and transfection efficiency of polyplexes

XTT assay was used to evaluate the cytotoxicity of the polyplexes. Cell viability has increased with the fluorination of the dendrimers and G5-PA showed superior viability than the naked G5 (Figure 4). Dose-dependent cytotoxicity was observed with increasing dendrimer concentrations but G5-PA showed cell viability above 90% for all 3 ratios. The percentage of Dy547-labeled miRNA positive cells was used to determine the transfection efficiency of the prepared G5-PA dendrimers. Transfection efficiencies were 71.6% and 98.3% at a w/w ratio of 6× for G5 and G5-PA, respectively (Figure 5). Confocal laser scanning microscopy images were also obtained after transfections in order to check the cellular distribution of the miRNAs. Bright green fluorescent signals of Dy547 were captured and confirmed the intracytoplasmic localization of the miRNAs (Figure 6). Major problem of the transfection reagents is the correlation between efficiency and toxicity and their efficiency and toxicity are cell type dependent. As seen in Figure 4 and 5, G5-PA provided both efficient transfection and above 90% cell viability after miRNA transfection.

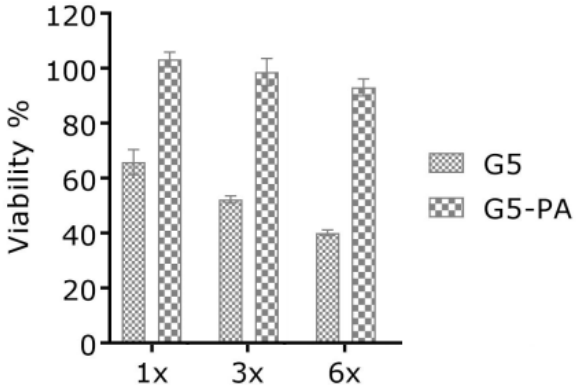


Figure 4: Cytotoxicity of the polyplexes.

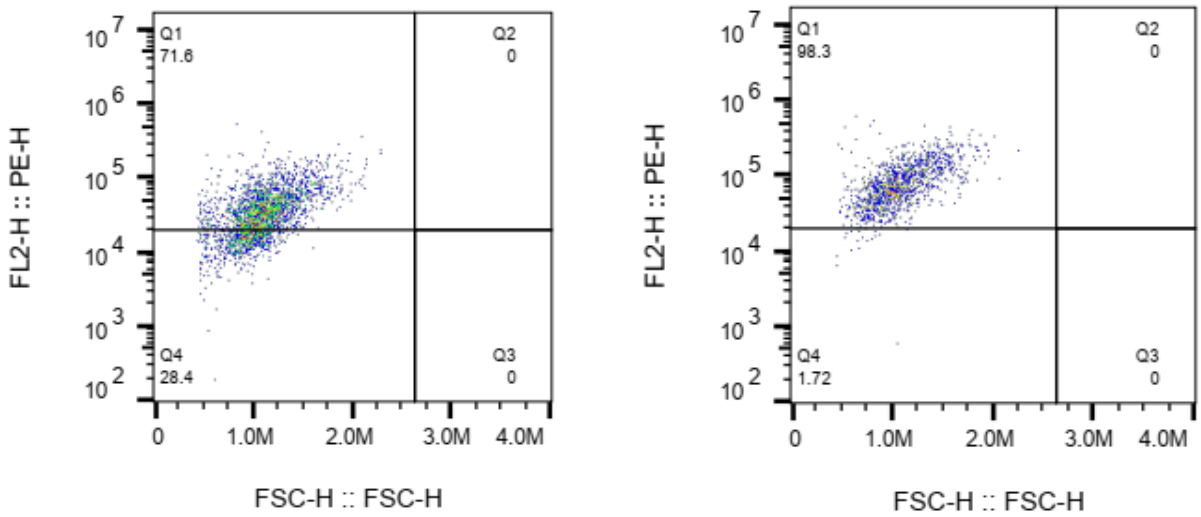


Figure 5: Flow cytometry results of the polyplexes: (Left) G5/miRNA, (Right) G5-PA/miRNA.

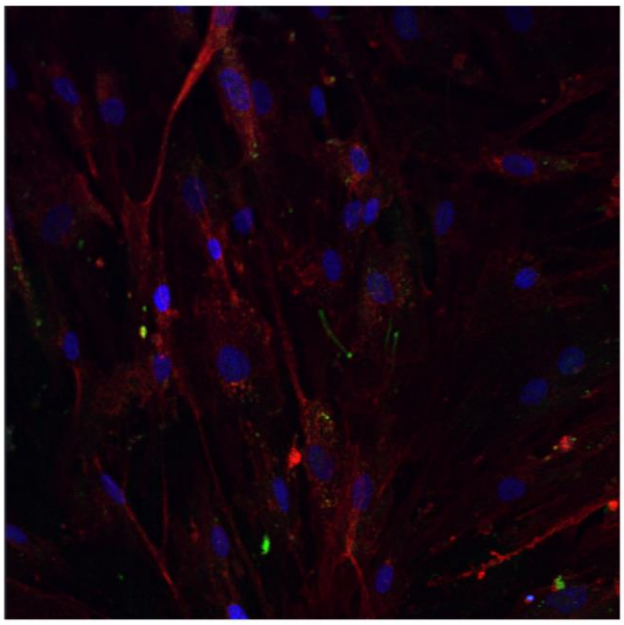


Figure 6: Confocal image of MRC-5 cells transfected with G5-PA/miRNA; Red: plasma membrane stain, WGA Alexa Fluor® 647 conjugate; Blue: nucleus stain, Hoechst 33342; Green: Dy547-labelled miRNA.

In silico calculations for effect of fluorination on transfection

Global energy, ACE, and TPSA values obtained from molecular docking and QSAR calculations are given in Table 2. As seen in Table 2, while G5-PA/miRNA polyplexes showing better ACE (-73.43 kcal/mol) and global binding energy (-198.82 kcal/mol), there was no significant difference between G5/miRNA and G5-PA/miRNA for TPSA values. Low ACE and global binding energy values point out strong and stable dendrimer/miRNA complexation and complex stability seems to arise from van der Walls interactions. TPSA values show that the sum of surfaces of polar atoms in a molecule and high values also indicate smooth penetration capability into the cell. All these directly shows the effect on transfection efficiency.

Table 2. Docking and QSAR results.

	Firedock				QSAR
Polyplex	**Global Energy[a] kcal/mol**	**aVdW[b], kcal/mol**	**rVdW[b], kcal/mol**	**ACE_2[c] kcal/mol**	**TPSA[d], Å²**
G5/miRNA	-21.92	-9.74	1.29	-6.38	11072
G5-PA/miRNA	-198.82	-87.62	46.46	-73.43	10610

[a]Binding energy of the docked solution; [b]Contribution of the van der Waals forces to the global binding energy; [c]Contribution of the atomic contact energy to the global binding energy; [d]Topological polar surface area.

CONCLUSION

The chemical structure is one of the key parameters effecting polymer/nucleic acid complexation, polyplex properties and transfection efficiency. In this study, the effect of fluorination with pentafluoropropionic anhydride on transfection was investigated and G5-PA PAMAM dendrimer showed higher performance in terms of cell viability, miRNA complexation and transfection efficiency. These obtained results encourage the use of pentafluoropropionic anhydride for new dendrimer modifications and future tests on different cell lines. Also, consistency of the theoretical and experimental results show that design of the new transfection agent could be discussed with in silico analysis.

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