Research article

A NANOFORMULATION OF siRNA AND ITS ROLE IN CANCER THERAPY: *In vitro* AND *in vivo* EVALUATION

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Abstract: Overexpression of anti-apoptotic *Bcl-2* is often observed in a wide variety of human cancers. It prevents the induction of apoptosis in neoplastic cells and contributes to resistance to chemotherapy. RNA interference has emerged as an efficient and selective technique for gene silencing. The potential to use small interfering RNA (siRNA) as a therapeutic agent for the treatment of cancer has elicited a great deal of interest. However, insufficient cellular uptake and poor stability have limited its therapeutic applications. The purpose of this study was to prepare chitosan nanoparticles via ionic gelation of chitosan by tripolyphosphate for effective delivery of siRNA to silence the anti-apoptotic *Bcl-2* gene in neoplastic cells. Chitosan nanoparticles loaded with siRNA were in the size range 190 to 340 nm with a polydispersive index ranging from 0.04 to 0.2. They were able to completely bind with siRNA, provide protection against nuclease degradation, and enhance the transfection. Cell culture studies revealed that nanoparticles with entrapped siRNA could efficiently silence the anti-apoptotic *Bcl-2* gene. Studies on Swiss albino mice showed that siRNA could be effectively delivered through nanoparticles. There was significant decrease in the tumor volume. Blocking the expression of anti-apoptotic *Bcl-2* can enhance

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the sensitivity of cancerous cells to anti-cancer drugs and the apoptosis rate. Therefore, nanoformulations with siRNA can be promoted as an adjuvant therapy in combination with anti-cancer drugs.

Key words: Chitosan, RNA interference, siRNA, *Bcl-2,* Apoptosis, Cancer, Nanoformulation

INTRODUCTION

Over the past three decades, substantial progress has been made in understanding the molecular basis and genetics of cancer [1, 2]. In essence, it has been established that cancer is a variety of distinct diseases caused by defective genes. These gene defects are diverse in nature and can involve either loss or gain of gene functions. Apoptosis and the genes that control it have a profound effect on malignant phenotyping. Defective apoptosis pathways lead to tumor formation, progression and metastasis, and to the occurrence of multidrug resistance during cancer therapy [2-4].

In recent years, it has become increasingly evident that tumorigenesis is not merely the result of excessive proliferation due to the activation of oncogenes, but to the same extent depends on the impairment of apoptosis checkpoints [5]. Intriguingly, many of the alterations that induce malignant transformation, such as oncogene-driven deregulated proliferation and invasion, actually sensitize a cell to apoptosis. Therefore, only those oncogenically transformed cells that additionally acquire defects in apoptosis pathways and are thus protected against cell death induction will survive and become malignant [6]. A transformed cell can gain protection against apoptosis through inappropriate activation or expression of anti-apoptotic proteins or through the inactivation of pro-apoptotic factors [4, 7].

Gaining insight into the mechanisms and alterations by which components of the apoptotic machinery contribute to pathogenic processes should allow the development of more effective and specific and therefore better-tolerable therapeutic approaches. These include the targeted activation of pro-apoptotic tumor suppressors or the blockade of anti-apoptotic oncogenes in cancer [4, 5, 7, 8]. The induction of apoptosis through the intrinsic pathway is a mitochondrial event controlled by proteins of the Bcl-2 family [9-11]. These proteins share at least one of four homologous regions known as Bcl homology (BH) domains (BH1 to BH4). Some members of the Bcl-2 family block apoptosis, such as Bcl-2, Bcl-xL, Bcl-w and Mcl-1. Others promote apoptosis, such as Bax and Bak in the multidomain subfamily and Bad and Bid in the BH3 single domain subfamily. Cell survival is maintained through balance between pro-apoptotic and anti-apoptotic stimuli [4, 12-15].

Overexpression of *Bcl-2* is considered to be one of the mechanisms by which tumor cells acquire resistance to apoptosis. High levels of *Bcl-2* gene expression are found in a wide variety of human cancers, including prostate, breast, olorectal, lung, gastric, renal, neuroblastoma, non-Hodgkin's lymphoma, and

acute and chronic leukemia [15, 16]. In addition, *Bcl-2* is implicated in chemo-resistance because over-expression of *Bcl-2* can inhibit the cytocidal effect of many currently available anti-cancer drugs by blocking the apoptotic pathway. The expression level of Bcl-2 proteins varies with the degree of resistance to chemotherapeutic drugs: the protein levels are higher when the degree of resistance is higher. Therefore, inhibition of the protective function of Bcl-2 protein in tumor cells is an attractive strategy for either restoring the normal apoptotic process in these cells or making them more susceptible to conventional chemotherapy or radiotherapy [15, 17].

The discovery of RNA interference (RNAi) led to the discovery that small interfering RNA (siRNA) could be used to down-regulate the expression of such overexpressed genes [18, 19]. However, unmodified, naked siRNAs are relatively unstable in blood and serum, as they are rapidly degraded by endo- and exonucleases, meaning that they have short half-lives [20]. In this study, an attempt was made to prepare chitosan nanoparticles for effective delivery of siRNA to cancerous cells to silence the anti-apoptotic *Bcl-2* gene. Chitosan is a naturally existing cationic polysaccharide composed of glucosamine and N-acetylglucosamine residues. It can be derived through partial deacetylation of chitin, which is generally obtained from crustacean shells. Commercially available chitosan has an average molecular weight ranging between 3800 and 20,000 Da and is 66 to 95% deacetylated. Chitosan, a material of choice for developing micro or nanoparticles, has many advantages, namely: it has the ability to control the release of active agents; it allows synthesis without the use of hazardous organic solvents since it is soluble in aqueous acidic solution; it is a linear polyamine containing a number of free amine groups that are readily available for cross linking; its cationic nature allows for ionic cross linking with multivalent anions; it has muco-adhesive character, which increases the residual time at the site of absorption; and so on. Chitosan is known to have a low toxicity and immunogenicity and be biocompatible and degradable by enzymes [21-23]. Chitosan has been widely used in many drug delivery applications, especially in gene delivery systems, due to its positively charged amines allowing electrostatic interactions with negatively charged nucleic acids to form stable complexes [22]. There have been many studies on the development of chitosan-based carriers for DNA and RNA delivery [24, 25]. This study was designed to develop chitosan-based siRNA nanoformulations to target the anti-apoptotic *Bcl-2* gene, with the purpose being to overcome biodegradation and stability problems to effectively deliver siRNA to target cells.

MATERIALS AND METHODS

Materials
Chitosan (molecular weight of 110-150 kDa, degree of deacetylation > 65%), Pentasodium tripolyphosphate (TPP), 3-(4, 5 dimethyl thiazole-2 yl)-2, 5-diphenyl tetrazolium bromide (MTT), protease inhibitor cocktail, minimum essential

medium (MEM), Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trypsin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) sodium salt buffer solution, sodium dodecylsulphate (SDS), dithiothreitol (DTT), bovine serum albumin (BSA) and agarose were purchased from Himedia Lab Pvt. Ltd. (Mumbai, India). Both the sense and antisense sequences of siRNA strands for the anti-apoptotic gene *Bcl-2* (sense 5'-GUA CAU CCA UUA UAA GCU G-dTdT-3' and antisense 5'-CAG CUU AUA AUG GAU GUA C-dTdT-3') were purchased in purified form from Genei (Bangalore, India). Annealing of the two sequences was performed as per the manufacturer's instructions. Trizol LS and 100-bp DNA ladder was purchased from Invitrogen Life Technologies Co. (Carlsbad, CA, USA). Forward and reverse primers for *Bcl-2* and *GADPH* were purchased from Bioserve Biotechnologies (Hydrabad, India). One-step RT-PCR Master Mix Kit and protein markers were purchased from Novagen/Toyobo (Darmstadt, Germany). Rabbit polyclonal IgG primary antibodies for *Bcl-2* (C-21), β-actin (H-196) and goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals used in the study were of analytical grade.

Cell lines and culture media
HEp-2 (human epithelial laryngeal carcinoma), HeLa (human cervical carcinoma) and Chang liver (non-malignant human liver epithelial) cell lines were purchased from the National Centre for Cell Sciences (Pune, India). Cells were cultured with suitable medium (MEM or DMEM) supplemented with 10% FBS and 50 µg/ml gentamycin sulfate and incubated at 37ºC in a humidified atmosphere with 5% CO_2.

Preparation of nanoformulation
siRNA-loaded chitosan nanoparticles were prepared by ionic gelation of TPP with chitosan [22]. Nanoparticles were obtained upon the addition of 1 ml of TPP aqueous solution to 3 ml of chitosan solution under constant magnetic stirring at room temperature. The particles were then incubated at room temperature for 30 min before use for further analysis. Nanoparticles were collected by centrifugation at $16,000 \times g$ for 10 min at 4ºC. The supernatants were discarded and nanoparticles were re-suspended in RNase-free water.

Particle size and zeta potential
The mean particle size and poly dispersive index (PDI) of nanoparticles were determined after re-dispersion of nanoparticles in water treated with diethylpyrocarbonate (DEPC) by dynamic light scattering using the photon correlation spectroscopy technique at 25ºC on a Malvern NanoZS (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 633 nm laser and 173º detection optics. The zeta potential of the re-dispersed nanoparticles was measured via the laser Doppler electrophoresis technique at 25ºC using

a Malvern NanaZS (Malvern Instruments Ltd., Worcestershire, UK). Based on the measured conductivity of the sample, the voltage used for zeta-potential measurement was selected automatically. Malvern DTS v.5.00 software (Malvern Instruments Ltd., Worcestershire, UK) was used for data acquisition and analysis [26].

Morphology
The morphology of the nanoparticles was determined using scanning electron microscopy (SEM). The nanoparticles were suspended in DEPC-treated water (5 mg/ml) and the particles were visualized using a scanning electron microscope with EDS (JEOL, Akishima, Tokyo, Japan).

Determination of siRNA loading efficiency
The loading efficiency of siRNA (%) entrapped or adsorbed onto the nanoparticles was obtained by determining the free siRNA concentration [23, 26]. Chitosan nanoparticles were centrifuged at $16,000 \times g$ for 15 min and supernatants were collected. Free siRNA content in the supernatant was measured by measuring absorbance with a UV-Visible Spectrophotometer at 260 nm. Supernatants recovered from blank nanoparticles (without siRNA) were used as a blank. The siRNA loading efficiency was measured as the percentage of entrapped or adsorbed siRNA to the total amount of siRNA added. The acid orcinol method was also used to measure the amount of siRNA.

Assay for serum stability
siRNA-loaded chitosan nanoparticles were incubated at 37ºC with an equal volume of DMEM supplemented with a 10% final concentration of FBS. At each predetermined time interval (0, 0.5, 1, 2, 4, 8, 24, 48 and 72 h), 30 μl of the mixture was removed and stored at -20ºC until gel electrophoresis was performed. To terminate serum activity, samples were incubated in a bath incubator at 80ºC for 5 min and 5 μl heparin (1000 U/ml) was added to displace the siRNA from the chitosan nanoparticles. The integrity of the siRNA was then analyzed using a 1.5% agarose gel containing ethidium bromide. Electrophoresis was carried out with $1\times$ tris acetate EDTA (TAE) buffer at a constant voltage of 50 V for 1 h. siRNA bands were visualized under a UV transilluminator at a wavelength of 365 nm [23, 26].

Cytotoxicity assay
The cytotoxic effects of blank and siRNA-loaded chitosan nanoparticles were measured with the MTT assay on HEp-2, HeLa and Chang liver cells, as previously described [26-28].

Transfection of siRNA-loaded chitosan nanoparticles to cells and expression studies
HEp-2 and HeLa cells were respectively routinely maintained in DMEM and MEM with 10% FBS at 37ºC in a humidified incubator containing 5% CO_2.

24 h before transfection, 1×10^5 cells were seeded in each well of 6-well plates (Greiner bio-one, Germany) with culture medium containing 10% FBS. After 24 h, the cells were treated with chitosan nanoparticles containing 50 ng siRNA per well for 24 to 48 h. After the treatment, RNA and total protein were extracted for subsequent analysis[26].

Reverse transcriptase polymerase chain reaction (RT-PCR)
Total RNA was extracted using trizol reagent as per the manufacturer's instructions. Subsequently, total RNA was treated with DNase to remove contaminating genomic DNA and quantified using a DNA protein enzyme analyzer (Shimadzu, Kyoto, Japan). cDNA synthesis and amplification were performed using PCR apparatus (Eppendorf, Hamburg, Germany) in a volume of 50 μl comprising 2 μl of 5 μg of total RNA, 2 μl of oligo(dT), 25 μl of one-step RT-PCR master mix, 3 μl of 50 mM $Mn(OAc)_2$, 2 μl of 0.5 μM of the appropriate forward and reverse primers, and 16 μl of RNAse free water. Polymerase activation was done at 90ºC for 30 s and reverse transcription was performed at 60ºC for 30 min. *Thermus thermophilus* DNA polymerase enzyme was used for the cDNA synthesis step and PCR amplification step. The sequences of the PCR primers for *Bcl-2* (NM_016993) were 5'-GGA GCG TCA ACA GGG AGA TG-3' (forward) and 5'-GAT GCC GGT TCA GGT ACT CAG-3' (reverse) and the sequences for GAPDH (NM_017008) were 5'-CCA AGA AGC TGA GCG AGT GTC TC-3' (forward) and 5'-CCT GCT TCA CCA CCT TCT TG-3' (reverse). The conditions for the PCR amplification cycle consisted of 40 cycles with denaturation at 94ºC for 1 min, annealing at 60ºC for *GAPDH* and 51ºC for *Bcl-2*, and extension at 72ºC for 1 min, with 1 cycle of final extension at 60ºC for 7 min. The predicted size of the amplified products of *Bcl-2* and *GAPDH* were 127 and 349 bp respectively. Equal amounts of the corresponding products of *Bcl-2* and GAPDH were analyzed on 1.5% agarose gel electrophoresis (Bangalore Genei, India) and optical densities of ethidium bromide-stained DNA bands were quantified using Alpha Innotech software (USA) [29-31].

Western blot analysis
Total protein was extracted from the transfected cells by homogenizing them in a lysis buffer containing 4 M NaCl, 1 M $MgCl_2$, 0.5 M EDTA, 1 M HEPES (pH 7.9), 0.5 mM DTT and a cocktail of protease inhibitors. Cell lysates were centrifuged at $16,000 \times g$ for 15 min at 4ºC. Protein content in the supernatant was calculated using the Bradford method. A 50 μg sample of protein was separated on 10% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel electrophoresis and transferred onto nitrocellulose membrane (0.2 μm pore size, Bio-rad, CA, USA). After blocking, nitrocellulose membrane was incubated overnight at 4ºC with rabbit anti-*Bcl-2* antibody and rabbit anti-β-actin antibody at a dilution of 1:500, and further incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at a dilution of 1:1000. Bound antibodies were

detected using the chromogenic substrate tetramethyl benzidine, which is specific for horseradish peroxidase enzyme [26, 32].

In vivo studies

Twenty-four 3- to 5-week old Swiss albino mice of either sex weighing around 25-30 g were used for the investigation. All of the animals were maintained under standard husbandry conditions with food and water *ad libitum*. The experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC), KMC, Manipal, India (No.IAEC/KMC/30/2010-2011). The animals were divided into four groups with six animals each. The first group was the control, the second with the standard drug, the third with naked siRNA, and the fourth with siRNA-loaded nanoparticles. Cisplatin (8 mg/kg) was used as standard drug. 5×10^7 HeLa cells were suspended in 200 µl PBS (pH 7.4) injected subcutaneously into the right flank of each mouse except those in the control group. When the tumor reached 5 to 7 mm in diameter, six mice in each group received the first intratumoral injection, and dosing was repeated on days 15, 16, 18, 21 and 25. The tumor diameters were measured using a sliding caliper for maximum and minimum diameter on the day before the treatment and 15, 16, 18, 21 and 25 days after the treatment, and the tumor volumes were calculated [33].

Statistical analysis

The data are expressed as the means ± SEM of the indicated number of experiments. Statistical analysis was carried out using one-way ANOVA (GraphPad Prism 5.00, Instat Software, San Diego, CA, USA) followed by Tukey's post hoc test. A value of $p < 0.05$ was considered to be significant.

RESULTS

Characterization of nanoparticles

Chitosan nanoparticles were prepared using the ionic gelation method. Particles in the size range of 190 nm to 340 nm were obtained with polydispersity values between 0.04 and 0.2. The zeta potential of all of the formulations in this study was found to be approximately +30 mV and above. The morphology of siRNA-loaded chitosan nanoparticles was visualized using SEM (Fig. 1). The formation of chitosan-TPP nanoparticles occurred upon the addition of the TPP ions into the chitosan solution. The results showed that the appearance of the chitosan solution changed from clear to opalescent when TPP ions were added. This indicated a change of the physical states of the chitosan to form nanoparticles, then microparticles, and eventually aggregates. The effects of chitosan concentration and TPP concentration were determined in relation to the mean particle size, surface charge and encapsulation efficiency (Tables 1 and 2). The comparative positive value of the surface charge of the chitosan-siRNA complexes increased with increasing concentrations of chitosan at a fixed siRNA concentration. The increment was due to an increase in the number of positive charges, which counteracts the negatively charged siRNA. The net positive charge
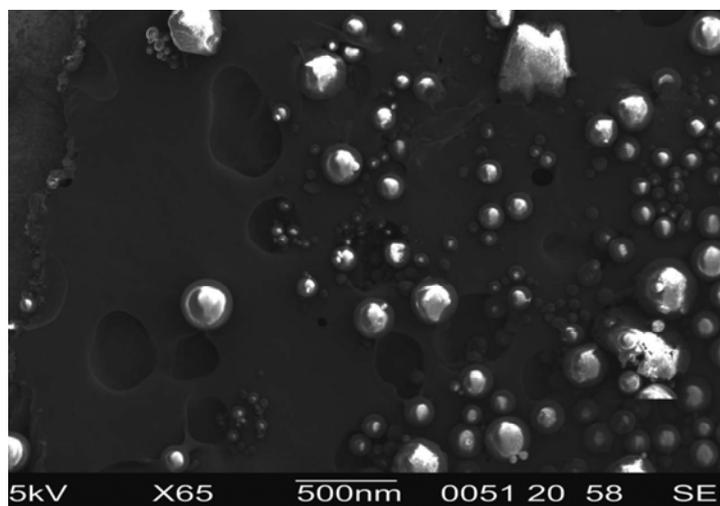
Fig. 1. Scanning electron microscopy of siRNA-loaded chitosan nanoparticles.

Table 1. The effect of chitosan concentration on mean particle size, PDI, zeta potential and % encapsulation.

Chitosan concentration [mg/ml]	Mean particle size [nm]	PDI	Zeta potential [mV]	Encapsulation [%]
1	181.6 ± 6.51	0.166 ± 0.04	18.53 ± 2.90	67.96 ± 1.65
2	236.6 ± 17.37	0.106 ± 0.02	36.30 ± 1.47	81.13 ± 3.1
3	251.86 ± 21.35	0.205 ± 0.02	39.06 ± 0.5	80.86 ± 4.2
4	265.4 ± 32.48	0.23 ± 0.04	41.73 ± 2.00	82.43 ± 1.9
5	303.03 ± 23.35	0.26 ± 0.05	43.73 ± 1.2	78.1 ± 2.78

Constant parameters: TPP concentration of 3 mg/ml and siRNA concentration of 19 μg/ml. The data are the means ± SEM of three independent experiments.

Table 2. The effect of TPP concentration on mean particle size, PDI, zeta potential and % encapsulation.

TPP concentration [mg/ml]	Mean particle size [nm]	PDI	Zeta potential [mV]	Encapsulation [%]
1	228.29 ± 17.06	0.285 ± 0.06	27.4 ± 3.27	69.86 ± 5.6
2	246.39 ± 46.5	0.13 ± 0.02	37.3 ± 1.49	82.33 ± 1.46
3	276.56 ± 6.04	0.18 ± 0.03	41.1 ± 1.37	83.27 ± 1.12
4	385.23 ± 15.17	0.21 ± 0.02	40.53 ± 2.21	84.33 ± 0.57
5	461.13 ± 24.39	0.27 ± 0.01	42.5 ± 2.46	83.66 ± 1.15

Constant parameters: Chitosan concentration of 2 mg/ml and siRNA concentration of 19 μg/ml. The data are the means ± SEM of three independent experiments.

of the particles was desirable to prevent particle aggregation and promote electrostatic interaction with the overall negative charge of the cell membrane. The final formulation used for further study was prepared with 2 mg/ml chitosan and 2 mg/ml TPP, with a mean particle size of 263.1 nm, PDI of 0.117 and zeta potential of +38.5 mV, giving an encapsulation efficiency around 83.88%.

siRNA loading efficiency
The amount of siRNA absorbed on the chitosan nanoparticles was measured using UV spectrophotometry and the acid orcinol method and compared with the total initial siRNA concentration. siRNA loading efficiency was found to be more than 80% by both methods.

Serum stability
siRNA must be protected from digestion by nucleases for maximum activity in cells, so the stability of the nanoparticle-siRNA complexes is crucial. Formulations were incubated with 10% FBS to evaluate their stability. On visual examination of the intensities of bands, naked siRNA was found to be intact for up to 1 h. Thereafter, partial degradation took place and at 6 h, complete degradation was observed. siRNA loaded in chitosan nanoparticles was found to be intact for up to 4 h. Partial degradation was observed at 12 h, and at 48 h siRNA was completely degraded. Chitosan nanoparticle-siRNA complexes were protected against nuclease digestion (Fig. 2).
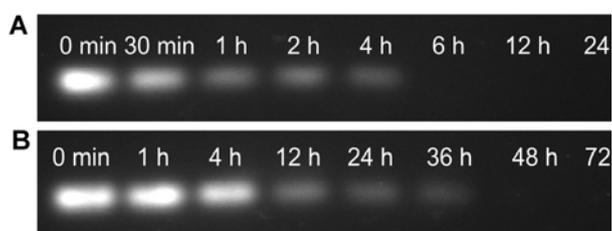

Fig. 2. Serum stability of naked siRNA (A) and siRNA-loaded chitosan nanoparticles (B) incubated in 10% serum over a period of time.

Cytotoxicity
The cytotoxicity of chitosan nanoparticles containing siRNA was evaluated using the MTT method for HEp-2, HeLa and Chang liver cells. Cells were treated with blank nanoparticles and nanoparticles loaded with siRNA and incubated for 48 to 72 h. Between 90 and 95% cells were viable after 48 h and 80 to 85% cells were viable after 72 h treatment with the blank formulations. For siRNA-loaded chitosan nanoparticles, there was a 14 to 18% loss of viability after 48 h and 24 to 32% loss of viability after 72 h (Fig. 3). TPP was not found to be toxic.
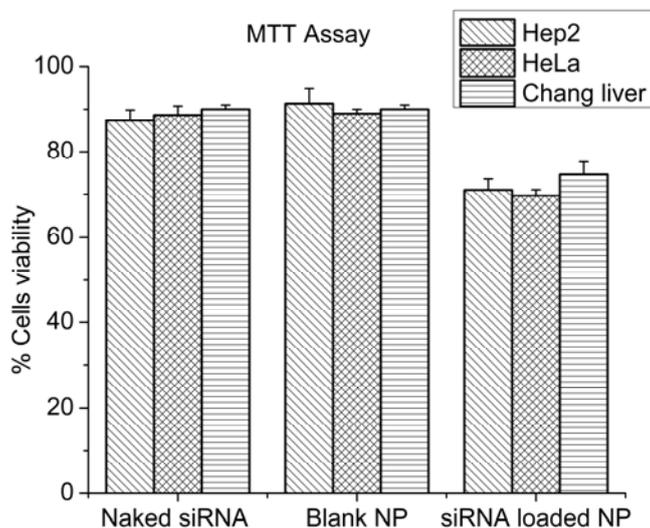
Fig. 3. *In vitro* cytotoxicity of naked siRNA, blank chitosan nanoparticles and siRNA-loaded chitosan nanoparticles evaluated using the MTT assay after 72 h of treatment. The data are the means ± SEM of five independent experiments.

Bcl-2 mRNA expression

After transfecting the cells with nanoparticles containing siRNA specific to *Bcl-2* mRNA, total RNA was isolated and RT-PCR was performed. As shown in Fig. 4, *Bcl-2* mRNA levels in nanoparticle-siRNA complex-transfected cells were significantly decreased compared to the levels for the control cells and naked siRNA-transfected cells. siRNA can be effectively prevented from nuclease-mediated degradation by preparing nanoparticle-siRNA complexes, which effectively reduces the mRNA level compared to that for cells transfected with naked siRNA.

Western blot analysis

Western blot was used to examine the expression of *Bcl-2* protein. As shown in Fig. 5, Bcl-2 protein levels in nanoparticle-siRNA complex-transfected cells were significantly lower than those for the control cells and naked siRNA-transfected cells for both cell lines.

In vivo studies

A study was conducted on Swiss albino mice to observe the effect of siRNA-loaded chitosan nanoparticles on tumor regression. The tumor growth in terms of volume (cm^3) was recorded from the first day of inoculation of HeLa cells. The tumor volumes were recorded on various days for the control group and groups treated with naked siRNA, siRNA-loaded chitosan nanoparticles, and cisplatin. As shown in Fig. 6, there was significant increase in the tumor volume in the control group and naked siRNA-treated group. In the cisplatin-treated group, there was a significant level of tumor regression. In the group transfected with

nanoparticles loaded with siRNA, there was also significant regression. Thus, chitosan nanoparticles can be used as an efficient carrier for *in vivo* delivery of siRNA.
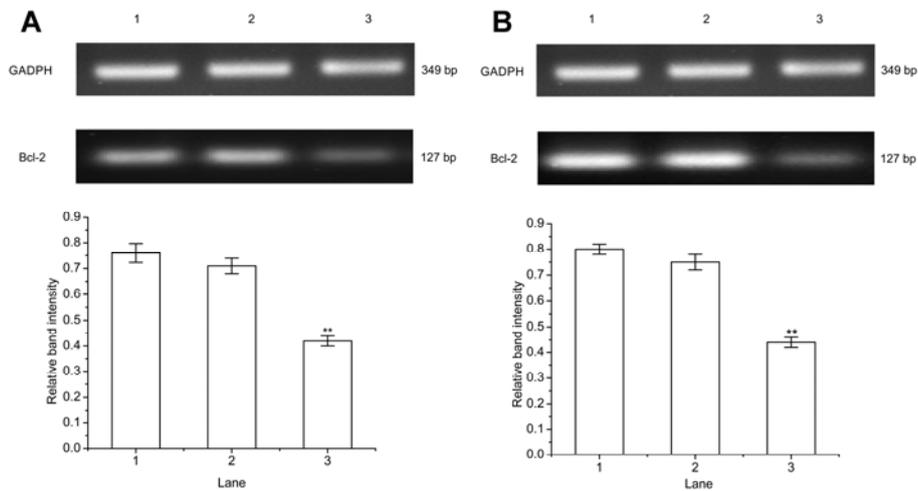

Fig. 4. mRNA expression of *Bcl-2* assessed by RT-PCR in HEp-2 cells (A) and HeLa cells (B). Lane 1: untreated cells; lane 2: naked siRNA-treated cells; lane 3: siRNA-loaded chitosan nanoparticles-treated cells. The data are the means ± SEM of five independent experiments. **Significant decrease over control and naked siRNA-treated cells, P < 0.001.
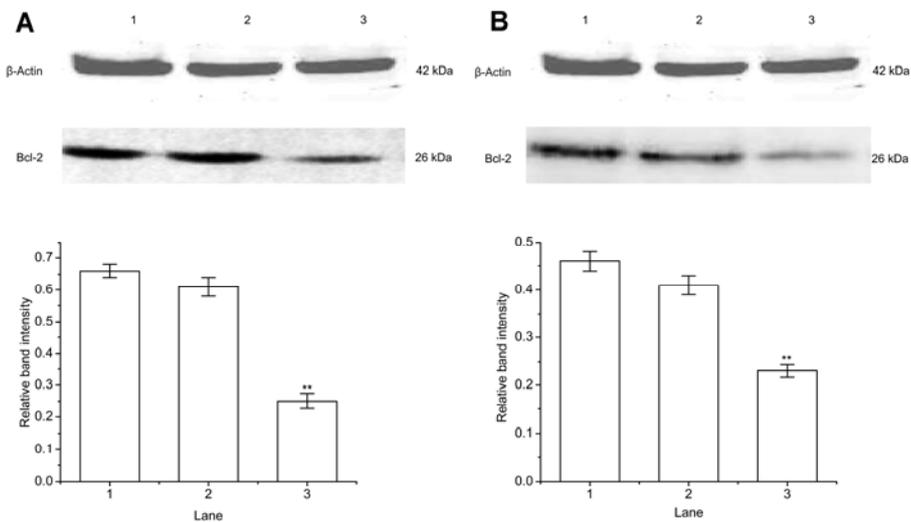

Fig. 5. Bcl-2 protein expression assessed by Western blotting in HEp-2 cells (A) and HeLa cells (B). Lane 1: untreated cells; lane 2: naked siRNA-treated cells; lane 3: siRNA-loaded chitosan nanoparticles treated cells. The data are the means ± SEM of five independent experiments. **Significant decrease over control and naked siRNA-treated cells, P < 0.001.
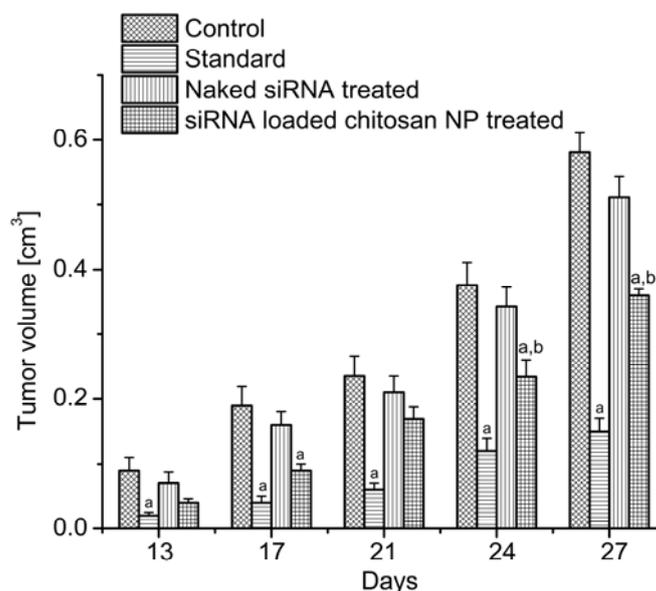
Fig. 6. *In vivo* studies on the effect of siRNA-loaded chitosan nanoparticles on tumor regression. Cisplatin (8 mg/kg) was used as a standard drug. Values are the means ± SEM; n = 6. Tukey's analysis of variance at each time showed a significant difference: [a]p < 0.05 compared to the control group and [b]p < 0.05 compared to the drug-treated control group.

DISCUSSION

Since apoptotic programs can be manipulated to produce massive changes in cell death, the genes and proteins controlling apoptosis are potential targets in cancer therapy. Alterations in apoptotic programs provide opportunities to target cell death in selective manner [1]. Bcl-2 is a potent pro-survival advocate, and it has been found to shield cancer cells from apoptotic instructions [34]. Up-regulation of *Bcl-2* is a common cause of tumorigenesis and chemotherapy resistance in multiple tumor types and it correlates with poor survival and disease progression [35].

For the last decade, RNAi research has grown very rapidly due to its huge potential as a therapeutic strategy for clinical applications in the treatment of many inheritable or acquired diseases by silencing undesirable gene expressions. However, for systemic use, the delivery of siRNA remains the greatest challenge. It is imperative that siRNA reach the cytoplasm of the target cell to become effective and induce silencing. A major limitation in the therapeutic use of siRNA is its rapid degradation in the plasma and cellular cytoplasm, resulting in a short half-life [36-38]. Furthermore, naked siRNA cannot passively diffuse through cellular membranes due to the strong anionic charge of the phosphate backbone and consequent electrostatic repulsion from the anionic cell membrane surface. Cellular entrance is also limited by its size. Therefore, the successful use of siRNA is largely dependent on the development of a delivery vehicle that could be administered efficiently, safely, and repeatedly [22, 39]. An ideal

carrier should be able to bind and condense siRNA, provide protection against degradation by nucleases, specifically direct siRNA to target cells, facilitate its intracellular uptake, escape from the endosome/lysosome into the cytosol, and finally allow the promotion of efficient gene silencing [21, 22, 40].

The use of chitosan as a siRNA delivery carrier is based on its cationic property. At acidic pH, below the pKa, the primary amines in the chitosan backbone become positively charged. These protonated amines facilitate binding of chitosan to negatively charged siRNA via an electrostatic interaction. Interaction between the positively charged chitosan backbone and negatively charged siRNA leads to the formation of nano-size complexes [41-43].

In this study, the ionic gelation method was used to prepare siRNA-loaded chitosan nanoparticles. Electrostatic interaction between polyanion TPP and cationic chitosan results in the formation of nanoparticles. Stable and smaller-sized nanoparticles were formed when lower molecular weights of chitosan (~110 kDa) were used instead of higher molecular weights (~270 kDa) [23]. The binding capacity of siRNA depends on the degree of deacetylation of chitosan, as the higher the degree of deacylation, the greater the siRNA-binding capacity. We used chitosan with a molecular weight of 110-150 kDa to produce smaller particles. The results of our study showed that siRNA can be incorporated successfully with high encapsulation efficiency into biodegradable chitosan nanoparticles. These siRNA-loaded nanoparticles were spherical in shape, had a smooth surface and a net positive surface charge. The particle size and the encapsulation efficiency in particular were influenced by concentrations of chitosan and TPP. An increase in the concentration of chitosan resulted in particles with larger size and a high positive charge. As particle size affects the transfection of nanoparticles into cells, chitosan concentration was fixed at 0.2 mg/ml. Our final formulation was able to protect siRNA from degradation by serum nucleases for up to 4 h in 10% FBS. Beyond this period, partial degradation was observed up to 36 h. At 48 h, siRNA was completely degraded.

We determined the *Bcl-2* mRNA levels in HEp-2 and HeLa cells transfected with siRNA-containing nanoparticles and naked siRNA. The levels of *Bcl-2* mRNA in HEp-2 and HeLa cells transfected with siRNA-loaded nanoparticles decreased significantly compared to those of naked siRNA-treated and control groups. The decrease in *Bcl-2* expression in the presence of nanoparticles containing siRNA correlated to a decrease in the protein levels, as evaluated by Western blot analysis. A decrease in anti-apoptotic Bcl-2 protein levels leads to an increase in free pro-apoptotic Bax protein levels in the cytoplasm, facilitating the release of cytochrome C from the mitochondria and the activation of the intrinsic pathway of apoptosis. The blank formulation (without siRNA) was found to be non-toxic to normal and cancerous cells. Loss of viability was observed among the cells treated with the siRNA nanoformulation. This may be due to activation of the intrinsic pathway of apoptosis, as explained above. *In vivo* studies showed that siRNA was effectively delivered through nanoparticles. There was a significant decrease in the tumor volumes after the

treatment with chitosan nanoparticles containing siRNA compared to the naked siRNA-treated group and control group. By reducing anti-apoptotic *Bcl-2* expression, the sensitivity of cancerous cells to anti-cancer drugs and the apoptosis rate can be enhanced. This method can be promoted as an adjuvant therapy in combination with anticancer drugs. The dose and side effects of anticancer drugs can be reduced to a certain extent with adjuvant siRNA therapy [44]. Chitosan can be used as an effective carrier for siRNA in order to achieve efficient delivery.

Conflict of interest. The authors declare that there is no conflict of interest.

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