IN VITRO ANTI-NEOPLASTIC POTENTIAL OF CHITOSAN NANOPARTICLES AGAINST CERVICAL CANCER

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Abstract: The efficacy of conventional chemotherapy remains limited owing to its inability to reach the specific site coupled with numerous side effects. We report positively charged chitosan nanoparticles (CHNP) prepared by ionic gelation method and cross-linked with sodium tripolyphosphate (sTPP) in the size range of ~115.4 nm with a PDI of 0.365. A high surface charge of +19.5±1.0 mV stabilized them against aggregation over a wide range of temperature and pH. FTIR of chitosan polymer indicated the characteristic peak of C-N (1030-1067 cm⁻¹) but in chitosan nanoparticles (CHNP) the peak shifted to 1417.59-1551.64 cm⁻¹ due to the wagging of (-NH₂) bond. The ionic interaction with the phosphate group of TPP indicated the conversion of chitosan polymer in the nano form, that forms a cross link with TPP. The strong and sharp peak of phosphate at 1030 cm⁻¹ in CHNP confirmed the involvement of TPP while making the nanoparticles. Doxorubicin (DOX), a broad spectrum anti-neoplastic drug, was entrapped at an encapsulation efficiency (E.E) of ~48%. A biphasic release pattern was observed with ~76% of the drug in pH 5.8 as against 80% in pH 7.4 within 24 h. Dose and time dependent in vitro cytotoxicity of void CHNP, DOX per se, and doxorubicin loaded chitosan nanoparticles (DLCHNP) after 24h, 48h and 72h was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on SiHa (cervical cancer) and HEK (Human Embryonic Kidney) cell lines. DLCHNP showed an increased cytotoxicity in SiHa cells as compared to DOX per se in 72h but in HEK, negligible cytotoxicity was observed. Cellular uptake of DLCHNP was enhanced in SiHa as compared to HEK cells.

Keywords: Anti-neoplastic, Chitosan nanoparticles, Doxorubicin, Release kinetics, Biopolymers, Cervical cancer.
INTRODUCTION

Developing a drug delivery system (DDS) that optimizes the pharmaceutical action of a drug with decreased toxicity in vivo is a challenging task\textsuperscript{[1]}. In the current scenario, controlled drug delivery formulations using nanoparticles have become more sophisticated with the ability to increase the drug delivery at the therapeutic site along with decreased toxicity of the drug. Biopolymeric nanoparticles have emerged as a DDS owing to their biodegradability, biocompatibility, and several added advantages such as enhanced circulation and controlled release \textsuperscript{[2]}. The DDS using nanoparticles are being currently used to alter the pharmacokinetic and biodistribution of anti-cancer drugs\textsuperscript{[3,4]}. The reported DDS exhibited enhanced encapsulation and release the drugs based on pH variation without compromising the efficacy of the drugs\textsuperscript{[5]}.

Chitosan is a multifunctional biopolymer with many interesting applications. Chitosan with its versatile characteristics like adhesiveness, non-toxicity and anti-tumour activity acts as a paramount carrier for cancer therapy. It has emerged as a promising nanocarrier for drug delivery as it can be manipulated easily into various forms, is biocompatible, and biodegradable\textsuperscript{[6,7,8]}.

DOX is an antineoplastic drug which is often used in multidrug chemotherapy regimens to treat various solid tumors, leukemias, various types of carcinoma and soft tissue sarcomas\textsuperscript{[9]}. Cardiomyopathy and myelosuppression\textsuperscript{[10]} are known side effects of DOX. The development of effective approaches to limit cardiac toxicity while maintaining the anti-cancer efficacy of DOX has become a focus in recent years. To minimize the serious side effects of DOX\textsuperscript{[11]}, continuous intravenous infusion or within liposomal based nanocarrier as a protective measure have been reported\textsuperscript{[12]}.

The drug release profiles of nanoparticles determine the biological behaviour of drugs. Release profiles of the drugs are attributed to the considerations of both the properties of carrier and the drug itself\textsuperscript{[13,14]}. The rationale of our study was to encapsulate DOX in chitosan nanoparticles and to evaluate the release profile at different pH. In vitro cytotoxicity and cellular uptake of these DLCHNP was also assessed in cervical cancer cells SiHa and non-cancerous Human Embryonic kidney cells- HEK.

MATERIALS AND METHODS

All the chemicals purchased were of analytical grade and used without further purification. Chitosan (85% deacetylated, MW~50 kDa), Doxorubicin hydrochloride (99.0%), tripolyphosphate (sTPP), (3-(4,5-dimethyathiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl sulphoxide (DMSO), 1-Chloro,2,4-Dinitrobenzene (CDNB), Sodium bicarbonate, HEPES
(Hydroxy ethyl piperazine ethane sulphonic acid), Dulbecco’s modified Eagle’s medium, Penicillin and Streptomycin purchased from Sigma-Aldrich (USA). Foetal Calf Serum was procured from Hi Media. The dialysis membrane, Spectra/Por-7; MWCO, (10 kDa) was purchased from Spectrum Laboratories Inc (USA). Human Embryonic Kidney (HEK) and Cervical cancer cells (SiHa) were procured from NCCS, Pune. All experiments were done using double-distilled water.

**Preparation of Chitosan Nanoparticles**

The chitosan nanoparticles were prepared by conventional ionic gelation method as per our previously described protocol[7]. In brief, 0.5% chitosan (minimum 85% deacetylated) was dissolved in double distilled water containing 0.1% acetic acid. 1% aqueous solution of sodium salt of tripolyphosphate (sTPP) was also prepared in double distilled water. The desired ratio of Chitosan:sTPP was 1:2 at a pH of 3.23 was maintained. The TPP titrated chitosan solution was further stirred for 24 h at room temperature. It was then centrifuged at 12,000 rpm for 20 min. The resulting pellet was collected and resuspended in 0.5 % acetic acid at a final pH of 6.5. The nanoparticles from 4-5 batches of 50 ml each were pooled, lyophilized and stored for all further experiments.

**Physico-chemical Characterization**

**Dynamic Laser Light Scattering (DLS)**

The size of nanoparticles were measured by Zetasizer Nano ZS (Malvern, UK) based on DLS. Briefly, nanoparticles were suspended in distilled water at a concentration of 0.05% in 1% acetic acid and vortexed for few minutes. Size measurements were performed at 25 °C with 173° scattering angle and the mean hydrodynamic diameters were determined by cumulative analysis[15].

**Zeta Potential**

Surface charge and electrophoretic mobility of nanoparticles was measured by the frequency shift of the scattered light at a 12° scattering angle using Zeta Nano-ZS (Malvern Instruments, USA).

**Transmission Electron Microscopy (TEM)**

The morphological characteristics of the nanoparticles were examined using high resolution TEM in a Philips EM300 instrument, at an accelerating voltage of 80kV using different magnification. A drop of the sample was mounted on a carbon-coated copper grid (mesh size 300). The grid was air dried, kept in a desiccator at room temperature before loading on the
microscope. Image contrast was provided by exposing the sample to 2% uranyl acetate or 2% phosphotungstic acid solution. Heavy metals are commonly used for negative staining as they produce high electron density which gives good image contrast[16].

Fourier Transformed Infra Red Spectroscopy (FTIR)

The transmission FTIR spectrum of the lyophilized powder of polymer and void CHNP were determined using Cary 630 diamond FTIR, Agilent Technologies.

Drug Loading & Entrapment Efficiency (EE %)

Entrapment efficiency was calculated as per previously published protocol[2]. Briefly, DOX was dissolved in water and loaded to a known amount of nanoparticles solution with vigorous stirring and bath sonication. Free drug was physically entrapped in nanoparticles. The DLCHNP were separated from un-entrapped DOX after passing the solution through a Millipore filter UFP2THK24 (100KDa cut off) and absorbance of free DOX was noted using UV visible spectrophotometer (BioTek Synergy HT, USA) at 480 nm. The EE% was calculated as:

\[
\text{EE} \, (\%) = \frac{[\text{Drug}]_{\text{total}} - [\text{Drug}]_{\text{free}}}{[\text{Drug}]_{\text{total}}} \times 100 \tag{i}
\]

Where, ([Drug] total and [Drug] free) are the amount of total drug added and free drug respectively.

Release Profile

The lyophilized CHNP encapsulating DOX were re-dispersed in PBS and real time release kinetics was observed at physiological pH 7.4 and acidic pH 5.8 using dynamic dialysis bag method (Spectra/Por-7; Mw: 10kDa)[17]. Briefly, a known amount of DLCHNP was put in the dialysis bag which was then placed in 500ml of PBS under constant magnetic stirring at a rotation speed of 500rpm using shaker. Definite aliquots of the dissolution medium were taken at specific time intervals and the same volume of fresh dissolution medium was added to the flask to maintain a sink condition. Known amount of samples were withdrawn and analyzed for the concentration of DOX using UV visible spectrophotometer (BioTek Synergy HT, USA) at 480 nm.

Cell culture

Human cervical cancer (SiHa) and non-cancerous Human Embryonic Kidney (HEK) cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum. All the cells were grown at 37 °C in a humidified atmosphere of 5% CO₂ in CO₂ incubator (Thermo Scientific, USA).
Cytotoxicity Evaluation

Cytotoxicity of DOX per se, void CHNP and DLCHNP was measured by the tetrazolium (MTT) method. Briefly, 5 x 10^3 cells/well were plated in 96 well microplate, supplemented with 10% FCS media. The cells were exposed to various concentrations of DOX per se, DLCHNP and void nanoparticles, and incubated for 24 h, 48 h and 72 h respectively, 20 µl MTT (5 mg/ml) was added, and the plates were incubated at 37 °C for 4 h. The formazan crystals formed by the cellular reduction of MTT were dissolved in 100µl of DMSO. After mixing with a mechanical plate mixer, the optical density was read at 540nm wavelength on an ELISA-reader (Synergy HT, Biotek, USA). All measurements were done in triplicates. The percent cytotoxicity values were determined by

\[
\% \text{ Cytotoxicity} = \frac{([A]_{\text{control}}-[A]_{\text{test}}) \times 100}{[A]_{\text{control}}} \tag{ii}
\]

Where, \([A]_{\text{test}}\) is absorbance of the test sample and \([A]_{\text{control}}\) is the absorbance of the control sample.

Cellular Uptake

Exponentially growing cells at a concentration of 5 x 10^4 cells/well were plated on 12mm round glass cover slip in a 24 well microplate supplemented with 500µl complete media. The cells were incubated with DOX per se, void CHNP and DLCHNP for 4 h and washed with PBS (pH 7.4) twice to eliminate un-internalized nanoparticles. The cells were fixed by 4% paraformaldehyde and mounted in DPX. Images were taken after visualization by fluorescent upright microscope (Nikon Eclipse 90i).

Statistical Analysis:

The results were expressed as mean ±SD. Comparison among groups were analyzed by Two-way ANOVA using Prism (5.0) software (Graphpad software Inc.CA). Levels of significance were accepted at ≤0.05 level.

RESULT AND DISCUSSION

Chitosan has been used in various pharmaceutical and biomedical applications as it is easily modified, biocompatible and biodegradable. sTPP is an extensively used, non-toxic, anionic, and multivalent cross-linking agent with five bonding sites on the molecules and has the ability to gel rapidly. This property of sTPP has been exploited to electrostatically interact with cationic chitosan by ionic gelation method. This simple and mild method can be carried out without using any toxic agents that cause undesirable side effects. In the present study, attempts
have been made to prepare nanoparticles using chitosan as a polymer and sTPP as a cross-linker by ionotropic gelation method[7] to achieve high drug entrapment efficiency.

The size of nanoparticles (diameter) measured by DLS was ~115.4 nm with a poly-dispersity index (PDI) 0.365. The surface charge of nanoparticles was ~ 19.5 mV ± 1.0. The transmission electron micrograph (TEM) of the nanoparticles has confirmed the nano-size of the particles (Fig. 1).

The FTIR of chitosan polymer and nanoparticles are presented in Fig. 2. The characteristic peak of C-N (1030-1067 cm\(^{-1}\)) was observed in FTIR of chitosan polymer, along with other peaks. But in CHNP the peak was shifted to 1417.59-1551.64 cm\(^{-1}\) due to the wagging of (-NH\(_2\)) bond. The ionic interaction with the phosphate group of sTPP indicated the conversion of chitosan polymer in the nano form, that formed a cross link with sTPP. The strong and sharp peak of phosphate at 1030cm\(^{-1}\) in CHNP confirmed the involvement of sTPP while making the nanoparticles. The entrapment efficiency of DOX in chitosan nanoparticles was ~ 48% as calculated by UV-Vis spectrophotometer (Fig. 3).
Altering pharmacokinetics and pharmacodynamics of drugs is the basis for effective drug delivery. Furthermore, the rationale of DDS is to release the drug at the therapeutic site maintain sustained levels of the drug concentration in the blood to enhance the efficacy of the treatment. The release kinetics of DOX from chitosan nanoparticles at various pH was studied to ascertain its release mechanism for correlating its behaviour in biological systems. At the physiological pH (pH 7.4) the release pattern was biphasic with ~20% release in the first hour, and reaching ~30% in the next four hours. In the second phase, a controlled release ~80% up to 24 h was observed (Fig. 4). At the acidic pH (pH 5.8) the release pattern was again biphasic with
~19% release in the first hour with not much increase (~23%) in the next four hours. In the second phase, a controlled release ~76% up to 24 h was observed (Fig. 4).

![Fig 4: Release of DOX from DLCHNP at pH 7.4 & pH 5.8](image_url)

During the first phase, an initial burst release of drug was observed that can be attributed to leaching and enhanced dissolution of the drug followed by its subsequent diffusion\(^{[20]}\). According to Peppas\(^{[21]}\), there are three primary mechanisms by which the release of active agents can be controlled i.e., erosion, diffusion and swelling followed by diffusion. The observed biphasic pattern (burst release and slow sustained release) are indicative of combined effect of diffusion and erosion mechanism for controlled drug release where the first burst release represent the adsorbed drug. Slow and sustained release may be attributed to the slow degradation/erosion of the nanoparticles via hydration by hydrolysis\(^{[22]}\).

The cytocompatibility of void CHNP and the anticancer activity of DLCHNP was assessed by MTT assay (Fig. 5). The IC\(_{50}\) was also calculated and the values are depicted in Table A. In DOX per se treated cells (0.1µg/ml), the IC\(_{50}\) was 1.62±0.02 and 27.72±0.06 at 72 h for HEK and SiHa cells respectively. For DLCHNP, there was no IC\(_{50}\) achieved at this dose in HEK cells but higher IC\(_{50}\) (25.04±0.03) in SiHa cells at 72 h was reported. These results indicated preferential killing of cancer cells as compared to normal cells by the DLCHNP.
Table A: IC\textsubscript{50} of cell growth in different cell lines

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<tr>
<th>DOX (0.1µg/ml)</th>
<th>HEK</th>
<th>SiHa</th>
<th>DLCHNP(0.1µg/ml)</th>
<th>HEK</th>
<th>SiHa</th>
<th>DOX (0.05 µg/ml)</th>
<th>HEK</th>
<th>SiHa</th>
<th>DLCHNP(0.05µg/ml)</th>
<th>HEK</th>
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<td>__</td>
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<td>1.62±0.02</td>
<td>1.62±0.02</td>
<td>9.34±0.08</td>
<td>9.34±0.08</td>
<td>2.68±0.05</td>
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<tr>
<td>2.68±0.05</td>
<td>9.08±0.02</td>
<td>5.11±0.14</td>
<td>3.81±0.09</td>
<td>29.46±0.07</td>
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<td>9.34±0.08</td>
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<td>25.04±0.03</td>
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*: no IC\textsubscript{50} was achieved at this dose of drug.
Cellular internalisation of DOX *per se* and DLCHNP was confirmed by fluorescent imaging indicating enhanced uptake of DLCHNP by MCF-7 cells as compared to HEK (Fig. 6a & b).

**CONCLUSION**

Chitosan nanoparticles have been successfully prepared by ionic gelation in the size range ~115.4 nm with PDI 0.365. The nanoparticles formed were positively charged (~19.5±1 mV).
Biopolymers are extensively used for controlled/sustained delivery of drugs from formulations. The rate of release was higher initially at the physiological pH (7.4) indicating a fast elimination where as in the second phase, slow and sustained release was observed. Internalization in SiHa cells was enhanced when compared to non-cancerous HEK. DLCHNP indicated enhanced anti-neoplastic activity in both HEK cells and SiHa cells respectively, making them a promising candidate in cancer therapy. Encapsulating DOX in nanoparticles obviates the disadvantages of conventional drug therapies by circumventing the side effects related to dosing and toxicity. However, further optimization studies including stabilization and targeting of the nanoparticles are warranted both in vitro and in vivo to understand their mechanism of action to evolve as a therapeutic DDS.

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REFERENCES


