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PEI-alginate nanocomposites: Efficient non-viral vectors for nucleic acids

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ABSTRACT

Branched polyethylenimine (PEI, 25 kDa) was ionically interacted with varying amount of alginic acid to block different proportion (2.6–5.7%) of amines in PEI to form a series of nanocomposites, PEI-Al. These nanocomposites, upon interaction with DNA, protected it against DNase I. Among various complexes evaluated, PEI-Al(4.8%)/DNA displayed the highest transfection efficiency in HEK293, COS-1 and HeLa cells that was ~2–8-folds higher than Superfect™, Fugene™, PEI (750 kDa)-Al(6.26%) and PEI alone. The projected nanocomposites were nearly non-toxic to cells *in vitro*.Furthermore, the concentration of PEI-Al(4.8%) needed to deliver GFP-specific siRNA in COS-1 cells was 20 times lower than PEI (750 kDa)-Al(6.26%). Intracellular trafficking of PEI-Al(4.8%) with or without complexed DNA in HeLa cells shows that both appear in the nucleus after 1 h.

1. Introduction

Increasing identification of genetic disorders has raised the possibility of cure by way of gene therapy involving target-specific delivery of therapeutic genes. So far, viral vectors, used to deliver genes, are efficient, but are plagued with safety issues (Servos, 1987; Yang et al., 1994; Miller and Vile, 1995). In contrast, non-viral vectors are preferable to viral vectors due to their reduced toxicity and lower immunogenicity and are easier to design and synthesize. The classes of polycationic polymers constitute such attractive candidates owing to their ability to condense DNA into nano-size polyplexes (Felgner et al., 1997). Although efficient as gene delivery vectors, cationic polymers are often highly toxic and not permissible for use in humans. Systemically injected cationic polyplexes interact with serum proteins resulting in their rapid clearance from the bloodstream (Nishikawa et al., 1998; Dash et al., 1999). Therefore, it is necessary to modify these polyplexes to eliminate their toxicity as well as interaction with serum proteins, before being used for systemic gene delivery.

One way to diminish toxicity and avoid the undesirable interactions of cationic polyplexes with serum proteins is to reduce positive charge by ionic or covalent binding to hydrophilic polymers such as polyethylene glycol (PEG) and polysaccharides (Choi et al., 1998; Baszkin and Norde, 2000). However, due to their functional groups, polysaccharides offer better option than PEG, which does not possess reactive groups required for ligand coupling (Erbacher et al., 1999; Passirani et al., 1998). These functional groups present in the polysaccharides can be utilized to graft ligands to achieve target-specific delivery (Lemarchand et al., 2004).

Alginic acid, a polysaccharide, popularly used in food and pharmaceutical industries (Dumitriu, 1996; Jiang et al., 2007) is used for cell immobilization and encapsulation due to its biocompatibility and gelation with divalent cations (Goosen, 1992). The nanocomposites of PEI (750 kDa) and alginic acid were explored earlier in our laboratory (Patnaik et al., 2006). The presence of alginic acid in nanocomposites not only decreased the toxicity but also efficiently transfected mammalian cells. Encouraged by these promising results, we thought of replacing PEI (750 kDa) in nanocomposites with PEI (b 25 kDa) as it is considered to be a gold standard (Zwiorek et al., 2004), which may further augment the transfection efficiency of the nanocomposites. Therefore, in continuation with our earlier studies, we prepared a series of PEI (b 25 kDa)-Al nanocomposites, evaluated them for transfection efficiency and cytotoxicity in various cell lines and compared with those of commercial transfection reagents. The transfection efficiency evaluated in various cell lines was found to be much superior to PEI (750 kDa)-Al(6.26%), PEI alone, Superfect™ and Fugene™. Cell cytotoxicity of the projected nanocomposites was almost negligible. We then studied the uptake and intracellular trafficking of labeled PEI (25 kDa)-Al(4.8%) nanocomposite in HeLa cells at different time points. We further showed that PEI (25 kDa)-Al(4.8%) nanocomposite effectively protected complexed DNA for 2 h *in vitro*.

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2. Materials and methods

2.1. Materials

Polyethylenimine (PEI, av. MW 25 kDa), alginic acid, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), agarose, Tris, ethidium bromide, xylene cyanol, tetramethylrhodamine isothiocyanate (TRITC), 4′,6-diamidino-2-phenylindole dilactate (DAPI) and bromophenol were procured from Sigma (USA). YOYO-1 for labeling plasmid DNA was purchased from Invitrogen (USA). Commercial transfection agents, viz., Superfect™ and Fugene™ were procured from Qiagen (France) and Roche Applied Science (USA), respectively. Cell culture products were purchased from Gibco-BRL-Life Technologies (UK). Plasmid isolation kit was purchased from Qiagen (France). The plasmid pEGFPN3 was procured from Clontech (USA). FTIR spectra of nanocomposites were recorded on a single beam Perkin Elmer (Spectrum BX Series), USA. The particle size and zeta potential of nanocomposites and their DNA complexes were determined on Zetasizer Nano-ZS (Malvern instruments, UK). The size of nanocomposites and DNA complexes was also determined by Atomic Force Microscopy (PicoSPM System, Molecular Imaging, USA). TEM images of nanocomposites were recorded on a Fei-Phillips, Morgagni 268D microscope (USA). GFP reporter gene expression was detected under Nikon Eclipse TE 2000-S inverted microscope (Japan). Green fluorescent protein (GFP) was analyzed on NanoDrop® ND-3300 Fluorospectrometer (USA) at an excitation wavelength of 488 nm and emission at 509 nm. Labeled nanocomposites and nanocomposite/DNA complexes were monitored on LSM 510 Meta fitted to an inverted microscope (Axio observer, Zeiss, Germany).

2.2. Synthesis of siRNA

For *in vitro* synthesis of GFP-specific siRNA, the oligonucleotide sequences, viz., d (TAA TAC GAC TCA CTA TAG) (T7 primer), d (ATG AAC TTC AGG GTC AGC TTG CTA TAG TGA GTC GTA TTA) (GFP sense) and d (CGG CAA GCT GAC CCT GAA GTT CTA TAG TGA GTC GTA TTA) (GFP antisense), were procured from TCGA (Delhi, India).

Using T7 primer sequence annealed separately to GFP sense and antisense oligonucleotides, GFP sense and antisense siRNA strands were synthesized by T7 RNA polymerase (Patnaik et al., 2006). The sense and antisense RNA strands were annealed and the double stranded siRNA was used for transfection.

2.3. Preparation of PEI-Al nanocomposites

The positive charge of branched PEI (25 kDa) was partially neutralized by interaction with alginic acid. The nanocomposites were prepared as reported earlier (Patnaik et al., 2006). Briefly, alginic acid (1.13 mg) was suspended in 113 ml water and stirred at 90 °C to solubilize it and added drop wise to a pre-heated aqueous solution of PEI (5 mg/500 ml) under constant stirring (90 °C, 4 h). The solution was filtered through a 0.22 μm membrane. The filtrate containing nanocomposite was lyophilized. Likewise, a series of nanocomposites were prepared by varying the alginate content.

The sugar content in the PEI-Al nanocomposites was determined by phenol-sulfuric acid method (Zhang et al., 2005). The amount of alginate in the nanocomposites was estimated by interpolation to a calibration curve for known amount of alginic acid standards. These nanocomposites were also characterized by FTIR.

2.4. Preparation of PEI-Al/DNA complexes

To prepare PEI-Al/DNA complexes, 5 μl of 20% dextrose was added to 1 μl pDNA (0.3 μg/μl) followed by the amount of nanocomposites to obtain the weight ratios, 0.66, 1.33, 2.0, 2.33, 2.66, 3.33, 4.16 and 5.0, and the final volume was made to 20 μl by adding water. The solutions were incubated for 25 min (room temp) to obtain PEI-Al/DNA complexes and subjected to measurement of size and ζ-potential, DNA retardation assay, transfection and cell toxicity.

2.5. Size and ζ-potential measurements

The particle size of PEI-Al nanocomposites was measured by dynamic light scattering (DLS) and atomic force microscope (AFM). Lyophilized PEI-Al (1 mg/ml of water) and PEI-Al/DNA complexes were subjected to particle size measurement on Zetasizer. Particle size was also examined by AFM in acoustic mode, using SPIP software and TEM. Zeta potential measurements were carried out in triplicates on Zetasizer in automatic mode for an average of thirty runs by Smoluchowski approximation.

2.6. DNA retardation assay

Plasmid DNA (0.3 μg) was complexed with P-Al nanocomposites and PEI at different weight ratios (w/w) in 5% glucose, incubated for 20 min (room temp) and electrophoresed on 0.8% agarose, as described in literature (Zhang et al., 2005). The electrophoretic mobility of DNA decreased with increasing concentration of nanoparticles/PEI in the polyplexes.

2.7. In vitro cell transfection

We examined transfectability of PEI-Al nanocomposites in HEK293, COS-1 and HeLa cells seeded in 96-well plates. After 16 h incubation, cells were washed with serum-free Dulbecco's modified Eagle's medium (DMEM). pDNA encoding GFP was complexed with PEI-Al at weight ratios 0.88, 1.33, 2.0, 2.66, 3.33, 4.18, 6.0, PEI (750)-Al (6.26%) at weight ratio described in literature (Patnaik et al., 2006) and PEI at weight ratios 0.83, 1.66 and 2.5, as described before. DNA complexes were also prepared with the commercial transfection reagents, viz., Superfect™ and Fugene™, at ratios (v:w) 2:1 and 4:1, respectively, as suggested by the manufacturers. DNA complexes were diluted with DMEM (with or without serum) to 80 μl (final volume), added to cells and incubated (37 °C). After 3 h, cells were washed, fresh growth medium (DMEM with 10% FCS) was added (200 μl/well) and incubated for 36 h.

In another experiment, 2 μl of 5 μM GFP-specific siRNA was added along with 1 μl of 1.4 nM pDNA to obtain PEI-Al(4.8%)/DNA/siRNA. Similarly, 200 μg/ml of PEI(750)-Al(6.26%) was complexed with 1 μl of 1.4 nM pGFPDNA and 2 μl of 5 μM GFP-specific siRNA to obtain PEI(750)-Al(6.26%)/DNA/siRNA and co-transfected in COS-1 cells. Cells treated with PEI-Al(4.8%)/DNA alone served as the control. The expression of GFP was visualised in an inverted fluorescent microscope.

2.8. Quantification of EGFP expression

To estimate GFP expression, after 36 h of transfection assay, the cells were washed with PBS (4× 1 ml) and lysed in 50 μl lysis buffer (10 mM Tris–HCl, 0.5% SDS and 1 mM EDTA, pH 7.4). The lysate was shaken (15–20 min, 25 °C) and GFP intensity was measured from 2 μl cell lysate on NanoDrop® ND-3300 spectrofluorimeter. The background and autofluorescence in mock treated cells were subtracted. The total protein content in cell lysate from each well was estimated by Bradford's test with bovine serum albumin, as a standard. Total fluorescent GFP in lysate was normalized against the amount of protein in the lysate and expressed in arbitrary units/mg protein along with the mean ± standard deviation from triplicate samples.

2.9. Percentage of cells transfected via fluorescence-assisted cell sorting (FACS)

The percentage of cells transfected was estimated by FACS analysis. COS-1 and HEK293 cells were incubated in a 24 well plate and transfection carried out, as described above. Post 36 h, the media was aspirated out and cells washed twice with phosphate buffer saline (PBS). The cells were then trypsinized followed by addition of 500 μl DMEM supplemented with 10% FCS and collected in a 1 ml eppendorf. Cells were centrifuged at 1200 rpm (5 min, 4 °C). Subsequently, cells were rinsed with PBS (2× 500 μl) and suspended in PBS (200 μl). The number of cells transfected was estimated by FACS. All experiments were carried out in triplicates.

2.10. Cytotoxicity assay

Cytotoxicity of PEI-Al/DNA complexes and commercial transfection reagents was assayed by colorimetric MTT assay (Pathak et al., 2007) in HeLa, HEK293 and COS-1 cells, 36 h post-transfection in an ELISA plate reader (MRX, Dynatech Laboratories) at 540 nm. Cells were transfected with PEI-Al/DNA, PEI/DNA, Superfect™/DNA and Fugene™/DNA complexes and, after 36 h, media was replaced with 0.2 ml MTT reagent (0.5 mg dissolved in 1.0 ml of DMEM) and incubated (1 h). The supernatant was removed and formazan crystals were suspended in 100 μl isopropanol containing 0.06 M HCl and 0.5% SDS, and the absorbance read at 540 nm. Non-transfected cells served as controls with 100% viability, while wells with MTT reagent alone served as blank. The cell viability (%) was estimated as $[abs]_{transfected}/[abs]_{control} \times 100$. All experiments were repeated at least thrice.

2.11. Fluorescence confocal microscopy

The trafficking of TR-PEI-Al nanocomposite and TR-PEI-Al/DNA-YOYO was studied in HeLa cells. PEI-Al(4.8%) (10 mg/ml of water) was treated with tetramethyl rhodamineisothiocyanate (TRITC) (1 mg/100 μl of DMF) overnight with stirring, followed by concentration in a Speed Vac. Unreacted/hydrolyzed TRITC was extracted with ethyl acetate to obtain tetramethyl rhodamine-labeled PEI-Al (4.8%) (TR-PEI-AL). DNA was labeled with the intercalating dye YOYO-1 (DNA-YOYO) (Cheng, 2000). HeLa cells were seeded at a density of 1.5×10^5 cells/well on circular glass coverslips in a 6-well plate and grown overnight to obtain ~70% confluence. Cells were then incubated (37 °C) with labeled nanocomposites (22 μg/ml of culture medium) and labeled nanocomposite/DNA complexes (w:w 3.33) for 0.5, 1, 2 and 4 h, washed with PBS (3× 1 ml) and fixed in 4% paraformaldehyde (1 ml/well, 10 min, r.t.). Cells were washed with PBS (3× 1 ml), stained with DAPI (20 μl of 10 ng/ml) and mounted on clean glass slides with fluorescence-free glycerol-based UltraCruz™ Mounting Medium (Santa-Cruz Biotechnology, USA) and examined in a confocal microscope.

Table 1
Percent of amines ionically linked to alginate in PEI-Al nanocomposites.

S. No.	Attempted ionic interaction (%)	Observed proportion of ionic interaction (%)
1	5	2.6
2	7	3.4
3	9	4
4	11	4.8
5	13	5.7

2.12. DNase protection assay

DNase I protection assay was carried out on 0.6 μg pDNA alone and PEI-Al (4.8%)/DNA complex (w:w, 3.33) in 25 μl (final volume) (Gebhart et al., 2002). Each sample was divided into 2× 10 μl aliquots. To the first was added 1 unit of DNase I (1 μl), while 1 μl PBS was added to the second, as control. Samples were incubated at 37 °C and withdrawn at regular intervals (15, 30, 60 and 120 min). The enzyme was inactivated by adding 0.6 μl of 100 mM EDTA followed by heating to 75 °C for 10 min. Subsequently, heparin (10 μl, 5 mg/ml) was added to the samples and incubated (2 h, r.t.) to dissociate the complex and release DNA. After adding xylene cyanol, samples were electrophoresed (100 V, 1 h) in 0.8% agarose, stained as before and photographed on an Alpha Imager (Alpha Innotech, San Leonardo, CA). The amount of supercoiled, linear and nicked closed circular forms of DNA in complexes, both before and after heparin treatment, was estimated by densitometry of the image using AlphaEaseFC software.

3. Results and discussion

A series of nanocomposites with varying amount of alginic acid were prepared by mixing the pre-heated solution (90 °C) of PEI and alginic acid. The proportion of amines interacting ionically with alginic acid was determined colorimetrically from the content of alginate in the nanocomposites. We found approximately 50% of the attempted ionic interaction in nanocomposites (Table 1), which is similar to our earlier data (Patnaik et al., 2006). FTIR analysis of PEI-Al nanocomposites yielded peaks at 3436 (amino stretching), 2927 and 1628 cm^{-1} (carbonyl stretching). The peak at 1628 cm^{-1} is indicative of the presence of carbonyl group of alginic acid in the PEI-Al nanocomposites.

The particle size and morphology of PEI-Al nanocomposites were determined by DLS and AFM studies. The size of PEI-Al nanocomposites was found to be in the range of 53–125 nm (Table 2). It was also observed that on increasing alginate content, the nanocomposite size decreased, which we attributed to an effective charge interaction between amines of PEI and alginate content resulting in more compact structures. The AFM images of PEI-Al(4.8%) nanocomposite showed spherical particles with a size of ~55 nm (Fig. 1). The average particle size obtained by AFM was found to be smaller than that obtained with DLS, which might be

Table 2
Size and zeta potential of PEI-Al nanocomposites.

S. No.	Samples	Average particle size in nm (PDI)		Zeta potential (+mV)		Ratio of nanocomposites: DNA (w/w)
		Nanocomposites (in H_2O)	DNA loaded complex (in H_2O)	Nanocomposites (in H_2O)	DNA loaded complex (in H_2O)	
1	PEI-Al (2.6%)	125 (0.316)	137 (0.432)	21.9	17.09	2.5
2	PEI-Al (3.4%)	111 (0.208)	122 (0.208)	19.04	12.94	2.5
3	PEI-Al (4%)	81 (0.405)	110 (0.315)	16.4	10.46	2.5
4	PEI-Al (4.8%)	73 (0.192)	89 (0.247)	14.07	8.47	3.33
5	PEI-Al (5.7%)	53 (0.241)	76 (0.271)	11.24	7.05	6.66

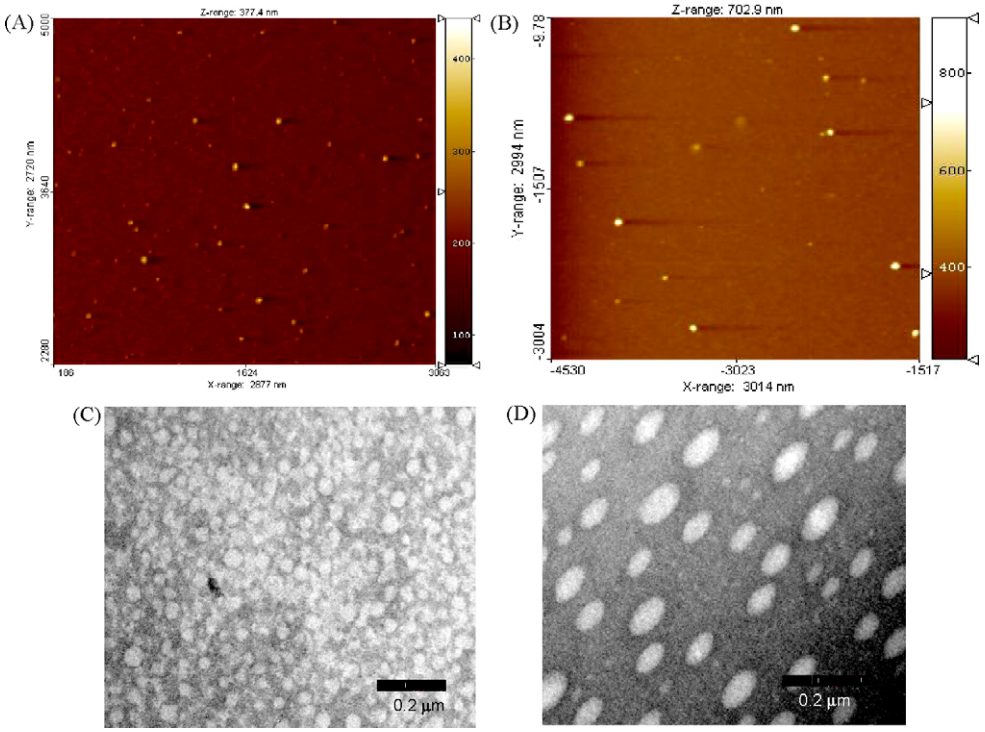

Fig. 1. AFM images of (A) PEI-Al(4.8%) alone, (B) PEI-Al(4.8%)/DNA. 2–3 μl of each polyplex solution was deposited on a freshly split untreated mica strip and images were recorded in acoustic mode. TEM images of (C) PEI-Al(4.8%) alone, and (D) PEI-Al(4.8%)/DNA. The size of nanocomposites was observed to be ~55 nm and that of polyplexes to be ~70 nm.

attributed to the contribution of hydrodynamic diameter in DLS measurements.

The presence of alginate in PEI-Al nanocomposites was also determined by measuring the zeta potential of nanocomposites in water. The zeta potential of PEI was +31 mV, while that of PEI-Al nanocomposites was in the range of +20–10 mV (Table 2). The lower value of zeta potential in nanocomposites compared to PEI is suggestive of the presence of alginate in PEI-Al nanocomposites. The zeta potential of PEI-Al nanocomposites decreased with increase in alginate content, which showed a further decline after complexation with plasmid DNA (pDNA). At different weight ratios of nanocomposites/DNA complex, the zeta potential showed an increase with increasing weight ratios (Table 3). This is due to an increase in cationic charge at higher weight ratios. However, at same weight ratios, the nanocomposites/DNA complex had different zeta potential values; the nanocomposites with higher alginate content had a lower zeta potential. Nevertheless, nanocomposites/DNA carries sufficient positive surface charge

Table 3
Zeta potential of PEI-Al/DNA complexes at various w/w ratios.

Samples	Sample/DNA weight ratios (w/w)					
	1.66	2.5	3.33	5	6.66	7.5
PEI-Al (2.6%)	15.99 ± 0.86	17.49 ± 0.53	18.06 ± 0.61	18.71 ± 0.72	19.14 ± 1.4	20.04 ± 1.65
PEI-Al (3.4%)	11.95 ± 0.98	12.71 ± 0.62	13.05 ± 0.88	13.9 ± 1.2	16.74 ± 0.70	17.41 ± 0.94
PEI-Al (4%)	9.51 ± 0.49	10.93 ± 0.91	11.2 ± 1.03	12.5 ± 0.92	13.82 ± 0.42	14.5 ± 0.99
PEI-Al (4.8%)	6.23 ± 0.65	6.91 ± 0.51	8.12 ± 0.43	10.07 ± 0.34	12.65 ± 0.82	12.98 ± 1.05
PEI-Al (5.7%)	2.14 ± 0.62	2.99 ± 0.56	4.5 ± 0.52	5.42 ± 0.55	7.22 ± 0.64	9.87 ± 0.54

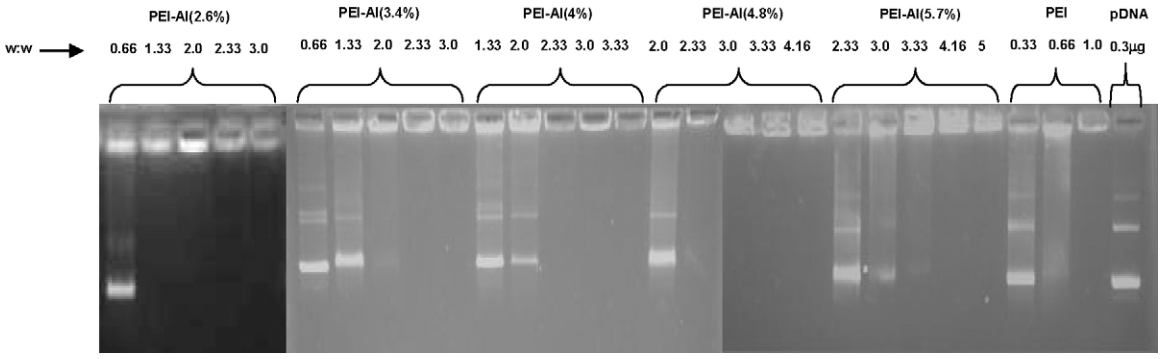

Fig. 2. Gel retardation assay of PEI-Al/DNA and PEI/DNA complexes. The values mentioned at the top of gel represent w/w ratio of nanocomposites/DNA in a 20 μl reaction.

needed to interact with endosomal membrane causing its disruption after internalization (Walker et al., 2005).

We found complexing of pDNA (0.3 μg) with PEI and PEI-Al nanocomposites at different weight ratios led to decreased electrophoretic mobility of DNA (Fig. 2). At w:w ratio 1.0, PEI completely retarded pDNA, while a higher weight ratio was required by PEI-Al nanocomposites to retard the same amount of pDNA (Fig. 2). Thus, compared to PEI-Al nanocomposites, PEI retards pDNA (0.3 μg) at a lower w:w ratio due to higher cationic charge. In any case, the interaction between pDNA and PEI-nanocomposite leads to neutralization of anionic phosphate backbone of DNA so that the electrophoretic mobility of the complex decreases.

To assess the transfection efficiency of the projected nanocomposites, we have compared the level of GFP expression in cells transfected with the nanocomposite/DNA complexes. The *in vitro* expression of PEI-Al/pDNA was evaluated in HEK293, COS-1 and HeLa cells and compared with the standard transfection reagents, viz., Superfect™ and Fugene™ loaded with pDNA. We found that on increasing alginate content in PEI-Al nanocomposite/pDNA, the GFP expression increased till 4.8% amines were blocked (Fig. 3) and beyond this, it decreased. GFP intensity at various w/w ratios of nanocomposites/DNA complex showed that PEI-Al(2.6–4%) formulations gave a higher fluorescence intensity at w/w 2.5, whereas for PEI-Al(4.8%) and PEI-Al(5.7%) it was 3.33 and 6.66, respectively (Fig. 3a). The results might be attributed to the presence of alginate moiety in the nanocomposites, similar to that shown earlier for PEI(750)-Al/DNA (Patnaik et al., 2006). In HEK293 cells, in serum-free conditions, GFP expression was 3.7- and 3-folds higher

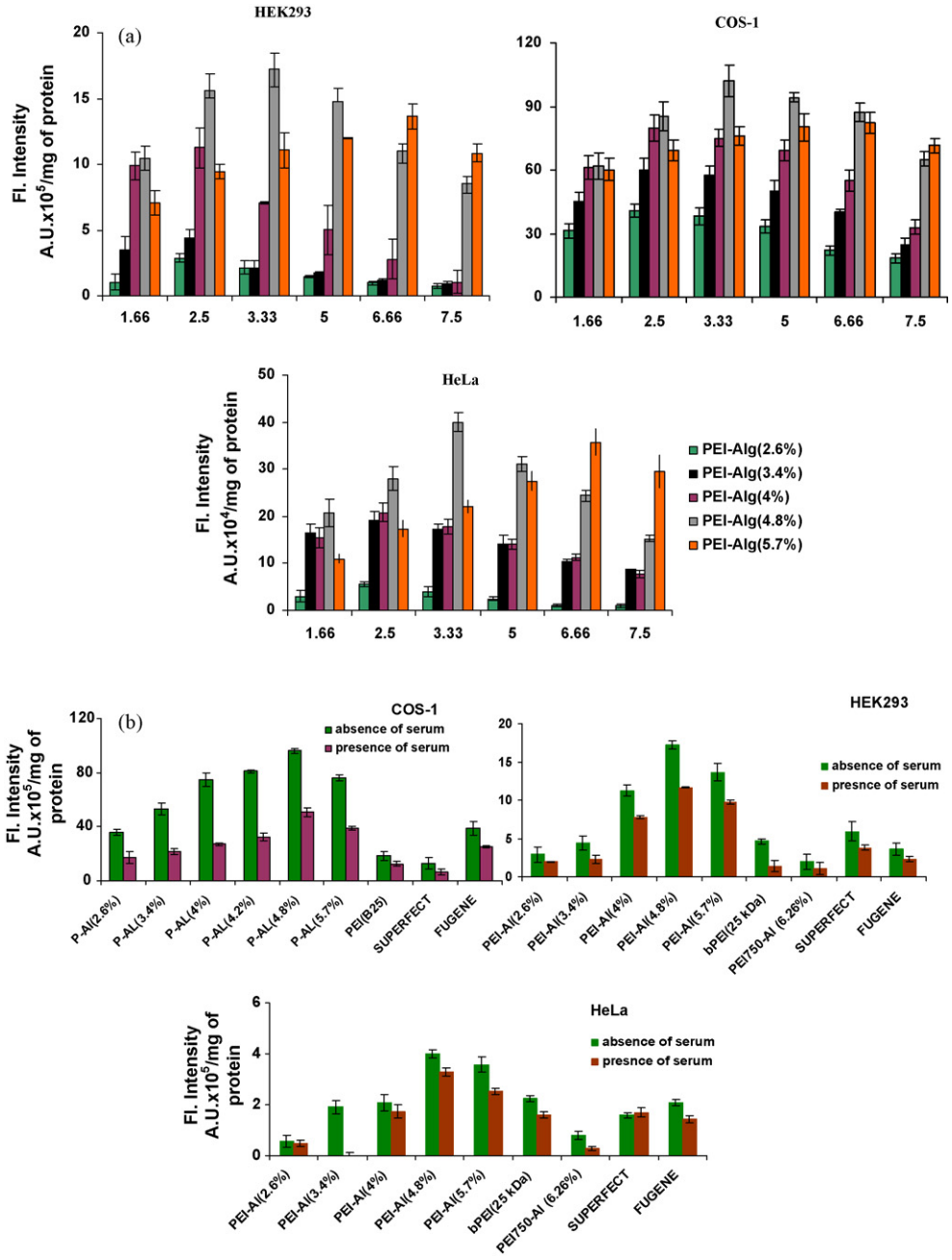

Fig. 3. GFP fluorescence intensity in COS-1, HEK293 and HeLa cells transfected with PEI-Al/DNA, PEI/DNA, Superfect™/DNA and Fugene™/DNA complexes in DMEM (supplemented with or without FCS). (a) GFP fluorescence intensity at different weight w/w ratios. (b) GFP fluorescence intensity of nanocomposites/DNA complexes at w/w ratio giving highest GFP intensity. The results represent the mean of three independent experiments performed in triplicates. The error bars is representative of standard deviation calculated for experiments carried out in triplicates.

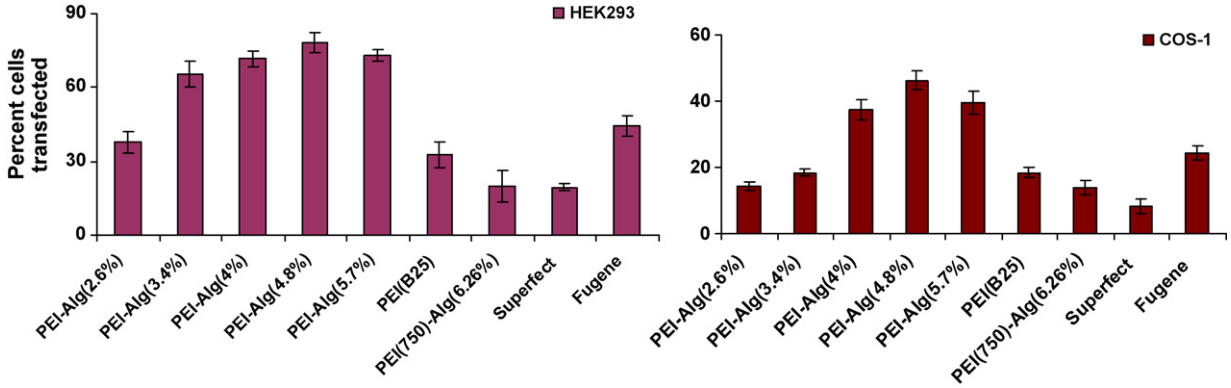

Fig. 4. Percent cells transfected with PEI-Al/DNA, PEI/DNA, Superfect™/DNA and Fugene™/DNA complexes in HEK293 and COS-1 cells. The error bars is representative of standard deviation calculated for experiments carried out in triplicates.

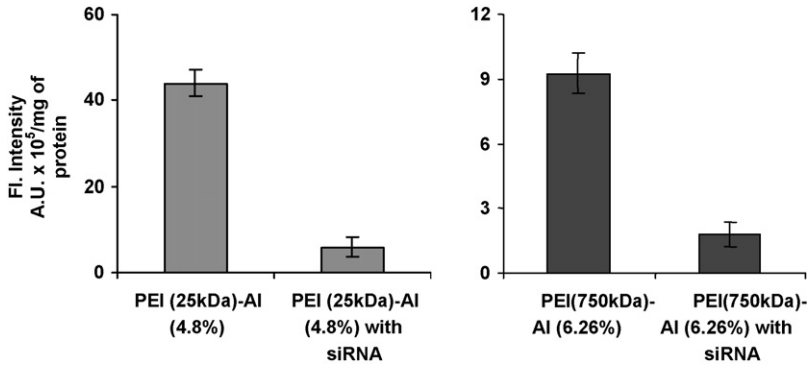

Fig. 5. PEI-Al(4.8%) and PEI(750)-Al(6.26%) were checked for its ability to deliver GFP-specific siRNA into COS-1 cells. The results represent the mean of three independent experiments performed in triplicates and the standard deviation is represented as error bars.

in PEI-Al(4.8%) nanocomposites compared to PEI and Superfect™, respectively (Fig. 3). Under similar conditions, HeLa cells expressed GFP 2- and 1.4-folds better than Superfect™ and Fugene™, respectively (Fig. 3). Finally, GFP expression in all cell lines transfected with PEI-Al(4.8%)/DNA complex was 8-folds higher than PEI (750)-Al(6.26%)/DNA complex (Fig. 3).

In the presence of serum, GFP expression in all cell lines transfected with nanocomposites was greater than PEI, Superfect™ and Fugene™. In COS-1 cells, GFP expression with PEI-Al(4.8%)/DNA complex was 4-folds higher than PEI. In HEK293 cells, PEI-Al(4.8%)/DNA complex exhibited GFP expression 3–6-folds higher than PEI, Superfect™ and Fugene™ (Fig. 3), whereas in HeLa cells, ~2-folds higher expression was observed as compared to Superfect™ and Fugene™ (Fig. 3).

In these studies, GFP expression being dependent on the transfection of cells with pGFP, the observed results indirectly demonstrate and compare transfection efficiency with various nanocomposite carriers showing that PEI-Al(4.8%)/DNA performs the best.

To know the percentage of cells transfected by PEI-Al/DNA complex, FACS was carried out in COS-1 and HEK293 cells. We observe that PEI-Al (4.8%) transfected a higher percent of cells compared to other formulations (Fig. 4). The percent of cells expressing GFP increased as the alginate moiety increased in nanocomposites/DNA complex which is in agreement with spectrofluorometric data (Figs. 3 and 4). Also, on comparing with commercial reagents, PEI-Al (4.8%) formulation gave a higher percent of cells expressing GFP. The FACS and GFP quantification spectroflurometrically suggest that PEI-Al(4.8%) is an efficient transfection agent.

Cationic polymer-aided delivery of siRNAs not only allows testing the efficiency of expression of a foreign gene, but also offers a novel approach in genomedicine. Therefore, the ability of PEI-Al(4.8%) to co-transfect and deliver DNA and siRNA was investigated in COS-1 cells and compared with PEI (750)-Al(6.26%). As shown in Fig. 5, PEI-Al(4.8%)/siRNA suppressed GFP expression 80–82%. Finally, it is important to mention that to achieve the identical degree of GFP suppression as that with PEI-Al(4.8%)/siRNA, 20 times greater amount (200 μg/ml) of PEI (750)-Al(6.26%)/DNA/siRNA was required as compared to PEI (25 kDa)-Al (4.8%)/DNA/siRNA (Fig. 5).

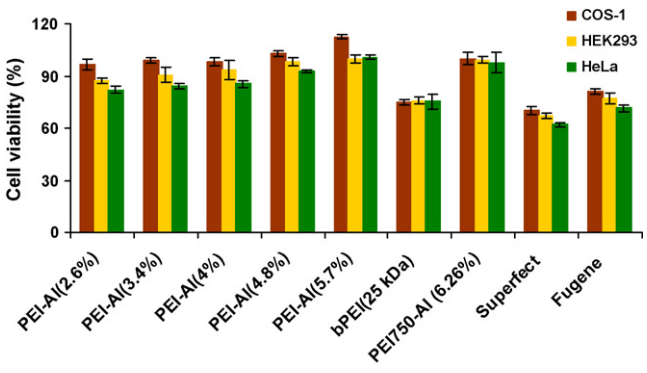

Fig. 6. Cytotoxicity of PEI-Al/DNA, PEI/DNA, Superfect™/DNA and Fugene™/DNA complexes in HeLa, HEK293 and COS-1 cells. Cells were treated with DNA complexes under conditions described in transfection, and cytotoxicity was determined by MTT assay. Percent viability of cells is expressed relative to control cells. Each point represents the mean of three independent experiments performed in triplicates. The error bars in the figure represents standard deviation calculated for experiments carried out in triplicates.

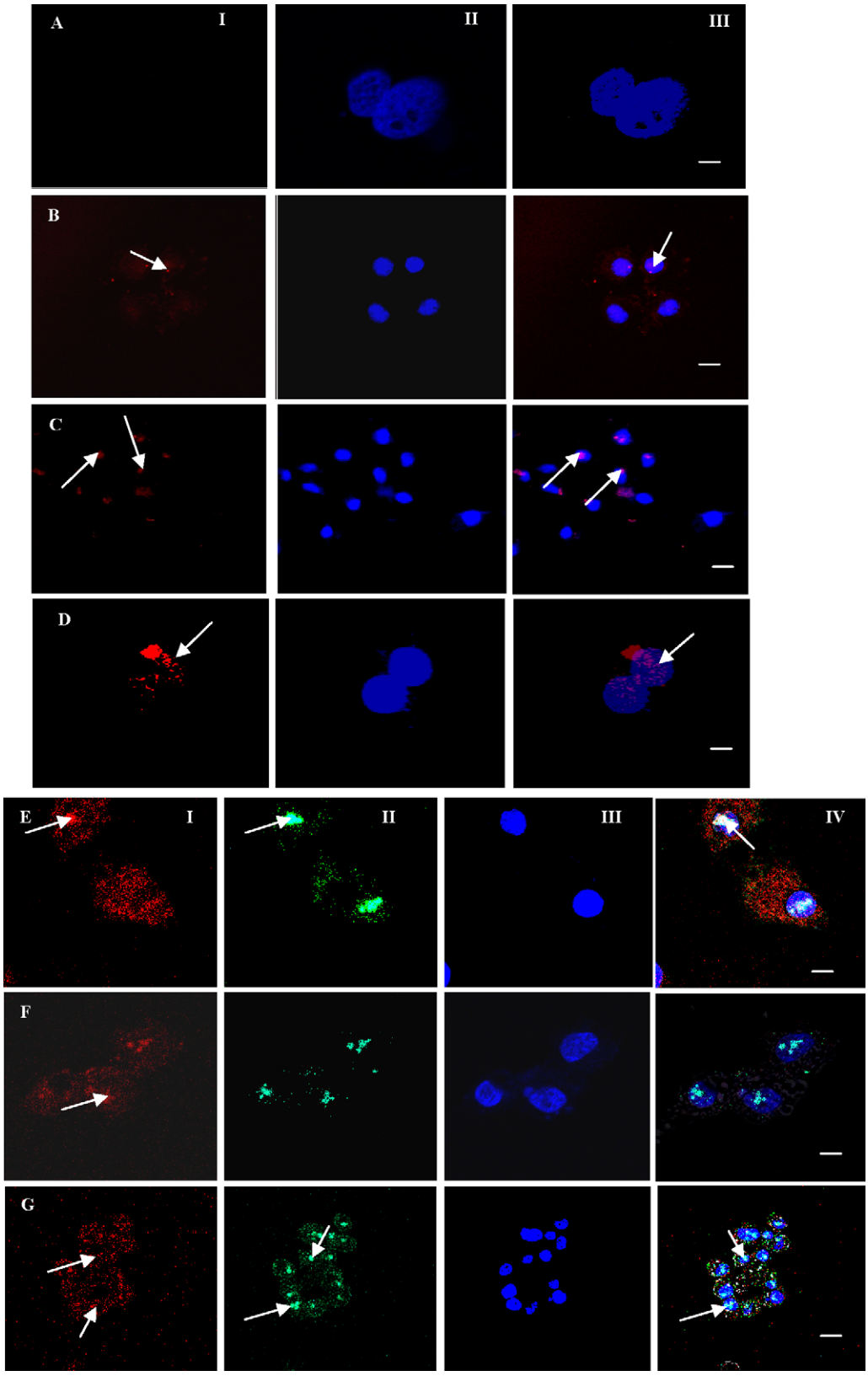

Fig. 7. Intracellular trafficking of labeled PEI-Al(4.8%) alone and its complex with YOYO-1-DNA in HeLa cells by confocal microscopy. A–D show HeLa cells treated with labeled PEI-Al(4.8%) alone at different time points: (A) control, scale bar: 10 μm (B) 1 h, scale bar 25 μm (C) 2 h, scale bar 25 μm (D) 4 h, scale bar 25 μm. E–H show images of HeLa cells treated with labeled PEI-Al(4.8%)/YOYO-1-DNA at different time points: (E) 1 h, scale bar 20 μm (F) 2 h, scale bar 20 μm (G) 4 h, scale bar 25 μm. In images A–D, the first quadrant (I) shows the cells observed under rhodamine filter (laser Helium–Neon 1 mW), the second quadrant (II) shows images captured under DAPI filter (laser diode 25 mW) and the third quadrant (III) represents the overlaid images. In E–G, the first quadrant (I) shows the cells observed under rhodamine filter, the second quadrant (II) shows images captured under fluorescein filter (laser argon 30 mW), the third quadrant (III) shows images captured under DAPI filter and the fourth quadrant (IV) represents the overlaid images.

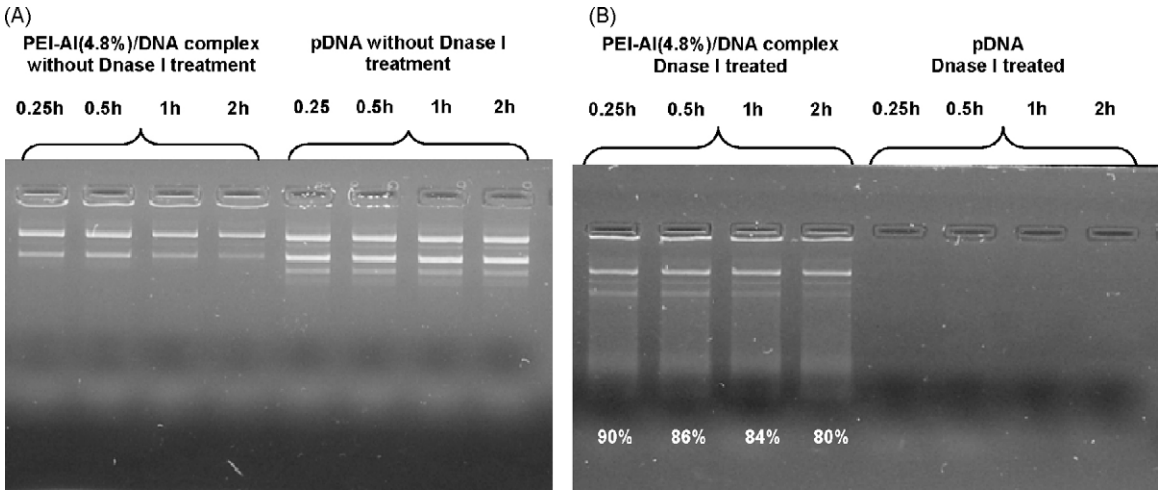

Fig. 8. PEI-Al(4.8%)/DNA complex was treated with DNase I at a w/w 10:3 for different time intervals. The complexed DNA was released by treating the samples with heparin. The release of DNA was monitored in 0.8% agarose. The amount of DNA protected (%) after DNase treatment is calculated as the relative integrated densitometry values (IDV) quantified and normalized by that of pDNA values (not treated with DNase I) using AlphaEaseFC software.

Although branched PEI (25 kDa) is an efficient transfection agent, its charge associated cytotoxicity limits the application *in vivo* (Kircheis et al., 2001). By partially masking the charge on PEI with alginic acid, mammalian cell viability profiles improved (Fig. 6). The PEI-Al/DNA complexes showed more than 90% cell viability similar to those exhibited by PEI (750 kDa)-Al/DNA complexes as compared to 65% in PEI (Goosen, 1992). Furthermore, the cytotoxicity of Superfect™ and Fugene™ was found to be more or less comparable to PEI (Fig. 6). The higher cell viability might be attributed to the masking of free amino groups in PEI by alginate leading to a reduction in net positive charge on polymer and the resulting nanocomposites performed far better as compared to Superfect™ and Fugene™.

To follow the intracellular passage of nanocomposites, HeLa cells were treated with TR-PEI-Al nanocomposites and TR-PEI-Al/DNA-YOYO complex. After 1 h, fluorescent nanocomposite particles appeared inside the cytoplasm with a faint fluorescence in the nucleus. Likewise, the dual labeled PEI-Al(4.8%)/pDNA complex also enters the nucleus after 1 h (Fig. 7). Godbey et al. (1999) have earlier reported that PEI has the property of nuclear localization. The intracellular trafficking of PEI-Al nanocomposites observed under confocal microscope showed the presence of nanocomposites alone as well as DNA complex inside the nucleus. This implies that presence of alginate has not hampered the nuclear localization property of PEI.

An important feature of efficient transfection reagent is its ability to protect complexed DNA from degradation by nucleases present in the extracellular and intracellular environment. Therefore, PEI-Al(4.8%)/DNA complex was treated with DNase I (Fig. 8). Heparin competitively displaced DNA from PEI-Al(4.8%)/DNA complex (Fig. 8). The amount of pDNA released after heparin treatment, as estimated by densitometric analysis, was found to be 90% after 30 min, which declined to 80% after 2 h (Fig. 8). It was concluded that PEI-Al(4.8%) protects DNA against nucleases for a significant period of time.

4. Conclusions

We have examined the polycation-based gene delivery with PEI-Al nanocomposites, *in vitro*, both in the presence and absence of serum and found that these not only exhibited lower cytotoxicity but also delivered nucleic acids (pDNA and siRNA) more efficiently than the standard transfection reagents (Superfect™ and Fugene™). Also, the protection imparted by PEI-Al nanocomposites to complexed DNA against DNase makes it a potent transfection agent. We suggest these results as forerunner of successful delivery of specific genes to defined targets.

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