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Oligoamine-tethered low generation polyamidoamine dendrimers as potential nucleic acids carriers

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In recent years, dendrimers have emerged as the most widely explored materials for theranostics emphasizing their potential in therapeutic delivery and diagnostics as well as in pharmaceutical technology. Amongst, cationic PAMAM dendrimers have been extensively studied for their prospective in various biomedical applications due to their defined structure, distinctive features such as monodispersity, uniformity and amenable to functionalization. Here, low generation PAMAM dendrimers (G2-G4) have been modified via Michael addition reaction followed by amidation with an oligoamine linker, tetraethylenepentamine (TEPA). Subsequently, these modified dendrimers were characterized by physicochemical techniques and evaluated for their capability to transfer nucleic acids in vitro. The results displayed significant improvements in the transfection efficiency in both HeLa and A549 cells maintaining higher cell viability. Sequential delivery of GFP-specific siRNA resulted in ~73% suppression of the target gene. Flow cytometry results revealed that one of the formulations, mG3/pDNA complex, exhibited the highest gene transfection (~49-68%) outperforming pDNA complexes of native dendrimers and the standard transfection reagents, Lipofectamine and Superfect (~32-36%). All these results ensure the potential of the modified dendrimers as effective vectors for future gene delivery applications.

1. Introduction

Dendrimers are highly branched and regular synthetic macromolecules with well-defined structures, which are finding widespread use as functional nano-biomaterials.[1,2] The unique architecture and properties have made them useful for various biomedical applications including drug and gene delivery, imaging and diagnostics.[3,4] Recently, gene therapy has shown potential to treat various diseases ranging from inherited disorders to acquired diseases.[5-8] Nonetheless, challenges associated with gene therapy such as gene delivery efficiency, gene expression / regulation, and toxicity still exist mainly due to lack of safe and efficient gene vectors. Viruses have been shown to overcome these challenges,[9,10] however, severe complications such as immunogenic response, pathogenicity and difficulties in large scale production have led to lethal outcome, which limit their applications. Non-viral gene delivery vectors, on the other hand, suggest structural and chemical versatility for manipulating their properties to modulate gene carrying capacity.[11-13] The rich collection of polymeric vectors and their designing strategies are receiving attention of the researchers nowadays. Various synthetic and natural vectors have been

shown to possess safety and scale-up capability, though, suffer from low transfection efficiency and high cytotoxicity.[14-18] Of these, branched polyethylenimine (bPEI, 25 kDa) has been the most widely used vector and considered as a gold standard for the delivery of therapeutic genes to various cells.[13,16] PAMAM dendrimers have also been demonstrated as potential candidates in the design and synthesis of efficient and biocompatible gene delivery vectors.[19-23] The high degree of branching, high charge density, globular architecture, well defined molecular weight and tunable surface functional groups of dendrimers allow efficient interactions with the therapeutic molecules. Moreover, primary amines on outer surface of the dendrimers allow electrostatic interaction with the negatively charged nucleic acids and condense them into nano-sized complexes (dendriplexes) as well as protect the cargo against nucleases. On the contrary, tertiary amines in the core get protonated at acidic pH and facilitate the endosomal escape through proton-sponge mechanism. PAMAM dendrimers of higher generations (G5-G10) are more toxic than lower generation ones (G2-G4) but exhibit higher transfection efficiency. It is well reported that the cytotoxicity of higher generation cationic PAMAM dendrimers is the result of interactions between positively charged dendrimers and negatively charged molecules located on the cell surface. Therefore, several modifications have been suggested to reduce the toxicity and enhance their gene transfer efficacy.[24-31] Additionally, to reduce the cost and time of the production of higher generation dendrimers, non-toxic lower generation PAMAM dendrimers have been modified by tethering polyethylenimine (PEI), diethylenetriamine, histidine, guanidine and spermine or by crosslinking using disulfide linker. These modifications have resulted in improved transfection efficiency compared to their native counterparts but almost comparable to Lipofectamine or branched PEI (25 kDa).

In the present investigation, low generation PAMAM dendrimers (G2-G4) have been modified via Michael addition of methyl acrylate onto the surface amine groups followed by amidation reaction with a flexible oligoamine linker, TEPA, to improve transfection efficiency of the modified dendrimers without compromising on cytotoxicity. Modified PAMAM dendrimers (mG2-mG4) were characterized physicochemically and then evaluated for their gene carrying capacity and cell viability. The best formulation among all was assessed for its ability to deliver siRNA by quantifying the suppression of the target gene expression. The results were compared with native dendrimers and commercially available efficient transfection reagents, Lipofectamine 2000 and Superfect.

2. Materials and methods

2.1 Synthesis of modified PAMAM dendrimers (mG2-mG4)

Poly (amidoamine) (PAMAM) dendrimers (G2-G4) were conjugated with tetraethylenepentamine (TEPA) in a two step process following a reported method.[32] Briefly, to an ice-cold solution of PAMAM dendrimer (G2, 50 mg, 0.015 mmol) in methanol (2.5 ml), a large excess of methyl acrylate (5.76 mmol), taken up in methanol (2.5 ml), was added dropwise and the reaction mixture was stirred for 72h at 37˚C. The excess of methanol and methyl acrylate was removed under reduced pressure on a rotary evaporator to obtain a viscous syrupy residue (G2.5), which was again dissolved in methanol (5 ml) and cooled in an ice-salt bath. A pre-ice-cold solution of TEPA (1.2 g, dissolved in 10 ml of methanol) was added dropwise over a period of 15 min and the reaction mixture was stirred for 72h at 50^{o}C. Then the solvent was evaporated on a rotary evaporator, the syrupy residue, so obtained, dissolved in deionized water (dH_2O) and subjected to dialysis against water for 48h with intermittent change of water. The dialyzed sample was collected and lyophilized in a speed vac to obtain modified G2 (mG2) dendrimer in ~80% yield. Similarly, PAMAM G3 and G4 dendrimers were modified to obtain mG3 ad mG4 in ~77% yield. These modified dendrimers were subjected to characterization by ^{1}H-NMR. ^{1}H-NMR (D_2O) δ (ppm): 2.25 (-CH_2CONH-), 2.5 (>N-CH_2-), 2.65 (-NCH_2-), 2.85 (-NCH_2, TEPA), 3.15 (-NCH_2CH_2CO). Percent substitution of TEPA onto G2-G4 dendrimers was determined by the standard TNBS method.[33]

2.2 Preparation of modified dendrimers (mG2, mG3, mG4)-pDNA complexes

Aqueous solutions of mG2 / mG3 / mG4 (1mg/ml of each) were separately mixed with 0.3μg pDNA at various w/w ratios (0.16, 0.33, 0.5 and 0.66) in presence of 20% dextrose (5 μl) and the final volume was made upto 20 μl with deionized water. The resulting samples were then vortexed and incubated for 30 min at room temperature (25±1 ^{o}C) prior to their use in biophysical studies.

2.3 DNA binding assay by gel electrophoresis

DNA binding ability of PAMAM dendrimers (G2, G3, G4) and modified PAMAM dendrimers (mG2, mG3, mG4) was determined by gel electrophoresis. All the native and

modified dendrimers-pDNA complexes were prepared at w/w ratios of 0.16, 0.33, 0.50 and 0.66 keeping the amount of pDNA fixed (300 ng). These complexes were vortexed, incubated for 30 min at 37 ^{o}C, mixed with 2µl Orange G dye and loaded in to the wells of 0.8% agarose gel pre-treated with ethidium bromide. The samples were electrophoresed (100V, 1h) and the bands visualized in Gel Doc System (G:box UV illuminator, Syngene, UK).

2.4 Acid-base titrations

Buffering capacity is usually measured by acid-base titration. Here, in this study, we have compared the buffering capacity of native (G2-G4) and modified dendrimers (mG2-mG4). Each dendrimer (3 mg) was dissolved in 0.1M NaCl solution (30 ml, 0.1mg/ml) and pH was adjusted to 10.0 by the addition of 0.1N NaOH. Then, the pH of each of the solution was brought to 3.0 by adding 25µl aliquots of 0.1N HCl (titrant) and after each addition, pH of the solution was recorded. Finally, a graph was plotted between pH and amount of aq. HCl used (µl).

2.5 Stability of native and modified dendrimers-pDNA complexes

Native PAMAM dendrimers (G2-G4) and modified PAMAM dendrimers (mG2-G4) (1mg/ml) were complexed with pDNA at their best working w/w ratios (at which these complexes exhibited the highest transfection efficiency) and incubated for 30 min. Subsequently, heparin, a strong polyanion, was added in increasing amounts from 0-3.0 U, which competed with pDNA and released it from native and modified PAMAM dendrimers. The samples were further incubated for 30 min, mixed with 2 µl Orange G dye, electrophoresed (100V, 1h) in 0.8% agarose gel containing EtBr and bands visualized in Gel Doc System (G:box UV illuminator). Percent release of pDNA from the complexes was determined by densitometry.

2.6 In vitro transfection assay

HeLa and A549 cells were seeded into 96-well culture plates in DMEM with 10% FBS and incubated at 37˚C in a humidified 5% CO_2 environment. Transfection was carried out at ~70-75% confluency in the absence and presence of serum. Native PAMAM-pDNA and modified PAMAM/pDNA complexes were formed at various w/w ratios (1.66, 3.33, 5.0, 6.66, 10.0 and 16.66) and diluted separately with complete and incomplete media. bPEI/pDNA complex was prepared at w/w ratio of 1.6. Similarly, pDNA complexes were also made with

commercially available transfection reagents, Lipofectamine 2000 and Superfect, according to manufacturers' protocols. These complexes were then gently added onto the cells and kept the plates at 37˚C in CO_2 incubator. After 3h, the media was aspirated out and fresh complete medium was added onto the cells in each well. Post-36h, the cells were observed for GFP expression under inverted fluorescent microscope at 10x magnification.

In another experiment, siRNA was sequentially delivered by the modified PAMAM dendrimers (mG2-mG4) and compared the results with Lipofectamine and Superfect mediated siRNA delivery. HeLa cells were first treated with modified PAMAM (mGn)-pDNA complexes at w/w ratio of 6.66 and after 3h of incubation, the complex was aspirated out followed by addition of modified PAMAM (mGn)-siRNA complex (0.3 μM/μg of mGn). Again, after 3h, it was replaced by complete medium (DMEM with 10% FBS). The plate was kept in an incubator for 36h and then suppression levels were monitored by GFP quantification. All the experiments were repeated three times.

2.7 Quantification of GFP expression and total protein content

Quantitative analysis of green fluorescent protein (GFP) protein in the transfected cells was performed on Nanodrop ND-3000 spectrofluorometer. Post-transfection, the cells were washed with 1x PBS and incubated with cell lysis buffer for 45min at 37˚C. After centrifugation, 2μl of cell lysate from each well was dropped on Nanodrop to estimate protein spectrophotometrically. 1x PBS was used as a blank to calibrate the spectrofluorometer to zero reading and corrected for background. The total protein content was estimated at 590nm using Bradford reagent (BioRad) with BSA (Bangalore Genei, India) as a standard. Coomassie Brilliant Blue G dye present in Bradford reagent binds to proteins and results in the shifting of the absorption maxima. The fluorescence intensity was estimated in triplicates and expressed as arbitrary fluorescence units/mg protein.

2.8 Cytotoxicity assay

MTT colorimetric assay was performed to evaluate the cytotoxicity of the native and modified dendrimers-pDNA complexes on HeLa and A549 cells and the results compared with pDNA complexes of bPEI (25 kDa), Lipofectamine and Superfect. Transfection was carried out as described above and after 36h of incubation, MTT (100μl, 1mg/ml) was added to cells in each

well and the plate was kept at 37˚C in an incubator under humidified 5% CO_2 environment for 2h. Then, the supernatant was aspirated out and the formazan crystals, so formed, were dissolved in 100µl isopropanol containing 0.5% SDS and 0.06M HCl. The color intensity was then measured spectrophotometrically at 540nm on an ELISA plate reader. Untreated cells with 100% viability were taken as control. IC_{50} value or the concentration of native and modified dendrimer/pDNA complexes, at which the HeLa cell viability reaches 50%, was determined at their w/w ratio 10.0 and 6.6, respectively. All the experiments were carried out in triplicates.

2.9 Hemolytic activity

To examine the activity of modified PAMAM dendrimers on human red blood cells (hRBCs), hemolysis assay was performed. Human blood was collected and RBCs isolated and washed with PBS (pH 7.2) thrice and then suspended to 4% (v/v) in PBS. 100 µL of hRBC suspension was added into each well of a sterilized 96-well plate and treated with varying concentrations (5, 10, 20, 40, 50 and 100 µg/ml) of modified dendrimers and their native counterparts. The plates were incubated for 1h at 37ºC and centrifuged at 800 g for 10 min. Further, aliquots of 100 µL of supernatant were transferred to fresh 96-well plates and hemoglobin release was monitored on an ELISA plate reader by measuring the absorbance at 540 nm. Triton X-100 (2%, w/v) was used as control causing 100% hemolysis.

2.10 Enzymatic assay

To examine the ability of modified PAMAM dendrimers to provide protection to the condensed pDNA against nucleases, DNase I protection assay was carried out. mGn-pDNA complexes were separately incubated with DNase I in a buffer (100nM Tris, 25mM $MgCl_2$ and 5mM $CaCl_2$) at 37 ^oC for 0.25, 0.5, 1.0, 2.0 h. Complexes in PBS alone served as controls. After stipulated time of incubation, 5µl EDTA (100mM) was added to the reaction mixture to quench the activity of DNase I followed by heating to 75 ^oC for 10 min. The mixture was further incubated for 1 h at ambient temperature with 10 U of heparin to release bound pDNA from mGn-pDNA complexes. Subsequently, the samples were mixed with 2µl Orange G dye, electrophoresed (100V, 1h) in 0.8% agarose gel containing EtBr (2µl/100ml gel) and bands visualized on Gel Doc System (G:box UV illuminator). The amount of pDNA released from complexes was estimated by densitometry.

2.11 Fluorescence activated cell sorting (FACS) analysis

For examining GFP expression at the individual cell level, FACS analysis was performed post-36h of incubation. Briefly, HeLa and A549 cells after seeding in 24-well plates for 24h were washed with 1x PBS (pH 7.4) followed by removal of the media. Native PAMAM (G2, G3 and G4) and modified PAMAM (mG2, mG3 and mG4) dendrimers were complexed with pDNA (1.5μg) at w/w ratios of 6.66, 10, 16.66 and 3.33, 6.66, 10, 16.66, respectively, and incubated for 30 min at room temperature. Similarly, pDNA complexes were prepared with bPEI (w/w ratio 1.6) and Superfect (following manufacturer's protocol). These complexes, diluted with DMEM with and without 10% FBS, were added gently onto the cells for 3h at 37 ^{o}C. Then, the media was replaced by complete media (containing 10% FBS) and kept the plates in an incubator. After 36h, the plates were observed under a fluorescence microscope and cells subjected to trypsinization. An equal volume of complete medium (DMEM with 10% FBS) was added to each well and centrifuged at 5000 rpm for 5 min at 4 ^{o}C. The supernatant was removed and the pellet washed with 1x PBS. Subsequently, the pellet was resuspended in 1x PBS (1 ml), transferred to a cuvette and subjected to flow cytometric analysis. The cells without sample treatment (i.e. non-transfected cells) were used as a negative control with their fluorescence set to 1%. For each sample, 5000 events were counted by a flow cytometer (BD, USA) equipped with Cytosoft Software (Guava_EasyCyte_Plus Flow Cytometry System).

3. Results and discussion

Synthetic polymers with defined structure, shape and size are finding increasing attention in the development of drug and gene delivery systems. The unique structure and shape of PAMAM dendrimers have received the most attention to develop as potential gene and drug delivery systems. These molecules possess highly branched structure, modifiable functional groups on the surface, mono-dispersity, fixed molecular weight and have ability to bind negatively charged nucleic acids and protect them from degradation, however, their gene carrying capacity and cytotoxicity is generation dependent i.e. higher generation PAMAM dendrimers (G5-G10) transfer these biomolecules more efficiently but cytotoxic as compared to lower generation ones (G2-G4), which are relatively non-toxic but exhibit poor transfection. Therefore, modifications have been incorporated in their structures to improve transfection efficiency and cell viability. In the present investigation, we assumed that the transfection

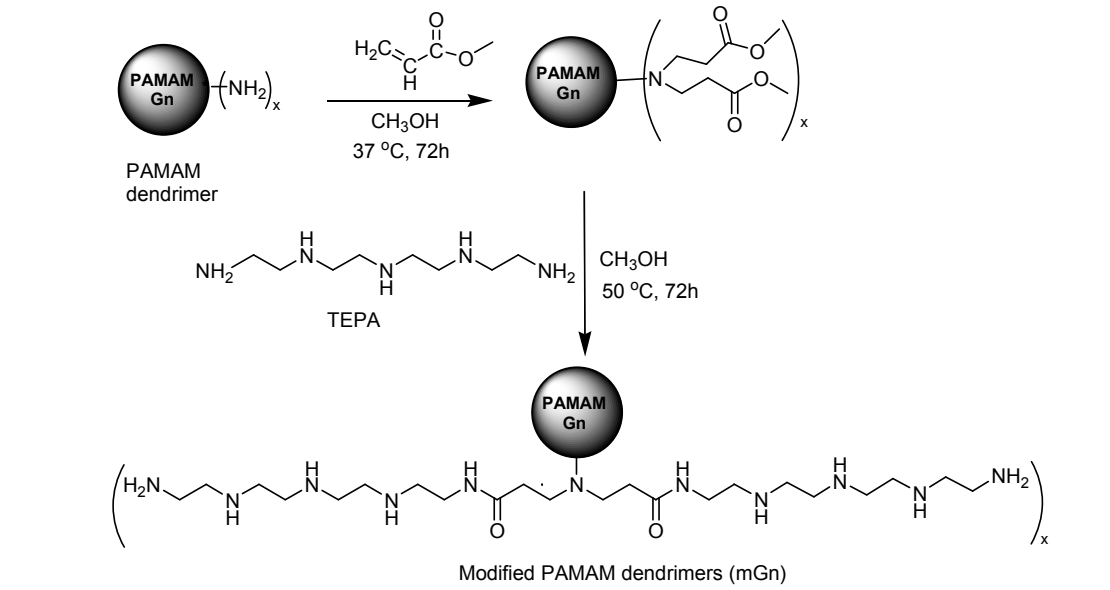
efficiency of the lower generation (G2-G4) dendrimers could be enhanced by tethering a flexible oligoamine linker, tetraethylenepentamine (TEPA). The projected commercially available linker was selected mainly for the following reasons, viz., (i) on conjugation, it would provide primary amines on the surface away from the branched structure for interaction with negatively charged nucleic acids, (ii) the secondary amines (3 / linker molecule) in the backbone would not only improve the buffering capacity but also, to an extent, participate in the cooperative binding of nucleic acids via electrostatic interactions, and (iii) the incorporated secondary amines would assist in controlling the cytotoxicity of the modified dendrimers. The cooperative binding would strengthen the DNA binding ability and suppress the degradation in the cellular milieu. Keeping these points in mind, we synthesized TEPA conjugated G2, G3 and G4 dendrimers (mG2, mG3 and mG4) via a two- step reaction, as depicted in Scheme 1.

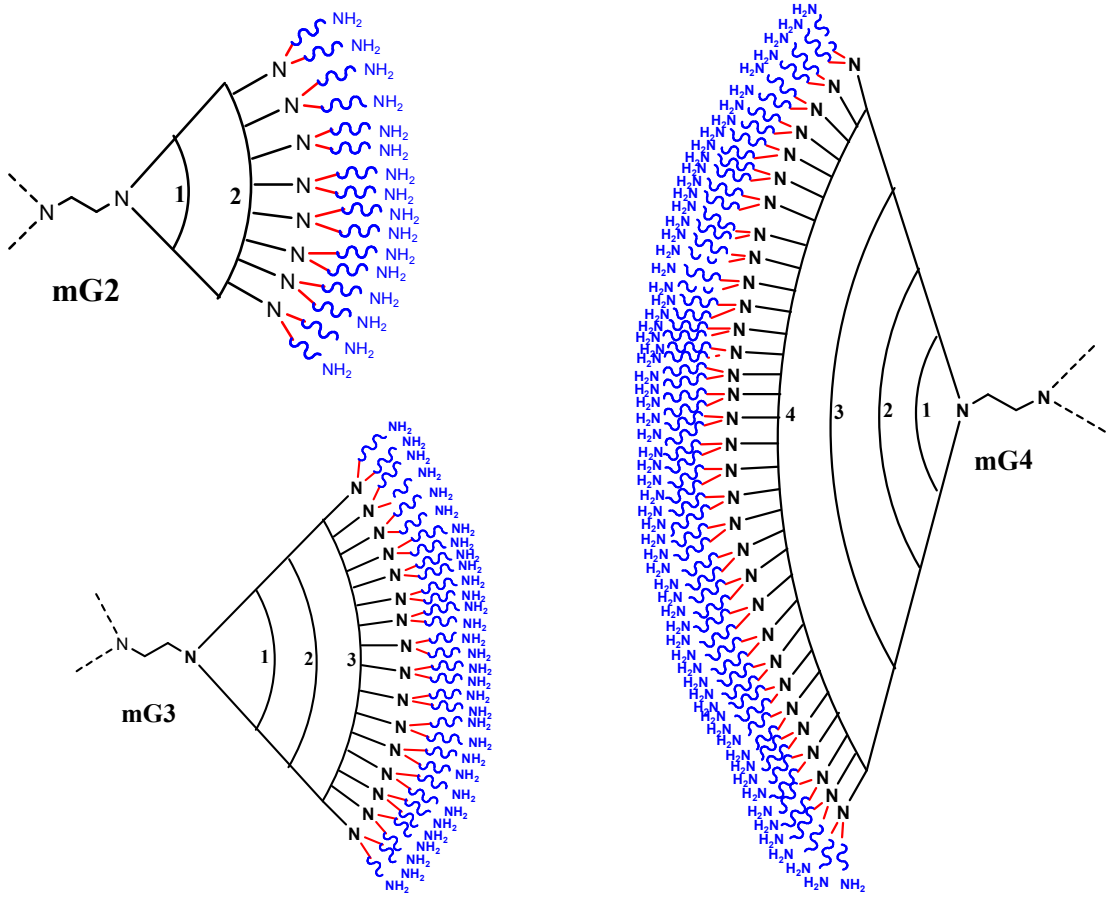
Preparation of modified dendrimers begins with Michael addition reaction involving methyl acrylate and surface amine groups of PAMAM dendrimers (G2, G3 and G4) following the standard method.[32] Each terminal nitrogen atom serves as a branching point. The reaction resulted in the generation of G2.5, G3.5 and G4.5 dendrimers, which subsequently subjected to reaction with TEPA in large excess to yield a new set of surface primary amine groups and secondary amines in the backbone of the flexible oligoamine linker (Table 1).

Table 1: Density of primary, secondary and tertiary amines in native and modified PAmAM dendrimers

Dendrimer gen. **Types of amine groups**	**G2**	**G3**	**G4**	**mG2**	**mG3**	**mG4**
Primary	16	32	64	32	64	128
Secondary	---	---	---	96	192	384
Tertiary	8	16	32	24	48	96

The attempted conjugation was determined by TNBS method using UV-VIS spectrophotometry.[33,34] A soluble yellow-colored product was formed between 1^{o} amines of mGn dendrimers and TNBS, which was measured at 335 nm. Analysis results revealed quantitative generation of TEPA-conjugated G2, G3 and G4 dendrimers. Modified dendrimers were also characterized by ^{1}H-NMR, peak at δ 2.85 due to $-NCH_2$ in TEPA confirmed the incorporation of the linker in the modified dendrimers (**Figure S1**).





Scheme 1. Schematic representation of synthesis of modified PAMAM dendrimers (mGn). n=2, x = 16; n = 3, x = 32; n = 4, x = 64.

3.1 Mobility shift assay

The positive charge on native PAMAM (G2, G3 and G4) and modified PAMAM (mG2, mG3 and mG4) dendrimers and negatively charged backbone of DNA interact with each other through electrostatic interactions and form nanosized complexes (dendriplexes). DNA retardation assay was executed to find out the amount of cationic polymer required to completely neutralize the charge on the fixed amount of DNA. The mixing was done at different w/w ratios of dendrimers and pDNA and the samples run on the gel. **Figure 1** shows DNA binding to PAMAM dendrimers at different w/w ratios. The complete retardation of mobility of 0.3µg of pDNA by PAMAM (G2)-pDNA complex occurred at w/w ratio of 0.66:1, while PAMAM (G3 and G4) and PAMAM (mG2, mG3 and mG4)-pDNA complexes retarded at w/w ratio of 0.5:1. This might be due to the fact that after amidation, pDNA interacted mainly with the surface amine groups and the modified dendrimers behaved in similar fashion as the unmodified ones. Further, to determine the binding affinity between dendrimers and pDNA, apparent equilibrium dissociation constant (K_d) was calculated using gel retardation assay following a reported protocol.[35] As expected, the results revealed the highest affinity of mG4 and it decreased from mG4-mG3-mG2-G4-G3-G2 on the basis of their K_d values (**Table S1**).

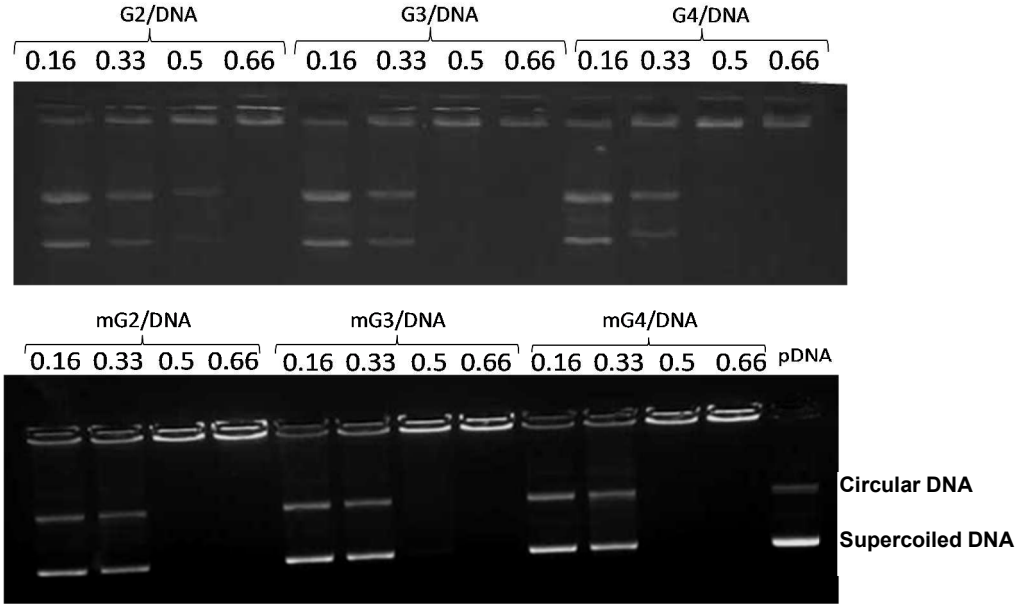


Figure 1. DNA mobility shift assay of native and modified PAMAM dendrimers/pDNA complexes. Dendrimers were mixed with pDNA at various weight ratios and electrophoresed in 0.8% agarose gel.

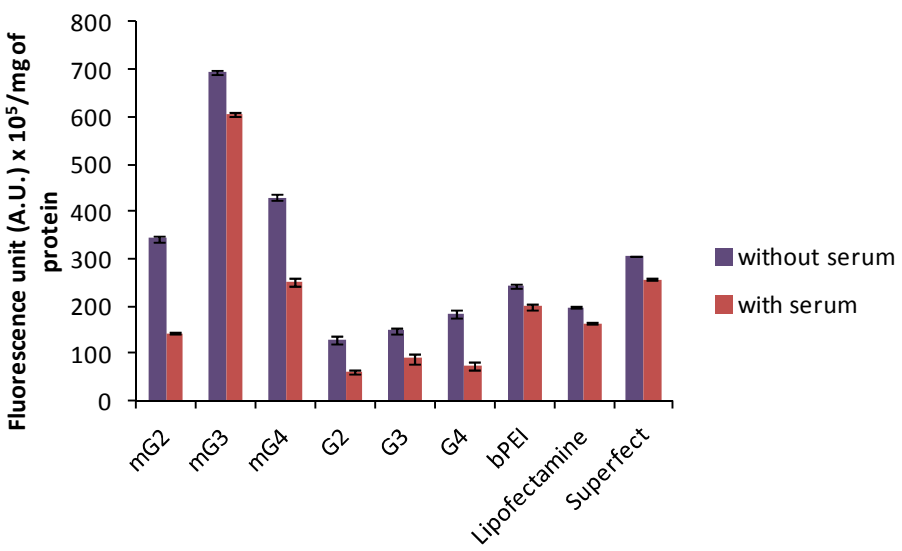
3.2 In vitro transfection in mammalian cells

The gene delivery efficacy of the modified PAMAM dendrimers (mG2, mG3 and mG4) was evaluated by *in vitro* transfection experiments onto HeLa and A549 cell lines. The results were compared with the native PAMAM dendrimers (G2-G4) and commercial transfection reagents, viz., Lipofectamine and Superfect. The assay was performed at different w/w ratios of dendrimer (modified dendrimers)/pDNA complexes slightly higher than those used in the gel retardation assay so as to keep the overall charge positive on the complexes. The transfection results on A549 and HeLa cell lines, in the absence and presence of serum (10%), are presented in **Figure 2 (a-c)**. It was observed that pDNA complexes of modified dendrimers showed significantly enhanced transfection efficiency (~2.2-8.6 folds) than pDNA complexes of native PAMAM dendrimers, bPEI and commercially available Lipofectamine and Superfect. The transfection efficiency was found to be cell line dependent and showed higher transfection in HeLa cells (**Figure 2a**). Also, the transfection efficiency varied with w/w ratio of dendrimer/pDNA in the complexes. Native and modified PAMAM dendrimer/pDNA complexes exhibited the highest transfection efficiency at w/w ratio of 6.66:1. Initially, transfection efficiency increased with an increase in the w/w ratio from 1.66 to 6.66 and after attaining the maximum at this ratio, it started decreasing. Among the modified mGn/pDNA complexes, mG3/pDNA complex showed the highest transfection efficiency in both the cell lines. mG3/pDNA complex displayed ~3.5 and 2.3 folds higher transfection efficiency than Lipofectamine/pDNA and Superfect/pDNA complexes, respectively, in HeLa cells, while in A549 cells, it was ~4.4 and 2.2 folds higher than Lipofectamine/pDNA and Superfect/pDNA complexes, respectively. Compared to native G3/pDNA complex, mG3/pDNA complex displayed ~8.6 and 4.7 folds higher transfection in A549 and HeLa cells, respectively. In the presence of serum, similar trend was observed, however, as expected, the transfection efficiency decreased marginally, which might be attributed to non-specific interactions between the positively charged complexes and negatively charged serum proteins. Still, mG3/pDNA complex performed the best and exhibited ~1.8-3.9 folds higher transfection as compared to

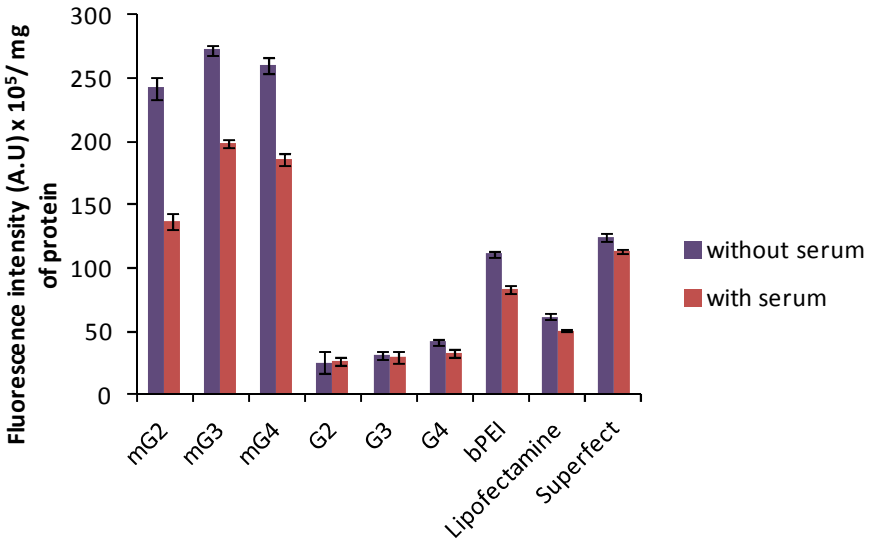
Lipofectamine/pDNA and Superfect/pDNA complexes, respectively, in both HeLa and A549 cells. With respect to native dendrimers/pDNA complexes, mG3/pDNA complex showed ~6.8 folds higher gene delivery activity in both the cell lines. Surprisingly, in HeLa cells, the decrease in transfection efficiency of the native G3/pDNA complex was higher than that observed in mG3/pDNA complex. In both the absence and presence of serum, the modified dendrimers displayed considerably higher transfection efficiency compared to their unmodified (native) counterparts. In A549 cells, a surprising result was obtained in the absence and presence of serum, both mG3/pDNA and mG4/pDNA complexes exhibited comparable transfection efficiency (**Figure 2b**).

The higher transfection efficiency displayed by the modified dendrimer/pDNA complexes might be attributed to several factors such as higher buffering capacity, improved binding affinity, stability under cellular environment (i.e. enzymatic stability) and higher charge density. Consequent to conversion of terminal primary amines of the native dendrimers to tertiary amines as well as incorporation of secondary amines of the oligoamine linker improved the buffering capacity marginally but good enough to enhance the endosomal escape capacity.[36] Previously, it has also been demonstrated that modification of polypropylenimine with oligoethylenimine, transfection efficiency increased, which might be due to change in the molecular weight and number of secondary and tertiary amines incorporated post-modification implying enhanced proton sponge effects.[37,38] Besides, higher charge density (i.e. zeta potential) is also important during interactions with the cell membrane and endosomal release of the complexes. It has been shown that increased zeta potential enhances cell association to promote the cellular uptake and thereby transfection efficiency.[39-41] Internalization and accumulation of positively charged complexes in the endosomes induce protonation of the surface amine groups via buffering mechanism, which eventually triggers the entry of Cl^- ions into the endosomes resulting in an increase in the endosomal osmotic pressure followed by its rupturing to release the complexes into the cytoplasm of the target cells.[42] All these factors are cumulatively responsible for enhanced transfection efficiency of the modified dendrimer complexes outperforming the efficacy of the native dendrimers and commercial transfection reagents.

(a)


Fluorescence unit (A.U.) x 10^5/mg of protein
800
700
600
500
400
300
200
100
0
mG2
mG3
mG4
G2
G3
G4
bPEI
Lipofectamine
Superfect
without serum
with serum

(b)


Fluorescence intensity (A.U) x 10^5/ mg of protein
300
250
200
150
100
50
0
mG2
mG3
mG4
G2
G3
G4
bPEI
Lipofectamine
Superfect
without serum
with serum

(c)

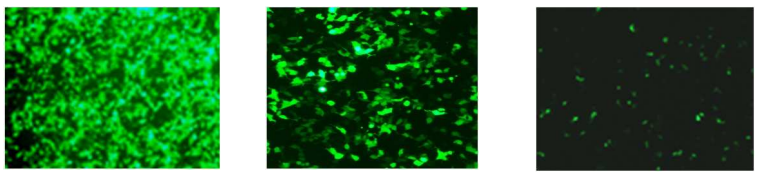

mG3
Superfect
G4

Figure 2. In vitro transfection efficiency of native and modified dendrimer-pDNA complexes in the absence and presence of serum in (a) HeLa cells (w/w ratios of native dendrimers/pDNA complexes 10 and modified dendrimers/pDNA complexes 6.66), and (b) A549 cells (w/w ratios of native dendrimers and modified dendrimers/pDNA complexes 6.66). The fluorescent intensity of GFP in the cell lysate was measured on a NanoDrop spectrofluorometer and the results are expressed in terms of arbitrary units (A.U.) / mg of total protein. The results represent the mean of three independent experiments. (c) Fluorescence microscopic images, captured at 10X, of transfected HeLa cells with mG3, Superfect and G4/pDNA complexes. Cells were incubated with pDNA complexes of mG3, Superfect and G4 for 3h and expression of GFP was monitored post 36h of incubation.

Further, in order to determine percent cells transfected with pDNA complexes of native dendrimers, modified dendrimers, bPEI (25 kDa) and Superfect, FACS analysis was carried out. It was found that modified dendrimers/pDNA complexes transfected higher percentage of cells as compared to native dendrimers/pDNA complexes. Among modified dendrimers, mG3/pDNA complex transfected the highest percentage of cells (~49-68% cells) at w/w ratio of 6.66, while PAMAM G4/pDNA, bPEI/pDNA and Superfect/pDNA complexes could transfect only ~2-8%, ~18-20% and ~32-36% cells, respectively (**Figure 3**), in both the cells. Even mG2/pDNA and mG4/pDNA complexes transfected higher number of cells (~13-28%) compared to unmodified dendrimers/pDNA complexes in both HeLa and A549 cells. Interestingly, in contrast to FACS results in A549 cells, wherein mG2/pDNA, mG3/pDNA and mG4/pDNA complexes displayed marked difference in the number of transfected cells (~18%, 49% and 28%, respectively), quantification results (using spectrofluorometry) of GFP showed more or less comparable transfection (**Figure 2b**). This anomaly could be explained that once transfected, mG2 and mG4 facilitated more efficient gene expression than mG3 specifically in A549 cells, i.e. higher GFP expression in less number of cells. The results advocate the potential of these modified dendrimers as promising gene carriers.

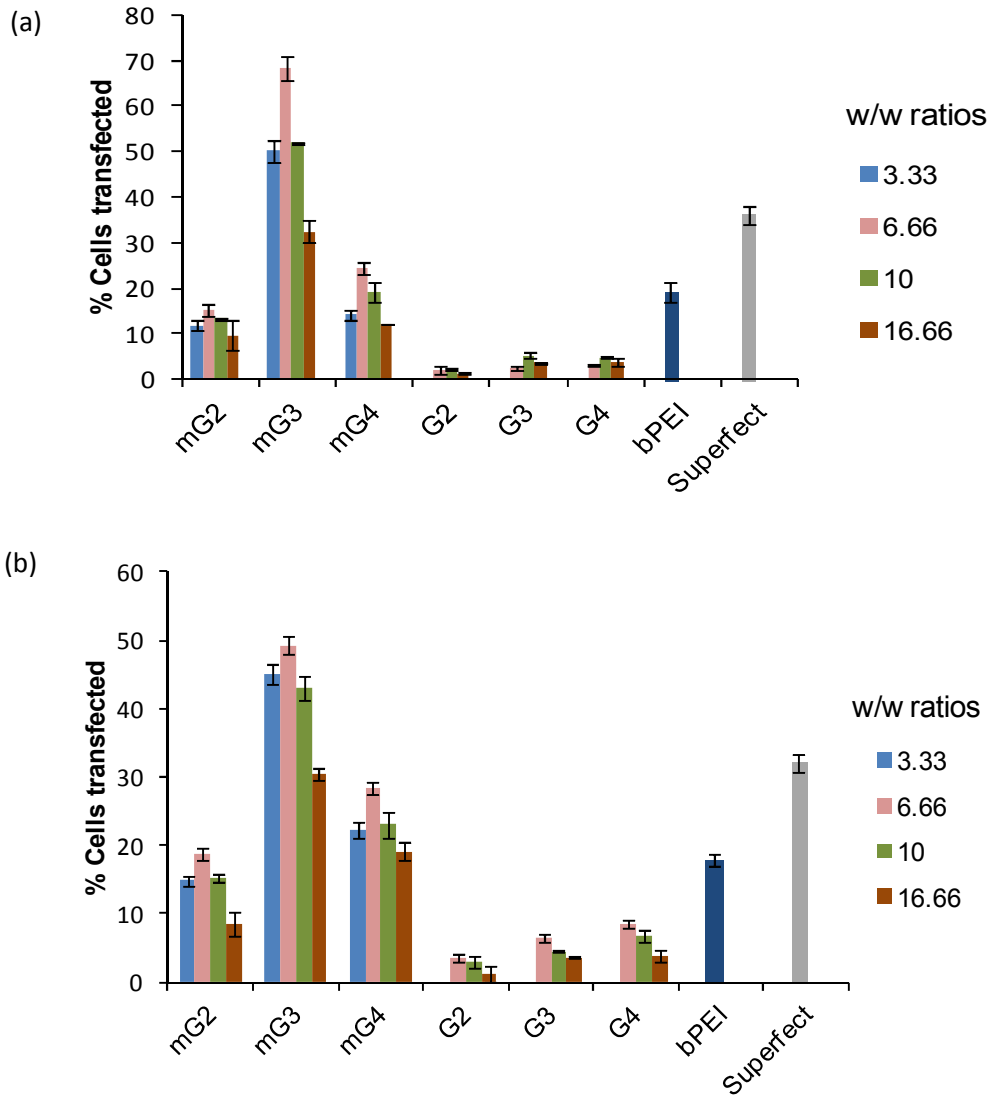


Figure 3. Percent transfection efficiency of pDNA complexes of native and modified dendrimers-pDNA complexes at various weight ratios in (a) HeLa, and (b) A549 cells. Results were compared with bPEI/pDNA and Superfect/pDNA complexes. Experiment was repeated three times and error bars represent the standard deviation.

3.3 siRNA delivery

The versatility of mG3 vector was further established by sequential delivery of siRNA onto HeLa cells. The results showed that siRNA delivered by mG3 dendrimer suppressed expression of GFP by ~73% (**Figure 4**), while Superfect and Lipofectamine 2000 mediated

sequential delivery of siRNA resulted in ~47% suppression in the expression of the GFP gene implying that the modified G3 dendrimer can be used for efficient delivery of nucleic acids (DNA and RNA) in vivo.

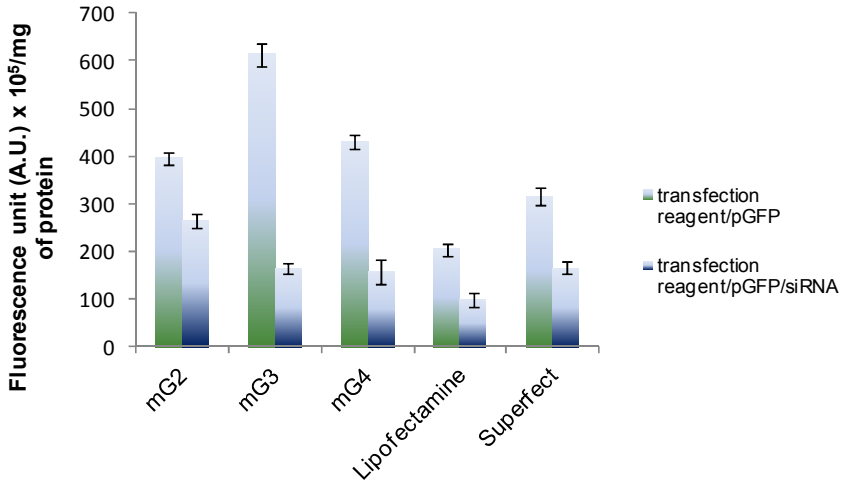


Figure 4. Sequential delivery of GFP-specific siRNA using mGn dendrimers in HeLa cells. Percent suppression of EGF expression by mGn mediated delivery was compared with the efficacy of Lipofectamine and Superfect-mediated sequential delivery. Experiments were repeated three times and error bars represent the standard deviation.

3.4 Particle size, morphology and zeta potential measurements

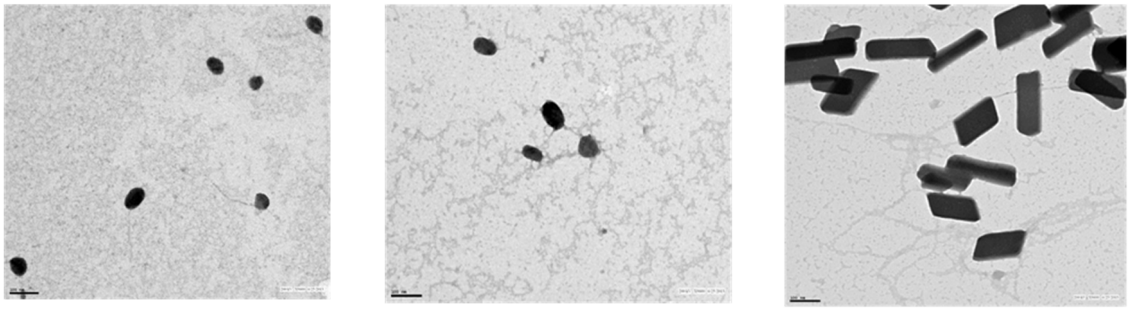
The particle size and zeta potential of pDNA complexes of G2, G3, G4, mG2, mG3 and mG4 complexes at their best w/w ratios are shown in Table 2. Interaction with pDNA yielded particles in nano-size range. It was observed that the size of modified mGn-pDNA complexes was higher in comparison to their respective native dendrimers, which might be due to tethering of an oligoamine linker, TEPA, with higher degree of branching in modified dendrimers. On complexation with pDNA, particle size found to be in the range 157-211 nm and in the presence of 10% serum, size of the complexes further decreased due to more compaction and ranged between 37-52 nm. The morphology and size of pDNA complexes of mG2-mG4 were also examined by TEM, which revealed that mG2 and mG3/pDNA complexes were almost spherical / oval in shape with sizes ~40 and 50 nm, respectively (**Figure 5**). However, surprising results were obtained in mG4-pDNA complex, it exhibited rectangular shaped particles of dimension 162 nm x 72 nm. The particles in DLS were found to be in bigger size as compared to that

obtained in TEM. This could be due to the measurement of hydrodynamic diameter in DLS, whereas TEM provides the size of the complex in dry state.[11,12,16]

Zeta potential studies showed a slightly higher +ve charge on the pDNA complexes of modified dendrimers as compared to pDNA complexes of native dendrimers. However, in presence of 10% FBS, the polarity of the complexes reversed i.e. all the complexes became negatively charged due to adsorption of serum protein around +vely charged complexes.

Table 2: Size and zeta potential measurements of native and modified dendrimers-pDNA complexes

Samples	**Average size ± S.D. (nm)**		**Average zeta potential ± S.D. (mV)**	
	pDNA complex in H_2O	*pDNA complex in 10% FBS*	*pDNA complex in H_2O*	*pDNA complex in 10% FBS*
G2	210.6±8.0	36.7±1.8	27.9±2.9	-22.3±2.6
G3	167.8±6.7	38.5±1.6	30.1±3.5	-22.2±3.1
G4	194.2±6.2	41.2±1.9	30.2±1.6	-21.6±2.2
mG2	156.7±5.7	49.8±1.7	32.6±2.1	-17.0±3.3
mG3	170.1±7.0	51.8±1.8	33.6±6.2	-15.1±1.2
mG4	194.1±7.5	51.4±1.9	34.5±4.4	-9.2±1.7



(a) (b) (c)

Figure 5. Transmission electron microscopic (TEM) images of pDNA complexes of (a) mG2, (b) mG3 and (c) mG4. Scale bar: 100 nm

3.5 Buffering capacity

Buffering capacity of the cargo is an essential factor for endosomal escape of the complexes into the cytoplasm for nuclear translocation of plasmid. Endosomal escape is generally accomplished by endosomal disruption by protonation of polymers (proton sponge effect) in endosomal compartments (low pH). Buffering capacity of the native PAMAM (G2-G4) and modified PAMAM (mG2-mG4) dendrimers was determined by an acid-base titration method in the pH range 3-10. From the plot (**Figure 6**), it was found that proton capturing tendency of the modified PAMAM (mG2, mG3 and mG4) dendrimers slightly increased compared to native PAMAM (G2, G3 and G4) dendrimers. This could be due to the facts, viz., (i) during Michael addition, the primary amines were converted into tertiary amines, and (ii) after reaction with the tetraethylenepentamine, secondary amines were introduced. Incorporation of secondary and tertiary amines in the modified dendrimers might have contributed a bit towards an increase in the overall buffering capacity, which destabilizes the endosomal membrane at acidic pH facilitating the effective endosomal escape.

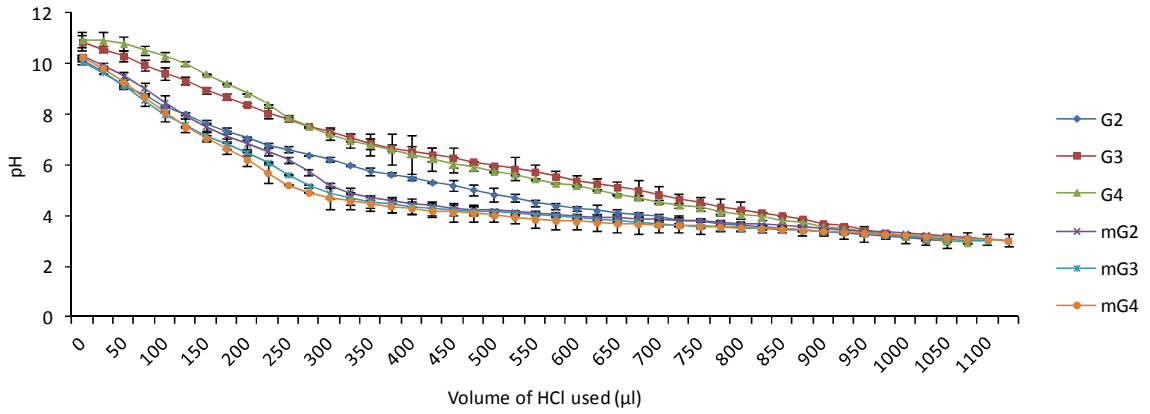


Figure 6. Acid-base titration curves of native and modified PAMAM dendrimers.

3.6 Stability of pDNA complexes of modified dendrimers

A cationic polymer capable of binding pDNA tightly significantly affects its release inside the cells. An ideal vector should bind pDNA sufficiently enough so that it can carry it to the target inside the cell without exposing it for degradation in the cellular environment. To mimic the release of pDNA inside the cells in vitro, pDNA complexes of native and modified

dendrimers were exposed to a highly negatively charged polymer, heparin, and by increasing its concentration (in terms of units), release pattern of pDNA was obtained. As shown in **figure 7**, ~86-90% pDNA was released from the native dendrimers (G2-G4) complexes. Under similar conditions, modified PAMAM (mG2-mG4) dendrimers-pDNA complexes released ~75-78% of complexed DNA. Interestingly, the binding ability of mGn-pDNA complexes was observed to be higher, which could be due to higher charge and branching in the modified dendrimers enabling better interactions with the complexed pDNA, whereas, loose or inefficient binding of pDNA with G2-G4 dendrimers might be one of the reasons for displaying lower transfection.

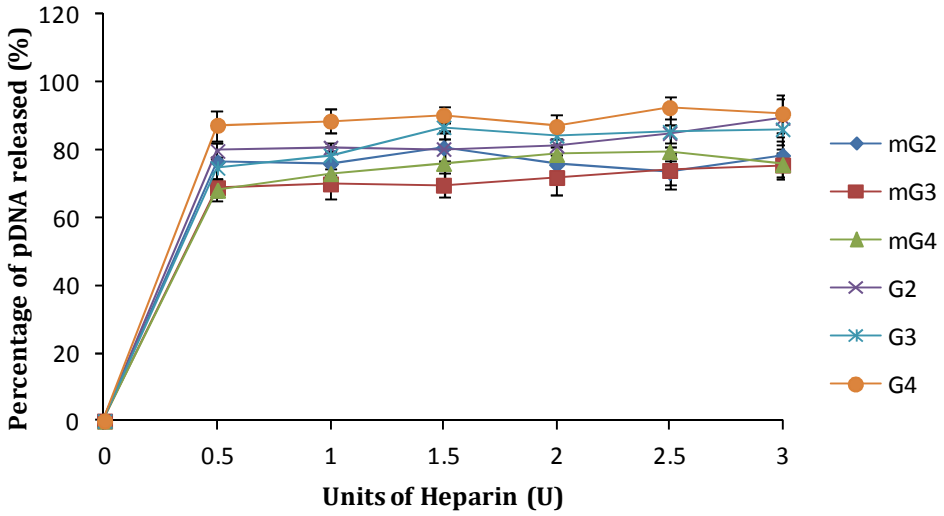


Figure 7. DNA release assay of native and modified PAMAM dendrimers-pDNA complexes. Complexes were treated with increasing amounts of heparin (0 - 3.0 U) and the amount of pDNA released was estimated by densitometry.

3.7 Cell viability assay

To demonstrate the cytotoxicity profiling of modified PAMAM dendrimers, MTT assay was carried out and results were compared with the native dendrimers, bPEI and the commercial transfection reagents, Lipofectamine and Superfect. It was observed that the cell viability did not change significantly after incorporation of oligoamine linker, TEPA. Modified PAMAM dendrimers-pDNA complexes exhibited cell viability ~75-89% comparable to PAMAM counterpart (~71-88%), but significantly higher than Lipofectamine/pDNA (~48-55%) and bPEI/pDNA (~62%) complexes. Superfect/pDNA complex exhibited 73-77% cell viability,

which is an activated G5-PAMAM dendrimer (**Figure 8**). As reported in the literature, higher generation PAMAM dendrimers exhibit higher toxicity due to strong interactions with the cell membranes. Here, in the present study, our major goal was to improve the transfection efficiency of the non-toxic lower generation PAMAM dendrimers without compromising on the cytotoxicity. As expected, the conjugation of TEPA did not result in the enhanced cytotoxicity of the modified dendrimers even after the density of primary amines increased. This could be due to simultaneous increase in the density of secondary and tertiary amines in the modified dendrimers, which, to an extent, took care of the effect of an increase in the cytotoxicity due to an increment in the density of primary amines. Literature also records that cationic polymers, such as linear polyethylenimine,[43] end-modified poly (β-aminoesters),[44] linear poly(amido amine)s,[45] having secondary or tertiary amines or both, exhibit minimal cytotoxicity. Even branched polyethylenimine (25 kDa), which is highly toxic, shows very low toxicity when the primary amine density is converted into secondary or tertiary amines.[16,46,47] The IC_{50} values further revealed that the native and modified dendrimers were non-toxic (**Table S2**).

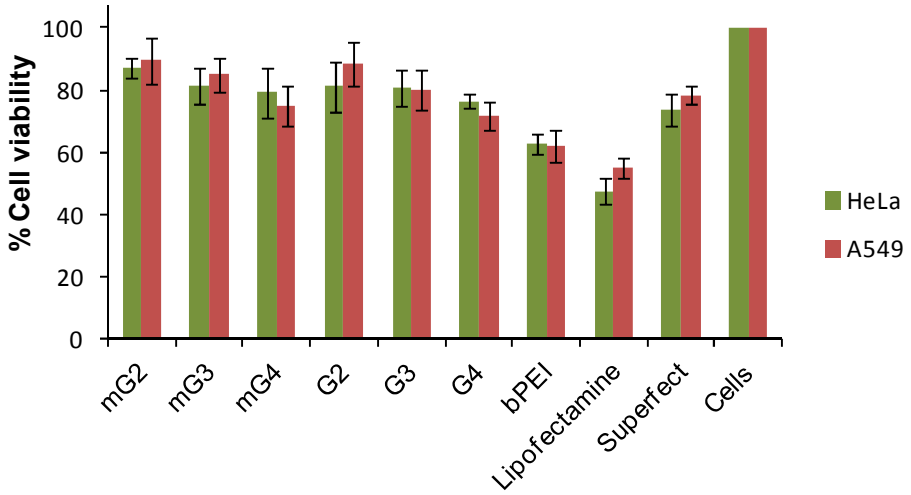


Figure 8. Cell viability profiles of native and modified PAMAM dendrimers, bPEI (w/w 12), Lipofectamine and Superfect-pDNA complexes their respective best working w/w or v/w ratio in HeLa and A549 cells (HeLa cells - w/w ratios of native dendrimers/pDNA complexes 10 and modified dendrimers/pDNA complexes 6.66; A549 cells -w/w ratios of native dendrimers and modified dendrimers/pDNA complexes 6.66). Cells were treated with pDNA complexes and their viability was determined by MTT assay. Percent viability of cells is expressed relative to control cells. Each point represents the mean of three independent experiments performed in triplicate.

3.8 Hemolytic activity

The hemolysis of human red blood cells was measured at varying concentrations of dendrimers and the results have been presented in **figure 9**. All the modified dendrimers (mG2-mG4) showed low hemolytic activity upto 100µg/mL with less than 3% of human erythrocytes being lysed. The hemolytic activity was found to be almost similar to the activity of native PAMAM dendrimers (G2-G4). It was also noticed that modification did not result in the increase in the membrane disrupting effect, supporting the endosomal membrane disruption by proton-sponge mechanism. The low hemolytic activity at high dendrimers concentration along with their enhanced gene delivery efficiency advocates the suitability of the vectors for future gene delivery applications.

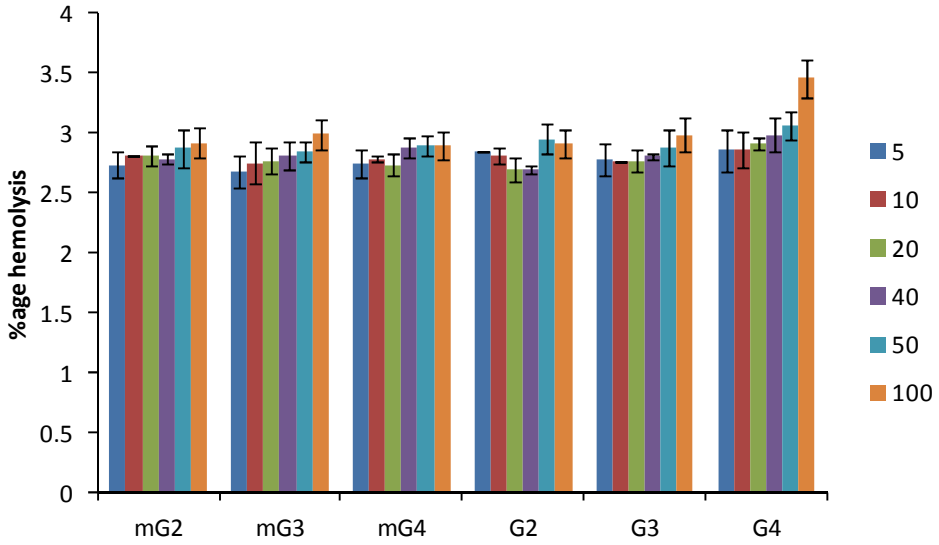


Figure 9. Hemolysis assay. Percent hemolysis caused by native and modified PAMAM dendrimers at various concentrations (µg/ml). Triton X-100 was used as control causing 100% lysis. Experiment was carried out in triplicate.

3.9 Protection of pDNA against nucleases

The protection of DNA is a prime requisite for successful entry of DNA inside the cells, as cellular milieu contains nucleases, which rapidly degrade native DNA. Therefore, DNase I assay was performed to reveal the ability of modified dendrimers (mG2, mG3 and mG4) to

protect the bound pDNA against nucleases. Naked DNA was found to be digested by DNase I within 15 min, whereas mG2, mG3 and mG4 dendrimers protected ~75%, 88% and 92%, respectively, of bound pDNA even after 2h of treatment (**Figure 10**). These results clearly imply that modified dendrimers have capability to provide protection to nucleic acids in the cellular environment and can serve as efficient vectors for nucleic acids to carry them in to the cells.

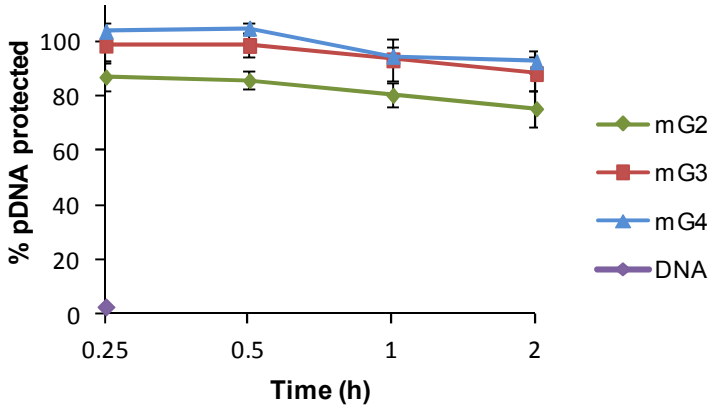


Figure 10. DNase I protection assay of mGn//pDNA complexes at w/w ratio 6.66.

4. Conclusions

In the present study, low generation PAMAM dendrimers (G2-G4) were conjugated with a flexible oligoamine linker via Michael addition of methyl acrylate followed by amidation to improve transfection efficiency without compromising on cell viability. These modified dendrimers not only showed improved buffering capacity, which facilitated their endosomal escape but also exhibited significantly higher transfection efficiency with cell viability almost comparable to native dendrimers. Increased positive charge density on the modified dendrimers was the prime factor for enhanced transfection efficiency which aided in binding of pDNA and protecting it against nucleases. The surface modification of dendrimers with the projected linker can be a simple and effective method to develop a proficient carrier system for the successful delivery of nucleic acids in vitro and in vivo.

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***Abbreviations**: PAMAM – poly (amidoamine); G2, G3, G4 – generation 2, 3 and 4; TEPA – tetraethylenepentamine; bPEI – branched polyethylenimine; GFP – green fluorescent protein; pDNA – plasmid DNA; EtBr – ethidium bromide; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TNBS – 2,4,6-trinitrobenzene sulfonic acid; FBS – fetal bovine serum; DMEM – Dulbecco's Modified Eagle Medium; SDS – sodium dodecyl sulfate; RBCs – red blood cells; EDTA – ethylenediaminetetraacetate.*

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GRAPHICAL ABSTRACT

Oligoamine-tethered low generation PAMAM dendrimers (mG2-mG4) have been synthesized, which displayed significantly higher transfection efficiency with minimal cytotoxicity in vitro.