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Research paper

Gellan gum blended PEI nanocomposites as gene delivery agents: Evidences from *in vitro* and *in vivo* studies

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ABSTRACT

Branched Polyethylenimine, 25 kDa (PEI), was blended with gellan gum, an anionic heteropolysaccharide, for partial neutralization of its excess positive charge to form gellan gum-polyethylenimine (GP) nanocomposites (NCs). Subsequently, we manipulated the amount of gellan gum for obtaining a series of NCs and characterized them for their size, charge and morphology. Among all the NCs, one member, named GP3, showed the best transfection efficiency in tested cell lines in comparison with the rest of the series, PEI, Lipofectamine and other commercial transfection agents and also exhibited minimum cytotoxicity. It was found to transfect primary cells of mouse skin with better efficiency than PEI and Lipofectamine and was able to protect the plasmid DNA from nucleases and serum proteins present in the blood. GP3 exhibited efficient intracellular delivery of plasmid as revealed by confocal studies while its intracellular presence was also confirmed by the knockdown of GFP expression (using GFP specific siRNA) and JNKII by quantifying proteins in cell lysates and by western blotting and hybridization, respectively. *In vivo* cytotoxicity studies in *Drosophila* showed lack of induction of stress response in the exposed organisms. Further, exposed organisms did not show any developmental delay or mortality and no morphological defects were observed in the emerged flies. *In vivo* gene expression studies in Balb/c mice revealed maximum expression of luciferase enzyme in spleen. The study suggests that GP3 may act as an efficient non-viral gene carrier with diverse biomedical applications.

1. Introduction

Of the non-viral vectors designed for the safe delivery of therapeutic genes to target sites, polymeric vectors are a leading class of gene carriers [1]. While viral vectors outnumber the non-viral gene vectors, the later offer several advantages including safety, low immunogenicity, and capacity to deliver larger genes and cost effectiveness [2,3]. The two leading classes of non-viral gene delivery systems involve the use of either cationic lipids [4] or cationic polymers [5]. Of these, cationic polymers are more attractive for their easily achievable synthesis and also they can be tailored to suit the special requirements such as biocompatibility, DNA binding ability and endosomolytic property for gene delivery [6]. Among the cationic polymers, PEI is particularly a promising candidate as a vector for its relatively high level of transfection in a number of target organs by various routes of delivery [7–9]. PEI is often considered to be a 'proton sponge' since its charge density is dramatically pH-dependent [10]. This unique ionization property is thought to be a key factor that contributes to its high transfection efficiency. Conversely, the very high positive charge density also appears to be the primary cause of its marked toxicity, consequently limiting its use as a gene delivery vector *in vivo*.

To circumvent PEI toxicity, we introduce in this study an anionic sugar polymer into PEI system to increase the transfection efficacy by mitigating its positive charge. The polyanion chosen for this purpose is gellan gum (GG), a linear, anionic heteropolysaccharide, produced by a microorganism, *Sphingomonas elodea*. Its structure is based on a tetrasaccharide repeating unit composed of (1-3)-β-D-glucose, (1-4)-β-D-glucuronic acid, (1-4)-β-D-glucose, and (1-4)-α-L-rhamnose as the backbone. It is a non-toxic food-additive commonly used in confectionary and different food and dairy products. Apart from the above, GG has also been used as a material for tissue engineering applications [11] and as a bioadhesive material in drug delivery [12–15].

Previous reports on the use of sugar-based polymers in gene delivery show promising results [16–18]. The rationale for using GG as a blending agent in this study is for the following reasons: (i) it is an anionic polysaccharide containing only one carboxylic acid moiety among the four units (repeat units) so the binding

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properties would be different from the other polysaccharides, (ii) it is readily soluble in water on heating so scaling up of the reaction conditions is easy and (iii) it is a biodegradable polymer and has not been explored for gene delivery so far. Considering the above, we, therefore, explored the effect of this polysaccharide on the transfection ability of PEI by manipulating the weight ratio of GG to prepare a series of GG blended PEI nanocomposites (GP-NCs). Subsequently, transfection efficiency of the GP-NCs was compared with the existing commercially available transfection reagents.

To succeed as a transfection agent, it is pertinent to test its efficiency on different cellular matrices. This requires improvement at various steps prior to beginning clinical trial studies. In this context, we have tested our system in both *in vitro* and *in vivo* models which include toxicity studies in *Drosophila* and expression of luciferase in mice model. Here in, we have succeeded in developing a transfection reagent that can be efficiently used both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture and materials

HEK293, HeLa and HepG2 cells (obtained from NCCS, Pune, India) were maintained (37 °C, 5%CO_2–air) in Dulbecco's Modified Eagle's Culture Medium (DMEM) (Sigma, USA) with 10% heat-inactivated fetal calf serum (GIBCO-BRL-Life Technologies, UK) supplementation and 1% antibiotic cocktail of streptomycin and penicillin. Specialized chemicals and reagents, used in the present study, were procured from their respective suppliers such as PEI (Mw 25 kDa), gellan gum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and high retention dialysis tubing (Sigma–Aldrich Chemical Co., USA), transfection agents such as Superfect™ (Qiagen, France), Fugene™ (Roche Applied Science, USA), Lipofectamine™ (Invitrogen, USA) and GenePORTER 2™ (Genlantis, USA), plasmid purification kit (Qiagen, France) and the plasmid pEGFPN3 (Clontech, USA). The qualitative functional group analysis of GP NCs was carried out by FTIR (a single beam Perkin Elmer, Spectrum BX Series, USA with the following scan parameters: scan range, 4400–400 cm^{-1}; number of scans, 16; resolution, 4.0 cm^{-1}; interval, 1.0 cm^{-1}; unit, %T).

2.2. Animals

Six- to seven-week-old male and female Swiss albino mice pairs (20 ± 3 g) and male Balb/c mice (25 ± 3 g) were used for primary cell cultures and *in vivo* luciferase gene expression, respectively. These animals from the animal breeding colony of Indian Institute of Toxicology Research (IITR), Lucknow, were acclimatized under standard laboratory conditions and given a commercial pellet diet (Ashirwad Industries, Chandigarh, India) and water *ad libitum*. Animals were housed in plastic cages on rice husk bedding and maintained at 22 ± 2 °C with 12 h dark/light and 50–60% humidity as per rules laid down by Animal Welfare Committee of IITR. Animals were cared for according to the instructions laid by the Institutional (IITR) Ethical Committee.

2.3. Primary cell culture

One to two-day-old pups (4–5 nos.) were used for establishing primary cultures of keratinocytes, as described previously [19,20]. Briefly, these 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 chloride and 10% fetal bovine serum. The cells were allowed to attach at 37 °C in 5% CO_2 for 2.5 h, and medium was then replaced with serum-free keratinocyte growth medium-2 (KGM-2) (Lonza, Basel, Switzerland) containing 0.03 mM calcium chloride.

2.4. Hydrolysis of ester functionality present in GG

A solution of GG (200 ml, 1.0 mg/ml) in double distilled (dd) water in the presence of 0.125 M NaOH was heated to 90 °C. After 24 h of stirring at the same temperature and volume reduction (up to one third) through rotary evaporation, the solution was dialyzed against dd water (24 h, till the solution became neutral) followed by dialysis against 0.5 N HCl (24 h) and then dd water (24 h). After lyophilization, hydrolyzed GG was obtained as a white powder, which was further used for all studies.

2.5. Preparation and purification of GP NCs

PEI was ionically cross-linked with GG to prepare a series of GP (GP1–GP6) NCs (Scheme-1). Briefly, for the preparation of GP1 NC, to a pre-heated solution of PEI (50 ml, 1.0 mg/ml) at 90 °C was added dropwise a pre-heated solution of GG (11.5 ml, 1 mg/ml) at 90 °C over a period of 30 min with continuous stirring. The stirring was continued for 4 h at the same temperature and then the volume of the reaction mixture was reduced to one third of the total volume on a rotary evaporator, which was subjected to dialysis against water for 72 h with intermittent change of water. Thereafter, the solution was lyophilized in a speed vac to obtain a white residue of GP1 NC. For the purification of GP1, the solution (1 mg/ml) was passed through a Centricon filter (100 kDa cutoff, Millipore Inc., USA) and the residue was re-dissolved in dd water to obtain GP1 in ~70% yield. Likewise, other GP NCs (GP2, 20%; GP3, 40%; GP4, 60%; GP5, 80% and GP6, 100% cross-linking) were prepared and purified to obtain a ~70–75% yield. The purified NCs were then characterized by Infrared Spectroscopy (IR), Dynamic Light Scattering (DLS), Zeta potential and Atomic Force Microscopy (AFM).

The polysaccharide content in NCs was modulated by varying the proportion of GG in the reaction with a constant amount of PEI. The polysaccharide concentration in the NCs was determined by the phenol–sulfuric acid method [21]. Briefly, 25 μl of GP1 NC (1 μg/μl) was mixed thoroughly with 15 μl fresh 5% (w/v) phenol solution in dd water and 90 μl concentrated H_2SO_4 (95–97%). The mixture was incubated for 30 min at 25 ± 1 °C, diluted to 1.0 ml and the absorbance was measured on a Perkin Elmer Lambda Bio 20 UV–VIS spectrophotometer at 490 nm. Similarly, absorbance values of other NCs (GP2–GP6) were recorded. The content of polysaccharide in the NCs was calculated from a standard calibration curve derived from known concentrations of GG.

2.6. Formation of NC/DNA complexes

N/P ratio is defined as the ratio of moles of the amine groups of cationic polymer to those of the phosphate groups of DNA. To form NC/DNA complexes, an aqueous solution of GP NCs (1.0 mg/ml) was added to 1 μl of DNA (0.3 μg/μl) at various N/P ratios (7, 10, 15, 20, 25, 30) and the final volume was made up to 20.0 μl with water. For *in vitro* transfection assay, 5.0 μl of 20% dextrose solution was added before making up the final volume to 20.0 μl with water. The resulting samples were gently vortexed and incubated for 30 min at 25 ± 1 °C prior to their use in biophysical studies or transfection experiments.

2.7. Physical characterization of NCs

The prepared NCs were characterized for their morphology, size and zeta potential. The hydrodynamic diameter of NCs (1 mg/ml)

and their DNA complexes, suspended in water and 10% serum, were measured by DLS in triplicates using Zetasizer Nano ZS (Malvern Instruments, UK). The data analysis was performed in automatic mode and measured sizes were presented as the average value of 20 runs.

To characterize the morphology of NCs and their DNA complexes using AFM, a suspension of NCs (2.0–3.0 μl, 0.1 μg/ml) was deposited on a freshly split untreated mica strip, dried for 5 min at 25 ± 1 °C and imaged. Particle size was estimated using an image analyzing software package (SPIP) (PicoSPM System, Molecular Imaging, USA) for scanning probe microscopy.

Zeta potential measurements of NCs and DNA complexes in water and 10% serum were measured on a Zetasizer Nano ZS, carried out 30 runs in triplicates and the average values were estimated by Smoluchowski approximation from the electrophoretic mobility [22].

2.8. DNA retardation assay

PEI and GP NCs were complexed with pDNA (0.3 μg/μl) at N/P ratio 1.5, 2.0, 3.0 and 4.0 for the former and 3.0, 5.0, 7.0 and 10.0 for the later. DNA complexes (20 μl) were mixed with 2 μl xylene cyanol (in 20% glycerol), electrophoresed (100 V, 1 h) in 0.8% agarose, stained with ethidium bromide and visualized on a UV transilluminator using a Gel Documentation system (Syngene, UK).

2.9. Toxicity studies

2.9.1. In vitro cytotoxicity

Cytotoxicity of PEI/pDNA, GP NCs/pDNA as well as Superfect™, Fugene™, GenePORTER 2™ and Lipofectamine™/pDNA complexes was evaluated on HEK293, HepG2 and HeLa cells by MTT colorimetric assay. This assay involves the reduction of yellow MTT by mitochondrial succinate dehydrogenase in live cells into colored (dark purple) formazan product. The formazan crystals so formed are solubilized in organic solvents, and intensity is measured spectrophotometrically at 540 nm.

The cells were transfected as done in transfection experiments. After 36 h, MTT (200 μl, 0.5 mg/ml in DMEM) was added to the cells and the plate was kept in an incubator for 2 h at 37 °C. The supernatant was aspirated, and the formazan crystals were suspended in 100 μl isopropanol containing 0.06 M HCl and 0.5% SDS. The intensity of color was measured spectrophotometrically on an ELISA plate reader (MRX, Dynatech Laboratories) at 540 nm. Untreated cells were taken as control with 100% viability, and cells without the addition of MTT were used as blank to calibrate the spectrophotometer to zero absorbance. Similarly, cell viability was calculated for GP3, PEI and Lipofectamine in primary mouse keratinocytes also using MTT assay. The relative cell viability (%) compared to control cells was calculated by $[abs]_{sample}/[abs]_{control} \times 100$. IC_{50} value, at which the HEK293 cell viability reaches 50%, was estimated for GP nanocomposite/DNA and PEI/DNA complexes at N/P ratio of 15 and 10, respectively. The transfection efficiency for GP NCs and PEI was found to be highest at these N/P ratios.

2.9.2. In vivo toxicity assessment of GP3 in Drosophila melanogaster

To assess the *in vivo* toxicity of GP3, third instar larvae of *D. melanogaster* (Oregon R^+) arising from synchronized eggs/cultures were exposed to GP3 NC for 36 h as paralleled in transfection studies *in vitro*. However, unlike transfection conditions, larvae, kept in an Eppendorf tube, were exposed to 5% sucrose medium containing GP3 [20 μl, 19.3 μl 5% sucrose solution with 0.7 μl GP3 (1 mg/ml, in 5% sucrose)]. Third instar larvae, exposed to 5% sucrose alone, formed the negative controls. The particle size of GP3 NC in 5% sucrose solution was determined and confirmed to be in the nano-range, prior to exposure (data not shown). As in transfection studies, here too, we exposed third instar larvae to Lipofectamine (2.0 μl) (Invitrogen, USA) in the presence of 5% sucrose solution (18 μl) as positive control. After 36 h of exposure, both control and treated larvae were washed thoroughly in 10 mM PBS and total RNA was extracted from them using Trizol-RT extraction method (Life Technologies, USA). Subsequently, cDNA was synthesized (Fermentas, USA) as per the manufacturer's protocols. The transcripts of different stress genes, namely, *hsp70* (heat shock protein 70), *hsp60, hsp83 and hsp23* were amplified, as described in Singh et al. [23]. As an internal control for the quality/quantity of cDNA, RPL32 levels were analyzed [24]. The amplicons were resolved in 1% agarose gels. The transcript profiles were documented using versadoc, and their levels were analyzed semi-quantitatively by Quantity One software (Bio-Rad, USA). Each group contained 30 larvae/replicate, and the exposures/analyses were carried out in triplicate.

In another set of experiments, after a 36 h exposure, larvae of both control and treated groups were kept on normal food and observed for the emergence of adults.

2.10. DNA release assay

PEI and GP nanocomposites were complexed with pDNA (300 ng) at N/P ratio of their highest transfection efficiency and incubated for 30 min, as described in Section 2.6. Heparin, a polyanion, was added in different amounts varying from 0.1 to 2.0 U for the release of plasmid DNA, which is bound to the polycation. The samples were then incubated for 20 min and were electrophoresed (100 V, 1 h) in a 0.8% agarose gel, stained with ethidium bromide and visualized on a UV transilluminator using a Gel Documentation system. The quantitative analysis, after heparin treatment, was carried out by densitometry using Gene Tools software from Syngene.

2.11. DNase I protection assay

For assessing the ability of the nanocomposites (GP3) to protect the condensed pDNA from nucleases, DNase I protection assay was performed. Native pDNA and GP3/pDNA complex (at N/P ratio 15) were incubated at 37 °C for 0.25, 0.5, 1.0 and 2.0 h with DNase I (Sigma, USA) (1 μl, 1 unit/μl in a buffer containing 100 mM Tris, 25 mM $MgCl_2$ and 5 mM $CaCl_2$). Treatment with PBS alone served as the control. After incubation, 5 μl EDTA (100 mM) was added to the reaction mixture to quench the activity of DNase I and the mixture was further incubated at 75 °C for 10 min to degrade DNase I. The mixture was further incubated for 2 h at 25 ± 1 °C with 10 units (U) of heparin (1 U/μl) to release bound DNA from the GP3/pDNA complex. Subsequently, samples were electrophoresed and visualized, as described in Section 2.10. The amount of pDNA released from complexes after heparin treatment was estimated by densitometry using Gene Tools software from Syngene.

2.12. Protein adsorption

Protein adsorption to the surface of native PEI and GP3 was examined, as described in literature [25]. In brief, PEI and GP3 were complexed with pDNA at their best working N/P ratios. Samples were incubated with standard bovine serum albumin (BSA, Bangalore Genei, India) for 3 h at 25 ± 1 °C, and final pDNA and BSA concentrations were 3.0 μg/ml and 1.0 mg/ml, respectively. The samples (nanoparticle/BSA) were centrifuged at 14,000*g* for 2 h at 4 °C. The supernatant was removed, and the pellet was washed with MilliQ water (10 μl) to remove unbound BSA. The pellet was resuspended in MilliQ water (10 μl), added loading buffer (0.2 M Tris–HCl pH 6.8, 10% w/v sodium dodecyl sulfate (SDS),

20% v/v glycerol, 0.05% w/v bromophenol blue, 10 mM dithriothreitol) (10 μl) and incubated at 100 °C for 10 min to extract bound BSA from samples. Extracted solutions were separated in a 12% denaturing SDS–polyacrylamide gel after a run of 2 h at 25 mA and stained with RAPID stain to visualize BSA that had adsorbed onto samples.

2.13. In vitro transfection

HEK293, HepG2 and HeLa cells were seeded in 96-well plates, and after 16 h, media were aspirated and cells washed with phosphate buffer saline (PBS). PEI and GP NCs were complexed with pDNA (0.3 μg) at N/P ratios ranging from 7 to 30 and incubated for 30 min at 25 ± 1 °C. Similarly, pDNA complexes were prepared with Superfect™, Fugene™, GenePORTER 2™ and Lipofectamine™ following manufacturers' protocols. DNA complexes were diluted with serum-free DMEM (60 μl) and gently added to HEK293, HepG2 and HeLa cells. In another set, DNA complexes were diluted in DMEM supplemented with 10% FBS (to observe the effect of serum on transfection efficiency) and treated the cells, as described above. After 4 h, the transfection medium was replaced by 200 μl fresh DMEM-10% FBS and cells were incubated for 36 h.

To observe the knockdown of Green Fluorescent Protein (GFP) expression using GFP specific siRNA, pDNA complexes were made and transfected in HEK cells as above. After 3 h, the medium was replaced with 20 μl of siRNA/GP3 complex (2.5 μM/0.7 μg) mixed with 60 μl serum-free DMEM. The cells were again incubated for 3 h after which the medium was replaced with 10% serum containing DMEM (200 μl) and GFP expression was observed after 36 h. GP3/DNA nanoplex alone was used as a control. Fugene™/DNA complexes were also prepared, and all of these formulations added on to seeded HEK293 cells. The expression levels were monitored by quantifying GFP. All experiments were performed at least in triplicate.

2.14. Quantification of enhanced green fluorescent protein (EGFP) expression

EGFP expression in mammalian cells was quantitatively estimated on NanoDrop ND-3000 spectrofluorometer. After 36 h of transfection, cells in each well were washed with PBS (2 × 100 μl) and incubated with cell lysis buffer (10 mM Tris, 1 mM EDTA and 0.5% SDS pH 7.4, 100 μl) for 20 min at 37 °C. The cell lysates were centrifuged, and 2 μl of lysate was used for measuring EGFP spectrofluorometrically. The fluorescence intensity values for background and auto-fluorescence were determined using mock-treated cells. Total protein content in cell lysate from each well was estimated using Bradford reagent (Bangalore Genei, India) with BSA as the standard. The level of fluorescence intensity of GFP was calculated by subtracting the background values and normalized against protein concentration in cell extract. The data are reported as arbitrary unit (AU)/mg of cellular protein and represent mean ± standard deviation for triplicate samples.

2.15. Fluorescence activated cell sorting (FACS) analysis

For examining GFP expression at the individual cell level, FACS analysis was performed at 36 h post-transfection. Briefly, HEK293 cells after seeding in 24-well plates for 16 h were washed with phosphate buffer saline (PBS) following aspiration of the medium. PEI and GP NCs were complexed with pDNA (1.5 μg) at N/P ratios of 5, 10, 15 and 20 and incubated for 30 min at 25 ± 1 °C. Similarly, pDNA complexes were prepared with Lipofectamine™ following manufacturer's protocol. Formulations were diluted with serum-free DMEM (300 μl) and gently added on to HEK293 cells. After 4 h, the transfection medium was replaced by fresh DMEM-10% FBS (1 ml) and cells were incubated for 36 h. Subsequently, transfected cells were washed with PBS (1 × 1 ml) and harvested by trypsinization. Cells were recovered by centrifugation at 5000 rpm for 5 min at 25 ± 1 °C, the supernatant removed, the pellet was washed with 1× PBS (2 × 500 μl) and resuspended in 1× PBS (1 ml). Percentage of EGFP-expressing cells was determined to quantify the transfection efficiency by flow cytometry equipped with Cytosoft Software (Guava® EasyCyte™ Plus Flow Cytometry System, USA). The percentage of transfected cells was obtained by determining the statistics of cells fluorescing above the control level, where non-transfected cells were used as the control. Five thousand cells were analyzed to generate data for statistical purposes.

2.16. Transfection in primary mouse keratinocytes

Mouse keratinocytes were seeded in 6-well plates at a density of 1×10^6 cells/well as described above. After 48 h, the cells, following aspiration of medium, were washed with PBS. PEI and GP3 nanocomposite-pDNA (2 μg) complexes [at N/P ratio of 10 and 15, respectively] were made and incubated for 30 min at 25 ± 1 °C. Similarly, pDNA complex was also prepared with Lipofectamine™ following manufacturer's protocol. DNA complexes were diluted with serum-free KGM-2 containing 0.03 mM calcium chloride (500 μl) and gently added to the cells. After 3 h, the transfection medium was replaced by 1 ml fresh KGM-2 containing 0.03 mM calcium chloride and cells were incubated for 24 h after which FACS analysis was performed on a BD, FACS Calibur (Bioscience, USA), as described in Section 2.15, except that in this case 15,000 cells were used for analyses.

2.17. Confocal laser-scanning microscopy (CLSM)

To monitor intracellular trafficking, GP3 was labeled with tetramethylrhodamine isothiocyanate (TRITC). Briefly, 1 ml GP3 NC (10 mg/ml, in H_2O) was allowed to react with 26 μl TRITC (1 mg/100 μl, in DMF) overnight to block 1% of total amines in NCs. The solution was concentrated in a speed vac and unreacted/hydrolyzed TRITC was removed by triturating with ethyl acetate (3 × 2 ml). For labeling of pDNA, YOYO-1 iodide (2 μl, 1 mM solution in DMSO) was added to pDNA (0.3 mg) and stirred for 2 h at 25 ± 1 °C in dark, then stored at −20 °C. HeLa cells were seeded (1.5×10^5 cells/well) on circular glass coverslips in a 6-well plate and grown overnight to ~70% confluence. A solution of nanoplex (500 μl) containing 0.3 μg of pDNA in DMEM was added to each well. After incubation for 0.5, 1.0, 2.0 and 4.0 h, the cells were washed with 1× PBS (3 × 500 μl) and fixed with 4% paraformaldehyde for 10 min at 4 °C. The fixed cells were counter-stained with a blue nuclear dye, DAPI (4′,6-diamidino-2-phenylindole), and coverslips were mounted on glass slides 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 (UK).

2.18. Knockdown of JNK II using GP3

HEK293 Cells were seeded in 6-well plates one day before the transfection; after that, they were incubated with GP3/JNK II siRNA (10 nm) (cell signaling technology, USA) and GP3/siRNA (scrambled sequence) in minimal essential medium (minus serum) for 3 h. Thereafter, the medium was replaced with 10% serum containing medium followed by 36 h incubation. The cells were then washed with 1× PBS and lysed in 100 μl of cell lysis buffer (Sigma, USA) supplemented with 1× protease inhibitor cocktail (Sigma). Equal amounts of protein were resolved through SDS–PAGE and then transferred onto nitrocellulose membranes. The blots were

blocked for 2 h in TBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20) containing 5% BSA then probed overnight with primary antibody; JNK (1:1000). Immunoreactive bands were detected with appropriate secondary antibodies conjugated to alkaline phosphatase and detected with BCIP/NBT (Sigma). Densitometric analysis was carried out using Quantity One software (Bio-Rad, USA).

2.19. Intravenous injection of GP3 in Balb/c mice

Luciferase vector, pGL3 (25 μg), was complexed with GP3 and PEI at an N/P ratio of 15 and 10, respectively, with a final volume of 100 μl using normal saline. The complexes were incubated for 30 min at 25 ± 1 °C. Similarly, naked DNA (25 μg) in 100 μl normal saline was incubated for 30 min at 25 ± 1 °C. Three Balb/c mice were injected with the above made complexes i.e., GP3, PEI and naked DNA through the tail vein using a syringe of 40 U (insulin syringe, 1 ml) with needle of size 0.30 × 8 mm. After 7 days on normal diet, the animals were sacrificed by cervical dislocation and all the vital organs were dissected out. The organs were washed with chilled normal saline, weighed, chopped and made 25% w/v homogenate in 1× cell lysis buffer (promega, USA) containing 1× protease inhibitor cocktail (Sigma, USA). After 3 cycles of freezing and thawing (liquid nitrogen and 37 °C respectively), the whole homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C. One hundred microliters of the cell lysate from each sample was assayed for luciferase activity by adding 100 μl luciferin substrate on the luminometer (Berthold, Germany). Standard curve was made using luciferase enzyme provided with the kit. The experiment was repeated twice independently.

2.20. Statistical analysis

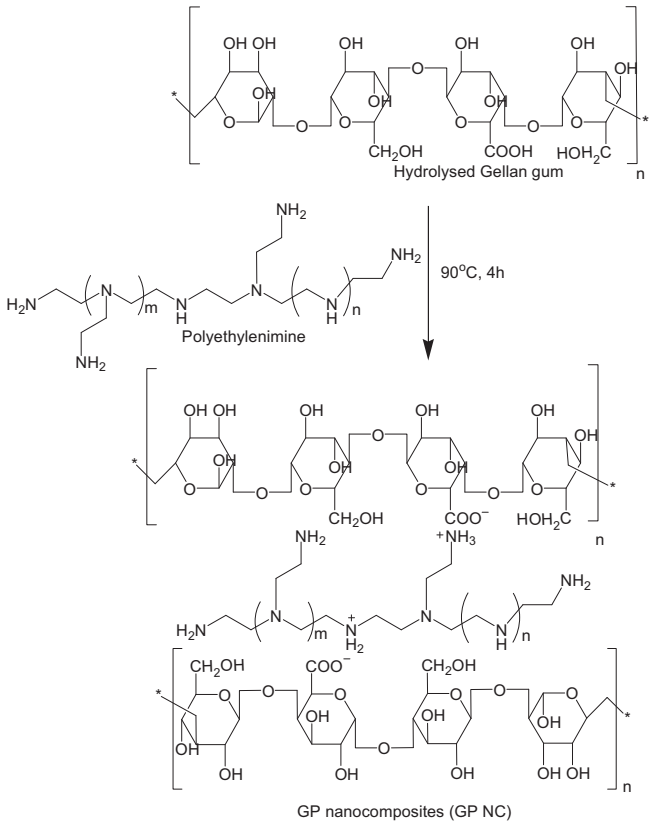
Statistical analysis wherever needed was carried out by one-way analysis of variance followed by Student's *t*-test after ascertaining homogeneity of variance and normality of data. A value of $P < 0.05$ was considered statistically significant. JMP version 6.0.0, Statistical Discovery™ (from SAS) was used for analysis.

3. Results and discussion

PEI has been used for gene delivery purposes owing to its high buffering capacity but the presence of high cationic charge limits its use *in vivo*. Polysaccharide blending helps in overcoming the commonly encountered problems with the use of PEIs, viz., reduces high charge density of PEI thus making it less toxic, lowers the interaction with blood serum proteins to allow intracellular distribution and provides target-specificity. The present investigation demonstrates that PEI interacts electrostatically with the acidic group of GG, an anionic heteropolysaccharide, to form NCs (125–322 nm) that efficiently allow *in vivo* gene delivery.

3.1. Preparation, purification and characterization of GP NCs

The blending of PEI with varying amount of GG formed a series of well defined GP NCs (GP1–GP6) (Scheme 1). Zeta potential of these NCs was found to be positive even after a filtration through 100 kDa cutoff filter, thereby indicating that GG is blended with PEI. Characterization of purified particles using infrared spectroscopy showed the peak at 1785 cm^{-1} (for carbonyl) and a broad peak at 3345 cm^{-1} (for amines) to confirm the presence of both the polymers in the NCs. As GG binds PEI electrostatically, it was mandatory to measure the amount of GG associated with PEI. We found ~23–31% of the attempted substitution of GG, as estimated by colorimetry indicating successful formation of NCs



Scheme 1. Schematic representation of preparation of GP NCs.

(Table 1). Further, to measure the size of these NCs, DLS was performed and we observed the average size of NCs in the range 125–322 nm (Table 2). Interestingly, size of the NCs was found to increase with an increase in polysaccharide content, which might be due to the association of increasing amount of hydrophilic polymer, GG, to PEI from GP1 to GP6. Moreover, it was found that the size of the NCs decreased after complexation with pDNA which further reduced in the presence of 10% FBS. The size of NC/DNA complexes measured by DLS decreased in the presence of serum, which might be due to adsorption of water molecules from the complexes by the anionic serum proteins resulting into dehydration of the same and subsequently leading to decrease in hydrodynamic diameter. Moreover, to examine the stability or aggregation behavior of particles over time in 10% serum, it was noticed that the particles size remained almost constant, which implied that the presence of sugar moiety in the NCs inhibited the non-specific binding or aggregation of particles.

We observed a decrease in zeta potential with an increase in percentage of cross-linking confirming successful blending of polysaccharide with PEI (Table 2). After making NCs-pDNA complexes,

Table 1
The percentage of amino groups blocked in PEI by gellan gum as estimated by colorimetry. The value in bracket (*n*) shows the no. of times the experiment was performed.

S. no.	Nanocomposites	Attempted substitution of GG (%)	Realized substitution of GG (%)(*n*)
1	GP1	10	2.25 ± 0.23(3)
2	GP2	20	6.23 ± 0.56(3)
3	GP3	40	10.62 ± 1.26(3)
4	GP4	60	17.16 ± 1.82(3)
5	GP5	80	19.41 ± 2.18(3)
6	GP6	100	26.23 ± 2.29(3)

Table 2
Particle size and zeta potential measurements of GP NCs and their corresponding DNA complexes in water and serum at N/P ratio of 15 for GP NCs/DNA and 10 for PEI/DNA.

	Average particle size (nm) ± S.D. (PDI)			Zeta potential (mV) ± S.D.			
Samples	Nanocomposites (in H_2O)	DNA loaded Nanocomposites (in H_2O)	DNA loaded Nanocomposites (in 10% serum)	Nanocomposites (in H_2O)	DNA loaded Nanocomposites (in H_2O)	DNA loaded Nanocomposites (in 10% serum)	Ratio of composite S: DNA (N/P)
GP1	122 ± 7.3 (0.123)	101.8 ± 6.2 (0.161)	95 ± 4.2 (0.106)	31.5 ± 1.5	22.9 ± 1.8	−10.5 ± 0.3	15
GP2	136 ± 7.8 (0.135)	125.2 ± 4.9 (0.127)	109 ± 6.9 (0.112)	28.3 ± 1.2	21.3 ± 0.2	−9.5 ± 0.1	15
GP3	149 ± 8.1 (0.139)	136.9 ± 7.2 (0.152)	121.8 ± 2.6 (0.129)	26.4 ± 1.4	19.5 ± 1.6	−9.8 ± 0.6	15
GP4	206 ± 9.7 (0.261)	187.1 ± 4.3 (0.110)	145 ± 7.4	23.5 ± 1.5	17.7 ± 1.4	−10.5 ± 0.8	15
GP5	253 ± 8.5 (0.293)	210.8 ± 9.4 (0.118)	203.8 ± 4.3 (0.102)	22.2 ± 1.7	16.2 ± 1.1	−9.8 ± 0.6	15
GP6	322 ± 5.2 (0.284)	223.0 ± 2.6 (0.109)	131.8 ± 16.8	21.5 ± 1.5	8.9 ± 0.8	−9.7 ± 0.7	15
PEI	–	396.0 ± 8.8 (0.539)	271.0 ± 12.3.8 (0.698)	41.1 ± 6.2	29.7 ± 1.3	−9.3 ± 0.8	10

we observed a decrease in their zeta potential due to neutralization of charge by DNA in the said complexes (Table 2). The results are in accordance with our observation with gel retardation assay. Conversely, zeta potential of the NC/DNA complexes in 10% serum was found to be negative since these proteins have overall negative charges. This observation finds support from earlier studies [16,22].

To determine the surface morphology and size of these NCs (GP1–GP6) and the DNA complexes, AFM analysis for one of the members, viz. GP3, was performed. We observed the size of GP3 and GP3/DNA complex to be approximately 70 nm and 90 nm, respectively (Fig. 1). A discrepancy in the observation of size measurement of GP3 and GP3–DNA complex by AFM and DLS (larger size shown by DLS and smaller by AFM analyses) may be due to the measurement of dehydrated form of NC by the former and hydrodynamic diameter of the particles by the later. Further, AFM analyses showed a nearly spherical shape of this NC. In AFM, the broadness of distribution was calculated by measuring the size of the nanocomposites (i.e., GP3) manually. It was found that 70% of the total population of NCs was in the size range of 40–80 nm and 30% in 80–120 nm. However, on complexation with pDNA, the particle size distribution was found to be changed and it was observed that 90% of the total population was in the size range of 30–50 nm and only 10% in 50–70 nm range.

3.2. Mobility shift assay

Neutralization of charge between GP NCs and pDNA resulted in the retardation of mobility of pDNA under the influence of electric field. Fig. S1 (see Supplementary material) shows DNA binding to GP NCs at different N/P ratios in gel retardation assay. We observed a complete retardation of 0.3 μg of pDNA- native PEI complex at N/P 4 and GP NCs at N/P ratio ranging from 5 to 7. At N/P ratios of 1.5 and 3 (for PEI and GP3 respectively), complete binding was not observed and a free DNA band could be seen in the gel. Increasing amounts of GP NCs (GP1–GP6) in DNA complexes were required to retard the electrophoretic mobility of a fixed amount of DNA. Such a difference in retardation of DNA mobility might be due to reduction in charge of PEI after blending with increasing amounts of GG.

3.3. Toxicity assessment of GP NCs

3.3.1. In vitro

One of the major setbacks faced in gene therapy is the cytotoxicity of the non-viral vectors [26]. To address the issue, we examined the viability of HEK293, HeLa and HepG2 cells by MTT assay after exposing the cells to GP NCs, PEI and commercial transfection agents for 36 h (the approximate time of the transfection studies). In GP NCs exposed cells, 100% or more cell viability was observed in comparison with control (taken as 100% viability) (Fig. 2a). The cell viability increased with increase in sugar content i.e., from GP1 to GP6, which might be due to the presence of sugar in the NCs that help in the proliferation of cells. Out of the three cell lines used, HEK293 displayed highest cell viability, given for a particular NC, followed by HepG2 and HeLa. In contrast, lipofectamine and PEI treatments caused 32% and 34% cell death, respectively. A significant ($P < 0.05$) increase in cytotoxicity was observed in primary mouse keratinocytes exposed to PEI and lipofectamine, while GP3 did not induce any toxicity in comparison with control, as evident by MTT assay (Fig. 2b). Further, IC_{50} values for GP NCs were determined based on MTT assay and were found to be significantly ($P < 0.05$) higher (~10 folds) as against PEI (Table 3).

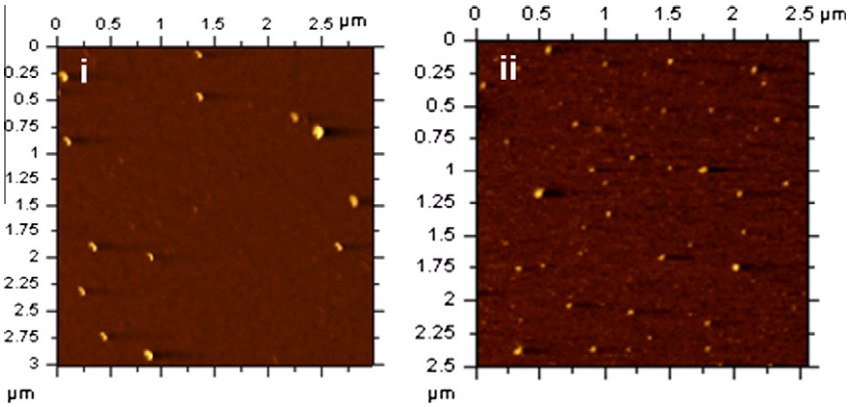


Fig. 1. Characterization of GP3 NC by Atomic Force Microscopy. AFM image of (i) GP3 NC (~90 nm average size) and (ii) GP3/DNA nanoplex (~70 nm average size). 2–3 μl of NC solution was deposited on a freshly split untreated mica strip and images were recorded in acoustic mode. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

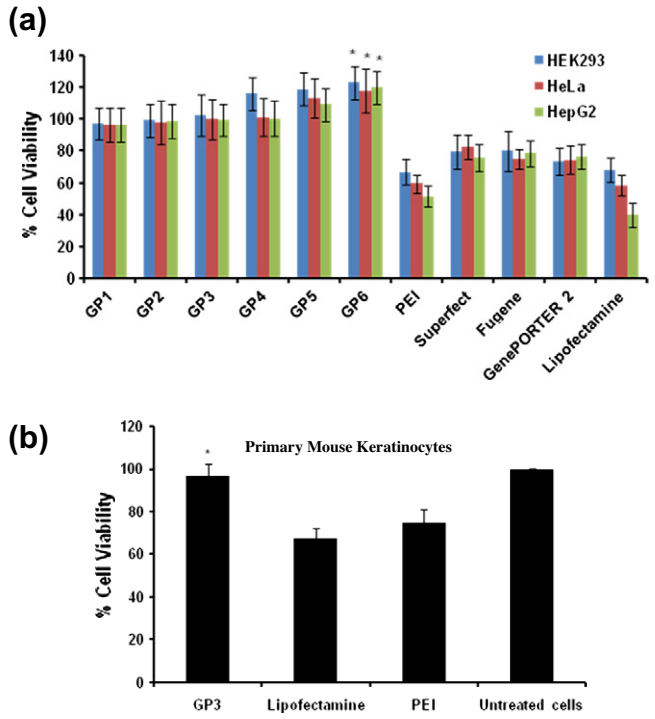


Fig. 2. (a) Cell viability profile of GP NCs/DNA nanoplexes (N/P ratio of 15) and Superfect™/DNA, Fugene™/DNA, GenePORTER 2™/DNA and Lipofectamine™/DNA complexes in HEK293, HeLa and HepG2 cells. (b) Cell viability profile of GP3 NC/DNA complex (N/P ratio of 15), PEI/DNA (N/P ratio of 10) and Lipofectamine™/DNA complexes in Primary Mouse Keratinocytes. Cells were treated with DNA complexes under conditions described for transfection assay, and cytotoxicity was determined using MTT assay. Percent viability of cells is expressed relative to control cells. Each point represents the mean of three independent experiments performed in triplicates. $^{*}P < 0.05$ vs. PEI and commercial transfection reagents. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
IC_{50} values for PEI and GP NCs in HEK293 cells.

Sample	IC_{50} value (mg/L)
GP1	156.2
GP2	163.4
GP3	190.4
GP4	195.3
GP5	200.1
GP6	210.3
PEI	17.5

3.3.2. In vivo

Likewise, *in vivo* toxicity of GP3 NC was tested in *D. melanogaster*, as a model organism, well known for its developmental and genetical studies for over a century. We did not observe any mortality in GP3 exposed organisms after 36 h (exposure time same as *in vitro*), when compared to their respective controls. Instead, we observed a significantly ($P < 0.05$) higher (20% increased) survival of the larvae exposed to GP3 in comparison with the control (Fig. 3). Further, we analyzed the expression of heat shock proteins (hsps), viz., *hsp70, hsp60, hsp83* and *hsp23* in GP3 or lipofectamine exposed larvae, as hsps, especially *hsp70*, has been proposed and shown as first tier bio-indicators of cellular stress or damage [27,28]. Only lipofectomine exposed larvae revealed a significant ($P < 0.05$) induction (5-fold higher compared to its respective control) of *hsp70* and not the other tested members of Hsp family, while GP3 did not induce any of the tested members of this family

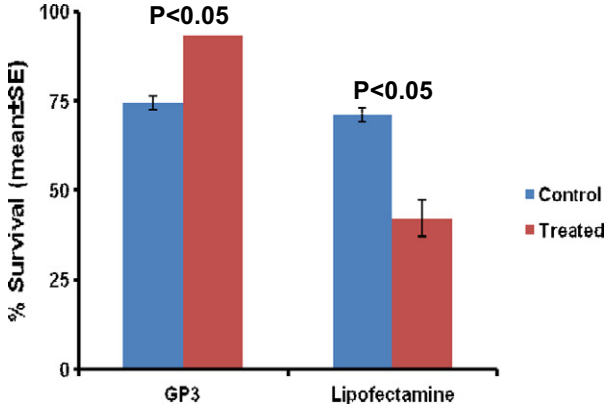


Fig. 3. Percent survival of Drosophila after being treated with GP3 NC and Lipofectamine at third instar larval stage and put on normal food thereafter. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4). Conversely, a recent study on cellular toxicity of silver nanoparticles using Drosophila showed the induction of *hsp70* [29]. This further supports our finding on non-induction of cellular toxicity by the tested NC *in vivo*. Interestingly, when larvae treated with GP3 or lipofectamine for 36 h were put back on normal food and observed for their development to adults, GP3 treated organisms did not show any significant ($P > 0.05$) increase in mortality (93% survival) while lipofectamine treated ones showed 60% mortality when compared to their controls. Moreover, neither a delay in fly emergence nor any morphological disorder was observed in GP3 treated organisms (Fig. 3). Lack of induction of hsps and healthy development of the organisms following GP exposure is indicative of non-cytotoxicity of the NC *in vivo* further supporting our *in vitro* observations.

3.4. Protection of pDNA against nucleases by GP3

To achieve successful entry of DNA in a cell through transfection, its protection from nucleases is a pre-requisite since these nucleases rapidly degrade native DNA [30]. Therefore, we studied the capacity of GP3 towards protecting pDNA from nucleases by DNase I protection assay. Naked DNA was found to be digested by DNase I within 15 min, whereas in GP3–pDNA complex, ~73% pDNA was isolated intact even after 2 h of treatment (Fig. 5). These observations clearly suggest that GP3 can be used as an efficient vector for taking the DNA to the cellular milieu without much degradation.

3.5. DNA binding ability of GP3

After achieving a substantial protection of pDNA following its complex formation with GP3, we further studied the extent of its binding with GP3 for its timely and targeted release in the cell. For such a desired function, it is pertinent to have a fine balance between positive and negative charges on GP3 and the pDNA. PEI is known to bind DNA very tightly, which may eventually affect the release of DNA during transfection [31,32]. In order to assess the DNA release from GP NCs, we compared the binding ability of GP3 and PEI with pDNA using heparin release assay. After using an increasing concentration of heparin (0.1–2.0 U) in the reaction mixture, we observed a maximum release (80%) of DNA in the presence of 2 U of heparin by GP3. Under the similar conditions, PEI released only 55% of DNA (Fig. 6). The results implied that GP3NC/pDNA complex was neither too tightly nor loosely bound. Moreover, it might be due to the excessive positive charge on PEI as well as its open structure, which is responsible for tight binding

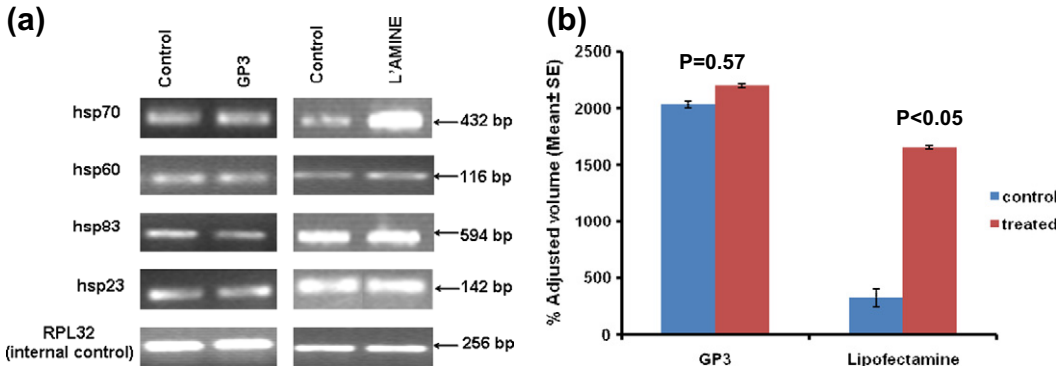


Fig. 4. (a) Transcript levels of different hsps in control and organisms exposed to GP3 and Lipofectamine™ (L'amine). Drosophila larvae were exposed individually to GP3 and Lipofectamine™ in 5% sucrose and hsp70, hsp23, hsp60 and hsp83 transcript levels were analyzed through RT-PCR. Drosophila larvae exposed to 5% sucrose alone formed the controls. RPL32 was used as an internal control for the RNA quality as well as quantity. (b) Densitometric analysis of transcript levels of hsp70 in control and organisms exposed to GP3 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.)

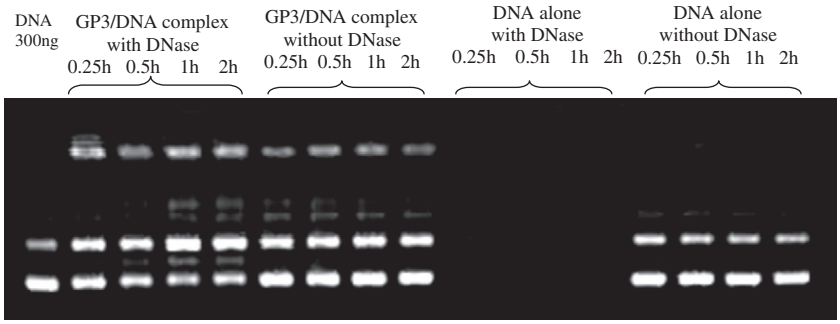


Fig. 5. DNase I protection assay. GP3/DNA nanoplex (N/P ratio 15) was treated with DNase I for different time intervals. The complexed DNA was released by treating the samples with heparin. The amount of DNA protected (%) after DNase treatment is calculated as the relative integrated densitometry values (IDV) quantified and normalized by that of pDNA values (untreated with DNase I) using Gel Documentation system (Syngene, UK).

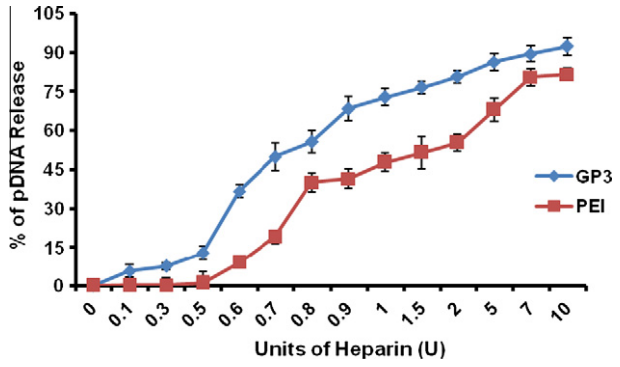


Fig. 6. DNA release assay of PEI and GP3 NCs. To a 20 μl of GP3/DNA nanoplex, heparin, in increasing concentration, was added and incubated for 20 min at 25 ± 1 °C. The samples were run on 0.8% agarose gel at 100 V for 45 min. Error bars represent ±standard deviation from the mean. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of DNA, whereas NCs are compact. In this context, due to polysaccharide blending, a certain amount of charge has been embedded inside the core of the NCs and is not available for interaction with pDNA. This may possibly explain an enhanced capability of GP3 over PEI towards the release of DNA following successful transfection with the former.

3.6. Interaction of GP3 with serum proteins

In addition to the above, several proteins especially serum proteins present in the body fluid may also affect the release of DNA due to their binding with the carrier systems [33]. To obviate this possibility, we tested the extent of GP3 binding with proteins by carrying out protein adsorption assay using BSA as a standard. In this context, earlier studies have reported that polysaccharides reduce the non-specific binding to proteins [34]. When compared with PEI, GP3 binding with serum was found to be ~50%, indicating a reduction in non-specific binding of GP3 to serum proteins, thereby a possibility of improved gene carrier activity of the NCs over PEI (Fig. 7). These observations further suggest that GP3 NC has a better potential as a gene carrier due to reduction in non-specific binding.

3.7. Transfection in transformed cells and primary mouse keratinocytes

Following our observation that GP NCs are non-cytotoxic both *in vitro* and *in vivo*, we carried out transfection studies in HeLa, HEK293 and HepG2 cells and also in primary mouse keratinocytes. *In vitro* transfection was carried out with GP NCs, PEI and selected commercial transfection agents in the presence or absence of serum. To keep the overall charge as positive for the DNA complex, a series of N/P ratio, viz., 7, 10, 15, 20, 25 and 30 were used. All the cell lines showed fairly high level of GFP gene expression for GP NCs and GenePORTER 2 in comparison with the rest (Fig. S2, see Supplementary material). Among all the NCs, the highest level of gene expression was observed with GP3. GP3/DNA complex enhanced the gene transfer efficacy by ~1.5–8.4 folds when compared to PEI (25 kDa, gold standard) and commercial transfection reagents in HEK293 cells in the absence of serum with a similar trend in HeLa and HepG2 cells also (Fig. 8a). However, for *in vivo*

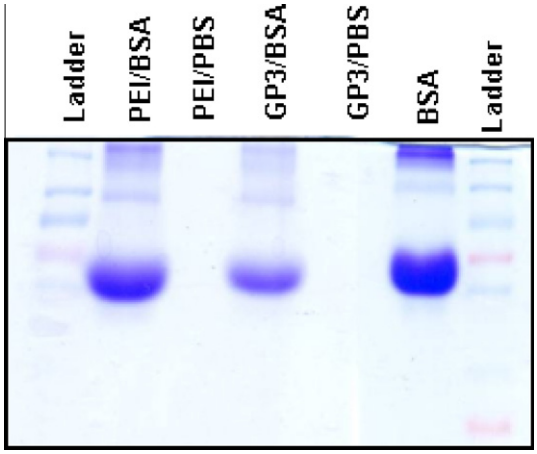


Fig. 7. Protein adsorption onto surface of GP3 NC compared to adsorption onto native PEI. Unbound BSA was removed by washing and centrifugation. Bound BSA was removed from the particles and run on a SDS–PAGE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

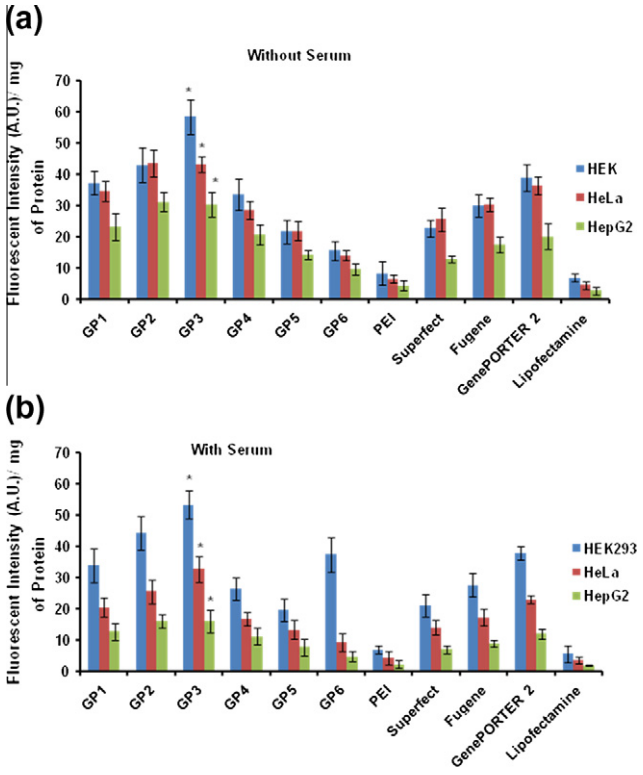


Fig. 8. GFP fluorescence intensity in HEK293, HeLa and HepG2 cells in (a) absence of serum and (b) presence of serum, transfected with GP NCs/DNA, PEI/DNA, Superfect™/DNA, Fugene™/DNA, GenePORTER 2™/DNA and Lipofectamine™/DNA complexes. The transfection profiles show fluorescence intensity expressed in terms of arbitrary units/mg of total cellular protein obtained at an N/P ratio of 15 and 10 for GP NCs/DNA and PEI/DNA polyplex, respectively. Cells were incubated with the complexes for 4 h, and the expression of GFP was monitored after 36 h. The fluorescent intensity of GFP fluorophore in the cell lysate was measured on spectrofluoremeter. The results represent the mean of three independent experiments performed in triplicates. $^{*}P < 0.05$ 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.)

transfection studies, serum remains a contributing factor due to its presence in circulatory fluid. Serum is also known to inhibit the transfection efficiency through binding with the cationic polymer/DNA complex and limiting its ability to deliver the specific gene [33]. Therefore, efforts were made to modulate the properties of cationic polymers to make them work efficiently in the presence of serum. Polysaccharides have been reported to reduce the non-specific interactions with the serum proteins, thereby increasing the transfection efficiency in *in vivo* conditions [34]. Interestingly, adding serum in the medium had little effect on the release of DNA from the GP NCs or on the transfection efficiency of GP/DNA complexes (Fig. 8b), indicating the efficient working of these NCs. We further demonstrated that degree of

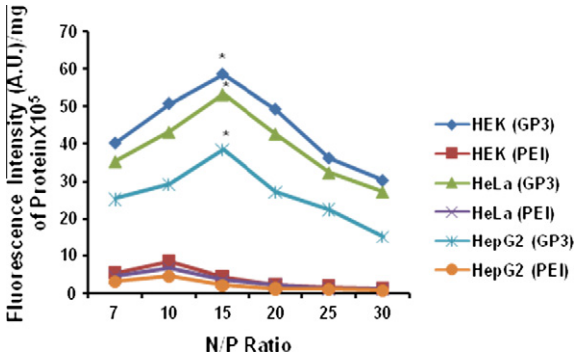


Fig. 9. Dose dependent GFP fluorescence intensity of GP3, and PEI/DNA complex, at the same N/P ratios in HEK293, HeLa and HepG2 cells. The transfection profiles show fluorescence intensity expressed in terms of arbitrary units/mg of total cellular protein. Cells were incubated with the complexes for 4 h, and the expression of GFP was monitored after 36 h. The fluorescent intensity of GFP fluorophore in the cell lysate was measured on spectrofluoremeter. The results represent the mean of three independent experiments performed in triplicates. $^{*}P < 0.05$ vs. PEI. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

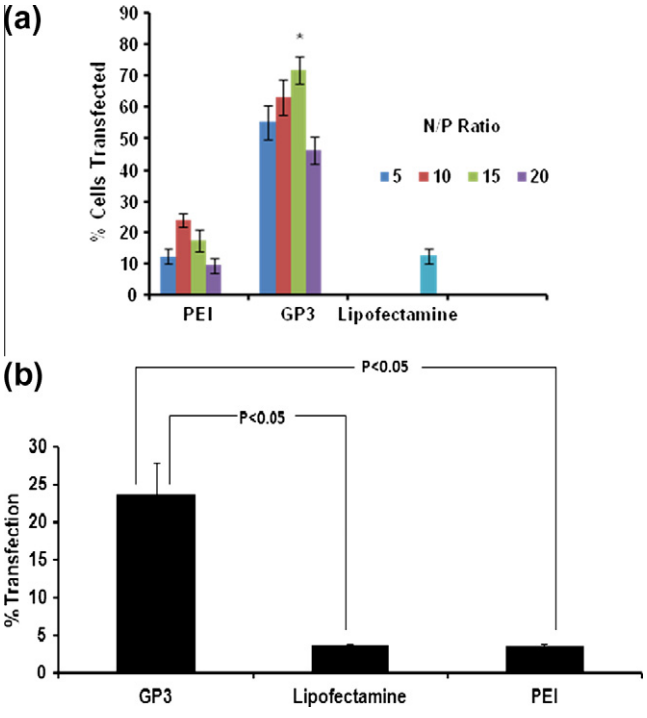


Fig. 10. (a) Percent transfection efficiency of GP3 NC/DNA complexes determined using FACS in HEK293 at various N/P ratios and compared with PEI and Lipofectamine™. (b) Percent transfection efficiency of GP3 NCs as determined using FACS in primary mouse keratinocytes and compared with Lipofectamine™ and PEI. $^{*}P < 0.05$ 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.)

polysaccharide blending and the N/P ratio of complexes affected the transfection efficiency, and it increased with increasing N/P ratio while showing a decrease beyond an optimal value (Fig. 9).

For quantitative measurement of GFP positive cells through transfection, we performed FACS analysis in GP3 NC and compared the same with that observed with PEI and Lipofectamine. Four different N/P ratios, viz., 5, 10, 15 and 20 each for GP3 NC and PEI were used, respectively. For PEI, a maximum transfection efficiency was observed at the N/P ratio of 10 (23.9 ± 2.4%), which decreased by ~5–10% at higher charge ratios, while the same in GP3 at N/P ratio of 15 was 72.0 ± 5.5% (Fig. 10a) which decreased on deviating from this N/P ratio on either side. Lipofectamine showed a transfection efficiency of only 12.4 ± 3.5%. All these results indicate that N/P ratio plays an important contributing role in determining the transfection efficiency, mediated through NCs. Concurrent with our transfection studies in transformed cells, we also performed transfection of EGFP gene using GP3, PEI and Lipofectamine in primary mouse keratinocytes. Transfecting the rapidly dividing cells is easier due to better permeability of the nuclear membrane during mitosis. Since primary cells divide slowly, transfection in these cells is a limiting step. In this study, a 23.6 ± 4.2% transfection by GP3 in comparison with that by PEI (3.5 ± 0.4%) and Lipofectamine (3.7 ± 0.1%), respectively, is indicative of one of the added advantages of the NC over the rest (Fig. 10b). It is tempting to speculate that such a transfection efficiency achieved by GP3 NC may be useful for its application in *in vivo* systems. This is more so as GP3 possesses a unique property of proton sponge that buffers the endosomal pH, induces continuous proton influx thus resulting into endosomal osmotic swelling and finally its rupture leading to release of DNA into the cytoplasm.

3.8. Intracellular localization of GP3/DNA complex

Once successful transfection is achieved, it is pertinent to track the path of cellular entry of a NC especially for its nuclear localization for its purposeful use in gene therapy. For intracellular localization of GP3/DNA complexes after transfection, HeLa cells following incubation with the above were examined by confocal laser-scanning microscopy. Both red and green fluorescence (red for GP3-TRITC and green for DNA-YOYO-1) were observed in the cytoplasm and nucleus of the cell within 1 and 2 h of addition of the complexes, respectively. DAPI staining (blue) was carried out for visualization of nucleus (Fig. 11). These observations clearly demonstrate the efficient intracellular delivery of DNA using GP3,

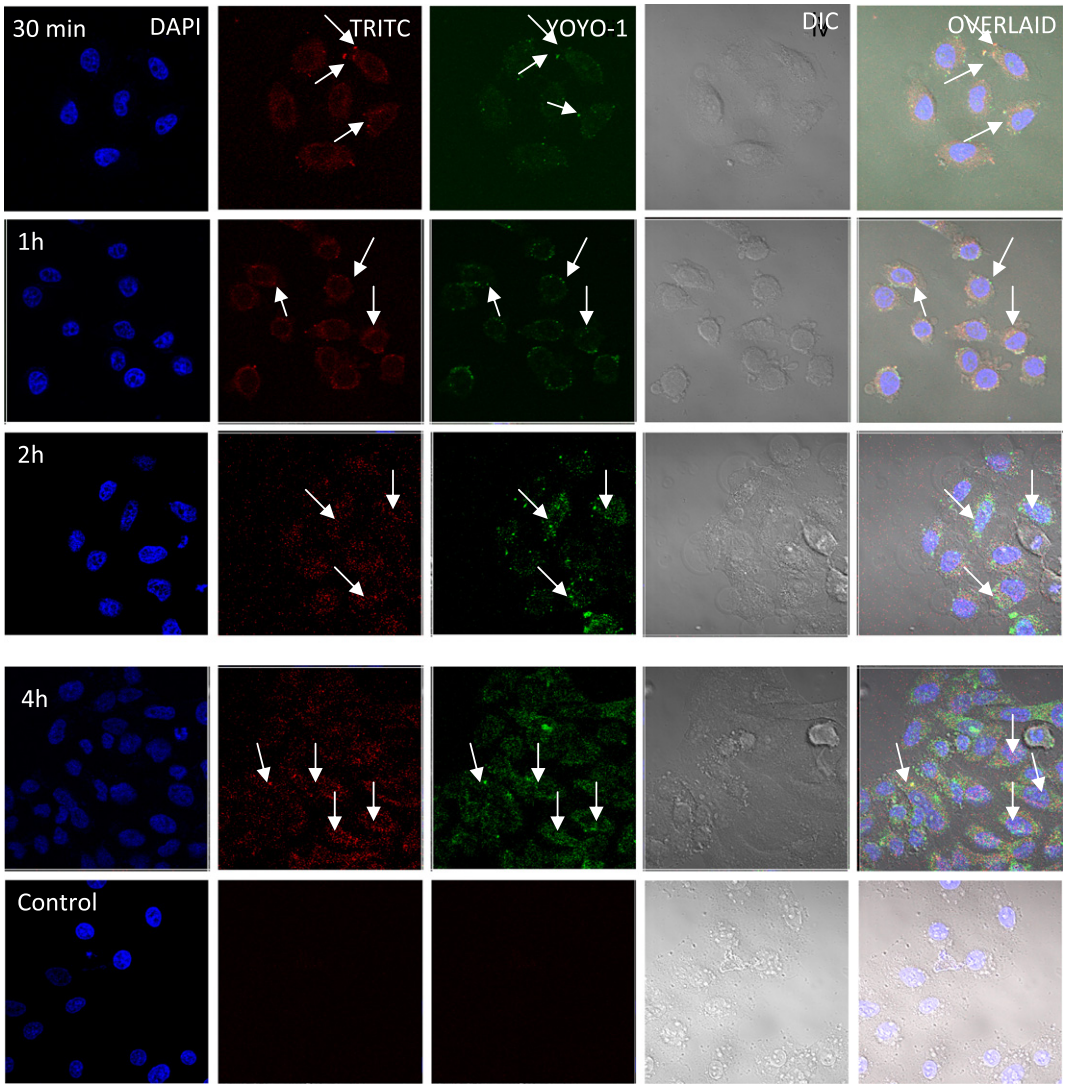


Fig. 11. Confocal microscopic images of HeLa cells treated with tetramethylrhodamine-GP3/YOYO-1-pDNA nanoplex at different time points. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

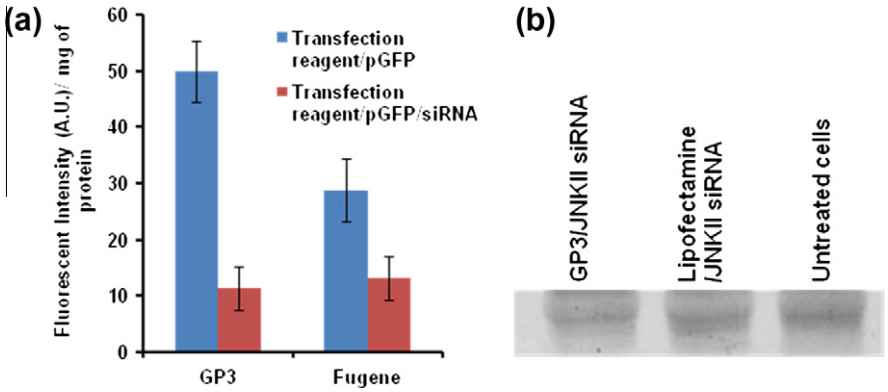


Fig. 12. (a) GP3 NC was checked for its ability to deliver siRNA in HEK293 cells by transfecting pEGFP DNA along with GFP specific siRNA. The expression of GFP in cells was reduced by more than 77% in comparison with Fugene™/pGFP DNA/siRNA, which suppressed GFP expression by only 54%, as monitored by measuring fluorescence on a spectrofluorometer. All the experiments were performed at least thrice and error bars represent the standard deviation. (b) Immunobloting assay for GP3 to see the JNKII knockdown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

which is in agreement with a previous study reporting that PEI delivers nucleic acids to the nucleus [35].

3.9. Delivery of siRNA using GP3

Concurrent with any successful transfection, it is important to demonstrate loss of function of the targeted gene through knockdown. In this context, RNA interference (RNAi) technology offers a supportive platform by which one can target a specific gene by using a siRNA for its suppression [36,37]. Therefore, we used GFP specific siRNA with an aim to knock down the expression of this particular gene in the cell. For this, we first transfected HEK293 cells with GP3–EGFP complexes and after 3 h, added GP3–siRNA. Post 36 h incubation, we observed ~77% knockdown of GFP expression using GP3/pDNA/siRNA as a carrier in comparison with a 54% knockdown by Fugene™/pDNA/siRNA complex (Fig. 12a). Further, to test the efficacy of the test system, we examined the knocking down of one of the major cellular stress kinases, JNK. We delivered JNK II siRNA with GP3 in HEK293 cells (as a model) and observed a 60% knockdown of JNK and a 55% knockdown by Lipofectamine as evidenced by Western hybridization (Fig. 12b). This further strengthens the utility of GP3 in having diverse applications for the delivery of different types of model vectors.

3.10. In vivo gene expression

To validate the *in vitro* transfection by GP3 and successful gene expression, it is pertinent to validate the same *in vivo*. Following intravenous injection of GP3 NC, naked DNA and PEI in Balb/c mice, luciferase gene expression was measured in these groups 3 days and 7 days post-injections. A non-significant yet increased luciferase gene expression was observed in the tested vital organs of the mice after 3 days (data not shown). Seven days post-injection, we observed a significant higher luciferase expression ($P < 0.05$) with GP3 NC when compared to PEI and naked DNA and maximum expression in spleen followed by heart and brain. An insignificant gene expression was observed with naked DNA group which could be due to the degradation of the DNA by the nucleases present in the blood [38]. We observed GP3 NC as the best delivery agent among the three, which may be due to the presence of polysaccharide moiety that prevents interactions with the blood serum proteins (see Section 3.6) thereby increasing the blood circulation time and resulting in more expression of the targeted gene. However, in other organs like lungs, liver and kidney, appreciable luciferase expression was not observed in any of these groups (Fig. 13). Previous studies on the biodistribution of polymers also support

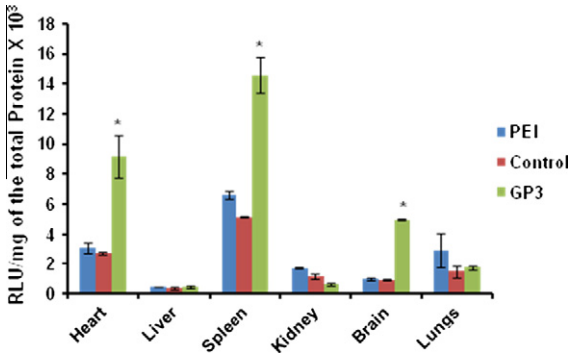


Fig. 13. *In vivo* gene expression analysis in Balb/c mice 7 days post-intravenous injection using pGL3 control vector as a reporter gene. The mice were sacrificed 7 days post-injection, and heart, liver, spleen, kidney, brain, and lungs were dissected out. The organs were homogenized in lysis buffer, and the lysate was read on luminometer to quantify the luciferase gene expression. $^{*}P < 0.05$ 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.)

our findings of observing maximum gene expression in the spleen [39]. Earlier studies reported gene expression dependency on size of the complexes [40]. Summarizing those studies, it is apparent that in most tissues, the endothelial barrier is tight, and only organs and tissues with an irregular fenestration, such as the liver, spleen, bone marrow, and certain tumors, have endothelia with large meshes, which allow extravasation of molecules up to 0.1–1 μm [41]. It is intriguing to note that we observed very low levels of reporter gene expression in the liver with GP3 NC and at present, we are unable to provide any plausible explanation for the same.

4. Conclusions

Taken together, the GG blended PEI NCs have improved transfection efficiency in comparison with PEI and the standard commercial transfection reagents in all the cell lines and also in primary mouse keratinocytes with negligible toxicity. Of the six GP-NCs, GP3 with optimum polysaccharide blending (~10.6%) shows the highest transfection efficiency. These NCs adequately protect the pDNA from enzymatic cleavage, reduce interaction with serum proteins and knockdown the GFP expression by ~77% using GFP specific siRNA and JNK (60%). GP3, when tested for *in vivo* cellular and organismal toxicity in *Drosophila*, showed non-significant induction of cellular stress in larvae and no

developmental adversities in larvae or adults. This further demonstrates its potential as a non-toxic gene delivery agent. *In vivo* gene expression studies in mice revealed maximum reporter gene expression in the spleen. Thus, GG blended PEI NCs hold great promise for future applications in gene delivery both *in vitro* and *in vivo*.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2011.01.009.

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