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Research paper

Polyglutamic acid-based nanocomposites as efficient non-viral gene carriers *in vitro* and *in vivo*

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ABSTRACT

A series of polyethylenimine (PEI) and γ-polyglutamic acid (PGA) nanocomposites (PPGA) was prepared and evaluated in terms of their cell viability and transfection efficiency *in vitro* and *in vivo*. On complexion with pDNA, the positively charged PPGA/DNA nanocomposites resulted in a higher level of *in vitro* reporter gene transfection (2.7–7.9-fold) as compared to native PEI, and selected commercial reagents and >95% cell viability in HEK293, HeLa and HepG2 cell lines. Further, PPGA-5 nanocomposite (the best working system in terms of transfection efficiency among the series) was found to efficiently transfect primary mouse keratinocytes up to 22% above the control level. PPGA-5, when tested for *in vivo* cytotoxicity in *Drosophila*, did not induce any stress in the exposed larvae in comparison with control. *In vivo* gene expression using PPGA-5 showed the highest transfection efficiency in spleen of mouse closely followed by heart tissues after intravenous injection through tail vein. Besides, these nanocomposites also delivered siRNA efficiently into mammalian cells, resulting in ~80% suppression of EGFP expression. These results together demonstrated the potential of the projected nanocomposites for *in vivo* gene delivery.

1. Introduction

Gene therapy has achieved vast significance as a potential method for treating genetic diseases over the past two decades. Initial research focused on using viral vectors for their high transfection efficiency. However, fundamental problems associated with viral vectors such as toxicity, immunogenicity and limitations with respect to scale up procedures encouraged the development of other potential gene carriers [1]. Delivery of nucleic acids by non-viral vectors has shown promise for their diverse applications in different areas including therapies for genetic and acquired diseases. However, development of a safe and efficient delivery system is the key to its success [2]. At present, synthetic compounds such as cationic polymers, lipids, peptides and dendrimers are being exploited extensively for their wider use in generating non-viral delivery systems [3–6]. While these molecules possess most of the functions essential for transfection [7], an ideal delivery system with low toxicity and high transfection efficiency still remains as a challenge.

Of the various chemical vectors, PEI is the most widely used cationic transfection reagent *in vitro* and *in vivo* although showing measurable cytotoxicity [8–10]. Added advantage of PEI is its properties of (i) excellent condensation of DNA, (ii) endosomal buffering capacity, and (iii) protection of pDNA from enzymatic degradation [11–13]. It also promotes nuclear localization of a transgene for its efficient and targeted expression [14]. In order to improve the biocompatibility and efficient gene transfer, several strategies using PEI have been adopted such as coating with human serum albumin [15], conjugation with dextran [16], PEGylation and acylation [10]. Besides, the adverse effects of the PEI-based systems have also been overcome by allowing electrostatic interactions with anionic compounds (alginic acid, hyaluronic acid, chondroitin sulfate, etc.) to make them safe and efficient delivery agents [17,18]. In the same context, gamma-polyglutamic acid (γ-PGA), a biodegradable polypeptide, which significantly improves cytocompatibility and reduces the cytotoxicity of the carriers, has also been used for gene delivery purposes involving ternary complexes of pDNA, cationic polymer and γ-PGA [19,20]. In one of such reports,

Abbreviations: AFM, atomic force microscope; CLSM, confocal laser scanning microscopy; DLS, dynamic light scattering; DMEM, Dulbecco's modified Eagle's medium; DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; EtBr, ethidium bromide; FACS, fluorescence activated cell sorting; hsp, heat shock protein; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; IR, infrared; IC_{50}, half maximal inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RLU, relative luminescence unit; TRITC, tetramethylrhodamine isothiocyanate.

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Kurosaki et al. reported a two-step formation of ternary complex, constructed with pDNA, PEI and γ-PGA, which were efficiently taken up by the cells via γ-PGA-specific receptor-mediated energy-dependent process [21,22]. The results indicated that the complex exhibited low toxicity but insignificant improvement in the transfection efficiency as compared to PEI–DNA complex. In another recent report, Jing et al. have shown enhanced gene carrying capacity of PGA-graft-oligoethylenimines [23]; however, this protocol involves time-consuming synthesis and purification of intermediates. To circumvent the above-mentioned limitations and to prepare improved PEI-based delivery agents, here, in the present study, we have evaluated PPGA nanocomposites, prepared through electrostatic interactions, for their ability to protect complexed pDNA, transfection efficiency and cell viability in various cell lines as well as primary cultures and compared the results with the standard commercially available transfection reagents. We show that the best formulation, PPGA-5, displayed improved transfection efficiency (2.7–7.9 folds in comparison with PEI and the standard transfection reagents) in transformed and primary cells with negligible toxicity. Further, efficacy of this formulation was validated by delivery of siRNA and intracellular trafficking. The *in vitro* observations were corroborated with *in vivo* studies, where in PPGA-5, nanocomposite exhibited high transfection efficiency in spleen followed by heart of mice after intravenous injection.

2. Materials and methods

2.1. Materials and chemicals

Branched PEI (25 kDa), (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT), poly-L-glutamic acid (avg. MW 13,000 Da), agarose, Tris, ethidium bromide (EtBr), high retention dialysis tubing (cut off = 12 kDa), xylene cyanol, bromophenol, and tetramethylrhodamine isothiocyanate (TRITC) were purchased from Sigma–Aldrich Chemical Co., St. Louis, MO, USA. Cell culture products and FBS were procured from GIBCO (Invitrogen, USA).

2.2. Cell culture

HEK293 (human embryonic kidney), HeLa (human cervical adenocarcinoma) and HepG2 (human hepatocellular liver carcinoma) cell lines (obtained from NCCS, Pune, India) were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50 mg/ml gentamicin.

2.3. Animals

Six- to seven-week-old male and female Swiss albino mice pairs (20 ± 3 g) for primary culture and male Balb/c mice (25 ± 3 g) for *in vivo* experiments were procured from the animal facility of Indian Institute of Toxicology Research (IITR), Lucknow. They were acclimatized under standard laboratory conditions with 12 h dark/light and 50–60% humidity as per rules laid down by Animal Welfare Committee of IITR. The animals, housed in plastic cages having rice husk as bedding, were given commercial pellet diet (Ashirwad Industries, Chandigarh, India) and water *ad libitum*. The animals were cared humanely according to the guidelines laid down by the Institutional Animal Ethics Committee.

2.4. Primary cell culture

New-born pups (1/2 days old, 4–5 nos.) were used as a source for primary cultures of keratinocytes as previously reported [24,25]. In brief, pups were euthanized and washed in 70% ethanol. The skin was stripped off and floated on 0.25% trypsin overnight at 4 °C. The epidermis after separating from the dermis was chopped in Waymouth's medium (Lonza, Basel, Switzerland) containing 1.2 mM calcium and 10% fetal bovine serum. The cells were allowed to attach at 37 °C in 5% CO_2 for 2.5 h, and the medium was replaced with serum-free keratinocyte growth medium-2 (KGM-2) (Lonza, Basel, Switzerland) containing 0.03 mM calcium chloride.

2.5. Synthesis of siRNA

The siRNA for knockdown of green fluorescent protein (GFP) gene expression was generated using a previously reported method from this laboratory [26].

2.6. Plasmid preparation

We used two plasmids. pEGFPN3 (Clontech, USA), used for transfection assay, essentially encoded an enhanced green fluorescent protein (EGFP), and pGL3 (Sigma, USA), used for *in vivo* gene expression studies, encoded a firefly luciferase enzyme under the regulation of a cytomegalovirus (CMV) immediate early promoter. These were propagated into competent *Escherichia coli* bacterial strain DH5α, and endotoxin-free plasmid preparation was carried out as per manufacturer's instructions (Qiagen, Courtaboeuf, France).

2.7. Preparation of PEI-poly-L-glutamic acid nanocomposites

To an aqueous solution of PEI (25 mg, 1 mg/ml), PGA (1.71 mg, for 1% substitution) was added in small portions with vigorous stirring. The reaction was allowed to stir overnight at 25 °C followed by its volume reduction (1/3) on a rotary evaporator. The solution was dialyzed against water for 48 h using a molecular weight cut-off (12 kDa). After lyophilization, the resultant product PPGA-1 was obtained in ~80% yield. Similarly, nanocomposites with 2% (PPGA-2), 2.5% (PPGA-3), 3% (PPGA-4), 3.5% (PPGA-5) and 4% (PPGA-6) substitution were prepared in ~70–80% yield and characterized by infrared (IR) spectroscopy (recorded on a single beam Perkin Elmer, Spectrum BX Series, USA) and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy (Bruker Avance 400 MHz).

2.8. Formation of nanocomposite/DNA complexes

An aqueous solution of PPGA (1 mg/ml) was added to pDNA (0.3 μg/μl) at various N/P ratios (4, 8, 12, 16, 20 and 30) to form the nanocomposite/pDNA complexes in 5% dextrose (5 μl), and the final volume was made to 20 μl with water. The resulting complexes were homogenized using vortex and incubated for 30 min at room temperature prior to their use in all biophysical studies or transfection experiments. Similarly, the polyplexes were also prepared for PEI/pDNA at the same N/P ratios.

2.9. Physical characterization of nanocomposites

The formed nanocomposites were characterized for morphology, size and zeta potential. The size of nanocomposites (1 mg/ml) and their DNA complexes, suspended in water and 10% serum separately, was determined as hydrodynamic diameter by dynamic light scattering (DLS) in triplicate using Zetasizer Nano ZS (Malvern instruments, UK). All the measurements were performed in automatic mode and presented as the average value of 20 runs. The morphology of nanocomposites and their DNA complexes was characterized using atomic force microscopy (PicoSPM System, Molecular Imaging, USA). Particle size was estimated by an image analyzing software (SPIP) for scanning probe microscopy. Zeta

potential measurements of nanocomposites and DNA complexes in water and 10% serum were estimated using Zetasizer Nano ZS. Each measurement was presented as an average value of 30 runs and was performed in triplicate, and the average values were estimated by Smoluchowski approximation from the electrophoretic mobility.

2.10. DNA mobility shift assay

DNA (0.3 μg/μl) was complexed with native PEI at N/P ratio 1.5, 3, 5 and 8 and PPGA nanocomposites at 2, 4, 6, and 8 as described earlier. DNA complexes (20 μl) were mixed with 2 μl xylene cyanol (in 20% glycerol), electrophoresed (100 V, 1 h) on 0.8% agarose gel, stained with EtBr and visualized on a UV transilluminator using a gel documentation system (Syngene, UK).

2.11. DNA release assay

PEI was complexed with pDNA, (5 μl, 0.3 μg/μl) at N/P ratio of 8 (at which highest transfection efficiency was observed) and incubated for 30 min, as described earlier. Heparin, a polyanion, was added (amounts varying from 0.2–10.0 (1 U/μl) units), which competes with pDNA for the nanocomposite and releases plasmid DNA, which is bound to the polycation. The samples were then incubated for 20 min, electrophoresed (100 V, 1 h) and visualized as described earlier. The amount of DNA released from complexes after heparin treatment was estimated by densitometry. Likewise, the assay was repeated with PPGA-5 nanocomposites.

2.12. Toxicity assessment

2.12.1. In vitro

Toxicity of pDNA complexes of PPGA nanocomposites, PEI, commercial transfection reagents, viz., Superfect™, Fugene™, GenePORTER 2™ and Lipofectamine™ was assessed on HEK293, HepG2 and HeLa cells by MTT assay [27]. In brief, after 36 h of transfection, cells were treated with MTT reagent for 2 h and the formazan crystals so formed were dissolved in MTT lysis buffer. The plate was read at 540 nm by an ELISA plate reader (MRX, Dynatech Laboratories). The cell viability (%) was calculated by using the formula as reported previously [28].

The half maximal inhibitory concentration (IC_{50}) of PPGA-5/DNA and PEI/DNA complexes was estimated at N/P ratio 16 and 8, respectively, which is the best working concentration for these two formulations on HEK293 cells. Similarly, cell viability test was performed for PPGA-5, PEI and Lipofectamine™ in primary mouse keratinocytes using MTT assay in triplicate.

2.12.2. In vivo

PPGA-5 was further assessed for its *in vivo* toxicity using Drosophila as a model as reported earlier [29a]. Briefly, third instar larvae were separately exposed to PPGA-5 (0.71 μl of 1 mg/ml in 19.29 μl of 5% sucrose) and Lipofectamine™ (2 μl and 18 μl of 5% sucrose) for 36 h at 22 ± 1 °C. After 36 h of exposure, larvae were washed thoroughly with 10 mM PBS, total RNA was extracted from the whole larvae using Trizol-RT extraction method (Life Technologies, USA), and subsequently, cDNA was synthesized (Fermentas, USA) as per manufacturers' protocols. The transcripts, namely *hsp70* (heat-shock protein 70), *hsp60*, *hsp83* and *hsp23*, were amplified following a protocol reported by Singh et al. [29b]. RPL32 levels were analyzed as an internal control for the quality/quantity of cDNA [30]. The amplicons were resolved on 1% agarose gel, documented using versadoc (Bio-Rad, USA) and analyzed by densitometry using Quantity One Software (Bio-Rad, USA). In another set, the third instar larvae were treated as described earlier but were grown on normal food after 36 h exposure and observed for emergence of adults.

2.13. In vitro transfection

2.13.1. In transformed cells

HEK293, HepG2 and HeLa cells were seeded into 96-well plates in 200 μl of growth medium (10% FBS), incubated at 37 °C, and transfection was carried out at 70–75% confluence as reported earlier [29a]. Briefly, nanocomposites/DNA and PEI/DNA complexes were made at various N/P ratios (4, 8, 12, 16, 20, 30), similarly, pDNA complexes were also made with Superfect™ (Qiagen, France), Fugene™ (Roche Applied Science, USA), GenePORTER 2™ (Genlantis, USA) and Lipofectamine™ (Invitrogen, USA) following manufacturers' protocols and gently added onto the cells in presence or absence of serum. After 36 h, the cells were observed under fluorescent microscope to monitor GFP expression in the cells.

Further, siRNA delivery efficiency was estimated by transfecting pEGFP (1 μl, 1.4 nM) with PPGA-5, as described earlier and subsequently transfected with GFP-specific siRNA (2 μl, 2.5 μM) complexed with PPGA-5 [29a]. Cells transfected with PPGA-5/DNA complex alone served as control. Similarly, Fugene™/DNA and Fugene™/siRNA complexes were also prepared and added to the HEK293 cells in a 96-well plate. After 36 h, the GFP expression was quantified using nanodrop. All experiments were performed in triplicate.

2.13.2. In primary mouse keratinocytes

Transfection in mouse keratinocytes was performed as reported earlier [29a]. Briefly, PEI and PPGA-5 nanocomposites were complexed with pDNA (2 μg) at the optimized N/P ratio of 8 and 16, respectively. Similarly, pDNA complex was prepared with Lipofectamine™ following manufacturers' protocol and added onto the cells. After 24 h of incubation, quantitative transfection efficiency of the control and the treated cells was estimated by FACS analysis (BD FACS Calibur, Bioscience, USA).

2.14. Evaluation of EGFP expression

Total EGFP expression in cell lysates was quantified (λ_{ex}: 488 nm; λ_{em}: 509 nm) spectrofluorometrically using NanoDrop™ (ND-3300, USA) as reported earlier [18], and values are reported as arbitrary unit (AU)/mg of cellular protein and represent mean ± standard deviation for samples taken in triplicate.

2.15. In vivo gene expression

PPGA-5 was complexed with 25 μg pGL-3 control vector in normal saline at optimal N/P ratio (16) for *in vivo* gene expression. A 100-μl aliquot of the complex was injected intravenously through mice tail vein using a syringe of 40 U (insulin syringe) with needle size of 0.3 × 8 mm. At predetermined time periods after injection, the mice were sacrificed, and liver, spleen, kidney, lung, heart and brain were excised. These organs were rinsed with chilled normal saline, weighed and made 25% w/v homogenate in lysis buffer (Promega). After three cycles of freezing and thawing, homogenates were centrifuged at 10,000*g* for 10 min at 4 °C and 100 μl of supernatant was used to measure luciferase activity. Luciferase activity was represented as RLU/gram of tissue. All the experiments were carried out in duplicate.

2.16. Fluorescence-activated cell sorting (FACS) analysis

Quantitative transfection efficiency was performed by flow cytometry (FACS), 36 h post-transfection. Briefly, 1×10^6 HEK293 cells/well were seeded into 24-well plates 1 day before transfection. PEI/DNA, PPGA-5/DNA (at N/P ratios of 8, 12, 16 and 20), Superfect™/DNA and Lipofectamine™/DNA complexes were diluted with 300 μl serum-free medium and added onto the cells.

Table 1
Characterization of PPGA nanocomposites via DLS and ^{1}H NMR.

Sample ID	Average particle size in nm ± SD (PDI)			Zeta potential in mV ± SD			Ratio of Nanocomposite: DNA (N/P)	Attempted substitution of PGA (%) on PEI	Realized Substitution of PGA as estimated by ^{1}H NMR (%)
	Nanocomposite (in H_2O)	DNA-loaded Nanocomposite (in H_2O)	DNA-loaded Nanocomposite (in DMEM)	Nanocomposite (in H_2O)	DNA-loaded Nanocomposite (in H_2O)	DNA-loaded Nanocomposite (in DMEM)			
PPGA-1	202.2 ± 0.52 (0.31)	255.3 ± 0.30 (0.19)	137 ± 12.2 (0.22)	37.7 ± 0.87	29.7 ± 1.3	−11.56 ± 2.2	16	1	0.79
PPGA-2	180.4 ± 1.56 (0.28)	205.6 ± 9.61 (0.21)	105 ± 12.4 (0.2)	35.5 ± 4.82	24.3 ± 1.8	−10.23 ± 1.6	16	2	1.54
PPGA-3	176 ± 3.68 (0.3)	193.6 ± 10.86 (0.16)	98 ± 1.5 (0.19)	32.3 ± 0.96	21.7 ± 1.3	−10.38 ± 1.3	16	2.5	1.97
PPGA-4	170.6 ± 9.26 (0.26)	190.8 ± 6.02 (0.17)	95 ± 6.7 (0.2)	30.2 ± 2.96	20.9 ± 1.6	−10.86 ± 1.58	16	3.0	2.34
PPGA-5	146.5 ± 6.52 (0.22)	171.1 ± 3.63 (0.2)	90 ± 7.4 (0.23)	25 ± 1.73	18.6 ± 1.4	−10.76 ± 1.69	16	3.5	2.59
PPGA-6	126.3 ± 7.38 (0.26)	163.2 ± 2.56 (0.19)	84 ± 8.3 (0.18)	24.5 ± 2.0	17.3 ± 2.1	−9.48 ± 1.48	16	4.0	2.8
PEI	–	365.25 ± 15.89 (0.47)	268.19 ± 1.4 (0.52)	40.25 ± 2.1	35.59 ± 0.56	−14.23 ± 1.83	10	-	

After 36 h, the transfected cells were washed with 1 × PBS (1 ml) and harvested from each well by the trypsin–EDTA (0.25%) treatment. After centrifugation, cell pellet was washed with 1 × PBS, fixed with 4% paraformaldehyde for 10 min at 25 ± 1 °C and again washed with 1 × PBS before cells were resuspended in 1 × PBS (1 ml). Transfection efficiency was evaluated as the percentage of cells expressing GFP using Guava® EasyCyte™ Plus System, USA. Non-transfected cells were used as the control. Five thousand cells/samples were analyzed, and transfection was carried out in triplicate.

2.17. Knockdown of JNK II using PPGA-5

Delivery of JNK II siRNA was performed using PPGA-5 on HEK293 cells as reported earlier [29a]. Briefly, cells were transfected with PPGA-5/JNK II siRNA (10 nM) (Cell Signaling Technology, USA) in minimal essential medium (without serum) for 36 h, and thereafter protein samples were prepared and processed for Western blotting as described in Goyal et al. [29a].

2.18. Confocal laser scanning microscopy (CLSM)

CLSM was employed to monitor intracellular trafficking of PPGA-5 nanocomposite/pDNA complex. Briefly, PPGA-5 labeled with TRITC and pDNA labeled with YOYO-1 iodide were complexed, and a solution of dual labeled PPGA-5/pDNA (500 μl) in serum-free DMEM was added to each well containing HeLa cells (1.5×10^5 cells/well) at ~70% confluence. After incubation for 0.5, 1, 2 and 4 h, the cells were washed thrice with 1 × PBS and fixed with 4% paraformaldehyde solution for 10 min at 4 °C. Then, the fixed cells were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI), and the coverslips were placed with fluorescence-free mounting medium (UltraCruz™, Santa-Cruz Biotechnology, USA). All confocal images were captured using a Zeiss LSM 510 inverted laser scanning confocal microscope, Germany.

2.19. Protection of pDNA against DNase I

DNase I protection assay was performed to assess the ability of the nanocomposite (PPGA-5) to protect the condensed pDNA from nucleases [18]. Briefly, native pDNA and PPGA-5/pDNA nanoplex (at N/P ratio of 16) were incubated at 37 °C for 15, 30, 60 and 120 min with DNase I. Subsequently, 5 μl EDTA (100 mM) was added, and the mixture was incubated at 75 °C for 10 min to inactivate DNase I. The mixture was further incubated for 2 h at 25 ± 1 °C with heparin (10 μl, 5 mg/ml) to release bound DNA from the cationic polymer. Subsequently, samples were electrophoresed and visualized, as described earlier (DNA release assay, Section 2.11). The amount of pDNA released from complexes after heparin treatment was estimated by densitometry.

2.20. Statistical analysis

Results are expressed as the mean standard deviation of at least three experiments wherever carried out. Statistical analysis was performed using Student's *t-test* with significance ascribed at $P < 0.05$. JMP 6.0.0 Statistical Discovery™ (from SAS) was used for analysis.

3. Results and discussion

As evident from the literature, polyethylenimine (25 kDa) is one of the most widely used cationic polymers for gene transfection *in vitro* and *in vivo*. However, due to very high cationic charge density and due to the fact that every third atom is a protonable nitrogen, several research groups, including ours', have reported that PEI is highly toxic (ca. ~50–60% cell viability at the best working concentration of PEI [31–33]. To circumvent the above hurdle, a number of modifications that include laborious chemical synthesis have been attempted [34]. However, much success is still illusive. To achieve descent transfection vis-a-vis minimal modification, we have partially modified the cationic polymer, PEI, by blending with γ-polyglutamic acid (13 kDa), a hydrophilic, biodegradable and naturally occurring biopolymer, which results in the formation of stable complex by virtue of ionic interactions in order to exploit the beneficiary properties of both the polymers. The resulting PPGA nanocomposites are fairly stable and dispersible in water. These nanocomposites not only reduced the charge-based cytotoxicity significantly but also enhanced the transfection efficiency in both transformed and primary cell cultures.

3.1. Characterization of PPGA nanocomposites

A series of PPGA nanocomposites was prepared by mixing of PEI with varying amounts of PGA, where they interact electrostatically. The resulting nanocomposites were characterized by IR, where

bands at 3369 (NH-stretching), 2941 (NH_2-stretching) and 1654 cm^{-1} (C=O stretching) confirmed the incorporation of PGA in the PPGA nanocomposites. The percentage of cross-linking was estimated using ^1H NMR (Table 1), and it was found to be ~70–79% of the attempted cross-linking.

The size of nanocomposites was determined by DLS (Table 1), where the size of native PPGA nanocomposites was found to be in the range of 126–202 nm in water and 84–137 nm in transfection medium, while complexes made with pDNA showed increased size in the range of 163–255 nm. As expected, the size of the nanocomposites decreased on increasing the concentration of polyglutamic acid, which might be due to the formation of more compact structures. Similarly, zeta potential showed a gradual decrease on increasing the amount of PGA, which is obvious as the two polymers are oppositely charged. All the nanocomposites as well as pDNA complexes carried a positive zeta potential value, when measured in water, while in transfection medium, a negative zeta potential was observed (Table 1).

Further, AFM analysis was done for one of the nanocomposites (PPGA-5) for its morphology and size. We observed the size of PPGA-5 and PPGA-5/pDNA complex to be 69 and 114 nm, respectively (Fig. 1). AFM measurements showed a smaller particle size in comparison with DLS measurements, which might be due to the measurement of hydrodynamic diameter of the swollen nanoparticles in DLS studies.

Though blending reduces the zeta potential of nanoparticles to a value lower than that of PEI (~40 mV), it is still sufficient to allow them to interact directly with the inner surface of the endosome membrane and promote its disruption, which is consistent with the role of charge in endosomal release of DNA complex [34].

3.2. Retardation in pDNA mobility

To evaluate the DNA condensation ability of PPGA nanocomposites, retardation assay on 0.8% agarose gel was performed. pDNA complexes of PPGA nanocomposites and PEI, prepared at different N/P ratios, were electrophoresed. At N/P ratio of 3, PEI completely retarded the electrophoretic mobility of pDNA, whereas as expected, the PPGA nanocomposites required a higher N/P ratio (8) to retard the same amount of pDNA (Fig. 2). Presence of PGA in the

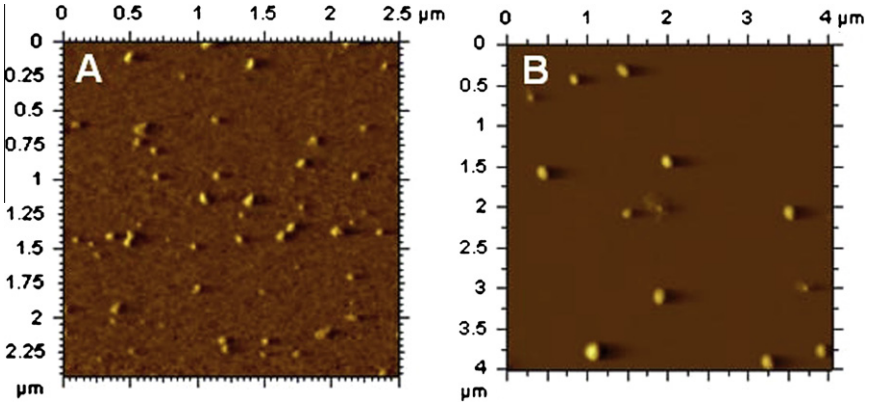

Fig. 1. AFM image of: (A) PPGA-5 nanocomposite (~69 nm average size) and (B) PPGA-5/DNA nanoplex (~114 nm average size). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

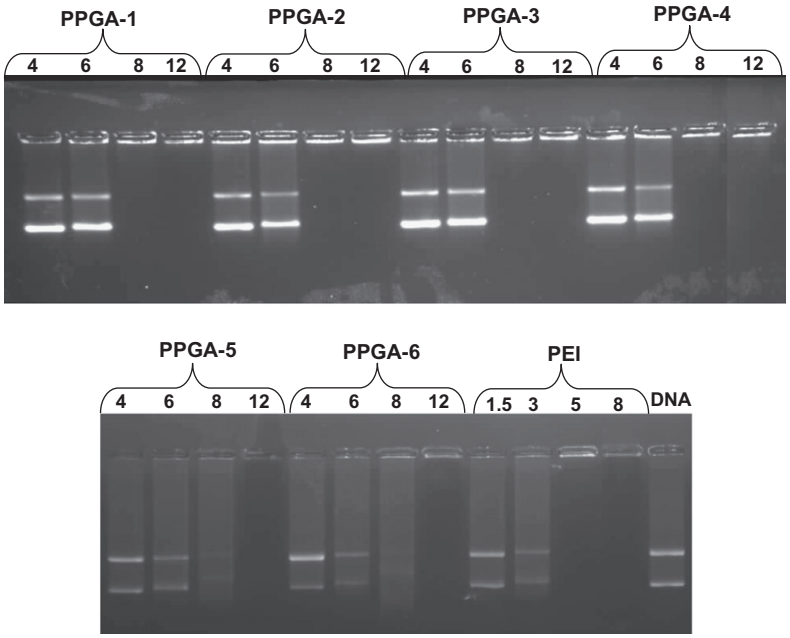

Fig. 2. DNA mobility shift assay of PPGA nanocomposite/DNA and PEI/DNA complexes.

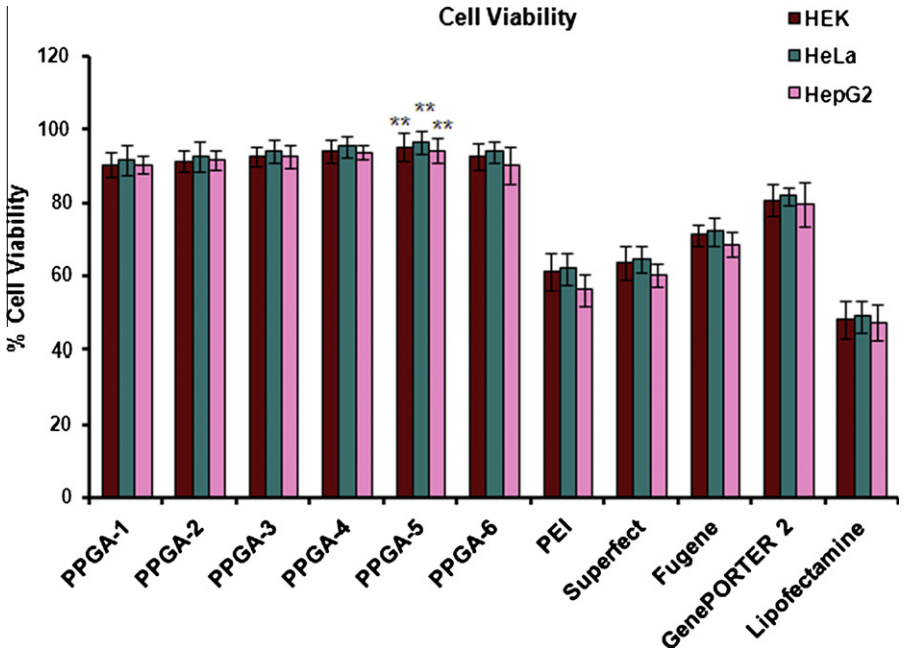

Fig. 3. Cell viability profile of PPGA nanocomposite/DNA nanoplexes at an N/P ratio of 16, PEI/DNA polyplex at an N/P ratio of 8, Superfect™/DNA, Fugene™/DNA, GenePORTER 2™/DNA and Lipofectamine™/DNA complexes in HEK293, HeLa and HepG2 cells. Each point represents the mean of three independent experiments performed in triplicate. Significance ascribed as $^{**}P < 0.01$ vs. PEI and Lipofectamine™ in all the cell lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nanocomposites partially masked the positive charge on PEI, resulting in the requirement of higher amounts of PPGA nanocomposites to retard electrophoretic mobility of pDNA. Therefore, on increasing the concentration of PGA in the series, the amount of nanocomposites required to retard a fixed amount of pDNA also increased.

3.3. Toxicity assessment

3.3.1. In vitro

Cationic polymers are known to electrostatically interact with cell membrane, destabilize and ultimately rupture it [35]. MTT assay was performed in order to demonstrate that the projected PPGA nanocomposites are safe and did not affect the cells (HEK293, HeLa, HepG2 and primary mouse keratinocytes) during internalization process. The comparative studies were carried out with PEI and the commercial transfection reagents. PEI exhibited very high cytotoxicity (~60%) at the best working concentration. In case of PPGA nanocomposites, >95% cell viability was observed (Figs. 3 and 4). The cell viability in case of PPGA-5 varied significantly ($P < 0.01$) in comparison with PEI. The increasing cell viability of PPGA nanocomposites might be due to partial masking of the primary amino groups on the surface of PEI and higher hydrophilicity of the surface of the nanocomposites, which prevented

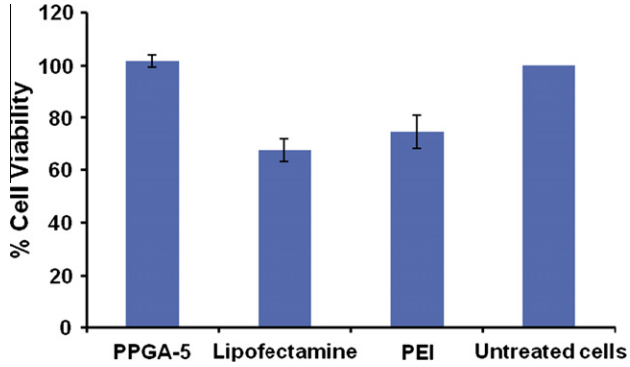

Fig. 4. Cell viability profile of PPGA-5/DNA nanoplexes (N/P ratio of 16), PEI/DNA and Lipofectamine™/DNA complexes in primary mouse keratinocytes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
IC_{50} values for PEI and PPGA-5 nanocomposites.

S. No.	Sample ID	$IC_{50} \pm SD$ (mg/L)
1.	PPGA-5	200 ± 12.23
2.	PEI	17.5 ± 2.38

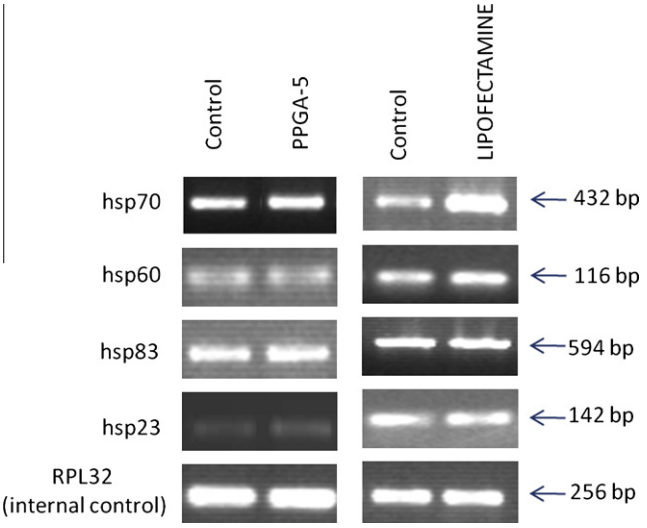

Fig. 5. Transcript levels of different hsps in control and organisms exposed to PPGA-5 and Lipofectamine™. RPL32 was used as an internal control for the RNA quality as well as quantity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

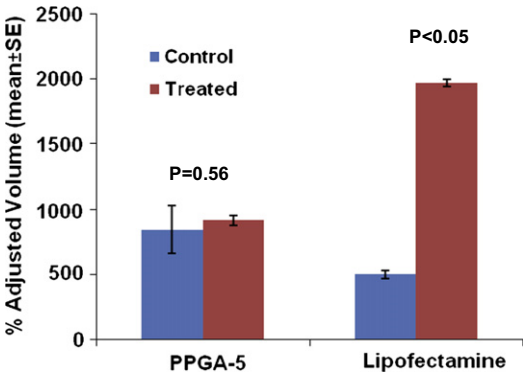

Fig. 6. Densitometric analysis of transcript levels of *hsp70* in control and organisms exposed to PPGA-5 and Lipofectamine™ for 36 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

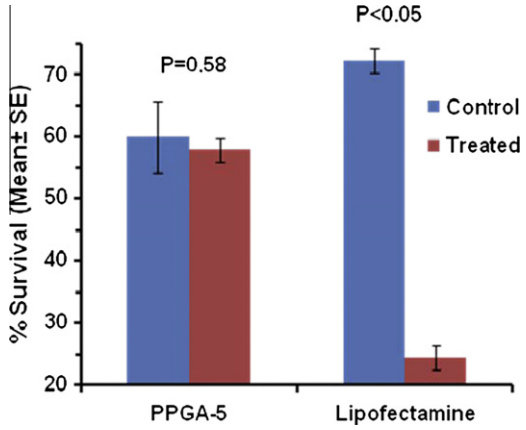

Fig. 7. Percent survival of *Drosophila* after being treated with PPGA-5 nanocomposite and Lipofectamine™ at third instar larval stage and kept on normal food thereafter. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

polymer aggregation and non-specific adhesion to the cell surface. These findings showed the potential of these nanocomposites for clinical applications.

IC_{50} value or the concentration of nanocomposite/pDNA complexes, at which the HEK293 cell viability reaches 50%, was estimated for the PPGA-5/DNA and PEI/DNA complexes at N/P ratio of 16 and 8, respectively. The values show that PEI remains toxic when compared with PPGA nanoparticles (Table 2).

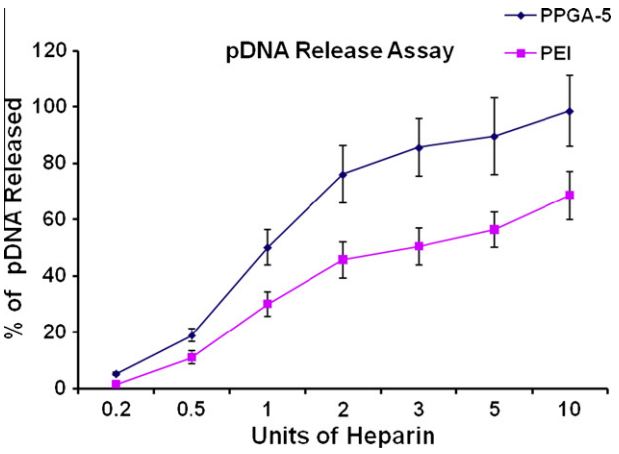

Fig. 9. DNA release assay of PEI and PPGA-5 nanocomposites. Data are represented as mean ± standard deviation (mean ± SD). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3.2. In vivo

To further confirm the safety of PPGA nanocomposites, the toxicity of PPGA-5 was assessed *in vivo* using *Drosophila melanogaster*, where we did not observe any mortality in organisms exposed to PPGA-5 during 36 h exposure period (exposure time is the same as *in vitro*) when compared to their respective controls. In contrast, Lipofectamine™ showed ~60% mortality with respect to the controls. Afterward, on analyzing the expression of heat-shock proteins (as they are excellent bio-indicators of cellular stress or damage), the transcript levels of *hsp60*, *hsp83* and *hsp23* in larvae exposed to PPGA-5 or Lipofectamine™ did not differ significantly from their respective sucrose controls ($P > 0.05$; Fig. 5). Additionally, the transcript levels of *hsp70* in larvae exposed to PPGA-5 were similar to those in their respective controls ($P > 0.05$; Figs. 5 and 6). However, the transcript levels of *hsp70* in larvae exposed to Lipofectamine™ were significantly higher when compared to those in their controls ($P < 0.05$; Figs. 5 and 6). Conversely, a recent study on cellular toxicity of silver nanoparticles using *Drosophila* showed the induction of *hsp70* [36]. This further supports our finding on *in vivo* non-cytotoxic properties of the tested nanocomposite. When the treated larvae were put on normal food and observed for their development to adult, no significant mortality was observed in PPGA-5-treated larvae with respect to their controls, and neither delay in fly emergence nor any morphological disorder was observed (Fig. 7). Lack of induction of hsps above the control level, no abnormality in development from larvae to adult in organisms exposed to PPGA-5 and no delay in emergence

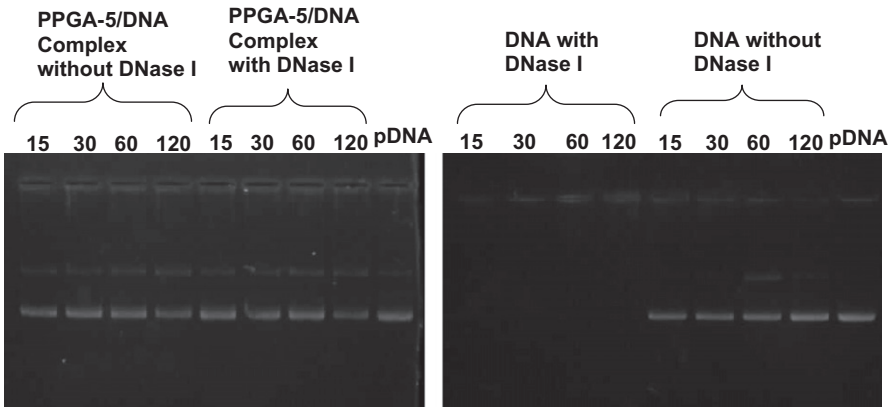

Fig. 8. DNase I protection assay of PPGA-5 nanocomposites at N/P ratio of 16 as compared with naked pDNA.

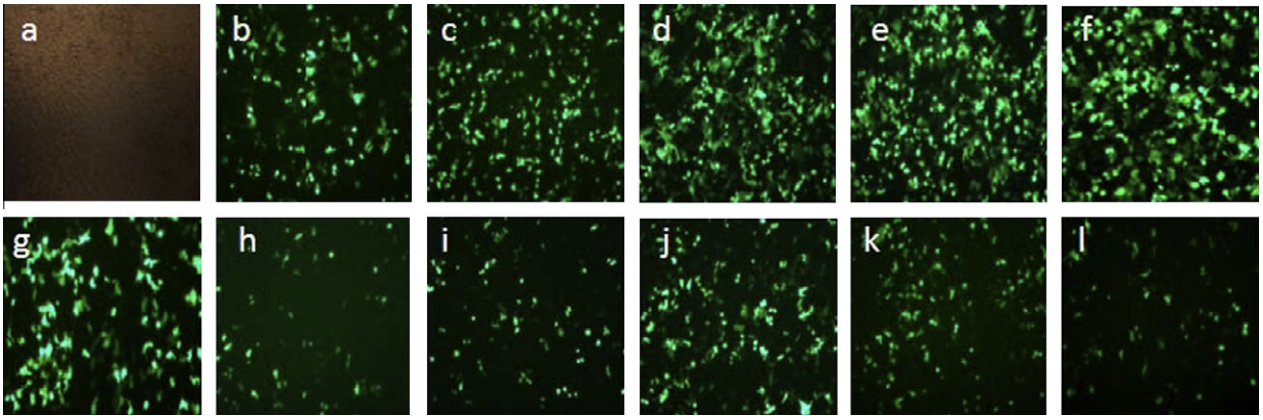

Fig. 10. Fluorescent microscopy of HEK293 cells transfected with (a) untreated HEK293 cells, (b) PPGA-1, (c) PPGA-2, (d) PPGA-3, (e) PPGA-4, (f) PPGA-5, (g) PPGA-6, (h) PEI, (i) Superfect™, (j) Fugene™, (k) GenePORTER 2™ and (l) Lipofectamine™/DNA complexes, at maximum transfection efficiency. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

suggested that PPGA-5 did not induce cellular stress *in vivo*. These results are in line with the findings of *in vitro* toxicity studies that PPGA-5 is relatively non-toxic.

3.4. Protection of pDNA against nucleases

Protection of pDNA from nucleases present in the extra- or intracellular environments is a prerequisite to achieve a successful transfection. Therefore, the ability of PPGA-5 to protect the bound DNA was tested by performing DNase I protection assay. The analysis was done by performing agarose gel electrophoresis. It was observed that naked pDNA was degraded within 15 min of incubation with DNase I, while no significant ($P > 0.05$) degradation (~20% after 2 h) was observed for pDNA bound with PPGA-5 (Fig. 8). These findings revealed that PPGA-5 provided protection to bound pDNA, which advocates that the designed nanocomposites may be used for the *in vivo* administration of nucleic acids.

3.5. DNA release assay

After successfully testing the protection ability of PPGA-5 from nucleases, we then tested the release efficiency of pDNA from PPGA-5/pDNA complex. These two bind with each other through electrostatic interactions between the amines of PPGA-5 and phosphates of pDNA. pDNA release efficiency was studied using a competitive anionic moiety, heparin, and the comparison was made with PEI. The complexes were incubated with increasing amounts of heparin, and pDNA release pattern was studied on 0.8% agarose gel. It was observed that ~68% and ~98% of pDNA was found to be released from PEI and PPGA-5 complexes, respectively, after addition of 10U heparin (Fig. 9). This may be due to tight binding of pDNA to PEI as compared to PPGA-5 nanocomposite, thereby requiring larger amounts of heparin to release the complexed pDNA from PEI/DNA. These results indicate that pDNA bound to PPGA-5 nanoconstruct was loosely held.

3.6. Transfection efficiency of PPGA nanocomposites

3.6.1. In transformed cell lines

Once the proposed system was assessed for toxicity and found to be non-toxic, we evaluated the gene transfer ability of the projected PPGA nanocomposites on HEK293, HepG2 and HeLa cells using plasmid containing reporter gene encoding green fluorescent protein (GFP), and the results were compared with native PEI and other commercial transfection reagents, viz., Superfect™, Fugene™, GenePORTER 2™ and Lipofectamine™. The experiments were carried out at N/P ratios ranging from 4 to 30 (see gel retardation assay). The N/P ratios were chosen in such a way to keep the overall charge positive on the PPGA/DNA complexes, which is required for efficient internalization of particles within the cells. After 36 h of transfection, GFP expression was observed through fluorescent microscopy (Fig. 10). On quantification of the protein in cell lysate, it was found that the transfection efficiency first increased with increasing N/P ratio and then decreased beyond an optimal value (Fig. 11). Therefore, to arrive at the optimal N/P ratio, various N/P ratios were tried and the best N/P ratio was used for the study. The transfection efficiency at N/P ratio 16 varied significantly ($P < 0.01$) with the transfection efficiency of PEI at N/P ratio of 8. Transfection studies were carried out both in the absence and in the presence of serum. In the absence of serum, PPGA-5 formulation displayed ~5.2 folds ($P < 0.01$) higher gene expression in HEK293 cells compared with PEI, whereas the expression was ~2.8, 4.5, 3.3 and 7.9 folds higher ($P < 0.01$) than GenePORTER 2™, Superfect™, Fugene™ and Lipofectamine™, respectively (Fig. 12a). In HeLa and HepG2 cells, the GFP expression of PPGA-5 formulation was ~2.3–6.6 and ~1.7–5.0 folds higher, respectively, as compared to PEI and commercial transfection reagents. More importantly, in the presence of serum, PPGA-5 formulation exhibited ~2.7–7.9 folds higher ($P < 0.01$) GFP expression

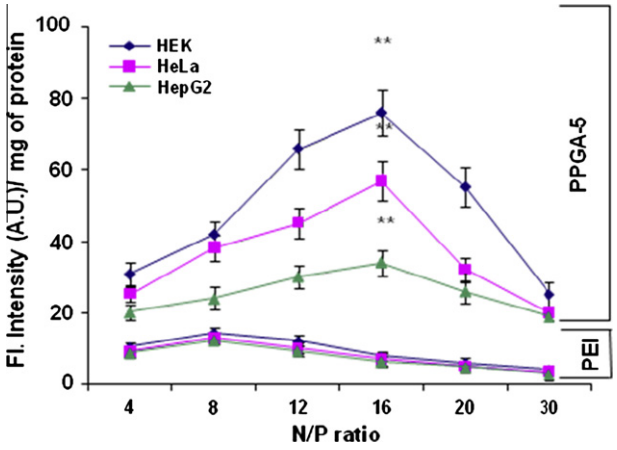

Fig. 11. Dose-dependent GFP fluorescence intensity of PPGA-5 nanoplex and PEI/DNA complex, at the various N/P ratios in HEK293, HeLa and HepG2 cells. Fluorescence intensity is expressed in terms of arbitrary units/mg of total cellular protein. Data represent the mean of three independent experiments performed in triplicate. Significance ascribed as $^{**}P < 0.01$ vs. PEI at maximum transfection efficiency (N/P ratio = 8). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compared with PEI and the standard transfection reagents in HEK293 cells (Fig. 12b). From these studies, it was also observed that transfection efficiency increased with increase in the percentage of PGA, reached up to a maximum for PPGA-5, and thereafter started decreasing. The transfection efficiency was also found to be cell line dependent showing highest expression in HEK293 followed by HeLa and HePG2.

Further, reporter gene expression was quantified by flow cytometry. The transfection efficiency of PPGA/DNA complex was compared with PEI and the commercial reagents, viz., Superfect™ and Lipofectamine™, in HEK293 cells. Using five different N/P ratios, viz., 4, 8, 12, 16 and 20, it was observed that bPEI showed the maximum transfection efficiency (24 ± 2.3%) at N/P ratio of 8, while PPGA-5 exhibited the maximum transfection efficiency (71 ± 3.1%) at N/P ratio of 16. It was also observed that on increasing the N/P ratio, the transfection efficiency first increased and then decreased beyond an optimal value (Fig. 13). The percent transfection efficiency of nanocomposites at N/P ratio of 16 varied significantly ($P < 0.01$) with that of PEI at N/P ratio of 8. A comparison of PPGA-5 with Superfect™ (30 ± 3%) and Lipofectamine™ (15 ± 4%) revealed a significantly ($P < 0.01$) higher percentage of GFP-positive cells in the former indicating potential of PPGA nanocomposites for future applications in gene delivery.

3.6.2. In primary mouse keratinocytes

Concurrent with our transfection studies in transformed cells, we performed transfection studies in primary mouse keratinocytes [37–39]. The best nanocomposite in terms of transfection efficiency i.e., PPGA-5 was able to efficiently transfect primary mouse keratinocytes up to 22% in comparison with PEI and Lipofectamine™ which showed only 3.5 ± 0.35 and 3.7 ± 0.1% transfection, respectively (Fig. 14). This shows the utility of PPGA-5 nanocomposite for use in *in vivo* models. Moreover, polyglutamic acid is functionally related to nucleoplasmin by its involvement in histone binding and nucleosome assembly [40].

3.7. In vivo transfection efficiency

In vivo transfection efficiency of the complexed PPGA-5 was examined in Balb/c male mice by luciferase activity in different tissues of the organism after 3 and 7 days of intravenous injection. While a slightly elevated luciferease activity was observed in spleen and heart of the exposed organisms after 3 days (non-significant gene expression as compared to controls; data not shown), a significant ($P < 0.05$) increase in luciferease activity was observed in these tissues after 7 days. PPGA-5 polyplex showed higher gene expression in terms of RLU/g of the tissue compared with unmodified PEI

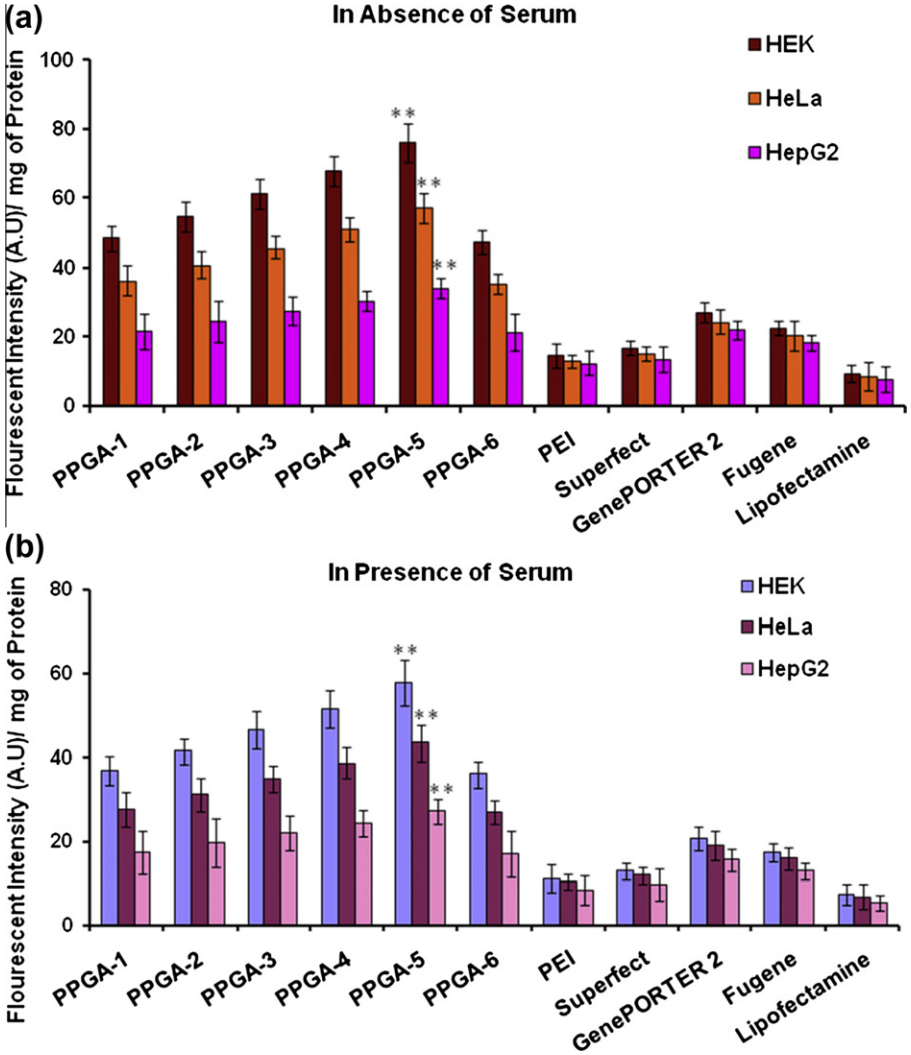

Fig. 12. GFP fluorescence intensity in HEK293, HeLa and HepG2 cells transfected with PPGA nanocomposite/DNA, PEI/DNA, Superfect™/DNA, Fugene™/DNA, GenePORTER 2™/DNA and Lipofectamine™/DNA complexes in (a) absence of serum and (b) presence of serum. Data represent the mean of three independent experiments performed in triplicate. Significance ascribed as $^{**}P < 0.01$ vs. PEI and commercial transfection reagents in all the cell lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

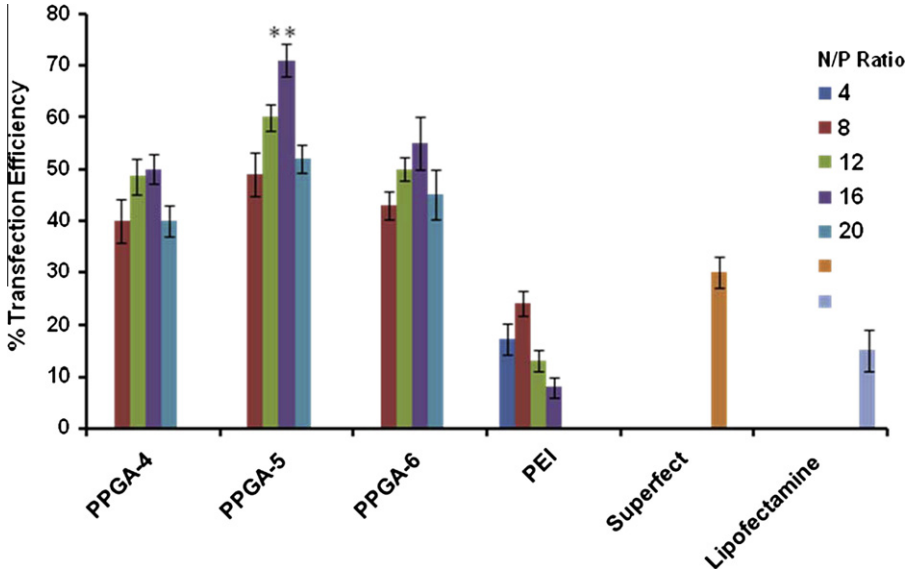

Fig. 13. Percent transfection efficiency of PPGA-4, PPGA-5 and PPGA-6 nanocomposite/DNA nanoplexes in HEK293 cells determined using FACS at various N/P ratios compared with PEI, Superfect™ and Lipofectamine™. Significance ascribed as $^{**}P < 0.01$ vs. PEI (at all N/P ratios), Superfect™ and Lipofectamine™. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

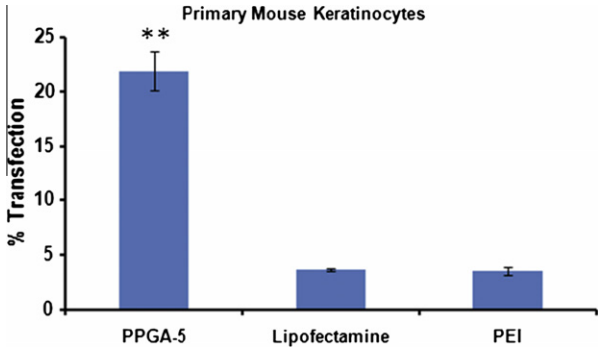

Fig. 14. Percent transfection efficiency of PPGA-5 nanocomposite as determined using FACS in primary mouse keratinocytes and compared with Lipofectamine™ and PEI. Significance ascribed as $^{**}P < 0.01$ vs. PEI and Lipofectamine™. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

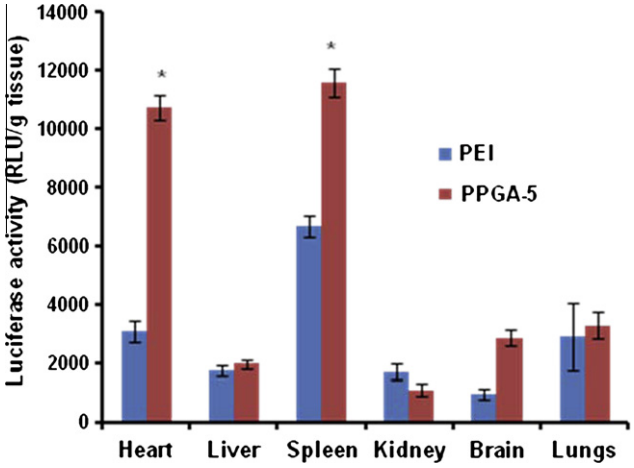

Fig. 15. *In vivo* gene expression analysis in Balb/c mice 7 days post-intravenous injection using pGL3 control vector containing luciferase as a reporter gene. Significance ascribed as $^{*}P < 0.05$ vs. PEI and control in the respective organs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 15). The surveillance of getting highest gene expression in spleen is supported by previously published reports. It was shown that PGA is a capsular component of *Bacillus anthracis* and is known to accumulate in the spleen after intravenous injection in mice [41,42]. Therefore, it is tempting to speculate that the present observation of higher gene expression by PGA-based nanocomposites in spleen may possibly be due to the same reason. However, at present, we are unable to provide a plausible mechanism for the same.

3.8. siRNA delivery

Once successful transfection is achieved, it is pertinent to analyze the knockdown of the targeted gene by using the test nanocomposite. Therefore, PPGA-5 nanocomposite was evaluated in terms of GFP-specific and JNK II siRNA delivery in HEK293 cells. GFP siRNA was transfected after transfection with EGFPpDNA for

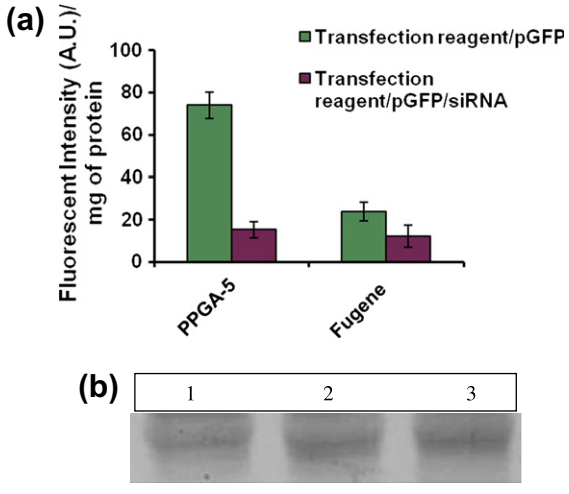

Fig. 16. (a) Efficient delivery of siRNA by PPGA-5 nanocomposite in HEK293 cells in comparison with Fugene™/pGFP DNA/siRNA. Data are represented as mean ± SD. (b) Western blot showing JNKII levels in control cells and in cells transfected with PPGA-5/siRNA. Lane 1: PPGA-5/JNKII siRNA, Lane 2: Lipofectamine™/JNKII siRNA, Lane 3: untreated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3 h and after 36 h of further incubation, the observed knockdown of GFP expression by PPGA-5/pDNA/siRNA and Fugene™/pDNA/siRNA was observed to be ~80% and 50%, respectively (Fig. 16a). A comparison of knockdown of the JNK II-specific protein by PPGA-5 with Lipofectamine™ was carried out by Western hybridization. Knockdown achieved by PPGA-5 was observed to be ~62% while that by Lipofectamine™ was ~52% (Fig. 16b). Hence, a quantitative analysis done in case of GFP-specific siRNA showed a significant decrease in knockdown efficiency of PPGA-5 nanocomposite in comparison with Fugene™, while a semi quantitative analysis done in case of JNKII showed a comparative knockdown efficiency with respect to Lipofectamine™. Hence, the delivery of GFP-specific siRNA oligonucleotides by the test nanocomposite down-regulated the targeted gene better than Fugene™ indicating its potential as an effective carrier for the delivery of siRNA.

3.9. Confocal laser scanning microscopy (CLSM)

Following successful transfection, it is pertinent to track the path of DNA entry into the cell. Therefore, PPGA-5/DNA complexes were added onto HeLa cells, and the cells were observed through confocal laser scanning microscopy to examine the epifluorescence of tetramethylrhodamine(TMR)-labeled PPGA-5 nanocomposite and YOYO-1-labeled pDNA. Nuclear staining was carried out using DAPI (blue). Red fluorescence (for PPGA-5) and green (for DNA) fluorescence were observed near the plasma membrane within 15 min of the addition of complexes to the cells. The PPGA-5 nanocomposite was able to carry DNA inside the cytoplasm within 30 min and to the nucleus within 1 h of the addition of complexes to the cells (Fig. 17). This observation was in agreement with a previous study reporting that PEI delivers nucleic acids to the nucleus [43]. As the substitution on the PEI was too small, the nanocomposite more or less behaved like native PEI. This finding clearly demonstrates that efficient intracellular delivery of DNA can be achieved using PPGA-5 nanocomposite.

4. Conclusions

Taken together, we conclude that a series of polyglutamic acid-coated PEI nanocomposites were prepared, and their transfection efficiency in various cell lines and primary cultures of skin origin was tested. It was established that these nanocomposites have the potential for efficient gene delivery with negligible toxicity to

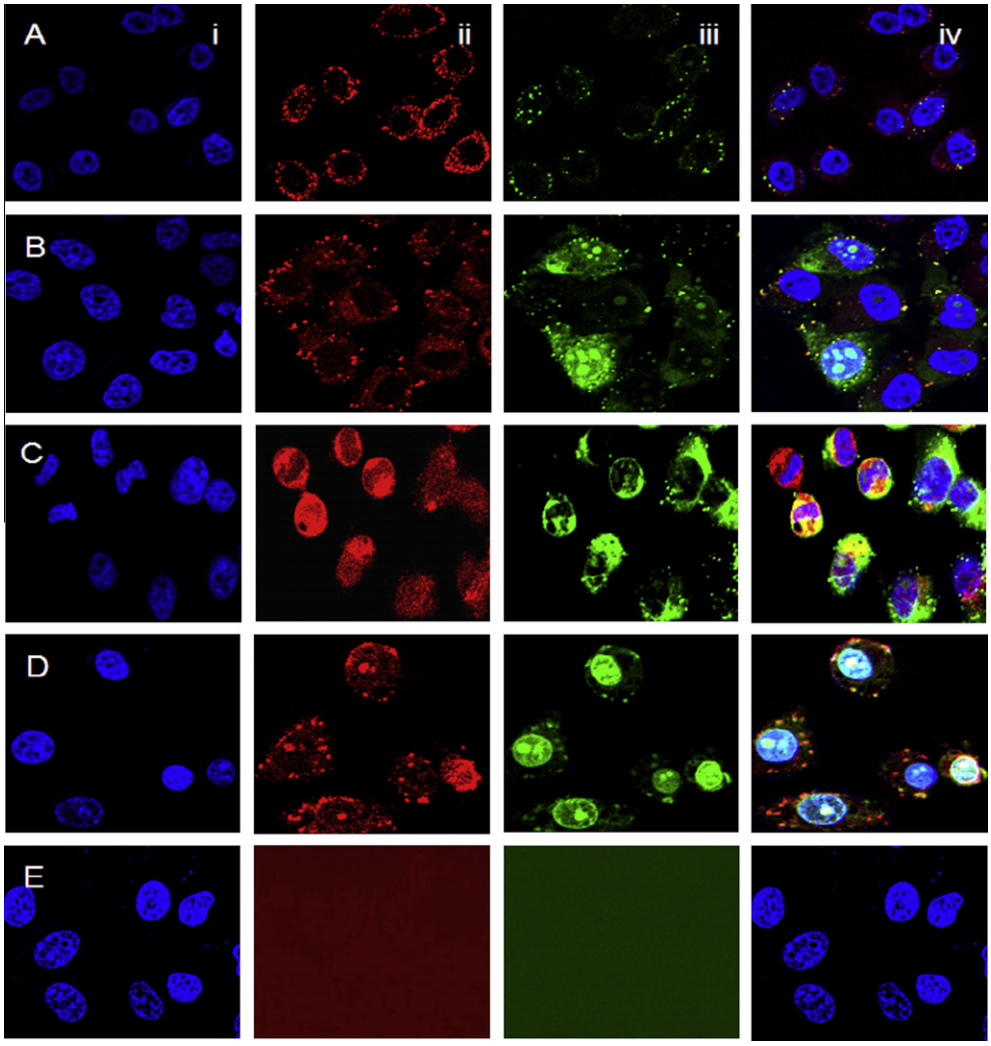

Fig. 17. Confocal microscopic images of HeLa cells treated with TMR-PPGA-5/YOYO-1-pDNA nanoplex at different time points: (A) 30 min, (B) 1 h, (C) 2 h, (D) 4 h, and (E) control. In each image, the first quadrant (blue) shows nuclear staining by DAPI in the cells, the second quadrant (red) shows TMR-PPGA-5, the third quadrant (green) shows YOYO-1-pDNA and the fourth quadrant (IV) represents the overlay of images, where yellow represents TMR PPGA-5/YOYO-1-pDNA nanoplex. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the cells. The tested nanocomposites efficiently (~80%) knocked down the GFP gene expression using GFP-specific siRNA and effectively protected pDNA from nucleases. The nanocomposite with 3.5% PGA content (PPGA-5) was proven to be the best among the series in terms of transfection efficiency and cell viability. Further, biocompatibility and biodegradability make the approach more efficient for use in *in vivo* models.

Acknowledgements

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