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Novel polyallylamine–dextran sulfate–DNA nanoplexes: Highly efficient non-viral vector for gene delivery

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Abstract

Polyallylamine (PAA), a less investigated polycationic polymer along with polyanionic dextran sulfate (DS) has been complexed with DNA into self assembling PAA–DS–DNA complexes with zinc as stabilizing agent. The complexes prepared were characterized by dynamic light scattering and atomic force microscopy and found to have average hydrodynamic diameter of 150 nm with polydispersity below 0.2, i.e. nanoplexes with narrow size distribution. As expected, the zeta potential values increased with the increase in the PAA in the nanoplexes. Further, PAA–DS–DNA nanoplexes were investigated for in vitro transfecting efficacy on HEK 293 cells. The nanoplexes were found to have better gene transfer efficacy compared to the DNA–PAA complex and commercially available transfecting agent lipofectin. The cytotoxicity was also decreased considerably as revealed by MTT colorimetric assay. The nanoplexes prepared with PAA to DS ratio of 2 were found to be most efficient transfecting agent.

Keywords: Polyallylamine; Dextran sulfate; Nanoplexes; Atomic force microscopy; Lipofectin

1. Introduction

For the past few decades viral vectors have been known to have efficient transfection efficiency, but disadvantages such as immunogenicity, pathogenicity and low gene carrying capacity have led to the search of non-viral delivery system (Verma and Somia, 1997; Chong and Vile, 1996; Otto et al., 1994; Song et al., 1997). These non-viral delivery systems are generally considered to be safer since they are typically less immunogenic and lack mutational potential.

Amongst various non-viral vectors cationic polymers and lipids have emerged as most promising candidate. These polycationic polymers interact with the polyanionic plasmid DNA to reduce them to nanometric particles. The ability of the polycation to condense DNA into nanoparticles is often critical for transfection efficiency. These systems carry net positive charge which helps to interact with negatively charged proteoglycans of the cell membrane, leading to cell surface tethering and enhanced endocytosis (Boussif et al., 1995; Haensler and Szoka, 1993; Mislick and Baldeschwieler, 1996; Zabner et al., 1995). The cationic polymers have been found to form more stable complexes than lipids, thus rendering more protection during cellular trafficking (Zabner et al., 1995; Hwang and Davis, 2001; Pollard et al., 1998). A large number of naturally occurring and synthetic polymers have been investigated for efficient gene delivery into mammalian cells.

Polyethylenimine and polyamidoamine dendrimers which possess endosome buffering capacity at acidic pH are efficient transfecting agents (Boussif et al., 1995). However, cationic polymers like poly-L-lysine can efficiently deliver DNA only when coupled with specific ligands which leads to receptor mediated endocytosis and used in presence of lysosomotropic agents such as chloroquine (Zenke et al., 1990; Wagner, 1998; Midoux et al., 1993; Zuaner et al., 1998). Modification of poly-L-lysine by grafting amino acids with side chains of lower pK_a values, such as histidine, increases the transfection efficiency to the PEI level (Pichon et al., 2001). One of the less investigated cationic polymers, polyallylamine (PAA) has high density of primary amino groups existing as free amine or as cationic ammonium salt. The polycationic nature of the PAA appears to be the main origin of its marked cytotoxicity, a property common with most polycationic polymers (e.g. polyethylenimine, poly-L-lysine). Attempts to decrease the toxicity by glycolation of PAA have considerable results

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along with improved transfection efficiency (Boussif et al., 1999).

In the present investigation PAA have been complexed with DS along with DNA with the aim to improve upon transfection efficiency and to reduce cytotoxicity. The polyanion dextran sulfate (DS), is a branched chain of anhydroglucose units which contains approximately 2–3 sulfate groups per glucosyl residue. Dextran sulfate is a biodegradable and biocompatible polymer that has been widely used in pharmaceutical applications. Since, the transfection efficiency has also been found to increase with the use of zinc ions; we have also incorporated zinc ions during the complex formulations (Pichon et al., 2002). DLS and AFM were employed for determining the size and surface charge of the prepared nanoplexes. The in vitro transfection efficiency and cytotoxicity experiments were carried out on human embryonic kidney 293 (HEK 293) cell line. Moreover, to investigate the role of polymer, experiments were carried out with nanoplexes prepared at different PAA to DS ratios.

2. Materials and methods

2.1. Materials

Dextran sulfate (MW 500,000), mannitol, zinc sulfate, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Tris, EDTA, ethidium bromide (ETBR), bromophenol blue (BPB) and xylene cyanol (XC) were all from Sigma (St. Louis, MO, USA). All other chemicals and reagents were procured locally. Nanoparticles were sonicated using Misonix 3000 sonicator, West Chester PA, USA, with total sonication time of 1 min (6× 10 s pulse each followed by a 10 s stop time) at 4 °C (ice-bath) with power set at 3 W using micro-tip probe. Cellulose ester dialysis tubing (molecular weight cut-off (MWCO) 1000 kDa) and regenerated cellulose dialysis membrane tubing (MWCO 15 kDa, 15 mm diameter) were obtained from Spectrum (Rancho Dominguez, CA, USA). Millipore Centricon YM-100 (100-kDa cut-off) was obtained from Millipore (Bedford, MA, USA). GFP protein expression was observed under Nikon Eclipse TE 2000-U inverted microscope, Kanagawa, Japan, fitted with C-Fl epifluorescence filter block B-2A consisting of excitation filter Ex 450–490 nm, Dichroic mirror DM 505 and barrier filter BA 520. Qiagen kit for plasmid isolation was purchased from Qiagen Inc., CA, USA. Cell culture media, Dulbecco's modified eagle's medium (DMEM), fetal calf serum (FCS) were from GIBCO-BRL-Life Technologies, Web Scientific Ltd., UK.

2.2. Preparation of PAA–DS–DNA nanoplexes

PAA–DS–DNA nanoplexes were prepared at room temperature. Three hundred microliters of PAA solution (0.1 mg/ml), pH 8 was added to a solution containing 100 μl of plasmid DNA (50 μg/ml in 10 mM Tris buffer, pH 8), 200 μl of DS aqueous solution (0.1 mg/ml) and 500 μl of a 10 mM Tris buffer, pH 8 with continuous vortexing at maximum speed. After 2 min, 25 μl of a zinc sulfate solution (10 μM) was added and the resulting nanoplexes were vortexed for another 5 min. The stabilized DNA nanoplexes were washed for 24 h in the dark by dialysis against a 5% (w/v) mannitol solution using Spectra Poor CE MWCO 1000 kDa membrane (Tiyaboonchai et al., 2003). The purified, DNA-loaded nanoplexes were lyophilized using speed vac. The lyophilized nanoparticles were stored in a desiccator at 2–8 °C. All the samples were prepared in duplicate. The lyophilized particles containing 50 μg/ml of DNA were reconstituted in 10 mM Tris, pH 7.4, and dialyzed against the same buffer at 4 °C in a dark room for 24 h before characterization.

2.3. Characterization of nanoplexes

The PAA–DS–DNA nanoplexes prepared in the present study were characterized by the following procedures.

2.3.1. Dynamic light scattering (DLS)

The hydrodynamic diameter of the PAA–DS–DNA nanoplexes was determined by dynamic light scattering (DLS) measurements. Nanoplexes were suspended in 1 ml of 10 mM Tris, pH 7.4 and sonicated prior to measurements. Size of nanoplexes was determined using Zetasizer, Nano ZS (Malvern instruments, UK) employing a nominal 5 mW HeNe laser operating at 633 nm wavelength. The scattered light was detected at 135° angle. The refractive index (1.33) and the viscosity (0.89) of ultrapure water were used at 25 °C for measurements. All the data analysis was performed in automatic mode. Measured sizes were presented as the average value of 20 runs.

2.3.2. Zeta potential measurements

The PAA–DS–DNA nanoplexes were suspended in 1 ml of 10 mM Tris, pH 7.4 as in case of size determination studies and further used for zeta potential measurements. Zetasizer Nano ZS was used for carrying out zeta potential studies. Zeta potential measurements were carried out in automatic mode and the values were presented as the average value of 30 runs. The Smoluchowski approximation was used to calculate zeta potential from the electrophoretic mobility.

2.3.3. Atomic force microscopy

The size and surface morphology of the nanoparticles was determined by atomic force microscopy using PicoSPM System (Molecular Imaging, Arizona, USA) operating in acoustic mode. Imaging was obtained by employing a medium scanner (scan range 30 μm × 30 μm). A 250 μm long magnetically coated cantilever (AAC lever) with a spring constant of 2.8 N/m and resonance frequency of ∼65 kHz was used. Lyophilized powder (∼0.5 mg) of nanoparticles was dispersed by sonication in double distilled water (1 ml) to obtain a clear solution, 2–3 μl of this solution was deposited on a freshly split untreated mica strip and allowed to dry for 5 min at room temperature. Subsequently, the mica surface was imaged. Particle size was obtained using SPIP software.

2.4. DNA incorporation efficiency

One of the nanoplexes sample (lyophilized without dialysis), prepared using above methodology, was dispersed in 1 ml

10 mM Tris buffer, pH 7.4. The solution was filtered through a Millipore Centricon YM-100 (100-kDa cut-off) membrane filter. The nanoplexes were retained while the free plasmid DNA passed through the filter. The amount of plasmid DNA present in the filtrate was determined spectrophotometrically using a Perkin-Elmer Lambda Bio 20 UV/VIS spectrophotometer at a wavelength of 260 nm. The entrapment efficiency (E, %) was calculated from the total concentration of the added amount of DNA present in the system ($[DNA]_T$) and that in the filtrate ($[DNA]_f$) using the equation:

$$E\,(\%) = \frac{[DNA]_T - [DNA]_f}{[DNA]_T} \times 100.$$

2.5. In vitro cell transfection studies

Human embryonic kidney 293 (HEK 293) cells were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum and 50 μg/ml gentamycin. The cells were grown at 37 °C in humidified 5% CO_2 atmosphere. HEK 293 cells were seeded prior to transfection into 96-well plates at a density of 3.5×10^3 cells/well and incubated for 16 h for adherence. After stipulated time the cells were washed once with serum free DMEM. Reporter gene CMV-GFP encoding for green fluorescent protein incorporated in the nanoplexes was used to assess the efficiency of PAA–DS–DNA nanoplexes mediated transfection. PAA–DS–DNA nanoplexes entrapping 0.5 μg of plasmid DNA were suspended in 20 μl H_2O, then diluted with serum free DMEM to a final volume of 80 μl and this transfection media was added to each well, followed by incubation at 37 °C in humidified 5% CO_2 atmosphere for 4 h. After 4 h, the transfection media was replaced with 150 μl of serum supplemented DMEM and cells were further incubated for 36 h under same conditions. Thereafter, the cells, transfected with GFP reporter gene, were observed under bright field and UV using GFP filter, at 10× magnification, under an inverted fluorescent microscope to observe the expression of green fluorescent protein.

2.6. Analysis of EGFP expression

The cells transfected with the plasmid DNA carrying reporter gene GFP were quantified by measuring the fluorescence intensity of the protein expressed. After 36 h of transfection, the cells were washed once with PBS followed by lysis in a buffer containing 10 mM Tris–HCl, pH 7.4, 0.5% SDS and 1 mM EDTA, and incubated in a shaker for 15–20 min at 25 °C. The lysate (50 μl) diluted to 1 ml in PBS was used to estimate the expressed reporter gene product, green fluorescent protein (GFP), spectrofluorometrically on Fluoromax-3 (Jovin Yvon Horiba, Tokyo, Japan) at an excitation wavelength 488 nm and emission at 508 nm. Background correction did using lysate of control cells i.e. cells without addition of plasmid DNA. The total protein content in cell lysate from each well was estimated using Bradford's reagent (Biorad) by taking BSA as a standard. The fluorescence intensity data is reported as arbitrary units (a.u.)/mg of cellular protein.

2.7. In vitro cytotoxicity studies

The toxicity of PAA–DS–DNA nanoplexes was estimated using MTT colorimetric assay (Mosmann, 1983). HEK 293 cells were seeded onto 96-well plates at a density of 8×10^3 cells/well and incubated for 16 h for adherence. The cells were incubated with PAA–DS–DNA nanoplexes as described above in the transfection experiment. After 36 h, 50 μl MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (2 mg/ml in DMEM) was added to the cells and incubated for another 2 h. The MTT containing medium was aspirated, and the formazan crystals formed by the living cells were dissolved in 100 μl isopropanol containing 0.06 M HCl and 0.5% SDS. Aliquots were drawn from each well after 1 h of incubation and the absorbance measured spectrophotometrically in an ELISA plate reader at 540 nm. Untreated cells were taken as control with 100% viability and cells without addition of MTT were used as blank to calibrate the spectrophotometer to zero absorbance. The relative cell viability (%) compared to control cells was calculated by $[abs]_{sample}/[abs]_{control} \times 100$.

3. Results

3.1. Physicochemical properties

3.1.1. Morphology

The structure of PAA–DS–DNA nanoplexes was examined by AFM. AFM images of DNA-loaded nanoplexes shows particles with a uniform spherical shape and a smooth surface distributed throughout the sample (Fig. 1). Completely condensed DNA itself tends to be roughly globular in shape while incomplete condensation tends to display DNA with folded and looped structures (Dunlap et al., 1997). The AFM images suggests of highly condensed nature of DNA within the nanoplexes. Polymer concentration and the ratio of PAA to DS were virtually found to have no effect on the images of the particles.

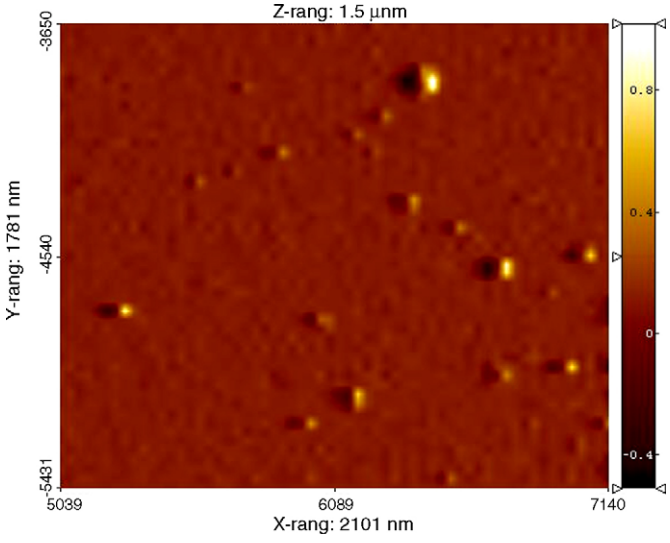

Fig. 1. Representative atomic force microscopy image of PAA–DS–DNA nanoplexes at polymer to DS ratio of 3. Average size is 125–135 nm.

Table 1
Size and zeta potential of PAA–DS–DNA nanoplexes

S. No.	Polymer ratios (PAA/DS)	Average particle size in nm by DLS in buffer	Polydispersity index	Zeta potential (mV) in buffer
1.	1:1	274	0.396	+22.8
2.	2:1	154	0.184	+24.1
3.	3:1	143	0.156	+26.6
4.	4:1	161	0.243	+35.8
5.	5:1	169	0.123	+54.2

Buffer: 10 mM Tris, pH 7.4.

3.1.2. Average particle size and zeta potential

The average particle size and polydispersity index of nanoplexes was studied by DLS. The ratio of PAA and DS had a significant effect on the nanoplex size and polydispersity index. With the increase in the PAA/DS weight ratio, the average hydrodynamic diameter and polydispersity index first decreased and then increased (Table 1). A polymer ratio of 1:1 yielded the largest average particle size (i.e. 274 nm) with a high polydispersity index of 0.396 (indicating a broad size distribution). Polymer ratios of 3 produced an average particle size of 143 nm with a polydispersity index of 0.156 indicating a narrow size distribution (Fig. 2). As expected the zeta potential also increased with the increase in the amount of polymer with respect to DS in the nanoplexes (Table 1).

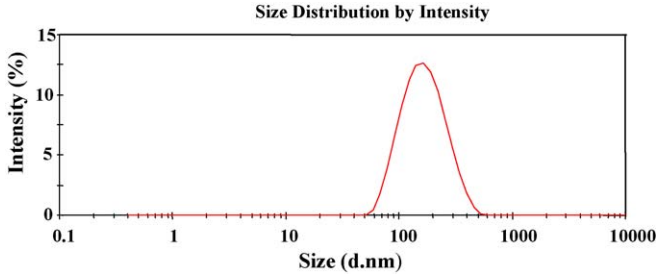

Fig. 2. Representative overlay of dynamic light scattering of PAA–DS–DNA nanoplexes at polymer to DS ratio of 3. Average size in this case is 143 nm.

3.2. Efficiency of DNA incorporation

The DNA entrapment efficiency was determined by filtering the PAA–DS–DNA nanoplexes suspended in 1 ml buffer through 100 kDa cut-off filter. The amount of DNA in filtrate calculated taking 1 O.D. at 260 nm equal to 50 μg of plasmid DNA. All nanoplexes prepared were found to have more that 93% DNA incorporation efficiency. Polymer ratios were not found to have a significant effect on the entrapment efficiency of DNA in the nanoplexes.

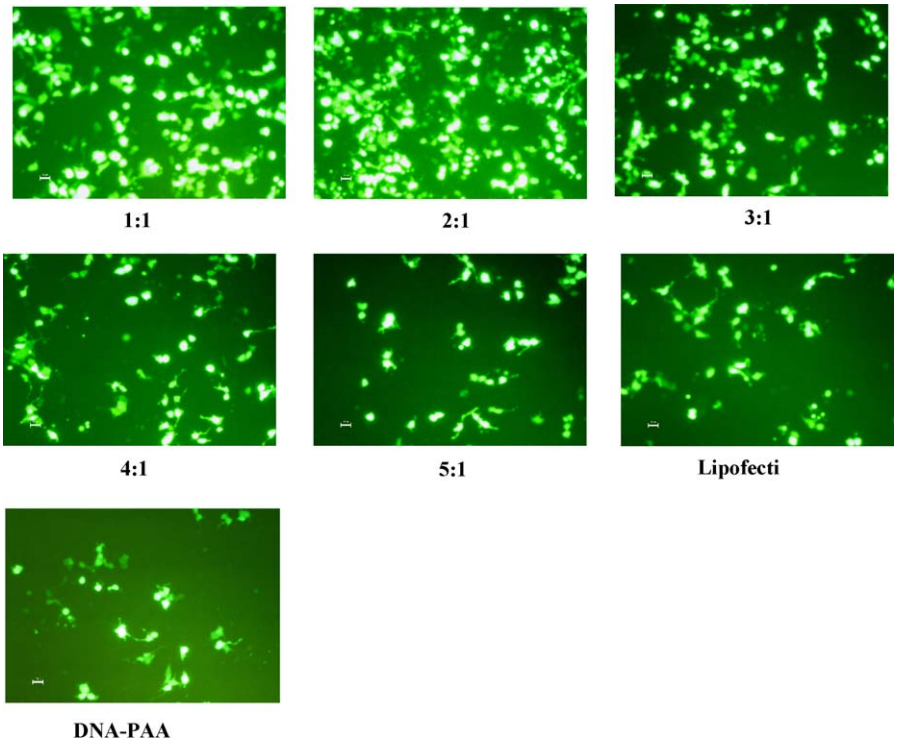

Fig. 3. Comparison of transfection efficiency of various PAA–DS–DNA nanoplexes. HEK 293 cells were incubated with PAA–DS–DNA nanoplexes entrapping 0.5 μg plasmid DNA for 4 h. The green fluorescent protein (GFP) expression was observed under fluorescent microscope at 10× magnification after 36 h.

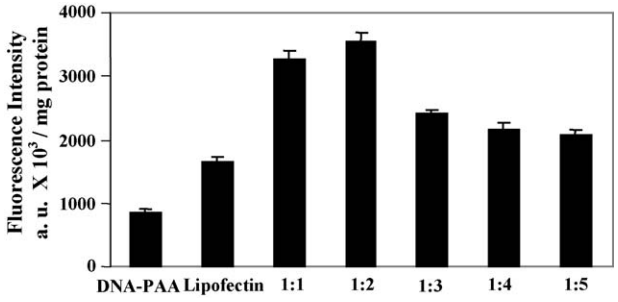

Fig. 4. Comparison of transfection efficiency of various PAA–DS–DNA nanoplexes. HEK 293 cells were incubated with PAA–DS–DNA nanoplexes entrapping 0.5 μg plasmid DNA for 4 h. The green fluorescent protein (GFP) expression was quantitated after 36 h using spectrofluorometer with excitation at 488 nm and emission at 508 nm. The results expressed in terms of fluorescent intensity (a.u.)/mg of protein. The assays were done in triplicate and standard error is shown.

3.3. In vitro cell transfection studies

In vitro studies, with both lipoplexes and polyplexes, have shown that electrostatic interactions between the negatively charged cell membranes and the positively charged DNA complexes are enhanced by increasing the overall charge of the complexes, which in turn is achieved by increasing the ratio of carrier to DNA (Felgner et al., 1997). The nanoplexes prepared at different polymer to dextran sulfate ratios were investigated for their transfection efficiency in HEK 293 cells. The cells were incubated with 0.5 μg DNA containing nanoplexes for 4 h in serum free medium. After 36 h, the GFP expression was visualized under fluorescent microscope and considerable amount of improvement in transfection efficacy was observed as compared with the native DNA–PAA (0.5 μg DNA/μg PAA) and commercially available transfecting agent lipofectin (Fig. 3). The transfection efficiency reached a maximum value with nanoplexes formulated at a PAA/DS ratio of 2. Later transfection decreased with the increase in the polymer ratio (Fig. 4).

3.4. In vitro cytotoxicity studies

The effect of complexation of PAA with DS on cytotoxicity was examined on HEK 293 cells using MTT colorimetric assay (19). Cells were incubated with different nanoplex formulation for 4 h (absence of serum) followed by further incubation for 36 h (presence of serum). Microscopic examination of DNA–PAA treated cells revealed considerable toxicity and cell morbidity (42% viability). PAA–DS–DNA nanoplexes were comparatively less toxic with only 13–24% loss in cell viability with formulations having maximum transfection efficiency. However, cell viability decreased with the increase in the polymer concentration in the higher PAA to DS ratio formulations (Fig. 5).

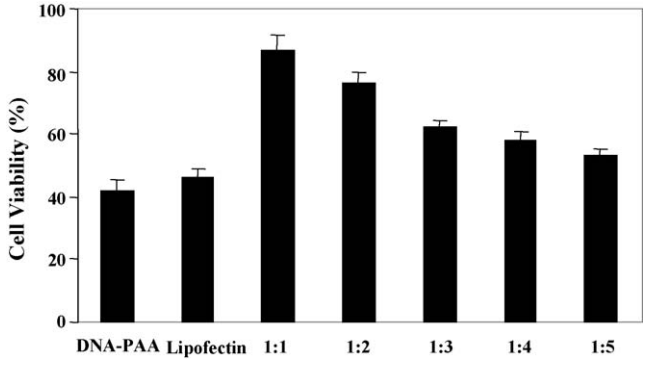

Fig. 5. Cell viability assay. HEK 293 cells were incubated with PAA–DS–DNA nanoplexes entrapping 0.5 μg plasmid DNA for 4 h. The cell viability was estimated after 36 h using MTT colorimetric assay. The assays performed in triplicate and standard error is shown.

4. Discussion

Till date a wide variety of cationic polymers have been used for in vitro and in vivo gene delivery purpose (Boussif et al., 1995; Haensler and Szoka, 1993; Kabanov et al., 1991). Due to the high cationic charge density, polycationic polymers (such as PEI) are able to effectively condense DNA and form nanometric particles capable of being endocytosed (Vinogradov et al., 1998). But unfortunately, the polycationic nature of polymers appears to be the primary origin of its toxicity. Cationic polymers such as PLL and PAA, which possess only primary amino groups, are relatively toxic along with poor gene delivery efficacy when complexed to DNA (Sgours and Duncan, 1990). When primary amines of PAA were substituted with glycolate from 50 to 70% transfection efficiency was found to increase considerably with increase in glycolylation (Boussif et al., 1999). However, the transfection efficiency decreased rapidly on glycolylation beyond 75%.

In the present investigation, a novel nanoplex gene delivery system employing PAA and DS with zinc sulfate as a stabilizing agent has been developed in an attempt to enhance PAA's transfection efficacy along with increase in cell viability. These nanoplexes are found to have enhanced transfection activity with the reduction in the intrinsic toxicity of the PAA component. In one of the earlier studies, polyethylenimine has been complexed with DS and significant improvement in transfection efficiency along with cell viability was found (Tiyaboonchai et al., 2003).

The size and surface charge of nanoparticulates are two essential parameters that control their uptake by cells (Zanta et al., 1997). The mean sizes of particles prepared using above methodology were found to be dependent on the PAA to DS ratio. The particle sizes reduced from 274 nm observed at a polymer ratio of 1:1 to 143 nm at a polymer ratio of 3:1. A zeta potential above +22 mV would, be expected to inhibit aggregation because of charge repulsion. The low values of polydispersity index observed in the study suggest uniform size distribution of particles. The particle size increases from 143 to 169 nm with the increase in the polymer ratio from 3:1 to 5:1. The increment in the size could be due to the formation of less compact structure because of increased electrostatic repulsions with the presence of large amount of cationic polymer.

Studies on size dependency of nanoparticle mediated gene transfection had shown that the smaller sized nanoparticles (<100 nm) has 27-fold higher transfection than the larger sized (>100 nm) in COS-7 cell line (Prabha et al., 2002). However,

high transfection efficiency of partially aggregated particles results from the conjunction of several features, such as large particles can sediment onto cell surface rapidly than small complexes, these contain large proportion of free cationic polymers in addition to those complexed with DNA which destabilizes the membrane favoring their entry into cells and they have high endosmolytic activity (Ogris et al., 1999; Tang and Szoka, 1997). Thus, it is expected that the 150 nm average diameter particles prepared by the PAA–DS–DNA blending should be suitable for uptake by endocytotic processes. Moreover, this particle size is obtained in low ionic strength media whereas under physiological salt concentrations (150 mM NaCl) particles rapidly aggregate into large particles (1 μm).

Complexing dextran sulfate with PAA results in reduction of intrinsic toxicity of PAA with moderate improvement in transfection efficiency. At PAA to DS ratio of up to 2 the cells were virtually resistant to nanoplexes even after 48 h. The cell viability was more than 70% at both 1 and 2 ratios of PAA to DS. As expected, the cell viability was found to depend upon the concentration of the polycationic polymer i.e. decreased with the increase in polymer concentration. The DS incorporation into nanoplexes has made convenient the use of PAA by at least 20 times higher than that used in DNA–PAA complexes.

We presumed that, by neutralizing or masking the cationic charge of the polymer by complexing with biodegradable, hydrophilic polymer, the toxicity could be reduced and its gene delivery efficiency could be enhanced. The nanoplexes prepared in this study with different formulations produced higher transfection efficiencies than DNA–PAA polyplexes (0.5 μg DNA/μg of PAA) and commercially available transfecting agent lipofectin. The reporter gene expression (green fluorescent protein) was maintained for 3 days after transfection and a gradual decrease was observed afterwards (Fig. 3). The increase in transfection efficiency was observed with the increase in the amount of DNA entrapped in nanoplexes but increase in toxicity prohibited the further study (data not shown).

5. Conclusions

We have prepared PAA–DS–DNA nanoplexes with different polymer to dextran sulfate ratios. This preparation methodology involves mild processing conditions and completely aqueous nature with very high DNA entrapment efficacy (>93%). As expected, the incorporation of DS into PAA have led to the reduction in cytotoxicity along with moderate improvement in transfection efficiency. This study suggests that PAA–DS–DNA nanoplexes can be further used for in vivo gene delivery experiments.

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