Journal of Experimental Nanoscience
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t716100757

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To cite this Article Dutta, Tathagata , Aghase, Hrushikesh B. , Vijayarajkumar, P. , Joshi, Mahendra and Jain, N. K.(2006) 'Dendrosome-based gene delivery', Journal of Experimental Nanoscience, 1: 2, 235 — 248
To link to this Article: DOI: 10.1080/17458080600647146
URL: http://dx.doi.org/10.1080/17458080600647146

Journal of Experimental Nanoscience,
Vol. 1, No. 2, June 2006, 235–248

Taylor & Francis
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Research Paper

Dendrosome-based gene delivery

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(*Received January 2006; in final form February 2006*)

Gene transfer to humans requires carriers for the plasmid DNA, which can efficiently and safely carry the gene into the nucleus of the desired cells. The purpose of the present study was to design dendrosomes as a novel, non-viral, vesicular, gene delivery vector and to carry out a comparative study of the relative transfection efficiencies of dendrosomes with standard non-viral, gene delivery vectors.

Fourth-generation PAMAM dendrimers were synthesized by double the Michael addition reaction and extensively characterized. The dendrimer–DNA complex was prepared and was confirmed by CD spectroscopy. The dendrosomes were prepared by the reverse phase evaporation method and the entrapment efficiency of the dendrosomal formulation was estimated. *In vitro* toxicity of the formulation was evaluated by hemolytic toxicity and cytotoxicity studies. Transfection efficiency of the dendrosomal formulations was compared to standard non-viral gene delivery vectors in HEK-293 cell.

The results of hemolytic toxicity cytotoxicity studies demonstrated that the dendrosomes possess negligible toxicity as compared to the other formulations and are suitable for *in vivo* administration. The results of transfection of HEK-293 cell with PGL2 showed that the dendrosomal formulation DF3 possesses superior transfection efficiency against other delivery systems under study.

Dendrosomes possess tremendous potential as a novel non-viral and non-toxic gene delivery vector.

Keywords: Dendrimers; Dendrosomes; Gene Delivery; Transfection

1. Introduction

Drug delivery research currently evaluates the potentials and benefits of synthetic gene carriers including cationic liposomes and polymers for gene therapy. While the cationic liposomes enjoy several advantages like spontaneous transfection due to net positive charge, high reproducibility of complex formation and non-immunogenicity, disadvantages like cytotoxicity and lack of stability of the lipoplex restrict their success [1].

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Journal of Experimental Nanoscience
ISSN 1745–8080 print/ISSN 1745–8099 online © 2006 Taylor & Francis
http://www.tandf.co.uk/journals
DOI: 10.1080/17458080600647146

With regard to polymers, much attention is paid to cationic polymers (CPs), which are able to condense large genes into smaller structures and to mask the negative charge of the DNA, the two most important necessities for transfection of most type of cells. Also neutral polymers like polyvinyl alcohol (PVA), which do not condense DNA, are evaluated to protect naked genes from extra cellular nuclease degradation and to retain them better at the site of injection after intramuscular injection [2]. Moreover, polymeric nano(micro) particles[3] which adsorbs or encapsulate oligonucleotides or genes, are under investigation as sustained release matrices for genetic drugs.

Dendrimers are highly branched and reactive three-dimensional polymers, with all bonds emanating from a central core. The term 'dendrimer' originated from the Greek word 'dendron' meaning tree and suffix 'mer' from meros, denoting smallest repeating units. In recent years dendrimers have attracted enormous attention among researchers working in the field of biomedical sciences. Attractive features like nanoscopic size, highly controllable molecular weigh, large number of readily accessible terminal functional groups and possibility of encapsulating a guest molecule in internal cavities give dendrimers a distinct edge over other polymers for the delivery of drugs [4]. The nanoscopic size not only helps the dendrimers to evade the reticuloendothelial system (RES) of the body but also make them extremely important for intracellular drug delivery. Inherent cationic charge and spherical shape associated with the amine terminated dendrimers make them highly potential for delivery of genes and immunogens [4]. The polyamidoamine (PAMAM) dendrimers are highly branched, globular dendrimers with primary amino groups at the periphery. Due to the presence of a definite number of primary amino groups, the PAMAM dendrimers possess a finite positive charge, which is responsible for condensation of the DNA and subsequent transfection [5, 6]. They are well-known cationic gene delivery vectors [7] and form a stable complex with DNA through electrostatic interactions. The dendrimer–DNA complex due to its net positive charge penetrates the cell membrane and thereby releases the DNA leading to subsequent gene expression [8–10]. However, the dendrimers are reported to possess hemolytic toxicity and cytotoxicity owing to their polycationic nature. Moreover, interaction with the oppositely charged macromolecules in plasma results in premature release of DNA within the blood [9, 11–13]. Also, degradation of the plasmid DNA by DNAase present in the plasma leads to poor gene expression *in vivo* [14–20]. The role of dendrimers in gene delivery has been extensively reviewed elsewhere [21–25].

We developed and investigated the transfection potential of dendrosomes, a novel non-viral gene delivery vector. The proposed dendrosomes are novel, vesicular spherical supramolecular entities, containing entrapped dendrimer–DNA complex, possessing negligible hemolytic toxicity, higher transfection efficiency and better *in vivo* acceptability.

2. Experimental

2.1. *Materials*

Methylmethacrylate, ethylenediamine and methanol were purchased from Fluka; phosphatidylcholine, cholesterol and 3-(4,5-dimehylthiazol-2-yl)-2,5-diphenyl

tetrazolium bromide (MTT) were obtained from Sigma Chemicals (St. Louis. MO). Diethyl ether and dimethyl sulfoxide (DMSO) were purchased from Rankem (Ranbaxy Labs. Ltd. India). Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM) was obtained from Nissui Pharmaceuticals (Tokyo, Japan). Foetal calf serum (10% FCS) was obtained from Biowhittaker (Walkersville, MD). Fourth-generation polyamidoamine (PAMAM) dendrimers were synthesized according to the method reported by Tomalia [5]. Fugene-6 (Roche Biomolecules, UK), a standard transfection reagent, was the benevolent gift of Dr. Uma Roy of CDRI Lucknow, India, and used without any further characterization.

2.1.1. Cells and plasmid. Human embryonic kidney cells (HEK-293) and PGL2 plasmid were the benevolent gift of Dr. Uma Roy of CDRI, Lucknow, India.

2.2. *Synthesis of fourth-generation PAMAM dendrimers*

PAMAM dendrimers were prepared according to the method reported earlier [5] using ethylenediamine as initiator core. In brief, methanolic methyl methacrylate solution (5% molar excess) was made to react with methanolic solution of ethylenediamine in a light-resistant round-bottomed flask to form ester-terminated dendrimer. The excess of methanol was evaporated using a rotary vacuum evaporator (Superfit, India) and the ester-terminated structure was treated with methanolic ethylenediamine (10 molar times) and kept for 55 h in dark. The excess of ethylenediamine was removed under high vacuum (5 mm of Hg) to yield 0.0G dendrimer. This reaction sequence was repeated required number of times to produce PAMAM dendrimers up to generation 4 (PAMAM G4). Completion of every step in the synthesis was confirmed by reacting with copper sulphate solution. The full generation gave a purple colour while half-generation gave a deep blue colour due to copper chelation at the terminal groups of the dendrimers. The synthesized PAMAM dendrimers were spectroscopically characterized. Prominent bands in IR and ^{1}H NMR spectra are presented in tables 1 and 2.

2.3. *Preparation of dendrimer–DNA complex*

The dendrimer–DNA complex was prepared [26] by addition of DNA to varying proportion of fourth-generation PAMAM dendrimers in phosphate buffer saline

Table 1. Major peaks in IR spectrum of fourth-generation PAMAM dendrimers.

S. No.	Wave No. (cm^{-1})	Functional group	Inference
1	3473.9	N–H asym stretch	Primary amine
2	3440	N–H sym stretch	Primary amine
4	2975.9	C–H stretch	–
5	1731.5, 1692.5	C=O stretch	Amide I band
6	1599.9	N–H in plane bending	Amide II band
7	1285.5	C–N stretch	Amines
8	630.1	OCN deformation	Amide IV band
9	1109.6, 1052.7	C–C bend	–

Table 2. ^{1}H NMR shifts of fourth-generation PAMAM dendrimer.

S. No.	Chemical shift (δ ppm)	Interpretation
1	2.36–2.39 (triplet)	R_2CH_2
2	2.64–2.69 (triplet)	$–CH_2CO–$
3	2.770–2.815 (triplet)	$>N–CH_2–CH_2–CO–NH$
4	3.026–3.067 (triplet)	$–CH_2NH_2$

Table 3. Zeta potential of various dendrimeric formulations at different charge ratios.

S. No.	Formulation code	Dendrimer : DNA charge ratio	Zeta potential (mV)
1	DGC10	10 : 1	+1.54
2	DGC25	25 : 1	+4.10
3	DGC50	50 : 1	+8.40
4	DGC75	75 : 1	+12.65
5	DGC100	100 : 1	+16.93

(PBS pH 7.4) and incubated at 25°C for 20 min to allow complex formation. By using a constant amount of DNA and varying the dendrimer concentration, different dendrimer–DNA charge ratios were obtained and their transfection efficiency and gene expression was evaluated.

2.4. *Zeta potential*

The zeta potential of various formulations of dendrimer–DNA complex having different charge ratios was determined using Zetasizer 3000HS (Malvern Instruments, UK).

2.5. *Circular dichroism*

The solution of pure DNA, dendrimer and dendrimer–DNA complex were prepared in PBS (pH 7.4). The CD spectroscopy was carried out in a Jasco, J-600 Spectropolarimeter (Jasco, Japan) using a sample cell path length of 1 mm and sample volume of 1.25 ml. The concentration of the sample solution was 1 mg/ml and the scanning range was 200–300 nm.

2.6. *Optimization of dendrimer–DNA charge ratio*

Human embryonic kidney (HEK-293) cells were grown in DMEM supplemented with 10% foetal calf serum (FCS). The cells were seeded in 96 well plates and left for 24 h to achieve 50–80% confluency. The cells were transfected with 0.08 μg of PGL2 (Promega) using either fourth-generation PAMAM dendrimers, dendrosomes or fugene-6 according to the transfection protocol described below.

The dendrimer–DNA charge ratio required for optimum transfection was determined by transfecting the cell lines with formulations of different dendrimer–DNA

charge ratios. The dendrimer–DNA complexes of varying charge ratios were dissolved (10%) in DMEM supplemented with 10% FCS. Microtiter plates were incubated for 20 min at 25°C. The growth medium was aspirated and replaced with fresh medium. Incubation was continued for 48 h followed by dual reporter gene assay. Separately the same protocol was carried out with fugene-6 as reference transfection agent and naked DNA as negative control.

2.7. *Preparation of dendrosomes*

Phosphatidylcholine (PC) and cholesterol (C) were dissolved in diethyl ether, in varying molar ratios (table 4), to which was added a solution of optimized dendrimer–DNA complex (100 µg/ml) in PBS (pH 7.4). The mixture was sonicated for 5 min with a pause of 1 min over an ice bath. Resulting thick emulsion was vortexed to remove traces of organic solvent. Dendrosome formation takes place by phase inversion (figure 2).

Table 4. Average particle size of various dendrosomal formulations.

S. No.	Formulation code	Molar ratio (PC : C)	Mean particle size (nm)
1	DF_1	9 : 1	130
2	DF_2	8 : 2	142
3	DF_3	7 : 3	157
4	DF_4	6 : 4	175
5	DF_5	5 : 5	177
6	DF_6	4 : 5	180

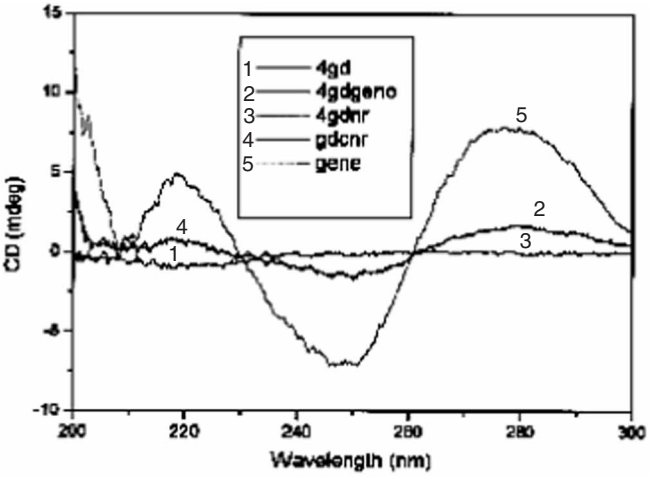


Figure 1. Circular dichroism spectra of dendrimer, naked DNA and dendrimer DNA complex. (1) 4gd: fourth-generation PAMAM dendrimer; (2) 4gdgenec: Dendrimer–DNA complex; (3) 4gdnr: fourth-generation PAMAM dendrimer noise reduced spectra; (4) gdcnr; noise reduced spectra of dendrimer DNA complex; (5) gene: naked DNA.

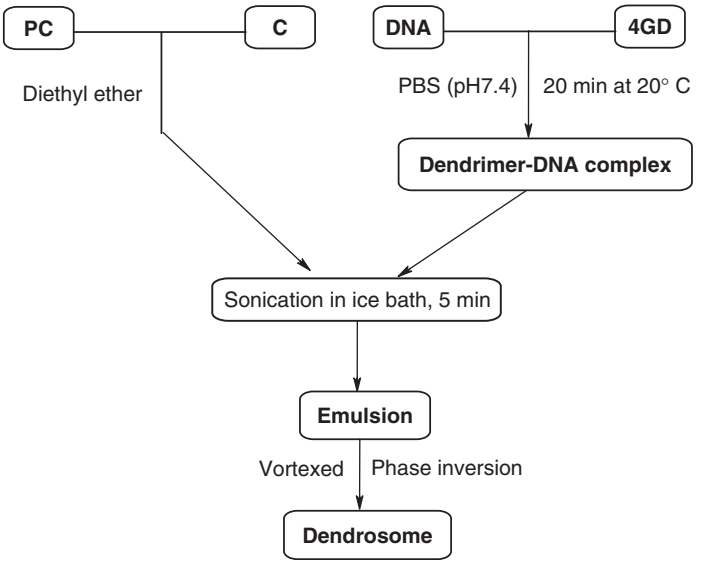


Figure 2. Schematic representation of dendrosome preparation (PC: Phosphatidylcholine, C: Cholesterol, 4GD: fourth-generation PAMAM dendrimer).

The dendrosomes were purified by passing them through a sephadex G 50 mini-column. The column was eluted with 0.5 ml PBS (pH 7.4). The size distribution of the dendrosomes was carried out using a dynamic light-scattering spectrophotometer (L-S 900 Otsuka Electronics, Japan).

2.8. *Determination of entrapment efficiency*

The entrapment efficiency (ratio of amount of dendrimer–DNA complex entrapped to the amount of complex added expressed in percentage) was determined by lysing dendrosomes with *n*-propanol. A dispersion of dendrosomes in PBS (pH 7.4) was mixed with an equal volume of *n*-propanol and vortexed for 10 min. The resulting solution was centrifuged for 15 min at 2500 rpm. Supernatant was withdrawn and analysed at 260 nm after appropriate dilutions against a PAMAM dendrimer solution as reagent blank.

2.9. *Hemolytic toxicity*

The study was designed based on the procedure reported by Malik *et al.* [27]. The objective of the study was to determine the haemolytic toxicity of dendrimer/fugene-6 and formulations based on them. 2% RBC suspension was prepared in PBS (pH 7.4). PAMAM 4G, dendrimer–DNA complex, fugene-6 (Roche Biomolecules, UK), fugene-6-DNA complex and dendrosomal suspension were added to RBC suspension (equivalent to 100 μg/ml of dendrimer/fugene-6). Triton-X-100 was used as positive control. All the samples were incubated at 37°C for 4 h and centrifuged

at 3000 rpm for 15 min. The supernatant was analysed at 550 nm spectrophotometrically (1601 UV–Vis spectrophotometer, Shimadzu, Japan). Percent haemolysis was calculated by considering absorbance of Triton-X-100 sample as 100% haemolysis.

2.10. *Transmission electron microscopy*

A drop of 0.001% w/v of methanolic solution of 4G dendrimer/dendrosomes was added to a carbon grid and stained with osmium tetroxide (negative stain) and viewed at an acceleration voltage of 50 kV in a transmission electron microscope (Hitachi, H-700, Transmission Electron Microscope).

2.11. *Scanning electron microscopy*

A drop of dendrosomal suspension was taken on a cover slip, dried and placed over a double adhesive copper tape and the entire assembly was fixed over an aluminium staff. The sample was gold coated by a spattering technique in an IB-2 ion coater. The photomicrographs were taken in a S-415 Hitachi Scanning Electron Microscope at 25 kV acceleration voltage.

2.12. *Transfection studies with dendrosomal formulations*

The same protocol, as for plain dendrimer–DNA complex, was used for estimating the transfection efficiency of optimized dendrosomal formulation.

2.13. *Luciferase assay*

After 48 h post transfection, the cells were harvested and assayed for the expression of the relevant gene. The medium was aspirated and cells were washed with 100 µl of PBS (pH 7.4). This was followed by the addition of luciferase substrate. The luciferase reaction was quantitated by measuring the intensity of emitted light in terms of a single photon count using a photoluminometer (Lumat LB 9507, Berthold Technologies, Germany). The total photon count in a given time was considered proportional to the amount of luciferase activity in the sample.

2.14. *Renillase assay*

After measuring the luciferase activity the plates were subjected to a renillase assay. 50 µl of renilite substrate was added to the plates along with calcium and magnesium ions. Renilla luciferase activity was quantitated as above.

2.15. *Cytotoxicity*

This was carried out as per the standard procedure for MTT assay described by Fischer *et al*. [28]. HEK-293 cell lines were used for assessment of cytotoxicity of different vectors. Cells were seeded in 96 well microtitere plates at a density of 1×10^5 cells per ml in serum containing media and left for 24 h for recovery. Test systems were

added (0.1 mg/ml) in fresh complete media to microtitere plates and incubated for 24 h or 72 h. Five hours before completion of the incubation period, 20 µl MTT (5 mg/ml) was added and incubation was continued. The medium was removed and 100 µl DMSO was added to dissolve formazan crystals. Optical density was measured at 550 nm using a plate reader (Power Wave X; BIO-TEK Instruments, Inc). Cell viability in the presence of polymers was expressed as a percentage of viability of cells in the absence of polymers.

2.16. *Statistical analysis*

Statistical analysis was performed by a Tukey–Kramer multiple comparison test. $p > 0.05$ was considered a statistically insignificant difference, whereas $p < 0.001$ was considered a very significant difference.

3. Results and discussions

The object of the present study was to study the transfection efficiency of dendrosomes. A fourth-generation PAMAM dendrimer was selected as that would make available the required positive charge but avoid the possible structural defects normally associated with generations higher than that [15, 26]. PAMAM 4.0G was synthesized by a reported method [5] and characterized spectroscopically. IR and NMR spectroscopic data presented in tables 1 and 2, respectively, confirm the synthesis of the dendrimer. Dendrosomes were prepared by entrapping the dendrimer–DNA complex by the reverse phase evaporation method. The dendrimer–DNA complex was prepared in PBS (pH 7.4) by incubating DNA with varying concentrations of dendrimer. The complexation of DNA was confirmed by circular dichroism (CD). The CD spectra (figure 1) of pure fourth-generation PAMAM dendrimer, pure DNA and dendrimer–DNA complex are shown in figure 1. Complexation of DNA with the dendrimer might cause a change in secondary structure of the DNA as a result of its decoiling and subsequent condensation on the dendrimer surface. Alteration in the CD spectrum could be attributed to this change in secondary structure of DNA. Tinoco *et al*. [29, 30] investigated the effect of polycations on the secondary structure of DNA and found it to change upon interaction with polycations leading to changes in the CD spectrum. Our results are in confirmation with these results. Zeta potential data (table 3) revealed that the net charge on the complex became increasingly positive with increasing dendrimer to DNA ratio. The effect of net charge on the transfection efficiency was evaluated in HEK-293 cells by luciferase and renilase assays (figure 3). It was observed that with increasing positive charge transfection efficiency increased and reached a maximum at the dendrimer : DNA charge ratio 50 : 1 (DGC-50). The naked DNA showed a minimum amount of gene expression. The dependence of transfection efficiency on the net charge of the dendrimer:DNA is reported in [26]. The results of our study are in accordance with the available reports. There might be an optimum dendrimer : DNA charge ratio for maximum transfection as it decreases beyond DGC-50. DGC-50 was selected for further studies as it had the maximum transfection efficiency.

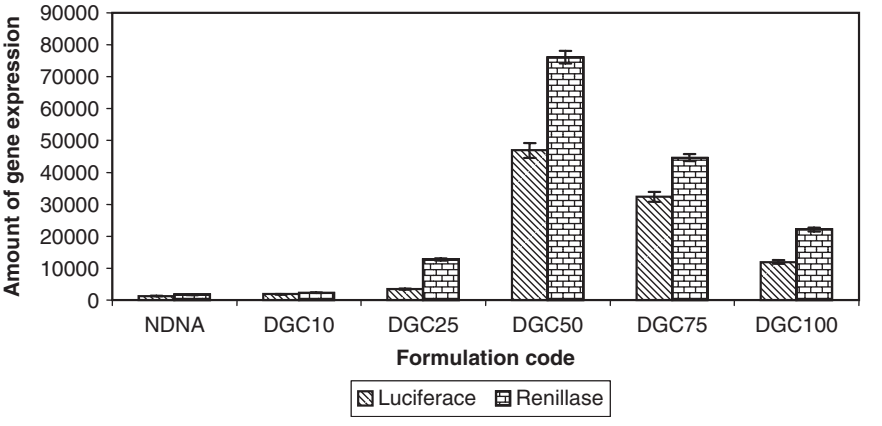


Figure 3. Expression of luciferase and renilase gene in HEK-293 cells transfected with various dendrimeric formulation having different charge ratio (NDNA = Naked DNA), DGC10 = DNA: Dendrimer (1 : 10); DGC25 (1 : 25); DGC50 (1 : 50); DGC75 (1 : 75); DGC100) (1 : 100). (Values = Mean ± S.D, $n = 6$, $p < 0.001$.)

3.1. *Preparation and characterization of dendrosome*

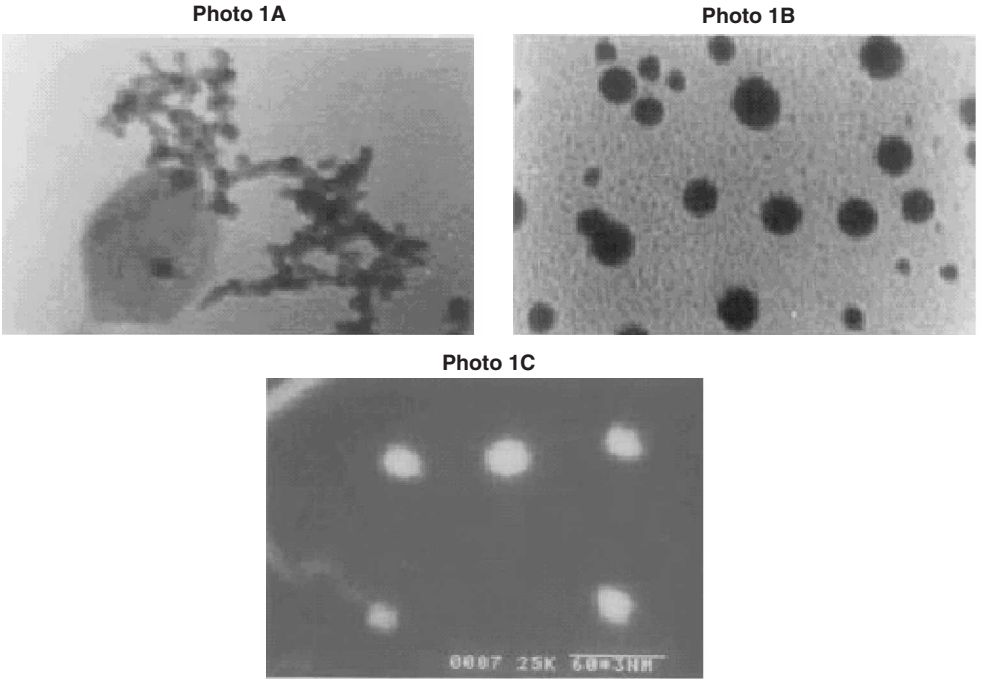
Dendrosomes were prepared by reverse phase evaporation and characterized further for their size distribution and surface morphology. Size distribution data revealed that with decrease in the ratio of PC : C the mean size of the dendrosomes increases (table 4). Even the highest mean size was 180 nm, which is quite suitable for transfection. TEM images (Photo 1A and 1B) show spherical structure, which could be an advantage as spherical structures are reported to be more suitable for transfection [11]. SEM images revealed a smooth surface of dendrosomes (Photo 1C). The selected DNA : dendrimer complex (DGC 50) was entrapped in dendrosomes and the entrapment efficiency determined (figure 4). Dendrosomal formulation (DF3) was found to have a maximum entrapment efficiency of 34.6 ± 2.8%, hence this formulation was used to study the transfection efficiency.

3.2. *Hemolytic toxicity*

Figure 5 shows the results of hemolytic toxicity studies of the various formulations. The hemolytic activity of fourth-generation PAMAM dendrimer alone was found to be the maximum (18.2%) while the dendrimer–DNA complex had much less hemolytic toxicity (3.7%). The prepared dendrosomal formulation was found to possess negligible hemolytic toxicity while the fugene-6 DNA complex does not posses any hemolytic toxicity at all. The low hemolytic toxicity of the dendrosomal formulation might be due to the masking of the terminal amino groups of dendrimers responsible for hemolytic toxicity.

3.3. *Transfection efficiency of dendrosomal formulations in HEK-293 cells*

The comparative tranfection efficiency of NDNA, dendrimer–DNA complex, dendrosomes (DF3) and fugene-6-DNA complex is shown in figure 6. The transfection



Photograph. 1A: TEM photograph of fourth-generation PAMAM dendrimer (×250 k); 1B: TEM photograph of dendrosomes (×100 k); 1C: SEM photograph of dendrosomes (×50 k).

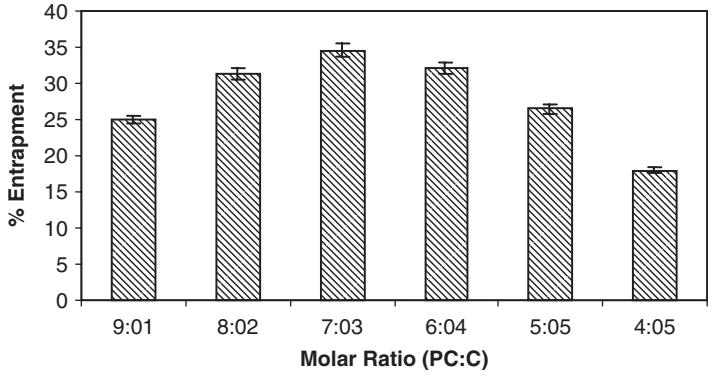


Figure 4. Optimization of entrapment efficiency of dendrosomal formulations with different molar ratio of phosphatidylcholine and cholesterol. (Values = Mean ± S.D, $n = 3$, $p < 0.001$.)

efficiency of NDNA was a minimum, which increased upon its complexation with the dendrimer. This observation suggested that overall the cationic nature had a positive impact on transfection efficiency, which might be due to higher uptake of the complex by cells compared to NDNA. The tranfection efficiency further improved on entrapment of dendrimer–DNA complex in dendrosomes. As is evident from the results of luciferase and renillase assay, the dendrosomal formulation possesses superior

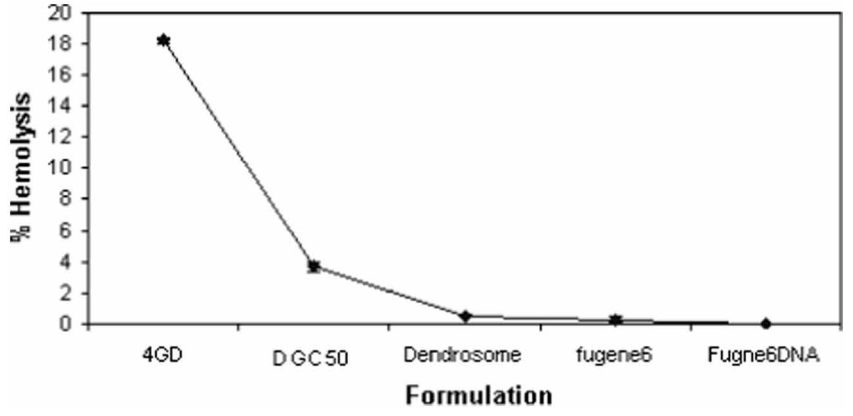


Figure 5. Hemolytic toxicity of various formulations (Values = Mean ± S.D, $n = 3$, $p < 0.001$).

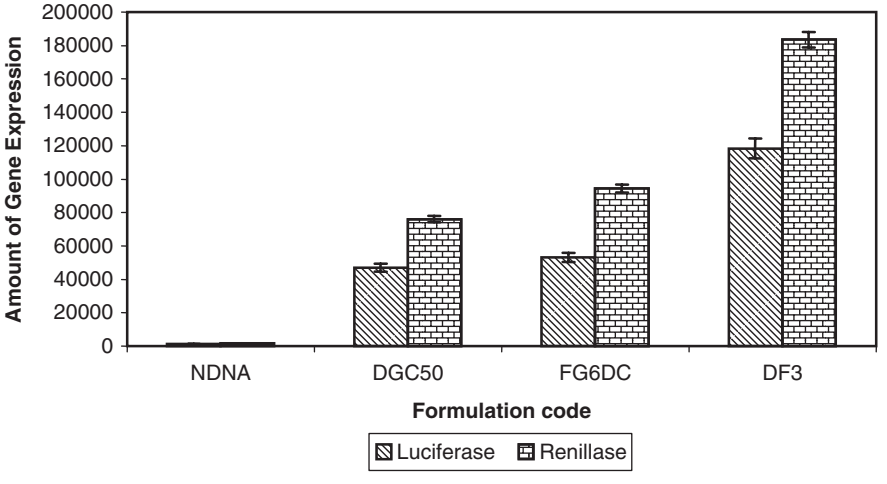


Figure 6. Comparative representation of luciferase and renillase gene expression produced by different optimized formulation. (Values = Mean ± S.D, $n = 6$, $p < 0.001$.)

transfection efficiency as compared to the DGC-50 and the fugene-6 formulation. DGC-50 and fugene-6 demonstrated similar transfection efficiency. The negative charge on the cell membrane might electrostatically accelerate the uptake of dendrosomal formulation. Moreover, the formulation due to an overall positive charge results in higher penetration through the cell membrane and greater gene expression. A major well-documented disadvantage of the dendrimer–DNA complex is its interaction with anionic blood components like proteins. In plasma, proteins interact competitively with DNA in the dendrimer–DNA complex causing its premature release. DNA thus released is subjected to denaturation under influence of DNAase. Dendrosomal formulation is thought to physically protect the dendrimer–DNA complex and prevent premature release of DNA [12].

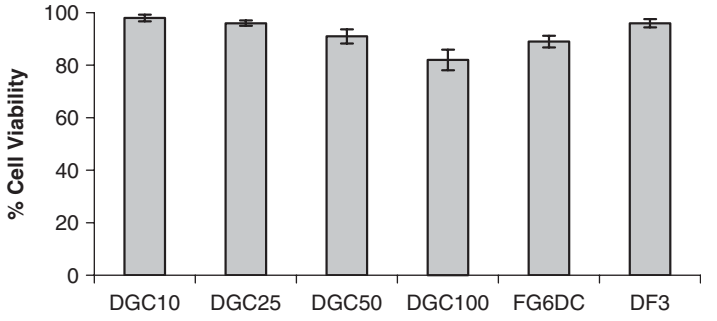


Figure 7. Cytotoxicity of formulations (Values = Mean ± S.D, $n = 3$, $p < 0.001$).

3.4. *Gene expression due to dendrosomal formulations in HEK-293 cells*

The comparative tranfection efficiency of NDNA, dendrimer–DNA complex, dendrosomes (DF3) and fugene-6-DNA complex is shown in figure 6. The dendrosomal formulation possesses superior transfection efficiency as compared to the dendrimer–DNA complex and the fugene-6 formulation as is evident from the results of luciferase and renillase assays. The overall cationic nature of the formulation might have a positive impact on its uptake but that might not be the only factor governing transfection efficiency. If the results of the haemolytic toxicity study are an indication then DGC50 should have more positive charge compared to dendrosomes; still the transfection efficiency of dendrosomes was found superior. The exact cause responsible for this enhanced tranfection still remains to be explored. There could be some optimum positive charge for better transfection efficiency. Beyond a particular level of positive charge the formulation could have become cytotoxic. Decreased cell viability as a result of increased positive charge could be one of the factors. In order to explore this possibility further, a separate experiment to evaluate cytotoxicity was planned. All the systems used in transfection efficiency study were incubated in equivalent concentrations with HEK-293 cells and cytotoxicity was evaluated by MTT assay (figure 7). It was observed that DGC 75 and DGC 100 had very significantly higher cytotoxicity than DGC 50 ($p < 0.001$). While DF3 has very significantly lower cytotoxicity than DGC 50 ($p < 0.001$), no significant difference in the cytotoxicity of DGC 50 and FG6DC was observed ($p > 0.5$). From these results it could be concluded that the positive charge associated with DGC 75 and DGC 100 increases their cytotoxicity to such an extent that they failed to induce gene expression as compared to DGC 50. The cytotoxicity of DGC 10 and DGC 25 was significantly lesser than that of DGC 50 ($p < 0.001$), still they failed to induce comparable gene expression. This made us to believe that there should be some optimum positive charge responsible for maximum gene expression and minimum cytotoxicity.

4. Conclusion

The results of the present study clearly indicate that the dendrosomes possess tremendous potential as a novel, vesicular, non-viral gene delivery vector in terms of

ease of preparation, higher transfection efficiency and negligible hemolytic toxicity. These properties make them potential carrier systems in gene therapy and DNA vaccination. *In vivo* studies are required to estimate its full potential, in terms of gene expression, absorption through various routes and to understand better their mechanism of gene transfer.

Acknowledgements

The authors would like to acknowledge their indebtness to Dr. Uma Roy of the Central Drug Research Institute, Lucknow, for providing plasmid DNA, HEK-293 Cells, and fugene-6 transfection reagent. Tathagata Dutta would like to thank the University Grants Commission, India, for providing financial support.

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