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Dextran–glycidyltrimethylammonium chloride conjugate/DNA nanoplex: A potential non-viral and haemocompatible gene delivery system

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ABSTRACT

Non-viral gene carriers have attracted great interests for their unique properties. Cationic polymers have been in focus nowadays. Dextran is one of the most widely studied polymer in terms of gene therapy and in vivo disposition. But its applications are limited by its own drawbacks. To overcome the drawback, we have modified dextran using glycidyltrimethylammonium chloride (GTAC) bearing cationic groups. Nanoplexes were prepared using the derivative and calf thymus DNA (ctDNA) by reducing the surface charge and size of ctDNA. Complexation and stability of the nanoplex was proved using agarose gel electrophoresis and by Ethidium bromide (EtBr) displacement assay. Acid base titration studies were done to determine its buffering capacity. Derivatization was confirmed using NMR. Protection of ctDNA from nuclease digestion was evaluated. Stability of the nanoplex towards plasma components was analyzed. Its interactions with blood components were tested by haemolysis and aggregation studies. *In vitro* cytotoxicity studies have been done to investigate the effect of nanoplex on HepG2 cells by MTT assay. This derivative has been proved to be feasible in transfection. The above investigations prove the capability of dextran modified with GTAC as a promising non-viral and haemocompatible gene delivery agent.

1. Introduction

For the past few decades, great interest has been stimulated in the treatment of human diseases by gene therapy. The success of gene therapy mainly depends on the development of transfecting agents that carry therapeutic genes into mammalian cells for gene expression (Suna and Kyung-Dall, 2008). Vectors or carriers play a major role in gene therapy due to the instability of naked plasmid DNA under in vivo conditions. The use of viral vectors as gene carriers remain associated with some dangerous adverse effects, inspite of their higher efficiencies for delivery and expression. Serious problems that limit their use include pathogenicity, immunogenicity, wild type reversion, toxicity, release of virus in the semen of treated patients and expensiveness. Conversely, non-viral vectors offer unique advantages like lower immunogenicity, safety profiles, low cost production and lack of mutational potential (Hosseinkhani et al., 2004; Kim et al., 2005; Suna and Kyung-Dall, 2008; Cavallaro et al., 2008; Tsuchiya et al., 2006). Therefore there arises a need to optimize non-viral vectors to enhance the transfection efficiency which generally do not reach adequate therapeutic levels.

The key technology in improving the efficiency of non-viral vectors lies in the involvement of cationic polymers as the therapeutic gene carriers. Polycations are the promising candidates in the field of non-viral vectors because of their chemical diversity (Cavallaro et al., 2008). They are able to condense genetic material into compact nanosized structures by forming polyelectrolyte complexes. They mask the negative charges of DNA enabling the transfection of many types of cells (Vroman et al., 2007).

Polycations used in gene delivery include polyethyleneimine (PEI), poly(L-lysine), poly(dimethyl aminoethyl methacrylate), pDMAEMA), poly(trimethyl aminoethyl methacrylate, p(TMAEMA), poly(vinylpyridine), chitosan, and diethylaminoethyl dextran (DEAE-dextran) (Hosseinkhani et al., 2004; Cavallaro et al., 2008). Several problems including low biocompatibility, toxicity, low biodegradability and low transfection efficiency still lie associated with polymeric gene delivery systems.

Much attention has been devoted to the preparation of biopolymer nanoparticles and their application in the field of pharmaceutics. Several reports proved that nanoparticles made up of polysaccharides are provided with a sheathing property and increased blood half time (Hosseinkhani et al., 2004). The main approach is the use of cationic polymers that loose their DNA binding properties within time. Many researchers were drawn to study and understand the importance of dextran and its derivatives due to its unique characteristics. Dextran is a biodegradable and biocompatible polymer. Its straight chain consists of α-1,6 glycosidic linkages with few α-1,3 glycosidic linkages as the branch linkage (Yalpani and Hedman, 1985). Dextran fractions are stable for five years. They are readily soluble in water and electrolyte solutions.

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It binds to erythrocytes and platelets increasing their electronegativity and thus reducing erythrocyte aggregation. Dextrans can be used as plasma expanders, for ophthalmic use, for intrauterine examinations, and also in creams and ointments (Hosseinkhani et al., 2003). This proves its excellence in biocompatibility. Cationization of dextran can increase its efficiency similar to that of PEI. Cationic dextran can be prepared with reagents carrying a positive charge.

The main aim of this work was to evaluate the haemocompatibility and efficiency of chemically modified dextran nanoparticles as therapeutic gene carriers.

2. Materials and methods

2.1. Materials

Dextran (MW 35,600), sodium hydroxide, Ethidium bromide (EtBr), 3-(4,5-dimethylthialzol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT), Dulbecco's modified eagle's medium (DMEM), trypsin, ethylenediaminetetraacetic acid (EDTA) and DNase I was purchased from Sigma–Aldrich Chemicals Co., USA. pGL3 control DNA was from Promega, USA. Deoxyribonucleic acid sodium salt from calf thymus (ctDNA) was from Worthington biochemical corp. Glycidyltrimethylammonium chloride (GTAC) (MW151.63) was purchased from Fluka, USA. Fetal bovine serum (FBS) was from GIBCO (USA). All other reagents were of analytical grade from Merck, India.

2.2. Cationization of dextran

100 mg of dextran (MW 35,600) was dissolved in 10 ml of 5% sodium hydroxide. 1.25 ml of Glycidyltrimethylammonium chloride was slowly added to the dextran solution. The reaction mixture was kept at 40 °C under continuous stirring for 24 h. The product was recovered by extensive dialysis against double distilled water until no 2,3-epoxypropyltrimethylammonium chloride was left in the solution. The dextran derivative (Dex-G) thus obtained was stored at 4 °C.

2.3. ^1H NMR studies

The polymer for study was dissolved in D_2O and then analyzed. The ^1H NMR spectra were obtained using NMR spectrometer (Bruker Avance DPX 300).

2.4. Degree of substitution

The modified effect of Dex-G was determined from the data procured from ^1H NMR spectra. The following equation was considered in the calculation of the degree of substitution:

$$DS(\%) = \left[\frac{[(CH_3)_3]}{[H] \times 1/9}\right] \times 100$$

where DS (%) is the degree of substitution in percentage, $[(CH_3)_3]$ is the integral of the quaternary ammonium group peaks at 3.4 ppm and [H] is the integral of ^1H peaks between 4.7 and 5.7 ppm (Wang et al., 2010).

2.5. Formulation of polyelectrolyte complexes

Nanoplexes were prepared using Dex-G and ctDNA at room temperature for biophysical studies. Constant amount of ctDNA (10 μg) and varying amounts of Dex-G was diluted in saline to a volume of 100 μl. The Dex-G solution was then added to the DNA solution and vortexed. The complexes were incubated for 60 min. The complexes contained increasing polymer/DNA weight ratios ranging from 0.5:1 to 5:1.

2.6. Zeta potential and particle size determination

The surface charge of the nanoplexes was determined using the Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at a temperature of 25 °C. The Smoluchowsky approximation was used to check the zeta potential. The complexes were prepared in saline with increasing polymer/DNA ratios from 0.5 to 5. The particle size of the nanoplexes was evaluated by dynamic light scattering measurement. Here, complexes with varying ratios were measured using the Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at 25 °C.

The effect of serum on the particle size and zeta potential of Dex-G/ctDNA complexes was also studied. The average particle size and polydispersity index was evaluated based on dynamic light scattering using the Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at 25 °C. Varying concentrations of Dex-G was added to a constant amount of ctDNA (10 μg) diluted to a volume of 200 μl with Dulbecco's modified eagle's medium containing 10% fetal bovine serum and incubated for 30 min at room temperature. The surface charge of the nanoplexes was determined by photon correlation spectroscopy using the Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at a temperature of 25 °C. The Smoluchowsky approximation was used to check the zeta potential.

2.7. Buffering capacity of polymer

Different concentrations of Dex-G were taken to test the buffering capacity. The pH of the polymer was adjusted to 10 using sodium hydroxide. Hydrochloric acid was sequentially added (20 μl) to the polymer solution. The pH change was recorded during the titration.

2.8. Agarose gel retardation studies

The condensation of ctDNA with increasing concentration of Dex-G was analyzed on 0.8% agarose gel containing Ethidium bromide in Tris–acetate–EDTA (TAE) buffer solution. Electrophoresis was carried out at 100 V for 90 min. The DNA bands were then visualized and photographed using MultiImageTM Light Cabinet (Alpha Innotech Corporation, San Leandro, CA, USA).

2.9. Stability of complexes in plasma

The stability of the complexes in the presence of plasma was checked by agarose gel electrophoresis. The complexes were prepared and incubated with 20 μl of plasma. The DNA release was determined after 1/2 h at room temperature.

2.10. DNase I protection assay

DNase was prepared in digestion buffer containing sodium acetate and magnesium sulfate at pH 7.4. Nanoplexes of varying ratios were prepared. The complexes were incubated with DNase of concentration 569 U/ml. The reaction was terminated using termination buffer containing 0.5 M EDTA. The protection rate of Dex-G was detected by visualizing the bands using agarose gel electrophoresis in a Bio-Rad electrophoresis system (Bio-Rad laboratories, CA, USA) (Douvas, 1978).

2.11. PAGE analysis of Dex-G with plasma proteins

Native PAGE analysis was performed to detect the interaction of Dex-G, dextran and PEI with plasma proteins. Plasma (20 μl) was

incubated with the polymer for 20 min and then centrifuged. Of the supernatant, 20 μl was loaded onto the PAGE system and then separated at 100 V in a Mini-PROTEAN II electrophoresis cell (Bio-Rad, CA, USA).

2.12. Blood and plasma

Whole blood was collected from a healthy volunteer and added to a tube containing sodium citrate (ratio of blood to anticoagulant taken was 9:1). Erythrocytes were isolated from the fresh citrated blood by centrifugation at 700 rpm for 10 min. White blood cells were isolated by centrifuging plasma at 1500 rpm for 10 min. (Kainthan et al., 2006).

2.12.1. Haemolysis

The RBCs were washed thrice with saline. Nanoplexes were prepared and incubated with 100 μl of RBCs for 2 h at 37 °C. The samples were then centrifuged at 1500 rpm for 5 min. After centrifugation, the supernatant was analyzed for haemoglobin release at 399 nm. Triton X 100 was used as the positive control (100% lysis) and 0.09% NaCl was used as the negative control (0% lysis).

2.12.2. RBC aggregation

Human erythrocytes were collected by centrifugation of whole blood at 700 rpm for 10 min and then diluted with saline at 1:10 ratio. To 100 μl of erythrocytes, varying ratios of the nanoplexes were added and incubated for 1 h at 37 °C. Aggregation was detected through phase contrast microscope (Leica DM IRB, Germany) at 40× magnification (Björn Neu and Meiselman, 2002).

2.12.3. WBC aggregation

Human leukocytes were isolated by centrifuging whole blood layered on the Histoplaque, for 30 min at 700 rpm. Varying ratios of the nanoplexes were incubated with 100 μl of WBCs and the mixture was kept for incubation at 37 °C for 30 min. Detection of aggregation was done through phase contrast microscope (Leica DM IRB, Germany) at a magnification of 40×.

2.13. EtBr displacement assay

The assay provides evidence for the stability of the nanoplexes formed between Dex-G and DNA. The sample containing ctDNA (10 μg/ml) and EtBr (0.06 mg/ml) was used to calibrate the fluorimeter to 100% fluorescence. Nanoplexes of different ratios were incubated in EtBr solution for 15 min. The fluorescence intensity was read at an excitation of 510 nm and emission of 590 nm using an automated microplate reader (Finstruments Micro plate Reader, USA) (Wiethoff et al., 2003).

2.14. Cytotoxicity

The evaluation of the viability and proliferation of cells against Dex-G was done by the cell viability assay. The main reagent, 3-(4,5-dimethylthialzol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT), was prepared in distilled water. Prior to assay, human hepatocellular liver carcinoma (HepG2) cell lines were subcultured from the stock culture and seeded into multi well plate. Cells were then incubated at 37 °C under 5% CO_2 atmosphere. Samples along with positive and negative controls were added in duplicates and incubated for 24 h at 37 °C in 5% CO_2 atmosphere. After 24 h of incubation, the samples were removed and MTT reagent (0.2 mg/ml) was added to each well and incubated for 3 h. The reagent was then removed and dimethyl sulfoxide (DMSO) was added to dissolve the MTT formazan crystals. Plates were shaken for 5 min. The absorbance of each solution was read at 570 nm using an automated microplate reader (Yun Xia Sun et al., 2008).

2.15. Flow cytometry analysis

The transfection efficiency of Dex-G was quantified by flow cytometry. Dex-G nanoparticles were prepared and fluorescently labeled using fluorescein isothiocyanate (FITC). The FITC labeled Dex-G was then subjected to a Sephadex G25 column. Nanoplexes were formulated with FITC labeled Dex-G and pGL3 plasmid at ratio 1:3. Hela cells were incubated with FITC labeled nanoplexes for 4 h at 37 °C in 5% CO_2 atmosphere. The cells were then washed with PBS. Trypsinization was performed by the incubation of cells with trypsin–EDTA for 4 min followed by the centrifugation of the trypsinized cells quenched in 2 ml of DMEM medium with serum. Supernatent was discarded and the cells were resuspended in PBS. The percentage of transfected cells was quantified by flow cytometric analysis on a BD FACSAria cell sorter (BD Biosciences) equipped with an argon laser tuned to 488-nm (for FITC). For detection, a bandpass filter of 515–545 nm was utilized. Data was evaluated by BD FACSDiva™ software (BD Biosciences) (Rekha and Sharma, 2009).

2.16. In vitro transfection activity

HepG2 cells were seeded in 24 well plate with DMEM medium containing 10% FBS and incubated at 37 °C for 24 h to get a confluency of 80%. The cells were then treated with nanoplexes prepared at ratios 1:4 and 1:7 with pGL-3 plasmid. The cells were incubated with the samples for 48 h in DMEM containing FBS.

The growth medium was then removed and the cells were washed with phosphate buffer saline. The cells were then lysed with the lysis reagent. The luciferase activity was measured by the addition of the luciferase assay substrate. The relative luminescence (RLU) was determined using the luminometer (Chamaleon, Hidex). Total protein was measured using bicinchonic acid (BCA) protein assay (Pierce, USA). Results of the luciferase activity were expressed as RLU (relative light units)/mg Cellular Protein (Yun Xia Sun et al., 2008).

The efficacy of transfection was also affirmed in the presence of serum with DMEM medium. Transfection was performed in the presence and absence of serum using HepG2 and Hela cell lines. Nanoplexes of Dex-G and pGL3 plasmid were prepared at ratios 1:4 and 1:3 in medium without serum, incubated for 30 min and then added to wells containing DMEM medium with and without 10% FBS. After 4 h, the medium was replaced and cells were further incubated with the samples for 48 h in complete medium. Cells were then washed with phosphate buffer saline. Quantitative luciferase expression was analyzed by the luciferase assay using a luminometer (Chamaleon, Hidex). Total protein was measured using the BCA protein assay kit. Results of the luciferase activity were expressed as RLU (relative light units)/mg Cellular Protein.

3. Results

3.1. Cationization of dextran

Water soluble cationized dextran derivative containing quaternary ammonium groups (Dex-G) were prepared by reacting dextran with glycidyltrimethylammonium chloride.

The NMR spectra obtained was analyzed to characterize Dex-G. The ^1H NMR spectrum of Dex-G was compared with the spectrum of dextran as in Fig. 1. Variations in the signals between the two spectra were detected. The characteristic peak of quaternary ammonium groups formed at 3.4 ppm appeared in the NMR spectrum of Dex-G measured in D_2O.

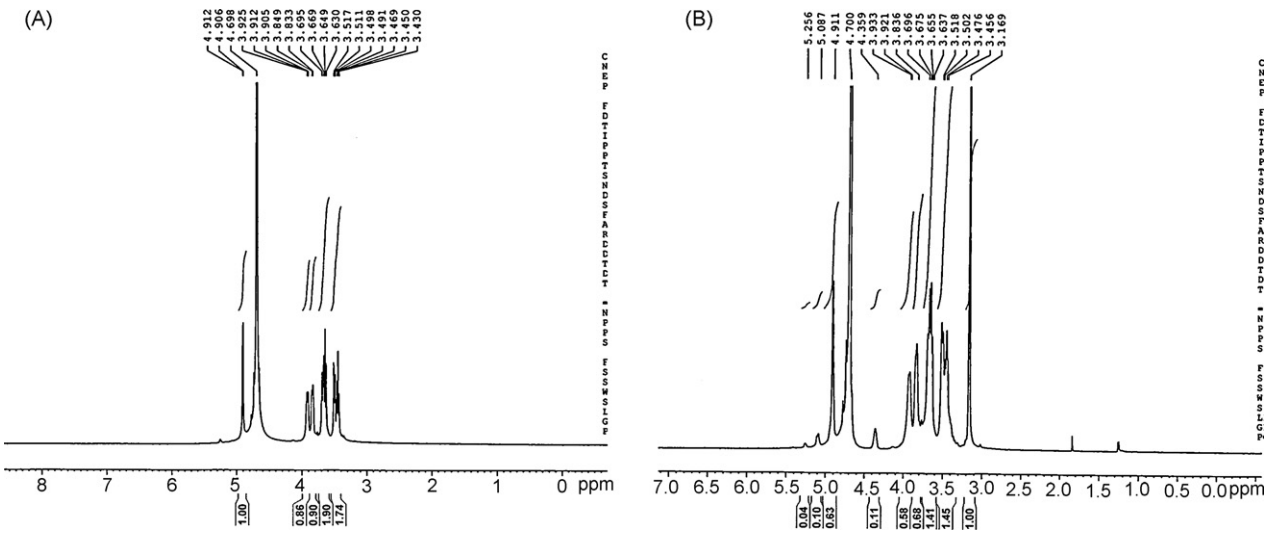

Fig. 1. ^{1}H NMR spectra of dextran (A) and of Dex-G (B).

3.2. Degree of substitution

The degree of substitution was evaluated from the ^{1}H NMR spectra as shown in Fig. 1. The signal obtained at 3.4 ppm signifies the presence of quaternized groups. The degree of substitution was calculated to be 20.92.

3.3. Nanoplex formation

The nanoplex was formed between Dex-G and ctDNA. Constant concentration of the DNA complexed with varying concentrations of the polymer. The DNA condensed to a small and compact nanometer size forming nanoplexes.

3.4. Zeta potential and particle size determination

Stability of a nanoplex is determined by its surface charge which is indicated by the zeta potential value. Zeta was recorded at 25 °C. The zeta value of dextran of molecular weight 35,600 was −2.97. The negative charge obtained was of the hydroxyl groups present in dextran. The surface charge of naked ctDNA was −14.7, whereas the surface charge for the complexes made of varying Dex-G and ctDNA ratios was found to increase from +11 mV to +22 mV as in Table 1. As the polymer concentration increased, the zeta potential value also increased. Dex-G acquired the positive surface charge from the quaternary ammonium bases.

Intact compaction and condensation of ctDNA within the polymer was asserted by the measurement of the particle size by dynamic light scattering performed at 25 °C. The mean hydrodynamic diameters of the complexes of different weight ratios, along with their polydispersity indices are given in Table 1. No significant difference was detected in the size distribution profile during complex formation. Size was seen in the range 150–270 nm. The least size was detected for the nanoplex of ratio 1:1.

The determination of the size and zeta potential of Dex-G/ctDNA complexes in the presence of serum is an important factor in the study of gene delivery vectors. As shown in Table 1, the changes observed in the size of the complexes on incubation in the presence of serum cannot be considered significant when compared to the zeta potential of the complexes observed in the presence of serum. The complexes showed a size distribution of 243.1–389.9 nm. No obvious change was seen in the polydispersity index which ranged from 0.076 to 0.360. Complexes in the presence of serum resulted in zeta potentials between 2.39 and 9.60 with significant variations.

3.5. Buffering capacity

The ability of Dex-G to protonate over a pH range of 10–5 was studied by the acid–base titration. The buffering capacity versus pH profile of Dex-G and PEI was monitored and compared as given in Fig. 2. In the pH range from 10 to 6, Dex-G showed a titration profile that was higher than PEI, but similar at pH 6. The titration was performed with varying concentration of Dex-G to obtain an optimal concentration with a favourable proton binding ability. Over the entire pH range from 10 to 5, the derivative exhibited a considerable buffering capacity with a moderate decrease after the pH 7. Upto addition of 600 μl of 0.1N HCl, no significant change in the pH was detected. Beyond this point, the pH decreased rapidly than PEI.

3.6. Gel retardation studies

The strength of complexation of Dex-G with ctDNA was ascertained by performing agarose gel electrophoresis. From Fig. 3, it was

Table 1
Zeta potential (mV), hydrodynamic diameter (nm) and polydispersity index of Dex-G–ctDNA complexes at different weight ratios.

Samples (polymer:DNA weight ratios)	In presence of 0.09% NaCl			In presence of transfection medium		
	Zeta potential (mV) ± sd	Average diameter (nm) ± sd	Polydispersity index (PDI) ± sd	Zeta potential (mV) ± sd	Average diameter (nm) ± sd	Polydispersity index (PDI) ± sd
Dex-G:DNA 0.5:1	11.7 ± 1.08	178.4 ± 2.042	0.118 ± 0.014	2.39 ± 0.238	251.5 ± 1.191	0.360 ± 0.008
Dex-G:DNA 1:1	13.1 ± 0.709	162.6 ± 0.969	0.406 ± 0.047	3.11 ± 0.427	243.1 ± 1.305	0.216 ± 0.027
Dex-G:DNA 1.5:1	18.5 ± 2.15	266.8 ± 3.04	0.180 ± 0.017	3.51 ± 0.140	389.9 ± 2.429	0.312 ± 0.065
Dex-G:DNA 2:1	20.6 ± 1.99	257.0 ± 1.736	0.300 ± 0.052	6.17 ± 0.326	360.2 ± 1.784	0.270 ± 0.053
Dex-G:DNA 3:1	20.7 ± 0.379	227.6 ± 1.266	0.311 ± 0.007	8.17 ± 0.598	333.1 ± 1.655	0.346 ± 0.011
Dex-G:DNA 4:1	22.3 ± 0.361	215.5 ± 1.584	0.348 ± 0.013	9.60 ± 0.429	268.4 ± 1.501	0.076 ± 0.039

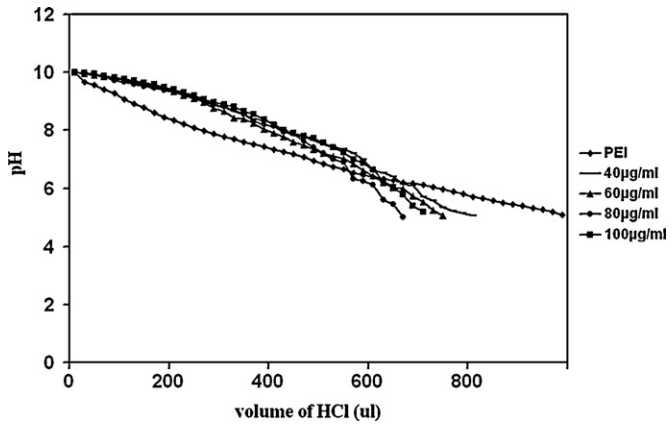

Fig. 2. Acid base titration curves of Dex-G of different concentrations (40 μg/ml to 100 μg/ml). The solution was titrated with 0.01N HCl. The titration curve of PEI (1 mg/ml) is given as reference.

observed that, as the weight ratio increased, the complexed DNA mobility towards the anode was retarded. The electrostatic interaction between the polymer and ctDNA restricted the free movement of the DNA. As the polymer concentration increased, the ctDNA gradually lost its mobility.

3.7. Stability of complexes in plasma

The stability of the complexes in the presence of plasma was checked on agarose gel by visualizing the mobility of the DNA along with its degradation as in Fig. 4. The DNA bands obtained were similar to that of the bands obtained in its absence. No extra release of DNA was observed as a result of the presence of plasma.

3.8. DNase I protection assay

Fig. 5 shows the DNase I challenge study on agarose gel. Uncomplexed and intact DNA were rapidly degraded by DNase I (Lane 2). As the concentration of the polymer increased, the pattern of fluorescence intensity was also found to increase. The protection assay was determined by the detection of the intensity of fluorescence of the DNA bands (Lane 3–7). Intense fluorescence was seen in some loading wells (Lane 6 and 7) whereas diffused fluorescence was also detected in other wells (Lane 3–5).

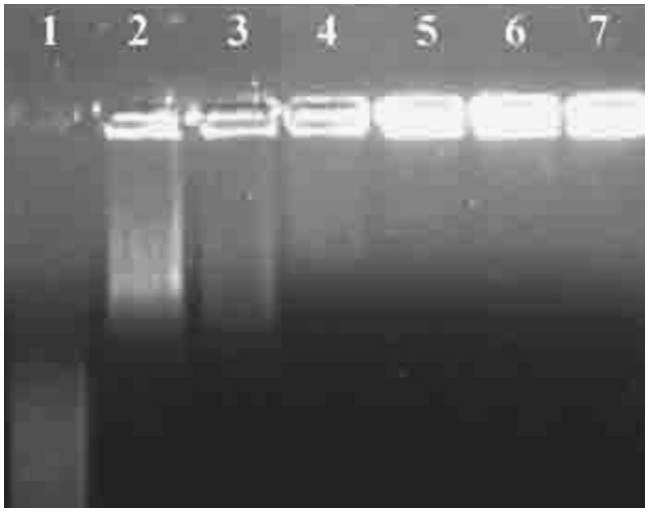

Fig. 3. Agarose gel electrophoresis of Dex-G-/DNA complexes at different weight ratios detected after 30′ of incubation compared with naked DNA. Lane 1 indicates calf thymus DNA. Lanes 2–7 indicates the polymer/DNA complexes (0.5, 1, 1.5, 2, 3 and 4).

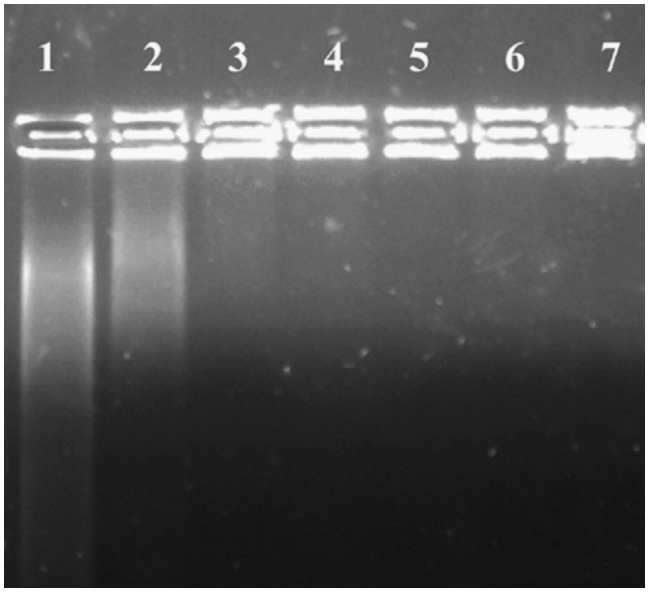

Fig. 4. Stability of Dex-G/DNA complexes after 30′ incubation with plasma at room temperature. Lane 1 indicates naked calf thymus DNA. Lanes 2–7 indicates the polymer/DNA complexes at different weight ratios (0.5, 1,1.5, 2, 3 and 4).

3.9. Native PAGE analysis of Dex-G with plasma proteins

The binding extent of plasma proteins with Dex-G was examined by performing native PAGE using samples incubated with plasma for 20 min as in Fig. 6. Dextran and PEI was also used as a comparative tool in the presence of plasma. PEI was found to bind to most of the plasma proteins present (Lane 5). This was detected by the absence of protein bands. Most of the plasma proteins did not bind itself to dextran (Lane 6 (Lane 2–4). Dex-G showed no interactions with the plasma proteins as all of the plasma protein bands were detected in the presence of Dex-G.

3.10. Blood compatibility studies

3.10.1. Haemolysis

No erythrocyte membrane disturbance was detected. No evidence of haemolysis was seen with the polymer itself in contact with the erythrocytes as in Table 2. The %haemolysis was detected to be less than 1%.

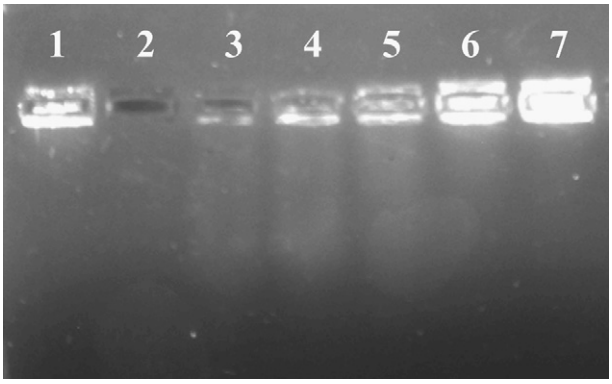

Fig. 5. Electrophoretic mobility data of Dex-G/DNA complexes following DNase I digestion. Lane 1: naked DNA; Lane 2: enzyme treated naked DNA; Lane 3–7: Dex-G/DNA complexes at different weight ratios (0.5, 1, 2, 3 and 4).

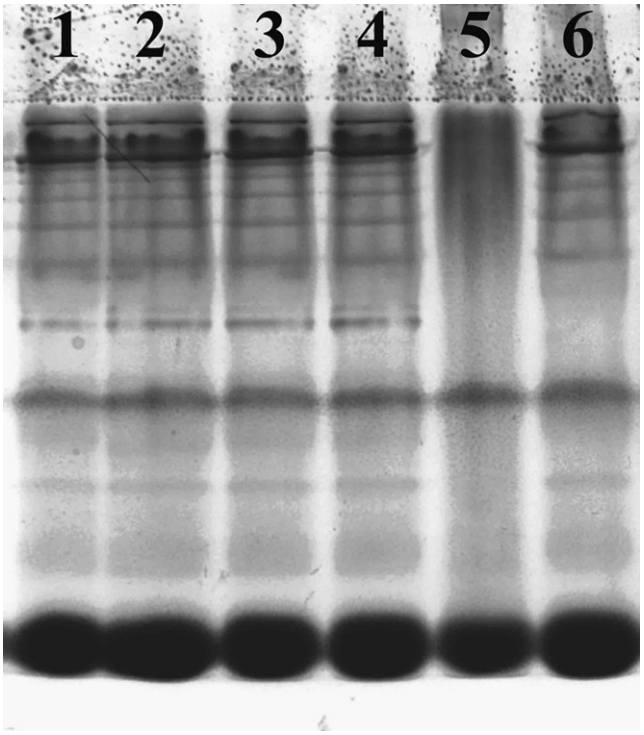

Fig. 6. Native Page analysis of Dex-G, PEI and Dextran with plasma after 20′ incubation at room temperature. Lane 1 indicates plasma proteins. Lanes 2–4 indicates the Dex-G and plasma. Lane 5 indicates PEI and plasma. Lane 6 indicates dextran and plasma.

3.10.2. RBC aggregation

RBC aggregation was performed for different weight ratios of Dex-G and PEI with ctDNA (ratios 1:2 and 1:4). RBCs suspended in the samples did not aggregate as shown in Fig. 7. They either had no demonstrable aggregation effect or inhibited aggregation when added to RBCs. PEI complex showed aggregation whereas Dex-G itself did not promote aggregation.

Table 2
Percentage of haemolysis of Dex-G/DNA complexes of different weight ratios measured after incubation with erythrocytes for 30′.Triton X 100 and 0.09% NaCl was taken as the positive and negative control respectively.

Samples	%Haemolysis	Standard deviation
Dex-G:DNA 1:0.05	0.0115	0
Dex-G:DNA 1:1	0.0690	0.0163
Dex-G:DNA 1:1.5	0.1380	0.0079
Dex-G:DNA 1:2	0.3221	0.0329
Dex-G:DNA 1:3	0.3796	0.0085
Dex-G:DNA 1:4	0.3911	0.0167
Dex-G:DNA 1:5	1.012	0.0258
Dextran derivative (100 μg)	0.7363	0.0333
Triton X 100	100	–
Saline	0	–

3.10.3. WBC aggregation

Different weight ratios of Dex-G and PEI with ctDNA were taken and incubated with WBCs for 30 min. Fig. 8 showed no evidence of aggregation for the derivative sample analyzed. No significant difference was seen in the ability of Dex-G complex to inhibit WBC's aggregation. PEI complex led to WBC aggregation.

3.11. EtBr displacement assay

The % fluorescence of EtBr DNA complex was taken as 100% whereas that of EtBr alone showed a % fluorescence of 37%. As the concentration of Dex-G increased, decrease in fluorescence was seen, as in Fig. 9. Stability was found to be retained till ratio 1:4.

3.12. Cytotoxicity

In cytotoxicity assay, three samples along with positive and negative controls were taken in duplicates. The % viability of dextran, Dex-G and PEI was compared in Fig. 10. As the negative control, Triton X 100 and as the positive control 10% Phenol was taken. The quantitative assessment of cytotoxicity by the MTT assay of cells after contact with the derivative showed 96.3% metabolically active cells. The viability test proved Dex-G to be the least toxic polymer on comparison with dextran and PEI. Dextran showed similar

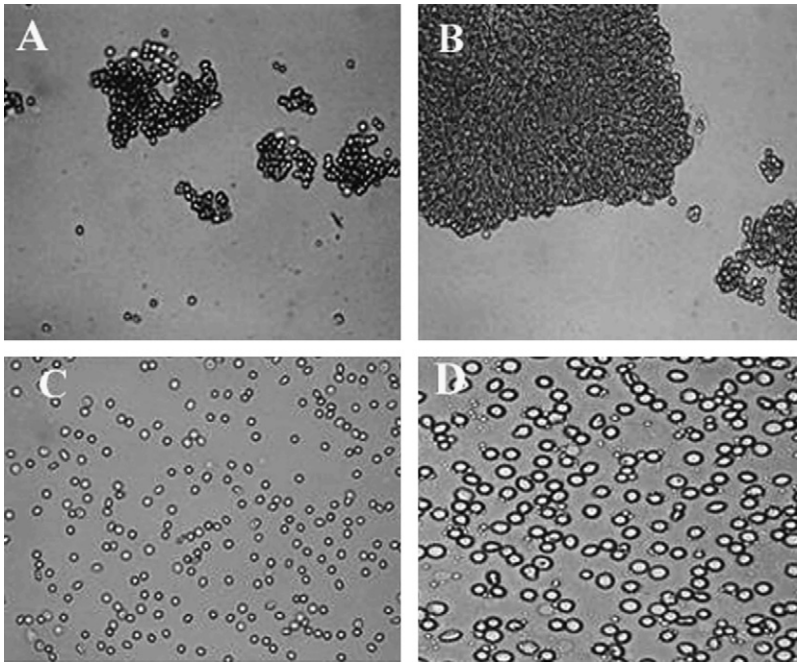

Fig. 7. Microscopic view of erythrocytes after incubation with PEI (A, B) and Dex-G (C, D) complexed with ctDNA at ratios 1:2 and 1:4 for 30′ respectively.

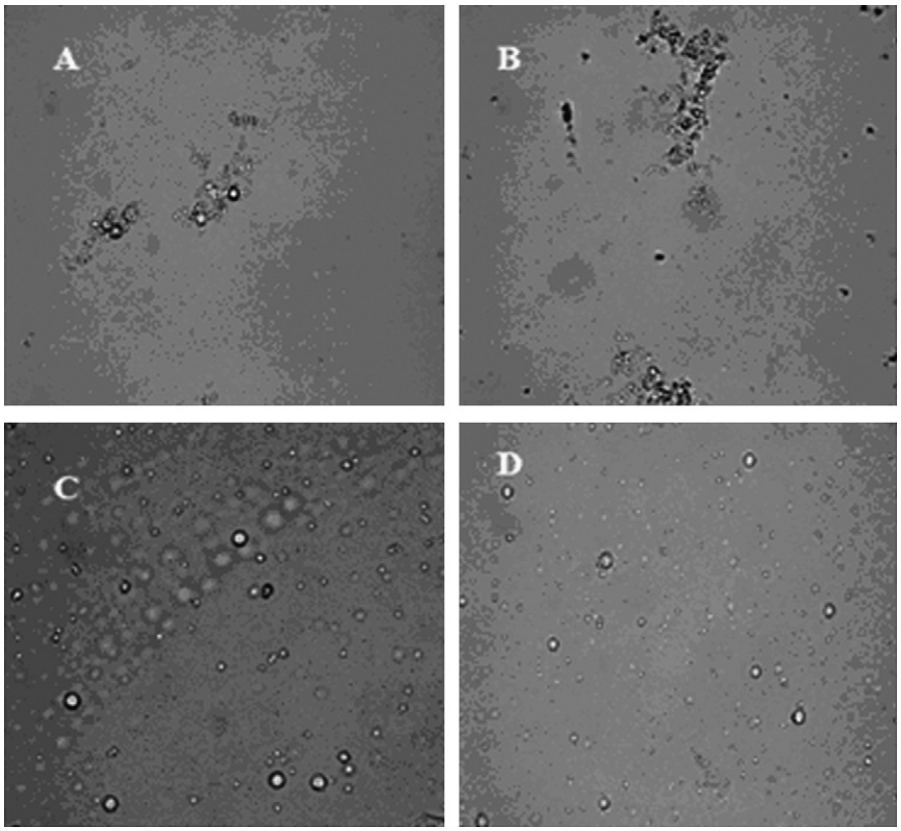

Fig. 8. Microscopic view of leukocytes after incubation with PEI (A, B) and Dex-G (C, D) complexed with ctDNA at ratios 1:2 and 1:4 for 30′ respectively.

results as that of Dex-G whereas PEI showed a lower percentage viability of 45.3% (Fig. 10).

3.13. Flow cytometry analysis

Fluorescence-activated cell sorting (FACS) analysis was performed as a reference to the transfection studies. A true picture of the appearance of transfected cells was visualized by flow cytometry which was based on the reaction between the isothiocyanate group of FITC and the amino groups of Dex-G. The samples were subjected to flow cytometry until 10,000 events has been counted. As control, population of cells without incubation with the nanoplex was evaluated. On quantification of the FITC fluorescence evaluated using cell lines, 45% of cells were detected to be FITC positive (Fig. 11) Quantification using HepG2 cell line also provided a favourable data.

3.14. In vitro transfection activity

Fig. 12 shows the transfection efficiency of the Dex-G when coupled to DNA in ratios 1:4 and 1:7. When compared to the control reagent PEI, Dex-G showed a considerable high level of transfection efficiency favourably in HepG2 cells. The level of gene expression differed among the two ratios used. The high level of gene expression was detected for the lower ratio (1:4) of Dex-G/DNA complex. As the ratio of the complex went higher, the electrostatic interaction decreased after a certain ratio, thus reducing transfection efficiency.

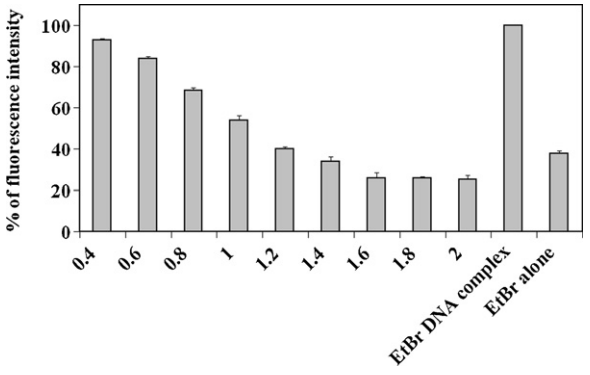

Fig. 9. The decrease in the relative fluorescence intensity was detected with the increase in the concentration of Dex-G. Complexes of Dex-G and ctDNA were taken in different ratios (0.4:1–2:1). The fluorescence intensity of EtBr and EtBr complexed with ct DNA was also detected.

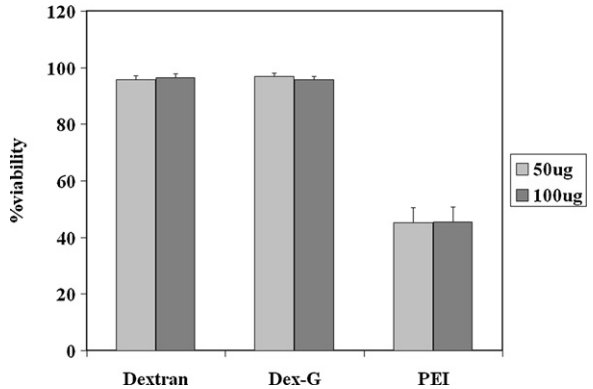

Fig. 10. Cytotoxicity of HepG2 cells after incubation of Dextran, Dex-G and PEI for 24 h. Cytotoxicity was evaluated by the MTT assay.

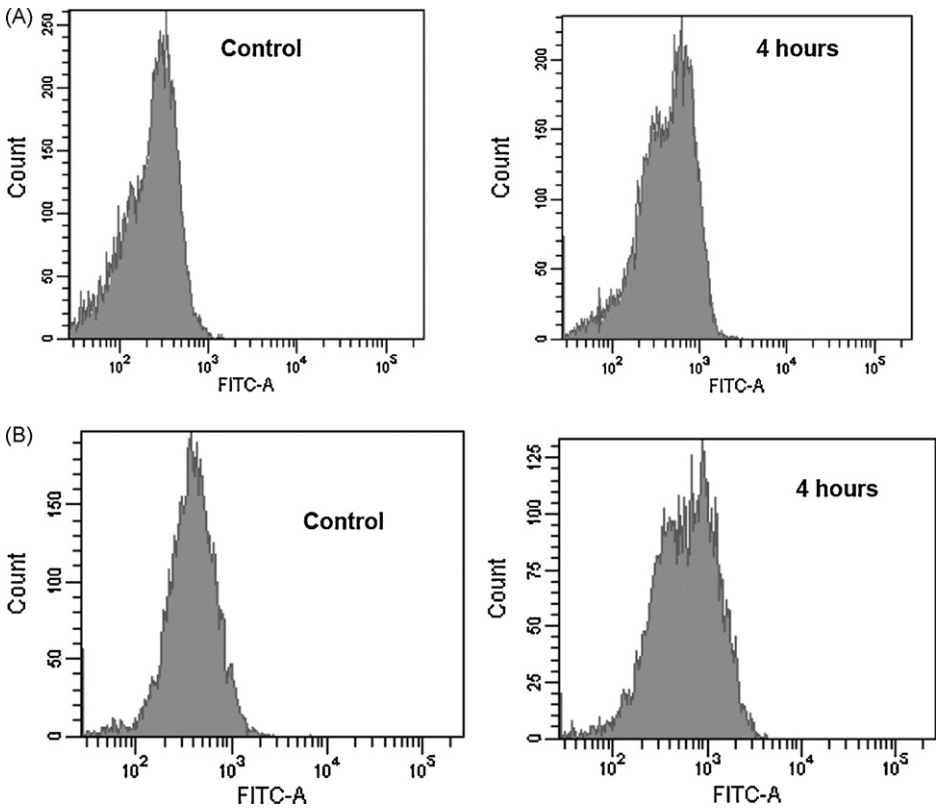

Fig. 11. Evaluation of transfection efficiency by FACS analysis of cells incubated with Dex-G/DNA complexes for 4 h with reference to control (A) L929 cells and (B) HepG2 cells.

The influence of serum on transfection efficiency of Dex-G/DNA complexes was assessed in both HepG2 and Hela cell lines using two different ratios of 1:3 and 1:4. Lower ratios were chosen because as observed from the above data with increasing ratios the transfection efficiency was found to be decreasing. As shown in the Fig. 13(A), the pattern of transfection in Hela cell line was found to be higher in the presence of serum. The level of gene expression differed among the two ratios used. In both the cell lines, transfection efficiency obtained was higher for the ratio of 1:3 and was comparable to the transfection efficiency of PEI in the case of HepG2 cell line. The best relative transfection efficiency was observed in the case of HepG2 cells at the ratio of 1:3 in the presence of serum as in Fig. 13(B). The efficiency was evaluated using PEI in ratios 1:3 and 1:4 as the control.

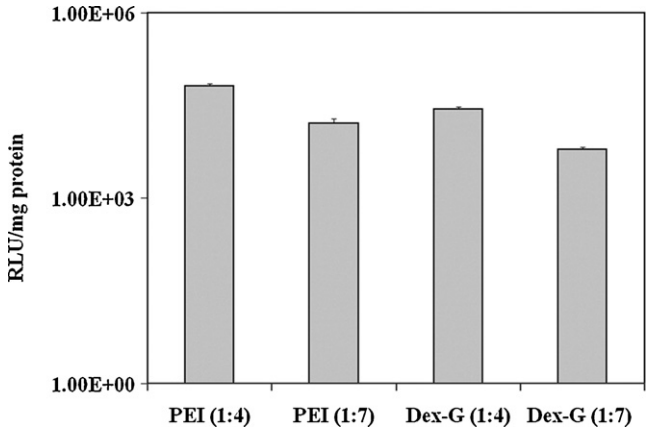

Fig. 12. Luciferase expression in HepG2 cells transfected by Dex-G/DNA complexes and PEI/DNA complexes taken at two different ratios (1:4 and 1:7).

4. Discussion

A large variety of cationic polymers have been studied in terms of their unique properties which can be used in gene therapy (Boussif et al., 1995; Kabanov et al., 1991; Haensler and Szoka, 1993). Many polycationic polymers (such as PEI) have been found to be good transfecting agent. But the drawback detected is that it is toxic. Of the lot, one of the most widely studied polymer is dextran. Inspite of its applications, dextran has its own drawbacks. Its high polarity provides difficulty in transcellular passages.

In this study, a dextran derivative (Dex-G) was prepared by modification of dextran with GTAC so as to enhance its use as an efficient gene carrier. Cationization of dextran was due to the attachment of quaternary ammonium groups to the polymer chain. A decrease in the epoxy groups was found to be accompanied with an increase in the free hydroxyl groups, thereby promoting conventional hydrolysis of epoxy groups (Joana et al., 2006).

On comparison of the peak intensities of both dextran and Dex-G NMR spectrum, the characteristic peak at 3.169 ppm which originated from GTAC was detected in the Dex-G spectrum. This confirmed the successful derivatization of dextran with GTAC.

The modification of Dex-G was also ascertained by calculating the degree of substitution. The presence of quaternary ammonium groups was confirmed from the ^{1}H NMR spectra. A one step reaction formulated Dex-G with a degree of substitution of 20.92 confirming the modification as required.

Complexes were prepared using Dex-G and ctDNA. The ctDNA concentration was kept constant whereas the concentration of Dex-G was varied. The electrostatic interaction between the positive groups of Dex-G and the negative groups of DNA led to the complexation. Several investigations were done using the complexes prepared in different weight ratios.

The two main parameters governing the use of the complexes in gene therapy is their size and zeta value (Zanta et al., 1997). Particles

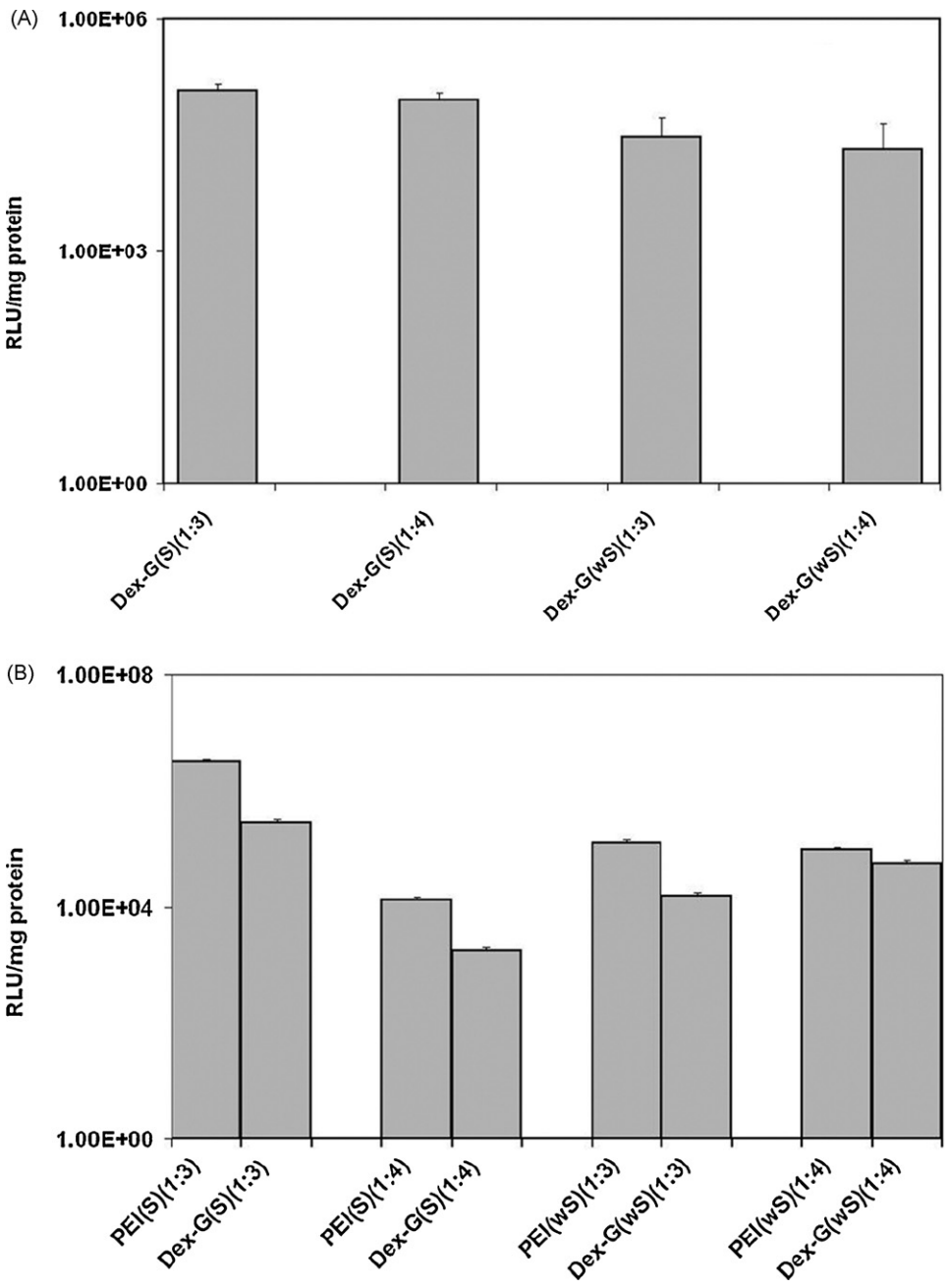

Fig. 13. (A) Transfection efficiency of Dex-G/DNA complexes in the presence (S) and absence (wS) of serum performed in HeLa cell line. The complexes were taken at two different ratios of 1:3 and 1:4. (B) Transfection efficiency of Dex-G/DNA complexes in the presence (S) and absence (wS) of serum performed in HepG2 cell line. The complexes were taken at two different ratios of 1:3 and 1:4 and compared with that of PEI.

in either large or small size have their advantages and disadvantages. If DNA is condensed into small complexes, it easily enters into the cell (Lin et al., 2006). However, larger particles may have a high loading capacity, but restrict proper cell entry. The Dex-G/ctDNA complex was found to be stable at ratio 1:1 which formed the smallest complex. Particle size detected showed the condensation of the nanoplex to a diameter range of several hundred nanometers which proved to be favourable for transfection. Several parameters including the concentration of DNA may influence particle size (Kircheis et al., 2001).

A positively charged DNA carrier helps in their interaction with the negatively charged cell membrane leading to the easy release of DNA into the cell (Kim et al., 2007). The cationization of dextran was affirmed by the positive zeta value obtained for its derivative after the reaction. This was also expected to allow transfection easily. The higher the polymer concentration, the more was the zeta potential value detected. This asserted the stability of the complex and its success of formulation.

A steady control of the size and zeta potential of the delivery vehicle in the presence of serum is of great importance. Possible interference by serum was checked by measuring the size and zeta potential of the complexes on incubation with 10% FBS. The presence of serum can lead to the disruption of complexation by the formation of aggregates or electrostatic interactions (Florea et al., 2002). The presence of serum neither led to aggregation of the complexes nor perturbed the formation of Dex-G/ctDNA complexes. This proves that the Dex-G/ctDNA complex formation is a strong and efficient process. The zeta potential decreased considerably on

the addition of FBS to the complexes. This reduction was due to the attachment of the negatively charged serum proteins onto the surface of the complexes (Finsinger et al., 2000; Trubetskoy et al., 1999).

The complexation ability of the Dex-G, stability of the nanoplexes in plasma and protection of ctDNA by Dex-G from nuclease digestion was determined by agarose gel electrophoresis. Nanoplexes with different polymer weight ratios were studied. Strong complexation between polymer and DNA was detected by the retardation of DNA in the well (Kabanov and Kabanov, 1995). As the polymer concentration increased, the ctDNA gradually lost its mobility due to the increase in the shielding effect of the polymer over DNA. The electrostatic interaction between the polymer and ctDNA restricted the free movement of the DNA (Wolfert et al., 1996). This is due to the charge neutralization between the negatively charged DNA and the positively charged polymer. It also confirmed the optimal ratios of complex formation.

The major consideration to be confronted in formulating efficient gene delivery vehicles is its stability in the physiological environment. The stability of the nanoplex in the presence of plasma components was also determined by the retardation of the DNA in the well. The complex showed good stability as similar DNA bands were obtained both in the presence and absence of plasma. Dex-G provided strong shielding effect over DNA and no leakages were detected. Plasma brought about no significant changes to the complexes.

Degradation of DNA by nucleases is one of the most important barriers faced by gene vectors (Hashida et al., 1996). Encapsulation of ctDNA was analyzed by examining the fragmentation of unprotected DNA as a result of the endonuclease activity of DNase I. Protection of ctDNA from nuclease digestion by Dex-G was confirmed by the intense fluorescence seen in the well when compared to the naked DNA digested with DNase I where no band was seen. This confirmed the stability of the nanoplex in the presence of nucleases. The detection of intense fluorescence proved the tight association between Dex-G and DNA and indicated the entrapment of the DNA within the polymer nanoparticles whereas diffused fluorescence was due to the leaching of small amounts of DNA from the nanoparticles.

The binding extent of Dex-G with plasma proteins was shown by Native PAGE. Absence of protein bands in the presence of PEI confirmed its interaction with plasma proteins. The lack of absence of plasma protein bands confirmed the stability of Dex-G in the presence of plasma proteins. Dex-G provided evidence for its inability to bind to plasma proteins confirming its safety to be used as a gene delivery agent.

Transfection efficiency correlates to the proton binding capacity of the polymer used as the gene delivery vehicle. Complexes need to escape from endosome in order for transfection to take place. The buffering capability of the polymer plays an important role in the escape mechanism (Park et al., 2005). Polyethyleneimine is considered as the best vector due to the presence of a high amount of amine functions of which only a small portion undergoes protonation under physiological conditions (Hashida et al., 1996). Dex-G showed a considerable buffering capacity when compared to PEI. A decrease in buffering was seen after pH 7. This proves its reduced transfection capability when compared to PEI. This affirms the polymer a capability to disrupt the endosome along with a slight decrease in its protonation due to the cationic behaviour extended to dextran on modification with quaternary ammonium groups. An increase in the buffering capacity detected in the beginning of the titration was due to the presence of the quaternary ammonium groups attached to Dex-G.

Condensation of ctDNA with Dex-G was confirmed by the EtBr displacement assay. Nucleic acids fluoresce in the presence of EtBr due to the intercalation of the dye with the minor groove of DNA helix (Warning, 1965). When polycations are added to the solution containing DNA intercalated with EtBr, EtBr gets excluded from DNA and the polymer–DNA complex is formed. A decrease of fluorescence was detected on exclusion of EtBr from the DNA. EtBr was first intercalated with ctDNA. On incubation with Dex-G, EtBr was found to be displaced from DNA due to the strong interaction between the polymer and DNA. This resulted in the decrease of fluorescence as EtBr was excluded.

Haemocompatibility is a major study which helps to detect the harmful effects of the polymer in the body. The polymer, after administered into the body, first comes into contact with blood and requires a good compatibility to ensure its role as a gene delivery system (Cavallaro et al., 2008). The damaging property of polymers on the cell membrane of blood cells was quantified by the haemolysis study. Haemolysis mainly occurs by the colloid osmotic mechanism where the net influx of solutes causes an osmotic imbalance leading to the lysis of the red blood cell (Murthy et al., 1999). Here, Triton X 100 and 0.9% NaCl solution are used as the positive and negative control respectively. Percentage of haemolysis detected was negligible. This proves that the polymer is not membrane lytic.

The major factor governing the delivery of genes into the body via efficient gene carriers is RBC aggregation (Björn Neu and Meiselman, 2002). Aggregation mainly occurs due to the electrostatic interactions between anionic cell membrane and cationic polymer. Aggregation studies were performed to detect the property of the polymer over blood cells visually. Major complications occur due to blood incompatibility (Gorbet and Sefton, 2004). Haemocompatibility was also determined by the detection of WBC aggregation. No aggregation was detected in both cases of RBC and WBC aggregation studies with Dex-G. This results due to the positive charge density attained by Dex-G which determines the extent of aggregation.

In vitro cytotoxicity was performed with Dex-G at two different concentrations. PEI was found to be highly toxic when compared to Dex-G. The cell viability detected for Dex-G was found to be higher when compared to PEI at a concentration of 100 μg/ml. This proved Dex-G to be least toxic. Cell viability of dextran and Dex-G was found to be similar. Modification of dextran has made it convenient to be used as a gene carrier.

FACS analysis was performed as a comparison to the transfection study. A true picture of the appearance of transfected cells can be visualized by flow cytometry which is based on the reaction between the isothiocyanate group of FITC and the amino groups of Dex-G. The samples were subjected to flow cytometry until 10,000 events has been counted. The percentage of cells transfected as per the flow cytometry was found to be 45%. According to Yuji Teramura, the decrease in fluorescence intensity may be due to the release of FITC labeled polymer from the cells to the culture medium. Cell division may also result in the same (Teramura et al., 2008).

A good transfecting vector is one who has the ability to condense and compact the genetic material, bind to the cell surface and facilitate its uptake (Kunath et al., 2003). The gene which it carries should be delivered to the targeted cells and provide a high gene expression. The stability of transfection governs the efficiency of the delivery system (Petersen et al., 2002). Transfection was done using HepG2 cells and nanoplexes, taken in two different ratios. Transfection by Dex-G showed a considerable high level of gene expression when compared to PEI. It was seen that transfection was favourable at a lower ratio when compared to the higher one. This maybe due to the strong complexation of the polymer with DNA at a lower ratio. As the ratio increases, the shielding effect of polymer over DNA decreases and thus leading to reduced transfection efficiency. This maybe due to the strong complexation between the polymer and DNA at a lower ratio. Reduction of transfection effi-

ciency can result by the inhibition of the non-specific interactions of the polyplex with the serum proteins. As the ratio of the complex goes higher, the electrostatic interaction may decrease after a certain ratio, thus reducing transfection efficiency. Transfection efficiency apparently depends on the particle size of the polyplexes (Xu et al., 2007). However, formation of nanoplexes was also found to affect transfection due to the conformational change undergone by DNA after its interaction with the polymer.

The effect of serum on transfection was investigated in two different cell lines using two different ratios of 1:3 and 1:4.The high level of expression detected for the lower ratio of 1:3 resulted due to the strong complexation between the polymer and DNA at a lower ratio. According to Elisa Brunette et al, several advantages persist on transfecting cells in the presence of serum like easier and less time consuming transfections, low experimental cost and avoidance of cells deprivation of serum (Brunette et al., 1992). In both the cell lines, transfection efficiency of Dex-G/DNA complexes enhanced in the presence of serum. The efficiency was evaluated using PEI as the control. Major factors influencing transfection efficiency are surface charge and size of the complexes used. When the size of the complex becomes too small, they may get easily trapped by the cells. Large particles facilitate the escaping of DNA from the complex into the nucleus. (Kunath et al., 2003; Tian et al., 2007). They also help to increase the sedimentation of complex which proves favourable for cell attachment and uptake thus promoting transfection efficiency. The increase in zeta potential of the complex may increase cytotoxicity thus lowering transfection efficiency (Kircheis et al., 1992). In this study, in the presence of 10% FBS, the size of Dex-G/DNA complexes slightly increased whereas the zeta potential decreased providing a smaller positive charge. The stability of Dex-G in serum and its high transfection efficiency signifies its role as a promising candidate in gene delivery.

From the above investigations, it is evident that cationization of dextran with GTAC can really help the polymer to act as a good gene carrier. This derivative showed a favourable transfecting capability along with reduced cell toxicity. Moreover it was identified to be haemocompatible and stable at different conditions provided. These nanoplexes have enhanced cell viability with reduction in transfection.

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