4.1 Synthesis of Polymer Conjugates

4.1.1 Solubility Studies of Polymers

In brief, approx. 50 mg quantity of material i.e. reactant (PEI2, PEI25, PAA15 or ChA) was taken in a test tube and vortexed for 5 min with 10 ml of different solvents. The test tubes were then kept at room temperature and observed after the period of 24 h. A visual solubility observation was carried out. This was necessary to figure out solvents for reaction and choosing method for separation of unreacted reactants, byproducts and polymer conjugates before proceeding to actual reactions. Table 4.1 shows the solubility data for reactants (PEI2, PEI25, PAA15 or ChA) used for polymer conjugate synthesis. The PEI2 (50% aqueous solution) was lyophilized in ModulyoD Lyophilizer (Thermo Scientific, USA) whereas PEI25 and PAA15 were used as received for solubility studies.

Table 4.1: Solubility studies for polymers (PEI2, PEI25 & PAA15 and ChA) used for polymer conjugate synthesis.

S. No.	Material	Solvents									
		Acetonitrile	**Ethyl acetate**	**MeOH**	**CHCl3**	**Water**	**Water: MeOH**	**THF**	**THF: MeOH**	**CHCl3: MeOH**	**DMF**
1	PEI2	.....	Insoluble	Soluble	Soluble	Soluble	.....	Insoluble	Soluble	Soluble	Insoluble
2	PEI25	.....	Insoluble	Soluble	Soluble	Soluble	.....	Insoluble	Soluble	Soluble	Insoluble
3	PAA15	Soluble	.....	Insoluble	Insoluble	Soluble	Soluble	Insoluble	.....	Insoluble	Soluble
4	ChA	Insoluble	Insoluble	Soluble	partially soluble	partially soluble	Soluble	Soluble	.....	Soluble	Soluble

4.1.2 Synthesis of PEI2 and PEI25 Conjugate Series

The conjugation of cholic acid (ChA) to PEI2 or PEI25 was achieved by covalent coupling via the N, N'-dicyclohexylcarbodiimide (DCC) chemistry (Han S. *et a*l-2001 and Hermanson G.T.-2008). In order to obtain conjugates with different substitution ratios, different feed ratios (i.e., lipid to polymer mol ratios) were employed for the synthesis (Wang D. *et al*-2002). A typical example for reaction with polymer:lipid feed ratio 1 is briefly described as follows.

A solution of 4 mL of ChA (40.85 mg, 0.1 mmol) in THF was taken in a round-bottom flask (ca.25 ml) and to this solution 2 mL of NHS (13.80 mg, 0.12 mmol) solution in THF was added under continuous stirring. After 5 minutes, 2 mL of DCC (24.74 mg, 0.12 mmol) solution in THF was added and allowed to react for 12 h at 4˚C with continuous stirring at moderate speed. This activated lipid solution was filtered and filtrate was added to the round-bottom flask containing 8 mL of polymer (200 mg, 0.1 mmol for PEI2) solution in $CHCl_3$. Reaction was continued at room temperature for 8 h with continuous stirring at moderate speed. Reaction mixture was concentrated to approximately half of its volume using rotary evaporator and the polymers were precipitated by drop-wise addition of excess quantity (400 mL) of diethyl ether with continuous stirring. The precipitate was washed two times with excess quantity (400 mL) of diethyl ether to remove impurities (unreacted ChA, DCC and NHS). Product was filtered and dried under vacuum for 24 h to remove traces of solvent. Table 4.2 and 4.3 shows actual quantities of reactants taken for synthesis of polymer conjugates of PEI2 and PEI25, respectively. Synthesized polymer conjugates were named with respect to their molecular weight and theoretical feed ratios. First three alphabets are for the type of polymer followed by a number that represents molecular weight of the polymer conjugated, ChA denotes the cholic acid and number in the parenthesis denotes the theoretical molar substitution ratio of lipid:polymer.

Table 4.2: Reactants and their quantities used for synthesis of polymer conjugates of PEI 2 kDa. Values in the bracket represents theoretical feed ratio chosen for actual reaction.

Sr. No.	Name of Polymer Conjugate	Polymer qty (mg)	Cholic acid qty (mg)	NHS qty (mg)	DCC qty (mg)
1	PEI2-ChA (0.2)	200 (0.1mmol)	8.16 (0.02 mmol)	2.76 (0.024 mmol)	4.94 (0.024 mmol)
2	PEI2-ChA (1)	200 (0.1mmol)	40.8 (0.1 mmol)	13.8 (0.12 mmol)	24.7 (0.12 mmol)
3	PEI2-ChA (2)	200 (0.1mmol)	81.7 (0.2 mmol)	27.6 (0.24 mmol)	49.48 (0.24 mmol)
4	PEI2-ChA (4)	200 (0.1mmol)	163.2 (0.4 mmol)	55.2 (0.48 mmol)	98.8 (0.48 mmol)

Table 4.3: Reactants and their quantities used for synthesis of polymer conjugates of PEI 25 kDa. Values in the bracket represents theoretical feed ratio chosen for actual reaction.

Sr. No.	Name of Polymer Conjugate	Polymer qty (mg)	Cholic acid qty (mg)	NHS qty (mg)	DCC qty (mg)
1	PEI25-ChA (0.2)	250 (0.01mmol)	0.817 (0.002 mmol)	0.276 (0.0024 mmol)	0.494 (0.0024 mol)
2	PEI25-ChA (2)	250 (0.01mmol)	8.16 (0.02 mmol)	2.76 (0.024 mmol)	4.94 (0.024 mmol)
3	PEI25-ChA (4)	250 (0.01mmol)	16.34 (0.04 mmol)	5.52 (0.048 mmol)	9.90 (0.048 mmol)
4	PEI25-ChA (16)	250 (0.01mmol)	65.36 (0.16 mmol)	22.08 (0.19 mmol)	39.60 (0.19 mmol)

4.1.3 Synthesis of PAA Conjugate Series

The ChA conjugation to polyallylamine 15 kDa (PAA15) was achieved by covalent coupling via the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) chemistry (Han S. *et a*l-2001 and Hermanson G.T.-2008). In order to obtain conjugates with different substitution ratios, different feed ratios (i.e., lipid to polymer mol ratios) were employed for

the synthesis (Wang D. *et al*-2002). Table number 8 shows actual quantities of reactants taken for synthesis of polymer conjugates of PAA15. A typical example for reaction with polymer:lipid feed ratio 5 is briefly described as follows:

A solution of 2.5 mL of NHS (2.76 mg, 0.024 mmol) in water: methanol (50:50) was added to 10 ml of ChA (8.17 mg, 0.02 mmol) solution in water:methanol (50:50) in a round-bottom flask containing under continuous stirring. Then, 2.5 mL of EDC (3.724mg, 0.024 mmol) in water: methanol was added and allowed to react at RT for 24 h with continuous stirring. After filtering, the activated lipid solution was added to 15 mL of PAA15 (60mg, 0.004 mmol) in the same solvent and allowed to react at room temperature for 24 h with continuous stirring. The reaction mixture was dialyzed (MWCO = 3,500; 8-h dialysis in each step) against 20% methanol in water first, followed by 5% methanol in water and then pure water, to remove unreacted species. The products were transferred in plastic tubes and freeze dried in ModulyoD Lyophilizer (Thermo Scientific, USA). Table 4.4 shows actual quantities of reactants taken for synthesis of polymer conjugates.

Table 4.4: Reactants and their quantities used for synthesis of polymer conjugates of PAA 15 kDa. Values in the bracket represents theoretical feed ratio chosen for actual reaction.

Sr. No.	**Name of Polymer Conjugate**	**Polymer qty (mg)**	**Cholic acid qty (mg)**	**NHS qty (mg)**	**EDC qty (mg)**
1	PAA15-ChA(1.25)	60 mg (0.004 mmol)	2.042 (0.005 mmol)	0.690 (0.006 mmol)	0.931 (0.006 mmol)
2	PAA 15K-ChA (2.5)	60 mg (0.004 mmol)	4.085 (0.01 mmol)	1.380 (0.012 mmol)	1.862 (0.012 mmol)
3	PAA 15K-ChA (5)	60 mg (0.004 mmol)	8.17 (0.02 mmol)	2.76 (0.024 mmol)	3.724 (0.024 mmol)
4	PAA 15K-ChA (10)	60 mg (0.004 mmol)	16.34 (0.04 mmol)	5.52 (0.048 mmol)	7.44 (0.048 mmol)

Synthesized polymer conjugates were named with respect to their molecular weight and theoretical feed ratios. First three alphabets are for the polymer followed by a number that

represents molecular weight of the polymer conjugated, ChA denotes the cholic acid and number in the parenthesis denotes the theoretical molar substitution ratio of lipid:polymer.

4.2 Characterization of Polymer Conjugates

4.2.1 Structural Analysis by NMR

The NMR spectra were recorded using Bruker Advance-II 400 MHz spectrometer in CDCl3 or DMSO solvents and expressed as δ ppm (coupling constant (J) has been expressed in Hz). Along with the NMR spectra for polymer conjugates, the NMR spectra for cholic acid (MW: 408.5714 g/mol) and unmodified polymers (PEI2, PEI25 & PAA15) were also obtained in CDCl3 (dutereated chloroform) or DMSO and expressed as δ ppm.

4.2.2 pDNA Binding to Polymer Conjugates

4.2.2.1 SYBR Green I Binding Assay

DNA binding capacity of the conjugates was assessed by SYBR Green I binding to plasmid DNA. SYBR Green I is an asymmetrical cyanine dye (Zipper H. et al-2004) used as a nucleic acid stain in molecular biology. SYBR Green I binds to double stranded DNA and the resulting DNA-dye-complex absorbs blue light (λmax = 497 nm) and emits green light (λmax = 520 nm). The stain preferentially binds to double-stranded DNA, but will stain single-stranded DNA with lower performance. The detection of nucleic acids by fluorescent dyes such as SYBR Green I has become increasingly important for a variety of analytic and diagnostic applications. The wide spectrum of applications of BR Green I and its overall convincing performance are due to its excellent properties. These include favorable photophysical properties, temperature stability, selectivity for dsDNA and high sensitivity (Vitzthum F. and Bernhagen J.-2002) Furthermore, SG assays for the quantification of dsDNA in solution can be optimized to result in easy, robust and reliable one-step procedures that display a dynamic linear range of up to four orders of magnitude using a single dye solution (Zipper H. et al-2003).

Briefly, 2 μL of gWIZ-GFP (a reporter plasmid encoding for green fluorescent protein) solution (0.025 μg/ μL) in 150 mM NaCl was placed in polypropylene tubes (in duplicate) containing HEPES buffered saline (pH 7). The polymer solution was prepared at two different concentrations (1X-0.625 μg/mL and 10X - 6.25 μg/ mL) in nuclease-free water so

that ionic strength of the resulting solution does not change significantly. 2 µL to 16 µL of either 1X or 10X (based on weight ratio calculation) was added to the tubes to give polymer:plasmid ratios ranging from 0.025 to 2. The final volume of the solutions was brought to 100 µL in all tubes. After 30 min of incubation at room temperature, 200 μL of 1 X SYBR Green I was added to the tubes and 300 μL of each sample was read on a 96-well plate (λ_{EX}, 485 nm; λ_{EM}, 527 nm) to quantify the amount of free DNA. The calculation of polymer or conjugates,, plasmid DNA and SYBR Green dye I as per volume and weight basis for pDNA binding studies by SYBR Green I assay is shown in table 4.5. The sigmoidal binding curves were generated by plotting %bound DNA vs polymer: plasmid ratio and the BC_{50} values were calculated, corresponding to 50% of the plasmid binding from the sigmoidal binding curves.

Table 4.5: Calculation of polymer, conjugates, plasmid DNA and SYBR Green dye I as per volume and weight basis for pDNA binding studies by SYBR Green I assay

Group	Weight basis			Volume basis			Nano plex (µl)	SYBR Green I (µl)	Total (µl)
	pDNA (µg)	Polymer /conjug ate (µg)	Polymer or conjugates: pDNA wt ratio	pDNA (µl)	Polymer /conjug ate (µl)	HEPES buffered saline pH 7.0			
A	0.05	0	0	2	0	98	100	200	300
B	0.05	0.00125	0.025	2	2	96	100	200	300
C	0.05	0.0025	0.05	2	4	94	100	200	300
D	0.05	0.0125	0.25	2	2	96	100	200	300
E	0.05	0.025	0.5	2	4	94	100	200	300
F	0.05	0.05	1	2	8	90	100	200	300
G	0.05	0.075	1.5	2	12	86	100	200	300
H	0.05	0.1	2	2	16	82	100	200	300

4.2.2.2 Electrophoretic Mobility Shift Assay (EMSA)

The ability of the conjugates to condense the plasmid DNA was evaluated by the EMSA (Neamnark A. et al-2009). The nanoplexes of pDNA with polymer (PEI2, PEI25 & PAA15) or conjugates of these polymer were prepared by adding 5 µL of plasmid DNA (gWIZ-GFP,

0.04 µg/µL) to polypropylene tubes containing HEPES buffered saline (pH 7). Different concentrations of polymer (from 1X-0.01 µg/µL and 10X-0.1 µg/µL dilutions) were added to tubes to obtain different polymer/plasmid weight ratios ranging from 0 to 1 (final solution volume: 35 µL). Table 4.6 shows the calculation of polymer or conjugates, plasmid DNA and gel loading dye as per volume basis for pDNA binding studies by EMSA. The nanoplexes (polymer/plasmid DNA complexes) were loaded into 0.8% agarose gel containing 1 µg/mL of ethidium bromide in Tris-Acetate/EDTA (x1) buffer, the agarose gel was run at 135 V for ~30 min, and the DNA bands were visualized under UV light (Alpha Innotech, Canada). A lane containing free plasmid DNA was run as control (i.e. no binding).

Table 4.6: Calculation of polymer or conjugates, plasmid DNA and gel loading dye as per volume basis for pDNA binding studies by EMSA

S. No.	Polymer or conjugates (µg)	pDNA (µg)	HEPES buffered saline (pH 7), µl	Gel loading dye (µl)	Total volume (µl)	Polymer or conjugates: pDNA wt ratio
1	0	0.2	25	5	35	0
2	0.0015	0.2	23.5	5	35	0.075
3	0.03	0.2	22	5	35	0.15
4	0.04	0.2	21	5	35	0.2
5	0.1	0.2	15	5	35	0.5
6	0.14	0.2	11	5	35	0.7
7	0.2	0.2	23	5	35	1

4.2.3 DNase I Protection Assay

Protection of plasmid DNA by unmodified polymers or their conjugates against treatment of DNase I for 1 hr was evaluated (Du Y. et al-2011). Table 4.7 shows the calculation of polymer, plasmid DNA and gel loading dye as per volume basis for DNase I protection assay. Polyplexes were prepared at various weight ratios (polymer/plasmid weight ratio of 1 to 10) and exposed to 1 µl of DNase I (1000 Kunitz/ml, in DNase buffer) for 1 hr at 37˚C. Activity of DNase I was stopped by incubating the polyplexes with 5 µl of ethylenediaminetetraacetic acid (EDTA) solution. To dissociate the DNA from polyplexes,

10 µl of heparin (5 mg/ml) was added to each tube and further incubated for 2 hr. Then samples were run on 0.8% agarose gel to observe the availability and integrity of protected plasmid DNA.

Table 4.7: Calculation of polymer or conjugates, plasmid DNA and gel loading dye for DNase I protection assay

S. No.	Polymer or conjugates (µg)	pDNA (µg)	HEPES buffered saline (pH 7), µl	Gel loading dye (µl)	Total	Polymer or conjugates: pDNA wt ratio
1	0	0.2	13	2	20	0
2	0.2	0.2	11	2	20	1
3	0.4	0.2	9	2	20	2
4	1	0.2	3	2	20	5
5	2	0.2	11	2	20	10

4.2.4 Heparin Challenge/Dissociation Assay

To determine the extent of polymer dissociation from polyplexes, the polymers/plasmid DNA complexes were challenged with heparin in the presence of serum proteins (Vader P. et al-2012 and Naik R. J. et al-2011). Briefly, polyplexes were prepared in 150 mM HEPES buffered saline using 0.4 µg of gWIZ-GFP and 1.6 µg of polymers at polymer:plasmid weight ratio of 4:1. Table 4.8 shows calculation of quantity of polymer conjugate, plasmid DNA and heparin used for preparation of nanoplexes in 150 mM HEPES buffered saline and table 4.9 for polyplexes in HEPES buffered saline with cell culture medium. To mimic transfection conditions, 10 µl of DMEM with 10% serum was added to polyplexes to give 5% final serum concentration (total volume: 20 µL). 5 µL of heparin sulfate was added to the polyplexes to give final heparin concentrations of 0.01 to 1 µg/µL. After incubating the complexes for 30 min at 37˚C, the samples were loaded on 0.8% agarose gel and analyzed by EMSA.

Table 4.8: Calculation of quantity of polymer conjugate, plasmid DNA and heparin used for preparation of nanoplexes in 150 mM HEPES buffered saline for anion challenge assay

S. No.	Polymer or polymer conjugate (µg)	Actual weight of pDNA (µg)	Heparin (µg)	HEPES buffered saline (pH 7), µl
1	4	0.4	0.01	30
2	4	0.4	0.05	30
3	4	0.4	0.1	30
4	4	0.4	0.25	30
5	4	0.4	0.5	30

Table 4.9: Calculation of quantity of polymer or polymer conjugate, plasmid DNA and heparin used for preparation of nanoplexes in 150 mM HEPES buffered saline for anion challenge assay

S. No.	Polymer/ polymer conjugate (µg)	Actual weight of pDNA (µg)	Heparin (µg)	HEPES buffered saline (pH 7)	DMEM + 10% FBS (µl)
1	4	0.4	0.01	20	10
2	4	0.4	0.05	20	10
3	4	0.4	0.1	20	10
4	4	0.4	0.5	20	10
5	4	0.4	1	20	10

4.2.5 Cytotoxicity Assay by MTT

The cytotoxicity of polymer conjugates was investigated in 293T cells using the MTT assay (Neamnark A. et al-2009). Table 4.10 shows calculation of quantity of polymer/ polymer conjugate for the study. In brief, 293T cells were seeded in 48 well tissue culture plates in 500 μL of10% FBS containing DMEM media. After overnight incubation, where the cells achieved ~70% confluency, solution of polymers or polymer conjugates (1 mg/mL in water) were added to the cells to give final concentrations of 2 to 20 µg/mL and incubated at 37 °C in CO_2 incubator. After 24 h incubation, 100 μL of MTT (5 mg/mL in HBSS) was added to each well. After incubation for 1 h, the medium was removed, 500 μl of DMSO was

added to dissolve the formed formazan crystals and the plate was read at 570 nm. The absorbance of polymer treated samples was normalized with the absorbance of control (untreated) cells (i.e., taken as 100 % cell viability) to calculate %cell viability in polymer-treated cells.

Table 4.10: Calculation of quantity of polymer/ polymer conjugate for cytotoxicity study in 293T cell line using MTT assay

S. NO.	Saline (µl)	Each substitution level of PEI2-ChA/ PEI25-ChA/ PAA15-ChA (µg)	PEI2/ PEI25/ PAA15 (µg)	Total (µl)
1	57	3	-	60
2	51	9	-	60
3	45	15	-	60
4	36	24	-	60
5	30	30	-	60
6	57	-	3	60
7	51	-	9	60
8	45	-	15	60
9	36	-	24	60
10	30	-	30	60
16	60	-	-	0

4.3 Formulation of Nanoplexes

The nanoplexes were formed by the polyelectrolyte complexation due to charge differences and the transferrin was also incorporated into the nanoplexes owing to charge difference (Nakase M. et al-2005). To prepare nanoplexes at different polymer/ pDNA (w/w) ratios, appropriate amounts of polymer and pDNA were each dissolved in HEPES buffered 150 mM saline. Next, polymer solution was added to pDNA solution in a low-binding tube, followed brief vortexing and incubation at 37˚C (Vader P. et al-2012). The nanoplexes were prepared according to the method described in Joshee N. et al i.e. by simultaneous gentle mixing of cationic species (polymer or its conjugate), pDNA and for transferrin followed by

short incubation (Joshee N. et al-2002). To obtain nanoplexes with transferrin, expressed as the weight ratio of Tf/ pDNA, different transferrin solutions and pDNA solutions were mixed prior to polyplex formation.

Appropriate quantity of unmodified polymer or polymer conjugate stock solution (1 mg/mL) was added to pDNA (0.8 µg) in 150 mM saline and the final volume was brought to 60 µL. The nanoplexes were formed at different polymer:pDNA weight ratios (ranging from 3.75 to 30). Nanoplexes were prepared in 1.5 ml microcentrifuge tube at different polymer:pDNA weight ratio.

4.4 Characterization of Nanoplexes

4.4.1 Dynamic Light Scattering (DLS) Studies

The hydrodynamic diameters of the nanoplexes of unmodified polymer or polymer conjugates and pDNA incorporated with or without transferrin were determined by dynamic light scattering using a NanoBrook 90Plus (Brookhaven, USA). The laser used was a nominal 5mW HeNe laser having a 633 nm wavelength. Scattered light was detected at a 90° angle. The refractive index (1.33) and the viscosity (0.89) of ultrapure water were used at 25°C for measurements. Data analysis was performed in automatic mode. Measured sizes were represented as the average values of 3 runs (Zheng M. et al-2012).

4.4.2 Zeta Potential

The Zeta-potential of the nanoplexes of unmodified polymer or polymer conjugates and plasmid DNA incorporated with or without transferrin were determined (Gratton S.A. et al-2008). After 30 min incubation, each nanoplex sample was diluted to a 10 ml final volume prior to measurements. Zeta-potential measurements were carried out using a NanoBrook 90Plus (Brookhaven, USA) at 25 °C. Data analysis was performed in automatic mode. Measured zeta potenial were represented as the average values of 3 runs.

4.4.3 Transmission Electron Microscopy

Samples were prepared by placing 10 µl of nanoplexes on pre-coated copper grid and then adding a drop of 0.1% phosphotungstic acid onto a sample placed on copper grid (Landry B. et al-2012). Excess sample was removed with the help of dry tissue paper after

exactly 1 minute of staining. Images were acquired on TEM, Philips/FEI (Morgagni) at two different magnifications (89000X and 28000X).

4.4.4 *In vitro* Transfection Studies

4.4.4.1 GFP Expression in 293T Cells by Fluorimetry

The polymer conjugates were evaluated for transfection efficiency in HEK-293T cells using the Green Fluorescent Protein (GFP)-coding gWIZ-GFP plasmid as a reporter gene. Figure 5.1 shows schematic overview of the suggested transfection protocol Cells were seeded in 48 well tissue culture plates in 10% FBS containing DMEM media. After 24 h, the cells were transfected with the polyplexes that were formed at different polymer/gWIZ-GFP weight ratios (ranging 1:3.75 to 1:30). Appropriate quantity of polymer stock solution in ultra pure distilled water (1 mg/mL) was added to plasmid DNA (0.8 µg) in 150 mM saline and the final volume was brought to 60 µL. The polyplexes were gently vortexed for 5 sec and incubated for 30 min at room temperature before addition to the cells (20 µL of polyplexes added in triplicate). Table 4.11 shows the treatment groups of nanoplexes prepared with different polymer or polymer conjugates in 150 mM saline.

Table 4.11: Treatment of 293T cells with nanoplexes prepared with different polymer or polymer conjugates in 150 mM saline

Sr. NO.	**Saline (µl)**	**PEI2-ChA/ PEI25-ChA/PAA15-ChA (1 mg/ml)**	**PEI2/ PEI25/ PAA15 (1 mg/ml)**	**gWIZ-GFP (µl)**	**Total (µl)**
1	55	3	3	2	60
2	49	9	9	2	60
3	43	15	15	2	60
4	34	24	24	2	60
5	28	30	30	2	60
6	60	--	--	--	60

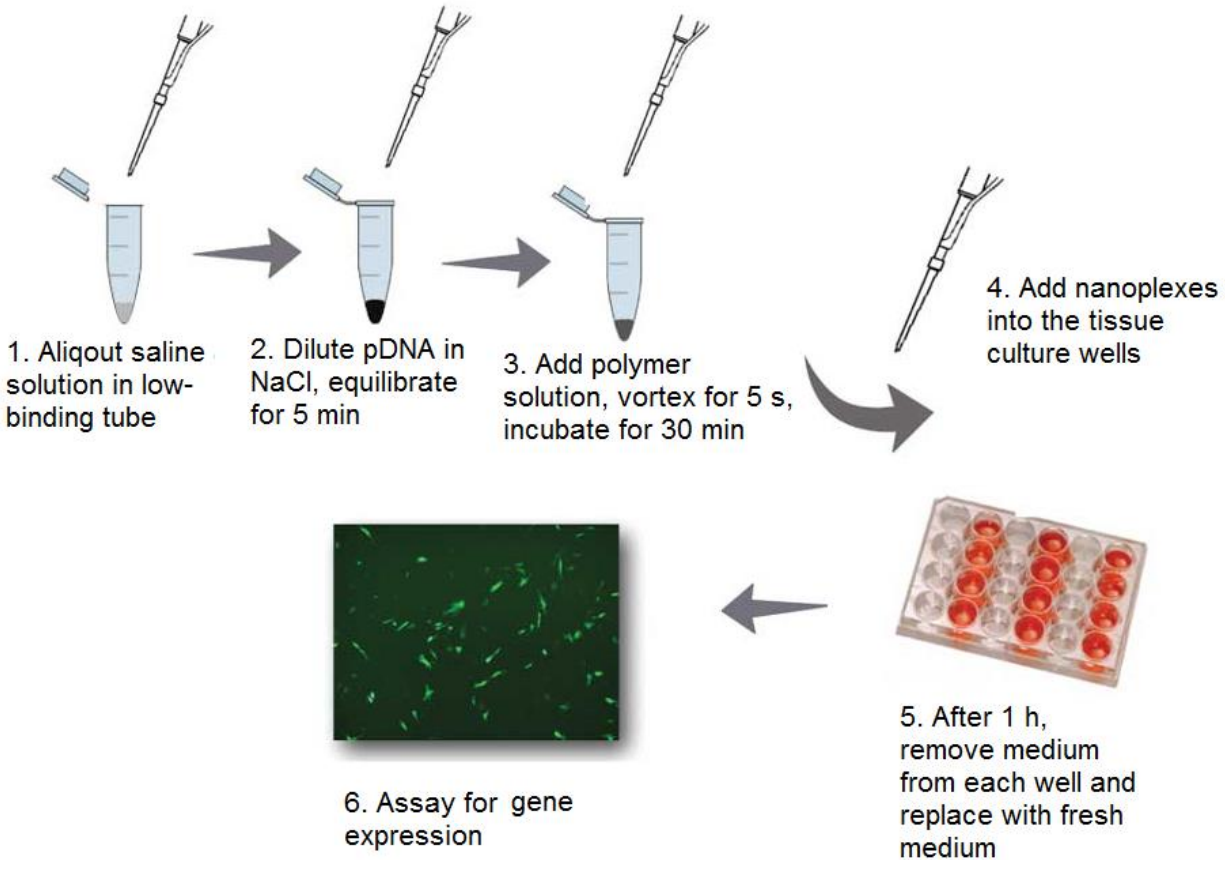

Figure 4.1: Schematic overview of the nanoplex preparation and transfection protocol (*adapted from*: Hsu C.Y.M. and Uludag H-2012)

The cells were allowed to incubate at 37 °C in a CO2 incubator for 24 h, after which the media was removed and the plates were read in a fluorescence plate reader (λEX, 485 nm, λEM, 527 nm). The results were summarized as GFP fluorescence (in arbitrary units) per well. Untreated cells or cells treated with naked gWIZ-GFP (i.e., with no polymers) were used as controls.

4.4.4.2 GFP Expression in 293T Cells by Flow Cytometry

The polymer conjugates were evaluated for transfection efficiency first in 293T cells using the Green Fluorescent Protein (GFP)-coding gWIZ-GFP plasmid as a reporter gene (Gersdorff K. et al-2006). Cells were seeded in 48 well tissue culture plates in 10% FBS containing DMEM media. After 24 h, the cells were transfected with the polyplexes that were formed at two different polymer/gWIZ-GFP weight ratios (15 and 30). Appropriate quantity of polymer stock solution (1 mg/mL) was added to plasmid DNA (0.8 µg) in 150 mM saline and the final volume was brought to 60 µL. The polyplexes were gently vortexed for 5 sec and incubated for 30 min at room temperature before addition of polyplexes to the cells (20 µL of polyplexes added in triplicate). The cells were allowed to incubate at 37 °C in CO2 incubator and, after 48 h, the media was removed and cells were trypsinized and fixed in 3.7% formaldehyde in HBSS (250 µL) so as to prepare samples for analysis by flow cytometry. The GFP expression was quantified by Beckman Coulter QUANTA SC flow cytometer using FL1 channel (3000-5000 events/sample). Instrument settings were calibrated for each run so as to obtain a background level of the GFP expression of 1% for the control samples (i.e., untreated cells). The mean GFP fluorescence for the total population and mean GFP fluorescence for GFP positive cells was also determined. In all the above experiments, both naked DNA as well as untreated cells were used as controls.

4.4.4.3 GFP Expression in CB-MSC Cells by Fluorimetry

The CB-MSC cells were seeded in 24-well tissue culture plates with 10% FBS DMEM (Incani V. et al-2009). Cells achieved ~70% confluency within 24 h, after which they were transfected with polyplexes formed at different polymer/plasmid DNA weight ratios ranging from 1.25 to 15. To prepare the polyplexes, appropriate quantity of polymer stock solution (1 mg/mL) was added to gWIZ-GFP (1.8 µg) in 150 mM saline with a final volume of 60 µL.

After vortexing gently for 5 sec and incubating for 30 min at room temperature, 20 µL of complexes were added to each well in triplicate. The cells were allowed to incubate at 37 °C in a CO2 incubator for 24 h, after which the media was removed and the plates were read in a fluorescence plate reader (λEX, 485 nm, λEM, 527 nm). The results were summarized as GFP fluorescence (in arbitrary units) per well. Untreated cells or cells treated with naked gWIZ-GFP (i.e., with no polymers) were used as controls.

4.4.4.4 GFP Expression in CB-MSC Cells by Flow Cytometry

The CB-MSC cells were seeded in 24-well tissue culture plates with 10% FBS DMEM and analyzed by flow cytometry (Thibault M. et al-2011). Cells achieved ~70% confluency within 24 h, after which they were transfected with polyplexes formed at different polymer/plasmid DNA weight ratios ranging from 1.25 to 15. To prepare the polyplexes, appropriate quantity of polymer stock solution (1 mg/mL) was added to gWIZ-GFP (1.8 µg) in 150 mM saline with a final volume of 60 µL. After vortexing gently for 5 sec and incubating for 30 min at room temperature, 20 µL of complexes were added to each well in triplicate. The cells were allowed to incubate at 37 °C in CO2 incubator and, after 48 h, the media was removed and cells were trypsinized and fixed in 3.7% formaldehyde in HBSS (250 µL) so as to prepare samples for analysis by flow cytometry. The GFP expression was quantified by Beckman Coulter QUANTA SC flow cytometer using FL1 channel (3000-5000 events/sample). Instrument settings were calibrated for each run so as to obtain a background level of the GFP expression of 1% for the control samples (i.e., untreated cells). The mean GFP fluorescence for the total population and mean GFP fluorescence for GFP positive cells was also determined. In all the above experiments, both naked DNA as well as untreated cells were used as controls.

4.4.4.5 GFP Expression in U87MG Cells by Flow Cytometry

The U87MG cells were seeded in 24-well tissue culture plates with 10% FCS DMEM. Table 4.12 shows the treatment of U87MG cells with nanoplexes prepared with different polymer or polymer conjugates with or without transferrin in 150 mM saline. Cells achieved ~70% confluency within 24 h, after which they were transfected with polyplexes formed at different polymer/plasmid DNA weight ratios ranging from 1.25 to 15. To prepare the

polyplexes, appropriate quantity of polymer stock solution (1 mg/mL) was added to gWIZ-GFP (1.8 µg) in 150 mM saline with a final volume of 60 µL. After vortexing gently for 5 sec and incubating for 30 min at room temperature, 20 µL of complexes were added to each well in triplicate. The cells were allowed to incubate at 37 °C in CO2 incubator and, after 48 h, the media was removed and cells were trypsinized and fixed in 3.7% formaldehyde in HBSS (250 µL) so as to prepare samples for analysis by flow cytometry. The GFP expression was quantified by Beckman Coulter QUANTA SC flow cytometer using FL1 channel (3000-5000 events/sample). Instrument settings were calibrated for each run so as to obtain a background level of the GFP expression of 1% for the control samples (i.e., untreated cells). The mean GFP fluorescence for the total population and mean GFP fluorescence for GFP positive cells was also determined. In all the above experiments, both naked DNA as well as untreated cells were used as controls.

Table 4.12: Treatment of U87MG cells with nanoplexes prepared with different polymer or polymer conjugates with or without transferrin in 150 mM saline.

S. No.	**150 mM saline (µl)**	**PEI2-ChA (4)**	**PEI25-ChA (4)**	**PEI25**	**Tf**	**DNA (µg)**	**polymer/ pDNA wt ratio**	**Tf/ DNA**
1	100	8	-	-	8	1.6	5	5
2	88.8	19.2	-	-	8	1.6	12	5
3	92	8	-	-	16	1.6	5	10
4	80.8	19.2	-	-	16	1.6	12	10
5	100	-	8	-	8	1.6	5	5
6	88.8	-	19.2	-	8	1.6	12	5
7	101.6	-	-	6.4	8	1.6	4	5
8	95.2	-	-	12.8	8	1.6	8	5
9	80.8	19.2	-		16	1.6	12	10
10	103.2	-	-	12.8	-	1.6	8	-
11	96.8	19.2	-	-	-	1.6	12	-
12	120 (NT)	-	-	-	-	-	-	-

4.4.4.6 GFP Expression in SKOV3 and NT8e cells by Flow cytometry

The SKOV3 and NT8e cells were seeded in 24-well tissue culture plates with 10% FBS DMEM. Cells achieved ~70% confluency within 24 h, after which they were transfected with

polyplexes formed at different polymer/plasmid DNA weight ratios ranging from 1.25 to 15. To prepare the polyplexes, appropriate quantity of unmodified polymer or polymer conjugate stock solution (1 mg/mL) was added to 1.8 µg of pDNA (gWIZ-GFP) in 150 mM saline with a final volume of 60 µL. After vortexing gently for 5 sec and incubating for 30 min at room temperature, 20 µL of complexes were added to each well in triplicate. The cells were allowed to incubate at 37 °C in CO2 incubator and, after 48 h, the media was removed and cells were trypsinized and fixed in 3.7% formaldehyde in HBSS (250 µL) so as to prepare samples for analysis by flow cytometry. The GFP expression was quantified by Beckman Coulter QUANTA SC flow cytometer using FL1 channel (3000-5000 events/sample). Instrument settings were calibrated for each run so as to obtain a background level of the GFP expression of 1% for the control samples (i.e., untreated cells). The mean GFP fluorescence for the total population and mean GFP fluorescence for GFP positive cells was also determined. In all the above experiments, both naked DNA as well as untreated cells were used as controls.

4.4.4.7 GFP Expression in SKOV3 and NT8e cells by Microscopy

The SKOV3 and NT8e cells were seeded in different 24-well tissue culture plates with 10% FBS containing DMEM. The cells achieved ~70% confluency within 24 h, after which they were transfected with nanoplexes formed at two different polymer/plasmid DNA weight ratios 10 to 12. To prepare the polyplexes, appropriate quantity of unmodified polymer or polymer conjugate stock solution (1 mg/mL) was added to gWIZ-GFP (1.8 µg) in 150 mM saline with a final volume of 60 µL. After vortexing gently for 5 sec and incubating for 30 min at room temperature, 20 µL of complexes were added to each well in triplicate. The cells were allowed to incubate at 37 °C in CO2 incubator and, after 48 h, the media was removed and the samples were observed under fluorescence microscopy (Clements B. A. et al- 2009). In this experiment, both naked DNA as well as untreated cells were used as controls.

4.4.5 Time Dependent Transfection Study

A time dependent transfection study was conducted to evaluate transfection efficiency of polymer conjugates as a function of time in human cord blood mesenchymal stem cells (CB-MSC). Polymer conjugates showing promising results in one time point transfection

experiments with CB-MSC were selected for this study. For the time course study, cells were treated with nanoplexes (with unmodified polymer or polymer conjugates) continuously during the experimental duration; cells were subcultured every 3 days to prevent over-growth. Subculturing was performed by dilution (x10) into fresh medium after resuspension (Landry B. et al-2012). All groups were subcultured with the same ratio regardless of cell concentration to ensure that the concentration of any remaining complexes stayed constant.

Cells were seeded in 12 well tissue culture plate in 1 ml of 10% FBS containing DMEM media. Cells achieved 70% confluency within 24 h, when they were transfected with polyplex formed with polymer or polymer conjugates and gWIZ-GFP prepared at different polymer/plasmid weight ratios ranging from 2.5 to 8.34. Appropriate quantity of polymer stock (1 mg/ml) was added to plasmid DNA in 150 mM saline, vortexed gently for 5 sec and incubated for 30 min at RT, essentially 20 μl of complexes were added to each well. The total amount of plasmid DNA loaded was maintained constant at 0.6 μg/well and the cells were allowed to incubate at 37 °C in CO_2 incubator. After predetermined time points (2, 4, 7 and 10^{th} day) post addition of complexes, media was removed and cells were trypsinized and fixed in 3.7 % formaldehyde in HBSS (250 µl) so as to prepare samples for analysis by flow cytometry. Cell samples from last two time points were fixed using 550 µl of 3.7 % formaldehyde in HBSS, as the cells were over confluent. The GFP expression was quantified by Beckman Coulter QUANTA SC flow cytometer using FL1 channel (3000-5000 events/sample). Instrument settings were calibrated for each run so as to obtain a background level of the GFP expression of 1-2% for the control samples (i.e., untreated cells). The mean GFP fluorescence for the total population and mean GFP fluorescence for GFP positive cells was also determined. In all the above experiments, both naked DNA as well as untreated culture was used as positive and negative controls, respectively.

4.4.6 Stability of Nanoplexes

After formation of the nanoplexes, the utility time frame for transfection is limited as the nanoplexes are unstable in the solution and will gradually form large aggregates over time. Aggregation can arise because of interaction with serum protein and/or interaction with other PEI complexes as a result of hydrophobic shielding (Hsu C.Y. *et al*-2012). One of the reasons for vast difference of transfection efficiencies in vitro and in vivo can be attributed to

complex stability. Systemic administration exposes polyplexes to physiological ionic strengths and anionically charged proteins, which can induce aggregation and destabilization by non-specific interactions (Petersen H. et al-2002). For in vitro experiments the complexes (nanoplexes) were incubated for 30 min before addition to the cells. But in in vivo application, the complexes need to remain stable and be able to transfect the cells after considerably longer period of time. Therefore, to assess this parameter an experiment was carried out in 293T cells using two types of complexes, one type being the complexes that were added immediately after nanoplexes preparation and without further incubation termed as "no incubation" group. The other type being the complexes which were incubated for further 24 hour after preparation of nanoplexes at 37˚C and are termed as "24 h incubation". The nanoplexes of PEI2-ChA conjugate with pDNA (gWIZ-GFP) and incorporated with transferrin were prepared at polymer:pDNA weight ratio of 10 in 150 mM saline solution. The nanoplexes of lipofectamine (commercially available standard transfecting agent) and PEI25 were also prepared with pDNA (gWIZ-GFP) at polymer:pDNA weight ratio of 10 in 150 mM saline solution. 20 µl of both types of nanoplexes were added to the 293T in 3 different wells (triplicate) cells and 72 h post nanoplexes addition, cells were harvested using routine procedure of trypsinization and cells were fixed in 3.7 % formaldehyde in HBSS (250 µl) so as to prepare samples for analysis by flow cytometry. The GFP expression was quantified by Beckman Coulter QUANTA SC flow cytometer using FL1 channel (3000-5000 events/sample). Instrument settings were calibrated for each run so as to obtain a background level of the GFP expression of 1-2% for the control samples (i.e., untreated cells). The mean GFP fluorescence for the total population and mean GFP fluorescence for GFP positive cells was also determined. The untreated cells were used as negative control.

4.4.7 BMP-2 Production in hBMSC cells

As a verification of transfection efficiency the human bone morphogenetic protein (BMP-2) production study was performed with polymer conjugates and plasmid BMP-2. It is a very sensitive assay and may give insights about synthesized polymer conjugate's ability of transfecting hard to transfect cells (Wang W. et al-2012). Therefore, the human BMP-2 production assay was performed in hBMSC cells using DuoSet ELISA Development Kit (#DY355). The nanoplexes were prepared with the polymers and plasmid BMP-2 with

weight ratios of 2, 4 and 8, and added to the 70 % confluent hBMSC cells in 24-well plates (in triplicate). The complexes were removed after 24 h and the cells incubated with fresh medium for 6 days. The supernatant were recovered for BMP-2 production. For assessment of the BMP-2 concentration, 96-well microplates were coated with a diluted capture antibody (100 µl) in PBS and incubated overnight at RT. Each well was washed with wash buffer (3×) and the remaining sites were blocked by incubating for 1 h at RT with 1% BSA solution (300 µl). The supernatants (100 µl) of the transfected cells or standards in 1% BSA solution were added to the wells and incubated for 2 h at room temperature. The wells were then washed and incubated with a biotinylated detection antibody (100 µl) for 2 h, followed by the addition of horseradish peroxidase-conjugated streptavidin solution (100 µl) for 30 min in the dark. After washing, 100 µl of substrate solution was added to the wells and the reaction was stopped after 20 min with 50 µl of 2N H2SO4. Immediately the absorbance in individual wells was measured at 450 nm using a microplate reader and converted into a BMP-2 concentration using a calibration curve generated using the manufacturer supplied BMP-2 standard.

4.4.8 Cell cycle analysis in NT8e cells

The cell cycle analysis was conducted to understand the effect of nanoplexes containing p53 on the cell cycle (Finkel E.-1999). The cell-cycle analysis is a method to distinguish cells in different phases of the cell cycle. This method requires propidium iodide (PI) and employs flow cytometry for the cell cycle analysis. Before analysis, the cells were permeabilized and treated with PI (a fluorescent dye that stains DNA quantitatively). As the DNA content of cells varies indifferent phases, the relative amount of cells in the different phases of cell cycle can be determined based on the quantity of DNA.

Reagents

RNAse: 10 µg/µl solution

Propidium Iodide: 1 mg/ml solution in PBS

Protocol

Cells were treated with polymer or polymer conjugates (at different weight ratios) and incubated for 24 hr. Cells were harvested, washed twice with PBS and fixed in chilled 70% ethanol. The fixed cell pellet was washed with PBS and re-suspended in 200 µl of PBS. Cells

were treated with 10 µl RNAse solution (0.5 mg/ml concentration) for 15 min at 37°C. The volume was made upto 1 ml and 50 µl propidium Iodide (50 µg/ml concentration) was added to it. Cells were incubated for 10 min at room temperature. 10,000 events were acquired on FACS Calibur and analyzed using Modfit software.

4.5 *In Vitro* Protein Expression Studies

Before evaluating the synthesized polymer conjugates in vivo, an in vitro protein expression study was necessary to demonstrate the ability of formulated complexes to synthesize the desired protein, p53. Therefore, in vitro protein expression studies were carried out in two cell lines viz. SKOV3 (p53 null) and NT8e (p53 mutant). Before actual protein expression was evaluated, it was necessary to calculate total amount of protein present in the cell lysate (Liu J. et al-1999).

4.5.1 Cell Lysate Preparation

- The medium was removed from each well and washed with 1X PBS (3 times).
- 150 µl of trypsin solution (0.25 mg/ml) was added to each well and kept in incubator (37˚C) for 5-10 min (observed for cell detachment)
- PBS solution (1 ml) was added to each well and centrifuged at 200 rpm in 4˚C for 10 min
- Supernatant was removed carefully (without touching the cell pellet) and cell pellet was dislodged.
- To each cell pellet 250 µl of cell lysis buffer, tapped gently and kept on ice for 45 min
- Each cell suspension was sonicated at low level to ensure complete lysis
- Samples were spun at 12000 rpm for 15 min at 4˚C and three aliquots (70 µl each) were kept in -80˚C until further analysis.

The Lowry's assay for total protein estimation was used as it is one of the most commonly performed colorimetric assays before proceeding to westen blot analysis. This procedure is sensitive because it employs two colour forming reactions. The principle behind the Lowry method of determining protein oncentrations lies in the reactivity of the peptide

nitrogen(s) with the copper (II) ions under alkaline conditions and the subsequent reduction of the Folin Ciocaltaeu reagent which contains phosphomolybdic complex which is a mixture of sodium tungstate, sodium molybdate and phosphate, along with copper sulphate solution and the protein, a blue purple colour is produced which can be assessed by measuring the absorbance at 650-700nm.

Reagents

A. 2% Na2CO3 in 0.1 N NaOH

B. 1% NaK Tartrate in H2O

C. 0.5% CuSO4.5 H2O in H2O

D.Reagent I: 48 ml of A, 1 ml of B, 1 ml C

E.ReagentII- 1 part Folin-Phenol [2 N]: 1 part water

BSA Standard- 1 mg/ ml

Procedure:

Five test tubes of 5 ml each were filled with 0.2 ml of BSA working standard the volume were made up to 1 ml using distilled water. One such test tube with 1 ml distilled water served as blank. 4.5 ml of Reagent I was added to these test tubes and incubated for 10 minutes. After 10 min incubation, 0.5 ml of reagent II was added and agin incubated for 30 minutes. The absorbance was measured at 660 nm and the standard graph was plotted for estimation of amount of protein present in the unknown samples (protein samples from NT8e and SKOV-3 cells). Table 4.13 shows quantities of standard protein (BSA) and reagents required for standard graph preparation which is required for total protein estimation using Lowry's protein estimation assay. Table 4.14 shows dilutions of cell lysates from the NT8e and SKOV-3 cells for estimation of total protein using Lowry's protein assay.

Table 4.13: Sample preparation for standard graph using bovine serum albumin (BSA) for total protein estimation using Lowry's protein estimation assay.

Sr. No.	Distilled water (µl)	BSA (µg/ µl)	Solution I (µl)	Solution II (µl)	Total (µl)
1	21	0	208.2	20.8	250
2	12	9	208.2	20.8	250
3	15	6	208.2	20.8	250
4	16.5	4.5	208.2	20.8	250
5	18	3	208.2	20.8	250
6	19.5	1.5	208.2	20.8	250

Table 4.14: Dilution of cell lysates from the NT8e and SKOV-3 cells for estimation of total protein using Lowry's protein assay.

Sr. No.	Treatment	Distilled water (µl)	Sample volume (µl)	Solution I (µl)	Solution II (µl)	Total (µl)
NT8e Cell samples						
1	PEI2-ChA-Tf (wt ratio 8)	17	5	208.2	20.8	250
2	PEI2-ChA-Tf (wt ratio 10)	17	5	208.2	20.8	250
3	PEI25 (wt ratio 5)	17	5	208.2	20.8	250
SKOV-3 Cell samples						
4	PEI2-ChA-Tf (wt ratio 8)	17	5	208.2	20.8	250
5	PEI2-ChA-Tf (wt ratio 10)	17	5	208.2	20.8	250
6	PEI25 (wt ratio 5)	17	5	208.2	20.8	250

4.5.2 Gel Casting and electrophoresis

Reagents

30% Acrylamide mixture (29.2% acrylamide + 0.8% bis-acrylamide) solution was prepared in D/W, filtered through Whatman filter # 3 and stored at 4ºC in an amber coloured bottle.

1.5 M Tris-HCl pH 8.8 (resolving buffer)

1M Tris-HCl pH 6.8 (stacking buffer)

10% SDS solution: 10 gm of SDS was added to 100 ml of water, heated to 70ºC for SDS to dissolve completely.

10% Ammonium persulphate (APS) solution

N,N,N',N'-Tetramethylethylenediamine (TEMED)

Pre-stained molecular protein marker

Tank Buffer: 196 mM Glycine, 50 mM Tris HCl and 0.1%SDS, pH 8.3

Agarose 1%

Mini gel dual assembly

Power Pack

i) Resolving gel recipe 30 ml volume (for 2 mini gels):

D/W	3.9 ml
Acrylamide mixture	8 ml
1.5 M Tris pH 8.8	7.5 ml
10% SDS	300 µl
10% APS	300 µl
TEMED	18 µl

ii) Stacking gel Recipe 10 ml volume (for 2 mini gels):

D/W	6.8 ml
Acrylamide mixture	1.7 ml
1M Tris pH6.8	1.25 ml
10% SDS	100 µl
10% APS	100 µl
TEMED	10 µl

Protocol

The glass plates were wiped with 70% alcohol and assembled. The bottom and the sides of the glass plates were sealed using 1% molten agarose. When the gel was solidified, the resolving gel mixture was poured gently, leaving some space for the stacking gel. The gel

mixture was overlaid with methanol to give a uniform gel front and kept for polymerization for 20-30 minutes. Thereafter, the methanol and the un-polymerized gel mixture were removed, washed with D/W. The stacking gel mixture was poured on top of the resolving gel and a comb was inserted. Stacking gel was allowed to polymerize, following which the wells were washed with D/W and marked. The assembly cathodic and anodic chamber was filled with tank buffer and the wells were loaded with the samples. A molecular weight marker was loaded in one well. Electrophoresis was carried out at 25 mA for the stacking and then the further run was carried out at 30 mA. Run was stopped when the dye front reached 1 mm above the gel end.

4.5.3 Protein Transfer

Reagents

Wet Transfer buffer: 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3

Poncue S solution: 0.1% Poncue powder in 5% acetic acid

Polyvinylidene difluoride (PVDF) membrane

Protocol

The PVDF membrane was cut according to the size of the gel and pre-wetted with methanol for 5 minutes before equilibration. The resolved gel and the membrane both were equilibrated with the transfer buffer for 20 minutes. Four whatman filter #3 sheets were cut according to the size of membrane. After equilibration, the membrane was kept over the gel and this was sandwiched between the folds of filter paper, two on either side. Care was taken to avoid any bubbles being trapped. The sandwich was then kept between the electrode plates of the transfer assembly (gel towards the cathode). The transfer assembly was filled with the transfer buffer. Transfer was carried out for 16 hr at constant 10 V or for 1 hr at 100 V in a cold room. The membrane was stained with Poncue solution till the pink bands appeared. The side containing the marker bands were marked (by small cut) and then de-stained by rinsing in D/W.

4.5.4 Western Blotting

Requirements

TBS(Tris-Buffered Saline): 50 mM Tris pH 7.5, 0.85% NaCl; pH was adjusted to 7.6, solution was autoclaved and stored at 4°C.

TBST (Tris-buffered saline with Tween 20): To 1 litre of TBS 1 ml of Tween 20 was added, stirred well and used.

Blocking Solution: 5% non-fat skimmed milk powder in TBST; kept at 37°C for 15 minutes to dissolve completely

Primary and Secondary antibody dilutions: The antibodies were diluted in 2.5% non-fat skimmed milk

Super-Signal West Femto kit (Thermao Scientific, USA)

Developing cassette (Bangalore Genei, India)

Plastic boxes

X-ray films

Protocol

The membrane was blocked with the blocking reagent for 2 h. The blots were then washed vigorously with TBST for 5 minutes. The blot was taken in a plastic bag, overlaid with primary antibody and kept on a rocker for 1 h. After incubation, blots were washed with TBST 3 times, for 10 minutes each. Blots were then incubated for 1 h with horse-raddish peroxide (HRP) conjugated secondary antibody. The nonspecific binding was removed by 3 TBST washes, 10 minutes each.

The blots were then taken to the dark room for visualization of the signal. Super-signal west femto developing reagent was made as per the manufacturer's instructions. The blot was kept on a clean glass plate and on the probed side Super signal west femto mixture was added and incubated for 5 minutes. After incubation, excess reagent was drained on the filter paper and the blot was kept in the developing cassette in between two transparent plastic sheets. The X-ray sheet was kept on the protein side and exposed for various times depending upon the signal intensity. The exposed films were developed in the developing machine. Equal loading was checked by studying β-tubulin expression.

4.6 Biodistribution Studies

All animal handling was performed in accordance with Animal Research Committee guidelines (CPCSEA Reg. Number 65/1999 dt. 11th March 1999) at Advanced Centre for Treatment, Research and Education in Cancer (ACTREC) Tata Memorial Centre, Kharghar, Navi Mumbai 410 210). The Animal Study was permitted by the Institutional Animal Ethics Committee (IAEC) of ACTREC (Proposal no. 24/2012, dt. Dec. 08, 2012)

Whole animal (in vivo) and organ (ex vivo) imaging:

The biodistribution studies were carried out in Swiss bare mice using plasmid DNA encoded for GFP (Yutaka Fujiki *et al*.-2008). Swiss bare mice were used so as to avoid interference from fur of the animal and for optimal visualization of fluorescence. Three groups of animals (3 animals in each group) were used. The animals were grouped as follows:

1. Plasmid DNA (gWIZ-GFP) in saline,
2. PEI2-ChA-gWIZ-GFP nanoplexes in saline (polymer conjugate:pDNA weight ratio 10:1), and
3. PEI2-ChA-gWIZ-GFP-Tf nanoplexes in saline (polymer conjugate:pDNA weight ratio 10 and pDNA:transferrin weight ratio 4:1)

The polymer conjugate:plasmid DNA weight ratio of 10 and plasmid/ligand weight ratio of 4 was chosen because these ratios were found to be efficient for transfection yet found to be relatively less toxic.

Protocol

The animals were injected with 100 µl of either pDNA in saline or nanoplexes without transferrin or nanoplexes incorporated with transferrin through tail vein. 100 µl of nanoplexes containing 15 µg of pDNA with or without transferrin (polymer:plasmid weight ratio 10:1) or naked plasmid DNA (15 µg) in saline were injected intravenously into the tail vein of Swiss bare mouse. The animals were provided water and food ad libitum. After 48 hours of injection, mice were anesthetized for the imaging procedure with an i. p. injection of mixture of ketamine 50 mg/ml and xylazine 2 mg/ml at 150 mg/kg of body weight (Caysa H. *et al*-2012). Animals were observed under animal imager after checking their reflexes. But as

the righting reflex of animals return in approximately 40-90 minutes, experiment was conducted accordingly.

Animals were placed in the IVIS Imaging System 200 and analyzed for fluorescence based on the manufacturer's recommendations (IVIS Lumina II, Caliper Life Sciences, UK). For both whole animal imaging and organ (ex vivo) imaging, excitation filter of λmax 465 nm and emission filter of λmax 520 nm was used. Each anesthetized mouse was focused and a grayscale image was taken with 10 or 30 ms exposure time under white light. Afterwards, the white light was changed to excitation light. The exposure time (ms) was measured automatically.

After imaging, the mice were sacrificed and major organs were removed for direct measurement of fluorescence from important organs for biodistribution viz. brain, lungs, liver and spleen. The fresh organs were placed on 10 cm plates and analyzed for fluorescence using the IVIS system (IVIS Lumina II, Caliper Life Sciences, UK). GFP was excited at 488 nm (filter range 445 to 490 nm) and detected at 510 nm. Data was recorded as photons/sec/cm2 using Living Image software v2.50 (Caliper Life Sciences, UK).

4.7 Tumor Regression Studies

All mice were cared for and maintained in accordance with Animal Welfare Regulations under an approved protocol by the Institutional Animal Care and Use Committee of Advanced Centre for Treatment, Research and Education in Cancer (ACTREC) Tata Memorial Centre, Kharghar, Navi Mumbai 410 210 and Animal Research Committee guidelines (CPCSEA Reg. Number 65/1999 dt. 11th March 1999) as per Animal Study Proposal permission no. 24/2012 Institutional Animal Ethics Committee (IAEC) dt. Dec. 08, 2012.

The NT8e xenograft tumors were developed in 6-8-week-old either sex nude mice (5 mice per group) by implanting a cell suspension of 5×10^6 NT8e cells (Xue W. et al 2007). The cell suspension in 100 µl of HBSS was injected s.c. on the right hind leg after the flanks were cleaned with 70% ethanol. Tumor growth was recorded once a week in three dimensions using a digital caliper. Tumors were allowed to grow and tumor volume was calculated [(length × width^2) / 2] and reported in mm^3. The treatment was planned to be

given after tumors reached the volume of ~100 mm^3. The animals with similar or comparable tumor volume were grouped and used for further experimentation.

Animals were divided into 2 groups (5 animals in each group):

1. Control (injection of plasmid p53 in saline),
2. PEI2-ChA-p53-Tf nanoplexes in saline (polymer:plasmid weight ratio 10:1 and plasmid:transferrin weight ratio 4:1)

As soon as the tumors developed ~100 mm^3 in size (~after 35 days), transferrin containing nanoplexes of novel lipopolymer (PEI2-ChA and plasmid p53 incorporated with Tf) in 100 µl of saline was injected intratumorally to investigate the tumor regression ability. The quantity of plasmid used was 8 µg at polymer:plasmid weight ratio of 10:1 and weight ratio of 4:1 of plasmid:transferrin was used. The animals were injected with either saline (control) or polyplexes (treatment) again after day 3 and 7 of first injection. Then again tumor volume was monitored for next 48 days. Finally mice were sacrificed on 48^{th} day and photographs were taken. The results were plotted as tumor volume vs. days post tumor transplantation.

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