Improved Transfection Efficiency of Chitosan-DNA Complexes Employing Reverse Transfection

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Received 16 April 2011; accepted 6 July 2011
DOI 10.1002/app.35179
Published online 21 October 2011 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: The present study was designed to systematically compare the conventional and reverse transfection methodologies for chitosan/DNA complexes using a low molecular weight (MW) chitosan. The hydrodynamic diameter of the complexes, measured by Dynamic Light Scattering (DLS) was found to be ~ 216 nm and TEM investigations showed spherical and compact complexes with an average size of 200 nm. The transfection efficiency of chitosan using the two methodologies was assessed by employing reporter gene coding for green fluorescent protein (GFP) and luciferase. More than 50% of HEK 293 cells were transfected when transfection done using reverse transfection strategy at pH 6.5 with 10% serum for 24 h followed by media replenishment with pH 7.4 with 10% serum for an additional 24 h period. Also, the cytotoxicity of chitosan/DNA complexes was also considerably lower than the commercially available transfection reagent lipofectamine. Our investigation concludes that maximal transgene expression levels could be achieved using reverse transfection where the chitosan/DNA complexes are preincubated on the plate surface followed by plating of cells at pH 6.5 with 10% serum for 24h and media resupplemented with pH 7.4 with 10% serum for an additional 24 h period. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: 1771–1777, 2012

Key words: biocompatibility; dynamic light scattering; electron microscopy; nanotechnology; particle size distribution

INTRODUCTION

Success of gene therapy and other gene regulation studies relies on targeted and efficient delivery of DNA to mammalian cells. Since, nonviral vectors offer numerous advantages, including stability, safety, low cost, and high flexibility for modification and size of the delivered transgene, they are rapidly emerging as preferred candidates.[1] However, nonviral vectors suffer from significantly low transfection efficiency (TE) as compared with viral vectors. Among various nonviral vectors, cationic polymers offer ease of preparation, purification, and chemical modification and a long shelf life.[2,3] Chitosan, a linear cationic polysaccharide comprising of *N*-acetyl-D-glucosamine and β (1,4)-linked D-glucosamine units has been widely investigated as a DNA carrier owing to its biodegradability, biocompatibility, and nonimmunogenicity.[4–9]

The TE of chitosan/DNA complexes have been found to depend on several factors such as the degree of deacetylation (DDA) and molecular weight (MW) of the chitosan, pH, protein interactions, charge ratio of chitosan to DNA (N/P ratio), cell type, nanoparticle size, and interactions with cells.[10] The DNA binding affinity and TE have been reported to be significantly manipulated by DDA or MW, while maximum protein expression levels are achieved by obtaining an intermediate stability through control of MW and DDA.[11,12] In addition to DDA and MW, the TE of chitosan/DNA complexes is also dictated by the pH of the culture medium, since chitosan is more protonated at acidic pH thus promoting binding not only to negatively charged DNA, but also to negatively charged cell surfaces. Lavertu et al. reported that the TE at pH 6.5 was higher than at pH 7.1 and was comparable to commercially available vectors such as Lipofectamine™ and Fugene® 6.[11] Sato et al. compared TE of chitosan/DNA complexes in A549 cells and also found it higher at pH 6.9 than at pH 7.6.[13] Also, a two to three times increase in gene expression level in the presence of serum as compared with without serum was observed and ascribed the effect as due to increased cell function.[13] Erbacher et al. also observed higher TE in HeLa cells in the presence of 10% serum than in the absence of serum.[14] In one of our previous studies, we reported high levels of transfection (>40%) when transfection was initiated at pH 6.5 with 10% serum for 8 to 24 h to maximize uptake and then the media was changed to pH 7.4 with 10% serum for an additional 24 to 40 h period.[15] Although a plethora of

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studies have been published, the optimum conditions for transfection of chitosan/DNA complexes in terms of method of transfection have not been reported.

The transfection procedure itself can be a critical factor dictating the transfection efficiency. The conventional transfection procedure involves preplating cells, i.e., the cells are allowed to attach, recover, and grow for 24 h before transfection. Reverse transfection involves simultaneously transfecting and plating cells, almost similar to procedures used for transfecting suspension cells. Ziauddin and Sabatini were the first to report a method for reverse transfection.[16] Reverse transfection as compared with conventional transfection allows rapid parallel analysis of large number of genes simultaneously. Two different strategies have been proposed for reverse transfection methods. It can be performed by loading desired DNA on the plates or glass slides and complex formation takes place when carrier and cells are added consecutively.[16] In another strategy, reverse transfection can be done by adding DNA/vector complexes first followed by addition of cells at the time of transfection.[17]

In the present investigation, we systematically evaluated the influence of method of transfection on *in vitro* TE of chitosan/DNA self-assembled complexes. The conventional method of transfection for chitosan/DNA complexes was compared with that of reverse transfection. We hypothesized that TE of chitosan/DNA complexes could be modulated by the method of transfection and higher TE could be achieved by enhancing the interaction of complexes with the cells. For this study, chitosan/DNA complexes were prepared employing commercially available chitosan with 85.3% DDA and 10 kDa MW and characterized for size and zeta potential. *In vitro* TE of these complexes was assessed on HEK 293 cells, by different methods of transfection employing two different plasmids containing enhanced green fluorescent protein (EGFP-C1) and luciferase protein (GL3-Luc) reporter genes, detected with flow cytometry and luminometry. Further, the cell viability was evaluated using the metabolic activity based alamar blue assay.

MATERIALS AND METHODS

Materials

HEK 293 cells were from ATCC Manassas, VA. HEPES, MES, Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium chloride, sodium bicarbonate, sterile 1*N* HCl cell culture tested were procured from Sigma Chemical Co., St. Louis, MO. Chitosan (MW = 10 K with deacetylation degree 85.3%) was purchased from Haidebei Marine Bioengineering, Jinan, China. Cell culture media, Dulbecco's modified eagle's medium (DMEM), Fetal calf serum (FCS) were from GIBCO-BRL-Life Technologies, Web Scientific, UK. Reporter plasmid pEGFP-C1, Lipofectamine™, 0.25% Trypsin-EDTA, Alamar Blue reagent, and Competent DH5α cells were from Invitrogen. Qiagen kit for plasmid isolation was purchased from Qiagen, CA. Plasmid for green fluorescent protein gene (EGFP-C1) and luciferase plasmid (pGL3-Luc) were procured from Invitrogen and Promega. All other chemicals and reagents were procured locally. Characterization was carried out on Fei-Philips Morgagni 268D Transmission Electron Microscope (TEM) from FEI Inc., Hillsboro, OR, and particle size analyzer PHOTOCOR FC from Photocor Instruments, College Park, MD. Luciferase activity was measured on Packard Lumicount microplate luminometer, Frankfurt, Germany.

Cell culture

The mammalian cell line, human embryonic kidney 293 (HEK 293) cells, was maintained as monolayer cultures in DMEM HG supplemented with 1.85 g/L of sodium bicarbonate, 10% FBS, and 50 μg/mL gentamicin at 37°C in 5% CO_2. In this study, cells with passage number lower than 30 were employed for all *in vitro* transfection experiments.

Plasmid DNA

All the transfection experiments were carried out using two different plasmids encoding enhanced green fluorescent protein gene (EGFP-C1) under the control of Simian virus 40 (SV 40) early promoter and luciferase plasmid (pGL3-Luc). The plasmid was transformed into *Escherichia coli* bacterial strain DH5α and extracted from the culture pellets using the Qiagen Endofree Maxi-Prep kit as per manufacturer's instructions.

Preparation of chitosan/DNA complexes

Complexes of chitosan/pDNA were prepared as described previously.[15] Briefly, chitosan was dissolved in hydrochloric acid at 0.5% (w/v) using an amine (from chitosan): HCl ratio of 1: 1 overnight on a rotary mixer. The chitosan solution hence obtained was further diluted with deionized water to obtain a different ratio of amine (chitosan glucosamine groups) to phosphate (N/P) when 100 μL of chitosan solution would be mixed with 100 μL of pDNA, the concentration of the latter always kept at 330 μg/mL in deionized water. Diluted chitosan solution was filter sterilized with a 0.2 μm syringe filter before mixing with pDNA. Complexes of chitosan/pDNA were prepared by adding 100 μL of sterile diluted chitosan solution to 100 μL of pDNA (330 μg/mL) at room temperature, vortexing gently. Complexes thus prepared were incubated for 30 min at room temperature before performing transfection experiments.

Physiochemical characterization

The complexes prepared for the present study were characterized by the following procedures.

Transmission electron microscopy

Complexes of chitosan/DNA were suspended in 1 mL double distilled water, which was later used for preparing samples for TEM. The diluted complexes (3 μL) was put on a formvar (polyvinyl formal) coated copper grid and air dried. To coat copper grids with formvar, a drop of 0.5% (w/v) solution of formvar in chloroform was placed on the water (previously degassed) surface. A thin film was formed on the water surface, onto which several clean copper grids were placed, with matty surface downwards. After 2 to 3 s, the grids along with the film were lifted off by a piece of filter paper with forceps and air dried. TEM pictures were taken on a Fei-Philips Morgagni 268D Transmission Electron Microscope. Before visualization of samples, a blank grid without sample was also scanned.

Dynamic light scattering (DLS)

The hydrodynamic diameter of the chitosan/DNA complexes was determined by DLS employing Photon Correlation spectrometer, PHOTOCOR FC fitted with argon ion laser operated at 632.8 nm as the light source using digital correlator. Measurements were carried out at an angle perpendicular to the incident light and data were collected over a period of 3 min. The mean complexes sizes were obtained from the method of cumulants. Instrument was calibrated with the monodisperse particle standards (silica, diameter 200 nm and latex beads, diameter 50 nm) supplied with the equipment before measuring complexes size in the present investigation. Complexes prepared by mixing 100 μL each of chitosan and pDNA were suspended in double distilled water (1 mL) before measurements.

Zeta potential measurements

Complexes of chitosan/pDNA were diluted as in size measurement experiments and subjected to zeta potential measurements on a Zetasizer Nano ZS employing disposable zeta cells with laser doppler velocimetry used to calculate the zeta potential from the electrophoretic mobility. Zeta potential measurements were also carried out in triplicates in automatic mode with the average of 20 measurements used for each sample within the triplicate.

In vitro transfection studies

The transfection experiments were done using two different strategies as detailed below.

Conventional transfection with chitosan/DNA complexes

For transfection experiments, HEK 293 cells were seeded in 24-well culture plates using 500 μL/well of complete medium and 50,000 cells/well incubated at 37°C, 5% CO_2. The cells were transfected the next day at ~50% confluency. Chitosan/DNA complexes containing 2.5 μg of DNA/well were used to transfect HEK 293 cells in a 24-well culture plates. Transfection media supplemented with 10% FBS was equilibrated overnight at 37°C, 5% CO_2, and pH adjustment performed with 1*N* sterile HCl before transfection. To maintain the pH stability of transfection media, 5 m*M* MES (for pH 6.5) were added to DMEM HG and sodium bicarbonate concentration was decreased to 10 m*M*. Chitosan/DNA complexes were prepared, as described above, incubated at room temperature for 30 min before proceeding with transfection. The complexes were diluted with transfection medium to have a final concentration of 2.5 μg DNA/500 μL of medium. Medium over cells was then aspirated and replenished with 500 μL/well of transfection medium containing chitosan/DNA complexes. Cells were then incubated with chitosan/DNA complexes until analysis at 48 h post-transfection or resupplemented with pH 7.4 medium with serum after 24 h and further incubated for another 24 h. After 48 h, cells were observed under a fluorescence microscope to monitor any morphological changes. Transfection efficiencies and transgene expression levels were then quantitatively assessed by flow cytometry for green fluorescent protein (GFP) and luminometry for luciferase. Lipofectamine was used as positive control following manufacturer's instructions and naked pDNA as a negative control. All experiments were done in duplicates, with a minimum of three separate experiments to demonstrate reproducibility. The data shown in graphs represent mean ± SD ($n = 3$).

Reverse transfection

The reverse transfection was done as reported earlier but with slight modifications.[16] Transfection was performed by adding complexes containing 2.5 μg of DNA/well to the cells at the time of plating cells in 500 μL complete media containing 10% FBS and 5 m*M* MES with pH adjusted to 6.5 and 1×10^5 cells. After 24 h of incubation, cells were resupplemented with fresh complete media pH 7.4 followed by further incubation for 24 h. In another strategy, complexes containing 2.5 μg of DNA/well was added to each of empty well of 24-well plate and incubated at room temperature for 15 min. At the end of incubation, 500 μL suspension of 1×10^5 cells in complete media containing 10% FBS and 5 m*M* MES with pH

adjusted to 6.5 added to each well and further incubated for 24 h at 37°C, 5% CO_2. Twenty-four hours post-transfection cells were replenished with fresh complete media pH 7.4 and incubated at 37°C, 5% CO_2 for another 24 h.

Transfection with lipofectamine

Complexes of lipofectamine/pDNA were prepared with 1: 2 ratio of pDNA (μg): lipofectamine (μL) according to manufacturer's protocol and were used as a positive control. For transfection in 24-well culture plates, lipofectamine was complexed with 0.5 μg of pDNA and incubated for 30 min for complexation. According to the manufacturer, cells were incubated for 4 h with lipofectamine/pDNA complexes in serum-free medium, replenished with complete media containing 10% serum, and analyzed after a total 48 h post-transfection.

Transfection efficiency measurements

Flow cytometry

Cells transfected with various methodologies followed by proper incubation were trypsinized (trypsin 0.25%-EDTA) for 2 min. After detachment, complete medium was added to inhibit trypsin activity. Cell suspensions were then transferred to 1 mL flow cytometry tubes and EGFP expression in the transfected cells quantified using a BD LSR Cytometer (BD Biosciences) equipped with a 488 nm argon laser for excitation. For each sample, 20,000 events were collected and fluorescence was detected through 510/20 nm (FL1) band pass filter for EGFP. In addition, forward scatter (FSC) and side scatter (SSC) were used to establish a collection gate to exclude dead cells and debris. The control sample (nontransfected cells only) was displayed on a dot plot (FL1 vs. FL2) and the gate drawn such that control cells were excluded. The percentage of positive events was calculated as the events within the gate divided by the total number of events, after excluding dead cells and debris.

Luminometry

Medium over the cells transfected with different methodologies was aspirated; cells were washed once with cold PBS and replenished with 100 μL of Glo Lysis Buffer followed by incubation at RT with shaking until complete lysis. Aliquots of 25 μL were transferred to 96-well white luminometer plates where an equal amount of Bright-Glo substrate was added just before measurement on a Packard Lumicount microplate luminometer. Another 10 μL aliquot of cell lysate was used to determine the protein content using Brad Ford's reagent (Bio Rad) taking BSA as a standard. The relative light units (RLU) were normalized to the protein content of each sample. The data is reported as relative light units (RLU)/min × mg of cellular protein and represent mean ± standard deviation for triplicate samples.

Cell viability

The toxicity of chitosan/DNA complexes was evaluated by colorimetric alamar blue assay.[18,19] The blue colored reagent alamar blue contains resazurin which is reduced to a pink colored resorufin by the metabolic mitochondrial activity of viable cells and can be quantified colorimetrically and fluorimetrically. HEK 293 cells were seeded onto 96-well plates using 100 μL/well of complete medium and at a density of 10,000 cells/well to yield ~50% confluency after 24 h of incubation. For 96-well plates, chitosan/DNA complexes having 0.5 μg of DNA/well, lipofectamine with 0.1 μg of DNA/well, and DNA alone as negative control at 0.5 μg DNA/well were used. After 48 h, 20 μL of alamar blue reagent, prewarmed at 37°C was added to each well and incubated for another 4 h. At the end of incubation 80 μL of media containing reduced alamar blue dye was transferred to 96-well plate and read on Elisa plate reader at 570 nm. Untreated cells were taken as control with 100% viability and cells without addition of alamar blue were used as blank. The relative cell viability (%) compared with control cells was calculated by $[\text{absorbance}]_{\text{sample}}/[\text{absorbance}]_{\text{control}} \times 100$.

Statistical analysis

All experiments were done in duplicates, with three separate experiments to demonstrate reproducibility. All data were presented as mean ± standard deviation (±SD) of all the experiments. Statistical analysis was performed using a Student's *t*-test. The differences were considered significant for $P < 0.05$ and $P < 0.01$ indicative of a very significant difference.

RESULTS AND DISCUSSION

Size and zeta potential of complexes

Particles size and surface charge dictates the interaction of complexes with the cells thereby leading to uptake and efficient transfection. It has been reported that polycation/DNA complexes having size larger than 100 nm mostly enters the cell by endocytosis or pinocytosis.[20] Also, the smaller-sized nanoparticles (mean diameter 70 nm) showed a 27-fold higher transfection than the larger-sized nanoparticles (mean diameter 202 nm) in COS-7 cell line and a four fold higher transfection in HEK-293 cell line.[21] It was shown earlier that chitosan 80-10-10

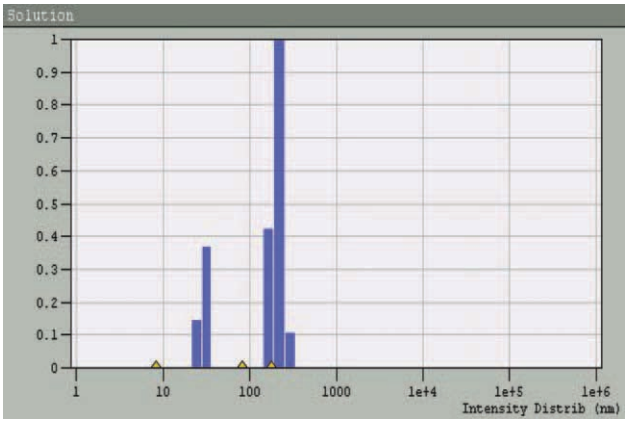

Figure 1 Representative dynamic light scattering (DLS) spectrum of chitosan/DNA complexes. The complexes were suspended in double distilled water and the average size of complexes is 216 nm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(DDA-MW-N/P ratio) gives comparable transfection efficiency to lipofectamine when transfection experiments done at pH 6.5, so we also prepared chitosan/pDNA complexes by mixing equal amounts of chitosan and pDNA to obtain an N/P ratio of 10 for our studies and performed transfection experiments at pH 6.5.[11,22] Complexes diluted in double distilled water gave an average hydrodynamic diameter of 216 ± 5.1 nm (Fig. 1). TEM investigation of complexes revealed spherical and compact particles with average size of 200 nm (Fig. 2). The images of complexes showed homogeneous distribution with a clear absence of aggregates. This variability in size is probably due to the two different methods used. DLS was performed on complexes suspended in water which makes them fully hydrated, whereas, TEM studies done on samples dried to a glass slide surface. Moreover, the DLS measurements provides a quantitative data for average size as it measures size of thousands particles per second whereas, TEM is quite qualitative as it visualizes only a small number of complexes. Hence, the use of these two different but complementary techniques allows an overall evaluation of both size and morphology. Later, we tried to determine the complex size in DMEM with and without 10% serum at pH 6.5 and found the poydispersity index to be higher than 0.7, which makes particles unsuitable for DLS measurements; hence data are not reported here.[23]

Zeta potential studies provide vital information about the surface charge of the complexes and the possible interaction with the cells. The surface charge of chitosan/DNA complexes depends on the concentration of DNA and chitosan as well as the pH and salt content of the suspension medium. The pKa of the amino groups in chitosan is ~6.5, hence the polymer's cationic charge density is greatly reduced by pH increases in the 5.5 to 7.5 region.[24] About 90% of the amino groups have been reported to be protonated at pH 5.5 to 5.7.[25] The zeta potential of complexes was measured in double distilled water (~pH 6.1) was found to be 36.8 ± 0.6 mV, which imparts sufficient electrostatic repulsive force to prevent aggregation between the cationic complexes.

In vitro transfection

The transfection efficiency of chitosan/DNA complexes was determined by preparing complexes of chitosan with plasmid pEGFP-C1 at N/P ratio of 10, transfections done at pH 6.5 on HEK 293 cells and compared with a commercially available transfection reagent, lipofectamine. The gene expression was evaluated quantitatively by flow cytometry for EGFP along with a luciferase assay employing luminometry. Initial transfection experiments were done in presence and absence of 10% serum at pH 6.5. The results showed that the transfection efficiency of complexes was higher in the presence of serum than in

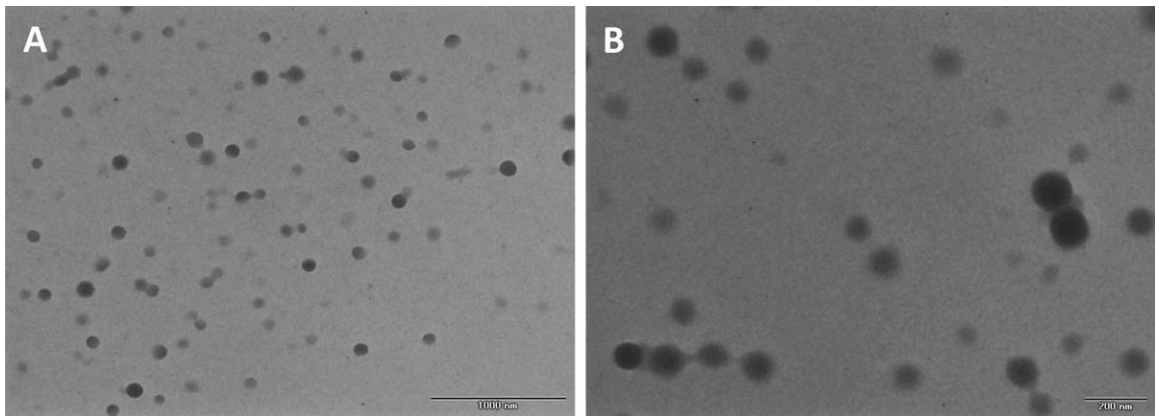

Figure 2 Transmission electron image (TEM) of chitosan/DNA complexes. The average size of complexes is 200 nm. The image A is at lower magnification with the bar equal to 1000 nm and image B is at higher magnification with the bar equal to 200 nm.

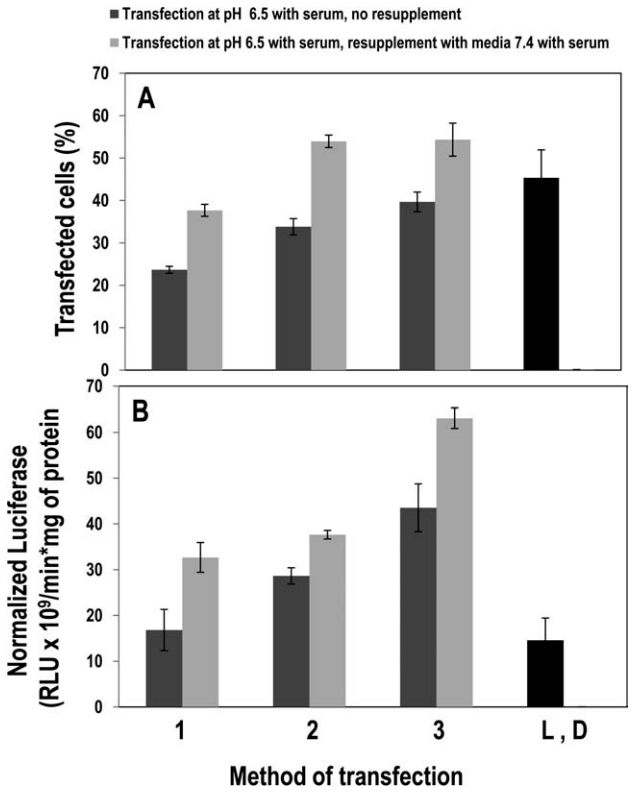

Figure 3 Transfection efficiency of chitosan/DNA complexes by different methods of transfection. HEK 293 cells were transfected with chitosan/DNA complexes in media with 10% serum, pH 6.5. After 24 h, transfection media was either resupplemented with media of pH 7.4 and serum content or left as it is and transfection quantified 48 h post-transfection. (A) The percentage of cells expressing GFP was estimated using flow cytometry and (B) the level of gene expression monitored by luminometry; 1, conventional transfection; 2, reverse transfection—transfection at the time of plating; 3, reverse transfection—transfection after preincubating complexes in plates, controls on the right are L: transfection with lipofectamine and analysis after 48 h, D: transfection with DNA only in medium pH 7.4 with serum. Values are mean ± SD, $n = 3$.

absence of serum (data not shown), which is in well agreement with our previous report and others.[13,15] Henceforth, all the transfection experiments here were carried out in the presence of 10% serum.

The influence of methods of transfection on the transfection efficiency of chitosan/DNA complexes was investigated by performing experiments using conventional and reverse transfection methodologies. Cells transfected using reverse transfection method at pH 6.5 with serum followed by replenishment with medium supplemented with 10% serum pH 7.4 after 24h showed the highest number of transfected cells i.e., 54.3% at 48 h post-transfection and the highest level of protein expression (Fig. 3). While employing conventional transfection strategy and medium resupplemented with 10% serum pH 7.4 resulted in only 37.7% of cells being transfected. The commercially available transfecting agent lipofectamine showed upto 45% GFP expressing cells. It is reported that, the uptake of complexes is greatly facilitated at pH 6.5, but once uptake is complete at about 24 h it is beneficial to return the cells to a physiological pH of 7.4 to allow completion of the transfection process to result in expression of the transgene.[15] Moreover, the high zeta potential values favors high uptake of chitosan/DNA complexes at pH 6.5 owing to its high cationic charge.[15] Also, the interaction of complexes with the cell membrane is one of the crucial parameters for efficient transfection. It is postulated that employing reverse transfection methodology, transfecting cells at the time of plating will increase the chances of interaction of complexes with the cells, thereby leading to higher uptake followed by high transfection efficiency. Further, the cells seeded to plates preincubated with complexes yielded highest level of gene expression (Fig. 3). This could possibly due to the fact that the reverse complex presentation to the cells prevents the problem of particle aggregation due to interaction with various serum proteins in the cell culture medium. It is postulated that the complexes preincubated on the plates onto which cells settle creates a mechanical stress on the cellular membrane and the nucleus thus promoting the entry of surface deposited particles into the cell.

Cell viability

The cytotoxicity of chitosan employed in this study was evaluated by estimating the cell viability. Microscopic examination of cells transfected with complexes indicated little toxicity at the levels of chitosan/DNA complexes used for transfection. Cellular metabolic activity was assayed by alamar blue, to quantitate the cell viability.[18,26] Cells transfected with control lipofectamine revealed considerable toxicity and cell morbidity during microscopic examination with viability reduced to ~35% after 48 h. However, the chitosan/DNA complexes were found to be only slightly toxic, where after 48 h of incubation more than 80% of cells were viable (Fig. 4). The cytotoxicity has been found to be dependent on the interaction of the polymers with cell membranes which increases with polycationic charge.[27] The polycationic polymers undergo strong electrostatic interaction with plasma membrane proteins, which can lead to destabilization and ultimately rupture of the cell membrane. Fischer et al. demonstrated that the cytotoxicity of different types of polycationic polymers depend on the number and arrangement of the cationic charges which determines the degree of interaction with the cell membranes and the cells exposed to cationic polymers first show membrane leakage followed by a decrease in the metabolic activity.[27] A comparative study between polycationic, neutral, and polyanionic polymers revealed that the polycationic polymers have the highest toxicity

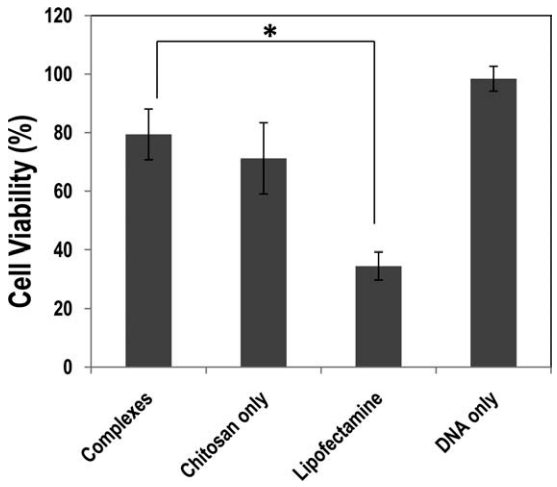

Figure 4 Cell viability—HEK 293 cells treated with chitosan/DNA complexes, chitosan at pH 6.5, 48 h post-transfection cell viability determined using alamar blue. Cells at pH 7.4 in complete media taken as 100% viable, controls include DNA alone and cells transfected using lipofectamine. An asterix (*) indicates of significant difference with $P < 0.05$. Values are mean ± SD, $n = 3$.

followed by neutral and anionic ones.[28] Our results are in well accordance with this theory as we observed slightly higher toxicity at cell incubated with chitosan only than with chitosan/DNA complexes, as the native polymer bears more positive charge compared with the polymer in complexes that is partly neutralized by binding to DNA (Fig. 4).

CONCLUSIONS

We have compared the conventional method of transfection for chitosan/DNA complexes with that of reverse transfection. Using reverse transfection methodology, the cells seeded to plates preincubated with chitosan/DNA complexes yielded highest level of gene expression. The cell viability studies revealed of more than 80% viability providing evidence of these complexes being biocompatible. The high *in vitro* transfection data of chitosan/DNA complexes opens up new avenue for use of these nanoparticles in *in vivo* studies.

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