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Evaluation of Chitosan-Tripolyphosphate Nanoparticles as a p-shRNA Delivery Vector: Formulation, Optimization and Cellular Uptake Study

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Abstract

Polysaccharides (especially chitosan) have recently attracted much attention as gene therapy delivery vehicles for their unique properties such as biocompatibility, biodegradability, low toxicity, and controlled release. Nanoparticles have strong potential as a carrier of plasmid short hairpin RNA (p-shRNA). This study aimed to find the optimum conditions for obtaining Chitosan/tripolyphosphate (TPP)/p-shRNA nanoparticles by the ionic gelation method, and investigating the cellular uptake of the optimized nanoparticles. After applying the central composite design of response surface methodology (RSM), the optimum conditions for preparation of nanoparticles with small size and high loading efficiency were: chitosan/TPP ratio = 10, pH = 5.5 and N/P ratio = 11. The resulting nanoparticles had an average size of 172.8 ± 7 nm and loading efficiency of $71.5 \pm 5\%$. SEM images showed spherical and smooth nanoparticles. The nanoparticles complexed with p-shRNA and may protect it against nuclease digestion. Cytotoxicity studies with HeLa and PC3 human cancer cells demonstrated that chitosan/TPP nanoparticles had low toxicity. Cellular uptake studies using HeLa cells showed that the nanoparticles entered the cells (cellular uptake) and delivered DNA, probably due to their favorable Zeta potential (approximately +28 mV) and small size.

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Keywords

Chitosan; Nanoparticle; Response Surface Methodology (RSM); p-shRNA; Cellular Uptake; Gene Delivery

INTRODUCTION

Polysaccharides and other natural biopolymers have recently received much attention in the pharmaceutical field due to their favorable properties such as being biodegradable, biocompatible, non-toxic, as well as facilitating controlled release of drugs and genetic materials. One of their recent most interesting applications is their use as a DNA carrier in gene therapy.¹

Gene therapy is a promising therapeutic approach for the treatment of genetic disorders, and intractable diseases such as cancer and infections.^{2, 3} Furthermore, it has the potential to overcome inherent problems associated with administration of protein drugs such as systemic toxicity, *in vivo* clearance rate, bioavailability and manufacturing costs. Gene therapy can be considered a promising alternative to conventional protein therapy.⁴

Although the main purpose of gene therapy is to increase the expression of a target protein through the use of nucleic acid vectors such as plasmids, it can also be applied to decrease the target protein production via the use of siRNA and antisense oligonucleotides.⁵ The main challenge in gene therapy is the delivery of the nucleic acid material which has yet to be resolved.^{3, 6} Two types of carriers are mainly utilized in gene delivery; viral carriers and non-viral carriers.^{7, 8} In spite of the high efficiency of viral delivery, the broad use of viruses has certain disadvantages such as the limited size of genetic materials, high immunogenicity, absence of targeting ability to cells of interest, and the possible induction of cancer if the virus integrates into a tumor suppressor gene. These limitations of viral gene delivery have favored exploration of non-viral gene vectors. Despite its low efficiency, non-viral gene delivery is now a promising approach due to its low immunogenicity, unrestricted DNA size, low production cost and reproducibility.^{2-4, 9} Non-viral gene delivery systems of recent interest are based on lipid, peptide, polymeric or dendrimeric carriers.¹⁰⁻¹³

Cationic polymers are among the most significant non-viral gene-delivery systems that generally possess amine groups in their backbone, which enable them to interact with the negative charge of nucleic acids. Due to their cationic and biocompatible properties, chitosan nanoparticles have drawn much attention for gene delivery in recent years.^{14, 15}

As an abundant natural N-acetylated polysaccharide, chitin exists in crustacean shells, yeast and fungi. Chitosan is composed of $\alpha(1-4)$ -2-amino-2-deoxy β -D-glucan monomers prepared by the partial N-deacetylation of chitin.¹⁶ It has been considered as a good candidate for gene delivery because it possesses many favorable properties: such as being biodegradable, biocompatible, non-toxic and inexpensive.¹⁷⁻²¹ From the technical point of view, the cationic polyelectrolyte nature of chitosan is promising for strong electrostatic interactions between negatively charged DNA and the positive charge of chitosan, and also protects the DNA from nuclease degradation.¹⁴ In summary, owing to its biopharmaceutical

characteristics, chitosan turns out to be a favorable carrier for gene delivery, oral protein delivery, and controlled released systems.

RNA interference (RNAi) is a post-transcriptional gene silencing technology achieved by double-stranded RNA (dsRNA)-mediated sequence complementarity to gene of interest. RNAi is found in a broad range of organisms such as plants, nematodes, flies and mammals.²² There is broadly conserved cellular machinery for dsRNA-induced gene silencing, which proceeds via a two-step mechanism. The first step consists of recognition and digestion of dsRNA into 21–23 base siRNAs (small interfering RNAs) by an RNase III family nuclease called “Dicer”. In the second step, these siRNAs are incorporated into a multi-component nuclease complex, RISC (the RNA induced silencing complex), which recognizes the targeted mRNAs through their homology to siRNAs and cleavages these cognate mRNAs leading to their destruction.²³

Two types of nucleic acid molecules are typically employed for RNAi: (A) chemically synthesized double-stranded small interfering RNA (siRNA); and (B) plasmid based short hairpin RNA (p-shRNA). In the present study we focus on shRNA, which is a plasmid-based strategy for silencing a target gene. shRNA is a RNA molecule with a hairpin structure, which is homologous to a region within the target gene. It is synthesized in the nucleus of cells, transported to the cytoplasm, modified and then incorporated into the RNA-induced silencing complex (RISC). Together RNAi and shRNA have found increasing therapeutic applications in recent years, especially for cancer treatment.²⁴ For example, chitosan nanoparticles have been used to deliver p-shRNA against the MDR1 drug efflux pump to increase taxol sensitivity in ovarian cancer cells.²⁵

To date, different methods have been proposed for preparation of chitosan nanoparticles, including ionic gelation, covalent cross-linking, desolvation and coacervation.¹⁴ The most popular method is probably ionotropic gelation of chitosan by the addition of TPP (sodium tripolyphosphate). TPP is a small negative ion that contains three negative charges at physiological pH²⁶ and its non-toxicity²⁷ favors its medical application. This compound is able to create five ionic cross-linking points with cationic groups of chitosan.^{28, 29} These characteristics make TPP one of the best cross-linking agents for preparation of chitosan nanoparticles.

To our knowledge there is no previous report on the systematic optimization of chitosan/TPP/p-shRNA nanoparticle preparation. As the use of RNAi and shRNA is an emerging approach with potentially wide therapeutic application, optimizing nanocarriers for delivery of these molecules is of some significance. Response surface methodology (RSM) is a commonly used statistical method that attempts to find the optimum conditions for performing a process with many variables. RSM has advantageous properties such as reducing the number of experimental trials, evaluating the interactions among multiple factors, as well as being highly effective and precise, and these characteristics make it an excellent tool for the purpose of optimization.^{30–33}

In 2009, Nasti et al. studied the effect of varying parameters such as pH and chitosan/TPP mass ratio on the size and zeta potential of chitosan/TPP nanoparticles.²⁶ However, since

genetic material was not used in their study they could not assess the impact of N/P ratio (molar ratio of amino groups of chitosan to phosphate groups of DNA) on the chitosan/TPP nanoparticles.

In the present study, the effects of three key factors (mass ratio of chitosan to TPP, pH and N/P ratio) on the size and loading efficiency of chitosan/TPP nanoparticles containing p-shRNA-EGFR were investigated by response surface methodology. The interaction of DNA with chitosan strongly affects the physico-chemical characteristics of nanoparticles, including their size and zeta potential. Generally a higher N/P ratio improves the results and provides better nucleic acid delivery. Furthermore, due to interaction of the N/P ratio with pH and chitosan/TPP ratio, changing the N/P ratio may modify the optimum values for pH and chitosan/TPP ratio calculated in the absence of genetic material. Finally, cytotoxicity and cellular uptake of the optimized nanoparticles were investigated on human cancer cell lines.

MATERIALS AND METHODS

Materials

Pentasodium tripolyphosphate (Sigma Aldrich), HCl (Merck), NaOH (Merck) were purchased. Chitosan ("low MW": Cat. No. 448869, Sigma Aldrich) was used after purification as will be described in Section (Purification of chitosan). psiRNA-hH1GFPzeo (Invivogen, San Diego, USA) and EGFR oligonucleotides (5-GTACCTCAGGAATTAAGAGAAGCAACATTCAAGAGATGTTGCTTCTCTTAATTCCTTTTGGAAA-3, 5-AGCTTTTCCAAAAAGGAATTAAGAGAAGCAACATCTCTTGAATGTTGCTTCTCTTAATTCCTGAG-3) and 5-FITC labeled phosphorothioated anti Bcl2 oligonucleotide (5-TCT CCC AGC GTG CGC CAT-3) were purchased from Eurofins MWG Operon (Huntsville, AL) and used as received.

Purification of Chitosan

Chitosan purification was performed as previously described with some modifications.²⁶ Briefly, 3 g of chitosan was dissolved in 300 mL of acetic acid solution (2% w/v) in double distilled water and stirred overnight. To denature and precipitate any proteic contaminant, the solution was boiled for 15 min. In order to separate any possible aggregated or denatured proteic contaminant, the resultant mixture was then centrifuged at 4500 rpm for 10 min. Afterwards, the supernatant was removed and passed through 0.4 μ m pore size filters. Chitosan was subsequently precipitated from the aqueous phase by adjusting the pH of the solution to 9 by adding 1 N sodium hydroxide. After centrifugation, the precipitate was redispersed in water at pH = 9 and again twice sedimented by centrifugation. The procedure was repeated with double distilled water until the conductivity and pH values became equal with those of pure water. The sample was stored at 4 °C after freeze-drying.

Preparation of p-shRNA-EGFR

In order to construct p-shRNA-EGFR, first the double-stranded (ds) oligonucleotide was hybridized by combining equal amounts of sense and antisense strands (500 pmol) in 50 μ l of annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM Mg-

acetate). The oligonucleotides were then annealed by holding the reaction at 97 °C for 2 min, and then cooling slowly to 30 °C with a ramp rate of 0.1 °C per second and hold at 30 °C for 30 min.

psiRNA-hH1 was digested with restriction enzymes Acc 65I and Hind III, and the resulting 3332 nucleotide fragment was ligated to the annealed ds oligonucleotide. The construct (Fig. 1) was transformed into *E. coli* and the obtained clones were confirmed by DNA sequencing.

Nanoparticle Preparation

Stock Solution—Stock solutions of chitosan and TPP were prepared at different pH values at concentration of 1 mg/ml. The pH values of the solutions were adjusted by 0.1 M NaOH and 0.1 M HCl. The stock solution of p-shRNA-EGFR was 1000 ng/μl.

Preparation of Chitosan/TPP/p-shRNA Nanoparticles—The mechanism of ionotropic gelation method for nanoparticle preparation is based on electrostatic interaction between negatively charged and positively charged molecules such as polyanions and cationic polymers. In the case of chitosan nanoparticles, the chitosan amino groups interact with anionic groups of tripolyphosphate.

For each run, the concentration of DNA was fixed and the concentration of chitosan varied to reach the corresponding N/P ratios. After combination of the appropriate amount of chitosan and DNA, the solution was mixed for 10 min and its volume was 1.5 ml. Then, 1 ml TPP with the concentration required for reaching the corresponding chitosan/TPP ratio was added in a dropwise manner. The resulting solution was stirred for 30 min, and then centrifuged in 4000 g for 5 min. The supernatant was separated in a new tube and used for subsequent analysis.

Nanoparticle Characterization

The size and zeta potential of the prepared nanoparticles were measured by photon correlation spectroscopy (PCS) using a Malvern Zetasizer ZS series and Scattering Particle Size Analyzer (Malvern Co., UK). The samples were sonicated for 5 min in a bath ultrasonicator (Wisd, WUC-D10H) before being analyzed and immediately used for measurements.

Analytical SEM studies of morphological features were performed for evaluation of shape, size and aggregation phenomena. To this end, nanoparticle samples were mounted on metal subs which were gold coated under vacuum, and were then examined on a FE-SEM (JSM-6700F; JEOL Ltd., Tokyo, Japan).

Loading Efficiency

To determine the loading efficiency, nanoparticles with different N/P ratios were centrifuged at 60000 g and 15 °C for 60 min and the amount of free p-shRNA-EGFR was determined in supernatant by Nanodrop 2000c spectrophotometer (Nano-drop Technologies, Wilmington, DE) at 260 nm using supernatant of non-loaded nanoparticles as basic correction. The

amount of incorporated p-shRNA-EGFR was calculated by the difference between the initial total amount of p-shRNA-EGFR and the measured amount in the supernatant. The loading efficiency of nanoparticles was determined by using the following equation:

$$\text{Loading efficiency (LE\%)} = \frac{T_{\text{p-shRNA}} - F_{\text{p-shRNA}}}{T_{\text{p-shRNA}}} \times 100 \quad (1)$$

where $T_{\text{p-shRNA}}$ and $F_{\text{p-shRNA}}$ stand for total amount and free amount of p-shRNA, respectively.

Gel Retardation Assay

The binding strength of p-shRNA with chitosan was evaluated by agarose gel electrophoresis. Different N/P ratios (0.5, 5, 12, and 19) of chitosan/TPP/p-shRNA at constant ratio of chitosan/TPP (10) were loaded (20 μl of the sample containing 13 μg of p-shRNA). After addition of loading dye with dilution of 1:6, electrophoresis was carried out at a constant voltage of 75 V for 90 min in TAE buffer (40 mM Tris-acetate/1 mM EDTA, pH 8) containing 0.6 $\mu\text{g/ml}$ ethidium bromide. UV transilluminator at the wavelength of 365 nm was used for visualization of p-shRNA bands.

FTIR Analysis

FTIR spectra of purified chitosan and freeze-dried chitosan/TPP nanoparticles were measured using Nicolet IR100 FT-IR Spectrometer. The samples were mixed with pure KBr as the background and compressed into discs using a manual tablet press.

Experimental Design

Central composite design was selected to optimize the formulation parameters in preparation of chitosan/TPP/ p-shRNA nanoparticles for maximum loading efficiency and minimum diameter. In this study, a 3-factor, 5-level central composite design was used to optimize nanoparticles preparation with independent factors consisting of chitosan/TPP ratio (X_1), pH (X_2) and N/P ratio (X_3) and the five levels as described in Table I.

Design Expert (STAT-EASE, 7.0.0) software was used for generation and evaluation of the statistical experimental design. The design matrix was constructed which included 20 experimental runs. An interactive second-order polynomial model was utilized to evaluate the response variables:

$$y = \beta_{k0} + \sum_{i=1}^3 \beta_{ki} X_i + \sum_{i=1}^4 \beta_{kii} X_i^2 + \sum_{i < j=2}^3 \beta_{kij} X_i X_j \quad (2)$$

Where y represents the response function, β_{k0} denotes the intercept, β_{ki} , β_{kii} and β_{kij} stand for the coefficients of the linear, quadratic and interactive terms, respectively. X_i and X_j represent the coded independent variables. For regression analysis of the obtained data as well as estimation of the coefficients in the regression equation, a statistical program in Design Expert 7.0.0 software was used. The equations were validated by ANOVA statistical test. In order to determine the individual and interactive effects of test variables on the

responses, response surfaces were plotted. Additional confirmation experiments were then performed so as to verify the validity of the statistical experimental design.

Cytotoxicity of Prepared Nanoparticles

To assess the cytotoxicity of the prepared nanoparticles, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used. Mitochondrial dehydrogenases of viable cells are able to cleave of the tetrazolium ring of MTT and producing the purple formazan that is soluble in organic solvent such as dimethyl sulfoxide (DMSO). For performing the MTT assay, HeLa and PC3 cell lines were seeded in a 96-well plate at a density of 1×10^5 cells/mL in 150 μ L of RPMI1640 containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 ng/ml streptomycin at 37 °C in humidified air containing 5% CO₂. After that the medium was replaced with fresh medium without FBS and different concentrations of prepared nanoparticles (5–100 μ g/ml of nanoparticles) were added. Control wells had no nanoparticles. To provide statistically significant results, all conditions including controls were placed in five wells. After 24 h of incubation cells were washed with PBS and 100 μ L of a MTT solution (0.5 mg/ml in DMEM) was added to each well. After 3 h incubation at 37 °C and 5% CO₂, the MTT solution was removed carefully and the formed formazan crystals were dissolved in DMSO. The absorbance was measured at 550 nm in a microplate reader (Infinite M200, Tecan, Austria). To determine relative cell viability the following equation was used:

$$\text{Cell viability}\% = \frac{\text{Abs test}}{\text{Abs Control}} * 100 \quad (3)$$

Where Abs test and Abs Control are stand for the absorbance value obtained for treated cells and untreated cells with nanoparticles respectively.

Cell Uptake of the Nanoparticles

Cellular uptake of optimized nanoparticles were investigated in the HeLa cell line by using FITC-labeled Bcl-2 antisense embedded in chitosan/TPP nanoparticles with N/P 5 and chitosan/TPP ratio 10. The HeLa cells were seeded at 1.2×10^5 cells per well in glass bottom dishes and incubated overnight at 37 °C and 5% CO₂. Then medium was removed and washed with PBS three times before adding 500 μ L of FBS-free RPMI containing chitosan/TPP nanoparticles containing FITC-labeled Bcl-2 antisense to each well with a final concentration of 500 nM FITC. The cells were incubated for 4 h at 37 °C and 5% CO₂ at dark. Then the medium of each well was removed and washed by PBS. Following each treatment, the wells were subsequently evaluated by confocal microscopy (Olympus FV1000-MPE).

In order to carry out quantitative uptake study, flow cytometry was used. The HeLa cells were seeded at 1.2×10^5 cells per well in 24 well plates. To determine chitosan/TPP nanoparticle uptake, FITC labeled Bcl-2 anti-sense were used and the cells were incubated with labeled chitosan/TPP nanoparticles with final concentration of 500 nM in serum and antibiotics free medium for 4 h. Treated and untreated cells were washed three times with PBS and detached by trypsinization. Then the pellet of cells was dispersed in 500 μ L PBS.

Finally, green fluorescence was measured by flow cytometry (BD FACSCalibur System) and results were analyzed with FlowJo (v.7.6.1).

RESULTS AND DISCUSSION

Particle Size

In this study, the ionic gelation method was chosen to prepare nanoparticles in the 20 different designed experimental conditions since it is a relatively simple and mild method. The range of nanoparticle sizes was between 170 ± 6 and 279 ± 10 nm through different experiments (Table II). SEM images of spherical nanoparticles are shown in Figure 2.

The size and shape of nanoparticles have a critical role in transferring genetic materials into the cells and the distribution in the body.³⁴ It has been reported that nanoparticles have a comparatively higher intracellular uptake than microparticles.^{24, 35, 36} One could assume that a spherical structure is more favorable for gene delivery by chitosan.³⁷

TPP was used for preparation of chitosan nanoparticles as a cross-linker, as it has some advantages including its small molecular weight with triple negative charge, low-toxicity, and its quick gelling ability.³⁸ The negatively charged phosphate groups of TPP can form cross-links with positively charged amino groups of chitosan via ionic interaction. The charge densities of chitosan and TPP are pH-dependent; thus pH is an important factor that influences the ionic interaction between TPP and chitosan. In other words, the formation of chitosan/TPP nanoparticles is highly pH-dependent. At pH = 6 and pH = 3.4, 40% and 100% of the chitosan amino groups are protonated, respectively. Since the pK_a of chitosan is around 6.5, most of its amino groups have a positive charge in the pH range of 3.5 to 5.5. According to the pK_a of TPP ($pK_{a3} = 2.3$), its charge density increases as the pH increases (at pH = 3 approximately 20% of the molecules are only doubly charged). Therefore TPP addition in a higher pH range more efficiently forms chitosan nanoparticles. Another key factor that influences the size of nanoparticles is the mass ratio of chitosan to TPP (chitosan/TPP ratio). A decrease in chitosan/TPP ratio until reaching a threshold causes a reduction in the size of nanoparticles; however, a further decrease of the chitosan/TPP ratio leads to aggregation or the formation of nanoparticles with larger particle sizes. Previous reports have shown important effects of chitosan/TPP ratio on the nanoparticle size.^{39, 40}

Likewise, N/P ratio has a considerable effect on size of nanoparticles. MacLaughlin et al. showed that by increasing the plasmid concentration (which in turn reduces the N/P ratio), the diameter of the nanoparticles increased.⁴¹

Surface Charge

The surface charge of the nanoparticles is an important factor in determining the stability of nanoparticulate suspensions. The efficiency and route of cellular uptake, affect the *in vivo* fate of nanoparticles.^{42–46} The ability of nanoparticles to escape the endo-lysosomes after cell uptake has been reported to be dependent on the surface charge of the nanoparticles.^{47, 48} Recently, the role of positive charge of nanoparticles in cytoplasmic trafficking was studied and it was shown that positive nanoparticles are capable of binding to anionic microtubules or molecular motor proteins and can move towards the cell nucleus

along the cytoskeletal network.⁴⁹ In our study, the surface charge of the chitosan-TPP-p-shRNA nanoparticles was measured by Malvern Zetasizer ZS (data not shown). The zeta potential was found to be in the range of -5 ± 3 to $+50 \pm 5$. It was observed that pH, chitosan/TPP ratio, and N/P ratio all had significant effects on the surface charge of the nanoparticles. In agreement with our data, similar effects of these factors on the zeta potential of nanoparticles have been previously reported.^{14, 40, 50, 51}

Thus, we suggest that the nanoparticles prepared in optimized conditions could be a good candidate for delivery of p-shRNA molecules because of their positive zeta potential which enables them to bind with cell membrane and increases the cellular uptake.

Interaction of p-shRNA with Chitosan/TPP Nanoparticles

In order to investigate the interaction of p-shRNA and chitosan/TPP nanoparticles, a gel electrophoresis retardation assay was used. In all the three levels of N/P ratio considered in RSM design, i.e., N/P = 5, 12 and 19, complete binding of p-shRNA with chitosan nanoparticles was observed (Fig. 3). This implies that in all of these N/P ratios in RSM design, a strong interaction exists between p-shRNA and chitosan/TPP nanoparticles. It should also be noted that at N/P = 0.5, only a weak interaction of p-shRNA with chitosan nanoparticles was observed, which may be attributed to high amount of p-shRNA compared to that of chitosan. As Katas et al. showed, the strength of interaction between chitosan and siRNA was higher when the nanoparticles were prepared by ionic gelation with TPP in comparison with simple complexation.³⁴ Other reports have shown the association of ODNs with nanoparticles to be more efficient when ionic gelation was used to prepare them instead of simple complexation.⁵² The obtained results in this study demonstrated the existence of a strong interaction between p-shRNA and chitosan/TPP nanoparticles in all tested N/P ratios in fixed chitosan/TPP ratio at 10 and pH = 5.5; thus the prepared nanoparticles provided a medium with satisfactory performance for maintenance of p-shRNA and may protect it against nuclease digestion.

Central Composite Design and Response Surface Methodology

The response surface method (central composite design) was used to determine the optimum conditions for preparation of chitosan/TPP/p-shRNA nanoparticles. A central composite design (CCD)⁵³ was applied to find the best experimental conditions of the three independent factors affecting the nanoparticles preparation process, including chitosan/TPP ratio, pH, and N/P ratio. As we studied three important variables, the design matrix consisted of 20 experiments; and the corresponding experimental data is given in Table II. The results also indicated that the size and loading efficiency of particles ranged from 170.7 to 278.3 nm and 47.66 to 85.66%, respectively.

By applying multiple regression analysis on the experimental data, the predicted models were developed by Eqs. (1) and (2). The analysis of variance (ANOVA) for the quadratic models showed that the regression sum of squares (R^2) for both of the models, i.e., the models developed for size and loading efficiency, was statistically significant. To evaluate the statistical significance of the regression models, F -test and p -value were used (Table III).

The *P*-value of the “size” model was lower than 0.05, showing that the model was statistically significant. However, the *P*-values of X_1 , X_3 and X_2^2 in this model were higher than 0.05 and their removal from the corresponding equation made the model more significant. Therefore, the best equation for particle size prediction was as follows:

$$Y_1 = +185.72 + 19.67X_1 + 0.16X_2 - 20.13X_3 + 6.59X_1X_2 - 7.21X_2X_3 + 9.03X_1^2 + 22.76X_3^2 \quad (4)$$

where Y_1 represents the nanoparticle size, and X_1 , X_2 and X_3 denote chitosan/TPP ratio, pH and N/P ratio, respectively. The values of R^2 and adjusted R^2 of the model were 0.9680 and 0.9493, respectively. The value of R^2 for size indicated that the model was sufficient for prediction of nanoparticles size within the selected range of experimental factors, and implied that the sample variation of 96% for the size of nanoparticles was attributed to the independent variables, and only 4% of the total variation cannot be explained by the model. Furthermore, the similarity between R^2 and adjusted R^2 indicated the adequacy of the model to predict the response by the optimization process. The *F*-value of the model of 51.86 implied that the fitted model was significant and there was only a 0.01% chance that an *F*-value as high as the obtained one could occur due to chance. Here, a lower value of CV (3.56) showed a better precision and reliability of the conducted experiments.^{29, 30, 54} It can be therefore concluded that the developed model could adequately represent the real relationships among the parameters chosen.

Figure 4(A) illustrates the effects of the chitosan/TPP ratio and pH on the particle size when the N/P ratio was fixed at 12. At this fixed N/P ratio, the minimum particle size (168 nm) was obtained when a chitosan/TPP ratio of 10 and a pH of 5.5 were employed. The interpretation may be that in the selected pH range, the TPP charge density had a more important role than that of chitosan on the nanoparticles size. At pH = 3.5, the charge density of chitosan is higher than that at pH = 5.5. Conversely, the TPP charge density is lower at pH = 3.5 when compared to that at pH = 5.5. Thus, the most favorable particle size was achieved in upper level of pH and lower level of chitosan/TPP ratio in the tested ranges for pH and chitosan/TPP ratio. Previous studies have also reported an increase in size of nanoparticles in case of an increased chitosan/TPP ratio.^{39, 40} Such an increase in size may be attributed to insufficient TPP leading to poor gelation of the chitosan solution.³⁹

In Figure 4(B), at a fixed pH of 4.5, the minimum particle size was achieved when the chitosan/TPP ratio of 10 and N/P ratio of 12 were used. At all values of the N/P ratio, the minimum particle size was attained when a chitosan/TPP ratio of 10 was employed, while a N/P ratio of 12 caused a minimum size of nanoparticles at all values of chitosan/TPP ratio. Mehrotra reported a decrease in particle size with increasing amount of TPP because the cross-linking agent hardens the chitosan nanoparticles which in turn decreases water absorption.⁵⁵

As Figure 4(C) demonstrates, at all values of pH, the minimal size of the nanoparticles was achieved when a N/P ratio of 12 was used. The global minimum of the particle size plotted versus pH and N/P ratio was located at pH = 5.5 and N/P ratio = 12. Considering the significant role of the positive charge of chitosan and negative charges of DNA on nanoparticles size, the effect of pH and N/P ratio as well as their interaction on particle size

can be explained. N/P ratio was defined in the previous sections as molar ratio of chitosan amino group to phosphate group of DNA; this ratio implies the charge ratio of chitosan to DNA *at a specific condition*, and if a change occurs in the condition at which the N/P ratio was calculated, then the resulting charge ratio of chitosan to DNA would be different from the molar ratio of chitosan amino group to phosphate of DNA.

The loading efficiency of the DNA is among the key factors, and should be considered in the preparation of nanoparticles containing genetic materials. Its range was found to be between 47.6 and 85.6% through the experiments. The *P*-value of the “loading efficiency” model was lower than 0.05, indicating the significance of the model. Nonetheless, *P*-values of $X_1 X_2$ and $X_1 X_3$ in this model were higher than 0.05, and were not included in the corresponding equation in order to make the model more significant. Consequently, the best equation for prediction of loading efficiency was as follows:

$$Y_2 = +42.21 - 0.96X_1 + 1.85X_2 - 7.74X_3 + 2.33X_2X_3 + 5.78X_1^2 + 10.31X_2^2 + 4.13X_3^2 \quad (5)$$

where Y_2 represents the loading efficiency, and X_1 , X_2 and X_3 denote chitosan/TPP ratio, pH and N/P ratio, respectively. The values of R^2 and adjusted R^2 of the model were 0.9557 and 0.9158, respectively.

Figure 4(D) indicates that maximum loading efficiency was achieved at pH = 5.5 and chitosan/TPP ratio = 10, when N/P ratio was fixed at 12, while minimum loading efficiency in this fixed N/P ratio was obtained at medium levels of both pH and chitosan/TPP ratio. The charge densities of DNA, cross-linking agent (TPP) and chitosan under the preparation conditions are expected to affect loading efficiency due to ionic nature of the nanoparticles. The loading efficiency was higher at upper and lower levels of pH than at middle pH; this may be attributed to high charge density of chitosan and TPP at pH = 3.5 and pH = 5.5, respectively. The binding constant of chitosan and DNA interaction was pH-dependent and was greater at lower pH due to the increased electrostatic attraction to DNA when chitosan is highly charged.⁵⁶ In addition, Koping-Hoggard et al. indicated that as the pH decreased, the DNA binding capacity of chitosan increased due to the resultant rise in positive charge density of the chitosan amino groups which facilitated its binding to negatively charged DNA.⁵⁷ When chitosan is highly charged, its binding affinity with DNA is high and it is therefore expected that the loading efficiency as well will be high.

Figures 4(E) and (F) show the effects of interaction between chitosan/TPP ratio and pH, respectively, with N/P ratio on the loading efficiency. pK_a of dsDNA phosphate groups is approximately 1.5.⁵⁶ Therefore only the charge density of chitosan and TPP changes over the selected pH range. It was expected that in high N/P ratios, the chance of interaction between chitosan and DNA are higher since the number of positive charges versus that of negative charges has increased, and a higher loading efficiency is expected. The opposite assumption was expected to apply at low N/P leading to lower loading efficiency. However, much to our surprise, we found that the highest loading efficiency was obtained at N/P = 5 (the low level of N/P ratio). This phenomenon may be explained as follows: the interaction between the chitosan chains and free DNA promotes overcharging, and therefore the subsequent binding between these positively charged complexes with other DNA molecules

will be encouraged, i.e., a higher degree of cooperativity of DNA-chitosan interactions is produced, leading to higher values of loading efficiency. Also, it seems that the contribution of the induction of overcharging to the overall loading efficiency is much greater at low N/P ratios than at high ones. The effect of chitosan/DNA complexes on the ionization of chitosan glucosamines was previously shown in another study. As Ma et al. reported, during complex formation between chitosan and DNA, some of the initially neutral glucosamines were protonated such that at pH 5.5, an additional 17% of total glucosamine units were protonated.⁵⁶ Similar observations have been reported previously for the interaction of polyethylenimine and other polycations with DNA.^{58, 59}

Another study reported that the maximum loading efficiency of chitosan nanoparticles was at N/P = 5, which is in agreement with our results; however, the authors attributed this high loading efficiency at N/P ratio = 5 to an increased effective surface area for binding of nucleic acids due to decreased size of nanoparticles.⁶⁰ This explanation does not hold for our study since we observed an increase in the size of nanoparticles at N/P ratio = 5, and the high loading efficiency cannot be related to the increased effective surface area. It is worthwhile to mention that this discrepancy may be due to the use of different genetic materials (p-shRNA instead of ODN).

Optimization

In this study, we tried to optimize the conditions, in a way to achieve minimum particle size with maximum loading efficiency. In order to find the optimal values of the selected variables, the regression equation was solved using the Design-Expert software. The best conditions for minimum particle size and maximum loading efficiency were pH = 5.5, chitosan/TPP ratio = 10 and N/P ratio = 11. For validation of the models, three experiments were conducted at the previously determined optimum conditions. The perfect agreement between the observed values for both of the responses and their values predicted by the related equations confirmed that both models were statistically significant. Therefore, these models have adequate precision for the prediction of optimum conditions in minimizing the particle size and maximizing the loading efficiency.

FTIR Evaluation

FTIR spectra of purified chitosan and chitosan/TPP nanoparticles are illustrated in Figure 5. According to Figure 5(A), three characterized peaks exist in the spectrum of purified chitosan, which are assigned as follows: 3432 cm^{-1} of $\nu(\text{OH})$, 1080 cm^{-1} of $\nu(\text{C O C})$ and 1647 cm^{-1} of $\nu(\text{NH}_2)$. However, a different spectrum was observed for chitosan/TPP nanoparticles (Fig. 5(B)) compared to that of purified chitosan (Fig. 5(A)). In chitosan/TPP nanoparticles, the 1647 cm^{-1} peak of -NH_2 bending vibration shifted to 1519 cm^{-1} and a new sharp peak appeared at 1632 cm^{-1} . The tripolyphosphate groups of TPP can therefore be supposed to be linked with ammonium groups of chitosan in nanoparticles. Our observation is in accordance with the findings of Wu et al. on chitosan/TPP nanoparticles,²⁴ and those of Knaul et al. on chitosan film modified by phosphate.⁶¹

Cytotoxicity Studies

The MTT assay was performed for seven different concentrations of prepared nanoparticles on HeLa and PC3 cells. When the highest two concentrations of nanoparticles were used, cell viability was around 85% compared with control samples, whereas in other concentrations it was around 95% (Fig. 6). It must be emphasized that the two highest concentrations of nanoparticles tested (80–100 $\mu\text{g/ml}$) are about five to ten times higher than the required concentration of Chitosan/TPP nanoparticles used for the transfection assays for gene delivery. Moreover in a transfection assay cells are usually treated for 4 hours whereas in the present MTT assay it was 24 h. There was no obvious difference between cellular toxicity observed between HeLa and PC3 cells. These results indicated that the prepared Chitosan/TPP nanoparticles in the range of concentration needed for transfection are nontoxic and have good biocompatibility. Previous studies have confirmed the obtained results where it was found that Chitosan/TPP and chitosan nanoparticles did not interfere with cell viability in the concentration range necessary for transfection.²⁶

Cellular Uptake Study

Many different factors such as the size of particles, surface charge and the incubation temperature have influence on *in vitro* cell uptake. Previous studies have shown that the size of particles is an important factor on the particle uptake and nano-sized particles showed relatively greater efficiency of cellular uptake than micro-sized particles. The particle uptake also depends on the incubation temperature, and cellular uptake at 37 °C is relatively higher than at 4 °C, suggesting the uptake process to be energy-dependent.^{62, 63} For investigation of *in vitro* uptake the HeLa cell line was used. Flow cytometry was used for quantification of cellular uptake of the Chitosan/TPP nanoparticles. During our experiments, we did not have fluorescently labeled p-shRNA plasmid. Therefore we used a FITC-labeled Bcl2 antisense oligonucleotide which we had obtained for a different set of experiments and incorporated it into Chitosan/TPP nanoparticles in the same manner as p-shRNA. The obtained results revealed that more than 80% of HeLa cells demonstrated nanoparticle uptake (Fig. 7). The confocal microscopy images confirmed the presence of fluorescent nanoparticles inside the HeLa cells (Fig. 8). According to both flow cytometry and confocal microscopy we can conclude that Chitosan/TPP nanoparticles have the ability to enter the cells.

CONCLUSION

p-shRNA-EGFR loaded chitosan/TPP nanoparticles were successfully prepared by the ionic gelation method. The effects of three important independent variables:

- (A) mass ratio of chitosan to TPP;
- (B) pH; and
- (C) molar ratio of amine groups of chitosan to phosphate groups of DNA (N/P ratio), on the two dependent variables (particle size and loading efficiency) was studied to optimize nanoparticle preparation using a central composite design (CCD).

The optimal conditions of the independent variables for minimizing the nanoparticle size and maximizing the loading efficiency were found to be at a lower level of chitosan/TPP ratio (10), near middle level of N/P ratio (11) and the higher level of pH (5.5). Under these optimized conditions, the nanoparticle size and loading efficiency were 172.8 ± 7 nm and $71.5 \pm 5\%$, respectively, and agreed closely with the experimental validation results. Cytotoxicity tests with HeLa and PC3 cells proved the optimized nanoparticles to have good biocompatibility and low-toxicity to cell lines. The results of *ex vivo* cellular uptake studies on HeLa cells showed high cellular uptake of the optimized nanoparticles and suggested favorable delivery of genetic materials. The high uptake may be due to their positive Zeta potential providing the ability to make good contact with cell membrane, and their small sizes. Accordingly, these nanoparticles can be considered as a promising carrier for shRNA, anti-sense oligonucleotides and plasmid gene delivery.

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Figure 1.
Schematic view of the constructed p-shRNA-EGFR plasmid.

Figure 2. Schematic diagram of chitosan/TPP/p-shRNA nanoparticles. The SEM image is shown in the lower-left part of the figure.

Figure 3.

Electrophoretic mobility of chitosan/TPP/p-shRNA nanoparticles with different N/P ratios.

1: ladder, 2: nanoparticles with N/P ratio = 0.5, 3: nanoparticles with N/P ratio 5, 4: nanoparticles with N/P ratio = 12.5: nanoparticles with N/P ration = 19.

Figure 4.

Response surface analysis. The plots (A, B, C, D, E and F) show the effect of chitosan/TPP ratio, pH and N/P ratio on the nanoparticles size and loading efficiency.

Figure 5.
FTIR spectra. (A) Purified chitosan and (B) chitosan/TPP nanoparticles.

Figure 6. Cytotoxicity of nanoparticles. MTT assay for different concentrations of the optimized nanoparticles with Hela and PC3 cell lines.

Figure 7. Cellular uptake of chitosan/TPP nanoparticles. HeLa cells treated with nanoparticles for 4 h and the uptake was assessed by flow cytometry.

Figure 8. Cellular uptake study. FITC-labeled chitosan/TPP nanoparticles after 4 h of incubation. (a) Nucleus of cells have been identified through Hoechst staining. (b) Fluorescent image of HeLa cells. (c) a and b merged.

Table I

Variables used in central-composite experimental design.

Independent variables	Symbol	Levels				
		-2	-1	0	1	+2
Chitosan/TPP ratio	X_1	4.9	10	17.5	25	30.1
pH	X_2	2.8	3.5	4.5	5.5	6.2
N/P ratio	X_3	0.2	5	12	19	23.8

Table II

Central composite design matrix (in coded level of three variables) and response values for nanoparticle size, loading efficiency and zeta potential.

Run	<u>Coded variable levels</u>			<u>Independent variables (Response)</u>		
	X_1	X2	X3	Size (nm)	Loading efficiency %	Zeta potential
1	1	-1	1	219.5	62.35	50.6
2	-1	1	1	172.4	70.62	11.3
3	-1	-1	1	198.4	63	17.6
4	0	0	1.68	208.7	47.60	18.8
5	0	1.68	0	179.1	81.46	8.45
6	1.68	0	0	238.3	64	12.2
7	-1	-1	-1	219.1	85.76	14.9
8	0	0	-1.68	278	74	-4.35
9	0	0	0	188.8	49	14
10	1	1	1	219.7	71.23	17
11	0	-1.68	0	181.4	75	43.3
12	0	0	0	185.7	50.36	9.47
13	1	1	-1	278.3	80.43	12.6
14	-1.68	0	0	170.7	67	21.3
15	-1	1	-1	221.8	83	21.8
16	0	0	0	176.2	55.66	15.7
17	1	-1	-1	249.1	80	46.9
18	0	0	0	181.2	50.33	21.1
19	0	0	0	190.7	55.56	25.5
20	0	0	0	191.5	53	16.1

Table III

ANOVA results for response surface quadratic model (“size” model).

Variables	Sum of squares	DF	Mean square	F value	p-value prob > F
Model	19875.81	9	2208.423	51.86051	<0.0001 ^a
X_1 -chitosan/TPP ratio	5282.341	1	5282.341	86.46864	<0.0001 ^a
X_2 -pH	0.364745	1	0.364745	0.005971	0.9399 ^b
X_3 -N/P ratio	5531.402	1	5531.402	90.54562	<0.0001 ^a
$X_1 \times X_2$	347.1613	1	347.1613	5.682814	0.0384 ^a
$X_1 \times X_3$	40.95125	1	40.95125	0.670347	0.4320 ^a
$X_2 \times X_3$	416.1613	1	416.1613	6.812301	0.0260 ^a
X_1^2	1187.149	1	1187.149	19.43289	0.0013 ^a
X_2^2	3.638488	1	3.638488	0.05956	0.8121 ^b
X_3^2	7499.248	1	7499.248	122.758	<0.0001 ^a
Residual	610.8967	10	61.08967		
Lack of fit	432.1484	5	86.42968	2.417636	0.1774 ^b
Pure error	178.7483	5	35.74967		
Cor total	20486.7	19			

^aSignificant ($p < 0.05$).^bNot significant ($p > 0.05$).