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Bioreducible polyethylenimine nanoparticles for efficient delivery of nucleic acids

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Recently, non-viral vectors for the nucleic acids delivery have received considerable attention. Among the various non-viral vectors, branched polyethylenimine (bPEI, 25 kDa) has been one of the most widely used carrier systems due to its high transfection efficiency, however, imparts high cytotoxicity. In this study, we have crosslinked bPEI with a bioreducible linker, 3,3'-dithiodipropionic acid (DTPA), via electrostatic interactions to obtain DTPA crosslinked bPEI (DP) nanoparticles. The crosslinking significantly reduced the cytotoxicity of the nanoparticles. To arrive at the best formulation in terms of nucleic acid transfection, a series of DP nanoparticles were prepared by varying the percent crosslinking. Dual action of DTPA, i.e. partial blocking of the charge density as well as crosslinking to convert bPEI into nanoparticles, did not alter pDNA condensation ability of the so formed nanoparticles rather the strategy favoured the unpackaging of the complexes inside the cells improving the release of pDNA, which resulted in higher transfection efficiency. All the formulations carried nucleic acids inside the cells and exhibited significantly higher transfection efficiency than native bPEI and commercial transfection reagent, Lipofectamine^{TM}. Sequential siRNA delivery displayed significant suppression in the target gene expression. All together, evaluation of delivery systems demonstrates that the newly synthesized DP NPs are quite promising as non-viral gene carriers.

Keywords: Transfection; bPEI; siRNA; pDNA; crosslinking, nanoparticles

Introduction

Gene therapy influences protein expression pattern by transferring exogenous nucleic acid into the diseased cells for the treatment and cure of acquired diseases. The clinical implementation of gene-based therapeutics requires safe and efficient delivery vectors. The uptake of nucleic acids into cells is a major challenge, as high molecular weight and negative charge on the nucleic acids made them poorly uptaken by the cells. Several gene delivery vectors, viz., viral and non-viral vectors, have been developed in order to overcome these barriers.[1-4] To conduct successful gene therapy, an ideal gene delivery vector should carry a gene of interest to its destination and release it efficiently to trigger its expression besides imparting low cytotoxicity, having high gene carrying capacity and modulating gene expression for desirable time period. Viral vectors are quite efficient but cause cytotoxicity, immunogenicity and tumorigenicity. These issues diverted the interest of researchers to the field of non-viral gene delivery systems, which included novel biocompatible materials designed and developed by innovative synthesis schemes.[5-7] Cationic polymers are generally exploited to form nanosized complexes with negatively-charged nucleic acids, which then interact with negatively charged lipid bilayers and are uptaken and internalized. There are numerous examples of cationic carriers that have been deployed in gene delivery applications including polyethylenimine (PEI), chitosan, polyamidoamine (PAMAM), poly(dimethylaminoethyl methacrylate) and polylysine.[8-10] Amongst them, branched polyethylenimine (25kDa) has been explored extensively for gene delivery purposes and considered as the gold standard in gene delivery, however, charge-associated toxicity has limited its applications. Extensive modifications have been incorporated in bPEI such as acylation, alkylation, pegylation, coating with sugar moieties and grafting of imidazolyl groups to suppress the associated cytotoxicity.[11-15] In order to develop a new category of safe and efficient gene delivery vectors, a few researchers have shown their interest to design and fabricate stimuli-responsive polymers.[16,17] Amongst them, disulfide-containing polymers constitute a novel class of polymers capable of undergoing reductive degradation intracellularly.[18-20] The disulfide linkages are redox sensitive functional domains, which are relatively stable in between oxidizing extracellular space and the reducing cellular

compartments. The inclusion of disulfide bonds in the polymers displayed higher stability in extracellular conditions and also maximum release of payload with minimal cytotoxicity than non-thiolated ones. Therefore, the introduction of disulfide bridges in polymers in form of grafting or crosslinks has been widely investigated for the design of bioreducible polymeric gene delivery carriers. Disulfide bonds have also been used to improve carrier stability and develop biocompatible high molecular weight (HMW) carriers for gene delivery applications.[18-21] Nowadays, RNA interference has also shown great potential in treating various diseases due to its ability to help in efficient and specific silencing of multiple genes.[22-24] Although small interfering RNAs (siRNAs) can help in the study and treatment of endothelial cells, but efficient siRNA delivery has so far been remained challenging.

To address these limitations, we report a delivery system based on nanoparticles while maintaining high gene-delivery efficiency and low cytotoxicity. A series of a bioreducible bPEI nanoparticles (DP) was produced by ionic crosslinking of amino groups on bPEI (25kDa) with 3,3'-dithiodipropionic acid (DTPA). These nanoparticles were characterized by physicochemical techniques and evaluated for their capacity to deliver nucleic acids in vitro. The DP nanoparticles exhibited 2- to 3-fold higher transfection efficiency than native bPEI. Interestingly, these polymers showed much lower toxicity in the cell line studied. The DP NPs also showed marked improvement over bPEI and Lipofectamine^{TM} in terms of siRNA transfection and therefore, hold great potential as transfection agents.

Results and Discussion

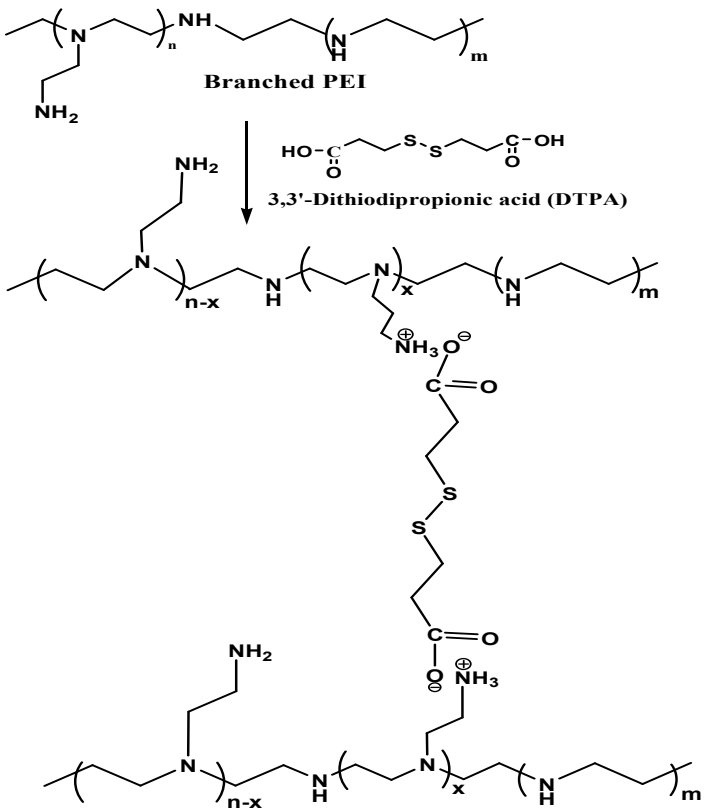
Inspite of several efforts towards development of an ideal gene delivery system, research is still moving around in overcoming barriers such as cellular entry, endosomal escape, cytoplasmic uptake and uptake by nucleus. bPEI is considered as the gold standard for gene delivery applications but due to high positive charge density (mainly because of primary amines), it suffers from charge-associated toxicity and non-specific interactions with serum proteins,[25,26] which have finally hindered its use as an efficient gene delivery vector in vivo. Reductively and hydrolytically degradable polymers with disulfide linkages are usually stable in blood circulation and can degrade rapidly to release DNA in reductive intracellular environment. Here, a dual purpose chemical modification was introduced to diminish its

positive charge density (main source of toxicity) as well as to convert the polymer into its nanoparticles, which have been shown to enter cells efficiently and exhibit high transfection efficiency and cell viability. The projected strategy was also explored to study the property of the DTPA linker to introduce disulfide linkages in order to maintain redox potential gradient in cellular premises and subsequent disassembly of the complexes to effect high gene expression.

Synthesis and physical characterization

DP nanoparticles were prepared by ionic crosslinking of bPEI with 3,3'-dithiodipropionic acid (DTPA) and a small series of DP-1, DP-2 and DP-3 nanoparticles with 3, 6 and 9% crosslinking, respectively, was prepared (Scheme 1). The percentage of crosslinking was altered by varying the amount of 3,3'-dithiodipropionic acid. The resulting nanoparticles were analyzed in terms of size and zeta potential by DLS. As expected, the size of DP nanoparticles decreased on increasing percent crosslinking from DP-1 to DP-3, however, this decrease in size from DP-1 to DP-2 was found to be substantial, but moving from DP-2 to DP-3, it was observed to reach a plateau around 160 nm. Size of bPEI has not been reported as it is a linear polymer with branching and exhibits variable size (~500 nm to microns) on measurement by DLS. However, on interaction with pDNA, it forms complexes in nanometer size range. Similarly, a decrease in zeta potential was also observed, as the crosslinking reduced the charge density on the resulting nanoparticles (Table 1). Further, these nanoparticles were allowed to complex with plasmid DNA (pDNA) at w/w ratio 2.33 (the best working weight ratio at which DP/pDNA complexes exhibited the highest transfection efficiency) and the size of the formed DP/pDNA complexes was found in the range of 132-222 nm (Figure 1 and Table 1). The average zeta potential of DP/pDNA complexes was observed in the range of +21 - +27 mV (Table 1). The zeta potential of the DP/pDNA complexes was found to be decreased in comparison to native bPEI/pDNA complex (prepared at the best working w/w ratio of 1.33). This confirmed the ionic crosslinking of bPEI with DTPA which resulted in reduced overall charge on DP nanoparticles. Size and zeta potential of DP/siRNA complexes (prepared at the ratio used in the transfection assay) were also measured (Table S1, *ESI*). These complexes showed size in the range of 451-548 nm while zeta potential in ~37-53 mV, which could be due to crosslinking between particles by siRNA, which resulted in accumulation of particles and

charge on them. In the presence of serum (10% FBS), both the parameters displayed a further decrease (i.e. size decreased to ~37-40 nm and zeta potential ~ -18 to -20 mV), which could be due to various factors, viz., (i) adsorption of serum proteins on the cationic surfaces inhibit aggregation among the nanostructures and stabilize individual particles, (ii) anionic serum proteins adsorb water from the cationic surface of the particles, which leads to partial dehydration around the particles, (iii) change of medium from water to 10% FBS (i.e. viscosity) restricts the swelling of the particles, and (iv) refractive index of the medium.[27-30] On analyzing DP nanoparticles in 10% FBS, we observed formation of a suspension with size range ~992-3164 nm, which became clear on increasing the concentration of serum to 50%. The size of DP nanoparticles in 50% FBS was found to be in the range of ~64-127 nm. The zeta potential of these nanoparticles in 50% FBS was found to around -10.0 mV (Table S2, Figures S1-S3, *ESI*). Transmission electron microscopy (TEM) further revealed the formation of nanoparticles and their DNA complexes. One of the formulations, DP-2 and its pDNA complex, prepared at w/w ratio of 2.33, was analyzed by TEM. The results as shown in figure 2 depict the formation of spherical shaped particles. Size of DP-2 nanoparticles was found in the range of ~45-50 nm while that of DP-2/pDNA complex in 35-45 nm (DP-2/pDNA complex) (Figure 2). The particle size was smaller than observed under DLS. The difference in size might be due to the fact that DLS measures the hydrodynamic diameter of the particles, while TEM provides the size in a dry state.



Scheme 1. Synthesis of DP nanoparticles.

Table 1. Particle size and zeta potential measurements of DP nanoparticles and DP/ pDNA complexes in water and serum

Sample ID	Average particle size in nm ± S.D.			Zeta Potential in mV±S.D.		
	DP Nanoparticles (in H_2O)	DNA loaded conjugate (in H_2O)	DNA loaded conjugate (in 10% FBS)	Nanoparticles (in H_2O) (+)	DNA loaded conjugate (in H_2O) (+)	DNA loaded conjugate (in 10% FBS)
bPEI (25kDa)	– –	195.8±8.9	38.94±2.5	31.3±0.7	26.5±0.4	-18.26±0.2
DP-1	262.5±15.64	222.13±1.6	39.67±3.4	27.4±1.1	23.9±0.3	-19.8±0.6
DP-2	160.4±4.39	158.13±2.9	37.32±0.3	26.5±0.9	23.7±0.6	-19.7±1.0
DP-3	157.6±2.39	132.03±1.4	39.18±0.6	25.9±0.6	22.7±2.0	-20.1±0.8

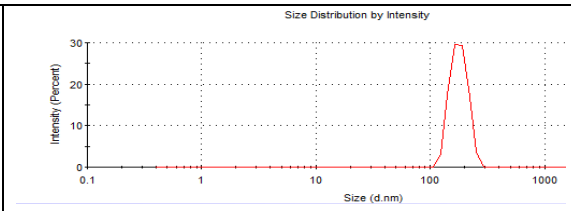
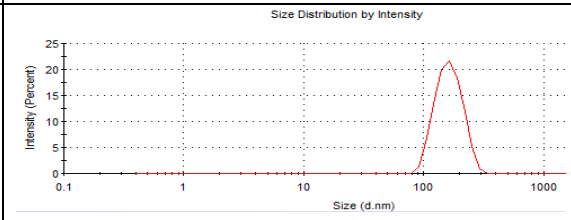
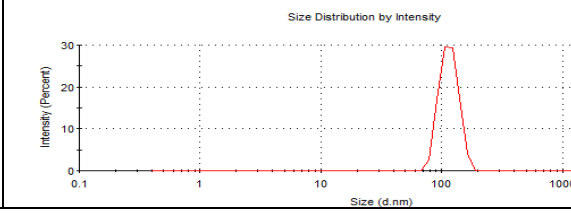
DP-1		Z-Average (d.nm): 222.1 PdI: 0.282
DP-2		Z-Average (d.nm): 158.1 PdI: 0.112
DP-3		Z-Average (d.nm): 132.0 PdI: 0.300

Figure 1. Particle size distribution of DP/pDNA complexes.

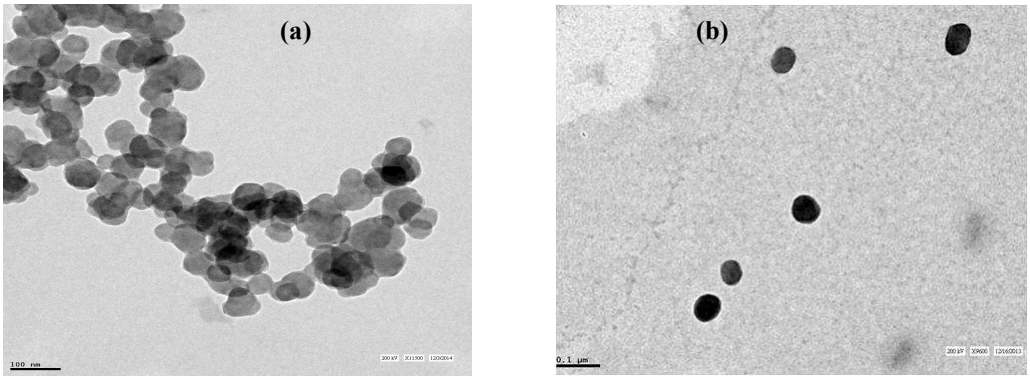


Figure 2. TEM images a) DP-2 nanoparticles, average particle size ~45-50 nm, and b) DP-2/pDNA complex, average particle size ~35-45 nm.

Electrophoretic Mobility Shift Assay

Agarose gel electrophoresis was performed to assess the pDNA binding efficacy of DP nanoparticles. The assay was carried out to determine the amount of DP nanoparticles /

native bPEI polymer required to completely neutralize the charge on plasmid DNA. So, the optimal binding concentration of the DP nanoparticles with pDNA (300ng/µl) was analyzed at different w/w ratios of 0.16, 0.23, 0.5, 0.66, 0.75, 1.0 and 1.2, while bPEI/pDNA complexes were prepared at w/w ratios of 0.16, 0.23, 0.30, 0.45, 0.5, 0.66 and 0.75. DP nanoparticles retarded pDNA at higher w/w ratio than bPEI. As depicted in figure 3, DP-1, DP-2 and DP-3 retarded mobility of pDNA at w/w ratio of 0.66, 0.66 and 0.75, respectively, whereas native bPEI retarded the same amount of pDNA at w/ w ratio of 0.3. This might be due to decrease in surface charge of DP nanoparticles after crosslinking with DTPA as well as burial of a certain amount of charge inside the pores of the nanoparticles, which was inaccessible for interaction with pDNA. DP-3 nanoparticles, containing the highest amount of crosslinker (DTPA), required the higher amount to retard the mobility of a fixed amount of pDNA as compared to others in the series. These observations also support the results obtained in DLS measurements (zeta potential).

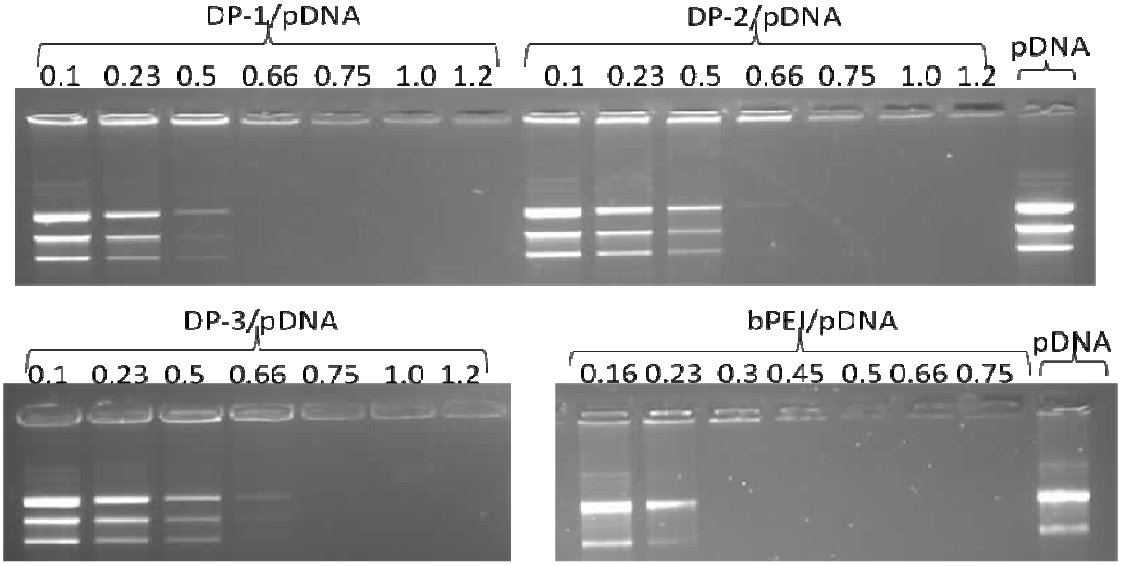


Figure 3. Electrophoretic mobility assay of pDNA in 0.8% agarose after binding to the native polymer bPEI (25kDa) and DP nanoparticles at different w/w ratios.

Heparin release assay

The binding of DP nanoparticles with pDNA is a prime factor but simultaneously it should be able to release the bound pDNA at the target from the complexes. In order to assess the pDNA release from DP/pDNA complexes, we compared the binding ability of complexes with bPEI/pDNA at their best working w/w ratio 2.33 and 1.33, respectively, using heparin

in increasing amount (0-3.0 U). The results showed DP nanoparticles exhibited maximum release of ~92% of pDNA whereas bPEI released ~60% of pDNA from bPEI/pDNA complex (Figure 4). These results suggest that PEI binds pDNA very strongly, which could be due to the presence of a high density of cationic charge (in particular, primary amines). Higher release in DP nanoparticles could be attributed to two factors, viz., (i) decrease in charge density as a result of electrostatic interaction with DTPA, and (ii) formation of nanoparticles resulted in burial of some charge inside the pores which was not accessible for binding pDNA. Hence, relatively loose complexes were formed. Several reports have also indicated that transfection efficiency depends on the pDNA binding ability of NPs.[12,15] The synthesized nanoparticles demonstrated the potential of not only carrying the bound pDNA efficiently inside the cell but also released it in sufficient amount in the cellular milieu to obtain enhanced gene expression.

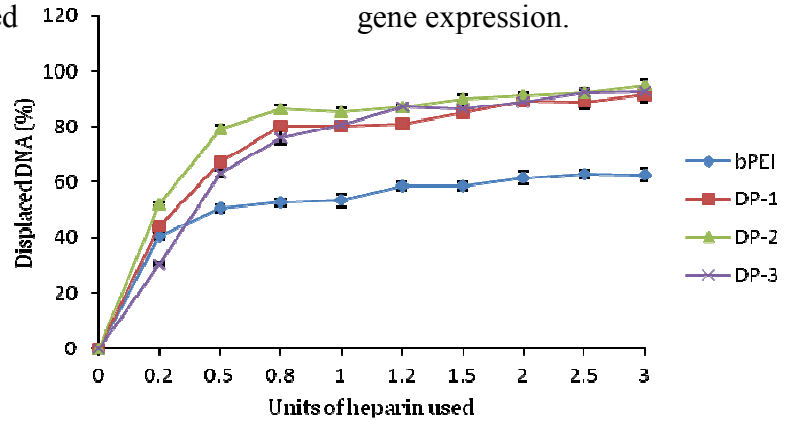


Figure 4. pDNA release assay of DP/pDNA complexes. To 20 µl of DP/pDNA (prepared at w/w 2.33) and bPEI/pDNA complexes (prepared at 1.33), heparin was added in increasing amounts and incubated for 30 min at RT. The samples were run on EtBr pre-stained 0.8% agarose gel at 100 V for 1 h and percentage release quantified by densitometry.

In vitro GFP expression

Gene transfer ability of DP nanoparticles was evaluated on mammalian cells. Transfection efficiency of pDNA complexes of DP nanoparticles, bPEI and Lipofectamine™ was evaluated on MCF-7 cells using EGFP (Enhanced Green Fluorescent Protein) as a reporter gene in the absence and presence of serum. The assay was carried out at higher w/w ratios (higher than the ratio at which DP nanoparticles and bPEI retarded the mobility of pDNA on

agarose gel) in order to keep overall charge on these complexes positive for efficient cellular internalization of the complexes. After 36h of incubation, cells were observed under fluorescence microscope for GFP expression. After quantifying fluorescence, it was observed that the transfection efficiency of DP/pDNA series was found to be ~2-3 folds higher than the bPEI and Lipofectamine^{TM}/pDNA complexes (Figure 5). Among the series of DP/pDNA complexes, transfection efficiency varied with percent crosslinking as well as w/w ratios. DP-2/pDNA complex performed the best in terms of transfection efficiency and scored ~2.5 and 3-fold higher transfection efficiency as compared to Lipofectamine^{TM}/pDNA and bPEI/pDNA complexes, respectively. Transfection efficiency also increased with an increase in the w/w ratio and it reached the highest at w/w ratios of 2.33 (in case of DP/pDNA complexes) and 1.33 (in case of bPEI/pDNA compex), beyond this value, the transfection efficiency decreased. Therefore, all other studies, such as size and zeta measurements, heparin release assay, DNase I protection assay, siRNA delivery, were carried out using DP/pDNA w/w ratio of 2.33. Similarly, bPEI/pDNA complex showed the highest transfection efficiency at w/w ratio of 1.33, Hence, this ration was used in all other experiments. Presence of 10% serum did not inhibit the transfection efficiency, which further implied the potential of modified nanoparticles for in vivo gene delivery applications. This might be attributed to its degradability via cleavage of disulfide bonds and release of cargo inside the cells. Thus, the obtained disulfide-containing DP nanoparticles might be suitable for further *in vivo* gene transfection study.

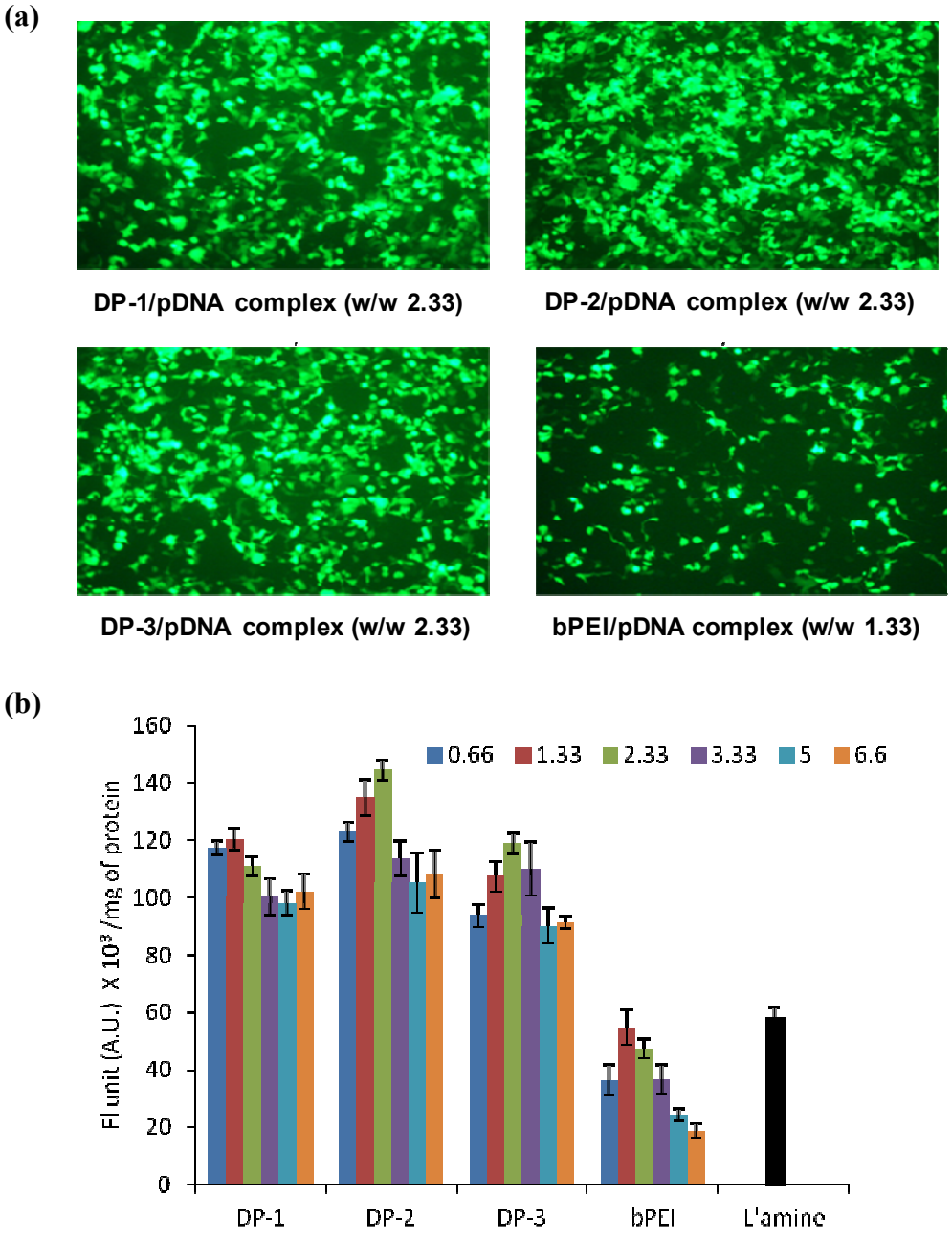


Figure 5. (a) Fluorescence microscopic images of GFP expression of pDNA complexes of DP nanoparticles and bPEI at different w/w ratios in MCF-7 cells. (b) GFP fluorescence intensity in MCF-7 cells in presence of serum, transfected with DP/pDNA, bPEI/pDNA and Lipofectamine^{TM}/pDNA complexes. The transfection profiles show fluorescence intensity expressed in terms of arbitrary units/mg of total cellular protein. The results represent the mean of three independent experiments performed in triplicates.

In vitro cytotoxicity

The cytotoxicity of the complexes was evaluated by the MTT assay. The viability of MCF-7 cells was examined by MTT assay after transfecting the cells with native bPEI/pDNA, DP/pDNA and commercial transfection reagent, viz., Lipofectamine™/pDNA, complexes (Figure 6). The results demonstrated that DP series exhibited >90% cell viability upto w/w ratios of 2.33, while bPEI and Lipofectamine^{TM} showed ~69% and 58% cell viability, respectively. The observed higher cell viability of DP series might be due to decrease in the charge density as a result of interaction with DTPA and partial inaccessibility of the charge hidden inside the pores of the nanoparticles.

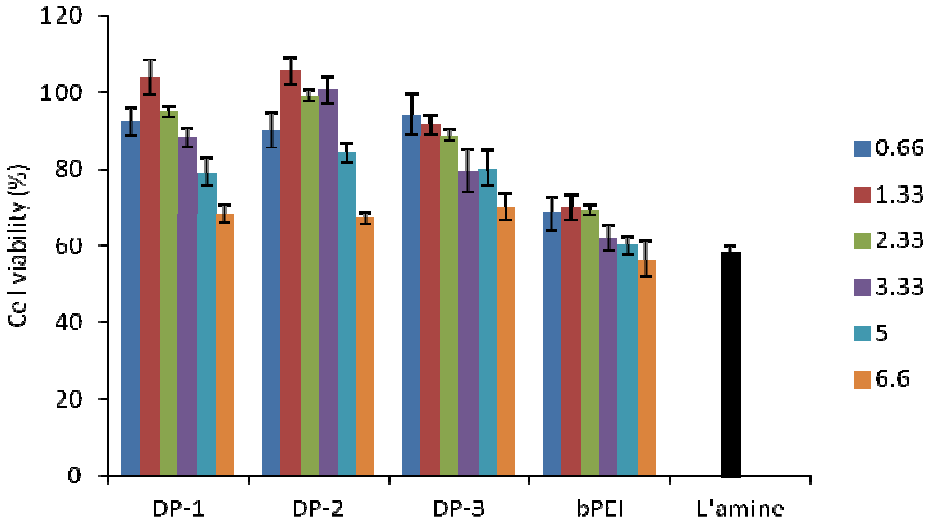


Figure 6. Cell viability profile of DP/pDNA, bPEI/pDNA and Lipofectamine^{TM}/pDNA complexes in MCF-7 cells. Experiments were carried out in triplicates and error bars represent the standard deviation.

DNase I protection assay

In order to determine the susceptibility of bound pDNA towards nucleases, DNase I enzyme protection assay was carried out. It is well known that cationic polymers binds pDNA to form small sized particles and protect the bound pDNA from nuclease degradation. Here, the assay was carried out at different time points and analyzed on agarose gel. In contrast to

the free pDNA, which was degraded completely by DNase I in 15 min, DP-2 effectively provided protection to bound pDNA and ~94% of pDNA was found to be intact even after 2 h of exposure (Figure 7). These observations clearly suggest that DP-2 can be used as an efficient vector for taking the pDNA to the cellular milieu without much of degradation.

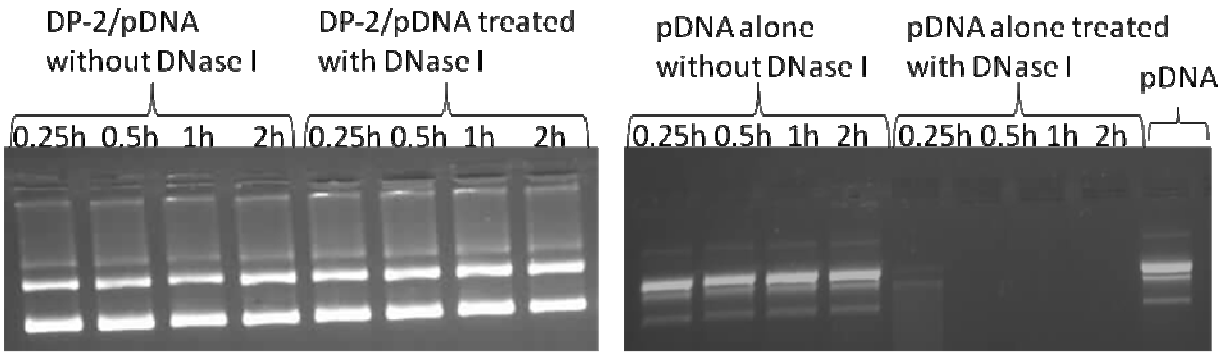


Figure 7. DNase I protection assay of DP-2/pDNA complex. The complex and native pDNA were treated with DNase I for different time intervals. pDNA was released from the complexes by incubation with heparin. The amount of pDNA protected was calculated by relative integrated densitometry analysis, quantified and normalized by pDNA values using Gel Documentation system Syngene UK.

siRNA transfection

The versatility of the DP vectors was further verified by sequential delivery of siRNA onto MCF-7 cells. The results showed that siRNA delivered by DP suppressed the expression of GFP by ~69-80% (Figure 8), while Lipofectamine^{TM}-mediated sequential delivery of siRNA resulted in ~47% suppression in the expression of the GFP gene. This implies that DP nanoparticles have higher efficacy that that of the commercial transfection reagent.

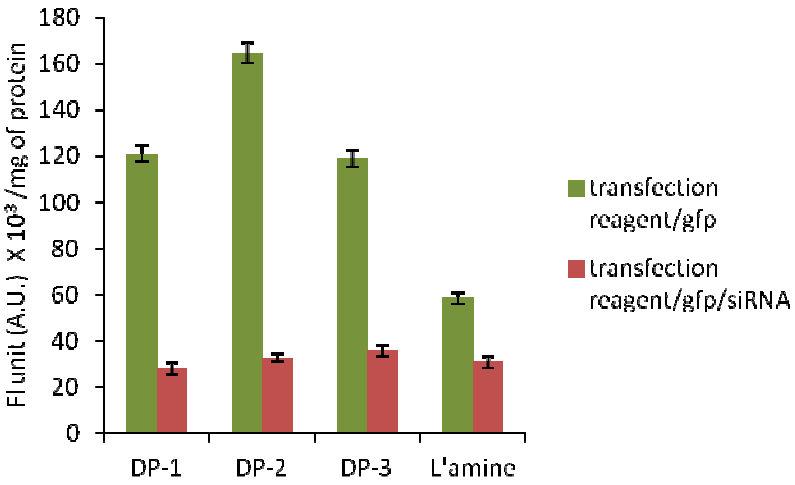


Figure 8. Sequential delivery of GFP-specific siRNA using DP nanoparticles. Percentage knockdown of GFP expression was compared with the efficacy of Lipofectamine™ mediated sequential delivery. Experiment was repeated three times and error bars represent the standard deviation.

Experimental section

Materials

Branched polyethylenimine (bPEI, 25kDa), 3,3'-dithiodipropionic acid (DTPA), agarose, deoxyribonuclease I (DNase I), 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and ethidium bromide (EtBr) were procured from Sigma-Aldrich Chemical Company, USA. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and Lipofectamine 2000™ were obtained from Invitrogen (USA). MCF-7 cell line was obtained from NCCS, Pune, India, and maintained as per the standard protocol.

Synthesis of 3,3'-dithiodipropionic acid (DTPA) crosslinked branched PEI (DP) nanoparticles

DP nanoparticles were synthesized via electrostatic interactions between bPEI (25 kDa) and 3,3'-dithiodipropionic acid (DTPA). Briefly, to an aqueous solution of bPEI (43mg, 1mg/ml), 3,3'-dithiodipropionic acid (6.31mg, 1mg/ml in DMSO for 3% crosslinking) was added dropwise and the reaction mixture was stirred for 4h at room temperature. The reaction mixture was then lyophilized to obtain the DTPA crosslinked bPEI (DP-1)

nanoparticles. Similarly, 6% and 9% DTPA crosslinked bPEI (DP-2 and DP-3) nanoparticles were synthesized. The nanoparticles, so formed, were characterized by dynamic laser scattering for their size and zeta potential and used for further studies.

Preparation of DP nanoparticles /pDNA complexes

An aqueous solution of DP nanoparticles (1mg/ml) was mixed with 1µl pDNA (300ng/µl) at various w/w ratios to form DP/pDNA complexes and the final volume was made up to 20µl by deionised water. Similarly, bPEI/pDNA complexes were prepared at different w/w ratios. For in vitro transfection assay, DP/pDNA and bPEI/pDNA complexes were prepared at w/w ratios of 0.66, 1.33, 2.33, 3.33, 5.0 and 6.66 (for corresponding N/P ratios, *pl. see Supplementary Information, Table S3*). 5µl of 20% dextrose solution was also added before making up the final volume to 20µl. Lipofectamine^{TM}/pDNA complex was prepared following the protocol supplied by the manufacturer (vol/w 4.0). The resulting samples were then vortexed and incubated for 30min at room temperature (25 $\pm$ 2 ^{o}C) prior to use in transfection experiments and other studies. All the complexes were prepared under identical conditions.

Size and zeta potential measurements

DP nanoparticle and bPEI solutions were prepared at the concentration of 1.0 mg/ml. Plasmid DNA was complexed with bPEI and DP nanoparticles as described above and the resulting complexes were characterised by using DLS on a Zetasizer Nano-ZS (Malvern Instruments, UK). DP/pDNA complexes were prepared at their best working w/w ratio of 2.33 (where these complexes exhibited the highest transfection efficacy) and incubated for 30 min at room temperature. The scattering of light was monitored at 173° to the incident beam and the mean hydrodynamic diameter was obtained from the diffusion coefficient using the Stokes-Einstein equation. The hydrodynamic diameter of nanoparticles and their complexes was determined by average value of 20 runs in triplicates. The surface charge of the nanoparticles was also determined by carrying out 30 runs in triplicates and estimated by Smoluchowski approximation from electrophoretic mobility.

For TEM imaging, grids were prepared using 10 µl solution of DP-2 nanoparticles and DP-2/pDNA complex (prepared at w/w ratio of 2.33) on carbon-coated copper grids. 1% Uranyl acetate was used for negative staining and images were observed at an accelerating voltage of 200 kV on HR-TEM (Tecnai G2 20 twin, Tecnai 200 kV twin microscope).

Electrophoretic mobility shift assay (EMSA)

To estimate the minimum amount of DP nanoparticles required to completely bind with the known amount of anionic charged pDNA, an agarose gel electrophoresis experiment was carried out. DP/pDNA and bPEI/pDNA complexes were formed at various w/w ratios of 0.1, 0.23, 0.5, 0.66, 0.75, 1.0 and 1.2, and 0.16, 0.23, 0.30, 0.45, 0.5, 0.66 and 0.75, respectively, with fixed amount of 1µl DNA (300ng/µl) and incubated for 30 min at room temperature. pDNA was taken as a reference standard. The complexes, thus formed, were mixed with 2µl Orange G dye, electrophoresed (100V, 1h) in 0.8% agarose, pre-stained with EtBr, in 1x TAE buffer and visualized in Gel Doc System (G:box UV transilluminator).

DNA release assay

The stability of the DP/pDNA complexes was assessed by heparin-mediated DNA release assay and compared with the stability of bPEI/pDNA complex. DP nanoparticles and bPEI were complexed with 0.3µg pDNA at their best working w/w ratios of 2.33 and 1.33, respectively (where these complexes exhibited the highest transfection efficiency), and incubated for 30 min. Then heparin, a polyanion, was added in increasing amounts varying from 0-3.0 U, which competed with pDNA and released it from the complexes. The samples were then incubated for 30 min, mixed with 2µl Orange G dye and were electrophoresed (100 V,1 h) in a 0.8% agarose gel, pre-stained with EtBr, and visualized on a UV transilluminator using Gel Doc System.

In *vitro* transfection studies

MCF-7 cells were seeded in a 96-well plate at a density of ~6 $x10^3$ cells/well and incubated overnight at 37^{o}C in a humidified environment in a CO_2 incubator. Post 24h, the media was aspirated and cells were washed with 1x PBS. bPEI/pDNA and DP/pDNA complexes were prepared at different w/w ratios (0.66, 1.33, 2.33, 3.33, 5.0, 6.66), incubated for 30 min at RT and diluted with media (DMEM with or without 10% serum). Similarly, pDNA complex was made with commercially available transfection reagent, Lipofectamine^{TM}, according to manufacturer's protocol at vol/w ratio of 4.0. These complexes were then gently added on to the cells and kept the plate at 37^{o}C in a humidified CO_2 incubator. After 3 h, the transfection mixture was aspirated out and fresh complete media (DMEM containing 10% serum) was added to the cells in each well. Cells were further incubated at 37^{o}C in CO_2 incubator for 36h. The cells were visually observed for GFP expression under fluorescence microscope.

Quantification of GFP expression

The quantification of the GFP expression in transfected cells was estimated by Nanodrop ND-3000 spectrofluorometer. After 36 h of transfection, the cells were washed with 1x PBS, incubated with 50µl of cell lysis buffer for 45 min at 37^{o}C and harvested. Then, 2µl of sample was used on nanodrop to estimate protein (λ_{ex}: 488nm; λ_{em}: 509 nm). 1x PBS was used as a blank to calibrate the spectrofluorometer to zero reading. The values were then normalized by using Bradford reagent with BSA as a standard and the total protein content was determined for each concentration and each well.

Cell viability assay

Cytotoxicity of bPEI/pDNA, DP/pDNA and Lipofectamine^{TM}/pDNA complexes was evaluated on MCF-7 cells by MTT colorimetric assay. This assay involves the reduction of tetrazolium group by mitochondrial succinate dehydrogenase in live cells into formazan crystals. After 36 h, MTT (1mg/ml) dissolved in DMEM was added to the cells and the plate was kept in a CO_2 incubator for 2 h at 37°C. After incubation, the supernatant was aspirated and the formazan crystals were suspended in 100 µl isopropanol containing 0.06M HCl and 0.5% SDS. The color intensity was measured spectrophotometrically on an ELISA plate reader (MRX, Dynatech Laboratories) at 540 nm. Untreated cells were used as control with 100% viability, and cells without addition of MTT were taken as blank to calibrate the spectrophotometer to zero absorbance.

siRNA transfection

In another experiment, GFP-specific siRNA was delivered using DP nanoparticles as a carrier. Cells were treated first with DP/pDNA complexes (w/w 2.33) for 3h, washed with 1x PBS and subsequently treated with 2µl GFP-specific siRNA (2.5 µM) using DP/siRNA formulations in 20µl reaction mixture. DP/pDNA complexes alone were used as control. Similarly, pDNA and GFP-specific siRNA were delivered using Lipofectamine^{TM} for the comparison purposes and quantified the GFP expression after 36h spectrofluorometrically.

DNase I Protection assay

To evaluate the potential of DP-2 nanoparticles towards protection of the bound pDNA from nucleases present in the cellular milieu, DNase I protection assay was executed at different time intervals. DP-2/pDNA complex, prepared at w/w ratio of 2.33, and pDNA alone were incubated for 0.25, 0.5, 1 and 2 h with 1 µl of DNase I (1U/µl) in a buffer containing 100

mM Tris, 25 mM $MgCl_2$ and 5 mM $CaCl_2$. After incubation, the DNase I was inactivated using 1 µl of EDTA (100 mM) and heated at 80 °C for 10 minutes. The reaction mixtures were further incubated with 10 U of heparin for 30 min to release the protected pDNA from DP-2/pDNA complexes. The reaction mixtures were electrophoresed for 1 h at 100V in 0.8% agarose gel pre-stained with EtBr and visualized on UV transilluminator using Syngene gel Documentation system. The amount of pDNA released from DP-2/pDNA complexes after treatment with heparin was estimated by densitometry.

Conclusions

In the present study, we have shown the effect of ionic crosslinking with a bioreducible agent, DTPA, which not only decreased the cationic charge density but also enhanced the transfection efficiency by several folds and cell viability. Electrostatic interactions between bPEI and DTPA resulted in the formation of DP nanoparticles. Heparin release assay showed that DP nanoparticles facilitated the release of pDNA from the complexes, which could be even higher inside the cells due to glutathione-mediated reduction of disulfide bonds. This could be one of the main reasons for obtaining higher transfection efficiency compared to bPEI and Lipofectamine^{TM}/pDNA complexes. siRNA delivery further established the versatility of DP-2 nanoparticles to carry nucleic acids efficiently inside the cells. These results altogether ensure the promising potential of these vectors in future gene delivery application in vitro and in vivo.

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