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DEVELOPMENT AND CHARACTERIZATION OF DOXORUBICIN AND siRNA ENCAPSULATED CHITOSAN NANOPARTICLES

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ABSTRACT

Objective: Chitosan nanoparticles (ChNP's) have been widely studied for drug and gene delivery. In this study, we prepared ChNP's for co-delivery of doxorubicin (DOX) and siRNA for cancer treatment.

Methods: The ionic gelation method was used to develop ChNP's. The positively charged DOX and negatively charged siRNA encapsulated into ChNP's. The particle size and zeta potential of the developed ChNP's were studied by particle size analyzer and morphology was examined by TEM. Encapsulation of DOX in ChNP's was confirmed by FTIR spectroscopy. The encapsulation efficiency and *in vitro* release of DOX were studied by UV-Vis spectrophotometry. The siRNA loading into ChNP's was confirmed by gel retardation assay.

Results: The developed ChNP's showed particle size ranged from 127±6.5 to 215±8.5 nm with zeta potential ranged from 16.5±0.3 to 25.8±0.3. Transmission Electron Micrograph showed DOX and siRNA encapsulated ChNP's are polydisperse and spherical in nature. FTIR study confirmed the binding of DOX with ChNP's with absorption peaks at 1016 cm^{-1}, 1316 cm^{-1}, 1412 cm^{-1}, 1645 cm^{-1} and 3370 cm^{-1}. The TPP:Ch ratio 0.1:0.5 showed the highest encapsulation efficiency 69±3.24%, with initial burst release and then sustained or slow release of DOX. Agarose gel retardation study confirmed the encapsulation of siRNA in ChNP's by retarded migration of siRNA-ChNP's in comparison with naked siRNA.

Conclusion: The developed ChNP's successfully encapsulated the DOX and siRNA and showed the sustain release of DOX. In conclusion, our study shown that ChNP's is having a potential of co-loading of DOX-siRNA as an efficient drug delivery system for the treatment of various cancers such as colorectal cancer, breast cancer etc.

Keywords: Multidrug Resistance (MDR), Chitosan, Doxorubicin, siRNA, Co-Delivery

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INTRODUCTION

Now a days, cancer is one of the main causes of death worldwide. Conventional therapies such as radiotherapy, surgery, chemotherapy and a combination of them are used to treat cancer [1]. Chemotherapy is one of the most commonly used treatment in which chemotherapeutics are used for treatment of cancer. Some most common chemotherapeutics such as 5-FU, paclitaxel, DOX are capable for suppression of tumor growth [2, 3].

DOX is an anthraquinone, hydrophilic anticancer drug that binds to topoisomerase, which induces apoptosis in different malignant cancer. However, problems related to acquired resistance and their acute cardiotoxicity, low penetration and limited distribution in solid tumor [4, 5]. Due to this side effects use of free DOX in the biomedical field is still limited. An alternative approach is to reduce side effects encapsulate DOX within a positively charged nanocarrier. Nanocarrier based drug delivery systems offer several advantages, including the greater surface area of nanoparticles, due to reduced size, sustained drug release rate, improvement in biodistribution, and reduction in side effects [6, 7]. Recently, nanocarriers are used for drug delivery are polymeric nanoparticles, micelles, dendrimers etc. Polymeric nanoparticles are most studied carriers in drug delivery, which exhibits pH-responsive, drug retaining ability during circulation and releasing it at the tumor site. A major problem with chemotherapeutic drugs is the gradually acquired multidrug resistance (MDR) due to the overexpression of active drug efflux pumps, which reduces the therapeutic efficacy. Even the use of siRNAs that target particular genes has been shown to reduce resistance problems. However, delivering siRNA to tumor cells is a major challenge because serum nucleases degrade siRNA and are immediately cleared by the reticuloendothelial system (RES) [8, 9]. The Discovery of RNA interference (RNAi)-based gene silencing has drawn much attention to cancer therapy due to its unique properties to silence a broad range of genetic targets. RNA interference (RNAi) is a cellular mechanism for gene suppression induced by siRNA in which 21-23 base pairs sequence of siRNA complementary to its target mRNA, which acts as a post-transcriptional regulator [10]. For clinical success, there is a major challenge associated with siRNA delivery at target site and cellular uptake. Some physiological and biological barriers are preventing their delivery at target site. But still, most practical applications need a carrier to transport the siRNA via systemic application to the target site [11-13].

Co-delivery of siRNA and encapsulated chemotherapeutic drugs is a promising approach to improve therapeutic efficacy. Various studies have shown that nanocarrier-based co-delivery of drugs with siRNA against the MDR genes suppressed P-glycoprotein (P-gp) expression which increases the retention of encapsulated drugs [8, 14].

Ch is a naturally occurring linear cationic polymer prepared through N-deacetylation of chitin isolated from seaweeds. Recently, it has increasing attention in pharmaceutical and biomedical applications because of its biocompatibility, biodegradability, non-toxicity, cationic properties and bioadhesive characteristics. Thus, ChNP's are of great significance as drug delivery systems for cancer therapy [5, 6]. The ChNP's can be formulated through various techniques, such as coacervation, co-precipitation, solvent evaporation, ionic gelation, and microemulsion. In ionic gelation method, several cross-linking agents such as sodium tripolyphosphate and glutaraldehyde are used, which provide an efficient network to entrap the drug molecules into the nanoparticles [7, 15]. Here, we developed DOX and siRNA encapsulated ChNP's in order to create an efficient drug delivery system. Physicochemical properties of the DOX and siRNA encapsulated ChNP's were studied, such as particle size, surface charge, percentage encapsulation efficiency and *in vitro*-drug release, gel retardation assay.

MATERIALS AND METHODS

Materials

Low mol. wt. Ch (Mol. Wt. 50,000–190, 000 Da, Deacetylation Degree 75-85%), sodium tripolyphosphate (TPP) was purchased from

Sigma-Aldrich (Mumbai, India), Acetic acid, Tween 80, 1 M NaOH, HCl, Agarose gel electrophoresis kit was purchased from Himedia Laboratories Pvt. Ltd, Mumbai, India. DOX Hydrochloride was received as a gift sample from Sun Pharma Advance Research co Ltd, India. The siRNA was purchased from Santacruze Biotech, India. All other chemicals used were of analytical grade. Double distilled water (DDW) and RNase free water used throughout the study wherever needed.

Preparation of ChNP's

ChNP's were prepared via ionic gelation method established by Calvo *et al.* [16] Ch solutions (0.3%, 0.4%, 0.5%, 0.6% w/v, pH-5.5) were prepared by dissolving Ch in 1% v/v glacial acetic acid. TPP solution (0.1% w/v) was prepared by dissolving TPP in deionized water. ChNP's were prepared by adding TPP solution dropwise into Ch solutions at room temperature with constant magnetic stirring for 1hr. Centrifugation was performed at 20,000 rpm at 10 °C for 30 min to collect nanoparticles. The supernatant was discarded and pellets of nanoparticles re-suspended in filtered deionized water.

Preparation of DOX encapsulated ChNP's

After optimization of Ch and TPP ratio 0.5**:**0.1 were selected for DOX encapsulation. To prepare DOX encapsulated ChNP's, DOX solution (Ch**:**DOX-10**:**1) were added into aqueous solution of TPP. Prepared TPP-DOX solution dropwise added into Ch solution under constant magnetic stirring for 1hr. at R. T.

Preparation of DOX and siRNA encapsulated ChNP's

For preparation of DOX and siRNA encapsulated ChNP's, DOX (Ch**:** DOX–10**:**1) and 5µl of siRNAs (20 µg/µl) was added to 1.5 ml aqueous solution of TPP (0.1% w/v), then this solution was dropwise added into Ch (0.5%, pH–5.5) solution under magnetic stirring at R. T. Then particles were incubated at room temperature for another 30 min before further analysis. All solutions were made in RNase-free distilled water and autoclaved before use. To prevent photodegradation of DOX, glassware's was used either amber color or covered with aluminum foil.

Characterization

Physicochemical characterization

Nanoparticles diameter, polydispersity index and zeta potential were determined by Malvern Zetasizer Ver. 7.12 (Malvern Instruments Ltd, UK). The aqueous dispersions of the nanoparticles were measured at 25 °C with a scattering angle of 90 °.

Morphology of nanoparticles

The ChNP's morphology was examined by transmission electron microscopy at 200 kv **(**JEOL Model JEM-2100).

Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared (FTIR) spectra were analyzed using an Alpha Bruker, Germany in the range 4000-400 cm^{-1}. The DOX, ChNP's, DOX-ChNP's samples were mixed with KBr and form a thin pellet to investigate the functional group.

Encapsulation efficiency of DOX

The encapsulation efficiency of DOX into ChNP's was measured using a UV-vis spectrophotometer at 480 nm. The free DOX in supernatant recovered from centrifugation (20,000 at 10 °C for 30 min) were quantified by measuring its absorbance at 480 nm wavelength with a dual beam Agilent Technologies Cary 60 UV-Vis spectrophotometer.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{C (sample)} - \text{C (supernatant)}}{\text{C (sample)}} \times 100$$

Where, C (sample) is the concentration of DOX added and C (supernatant) is the concentration of DOX in the supernatant (6).

***In vitro* release study of DOX**

4 ml of DOX loaded ChNP's were dispersed in a freshly prepared phosphate-buffered saline (PBS; pH = 7.4) as a release medium in a dialysis membrane sac (molecular weight cut-off 12 kDa). The ChNP's containing sac dialysis in 50 ml PBS (pH-7.4) as released media. The beaker was placed in a shaking incubator at 37°C under mild agitation. For each sample, 5 ml of the release medium was withdrawn at prearranged time intervals and replaced with the same amount of medium every time. The amount of released DOX in the medium was assessed at absorbance (λ = 480 nm) by UV-Vis spectrophotometry. The medium which was collected from plain NPs (without DOX) were used as the negative control. The percentage of cumulative DOX release was calculated according to the standard curve [10].

Confirmation of siRNA encapsulation in ChNP's by Agarose gel retardation assay

The binding of siRNA into ChNP's was confirmed by agarose gel retardation assay. Naked siRNA and prepared ChNP's, ChNP's-siRNA samples were taken for loading. Before loading into the agarose gel, all samples were incubated and shaken gently at 37 °C for 30 min; after that adding 5 µl of loading buffer into all samples. The samples were electrophoresed in 1% (w/v) agarose gel containing ethidium bromide (2 mg/ml) at a constant voltage of 120 V for 20 min. The results was observed under the UV transilluminator [17].

RESULTS AND DISCUSSION

ChNP's were prepared according to the ionic gelation method using negatively charged polyanion crosslinker TPP, which binds reversibly to positively charged Ch through electrostatic interaction at room temperature. This is an organic solvent-free method with a better ability to encapsulating hydrophilic drug DOX and siRNA [5, 15].

Physicochemical characterization

The DOX and siRNA encapsulated ChNP's were optimized on the basis of size distribution, surface charge, polydispersity index and encapsulation efficiency (table 1). The results revealed that Ch:TPP ratio 0.1:0.5 was found optimum on the basis of maximum percentage DOX encapsulation efficiency (69±3.24 %) with optimum size and distribution (192±8.1 and 0.62±0.05)**.**

Morphology of nanoparticles

The morphology of ChNP's was confirmed by TEM in which ChNP's were seen to be spherical with solid dense structure. The nanoparticles were appeared to be considerably smaller than the average particle size observed with the particle size analyzer.

Table 1: Optimization of DOX and siRNA encapsulated ChNP's

TPP: Ch (ratio)	Particle size (nm)	Zeta potential (mV)	PDI	Encapsulation efficiency
0.1:0.3	127±6.5	16.5±0.3	0.86±0.03	39±2.14 %
0.1:0.4	158±6.7	22.4±0.5	0.63±0.05	52±2.84 %
0.1:0.5	**192±8.1**	**24.6±0.3**	**0.62±0.05**	**69±3.24 %**
0.1:0.6	215±8.5	25.8±0.3	0.96±0.05	65±2.78 %

Values represent mean±SD (n = 3)

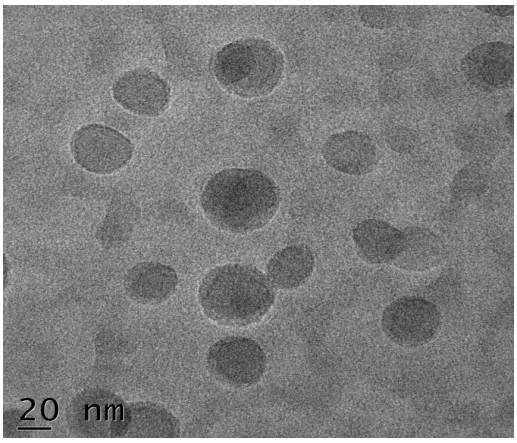

Fig. 1: Transmission electron microscopy of DOX and siRNA encapsulated ChNP's

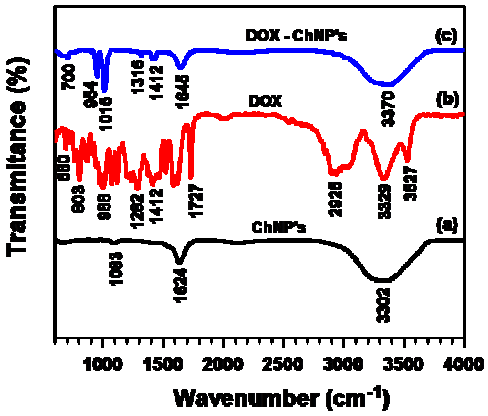

Fig. 2: FTIR spectra of a) ChNP's b) DOX c) DOX-ChNP's

FTIR analysis

FTIR spectroscopy is an important tool used to determine the functional groups in a molecule. In fig. 2 shown FTIR spectra of ChNP's, DOX, ChNP's-DOX. In ChNP's wider peak was observed at 3302 cm^{-1} due to the development of hydrogen bonding and shoulder peak at 1624 cm^{-1}. In DOX-loaded ChNP's the absorption bands appeared at 1016 cm^{-1} and 1645 cm^{-1} are related to the DOX [18].

Encapsulation efficiency and *in vitro* drug release

The DOX (DOX:Ch–1:10) encapsulated in all formulations of TPP: Ch. Encapsulation efficiencies shown in table 1. TPP:Ch (0.1:0.5) showed higher encapsulation efficiency (69±3.24 %). *In vitro* release of DOX were performed in phosphate buffer saline at pH–7.4 physiological pH of body fluids. The average cumulative release of DOX from nanoparticles at the prefixed time interval is shown in fig. 2. The release profile was biphasic. It was apparent that *in vitro* DOX release showed initial burst release and slower release of DOX in the second phase. After, 48 h it was shown 86.6±4.78 % of DOX released from ChNP's.

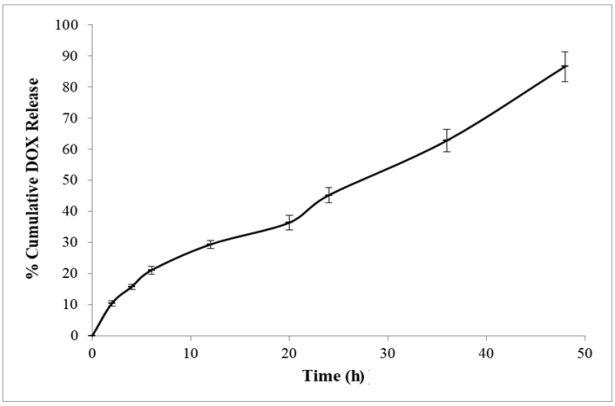

Fig. 3: *In vitro* release profile of DOX

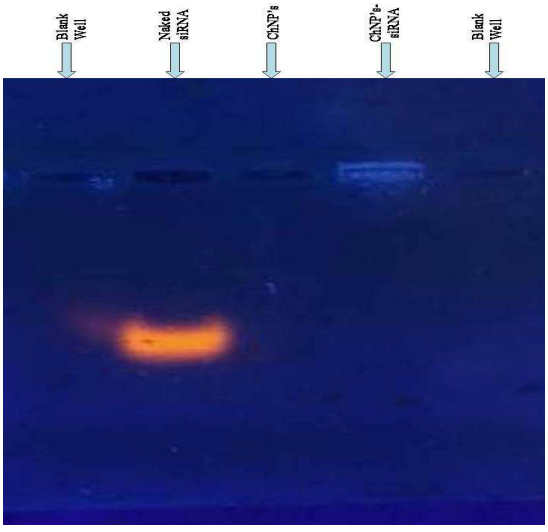

Fig. 4: Agarose gel retardation assay

Agarose gel retardation assay

The agarose gel retardation assay was used to confirm the loading of siRNA into ChNP's. The Naked siRNA and prepared formulations such as ChNP's and siRNA-ChNP's were loaded on agarose gel as shown in fig. 4. The results showed that naked siRNA moved with their electrophoretic mobility and shown as dark band on agarose gel. The ChNP's did not showed any migration and fluorescence. Whereas, siRNA-ChNP's showed retarded movement. The results clearly revealed that the siRNA was successfully loaded in ChNP's.

CONCLUSION

The ChNP's, DOX-ChNP's, DOX-siRNA-ChNP's were successfully prepared by ionic gelation method. The TPP**:**Ch ratio **1:5** was found optimum for ChNP's formulation on the basis of optimum particle size and highest encapsulation efficiency (69±3.24 %). *In vitro* release of DOX were showed initial burst release and prolong sustained-release i.e. 86.6±4.78 % at the end of 48 h. The loading of siRNA in ChNP's was confirmed by agarose gel electrophoresis retardation assay. The developed DOX-siRNA-ChNP's having wide scope for further research *in vitro* and *in vivo* to treat various types of cancer such as colorectal cancer, breast cancer and other solid tumors.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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