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RESEARCH

Enhanced Gene Delivery Mediated by Low Molecular Weight Chitosan/DNA Complexes: Effect of pH and Serum

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Abstract This study was designed to systematically evaluate the influence of pH and serum on the transfection process of chitosan–DNA complexes, with the objective of maximizing their efficiency. The hydrodynamic diameter of the complexes, measured by dynamic light scattering (DLS), was found to increase with salt and pH from 243 nm in water to 1244 nm in PBS at pH 7.4 and aggregation in presence of 10% serum. The cellular uptake of complexes into HEK 293 cells assessed by flow cytometry and confocal fluorescent imaging was found to increase at lower pH and serum. Based on these data, new methodology were tested and high levels of transfection (>40%) were achieved when transfection was initiated at pH 6.5 with 10% serum for 8–24 h to maximize uptake and then the media was changed to pH 7.4 with 10% serum for an additional 24–40 h period. Cytotoxicity of chitosan/DNA complexes was also considerably lower than Lipofectamine™. Our study demonstrates that the evaluation of the influence of important parameters in the methodology of transfection enables the understanding of crucial physicochemical and biological mechanisms which allows for the design of methodologies maximising transgene expression.

Keywords Chitosan · Complexes · Uptake · Transfection · Cytotoxicity

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Introduction

Targeted and efficient DNA delivery to mammalian cells remains an essential prerequisite for gene therapy and gene regulation studies. Research on non-viral vectors has gained momentum as they offer several advantages, including stability, safety, low cost and high flexibility for modification and to accommodate the size of the delivered transgene [1]. Amongst various non-viral delivery systems, 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 as it is biodegradable, biocompatible and non-immunogenic [4–9].

The transfection efficiency of chitosan/DNA systems depends 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 transfection efficiency have been found to increase with increase in DDA or MW while maximum protein expression levels are achieved by obtaining an intermediate stability through control of MW and DDA [11, 12]. High MW chitosans have been reported to degrade slowly in vivo, and have a risk of accumulation in the tissues over long period of administration [13]. In addition to DDA and MW, the transfection efficiency of chitosan/DNA complexes is very sensitive to 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. We previously reported that the transfection efficiency 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. [14] compared transfection efficiency of chitosan/DNA complexes in A549 cells and also found it higher at pH 6.9 than at pH 7.6. Additionally, Zhao et al. [15] investigated the effect of transfection medium pH on the transfection efficiency of chondrocytes using chitosan/DNA complexes and reported higher expression levels at pH 6.8 or 7.0 than at pH 7.4.

A large number of reports have been published showing transfection with chitosan/DNA complexes both in the presence and absence of serum in the transfection medium [14, 16, 17]. Sato et al. [14] demonstrated 2–3 times increase in gene expression level in the presence of serum compared to without serum and ascribed the effect as due to increased cell function. However, upon addition of 50% serum, a reduction in transfection efficiency was observed and ascribed to cell damage induced by the high content of serum. Erbacher et al. [18] reported higher transfection efficiency in HeLa cells in the presence of 10% serum than in the absence of serum. Despite several studies, the optimum conditions for transfection of chitosan/DNA complexes in terms of pH, serum and incubation time with cells have not been identified. No report to date has systematically examined the effect of pH and serum on physicochemical properties of complexes as well as in vitro cell uptake, transgene expression and cytotoxicity, and accounting for the kinetics of the transfection process.

This study was designed to systematically evaluate the influence of pH and serum on the physicochemical properties, cell uptake and in vitro transfection efficiency of chitosan/DNA self assembled complexes. We hypothesized that higher transfection efficiency of chitosan/DNA complexes at acidic pH is due to enhanced uptake owing to the presence of positive charge on non-aggregated nanometre size complexes which efficiently interact with the negatively charged cell membranes. Also, transfection efficiency would further depend on the processing of internalized complexes which would be higher in metabolically active cells at higher pH of 7.4 and in the presence of serum in contrast to acidic pH and absence of serum. Chitosan/DNA complexes were prepared using chitosan with 92% DDA and 10 kDa MW, which we previously reported as an efficient chitosan for transfection, and also characterized for size and zeta potential in various medium compositions and with varying pH and salt concentration [11]. In vitro transfection efficiency of these complexes was then assessed on HEK 293 cells, in different pH, with and without serum, employing a plasmid containing a fusion of an enhanced green fluorescent protein (EGFP) and a luciferase reporter gene, the former detected with flow cytometry and the latter with luminometry. Chitosan labelled with rhodamine isothiocyanate (RITC) and DNA with fluorescein or Cy3 was used to observe cell uptake via flow cytometry and confocal fluorescence microscopy as a function of pH and serum [19]. Cytotoxicity was evaluated using the Alamar Blue assay.

Materials and Methods

Materials

HEK 293 cells were from ATCC (ATCC #CRL 1573) Manassas, VA, USA. Dulbecco's Modified Eagle Medium high glucose (DMEM HG, Cat #12100-046), Fetal Bovine Serum (FBS, Cat #26140-079), Lipofectamine™ (Cat #18324-111), 0.25% Trypsin–EDTA (Cat #25200-056) and Competent DH5α cells (Cat #182630-12) were from Life Technologies, Carlsbad, California, USA. Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium chloride (Cat #D5652), HEPES (Cat #H4034), MES (Cat #M2933), sodium bicarbonate (Cat #S5761), sterile 1 N HCl (Cat #H9892) cell culture tested were obtained from Sigma-Aldrich, Oakville, Ontario, Canada. Bright-Glo™ Luciferase Assay System (Cat #E2620) and Glo Lysis Buffer (Cat #E2661) were from Promega, Madison, WI, USA. BCA™ Protein Assay Kit (Cat #23227) and Compat-Able Preparation Reagent Set (Cat #23215) were from Pierce Biotechnology, Rockford, IL, USA. The plasmid EGFPLuc was from Clontech Laboratories (Cat #6169-1) Mountain View, CA, USA. The EndoFree Plasmid Mega Kit (Cat #12381) was from Qiagen, Mississauga, Ontario, Canada. Label IT Fluorescein labelling kit full size MIR 3200, Label IT Cy3 labelling kit full size MIR 3600 was from Mirus, Madison, WI, USA. Alamar Blue reagent (Cat #DAL 1025) was procured from Invitrogen, Burlington, Ontario, Canada.

Cell Culture

The mammalian cell line, Human embryonic kidney 293 (HEK 293) cells, was maintained as monolayer cultures in DMEM supplemented with 10% FBS. In the present study, cells with passage number lower than 25 were employed for all in vitro transfection experiments.

Plasmid DNA

All complexes prepared with chitosan contained the plasmid pEGFPLuc (6.4 kb) which encodes a fusion of EGFP and luciferase from the firefly *Photinus pyralis* under the control of human cytomegalovirus (CMV) promoter [20]. The plasmid was transformed into *E. coli* bacterial strain DH5α and extracted from the culture pellets using the EndoFree Plasmid Mega Kit as per manufacturer's instructions. The purified pDNA was dissolved in

endotoxin-free water and the quality was assessed by restriction enzyme digestion, analysis on agarose gel and concentration/purity determined by UV spectrophotometry by measuring absorbance at 260/280 nm. For complex preparation, pDNA was suspended in deionized water to obtain the desired N/P ratio of 5, where N is moles amine from chitosan and P is moles phosphate from DNA.

Preparation of Chitosan/pDNA Complexes

Complexes of chitosan/pDNA were prepared as described previously [11]. Briefly, chitosan 92–10 (DDA–MW) was dissolved at 0.5% (w/v) in hydrochloric acid using an amine (from chitosan): HCl ratio of 1:1 overnight on a rotary mixer. This chitosan solution was further diluted with deionized water to obtain a ratio of amine (chitosan glucosamine groups) to phosphate (N/P) of 5 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. N/P of 5 was chosen since both in vitro and in vivo studies have shown this ratio to be efficient for chitosan 92–10 [11, 21]. Diluted chitosan solution was filter sterilized with a 0.2 µm syringe filter prior to mixing with pDNA; ninhydrin assays indicated that chitosan was not trapped in the filter [22]. 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, pipetting up and down and tapping the tubes gently. Complexes thus prepared were incubated for 30 min at room temperature before performing transfection experiments.

Physicochemical Characterization of Chitosan/pDNA Complexes

Size

The hydrodynamic diameter of the chitosan/pDNA complexes was determined by dynamic light scattering (DLS) measurements. Chitosan/pDNA complexes (30 µl) were diluted into various medium (600 µl) of different pH, salt and protein content followed by incubation of 30 min and size determined using a Zetasizer Nano ZS (Malvern instruments, UK) employing a nominal 5 mW HeNe laser operating at 633 nm wavelength. The scattered light was detected at 173°. The refractive index (1.33) and the viscosity (0.89) of ultrapure water at 25°C were used in data analysis performed in automatic mode using the instrument software (DTS 5.0). All measurements were done in triplicates with each of the triplicates measured 20 times to obtain an average. The particle size reported as hydrodynamic diameter obtained as intensity distribution by Cumulant analysis.

Zeta Potential

Chitosan/pDNA complexes were diluted as in size measurement experiments and subjected to zeta potential measurements on a Zetasizer Nano ZS but 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

Mammalian HEK 293 cells were cultured in DMEM HG supplemented with 1.85 g/l of sodium bicarbonate and 10% FBS at 37°C in 5% CO_2. Cells were maintained and sub-cultured according to ATCC recommendations without any antibiotics. The absence of mycoplasma was verified by fluorescence detection according to Hay et al. [23]. For transfection, 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.

Transfection with Chitosan/DNA Complexes

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 prior to transfection. To maintain the pH stability of transfection media, 10 mM HEPES (for pH 7.1) or 5 mM MES (for pH 6.5) were added to DMEM HG and sodium bicarbonate concentration was decreased to 20 and 10 mM, respectively. 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, as determined previously [11]. Medium over cells was then aspirated and replenished with 500 µl/well of transfection medium containing chitosan/DNA complexes, unless otherwise mentioned. For standard transfections, cells were then incubated with chitosan/DNA complexes until analysis at 48 h post-transfection. After 48 h, cells were observed under a fluorescence microscope (Zeiss Axiovert TV 100, GmbH Germany) to monitor any morphological changes. Transfection efficiencies and transgene expression levels were then quantitatively assessed by flow cytometry for green fluorescent protein (GFP) and by luminometry for luciferase assay, respectively. 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 ($n = 3$).

Transfection in Presence of Serum

Transfection was performed as above with complete transfection media containing 10% serum and 5 mM MES with pH adjusted to 6.5. After stipulated time points of 4, 8, 12 and 24 h, transfection media containing chitosan/DNA complexes was removed and cells replenished with fresh complete media containing 10% serum of either pH 6.5 or 7.4 followed by incubation to a total of 48 h post-transfection.

Initial Transfection in Absence of Serum

Cells transfected as above with transfection medium with 5 mM MES and pH adjusted to 6.5 but without serum. After stipulated time points of 4, 8, 12 and 24 h, transfection media containing nanoparticles was aspirated and cells re-supplemented with (1) medium without serum at pH 6.5 or 7.4 or (2) complete medium with 10% serum at either pH 6.5 or 7.4, and analyzed after a total of 48 h post-transfection.

Kinetics of Transgene Expression

Transfection was done as detailed above using complete transfection media containing 10% serum at pH 6.5. After stipulated time points of 12, 24, 48, 72 and 96 h cells were (1) trypsinized and analyzed for GFP expression by flow cytometry and (2) lysed followed by luciferase assay by luminometry.

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 treated with various transfection agents for 48 h of 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 5 ml flow cytometry tubes and EGFP expression in the transfected cells quantified using a MoFlo cytometer (MoFlo BTS, Beckman Coulter, Miami, FL, USA) equipped with a 488 nm argon laser for excitation (model ENTCII-621, Coherent, Santa Clara, CA, USA). 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. Signals were amplified in logarithmic mode for fluorescence and Summit software (v. 4.3, Beckman Coulter, Miami, FL, USA) was used to determine the GFP positive events by a standard gating technique. The control sample (non-transfected cells only) was displayed on a dot plot (FL1 versus 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.

Luciferase Assay

Culture medium of cells transfected at different conditions 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 prior to measurement on a Tecan Infinite™ M200 (Tecan Austria, Austria). Another 10 μl aliquot of cell lysate was treated with Compat-Able™ Preparation Reagent Set to remove interfering substances from the Glo Lysis Buffer prior to determining the protein content using BCA™ Protein Assay kit. The relative light units (RLUs) were normalized to the protein content of each sample.

Cell Uptake Studies

Flow Cytometry

To quantify cell uptake of the chitosan/DNA complexes, fluorescent complexes were prepared by labelling chitosan with RITC before complexation with DNA [19]. HEK 293 cells were seeded onto 24-well plate and transfected with complexes prepared from labelled chitosan as described above in the transfection experiment followed by incubation at 37°C. For chitosan only incubations, the amount of rhodamine labelled chitosan equivalent to that present in the complexes was used. Cells were also transfected with complexes prepared from chitosan and Cy3 labelled DNA

(DNA labelling according to manufacturer's protocol) to remove the effect of free chitosan in the measured cell uptake. After stipulated time points of 1, 2, 4, 8 and 24 h, uptake was terminated by replacing the medium containing transfection complexes with cold PBS. To visualize the internalization of the complexes, each cell monolayer was observed under fluorescence microscope. To determine the percentage and fluorescence intensity of cells internalizing complexes, cells were washed with cold PBS followed by trypsinization for 10 min. After complete detachment of cells from plate and from each other, DMEM supplemented with 10% FBS was added to inhibit trypsin activity. Initially, to quench the residual fluorescence out of the cell membrane, cells treated with 0.4% trypan blue for 2 min [24]. However, no difference in fluorescence level was observed between the cells treated with and without trypan blue, so later all samples were analyzed without trypan blue treatment. Cell suspensions were then transferred to 1.5 ml sterile centrifuge tubes, centrifuged and washed two times with cold PBS. The cells were finally suspended in 150 µl of PBS, transferred to 5 ml flow cytometry tubes and subjected to analysis on a MoFlo cytometer equipped with a 568 nm argon laser for excitation (model ENTCII-621, Coherent, Santa Clara, CA, USA). Rhodamine B has an excitation maximum at 551 nm and an emission maximum at 605 nm; Cy3 has an excitation maximum at 550 nm and an emission maximum at 570 nm, which can be analyzed by flow cytometry instruments using argon ion lasers. For each sample, 10,000 events were collected and Rhodamine B fluorescence was detected through 620/60 nm (FL4) band pass filter and Cy3 fluorescence detected through 580/30 nm (FL2) band pass filter. Also, FSC and SSC were used to establish a collection gate to exclude dead cells and debris. Signals were amplified in logarithmic mode for fluorescence and Summit software (v. 4.3, Beckman Coulter) was used to determine the rhodamine and Cy3 positive events by a standard gating technique. The control sample (non-transfected cells only) was displayed on a FL4 plot and the gate drawn such that control cells were excluded. The percentage of positive cells was calculated as the cells within the gate divided by the total number of cells, after excluding dead cells and debris. The mean fluorescence values of the positive cells internalizing complexes, obtained after proper gating was used to assess the level of uptake per cell.

Confocal Microscopy

HEK 293 cells were seeded 24 h prior to transfection in 35 mm MatTek's glass bottom culture dishes using 500 µl of complete medium and 50,000 cells/dish, incubated at 37°C, 5% CO_2. After 24 h of incubation, cells were transfected with complexes prepared from RITC labelled chitosan and fluorescein labelled DNA (DNA labelling according to manufacturer's protocol) as described above in the transfection experiment except that incubation time with complexes was only 8 h. After this time, the medium containing transfection complexes was removed and cells were washed twice with cold PBS solution. Cells were then stained for 5 min at 37°C with 1 ml of Cell Mask™ deep red plasma membrane stain at a concentration of 5 µg/ml in complete media followed by washing twice with cold PBS solution. Then 2 ml of complete media was added to the dish containing stained cells and imaged with a confocal microscope (LSM510 META, Carl Zeiss, GmbH, Germany) using the following excitation and emission wavelengths: fluorescein excitation 488 nm, emission band pass 510/520 nm; Rhodamine excitation 543 nm, emission band pass 565/615 nm; Cell Mask™ deep red excitation 633 nm, emission band pass 644/676 nm. Images were captured from randomly selected areas of each culture dish for each pH and analyzed using Z-sectioning.

Cytotoxicity of Chitosan/DNA Complexes

The toxicity of chitosan/DNA complexes was evaluated by colorimetric Alamar Blue assay [25, 26]. The blue coloured reagent Alamar Blue contains resazurin which is reduced to a pink coloured 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. Before estimating the cytotoxicity of chitosan/DNA complexes, a control experiment was done to show comparability of the Alamar Blue and MTT assay, by treating cells overnight with increasing concentrations of DMSO. For assessing the cell viability of complexes, cells were then incubated with 100 µl of medium containing chitosan/DNA complexes as described above in the transfection experiment. 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, pre-warmed 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 black corning 96-well plates and read on fluorescence plate reader with excitation 560 nm, emission 590 nm and cut off 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 to control cells was calculated by $[\text{Fluorescence}]_{\text{sample}}/[\text{Fluorescence}]_{\text{control}} \times 100$.

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

Complex Size and Zeta Potential

Chitosan/pDNA complexes were prepared by mixing a fixed amount of chitosan with pDNA to obtain an N/P ratio of 5 shown previously to be most effective for chitosan 92–10 [11, 21]. The complex formation occurs due to ionic interaction between the positively charged amino groups present on the glucosamine units of chitosan and the negatively charged phosphate groups of DNA [12]. The size of polycation/DNA complexes is a central parameter influencing cell uptake and gene transfection. It has been reported that polycation/DNA complexes having size larger than 100 nm mostly enter the cell by endocytosis or pinocytosis [27].

During in vitro transfection pH and serum content play vital roles in determining the efficiency of gene delivery in vitro. In order to study the effect of pH and serum proteins on the hydrodynamic diameter of complexes, zeta-sizing measurements were performed after exposing complexes to various media. Complexes diluted with double distilled water gave a uniform hydrodynamic diameter distribution of 243 ± 12 nm with a polydispersity index of 0.39 ± 0.07. Complex hydrodynamic diameter increased with increasing salt concentration from 391 ± 43.7 nm at 10 mM NaCl to 890 ± 71.6 nm in 150 mM NaCl. Chitosan/DNA complex hydrodynamic diameter in PBS was also found to be much greater than that in distilled water where PBS generated particle diameters near 1000 nm, i.e. 4–5 times larger than in distilled water. This increase in complex hydrodynamic diameter in PBS can be attributed to increased salt concentration and increased pH (from a pH of 6.1 measured in distilled water) both of which induce aggregation by reduced electrostatic repulsion between particles as is well known from DLVO theory of colloid stability [28]. This latter interpretation is also supported by the significant reduction in zeta potential seen from distilled water to PBS (Table 1).

Samples with very broad size distribution having polydispersity index values >0.7 are either not suitable for DLS analyses, or present agglomeration problems [29]. In this study, the polydispersity index of chitosan/DNA complexes in DMEM with or without 10% serum at all the three pHs was found to be more than 0.7, rendering particles unsuitable for DLS measurements, hence data are not reported here. For instance, in media with 10% serum, pH 6.5 three different populations with hydrodynamic diameter of 10.5, 643.1 and 4407 nm having PI of 0.742, were found due to presence of serum proteins and binding of serum proteins to chitosan/DNA complexes (Fig. 1).

To study the influence of salt and pH on the surface charge of chitosan/DNA complexes, the zeta potential was measured in different media (Table 1). The 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 pK_a of chitosan can be expressed as a linear function of its charge density with an intrinsic pK_a (pK_0) of 6.7 and, as for any polyelectrolyte, this charge density dependence of pK_a is reduced as ionic strength increases [30]. At 50% protonation, in the presence of 15 or 150 mM of NaCl, the pK_a of the amino groups is about 6.3 or 6.5, respectively, and the polymer's cationic charge density is greatly reduced by pH increases in the 5.5–7.5 region. For example, increasing from pH 5.5 to 7.5 results in a decrease of chitosan amine protonation from about 75 to 10% in 15 mM NaCl or a decrease from about 90 to 10% in 150 mM NaCl according to a recently developed molecular model of chitosan ionization [30]. Interestingly, the strong polyanionic nature of DNA in chitosan/DNA complexes facilitates the protonation of glucosamine units

Table 1 Hydrodynamic diameter, polydispersity index and zeta potential (mean ± SD, $n = 3$) of chitosan/pDNA complexes in various medium without serum

S.No.	Measurement medium	Hydrodynamic diameter (nm)	Polydispersity index	Zeta potential (mV)
1.	Double distilled water	243 ± 12	0.39 ± 0.07	41.4 ± 5.1
2.	10 mM NaCl	391 ± 43.7	0.41 ± 0.11	28 ± 5.2
3.	150 mM NaCl	890 ± 71.6	0.20 ± 0.08	23 ± 6.3
4.	PBS pH 6.5	911 ± 39.6	0.14 ± 0.07	11.4 ± 4.1
5.	PBS pH 7.1	1213 ± 84	0.10 ± 0.06	4.5 ± 1.0
6.	PBS pH 7.4	1244 ± 135.2	0.19 ± 0.5	−4.9 ± 3.5

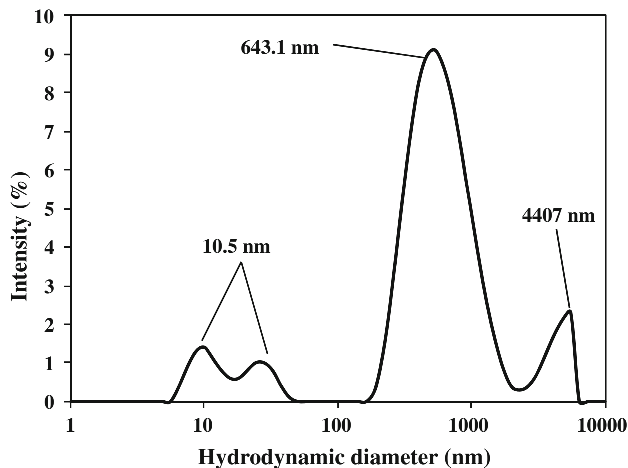

Fig. 1 Representative dynamic light scattering (DLS) spectrum of chitosan/pDNA complexes suspended in medium pH 6.5 with 10% serum showing three different populations with hydrodynamic diameter of 10.5 nm (serum proteins), 643.1 nm (binding of serum proteins to complex) and 4407 nm (large aggregates due to binding of serum to complex)

of chitosan due to proton transfer from the buffer to chitosan during complex formation, even at high pH such as 7.4 where chitosan would be largely uncharged in the absence of DNA [12]. The zeta potential of chitosan/DNA complexes in water was found to be 41.4 ± 5.1 mV and generates sufficient electrostatic repulsion to prevent aggregation of complexes during incubation times exceeding 120 min [11]. Zeta potential measurements indicate that particle surface charge was reduced by suspending complexes in PBS at pH 6.5 and even became negative at pH 7.4 (Table 1). It can be inferred from these results that an increase in pH of PBS reduces protonation levels of chitosan such that at pH 7.4 the overall charge on chitosan is less that the anionic charge of DNA in the complexes. This dependence of charge on pH is consistent with that previously reported where electrostatically neutral particles were found in the pH range of 7.0–7.4 using an N/P ratio of 6 while the zeta potential became −20 mV at pH 8–8.5 [31].

In order to investigate the effect of labelling with fluorescence dyes, the particle size was determined after labelling chitosan and DNA. However, no significant difference in hydrodynamic diameter was observed (unpublished data) hence it can be deduced that the effect of altered size on uptake and transfection would also be negligible. The dyes are relatively small molecules compared to chitosans and especially DNA, and both DNA and chitosan are labelled at approximately 1%, resulting in insignificant size change. The average MW of fluorescent

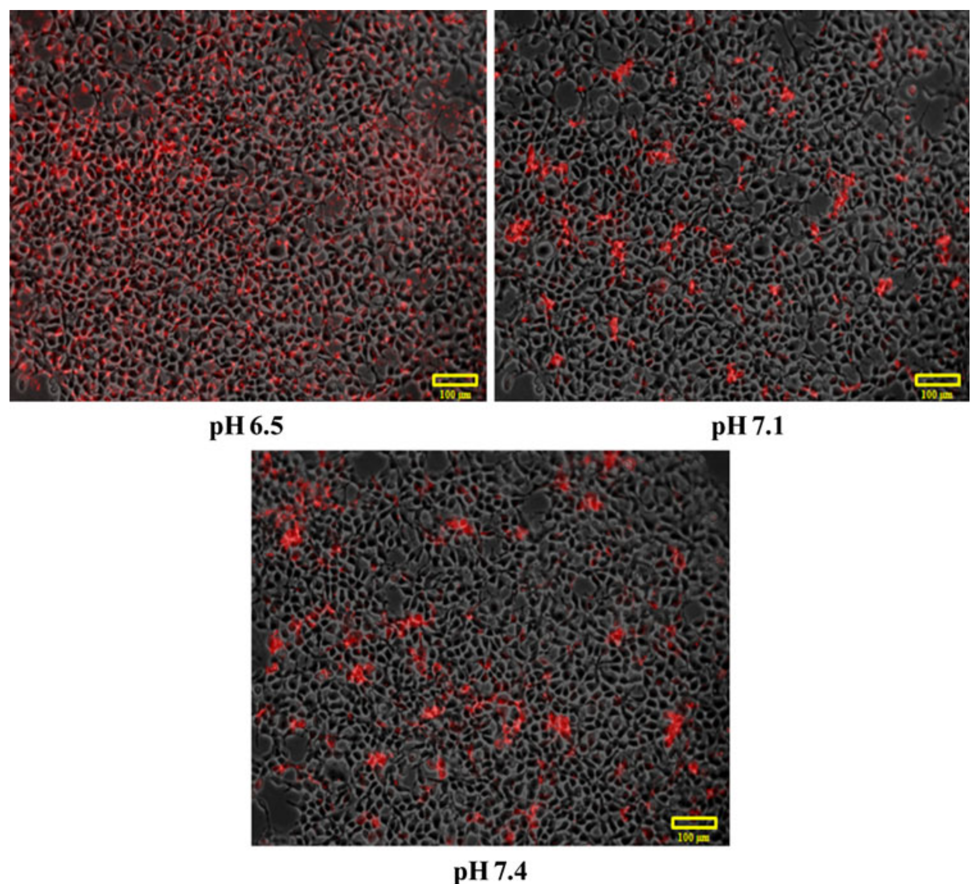

Fig. 2 Fluorescence microscope images of HEK 293 cells transfected at different pH. Cells were exposed to rhodamine labelled chitosan/DNA complexes, and 24 h post-transfection media was exchanged with PBS and cells with internalized or adsorbed complexes visualized under fluorescent microscope at ×10 magnification. Large aggregates present on cell surface at pH 7.4 while complexes were more uniformly distributed at lower pH 6.5

RITC–chitosan was shown in an article from our group to be similar to the unlabelled parent chitosan [4] and this was also found to be the case for labelled DNA compared to unlabelled DNA according to the manufacture's web site (Mirus corporation).

In Vitro Transfection

To estimate the transfection efficiency of chitosan/DNA complexes prepared by complexation of chitosan 92–10 (DDA–MW) with plasmid pEGFPLuc, transfection studies were carried out on HEK 293 cells and compared to a commercially available transfection reagent, Lipofectamine™. Transfection efficiency of complexes was assessed at different pH, i.e., 6.5, 7.1 and 7.4 in medium containing 10% serum. Cells incubated for 24 h with complexes prepared from rhodamine labelled chitosan revealed the presence of large aggregates at pH 7.4 adhering to cell surfaces while RITC–chitosan fluorescence appeared to be more uniformly distributed on the cells at pH 6.5 (Fig. 2). Transfection efficiency expressed as percentage of cells expressing EGFP was 26.3% at pH 6.5 and then dropped considerably at higher pH of 7.1 and 7.4 with 9.2 and 0.2%, respectively, also in agreement with earlier studies (Fig. 3) [11, 14]. This dependence of transfection efficiency on the pH of the transfection medium appears to be related to the protonation of amino groups in chitosan. The pK_a of the amino groups in chitosan is ~6.5, hence in the transfection medium of pH 6.5 chitosan is expected to be highly protonated and the chitosan/DNA complexes to be positively charged [30, 32]. Our zeta potential data in PBS without serum at pH 6.5 was 11.4 ± 4.1 mV and then decreased to 4.5 ± 1.0 mV at pH 7.1, suggesting a stronger affinity of complexes at pH 6.5 for the negatively charged cell membranes. This strong cationic charge of complexes at pH 6.5 could result in a high non-specific affinity for negatively charged cell membranes and consequently high cell uptake, thereby producing higher transfection efficiency than at higher pH of 7.1 or 7.4. Moreover, the complexes size also increased considerably at higher pH of 7.1 and 7.4 as compared to pH 6.5.

Cell Uptake of Complexes

Cell binding and uptake of chitosan/DNA complexes is the first step and an important barrier to efficient transfection. To study the effect of pH and serum on complex internalization, uptake experiments were done at different pH, and in the presence and absence of serum using complexes prepared with rhodamine labelled chitosan [19]. A comparative study was performed to evaluate cell uptake of chitosan presented in two forms (1) as a soluble macromolecule without DNA and (2) as a condensed chitosan/DNA complex. Flow cytometry was used to quantify the uptake of complexes by the cells, however, it does not generally discriminate between membrane bound and internalized fluorescent moieties, so that additional procedures were included to minimize any contribution of surface-bound particles in measuring uptake. Namely, a simple and efficient method involving treatment of the cells with trypsin followed by several washes before FACS analysis was used to remove surface-bound complexes and assess cellular uptake of fluorescent chitosan and complexes. Uptake of complexes by HEK 293 cells was pH and serum dependent, with maximum uptake occurring in medium at pH 6.5 supplemented with 10% FBS (Fig. 4).

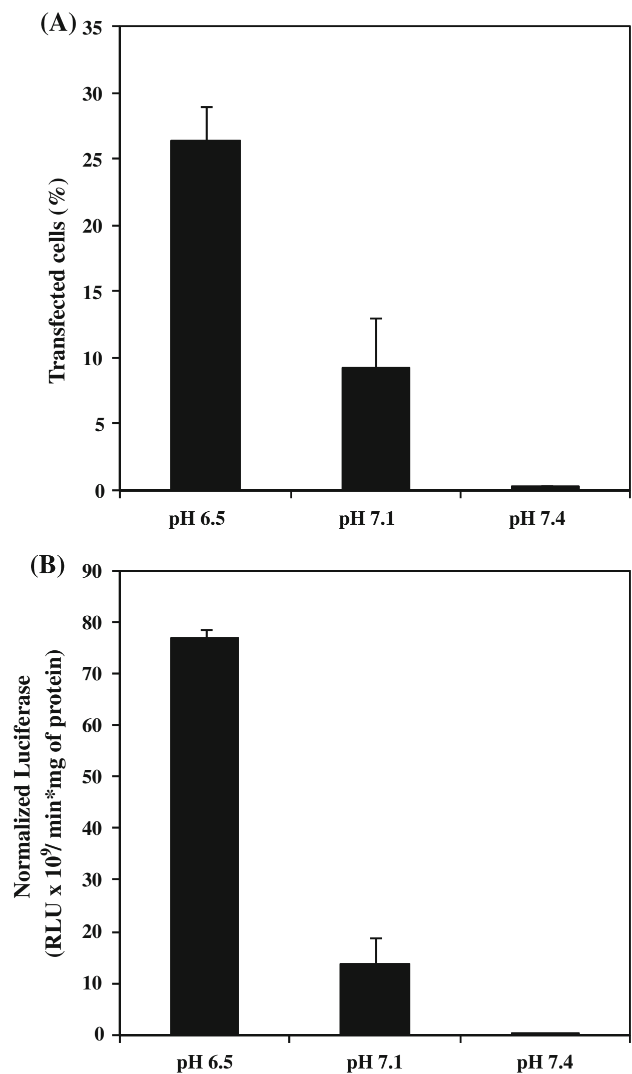

Fig. 3 Transfection efficiency of chitosan/DNA complexes at different pH. HEK 293 cells were transfected with chitosan/DNA complexes at different pH. After 48 h **a** GFP expression was quantified using flow cytometry and expressed as percentage of cells transfected, while **b** the level of gene expression determined by luminometry and expressed as RLU per minute per milligram of protein. Values are mean ± SD, $n = 3$

The uptake of complexes as well as of chitosan alone was higher in presence of serum at all the three investigated pHs. The percentage of fluorescent cells and the fluorescence intensity per cell was significantly enhanced by increasing incubation time from 1 to 24 h. At pH 6.5, almost 100% of cells internalized complexes after 4 h of incubation whereas at pH 7.1 only 50% cells were fluorescent with a lower level of fluorescence. Chitosan interacts with the cell membranes by non-specific electrostatic forces of attraction and any receptor specific to chitosan has not been identified in cell membranes [33]. A high positive surface charge on chitosan nanoparticles is thought to allow an electrostatic interaction with the negatively charged cellular membranes leading to internalization. Our results are in accordance with this theory with higher uptake at pH 6.5 compared to pH 7.1 and 7.4, as chitosan bears higher positive at lower pH (Table 1).

Our results show that the percentage of cells internalizing complexes after 1 h of incubation was less than chitosan alone at all the three pH (Fig. 4). After 4 h of incubation at pH 6.5 with serum, almost 100% of cells internalized complexes and chitosan alone whereas at pH 7.1 with serum only 55% internalized complexes while 100% cells internalized chitosan only. Separate studies underway suggest that approximately 60% of chitosan is free and undergoes uptake separately from the complexes. The level of uptake of chitosan only was much higher in contrast to complexes at all three pH in presence of serum. Also, the level of uptake of chitosan alone was higher with 10% serum versus without serum at all the three pH media. One possible reason for these observations is that positively charged chitosan forms small hydrodynamic diameter complexes with the negatively charged serum proteins which can then be efficiently internalized. To investigate the influence of serum proteins on chitosan, DLS measurements were performed after suspending chitosan in media supplemented with 10% serum. In media with 10% serum, two different populations with particle hydrodynamic diameter of 15 ± 3.6 and 71 ± 18.7 nm were found due to presence of serum proteins whereas in media containing chitosan a third population occurred with particle hydrodynamic diameter of 490 ± 111 nm, which appears due to complexation of chitosan with serum proteins (Fig. 5). These results suggest that, chitosan forms complexes/aggregates with serum proteins that could result in higher uptake when cells are incubated with chitosan in the presence of 10% serum whereas in contrast, very low uptake was observed for chitosan incubated in media without serum. A higher level of uptake was observed with complexes incubated at pH 6.5 media without serum as compared to chitosan only. This significantly higher uptake could be attributed to efficient binding of condensed chitosan to cell membrane due to presence of well defined particles at pH 6.5 in case of complexes as compared to

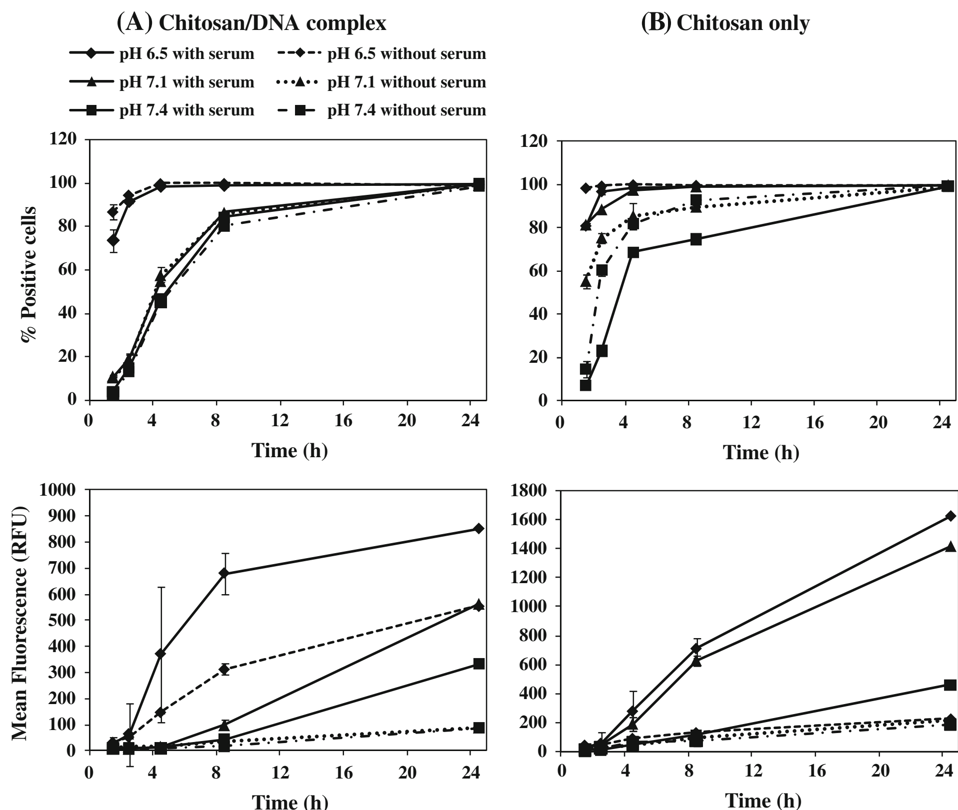

Fig. 4 Cellular uptake of rhodamine labelled chitosan/DNA complexes (**a**) and rhodamine labelled chitosan (**b**) at different pH. HEK 293 cells were incubated with labelled complexes or chitosan at different pH. After the stipulated time points, the percentage of cells with internalized label (*top*) and the mean fluorescence intensity of the label per cell (*bottom*) were determined by flow cytometry. Values are mean ± SD, $n = 3$

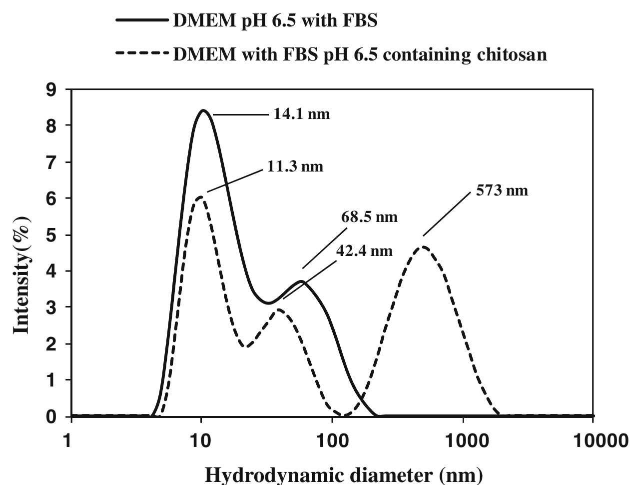

Fig. 5 Representative dynamic light scattering (DLS) spectrum of medium with 10% serum only and medium with 10% serum containing chitosan showing peaks corresponding to serum (11.3–68.5 nm) and chitosan/serum complexes (573 nm)

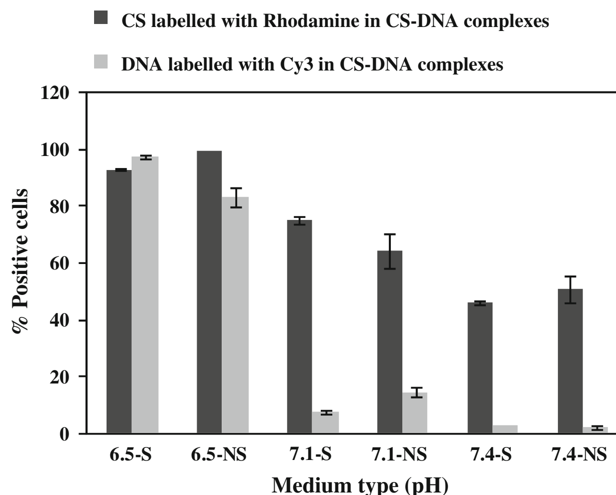

Fig. 6 Comparison of cellular uptake of complexes at different pH. Two different types of complexes were prepared one containing chitosan labelled with rhodamine and another containing DNA labelled with Cy3. After 8 h of transfection, the percentage of cells with internalized label was determined by flow cytometry. Values are mean ± SD, $n = 3$

chitosan only. This dependence of uptake on the presence of condensed particles is consistent with that previously reported where chitosan nanoparticles of hydrodynamic diameter 433 ± 28 nm were reported to have higher internalization than chitosan molecules with hydrodynamic diameter of 830 ± 516 nm [34].

At pH 7.4 with serum, transfection was almost absent while a high amount of uptake was observed after 24 h which was likely due to free chitosan not bound to DNA but rather to serum proteins. It can be speculated from these uptake data that either the amount of complexes internalized at higher pH, i.e., 7.1 and 7.4 is not sufficient enough to yield efficient transfection or was mostly free chitosan is internalized at these pH.

To more clearly distinguish between the uptake of free chitosan and chitosan/DNA complexes at all the three pHs, uptake experiments were also done with complexes prepared from Cy3 labelled DNA. Our results for 8 h of incubation with complexes show that the percentage of Cy3 positive cells at pH 6.5 with serum was almost equal to rhodamine positive cells at this pH for cells transfected with rhodamine labelled complexes (Fig. 6). For pH 7.1 and 7.4, however, the percentage of Cy3 positive cells was reduced to ~8 and 3% in contrast to about 90% rhodamine positive cells in presence of 10% serum. This difference in uptake comparing RITC–chitosan to Cy3–DNA can be explained by the presence of a significant amount of free chitosan in the complexes prepared at N/P ratio of 5. Thus the higher uptake of complexes prepared with RITC–chitosan at higher pH is most likely due to free chitosan since Cy3–DNA uptake was very much lower and in accordance with the lower values of transfection efficiency at these pHs (Fig. 3).

To fully substantiate the uptake results from flow cytometry analysis, cellular internalization of nanoparticles was assessed with confocal microscopy. Though, this technique is more qualitative than quantitative, it provides a direct observation of the localization of the fluorescent nanoparticles in the cells providing evidences of cellular internalization. During cellular tracking of the fluorescent chitosan/DNA complexes, fluorescence appeared to be distributed throughout inside the cells at pH 6.5 with serum (Fig. 7). However, cells incubated with the complexes at pH 7.1 and 7.4 with serum showed large aggregates found near the cell membranes with only small amounts that were internalized. At pH 7.1 and 7.4 without serum, internalization of complexes was almost absent, although small amount of chitosan uptake could be seen.

Kinetics of Gene Expression

The kinetics of gene expression was evaluated quantitatively by flow cytometry for EGFP along with a luciferase assay employing luminometry. At pH 6.5 in the presence of serum, an increase in the percentage of transfected cells was observed as a function of time up to 48 h and remained relatively constant thereafter (Fig. 8). After 12 h post-transfection, the percentage of cells expressing GFP was approximately 7.1% which increased to 26.7% for 48 h and 22.2% for 96 h. Similarly, the level of gene expression as measured by luciferase activity increased up to 48 h post-transfection and remained constant thereafter.

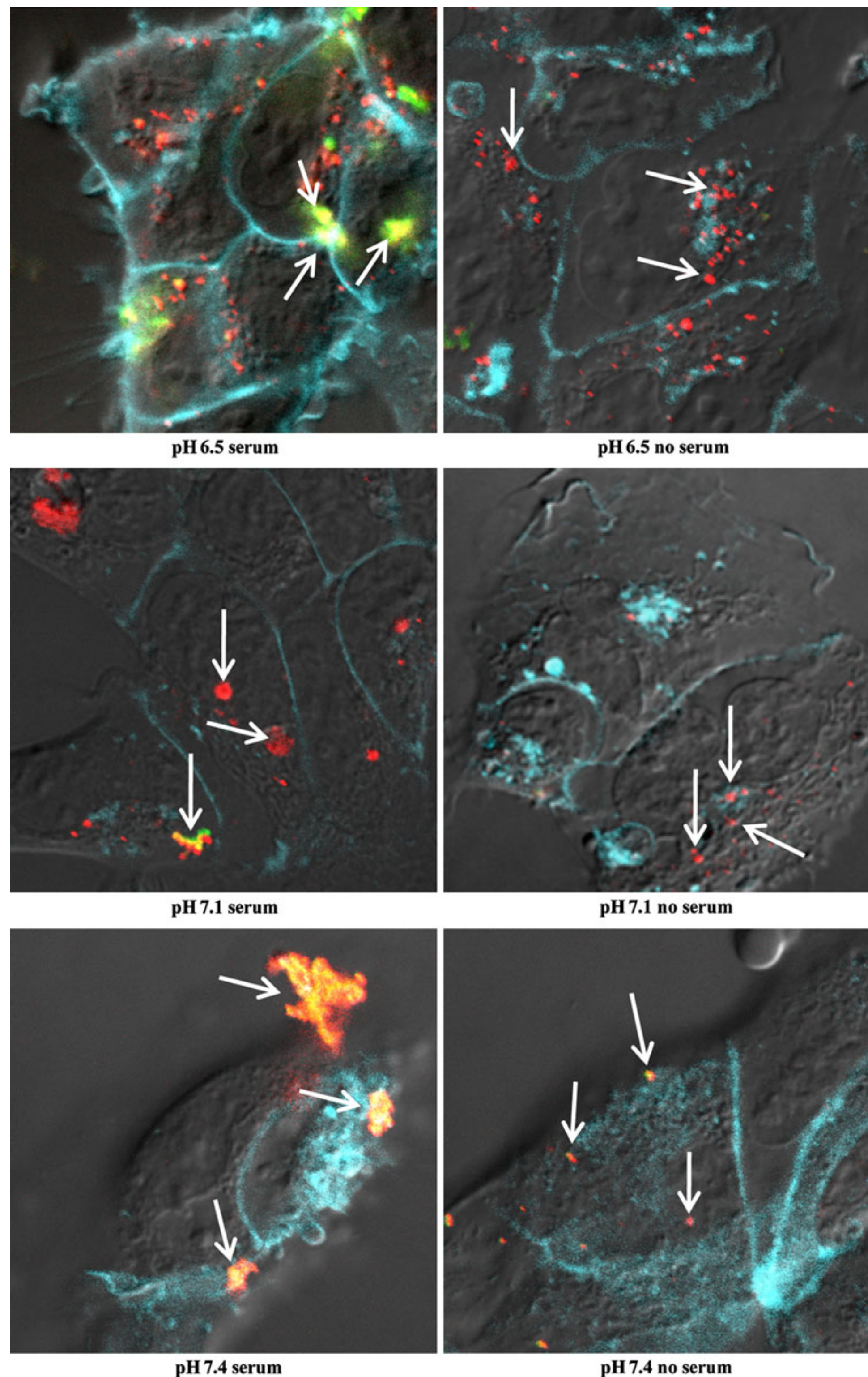

Fig. 7 Confocal microscope images of HEK 293 cells transfected at different pH. Cells transfected with chitosan/DNA complexes, where chitosan labelled with rhodamine and DNA with fluorescein, visualized under confocal microscope 24 h post-transfection. *Blue colour*—staining of cell membrane, *red colour*—rhodamine labelled chitosan only, *green colour*—fluorescein labelled DNA only and *yellow colour*—colocalization showing chitosan/DNA complex. At pH 6.5 with serum, a large amount of complexes are visible inside the cell (*white arrows*) whereas at pH 6.5 without serum, a large amount of free chitosan can be seen inside the cell (*white arrows*). At pH 7.1 with serum, a small amount of chitosan and complexes were visible (*white arrows*) whereas at pH 7.1 without serum only a small amount of free chitosan was seen inside cells (*white arrows*) without any internalization of complexes. At pH 7.4 with serum, large aggregates were observed outside the cells external to the cell membrane (*white arrows*) while at pH 7.4 without serum, a small amount of complexes were observed on cell membranes (*white arrows*). (Color figure online)

Optimization of Transfection Through Media Changes

To further investigate the effect of serum and pH on the transfection with chitosan/DNA complexes, and to optimize transfection efficiency, a series of experiments were performed by changing transfection media in the presence or absence of serum. Cells transfected in medium with serum at pH 6.5 for 8–24 h followed by replenishment with medium supplemented with 10% serum pH 7.4 produced the highest number of transfected cells, i.e., 41.8% at 48 h

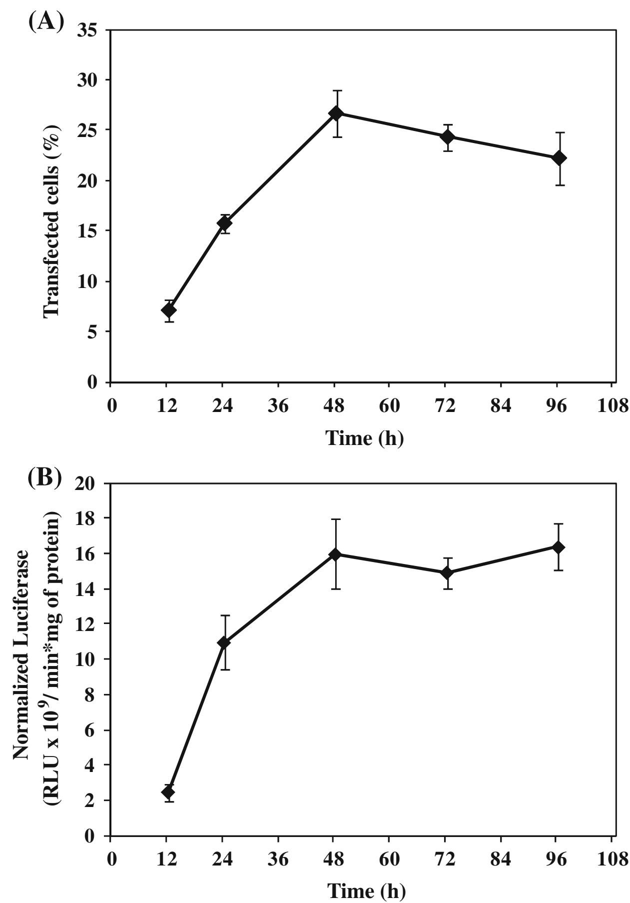

Fig. 8 Kinetics of gene expression for HEK 293 cells transfected with chitosan/DNA complexes at pH 6.5 with serum and analyzed by **a** flow cytometry for percentage of cells expressing GFP and by **b** luminometry for the expression of luciferase. Values are mean ± SD, $n = 3$

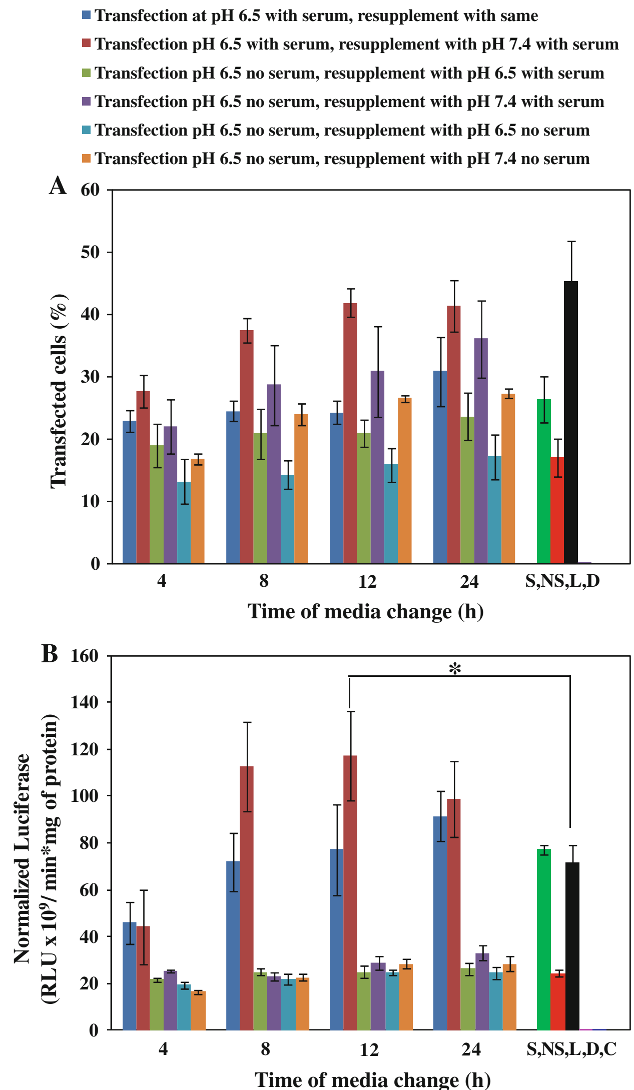

Fig. 9 Transfection efficiency of chitosan/DNA nanoparticles at different pH. HEK 293 cells were transfected with chitosan/DNA complexes in media with or without 10% serum. After stipulated time points, transfection media was replaced by the same media or by media of different pH and/or serum content and transfection quantified at 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. Controls on the right are *S*: transfection in medium pH 6.5 with serum and analysis after 48 h, *NS*: transfection in medium pH 6.5 without serum and analysis after 48 h, *L*: transfection with Lipofectamine™ and analysis after 48 h, *D*: transfection with DNA only in medium pH 7.4 with serum, *C*: non-transfected cells. An *asterisk* indicates very significant difference with $P < 0.01$. Values are mean ± SD, $n = 3$

post-transfection and the highest level of protein expression (Fig. 9). In contrast, maintaining cells at pH 6.5 for the entire 48 h transfection period resulted in only 26.4% of cells being transfected. Uptake of complexes is greatly facilitated at pH 6.5 (Fig. 6), but once uptake is complete at around 8–24 h (Fig. 4) it appears 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. It has been previously reported that a slightly acidic condition resulted in decreased cell metabolism leading to reduction of glucose metabolism and lactate production [35–37]. Also, as discussed below, our cell viability data shows higher cell viability at pH 7.4 as compared to pH 6.5 and 7.1 (Fig. 10).

One of the advantages associated with chitosan is that it is highly efficient in carrying out transfection in the presence of serum. Sato et al. [14] demonstrated that with chitosan/pGL3 complexes, at the serum content of 10%, gene expression level increased about 2–3 times as compared to medium without serum. Herein, we also observed that transfection efficiency is 20–50% lower in the absence of serum compared to with serum for time points of 8, 12 and 24 h at pH 6.5 or 7.4 (Fig. 9). The serum deprived cells continue to cycle until they complete mitosis, whereupon they exit into the G0 state and further division stops [38, 39]. The increase in transfection

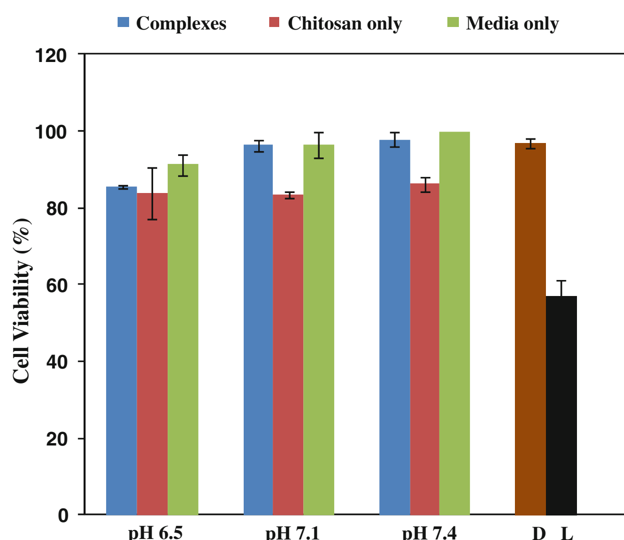

Fig. 10 Cell viability at different pH. HEK 293 cells treated with chitosan, chitosan/DNA complexes at different pH, 48 h post-transfection cell viability determined using Alamar Blue. Cells at pH 7.4 in complete media taken as 100% viable, other controls include *D*: cells incubated with DNA alone, *L*: cells transfected using Lipofectamine^{TM}. Values are mean ± SD, $n = 3$

efficiency in the presence of serum could thus also be attributed to serum promoting cell division.

Interaction of complexes with the cell membrane is the initial and one of the most significant steps for efficient transfection. In view of our zeta potential data and high uptake of chitosan/DNA complexes at pH 6.5 it appears that a cationic charge of the complex is a clear requirement for efficient transgene expression using these standard in vitro techniques of simple incubation of cells with media containing the complexes. One mechanism involved in this process could be non-specific electrostatic attraction to negatively charged cell membranes thereby promoting uptake [40]. A second possibility is the stabilization of hydrodynamic diameter at lower pH through interparticle electrostatic repulsion thus limiting particle aggregation to below 1 μm in diameter (Table 1) compared to fairly large aggregates that could be seen even visually at higher pH (Fig. 2).

Cytotoxicity

To assess potential cytotoxicity of high DDA (92%) and low MW (10 kDa) chitosan used in this study, cell viability was determined. Cell confluency, a qualitative measure of cell viability based upon cell coverage on the well surface, as judged by microscopy, indicated little toxicity at the levels of chitosan/DNA complexes used for transfection. To obtain a more quantitative measure of cell viability, we assayed cell metabolism activity using Alamar Blue [25, 26]. A comparative study between Alamar Blue and MTT assay revealed that the former is a single step procedure with higher sensitivity than MTT (data not shown). Microscopic examination of cells transfected with control Lipofectamine^{TM} revealed considerable toxicity and cell morbidity with cell viability reduced to ~57% after 48 h. However, the chitosan/DNA complexes were found to be only slightly toxic, where after 48 h of incubation more than 85% of cells were viable at pH 6.5 and 96% at pH 7.1 (Fig. 10). The lower cell viability at pH 6.5 could be due to increased charge density of chitosan/DNA complexes since cytotoxicity has been found to be dependent on the interaction of the polymers with cell membranes which increases with polycationic charge [41]. 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. [41] 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. A comparative study between polycationic, neutral and polyanionic polymers revealed that the polycationic polymers have the highest toxicity followed by neutral and anionic ones [42]. Our results are in accordance with this theory as we observed slightly higher toxicity at pH 6.5 where the complexes bear high positive charge and the lowest toxicity at pH 7.4 where the complexes were negative. Also, the viability of cells incubated with chitosan only was lower than with chitosan/DNA complexes, as the native polymer bears more positive charge compared to the polymer in complexes that is partly neutralized by binding to DNA (Fig. 10).

Conclusion

We have evaluated the combined effect of pH and serum on the size and zeta potential of chitosan/DNA complexes, in addition to examining cell uptake of complexes leading to transfection and/or cytotoxicity. The size of complexes was found to increase with the increase in pH from 6.5 to 7.4 whereas the zeta potential decreased. Maximum cell uptake and maximal transgene expression was observed when transfection was initiated at pH 6.5 supplemented with 10% serum, where complex size remains below 1 μm and complexes are positively charged. Optimization studies investigating uptake kinetics and media changes revealed that changing from pH 6.5 to 7.4 at 8, 12 or 24 h after initiating transfection produced the highest level of gene expression at 48 h, reaching 42% of cells being transfected and higher protein levels of transgene expression than

Lipofectamine™. High transfection efficiency attained when initiating transfection at pH 6.5 with 10% serum was due to the high cell uptake of positively charged complexes with limited aggregation. The presence of serum further enhances the transgene expression owing to a combination of increased uptake, and improved cell metabolism and division in contrast to cells without serum. This improved understanding of the transfection mechanisms of chitosan/DNA complexes allows for further development and optimization of these therapeutic vehicles as gene delivery vehicles.

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References

1. Felgner, P. L. (1997). Nonviral strategies for gene therapy. *Scientific American, 276*, 102–106.
2. Davis, M. E. (2002). Non-viral gene delivery systems. *Current Opinion in Biotechnology, 13*, 128–131.
3. Zhao, Z., Wang, J., Mao, H. Q., & Leong, K. W. (2003). Polyphosphoesters in drug and gene delivery. *Advanced Drug Delivery Reviews, 55*, 483–499.
4. Özgel, G., & Akbuga, J. (2006). In vitro characterization and transfection of IL-2 gene complexes. *International Journal of Pharmaceutics, 315*, 44–51.
5. MacLaughlin, F. C., Mumper, R. J., Wang, J., Tagliaferri, J. M., Gill, I., Hinchcliffe, M., et al. (1998). Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. *Journal of Controlled Release, 56*, 259–272.
6. Richardson, S. C., Kolbe, H. V., & Duncan, R. (1999). Potential of low molecular mass chitosan as a DNA delivery system: Biocompatibility, body distribution and ability to complex and protect DNA. *International Journal of Pharmaceutics, 178*, 231–243.
7. Borchard, G. (2001). Chitosans for gene delivery. *Advanced Drug Delivery Reviews, 52*, 145–150.
8. Guliyeva, Ü., Öner, F., Özsoy, S., & Haziroglu, R. (2006). Chitosan microparticles containing plasmid DNA as potential oral gene delivery system. *European Journal of Pharmaceutics and Biopharmaceutics, 62*, 17–25.
9. Mansouri, S., Lavigne, P., Corsi, K., Benderdour, M., Beaumont, E., & Fernandes, J. C. (2004). Chitosan–DNA nanoparticles as non-viral vectors in gene therapy: Strategies to improve transfection efficacy. *European Journal of Pharmaceutics and Biopharmaceutics, 57*, 1–8.
10. Mansouri, S., Cuie, Y., Winnik, F., Shi, Q., Lavigne, P., Benderdour, M., et al. (2006). Characterization of folate–chitosan–DNA nanoparticles for gene therapy. *Biomaterials, 27*, 2060–2065.
11. Lavertu, M., Methot, S., Tran-Khanh, N., & Buschmann, M. D. (2006). High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation. *Biomaterials, 27*, 4815–4824.
12. Ma, P. L., Lavertu, M., Winnik, F. M., & Buschmann, M. D. (2009). New insights into chitosan–DNA interactions using isothermal titration microcalorimetry. *Biomacromolecules, 10*, 1490–1499.
13. Nakamura, F., Onishi, H., Machida, Y., & Nagai, T. (1992). Lysozyme-catalyzed degradation properties of the conjugates between chitosans having some deacetylation degrees and methotrexate. *Yakuzaigaku, 52*, 59–67.
14. Sato, T., Ishii, T., & Okahata, Y. (2001). In vitro gene delivery mediated by chitosan. Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. *Biomaterials, 22*, 2075–2080.
15. Zhao, X., Yu, S. B., Wu, F. L., Mao, Z. B., & Yu, C. L. (2006). Transfection of primary chondrocytes using chitosan–pEGFP nanoparticles. *Journal of Controlled Release, 112*, 223–228.
16. Peng, S. F., Yang, M. J., Su, C. J., Chen, H. L., Lee, P. W., Wei, M. C., et al. (2009). Effects of incorporation of poly(gamma-glutamic acid) in chitosan/DNA complex nanoparticles on cellular uptake and transfection efficiency. *Biomaterials, 30*, 1797–1808.
17. Bozkir, A., & Saka, O. M. (2004). Chitosan–DNA nanoparticles: Effect on DNA integrity, bacterial transformation and transfection efficiency. *Journal of Drug Targeting, 12*, 281–288.
18. Erbacher, P., Zou, S., Bettinger, T., Steffan, A. M., & Remy, J. S. (1998). Chitosan-based vector/DNA complexes for gene delivery: Biophysical characteristics and transfection ability. *Pharmaceutical Research, 15*, 1332–1339.
19. Ma, O., Lavertu, M., Sun, J., Nguyen, S., Buschmann, M. D., Winnik, F. M., et al. (2008). Precise derivatization of structurally distinct chitosans with rhodamine B isothiocyanate. *Carbohydrate Polymers, 72*, 616–624.
20. Wet Wood, J. R.d., DeLuca, K. V., Helinski, D. R., & Subramani, S. (1987). Firefly luciferase gene: Structure and expression in mammalian cells. *Molecular and Cellular Biology, 7*, 725–737.
21. Jean, M., Smaoui, F., Lavertu, M., Methot, S., Bouhdoud, L., Buschmann, M. D., & Merzouki, A. (2009). Chitosan–plasmid nanoparticle formulations for IM and SC delivery of recombinant FGF-2 and PDGF-BB or generation of antibodies. *Gene Therapy, 16*, 1097–1110.
22. Prochazkova, S., Varum, K. M., & Ostgaard, K. (1999). Quantitative determination of chitosans by ninhydrin. *Carbohydrates Polymers, 38*, 115–122.
23. Hay, R. J., Caputo, J., & Macy, M. L. (1992). *ATCC quality control methods for cell lines* (2nd ed.). Rockville, MD: ATCC.
24. Hashimoto, M., Morimoto, M., Saimoto, H., Shigemasa, Y., & Sato, T. (2006). Lactosylated chitosan for DNA delivery into hepatocytes: The effect of lactosylation on the physicochemical properties and intracellular trafficking of pDNA/chitosan complexes. *Bioconjugate Chemistry, 17*, 309–316.
25. Nociari, M. M., Shalev, A., Benias, P., & Russo, C. (1998). A novel one-step, highly sensitive fluorometric assay to evaluate cell-mediated cytotoxicity. *Journal of Immunological Methods, 213*, 157–167.
26. Nakayama, G. R., Caton, M. C., Nova, M. P., & Parandoosh, Z. (1997). Assessment of the Alamar Blue assay for cellular growth and viability in vitro. *Journal of Immunological Methods, 204*, 205–208.
27. Wolfert, M. A., & Seymour, L. W. (1996). Atomic force microscopic analysis of the influence of the molecular weight of poly(L)lysine on the size of polyelectrolyte complexes formed with DNA. *Gene Therapy, 3*, 269–273.
28. Evans, D. F., & Wennerström, H. (1999). *The colloidal domain: Where physics, chemistry, biology and technology meet* (2nd ed.). New York/Weinheim: Wiley/VCH.

29. Donini, C., Robinson, D. N., Colombo, P., Giordano, F., & Peppas, N. A. (2002). Preparation of poly(methacrylic acid-g-poly(ethylene glycol)) nanospheres from methacrylic monomers for pharmaceutical applications. *International Journal of Pharmaceutics, 245*, 83–91.
30. Filion, D., Lavertu, M., & Buschmann, M. D. (2007). Ionization and solubility of chitosan solutions related to thermosensitive chitosan/glycerol–phosphate systems. *Biomacromolecules, 8*, 3224–3234.
31. Mao, H. Q., Roy, K., Troung-Le, V. L., Janes, K. A., Lin, K. Y., Wang, Y., et al. (2001). Chitosan–DNA nanoparticles as gene carriers: Synthesis, characterization and transfection efficiency. *Journal of Controlled Release, 70*, 399–421.
32. Anthonsen, M. W., & Smidsrod, O. (1995). Hydrogen ion titration of chitosans with varying degrees of N-acetylation by monitoring induced 1H-NMR chemical shifts. *Carbohydrate Polymers, 26*, 303–305.
33. Schipper, N. G., Olsson, S., Hoogstraate, J. A., deBoer, A. G., Varum, K. M., & Artursson, P. (1997). Chitosans as absorption enhancers for poorly absorbable drugs 2: Mechanism of absorption enhancement. *Pharmaceutical Research, 14*, 923–929.
34. Ma, Z., & Lim, L. Y. (2003). Uptake of chitosan and associated insulin in Caco-2 cell monolayers: A comparison between chitosan molecules and chitosan nanoparticles. *Pharmaceutical Research, 20*, 1812–1819.
35. Ozturk, S. S., & Palsson, B. O. (1991). Growth, metabolic, and antibody production kinetics of hybridoma cell culture: 2. Effects of serum concentration, dissolved oxygen concentration, and medium pH in a batch reactor. *Biotechnology Progress, 7*, 481–494.
36. Miller, W. M., Blanch, H. W., & Wilke, C. R. (1988). A kinetic analysis of hybridoma growth and metabolism in batch and continuous suspension culture: Effect of nutrient concentration, dilution rate, and pH. *Biotechnology and Bioengineering, 32*, 947–965.
37. Sauer, P. W., Burky, J. E., Wesson, M. C., Sternard, H. D., & Qu, L. (2000). A high-yielding, generic fed-batch cell culture process for production of recombinant antibodies. *Biotechnology and Bioengineering, 67*, 585–597.
38. Pardee, A. B. (1974). A restriction point for control of normal animal cell proliferation. *Proceedings of the National Academy of Sciences of the United States of America, 71*, 1286–1290.
39. Pardee, A. B. (1989). G1 events and regulation of cell proliferation. *Science, 246*, 603–608.
40. Huang, M., Fong, C. W., Khor, E., & Lim, L. Y. (2005). Transfection efficiency of chitosan vectors: Effect of polymer molecular weight and degree of deacetylation. *Journal of Controlled Release, 106*, 391–406.
41. Fischer, D., Li, Y., Ahlemeyer, B., Krieglstein, J., & Kissel, T. (2003). In vitro cytotoxicity testing of polycations: Influence of polymer structure on cell viability and hemolysis. *Biomaterials, 24*, 1121–1131.
42. Jevprasesphant, R., Penny, J., Jalal, R., Attwood, D., McKeown, N. B., & D'Emanuele, A. (2003). The influence of surface modification on the cytotoxicity of PAMAM dendrimers. *International Journal of Pharmaceutics, 252*, 263–266.