Chapter II
Materials and Methods
1. Sources of chemicals

Branched polyethylenimine (bPEI, 1.8 and 25 kDa), (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT), agarose, Tris, ethidium bromide, xylene cyanol, bromophenol blue, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), sodium cyanoborohydride, poly-L-glutamic acid (PGA, 13000 Da), N-(2-hydroxyethyl)-polyethylenimine (50kDa, 80% ethoxylated), tetramethylrhodamine isothiocyanate (TRITC), 4’,6-diamidino-2-phenylindole dilactate (DAPI), bovine serum albumin (BSA), deuterium oxide (D₂O), dextran (~40 kDa), sodium periodate, high retention dialysis tubing (12 kDa), polyethylene glycol diacid (600Da), paraformaldehyde, Dulbecco’s modified eagle’s medium (DMEM) were procured from Sigma Chemical Co. (USA). YOYO-1 for labeling pDNA was purchased from Invitrogen (USA). Commercial transfection agents, viz., GenePORTER 2™, Lipofectamine™, Superfect™ and Fugene™ were procured from Gelantis, (USA), Invitrogen (USA), Qiagen (France) and Roche Applied Science (USA), respectively. Alexa Fluor 488, Lysosensor Green DND-189 and Lysotracker Green DND-26 were purchased from Invitrogen (USA). Both the plasmid used in the study, viz., pEGFPN3 and luciferase plasmid (pGL3) control DNA, were procured from Sigma (USA). Rhodamine nucleic acid labeling kit was purchased from Mirus Bio (Madison, WI). Fetal calf serum (FCS) was purchased from Gibco-BRL-Life Technologies (UK). Plasmid purification kit was purchased from Qiagen (France). Chitosan was acquired from Fluka (Germany). Linear polyethylenimine (2.5 kDa) was obtained from Polysciences Inc. (USA). All other chemicals and reagents were procured locally.

2. Plasmid isolation

The plasmids, pEGFPN3, encoding for green fluorescent protein (GFP) and luciferase reporter plasmid (pGL3), were amplified and isolated using the following protocol:

3. Bacterial growth conditions

E. coli strain (DH5α) was grown in Luria Bertani (LB) medium supplemented with an antibiotic, ampicillin (100 µg/ml). The culture was grown with constant shaking at 200 rpm at 37 °C (Innova 4330, New Brunswick Scientific Co. Inc., Edison, USA).

4. Competent cell preparation

Competent cells of E.coli DH5α were prepared by the method of Cohen [1]. A single colony was inoculated in 5ml of LB medium and grown overnight at 37 °C, as primary inoculum. LB medium (100 ml) was inoculated as secondary inoculum i.e. 1% from the primary, and grown at 37 °C with constant shaking at 200 rpm. Once the absorbance (A₆₀₀) of
the medium reached ~ 0.4-0.6, the culture was centrifuged at 6000 rpm for 10 min at 4°C and the supernatant discarded. The pellet was resuspended gently in 5 ml of 0.1 M CaCl₂ solution (cold) and incubated for 20 min on ice, centrifuged at 6000 rpm (10 min, 4 °C). The supernatant was discarded and pellet re-suspended in 1.6 ml CaCl₂ (0.1M) + 10% glycerol solution. Aliquots of 50 μl were made and stored at -70 °C.

5. Transformation

Transformation was carried out following the method described by Mandel and Higa [2]. To a solution of competent cells (50 μl), DNA (100-200 ng) was added and incubated on ice for 30 min. Cells were then subjected to heat shock at 42 °C for 90 sec and incubated on ice (5 min). After incubation, 400-500 μl of LB medium was added to the cells and the sample was again kept on ice for 5 min, followed by incubation at 37 °C for 1 h with constant shaking at 200 rpm. Cells were centrifuged at 6000 rpm (5 min, at RT) and the supernatant discarded. The cells were resuspended in the remaining medium and plated on LB agar plates supplemented with ampicillin.

6. Preparation of plasmid DNA from E. coli transformants

6.1. Maxi preparation of DNA

Plasmid DNA was isolated on a large scale by using Qiagen Endofree Plasmid Maxi Kit. A single colony of recombinant E. coli was inoculated in 5 ml LB medium containing the antibiotic (ampicillin, 5 μl, 100 mg/ml) and the culture was grown for 8 h at 37 °C with constant shaking at 200 rpm. The primary culture was diluted to 5/500 in LB medium i.e. 500 ml LB medium was inoculated with 5 ml of the starter culture and the culture allowed to grow at 37 °C for 12-16 h with vigorous shaking at 300 rpm. Cells were harvested by centrifugation at 6000 x g at 4°C for 15 min in a SS-34 rotor (Kendro Laboratory Products, Newtown, Connecticut, USA). The bacterial pellet was homogenously suspended in 50 ml buffer P1 (50 mM Tris-Cl containing 10 mM EDTA and 100 μg/ml RNase A, pH 8.0) by pipetting up and down until no cell clumps remained. Then, 50 ml of buffer P2 (200 mM NaOH containing 1% SDS) was added and the solution mixed vigorously by inverting 4-6 times followed by incubation at RT for not more than 5 min. At the end of incubation period, 50 ml of chilled buffer P3 (3.0M potassium acetate, pH 5.5) was added and gently mixed by inverting 4-6 times. The lysate was poured into the barrel of QIA filter cartridge and incubated at RT for 10 min. The cell lysate was subsequently filtered and 12.5 ml of buffer ER added in order to remove endotoxins.
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Endotoxin-free ultrapure plasmid DNA
Isopropanol precipitate and resuspended DNA in endotoxin free resuspension buffer
Elute with QN buffer
Wash with QC buffer
Bind DNA
Incubate on ice for 30 min
Add endotoxin removal buffer
Clear lysates by filtration or centrifugation
Pelleted bacteria
Alkaline lysate

Figure 1. Schematic presentation of plasmid purification.

The solution was mixed by inverting the tube ~10 times and incubated on ice for 30 min. Meanwhile, a Qiagen-tip 500 was equilibrated by applying 10ml buffer QBT (750 mM NaCl containing 50 mM MOPS and 15% isopropanol, pH 7.0). The lysate containing ER buffer was allowed to enter the resin of equilibrated Qiagen-tip 500 by gravity flow. The resin containing the lysate was washed with buffer QC (1.25 M NaCl containing 50 mM Tris-
Cl and 15% isopropanol, pH 8.5) (3 x 80 ml). DNA was eluted with 35 ml of buffer QN (1.6 M NaCl containing 50 mM MOPS and 15% isopropanol, pH 7.0) in a 50 ml endotoxin-free tube. The DNA in elute was precipitated by adding isopropanol (24.5 ml), mixed and centrifuged immediately at 15,000 x g for 30 min at 4 °C. The supernatant was decanted carefully and the pellet was washed with 10 ml of endotoxin-free 70% ethanol. It was then centrifuged at 15,000 x g for 10 min. The supernatant was carefully decanted and pellet air dried. DNA was redissolved in 1 ml of endotoxin-free TE buffer (10 mM Tris containing 1 mM EDTA, pH 8.0). Yield and quality of plasmid DNA (pDNA) was determined by measuring the absorbance at 260 nm, taking the ratio of absorbance at 260/280 and quantitative analysis on an agarose gel.

7. Instruments

Following instruments were employed to carry out the proposed studies:

7.1 FTIR

Spectra of new compounds and nanocomposites / nanoparticles were recorded on 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.

7.2 \(^1\)H-NMR

\(^1\)H-NMR spectra were collected on Bruker 400 MHz specrophotometer. Deutrated solvents were used to dissolve the compounds and nanocomposites/nanoparticles. Chemical shifts (\(\delta\)) are presented in ppm.

8. Size and morphology of nanocomposites/nanoparticles and their DNA complexes

8.1. Zetasizer

The size of nanoparticles/nanocomposites and their DNA complexes, suspended in water and 10% serum separately, was determined as hydrodynamic diameter by dynamic light scattering (DLS) in triplicates using Zetasizer Nano-ZS (Malvern Instruments, U.K.). All the measurements were performed in automatic mode and presented as the average value of 20 runs, employing a nominal 5 mW He-Ne laser operating at 633 nm wavelength. The measurements were carried out at RT with the following settings: 14 measurements per sample; refractive index of water, 1.33; viscosity for water, 0.89 cP. The particle size reported in the present work is the average of three samples.

8.2. Transmission electron microscope (TEM)

The size and morphology of nanocomposites/nanoparticles were also examined by transmission electron microscope (TEM). It is a microscopic technique whereby a beam of
In the current study, 3 μl of sample solution (2 mg/ml in dd water) was put on a formvar (polyvinyl formal) coated copper grid and air-dried. The grid was then negatively stained with 2% phosphotungstic acid for few seconds. Samples were imaged with a Fei-Philips, Morgagni 268 D transmission electron microscope (USA).

### 8.3. Atomic force microscope (AFM)

Atomic force microscopy is a powerful tool allowing a variety of surfaces to be imaged and characterized at the atomic level. It provides a number of advantages over conventional microscopic techniques. AFM probe the sample and make measurements in three dimensions, viz., $x$, $y$, and $z$ (normal to the sample surface), thus enabling the presentation of three-dimensional images of a sample surface. They can be used in either an ambient or liquid environment. With these advantages, AFM has significantly impacted the fields of materials science, chemistry, biology, physics, and the specialized field of semiconductors. Microscope (PicoSPM System, Molecular Imaging, USA) was employed to characterize the morphology of nanocomposites with a force probe at nano-newton scale. Contact mode AFM is one of the most widely used scanning probe modes, and operates by rastering a sharp tip (made either of silicon or Si₃N₄ attached to a low spring constant cantilever) across the sample. An extremely low force is maintained on the cantilever, thereby pushing the tip against the sample as it rasters. Either the repulsive force between the tip and sample or the actual tip deflection is recorded relative to spatial variation and then converted into an analogue image of the sample surface. Imaging methods in AFM usually employs two general modes of operation, i.e. static mode or DC mode in which the static deflection of the cantilever is recorded and resonant or AC mode wherein the amplitude of the oscillation of the cantilever is kept at a set value while scanning the surface.

In the present study, the size and surface morphology of the nanocomposites/nanoparticles and their DNA complexes were examined by atomic force microscopy using PicoSPM System operating in MAC mode. A medium scanner (scan range
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30 μm x 30 μm) was used for imaging. A 225 μm long Au–Cr coated MAC cantilever with a force constant of 2.8 N/m and resonance frequency of ~ 65 kHz was used. The samples were imaged by depositing 2-3 μl (0.1 mg/ml) of samples solution on freshly untreated mica strip, dried for 5min at RT and imaged immediately. The AFM imaging of DNA complexes was also carried out, as described above. Prior to imaging, the nanocomposites/nanoparticles were incubated with DNA in 5% dextrose for 30 min. Particle size was estimated by an image analyzing software (SPIP) for scanning probe microscopy.

9. Zeta potential measurements

Zeta potential is physical property, which is exhibited by any particle in suspension and is a measure of the magnitude of the repulsion or attraction between particles. It is derived from the mobility distribution of a dispersion of charged particles when subjected to an electric field. Mobility is defined as the velocity of a particle per electric field unit and is measured by applying an electric field to the dispersion of particles and measuring their average velocity. Depending on the concentration of ions in the diluent, either the Smoluchowski (for high ionic strengths) or Huckel (for low ionic strengths) equations are used to obtain the zeta potential from the measured mobilities. Since zeta potential reflects the effective charge on particles, it is, therefore, related to the electrostatic repulsion between them. Moreover, surface charge is an important phenomenon to determine various characteristics such as the interaction of DNA with nanoparticles and also the complex of DNA-nanoparticles with the cell surface proteoglycans.

In the present study, zeta potential of nanocomposites/nanoparticles and their DNA complexes in water and 10% serum was estimated using Zetasizer Nano ZS. Each measurement was presented as an average value of 30 runs and was performed in triplicates and the average values are estimated by Smoluchowski approximation from the electrophoretic mobility. The Smoluchowski approximation was used to calculate zeta potential from the electrophoretic mobility.

10. DNA mobility shift assay

In order to estimate the amount of nanocomposites/nanoparticles required to completely neutralize the anionic charge of DNA, an agarose gel electrophoresis experiment was carried out. Binding of polycations to pDNA neutralizes the negative charge in the phosphate backbone of DNA, which retards its electrophoretic mobility. The location of DNA within the gel can be determined directly by visualizing the corresponding bands of DNA in gel after staining with low concentration of the fluorescent intercalating dye.
ethidium bromide. In the present work, complexes (pDNA-nanocomposites/nanoparticles) were formed at different w:w or N/P ratios. A known amount of DNA (0.3 μg) was mixed with various amounts of nanocomposites/nanoparticles in 5% dextrose and incubated for 30 min at RT. The complexes, thus formed, were mixed with loading buffer containing a tracking dye (bromophenol blue), loaded on a 0.8% agarose gel, electrophoresed at 100 V for 1 h in TAE (Tris-acetic acid-EDTA) buffer. The gels were stained with ethidium bromide and the bands corresponding to pDNA visualized on ultraviolet transilluminator.

11. DNA release assay

DNA release from the complexes is one of the important steps. DNA release assay was performed to examine the binding of nanoparticles and bPEI with plasmid DNA. All the nanoparticles were complexed with pDNA (0.3 µg/µl) w/w or N/P ratio, where these nanocomposites/nanoparticles-DNA complexes exhibited the highest transfection efficiency, and incubated for 30 min, as described in mobility shift assay. Subsequently, an aqueous solution of heparin (1 unit/µl), a strong anionic polymer, was added in increasing amounts in the above complex to release the bound pDNA. The samples were then incubated for 30 min and electrophoresed (100 V, 1 h) on a 0.8% agarose gel, stained with ethidium bromide and visualized on a UV transilluminator using a Gel Documentation System (Syngene, USA). The amount of DNA released from complexes after heparin treatment was estimated by densitometry.

12. DNase protection assay

The stability of the nanocomposites/nanoparticles DNA complexes toward digestion by nucleases was monitored using DNase I, assay as previously described [3]. Assay was carried out on 0.6 µg pDNA alone or nanoparticles/DNA complex in 25 µl (final volume). Each sample was divided into two aliquots of 10 µl each. The first aliquote was charged with 1 unit of DNase I (1 µl), while the second one was with 1 µl PBS and it served as control. Samples were incubated for 15, 30, 60 and 120 min at 37 °C. The enzyme was inactivated by adding 0.6 µl of 100 mM EDTA followed by heating to 75 °C for 10 min. Heparin (10 µl, 1 U/µl) was added to the samples and incubated (2 h, RT) to dissociate the complex and release DNA. After adding xylene cyanol, samples were electrophoresed (100 V, 1 h) in 0.8% agarose gel, stained as described earlier and photographed on Alpha Imager (Alpha Innotech, San Leonardo, CA). The amount of DNA protected (%) after DNase I treatment was calculated as the relative integrated densitometry values (IDV), quantified and normalized by that of pDNA values (not treated with DNase I) using AlphaEaseFC software.
13. Cell cultures

HEK293 (human embryonic kidney), HeLa (human cervical cancer), HepG2 (human hepatocellular liver carcinoma) and CHO (Chinese hamster ovary) cells were maintained in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 50 μg/ml gentamycin. The cells were cultured in a humidified 5% CO₂ atmosphere at 37 °C.

14. Cell viability assay

MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability is observed. The DNA complexes of nanocomposites/nanoparticles were assessed for in vitro toxicity in mammalian cells using MTT colorimetric assay [4,5]. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay is a laboratory test and a standard colorimetric assay for measuring cellular growth. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials. Yellow MTT is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active and, therefore, conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan, produced by cells treated with an agent, is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing cell death can be deduced. A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent, sodium dodecyl sulfate in dilute hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at 540 nm.

Cell viability was carried out by the MTT assay on HEK293, CHO, HepG2 and HeLa cells. Cells were transfected, as described in in vitro transfection studies and 36h post-transfection, MTT solution (200 μl, 0.5 mg/ml in serum free DMEM) was added to each well followed by incubation for 3 h in a humidified atmosphere of 5% CO₂ at 37 °C. The yellow tetrazolium salt was found to be reduced to a blue-colored formazan in viable cells.
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Subsequently, the medium was aspirated and the cells were washed with 1x PBS (2 x 100 μl) followed by the addition of isopropanol (200 μl) containing 0.04 M HCl and 0.5% SDS to each of the wells for 30 min to dissolve the formazan crystals. Aliquots were drawn from each well and the intensity of color was measured spectrophotometrically in an ELISA plate reader at 540 nm. Untreated cells were taken as control with 100% viability and wells with MTT reagent without cells were used as blank to calibrate the spectrophotometer to zero absorbance. The relative cell viability (%) compared to control cells was calculated by $\frac{\text{abs}_{\text{sample}}}{\text{abs}_{\text{control}}} \times 100$. All the experiments were carried out in triplicates.

15. In vitro transfection studies

For monitoring the in vitro gene expression in mammalian cells, green fluorescent protein encoding pDNA was transfected into the cells. In cell and molecular biology, the green fluorescent protein (GFP) gene is frequently used as a reporter of expression [6].

In the present work, mammalian cells were seeded in a 96-well plate at a density of ~$10^4$ cells/well depending on the cell type and incubated for 16 h for adherence. Subsequently, after that transfection was carried out at 70-75% confluence. Before addition of nanocomposites/DNA complexes at various w:w or N/P ratios in DMEM (total volume 80 μl), the cells were washed with 1 x PBS (1 x 100 μl) and the culture medium was changed with 60 μl DMEM (without serum). Similarly, pDNA complexes were made with Superfect™ (Qiagen, France), Fugene™ (Roche Applied Science, USA), GenePORTER 2™ (Genlantis, USA) and Lipofectamine™ (Invitrogen, USA) as well following manufacturers’ protocols and gently added to cells followed by incubation at 37 °C in humidified 5% CO₂ atmosphere. Likewise, in another set, DNA complexes were made and diluted with 10% serum containing growth medium to observe the effect of serum on transfection efficiency. After 4 h, the transfection medium was replaced by 200 μl fresh 10% FBS containing growth medium and thereafter, cells were further incubated for 36 h after which they were observed in fluorescent microscope to see GFP expression in the cells. GFP reporter gene expression was observed under Nikon Eclipse TE 2000-S inverted microscope (Kanagwa, Japan) fitted with C-F1 epifluorescence filter block B-2A consisting of an excitation filter, Ex 450-490 nm, Dichroic mirror, DM 505 and barrier filter, BA 520.

16. In vitro siRNA delivery

16.1. Synthesis of GFP specific siRNA

The following oligonucleotide sequences were synthesized using the standard phosphoramidite chemistry and purified on RP-HPLC [7]:
T7 primer: d (TAA TAC GAC TCA CTA TAG)
GFP sense: d (ATG AAC TTC AGG GTC AGC TTG CTA TAG TGA GTC GTA TTA)
GFP antisense: d (CGG CAA GCT GAC CCT GAA GTT CTA TAG TGA GTC GTA TTA)

After annealing T7 primer sequence to GFP sense or antisense oligonucleotides, complementary strands were synthesized by T7 RNA polymerase. The sense and antisense RNA strands were annealed and double stranded siRNA was used for transfection.

16.2. In vitro delivery of GFP specific siRNA

siRNA delivery efficiency was estimated by transfecting pEGFP (1 µl, 1.4 nM) with nanocomposites/nanoparticles as above and left for 4 h in the CO₂ incubator. After 4 h, medium was replaced with fresh incomplete DMEM and GFP specific siRNA (2 µl, 2.5 µM) complexed with nanocomposites/nanoparticles in a 20 µl reaction mixture was added onto the cells. After 4h of incubation with the siRNA, the medium was again replaced with 10% serum containing medium and cells were further incubated for 36 h. Nanocomposite/nanoparticle-DNA complex alone served as control. Similarly Fugene™/DNA and Fugene™/siRNA complexes were also prepared and added onto the HEK293 cells in a 96-well plate. After 36 h, the GFP expression was quantified using nanodrop. All the experiments were performed in triplicate.

17. Evaluation of EGFP expression

The fluorescence intensity was measured to quantify GFP expression in the transfected mammalian cells. Total EGFP expression in the cell lysates was quantified (λ_ex: 488nm; λ_em: 509 nm) spectrofluorometrically using NanoDrop™ (ND-3300, USA). Briefly, after 36 h of transfection, cells were washed with 1x PBS (2 x 100 µl, pH 7.4), lysed with 50 µl lysis buffer (10 mM Tris, 1mM EDTA and 0.5% SDS, pH 7.4) and agitated for 20 min at 37 °C. In a 2 µl cell lysate, EGFP was estimated spectrofluorometrically and corrected for background and auto-fluorescence in untreated cells. The values obtained from nanodrop were normalized using Bradford reagent with BSA (Bangalore Genei, India) as standard and the total protein content was estimated for each well. The level of fluorescence intensity of GFP was calculated by subtracting the background values and normalized against protein concentration in cell extract. The values are reported as arbitrary unit (AU)/mg of cellular protein and represents mean ± standard deviation for samples taken in triplicate.

18. Fluorescence activated cell sorting (FACS) analysis

Quantitative transfection efficiency was performed by flow cytometry, 36 h post-transfection. Briefly, 1x 10⁶ HEK293 cells/well were seeded in 24-well plates one day prior to transfection. Nanocomposites/nanoparticles and PEI were complexed with pDNA (1.5 µg) at different w/w or N/P ratios and incubated for 30 min at RT, diluted with 300 µl serum free
medium and added onto the cells. Similarly, pDNA complexes were prepared with the commercial transfection reagent following manufacturers’ protocols and added onto the cells. After 4 h, the transfection medium was replaced by fresh DMEM containing 10% FBS (1 ml) and cells were additionally incubated for 36 h. The transfected cells were washed once with 1 x PBS (1 ml) and harvested from each well by the trypsin-EDTA (0.25%) treatment. Post-centrifugation, cell pellet was washed with 1 x PBS (2 x 500 µl), fixed with 4% paraformaldehyde (1 x 500 µl), incubated for 10 min at RT, again washed with 1 x PBS (2 x 500 µl) and finally, the cells were resuspended in 1 x PBS (1 ml). Transfection efficiency was evaluated as the percentage of cells expressing GFP using Guava® EasyCyte™ Plus System, USA subtracting the background of untreated cells and by determining the statistics of cells fluorescing above the control level, where non-transfected cells were used as the control. Five thousand cells/samples were analyzed and transfection was carried out in the three independent experiments.

19. Cellular trafficking by confocal microscopy

The intracellular trafficking of nanocomposites/nanoparticles and their DNA complexes in culture cells was studied by confocal microscope at different time intervals. Confocal microscopy is an optical imaging technique used to increase micrograph contrast and/or to reconstruct three-dimensional images by using a spatial pinhole to eliminate out-of-focus light or flare in specimens that are thicker than the focal plane [8]. Confocal microscope has the capability of isolating and collecting a plane of focus from within a sample, thus eliminating the out of focus "haze" normally seen with a fluorescent sample. Fine detail is often obscured by the haze and cannot be detected in a non-confocal fluorescent microscope. Confocal microscope has a stepper motor attached to the fine focus, enabling the collection of a series of images through a three dimensional object. These images can then be used for a two or three dimensional reconstruction. Double and triple labels can be collected with a confocal microscope. Since these images are collected from an optical plane within the sample, precise localizations can be performed.

For confocal studies, the nanocomposites/nanoparticles were either labeled with FITC or TRITC. To a 1 ml suspension of nanoparticles (10 mg/ml), 100 µl FITC/TRITC (5 mg) dissolved in DMF (500 µl) was added and reaction stirred overnight. Subsequently, the solution was concentrated in a rotavapor and the labeled sample, thus obtained, washed with ethyl acetate in order to remove unreacted FITC/TRITC. pDNA was labeled with YOYO-1 [9] and complexed with labeled nanocomposites/nanoparticles. Labeled nanocomposites/nanoparticles or DNA complexes were administered to cells to monitor the entry of labeled samples into the nucleus. Cells were grown in DMEM supplemented with 10% FBS
overnight to ~70% confluency on circular glass coverslips in 6-well microplates. Cells were incubated with a suspension of dual labeled nanocomposite/nanoparticle-DNA complexes at 37 °C for different time point. Media was aspirated and plates washed three times with sterile 1x PBS (3x 1 ml). At predetermined time intervals, cells were fixed with 4% (w/v) paraformaldehyde in 1x PBS for 10 min at RT and washed four times with 1x PBS (4x 1 ml). Individual coverslips were stained with DAPI and then mounted on clean glass slides with fluorescence-free glycerol-based mounting medium (UltraCruz™ Mounting Medium, Santa Cruz Biotechnology, USA).

20. Animals

Six to seven weeks old male Swiss albino 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 laid down guidelines of Institutional Animal Ethics Committee.

21. In vivo gene expression

Nanocomposites/nanoparticles with 25 µg pGL-3 control vector were prepared at optimal N/P or w/w ratio for in vivo gene expression. Likewise, PEI-pGL3 complex was prepared and pGL3 alone was used as control. A 100 µl aliquots of complexes and naked DNA using normal saline solution was injected intravenously through mice tail vein using syringe of 40 U (insulin syringe) with needle size of 0.3 x 8 mm. At predetermined time periods after injection, the mice were sacrificed and liver, spleen, kidney, lung, heart and brain were excised and rinsed with chilled normal saline, weighed and made 25% w/v homogenate in lysis buffer (Promega). After three cycles of freezing and thawing, the homogenates were centrifuged at 10,000 x g for 10 min at 4°C and 100 µl supernatant was used to measure luciferase activity. Luciferase activity was represented as RLU /gram of tissue. All the experiments were carried out in duplicate.

22. 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.
REFERENCES