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Improvement of therapeutic efficacy of PLGA nanoformulation of siRNA targeting anti-apoptotic *Bcl-2* through chitosan coating

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ABSTRACT

Potential use of siRNA as therapeutic agent has elicited a great deal of interest. However, insufficient cellular uptake and poor stability limited its application in therapeutics. In our earlier study, we prepared PLGA nanoparticles for effective delivery of siRNA targeting *Bcl-2* gene to block its expression. Purpose of the present study was to improve effectiveness of PLGA nanoformulation of siRNA targeting anti-apoptotic *Bcl-2* gene through chitosan coating. We prepared chitosan coated PLGA nanoparticles by using the double emulsion solvent diffusion (DESE) method. Characterization of prepared chitosan coated nanoformulation was done followed by cytotoxicity studies, expression studies and *in vivo* studies. Particle size of chitosan coated nanoparticles was found to be increased compared to PLGA nanoparticles from 244 to 319 nm. Surface charge of chitosan coated nanoparticles was found to be positive facilitating transfection of nanoformulation into cells. *In vitro* studies indicated increased transfection of nanoparticles resulting in effective silencing of *Bcl-2*. Marked apoptotic lesions were observed in nuclear staining studies. On comparison of the results from the present study with those of previous study, it was found that the extent of silencing of *Bcl-2* gene by PLGA nanoformulation has improved significantly through chitosan coating. *In vivo* studies showed significant tumor regression in animals treated with chitosan coated PLGA nanoformulation of siRNA.

1. Introduction

RNA interference emerged as powerful and most widely accepted tool for post-transcription gene silencing induced by double stranded RNA (Fire et al., 1998; Dykxhoorn et al., 2003). RNAi can be made effective for therapeutic applications through delivery of shorter siRNA. This siRNA can be produced synthetically with high specific and potent gene silencing properties. siRNA has a great therapeutic potential in areas viz., cancer, infectious diseases to name a few and effective research tool to unravel the molecular and genetic basis of the diseases (Jagani et al., 2011). *In vivo* use is still a challenge that is primarily focused on the difficulty in delivery of siRNA to target cells. Enzymatic degradation in blood, interaction with blood components and nonspecific uptake by the cells are the major obstacles for delivering siRNA to the cells *in vitro* and *in vivo* (Kim, 2003). In addition, the immune responses against siRNA must be taken into account when considering the application of siRNA for therapies. siRNA delivery methodologies need to be standardized in order to establish them as a potential therapeutic tool. To achieve the knockdown by siRNA, many siRNA delivery systems based on physical and pharmaceutical approaches have been proposed. Presently, major delivery systems use either viral or non-viral vectors (Cheng et al., 2003).

Although the viral vectors transduce cells naturally and show very high transduction efficiency compared to transfection by non-viral methods, their major drawbacks are danger of viral toxicity and relatively strong host responses resulting from the activation of the human immune system and inflammatory potential. Non-viral vectors seem to be promising tools for gene delivery, because they can be modified by incorporating ligands for targeting specific cell types and are also relatively safe (Reischl and Zimmer, 2009; Hitesh et al., 2013). Nanosized particles such as polymeric micelles, polyplexes, liposomes, and lipoplexes are often studied as drug carrier systems for nucleic acid delivery. There are three major vectors that are convenient for non-viral siRNA delivery: lipid-based liposomes, polymer-based nanoparticles, peptide- or protein-based delivery systems (Aigner, 2007; Akinc et al., 2009).

Biodegradable polymer based nanoparticles have advantages over other carriers due to their increased stability and their controlled-release ability and low polydispersity. Poly

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(D, L-lactide-*co*-glycolide) (PLGA), biodegradable and biocompatible, is one of the most widely used polymers (Katas et al., 2009). PLGA has been widely investigated as carrier for siRNA due to small particle size, favorable safety profile, localized as well as target delivery and sustained-release characteristics. Major limitation of these nanoparticles is their negative surface charge, which confines the interaction with the negatively charged siRNA, the poor transport through the cell membrane (Koganti et al., 2013). Cationic surface modification can overcome these disadvantages and hence readily binds and condenses siRNA (Taetz et al., 2009). In the present study, we prepared siRNA loaded PLGA nanoparticles with cationic surface modification by coating PLGA nanoparticles with naturally occurring linear polysaccharide, chitosan. Chitosan coating on PLGA nanoparticles imparts beneficial qualities such as excellent biocompatibility, low immunogenicity, low toxicity, as well as high positive charge density. Surface charge and particle size are significant considerations while preparing formulations as these have major role in cellular uptake and trafficking of the nanoparticles. siRNA silencing efficiency was compared by delivering naked siRNA, siRNA loaded PLGA nanoparticles and siRNA loaded chitosan coated PLGA nanoparticles *in vitro* and *in vivo.*

2. Materials and methods

2.1. Materials

Poly (D, L-lactide-*co*-glycolide), [Purasorb® PDLG 5002A (Mw = 17 kDa, acid terminated, Lactic:Glycolic acid ratio L:G = 50:50] was provided as a gift sample from Purac Biomaterials (Gorinchem, Netherland). Polyvinylalcohol (PVA) (88% hydrolyzed, MW 22000), acridine orange, dimethylsulfoxide (DMSO), isopropanol and glacial acetic acid were purchased from Qualigens fine chemicals (Mumbai, India). Triton-X 100, trypsin, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) sodium salt buffer solution, sodium dodecylsulfate (SDS), dithiothreitol (DTT), bovine serum albumin (BSA), normal and low melting agarose were purchased from Himedia lab Pvt. Ltd. (Mumbai, India). Chitosan (molecular weight of 140–220 kDa), 3-(4,5 dimethyl thiazole-2 yl)-2,5-diphenyl tetrazolium bromide (MTT), sulphorhodamine B dye, protease inhibitor cocktail, dulbecco's modified eagle's medium (DMEM), minimum essential medium and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Both the sense and antisense sequences of siRNA strands for 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 manufacturer's instructions. Trizol LS and 100 bp DNA ladder was purchased from Invitrogen Life Technologies Co, (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.

2.2. Cell lines and culture media

HEp-2 (human epithelial laryngeal carcinoma cell line) and HeLa (human cervical carcinoma) were purchased from National Centre for Cell Sciences (Pune, India). Cells were cultured with minimum essential medium (MEM) or dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS and 50 μg/ml gentamycin sulfate and incubated at 37 °C in 5% CO_2 humidified atmosphere.

2.3. Preparation of nanoparticles

siRNA loaded chitosan coated PLGA nanoparticles were prepared by the double emulsion ($w_1/o/w_2$) solvent evaporation method (DESE) Jagani et al., 2011; Nafee et al., 2007. In brief, siRNA solubilized in 200 or 300 μl of Tris–EDTA buffer was mixed with 500 μl dichloromethane (DCM) containing various amounts of PLGA, and the mixture was emulsified to form primary w_1/o emulsion by sonication. Two ml of 2% (w/v) PVA containing various amount of chitosan in RNase free water were poured directly into the primary emulsion and further emulsified by sonication for another 60 s to form a $w_1/o/w_2$ double emulsion. The resulting emulsion was diluted with 4 ml of 2% (w/v) PVA and stirred for 3 h at room temperature to evaporate DCM. The nanoparticles were collected by ultracentrifugation at 16,000*g* for 10 min at 4 °C, washed twice with 6 ml of RNase free water, re-suspended in RNase free water, and freeze-dried for 48 h without use of a cryoprotectant. For freeze-drying, the samples were frozen to −42 °C over 2 h on a temperature controlled shelf. The primary drying was done at −42 °C and 1.030 mBar (103 pa) for 36 h, the shelf temperature was gradually increased to −10 °C over 12 h, and the final drying was done at −10 °C and 0.0010 mBar (0.1 pa) for 12 h, the temperature increased to +10 °C over 2 h and drying was continued for another 24 h.

2.4. Particle size and zeta potential

The mean particle size and poly dispersive index (PDI) of nanoparticles were determined after re-dispersion of freeze-dried nanoparticles in diethylpyrocarbonate (DEPC)-treated water by Malvern NanoZS (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 633 nm laser and 173° detection optics. The zeta potential of re-dispersed nanoparticles was measured by the laser Doppler electrophoresis technique at 25 °C using Malvern NanaZS (Malvern Instruments Ltd., Worcestershire, UK). Malvern DTS v.5.00 software (Malvern Instruments Ltd., Worcestershire, UK) was used for data acquisition and analysis (Jagani et al., 2011).

2.5. Determination of siRNA encapsulation efficiency

The encapsulation efficiency of siRNA (%) entrapped or adsorbed onto the Chitosan coated PLGA nanoparticles was obtained from the determination of free siRNA concentration. Freeze dried PLGA nanoparticles after re-dispersion in DEPC-treated water were centrifuged at 16,000*g* for 15 min and supernatants were collected. Free siRNA content in the supernatant was measured by measuring absorbance by UV–Visible Spectrophotometer at 260 nm. Supernatants recovered from blank nanoparticles (without siRNA) were used as a blank. siRNA encapsulation efficiency was the percentage of entrapped or adsorbed siRNA to the total amount of siRNA added. siRNA present in supernatant was also measured by acid orcinol method (Jagani et al., 2011; Katas and Alpar, 2006).

2.6. Assay for serum stability

siRNA loaded chitosan coated PLGA nanoparticles were incubated at 37 °C with equal volume of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% final concentration of fetal bovine serum (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 −20 °C until gel electrophoresis was performed. To detach siRNA from the nanoparticles, phenol/