Controlled Size Chitosan Nanoparticles as an Efficient, Biocompatible Oligonucleotides Delivery System

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ABSTRACT: Polymeric nanoparticles of chitosan crosslinked with glutaraldehyde have been prepared using reverse micellar system. An optically clear solution was obtained on redispersing these nanoparticles in aqueous buffer. The nanoparticles were characterized for their size and surface morphology employing dynamic laser scattering (DLS) and transmission electron microscopy (TEM). The TEM images showed spherical particles with smooth surface and narrow size distribution of about 90 nm, which was also supported by DLS data. Size and morphology of the particles remains the same on redispersing the lyophilized powder of these nanoparticles in aqueous buffer. Further, these nanoparticles were loaded with different synthetic oligonucleotides (ODNs). *In vitro* pH dependent release of the adsorbed oligonucleotides from these nanoparticles was also studied. At basic pH the release of oligonucleotides was found higher as compared with neutral and acidic medium. Cytotoxicity studies done on HEK 293 cells reveals that oligonucleotide loaded nanoparticles have high cell viability of nearly 76–88% whereas those of lipofectamine was about 35%. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 118: 2071–2077, 2010

Key words: nanoparticle; biodegradable; crosslinking; particle size distribution

INTRODUCTION

Recently, considerable interest has been shown in the use of antisense oligonucleotides to modify gene expression for therapeutic purposes. These have been used to inhibit the synthesis of cellular or viral proteins. Most of the studies of antisense oligonucleotides have been carried out in *in vitro*. The limiting factor is that oligonucleotide drugs are anionic macromolecules and cannot transit biological cell membranes and are rapidly degraded by nucleases.[1,2] To overcome these limitations, various chemical modifications of oligonucleotides were synthesized.[3] Chemical modifications that have been introduced to increase the stability of ODNs and their ability to penetrate the plasma membrane, including replacement of the nonbridging oxygen on the phosphodiester backbone by sulfur or a methyl group and replacement of the deoxyribose phosphate backbone as in peptide nucleic acids, are mainly focused on the phosphodiester backbone and/or the sugar moiety.[4–6]

Another strategy recently developed consists of encapsulation of oligomer in liposomes.[7] By this method, the delivery can be achieved by lipofusion. The limiting factor in this technology is the stability of liposomes. Therefore, to achieve better stability of the delivery system as well as that of oligonucleotides and their uptake, another system have been developed, which is based on polymeric nanoparticles. Among drug carriers, nanoparticles, usually biodegradable, have shown interesting potentialities to bind and deliver ODNs.[8] Nanoparticles being compact are well suited to traverse cellular membranes to mediate drug or gene delivery. It is also expected that due to smaller size, nanoparticles will be less susceptible to reticuloendothelial system clearance and will have better penetration into tissues and cells, when used in *in vivo* therapy. Using this strategy, it has been demonstrated that oligonucleotides adsorbed or entrapped onto/into nanoparticles are sufficiently resistant towards nucleases mediated degradation and results in increased uptake.[9–12] Several strategies have been explored so far in the development of these vehicles. Till date, a wide variety of chitosan based colloidal delivery vehicles, which have been described for the association and delivery of macromolecules.

Chitosan (poly(β-1-4)-2-amino-2-deoxy-D-glucopyranose or poly(D-glucosamine), a deacetylated form of chitin, is a natural biopolymer extracted from crustacean shells, such as prawns, crabs, insects, and shrimps. Due to its cationic nature, nontoxicity, and unique properties such as acceptable biocompatibility and biodegradability, chitosan has seen various pharamceutical applications including controlled

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drug delivery.[13–16] All these interesting properties of chitosan make this natural polymer an ideal candidate for controlled drug release formulations. It has also been proposed as biocompatible alternative cationic polymers that are suitable for nonviral gene delivery.[17] However, this system has a significant limitation, owing to its low transfection efficiency.[18] The transfection efficiency may depend on several factors, such as the chemical structure of polycations, cell type, nanoparticle size and composition, and interactions with cells.[19]

In this study, we have prepared chitosan nanoparticles with the aim to study the ability of nanoparticles prepared with size control parameters to carry oligonucleotides, which can be further used for *in vivo* studies. The nanoparticles were prepared in aqueous core of reverse micellar system as reported earlier.[20] The nanoparticles were characterized for their size and surface morphology employing dynamic laser scattering (DLS) and transmission electron microscopy (TEM). Subsequently, the studies related to adsorption and release of the oligonucleotides under *in vitro* conditions was carried out. These nanoparticles were further assessed for their biocompatibility by evaluating the cytotoxicity on mammalian cell line HEK 293 employing the MTT assay.

EXPERIMENTAL PROCEDURES

General methods

Chitosan (M.W. 400 kDa, DDA 83–85%), glutaraldehyde, surfactant i.e. sodium bis(ethylhexyl) sulfosuccinate (AOT), 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), were purchased from Sigma (St. Louis, MO). Cell culture media, Dulbecco's modified eagle's medium (DMEM), Fetal bovine serum (FBS) were from GIBCO-BRL-Life Technologies, Web Scientific, UK. The three oligonucleotides viz., 15 mer (5′-TTT TTT TTT TTT TTT-3′, ODN-1), 21 mer (5′-GCC GAG GTC CAT GTC GTA CGC-3′, ODN-2), and 20 mer (5′-GCC CAA GCT GGC ATC CGT CA-3′, ODN-3) in purified form were obtained from Bangalore Genei, India. All other reagents used were of analytical grade.

Cell culture

The mammalian cell line, HEK 293 cells (Human embryonic kidney 293), were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 50 μg/mL gentamicin.

Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared as reported earlier by Banerjee et al.[20] Briefly, to the 0.1 *M* AOT solution in *n*-hexane (210 mL), 1 mL of chitosan solution (prepared by dissolving 225 mg chitosan in 100 mL 10% acetic acid), liquor ammonia (30% v/v, 400μl), glutaraldehyde (25% v/v, 20μl), and 0.1*M* Tris-HCl buffer, pH 8.0 (1829.0 μL) were added with vigorous stirring under nitrogen atmosphere at room temperature. The stirring was continued for 24 h. After completion of reaction, solvent was evaporated off to get a semisolid. This was again suspended in 0.1*M* Tris-HCl buffer pH 8.0, (20 mL) and 30% calcium chloride solution (nearly 10 mL) was added drop-wise with continuous stirring to precipitate the surfactant as calcium salt of bis-(2-ethylhexyl)sulphosuccinate [$Ca(DEHSS)_2$]. The solution was centrifuged at 8000 rpm for 20 min at 4°C to get a pellet. The supernatant containing the nanoparticles was separated. The cake was redissolved in *n*-hexane (5 mL) and washed with 1 mL of 0.1*M* Tris-HCl, pH 8.0 to remove the nanoparticles adsorbed on [$Ca(DEHSS)_2$]. Aqueous layer was collected and mixed with the original centrifugate. Then the aqueous dispersion of nanoparticles was dialyzed for overnight using dialysis membrane (12 kD cut off) and lyophilized to get nanoparticles in powder form.

Characterization of nanoparticles

Dynamic light scattering

The hydrodynamic diameter of the chitosan nanoparticles was determined by dynamic light scattering (DLS) measurements. Lyophilized powder (2 mg) of nanoparticles suspended in 1 mL of double distilled water and sonicated before measurements. Nanoparticles size was determined using 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° angle. The refractive index (1.33) and the viscosity (0.89) of ultrapure water were used at 25°C for measurements. All the data analysis was performed in automatic mode. Measured sizes were presented as the average value of 20 runs.

Transmission electron microscopy

Lyophilized powder (2 mg) of chitosan nanoparticles was dispersed by sonication in double distilled water (1 mL) to obtain a clear suspension, which was later used for preparing samples for TEM. The sample solution (3μL) was put on a formvar (polyvinyl formal) coated copper grid and air dried. To coat copper grids with formvar, a drop of 0.5%

(w/v) solution of formvar in chloroform was placed on the water (previously degassed) surface. A thin film was formed on the water surface, onto which several clean copper grids were placed, with matty surface downwards. After 2–3 s, the grids along with the film were lifted off by a piece of filter paper with forceps and air dried. TEM pictures were taken on a JEOL JEM 2000 Ex 200 Model electron microscope. Before visualization of samples, a blank grid without sample was also scanned.

Stability of nanoparticles

The stability of the chitosan nanoparticles prepared by crosslinking with glutaraldehyde was examined in terms of the particle size. The lyophlized nanoparticles powder was stored at 4°C for 1–30 days and the size of the nanoparticles monitored by DLS.

Oligonucleotide loading on to the nanoparticles

The three oligonucleotides viz., 15 mer (5′-TTT TTT TTT TTT TTT-3′, ODN-1), 21 mer (5′-GCC GAG GTC CAT GTC GTA CGC-3′, ODN-2), and 20 mer (5′-GCC CAA GCT GGC ATC CGT CA-3′, ODN-3) have been used in this study. Oligonucleotide loading to the nanoparticle polymer was achieved by adsorption (electrostatic interaction). The nanoparticle suspension (10 mg/mL in 0.01*M* phosphate buffer, pH 7) was incubated with oligonucleotide (ODN-1/ODN-2/ODN-3) (10 μg/mL) at 25°C over a period of 24 h. The amount of oligonucleotide adsorbed to the nanoparticles was determined by measuring the optical density at 260 nm. The nonadsorbed oligonucleotides were separated from the particles by centrifugation at 10,000 rpm for 15 min. The amount of oligonucleotide bound to nanoparticles was taken as the difference between the amount of oligonucleotide added for incubation and the amount present in the supernatant. Similar experiments were carried out in different 0.01*M* phosphate buffers, pH 4 and 8. The loading efficiency (*E*%) was calculated from the total concentration of the added amount of oligonucleotide in the system ($[ODN]_T$) and that in the supernatant ($[ODN]_s$) using the equation:

$$E(\%) = [ODN]_T - [ODN]_s/[ODN]_T \times 100$$

Oligonucleotide release studies

A defined amount of nanoparticle (3.1 mg) used for oligonucleotide loading were separated from the dispersion medium by centrifugation and washed once with the buffer solution (0.01*M* phosphate buffer) and resuspended in 7 mL of buffer solution of different pH (4.0, 7.0, and 8.0). The suspension was then divided in 14 aliqouts of 500 μL each and left for incubation for at 37°C and the amount of oligonucleotide released from the nanoparticles was measured from time to time by spinning one tube and monitoring the optical density at 260 nm by UV–vis spectrophotometer of the supernatant. The percentage of oligonucleotides released from nanoparticles was calculated from the total amount of oligonucleotide in the nanoparticles ($[ODN]_T$) and that in the supernatant ($[ODN]_s$) using the equation:

$$\text{Release } (\%) = [ODN]_s/[ODN]_T \times 100$$

Cytotoxicity

The toxicity of ODN loaded chitosan nanoparticles was evaluated by MTT colorimetric assay.[21] HEK 293 cells were seeded onto 96-well plates at a density of 10×10^3 cells/well and incubated for 16 h for adherence. After stipulated time the plating media was removed and fresh 100 μL media containing ODN loaded nanoparticles was added to each well, followed by incubation at 37°C in humidified 5% CO_2 atmosphere for 6 h. Subsequently, the media was replaced with 100 μL of serum supplemented DMEM and cells were further incubated for 42 h under same conditions. 48 h post transfection, 50 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (2 mg/mL in DMEM) was added to the cells and incubated for another 2 h. At the end of incubation, the MTT containing medium was aspirated, and the formazan crystals formed by the living cells were dissolved in 100 μL isopropanol containing 0.06*M* HCl and 0.5% SDS. Aliquots were drawn from each well after 30 min. of incubation and the absorbance measured spectrophotometrically on a ELISA plate reader at 540 nm. Untreated cells were taken as control with 100% viability and cells without addition of MTT were used as blank to calibrate the spectrophotometer to zero absorbance. The relative cell viability (%) compared to control cells was calculated by $[abs]_{sample}/[abs]_{control}$ x 100. Lipofectamine, a commercially available transfection reagent, complexed with ODN 2 was used as positive control according to manufacturer's protocol.

RESULTS AND DISCUSSION

In this study, we evaluated the efficacy of chitosan as an oligonucleotide carrying system that can be further optimized for efficient *in vitro* and *in vivo* gene delivery system. The synthesis of chitosan-DNA complexes was facilitated by the formation of

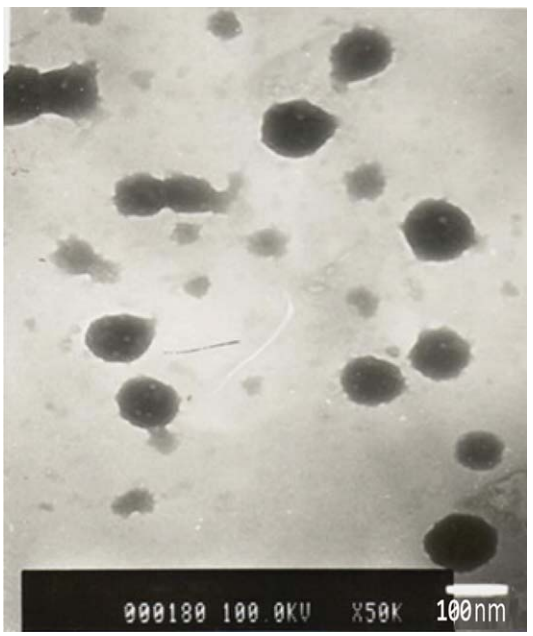

Figure 1 Transmission electron microscopy image of chitosan nanoparticles crosslinked with glutaraldehyde. The average particle size is 90 nm.

Schiff base between primary amino group of chitosan and aldehyde group of glutaraldehyde.

Preparation of chitosan nanoparticles

The chitosan nanoparticles employing glutaraldehyde as a crosslinker have been prepared by the method reported by Banerjee et al.[20] using aqueous core of the reverse micellar droplets. The water-in-oil (w/o) microemulsion system is thermodynamically stable and generally contains nanometer sized water droplets stabilized by a curved surfactant monolayer where the nanoparticle formation takes place. Chitosan solution in 10% acetic acid and glutaraldehyde were incorporated into 0.1*M* AOT surfactant in hexane leading to the formation of reverse micelles. The water to surfactant ratio (Wo) was adjusted to 8 by adding Tris-HCl buffer.

Characterization of chitosan nanoparticles

The size of nanoparticles has been found to play a pivotal role in *in vitro* transfections.[22] Prabha et al.[22] reported that the smaller-sized nanoparticles (mean diameter = 70 ± 2 nm) showed a 27-fold higher transfection than the larger-sized nanoparticles (mean diameter = 202 ± 9 nm) in COS-7 cell line and a 4-fold higher transfection in HEK-293 cell line. In majority of published reports, the nanoparticles of chitosan are prepared by simple complexation with DNA, which results in highly polydisperse particles. Size restrictions of polycations to nanometer range by preparing nanoparticles before complexing with DNA will provide an added advantage for improved gene delievry. So, in this investigation we have restricted the size of chitosan nanoparticles by using reverse microemulsion strategy.

The structure of chitosan nanoparticles was examined by TEM. TEM images of chitosan nanoparticles shows particles with spherical shape and a smooth surface distributed throughout the sample (Fig. 1). The size of chitosan crosslinked with glutaraldehyde nanoparticles, as evident from the TEM images was found to be ~ 90 ± 5 nm. DLS was employed to determine the size distribution of chitosan nanoparticles. In polydispersed systems, the final size distribution results depend on the method of fitting. Herein, the average hydrodynamic diameters of nanoparticles were calculated by employing NNLS (non-negative least squares) method, where different peaks are separated as multimodal distribution and provide more accurate results for samples with more than one size population than those compared with other methods. However, the effect of presence of dust was ruled out by taking average of numerous simultaneous measurements. DLS analysis of chitosan nanoparticles showed uniform size distribution in nanometer range (Fig. 2). The average particle size was found to be 102 nm with narrow size distribution having low polydispersity index (PDI) of 0.121. The DLS measures the hydrodynamic diameter by dispersing particles in aqueous phase or solvents whereas TEM measures the size of dried samples loaded onto to copper grids. It is speculated that the hydration and swelling of the particles in aqueous buffer or in the aqueous cores of reverse micellar droplets may be the possible reason for observing larger size by DLS measurements as compared to TEM.

Stability of nanoparticles

Stability while storage over a period of time is one of the important prerequisite for production of biological active molecules. It is well established that very small particles tend to aggregate among themselves to reduce the surface area, and hence to

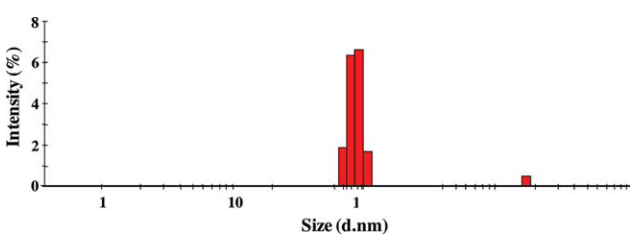

Figure 2 Representative dynamic light scattering spectrum of chitosan nanoparticles crosslinked with glutaraldehyde in buffer. The average hydrodynamic diameter in this case is 102 nm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I
Loading of Various Oligonucleotides Onto Chitosan Nanoparticles at Different pH

		Oligonucleotides loading on nanoparticle (10 mg)					
		pH = 4.0		pH = 7.0		pH = 8.0	
S.no.	Oligonucleotide	Amount (μg)	*E*%	Amount (μg)	*E*%	Amount (μg)	*E*%
1	ODN-1	6.53 ± 0.19	65.26 ± 1.86	6.19 ± 0.08	61.92 ± 0.80	4.09 ± 0.15	40.90 ± 1.47
2	ODN-2	9.28 ± 0.06	92.84 ± 0.59	9.14 ± 0.05	91.44 ± 0.54	6.60 ± 0.05	65.96 ± 0.50
3	ODN-3	7.75 ± 0.26	77.53 ± 2.65	7.47 ± 0.17	74.67 ± 1.72	5.27 ± 0.16	52.65 ± 1.56

E% - Loading efficiency

reduce the free surface energy. To check the stability of chitosan nanoparticles over a period time, nanoparticles were stored at 4°C and size determined after suspending in water at 1–30 days. A clear suspension was obtained by sonication when lyophilized powder of nanoparticles dispersed in water. The size of nanoparticles remain constant even after 30 days of storage, hence the nanoparticles were quite stable at 4°C.

Loading of oligonucleotides onto nanoparticles

Several factors influence the loading and release performance of chitosan nanoparticles. To study the DNA carrying capacity, ODNs were loaded onto the polymeric nanoparticles by adsorption. The charge of chitosan nanoparticles predominantly depends on the pH of the suspension medium. The pKa of the amino groups in chitosan is $\sim$ 6.5, hence the charge density reduces above this pH.[23] About 90% of the amino groups in chitosan have been reported to be protonated at pH 5.5–5.7.[14] Mao et al. reported electrostatically neutral nanoparticles in the pH range of 7.0–7.4 using N/P ratio of 6 and zeta potential of −20 mV at pH 8–8.5.[14] Herein, we also found maximum loading of oligonucleotide ODN-1 (65.26%) onto chitosan nanoparticles in the acidic pH as compared to neutral and basic pH with 61.92% and 40.90% (Table I). Similar pattern was observed with oligonucleotide ODN-2 and ODN-3. But the amount of ODN-2 and ODN-3 loaded to chitosan nanoparticle was found to be more as compared to ODN-1.

In vitro release studies of oligonucleotides from nanoparticles

One of the major objectives behind the preparation of these nanoparticles was to prepare particles that are stable under different physiological pH. So, to determine the stability, the ODNs loaded nanoparticles were suspended in different aqueous buffers. The *in vitro* release of adsorbed oligonucleotides onto chitosan nanoparticles was thus monitored under different pH conditions by measuring O.D. of supernatant at 260 nm. The cumulative percentage of ODNs released from nanoparticles at different time intervals has been shown in Figure 3. On the basis of release rate, the release of ODN can be divided into two stages (the slope of ODN releasing profiles). The initial stage comprises of first 8 h, the release rate is very fast (burst effect), especially in the cases of high pH media, i.e., pH 7.0 and 8.0. Rapid release at this stage could be attributed to the diffusion of ODN localized at the nanoparticles surface, which might be involved with the concentration gradient. However, the diffusion of ODN was further enhanced at high pH due to the deprotonation of chitosan amino groups which results in poor electrostatic or ionic interaction between the nanoparticles and ODN, leading to fast release. On the other hand, the electrostatic interaction between the cationic nanoparticles and ODN was strong in the low pH medium (pH < pKa), i.e., pH 4.0 buffer. In the second stage, the ODN release rate is relatively slow, especially at lower pH 4.0 buffer (Fig. 3). This is probably due to the small amount of ODN left complexed onto the surface of the nanoparticles.

It has been observed that the release of ODN-1 from nanoparticles in neutral pH was slow upto 4 h and about 95% released within 24 h whereas the release was fast in basic pH 8.0 and about 96% of oligonucleotide released within 8 h (Fig. 3). The release of ODN-1 in acidic pH was around 80% in 24 h. Similar pattern was observed for ODN-2 and ODN-3. The release of ODN-2 and ODN-3 were fast in basic pH as compared to neutral and acidic pH. The release was completed within 5–8 h in basic pH, but the almost complete release was observed after 24 h in neutral pH (Fig. 3).

Cytotoxicity

The cytotoxicity of materials is the major driving force to determine the suitability for pharmaceutical and biomedical applications. The cytotoxicity of the nanoparticles was estimated using MTT colorimetric assay. HEK 293 cells were incubated with ODN loaded chitosan nanoparticles at concentration of 10 μg/ml of ODN per well in a 96 well plate for 6 h (presence of serum), followed by further incubation for 42 h (resupplement with medium containing

serum). Microscopic examination revealed considerable toxicity and cell morbidity induced by Lipofectamine. However, cell morphologies observed by optical microscopy showed quite low cytotoxicity in case of cells treated with ODN loaded chitosan nanoparticles. It has been reported that chitosan was less toxic than other cationic polymers such as poly-L-lysine and polyethyleneimine *in vivo* and *in vitro*.[24,25] Our results are in well accordance with the literature. The ODN loaded chitosan nanoparticles were slightly toxic after 48 h nearly 76–88% cell viability was observed (Fig. 4). On the other hand cells treated with commercially available transfection reagent Lipofectamine showed only 35% viability. These results suggest that chitosan nanoparticles have low toxicity and can be used as biocompatible gene transfer reagent.

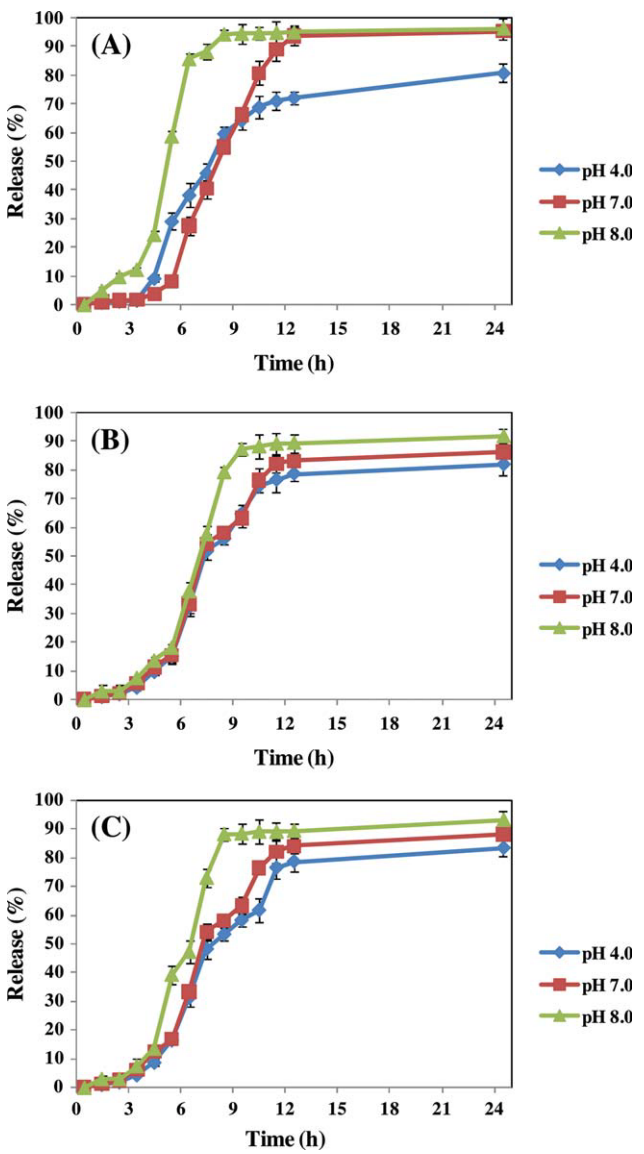

Figure 3 (A) The release studies of ODN-1 [d(TTT TTT TTT TTT TTT)], (B) The release studies of ODN-2 [d (GCC GAG GTC CAT GTC GTA CGD)], (C) The release studies of ODN-3 [d (GCC CAA GCT GGC ATC CGT CA)] at different pHs. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

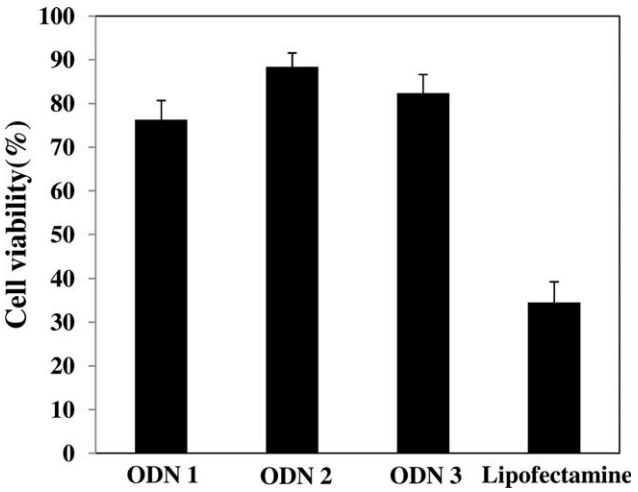

Figure 4 Cytotoxicity studies of ODNs loaded chitosan nanoparticles. HEK 293 cells treated with ODN loaded chitosan nanoparticles under transfection conditions followed my MTT assay.

CONCLUSION

We have prepared chitosan nanoparticles employing known methodology of reverse micellar system. Three different oligonucleotides were loaded onto the chitosan nanoparticles with maximum loading for ODN-2 in acidic pH. On monitoring the release of oligonucleotides from the chitosan nanoparticles it was observed that the release was maximum and rapid in basic medium as compared to neutral and acidic medium. The *in vitro* release kinetics data opens up new avenue for use of these nanoparticles in *in vivo* studies. The toxicity study provides evidence of these nanoparticles being biocompatible.

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