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Biodistribution and *In-vivo* Efficacy of Doxorubicin Loaded Chitosan Nanoparticles in Ehrlich Ascites Carcinoma (EAC) Bearing Balb/c Mice

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Abstract

Introduction: We report Doxorubicin loaded chitosan nanoparticles (DLCHNPs) prepared by ionic gelation of chitosan with tripolyphosphate (TPP). The polyelectrolytic behaviour of chitosan facilitates interaction with negatively charged cell surface and Doxorubicin(DOX) having both amphiphillic and amphoteric activity further facilitates binding to cell membranes, proteins for internalization leading to intercalation the DNA.

Methods and key findings: The size distribution assessed by nanoparticle tracking analysis was ~220 nm and PDI 0.365, having a zeta potential of +22.5 ± 1 mV, with an Entrapment Efficiency of 48%. 5% hemolysis was observed. Negligible difference in biodistribution was observed between free DOX and DLCHNPs in most of the organs, except heart where a significant decrease in the concentrations of DOX was observed. The distribution can be summarized as Spleen>Liver>Lung>Kidney>Heart.

Conclusion: The enhanced systemic circulation enables tumour accumulation and higher response. DOX *per se* reduced the tumour burden when compared to the controls, but significant tumour regression coupled with enhanced life span was observed with DLChNPs. Mice treated with *void* ChNPs showed 50% survival rate as compared to 100% survival rate when treated with DLChNPs.

Keywords: Chitosan nanoparticles; Doxorubicin; Biodistribution; Tumour regression

Introduction

Although numerous biomolecules appear therapeutically promising, the intrinsic properties that are responsive to changes in physicochemical and biopharmaceutical characteristics have not been satisfactorily exploited making their biomedical applications extremely challenging. The last few decades have seen an explosion of publications on the use of chitosan and its derivatives in the pharmaceutical industry. Chitosan is a linear polysaccharide that consists of ß-(1-4)-2-acetamido-d-glucose and ß-(1-4)-2-amino-d-glucose units derived from partial deacetylation of chitin [1,2]. The poor solubility of chitosan has been the major deterrent for its successful use in food and biomedical applications [3]. Biopolymeric nanoparticles have appeared as promising vehicles because of several attractive properties, such as an increased surface-to-volume ratio, which offers high potential for macromolecule association [4], drug interaction, the capacity to improve drug absorption and finally drug modification.

Chitosan exhibits various biological activities such as anti-bacterial [5,6], immuno-enhancing [7], antioxidant [8], matrix metalloproteinase (MMP) inhibition [9-11], anti-diabetic [12], anti-HIV [13], and anti-inflammatory activities [14]. It also has an antitumour role through improving the body's immune function. Maeda and Kimura [15] showed that low-molecular-weight chitosan and chito-oligosaccharide could inhibit tumour growth in S-180-bearing mice. Torzsas et al. [16] found that a diet containing chitosan could reduce the generation of precancerous lesions in colon cancer induced by azomethane compounds. Substantial efforts have been dedicated for the development and application of chitosan nanoparticles as vehicles for drug delivery [17-20]. Effect of chitosan nanoparticles have been assessed on tumour cells directly to interfere with cell metabolism, inhibit cell growth, or induce cell apoptosis [21]. The reactive hydroxyl and amine groups of chitosan facilitate the adherence to negatively charged surfaces, thereby enhancing cellular transport [19,20]. In this regard, therapeutic efficacy based on chitosan nanoparticle systems confers several advantages: i) nanospheres composed of highly concentrated chitosan molecules could serve as a "fusogen".

Doxorubicin (DOX) is composed of an aglycone moiety to which an amino sugar daunosamine is attached *via* a glycosidic bond. The aglycone part is a tetracyclic chromophore adiamycinone in which the B, C, and D rings form a planar anthraquinone system. These aromatic rings result in π-π stacking properties of DOX, which allows its intercalation into the DNA double helix (one of the mechanisms of action proposed for this anticancer drug). In addition, the amino sugar of DOX has a pKa value of 8.6, which confers alkaline properties onto this molecule and positive charge at neutral pH [22]. Because of the positive charge of DOX, DOX is expected to associate at a high level with polyanionic moieties.

Since, very few advanced drug delivery systems for doxorubicin have been approved by the FDA for the treatment of Kaposi's and sarcoma ovarian cancer [23]. PEGylation of liposomal formulation of doxorubicin (Doxil and Caelyx and a non-PEGylated formulation (Myocet) enhance the systemic circulation of encapsulated DOX thereby facilitating an increased tumour uptake providing a improved experimental response as compared to the unmodified DOX [24,25]. Our aim was to develop nanoparticles for delivery of DOX, and to explore the potential of nanoparticle tracking analysis (NTA) for

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the analysis of nanosized particles and to evaluate the haemolysis, biodistribution and tumour regression of the developed DLChNPs alongwith their *in-vivo* efficacy has been assessed.

Materials and Methods

Chitosan (~85% de-acetylated, Mw~50 kDa), tri-polyphosphate (TPP), 1-Chloro-2,4-Dinitrobenzene (CDNB), Doxorubicin hydrochloride (99.0%), Sodium bicarbonate, Sodium nitrite ($NaNO_2$) and HEPES (Hydroxy ethyl piperazine ethane sulphonic acid) were purchased from Sigma-Aldrich (USA). Triton X, Tri carboxylic Acid and Dichloromethane were purchased from M.P. Biotech, USA. Analytical grade chemicals were purchased and used without further purification.

Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared as per our previously described protocol [26]. In brief, 0.5% chitosan was dissolved in double distilled water (DDW) containing 0.1% acetic acid. 1% aqueous solution of sodium salt of tripolyphosphate (TPP) was also prepared in double distilled water. The desired ratio of Chitosan: TPP was 1:2 at a pH of 3 was maintained. The TPP titrated chitosan solution was further stirred for 24 h, centrifuged at 12,000 rpm for 20 min, and the resulting pellet was collected and resuspended in 0.5% acetic acid at a final pH of 6.5.

Nanoparticle tracking analysis (NTA)

NTA measurements were done using NanoSight NS 300 (NanoSight, Malvern, UK), equipped with a sample chamber with a 640-nm laser and a Viton fluoro-elastomer O-ring [27]. Using sterile syringes, requisite amount of samples were injected in the chamber. The data was captured using NTA 2.0 Build 127 and the samples were measured for 40s with manual shutter and gain adjustments. Three measurements of the same sample were performed and the error bars displayed on the NTA graphs were obtained by the standard deviation of the different measurements of each sample. The mean size and SD values obtained by the NTA software correspond to the arithmetic values calculated with the sizes of all the particles analyzed by the software.

Drug encapsulation and entrapment efficiency

Entrapment efficiency was calculated as per previously published protocol [28]. DOX was dissolved in water and loaded to a known amount of nanoparticles solution by 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 (100 KDa 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:

$$EE(\%) = \frac{[Drug]total - [Drug]free}{[Drug]total} \times 100 \quad \text{(i)}$$

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

Haemolysis

Haemolytic activity was done on whole blood and percent haemolysis calculated as *per* previously published protocol [29]. Briefly, the heparinized blood procured from normal human subjects were washed twice with phosphate buffered saline (PBS, pH 7.4) prior to the assay. The red blood cells (RBCs) were pelleted and centrifuged by centrifugation at 1200 rpm for 5 minutes. 100 µl of RBC were suspended in PBS at a 1:1 ratio, and were incubated with DOX, DLChNP and ChNP up to 4 hours at 37°C. The washing step was repeated to remove debris and serum protein. The absorbance of the lysed RBC was read in a UV visible spectrophotometer (Bio-Tek, USA) at 540 nm, 2 hours and 4 hours post-incubation. Deionised water was used as a positive control. The experiment was done in triplicates and percentage haemolysis was calculated using the following formula:

$$\text{Percentage haemolysis} = \frac{\text{Abs of positive control - Abs of sample}}{\text{Abs of positive control}} \times 100 \quad \text{(ii)}$$

Biodistribution

In bred, female 6-8 week old Balb/c (20-25 g) mice were obtained from Animal Holding Facility of Institution and studies were conducted as per previously standardized procedures [30]. All experiments were carried out in accordance with the Institutional Animal Ethical Committee under registration number 1666/PO/ac/12/CPCSEA. The animals were anesthetized with ether inhalation and samples were then administered *via* tail vein injection of DLChNP, DOX *per se* and ChNP. After the requisite time period, the mice were sacrificed by cervical dislocation and dissected to obtain various tissues (heart, lung, liver, spleen and kidney). For determining tissue distribution, whole tissue homogenate was prepared in PBS (pH 7.4). The drug was extracted with 1 ml dichloromethane (DCM) by vortexing for 3–4 min, and centrifuged at 6000 rpm for 4 min to collect bottom organic layer. 0.3 ml alkaline Borate buffer (pH 9.2) and 0.5 ml 5% TCA solution were added. The fluorescence of supernatant was measured at wavelengths of excitation at 480 nm and emission at 555 nm [31].

Tumour regression study

In bred, female 6 to 8 weeks old Balb/c (20 to 25 g) mice were used as per our previously published protocol [32]. The EAC cells were harvested by brief trypsinization and 2 $x10^6$ cells/100 µl were injected subcutaneously on the dorsal side of mouse in 100 µl DMEM. Measurable tumour appeared 7 days post inoculums (0.08 cc). Palpable tumours were measured in two perpendicular diameters using the vernier calipers and tumour volume was calculated by the formula.

$$V = \frac{LW^2}{2} \quad \text{(iii)}$$

where,

V= Tumour Volume, L= Tumour dimension at the longest point, W= Tumour dimension at the widest point.

Daily intravenous injection (post 7 days of inoculums was considered as day 1) of 1 mg/kg mice body weight of DOX *per se*, ChNP and DLChNP were given by tail vein injection up to day 21. The tumour volume was estimated on alternate days i.e. 7, 9, 11, 13, 15, 17, 19 and 21.

Statistical analysis

The results were expressed as Mean standard deviation. Comparison among groups were analysed by One-way ANOVA and means were separated by Tukey's test using Prism (5.0) software (Prism software Inc. CA). Levels of significance were accepted at ≤ 0.05 level.

Results

NTA enables sample visualization and provides approximate particle concentrations, which are very useful features (Figure 1a and 1b). Calculated entrapment of DOX in ChNP was 48%.

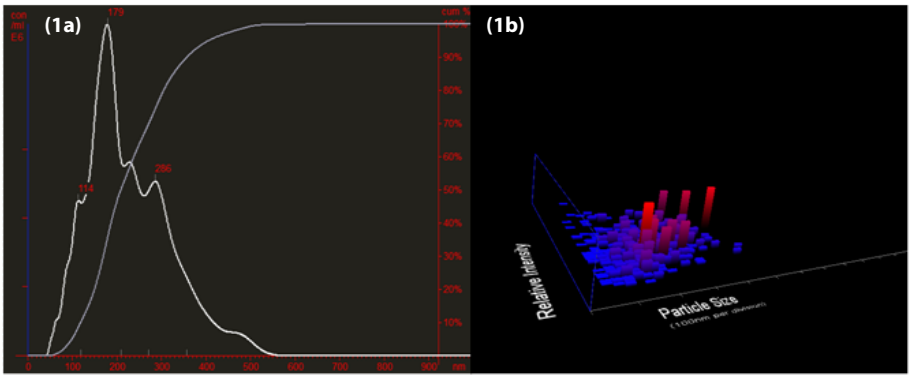

Figure 1: ChNPs measured with Nanosight Tracking Analysis (NTA). The size distribution (1a and 1b) 3D graph (size *vs.* intensity *vs.* concentration.

Biodistribution

Once the DLChNPs have been administered systemically in Balb/c mice, DOX gets distributed to all the organs. Biodistribution process involves the substance's absorption, distribution, metabolism and excretion. When the normal biodistribution pattern of a substance is known, any irregular pattern may suggest an abnormality or the presence of a disease [33]. The percent uptake of DOX *per se* and DLChNP was evaluated in the spleen, lungs, liver, kidneys, and heart. Biodistribution pattern was observed at an interval of 2 h, 4 h and 24 h respectively. The maximum injected dose was predominantly recovered from spleen and liver as shown in Figure 2. Negligible concentrations of DOX *per se* and DLChNP were found in kidneys, heart, but shows maximum accumulation in liver and spleen. The pattern of nanoparticle accumulation can be represented as follows: Spleen>Liver>Lung>Kidney>Heart. For DLChNP, the concentration of DOX in the liver was 75% post 1 hour that was reduced to 10% after 24 hours. As compared to DOX *per se*, DLChNP was metabolized to a significant extent, by the liver. Bio-distribution of DOX was highly altered when delivered *via* nanoparticles as there was minimal accumulation in heart tissue (Figure 2).

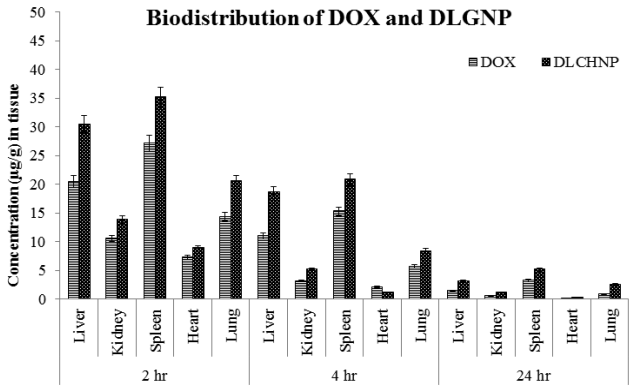

Figure 2: Graphs illustrates relationships between quantity of DOX (in milligrams) extracted from liver, kidney, spleen and heart. (Spleen> Liver> Lung> Kidney> Heart).

Haemolytic activity

For any therapeutic applications, it was imperative to assess the biocompatibility and toxicity of chitosan nanoparticles *in vivo* to prove their safety as a novel drug carrier to fulfil preclinical requirements. Haemolysis of DOX *per se*, DLChNP, ChNP were done on rat blood and 0.01% Triton X was used as a positive control to indicate 100% lysis. Less than 5% hemolysis was observed in all the groups which is regarded as nontoxic level (Figure 3).

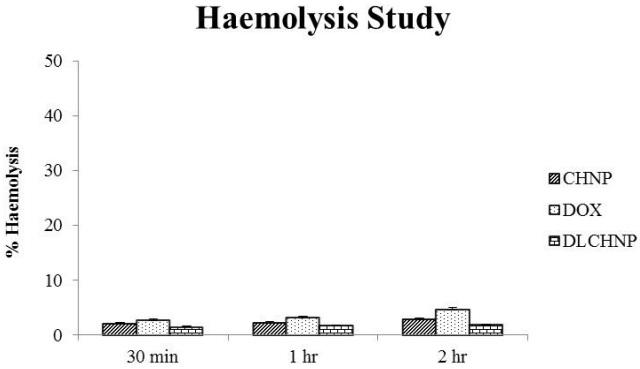

Figure 3: Effect of DOX *per se*, ChNP, and DLChNP on RBCs less than 5% was obseved The free DOX showed as high as 4.7% haemolysis whereas DLChNP gave as high as 1.95% and low 1.5% haemolysis at 2 hour and 30 min.

Tumour regression

To examine whether our *in vitro* observations could have therapeutic significance, animal studies were performed. The therapeutic potential of chitosan nanoparticles were assessed by tumour regression. 2 x 10^6 cells of Ehrlich ascites cells (EAC) were inoculated subcutaneously on the dorsal side of 20 g Balb/c mice and grown upto 10 mm^3 size (palpable tumour). The mice were divided into the following groups: ChNP, DLChNP, DOX *per se* and PBS. Intravenous injections were given once a day, regularly for 21 days. There was significant retardation of tumour in mice treated with DLChNP when compared to control i.e. PBS and ChNP (Figure 4).

Percentage survival

All mice in our experiments died with developing tumours between day 21 and day 30 post inoculation of tumour cells. However, no prolongation of the survival time was observed in either of the experimental groups. In the EAC solid tumour models, ChNPs potentiated the antitumour activity of DOX, resulting in retardation of tumour growth as compared with void ChNPs and DOX *per se*

suggesting augmentation of antitumour effectiveness in mice treated with nanoparticles encapsulating the drug. Percentage survival of mice was recorded over a period of 28 days. Mice treated with *void* ChNPs showed 50% survival rate as compared to 100% survival rate when treated with DLChNPs (Figure 5).

The treatment of the nanoparticles with doxorubicin dose of 12 mg/kg prolonged the life of the tumour-bearing mice from 10.3 to 14.8 days; the survivability rate is 43.0%, which is significantly higher than the treatment of 5 mg/kg dose of free doxorubicin. There were no behavioral changes or visible toxicity symptoms after continued intravenous injection for 21 days. None of the DLChNP-treated animals exhibited alopecia or weight loss, but the DOX treated animals had weight loss and alopecia throughout the duration of the experiment. This result proved that the nanoparticles are more effective than free doxorubicin to prolong the life of the tumour-bearing mice.

Discussion

Biomedical application of Chitosan has been investigated extensively so as to exploit its cationic functional amine group [34,35] to attach anionic molecules eg DNA, proteins. The complexes need to be delivered in biological systems hence their stability in physiological conditions must be evaluated [36]. Chitosan can be used to conjugate drugs and deliver at the solid tumour target site [37]. A successful nanodelivery system entails sustained and controlled release of the circulating drug in the blood. Earlier reports have indicated the dual role of DOX and camptothecin dendrimers that improved the biodistribution and antitumour efficacy compared with free drugs [38,39]. The EPR effect has been widely substantiated for an extensive array of drug delivery systems, such as liposomes, nanoparticles, micelles and even dendrimers [40].

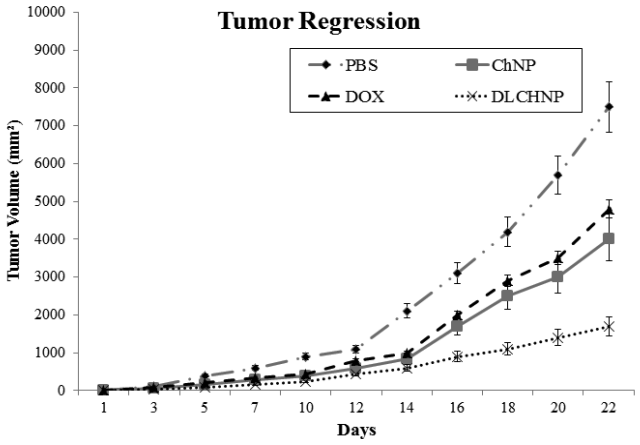

Figure 4: Tumour regression results showed that DLChNP reduces the tumour burden more rapidly in comparison to DOX *per se*, control (PBS) and ChNP.

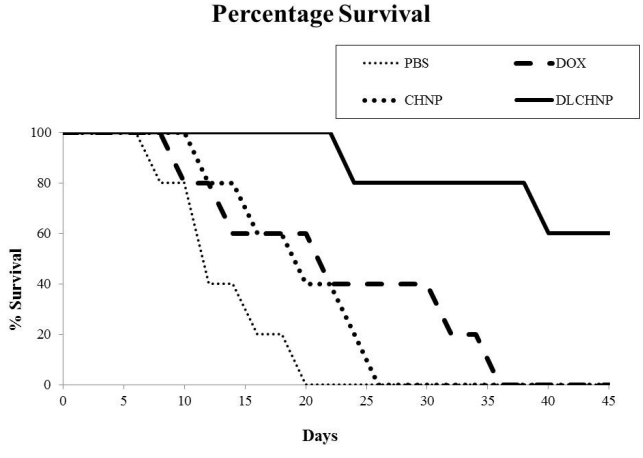

Figure 5: Percent survival study shows that in case of DLChNP and DOX, there was 100% survival, but in case of control survival rate decreases.

The numerous hydroxyl groups present in polysaccharides renders them as extremely interesting macromolecules for drug delivery as they have anchors for drug attachment and modification. The protonable groups in the DOX molecule may interact with the polysaccharide by the inter-molecular interactions, or by formation of weak hydrogen bond or by other weak forces as the van der Waals force or dipole–dipole interaction [34]. Since, chitosan is derived by the partial deacetylation of chitin, comprises of copolymers of glucosamine and N-acetyl-glucosamine in a linear chain, it is important to use a cross-linker to make particles by ionic gelation that results in nanoparticles [41,42]. Our recent work on chitosan/TPP nanoparticles has also established that the concentration of acetic acid used to dissolve chitosan and the temperature at which the crosslinking process occurs, strongly affect the polydispersity of the obtained nanoparticles [43]. Ionic cross-linking results in auto-aggregation between chitosan and its derivatives. The presence of a cross-linking agent eg sodium tripolyphosphate (TPP) facilitates the interaction between opposite charges of macromolecules to form stable nanopaticles. Nanoparticle formation is sensitive to temperature fluctuations as lower temperature enhances hydrogen bonds between the polar groups of chitosan and the surrounding water. This often leads to the formation of a hydration layer around the nanoparticles that may reduce the probability of nanoparticle collision.

Nanoparticle tracking analysis (NTA) is an innovative system for sizing particles from about 30 to 1,000 nm, with the lower detection limit being dependent on the refractive index of the nanoparticles. This technique combines laser light scattering microscopy with a charge-coupled device (CCD) camera, which enables the visualization and recording of nanoparticles in solution. The NTA software is then able to identify and track individual nanoparticles moving under Brownian motion and relates the movement to a particle size according to the following formula derived from the Stokes-Einstein Equation. One of the main constraints of DLS is the impact that few large particles, such as dust, may have on the average/mean size. The NTA technique is based on the tracking of single particles, whereas DLS measures a bulk of particles with a strong bias to the largest particles present in the sample. Therefore, the performance of NTA is expected to be less sensitive than DLS to the presence of minute amounts of large particles. The influence of large particles on NTA and DLS results have been summarized and reported earlier [27]. The high peak resolution and suitability for poly-dispersed samples make NTA a very useful and suitable technique.

A major limitation in chemotherapy is the infusion of excess amount of drug, indiscriminate killing of blood cells post systemic delivery, increase in the frequency of doses owing to the inability of the vehicle to deliver adequate doses of drugs to the affected areas in the body. Biopolymeric nanoparticles offer encouraging solutions by encapsulating drugs, thereby reducing toxicity and providing a protective barrier for the drug that limits its interaction with healthy cells [44-48]. However, studies have reported that chemical conjugation of doxorubicin to hydrophilic polymers have considerably improved the aforementioned attributes, which also improved drug loading [49,50].

Although, DOX is extensively and preferentially used for the treatment of breast cancer, cervical cancer, leukemias, Hodgkin's lymphoma, bladder, breast, stomach, lung, ovarian cancer. It has a five to ten minute half-life in the plasma [51]. Systemic toxicity of anti-cancer drugs limits their dose, while rapid clearance from circulation requires large doses to maintain optimal blood circulating levels in order to be effective. DOX contains acidic functions in the ring phenolic groups and a basic function in the sugar amino group which necessitates the use of nanoparticles for prolonged residence time of DOX.

The therapeutic potential includes reduced side effects, enhanced bioactivity, increased patient compliance due to decreased administration frequency, and the ability to co-deliver multiple drugs with synergistic effects to the target site [52,53]. The differences observed with the DLChNPs in blood clearance and biodistribution are mainly owing to the positively charged surface of chitosan, and partly due to the steric stabilization by the cross-linker.

Application of nanoparticles in cancer therapeutics is based on the premise that an optimal nanoparticulate delivery system would preferentially accumulate within the tumour *via* the EPR effect [50] and hence would result in an enhanced therapeutic efficacy, while considerably decreasing the toxicity in normal tissue [50]. Hence, formulation of a successful nanoparticulate drug delivery system necessitates a comprehensive and in-depth knowledge of its biodistribution, plasma clearance and tumour accumulation. DLChNPs effectively decreased the toxicity of doxorubicin and significantly increased the survivability of EAC tumour bearing mice.

The effects of doxorubicin nanoparticles on the treatment of murine ascites EAC were investigated, and DLChNP showed reduced tumour burden as compared to DOX *per se*. It may be possible, that the delayed release of the doxorubicin from the DLChNPs resulted in the decrease of the activity and toxicity. Further, increasing the dose of nanoparticles, equivalent to 12 mg/kg doxorubicin indicated enhanced toxicity, but the tumour inhibition rate does not improve. Kratz pointed that only the free drug could exert its pharmacological effect [53].

Conclusion

We evaluated NTA as a new method for analysing nanoparticles, as the sample scan was a useful feature of NTA, it should be used prudently to avoid false or biased results. We report CHNPs in the size range ~120 nm with PDI 0.365 prepared by mild procedures involving only hydrophilic environment specifically by ionic gelation. The zeta potential of the nanoparticles was ~+22.5 ± 1 mV. The Entrapment Efficiency of DOX was estimated ~48%. Less than 5% hemolysis was observed in all the groups which is regarded as nontoxic level.

Bio-distribution studies indicated reduced cardiotoxicity when administered in nanoparticles. It can be summarized as Spleen>Liver>Lung>Kidney>Heart. DLChNP reduces the tumour burden significantly when compared to DOX. It is effective in prolonging the life-span of mice, as compared to ChNP. Highest survival rate (%) was observed in DLChNP followed by DOX *per se*, ChNP and PBS control.

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Conflict of Interest

The authors have no conflict so far.

References

1. Je JY, Kim SK (2005) Water-soluble chitosan derivatives as a BACE1 inhibitor. Bioorg Med Chem13: 6551-6555.
2. Kim SK, Rajapakse N (2005) Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. Carbohydr Polym 62: 357-368.
3. Kumar MNVR (2000) A review of chitin and chitosan applications. React Funct Polym 46: 1-27.
4. Silva GA, Coutinho OP, Ducheyne P, Shapiro IM, Reis RL (2007) Starch-Based Microparticles as Vehicles for the Delivery of Active Platelet-Derived Growth Factor. Tissue Eng 13: 1259-1268.
5. Wang Y, Zhou P, Yu J, Pan X, Wang P, et al. (2001) Antimicrobial effect of chitooligosaccharides produced by bioreactor. Carbohydr Polym 44: 71-76.
6. Suzuki K, Mikami T, Okawa Y, Tokoro A, Suzuki S, et al. (1986) Antitumor effect of hexa-N-acetylchitohexaose and chitohexaose. Carbohydr Res 151: 403-408.
7. Jeon JY, Park PJ, Kim SK (2004) Free radical scavenging properties of hetero-chitooligosaccharides using an ESR spectroscopy. Food Chem Toxicol 42: 381-387.
8. Rajapakse N, Kim MM, Mendis E, Huang R, Kim SK (2006) Carboxylated chitooligosaccharides (CCOS) inhibit MMP-9 expression in human fibrosarcoma cells via down-regulation of AP-1. Biochim Biophys Acta 1760: 1780-1788.
9. Kim MM, Kim SK (2006) Chitooligosaccharides inhibit activation and expression of matrix metalloproteinase-2 in human dermal fibroblasts. FEBS Lett 580: 2661-2666.
10. Q VT, Kim MM, Kim SK (2006) Inhibitory Effect of Chitooligosaccharides on Matrix Metalloproteinase-9 in Human Fibrosarcoma Cells (HT1080). Mar Biotechnol 8: 593-599.
11. Liu B, Liu WS, Han BQ, Sun YY (2007) Antidiabetic effects of chitooligosaccharides on pancreatic islet cells in streptozotocin-induced diabetic rats. World J Gastroenterol 13: 725.
12. Artan M, Karadeniz F, Karagozlu MZ, Kim MM, Kim SK, et al. (2010) Anti-HIV-1 activity of low molecular weight sulphated chitooligosaccharides. Carbohydr Res 345: 656-662.
13. Yang EJ, Kim JG, Kim JY, Kim SC, Lee NH, et al. (2010) Anti-inflammatory effect of chitosan oligosaccharides in RAW 264.7 cells. Cent Eur J Biol 5: 95-102.
14. Maeda Y, Kimura Y (2004) Antitumor Effects of Various Low-Molecular-Weight Chitosans Are Due to Increased Natural Killer Activity of Intestinal Intraepithelial Lymphocytes in Sarcoma 180-Bearing Mice. J Nutr 134: 945-950.
15. Torzsas T, Kendall CW, Sugano M, Iwamoto Y, Rao AV (1996) The influence of high and low molecular weight chitosan on colonic cell proliferation and aberrant crypt foci development in CF1 mice. Food Chem Toxicol 34: 73-77.
16. Gan Q, Wang T (2007) Chitosan nanoparticle as protein delivery carrier-systematic examination of fabrication conditions for efficient loading and release. Colloids Surf B Biointerfaces 59: 24-34.
17. Janes KA, Fresneau MP, Marazuela A, Fabra A, Alonso MJ, et al. (2001) Chitosan nanoparticles as delivery systems for doxorubicin. J Cont Rel 73: 255-267.
18. Roy K, Mao HQ, Huang SK, Leong KW (1999) Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. Nat Med 5: 387-391.
19. Illum L, Farraj NF, Davis SS (1994) Chitosan as a novel nasal delivery system for peptide drugs. Pharm Res 11: 1186-1189.
20. Chung YC, Su YP, Chen CC, Jia G, Wang HL, et al. (2004) Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. Acta Pharmacol Sin 25: 932-936.
21. Allen TM, Martin FJ (2004) Advantages of liposomal delivery systems for anthracyclines. Semin Oncol 31: 5-15.
22. Addeo R, Faiola V, Guarrasi R, Montella L, Vincenzi B, et al. (2008) Liposomal pegylated doxorubicin plus vinorelbinecombination as first-line chemotherapy for metastatic breast cancerin elderly women or 65 years of age. Cancer Chemother Pharmacol 62: 285-292.
23. Kaminskas LM, McLeod VM, Kelly BD, Sberna G, Boyd BJ, et al. (2012) Comparisonof changes to doxorubicin pharmacokinetics, antitumor activity, andtoxicity mediated by PEGylated dendrimer and PEGylated liposomedrug delivery systems. Nanomedicine 8: 103-111.

24. Gabizon A, Shmeeda H, Barenholz Y (2003) Pharmacokinetics of pegylated liposomal doxorubicin: Review of animal and human studies. Clin Pharmacokinet 42: 419-436.

25. Verma AK, Kumar V, Agarwal S, Leekha A, Tyagi A, et al. (2013) Interplay of Immune Response and Oxidative Stress Induced By Chitosan Nanoparticles to Possibly Combat Inflammation. World Res J Biosci 1: 28-34.

26. Filipe V, Hawe A, Jiskoot W (2010) Critical Evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the Measurement of Nanoparticles and Protein Aggregates. Pharmaceutical Res 27: 36.

27. Verma AK, Pandey RP, Chanchal A, Sharma P (2011) Immuno-potentiating role of encapsulated proteins of infectious diseases in biopolymeric nanoparticles as a potential delivery system. J Biomed Nanotech 7: 63-64.

28. Gulyaev AE, Gelperina SE, Skidan IN, Antropov AS, Kivman GY, et al. (1999) significant transport of doxorubicin into the brain with polysorbate 80-coated nanoparticles. Pharm Res 16: 1564-1569.

29. Verma AK, Kumar S (2008) Novel Hydrophilic Drug Polymer Nano-Conjugates of Cisplatin Showing Long Blood Retention Profile - Its Release Kinetics, Cellular Uptake and Bio-Distribution. Curr Dru Deliv 5: 120-126.

30. Reddy LH, Sharma RK, Chuttani K, Mishra Ak, Murthy RR (2004) Etoposide-incorporated tripalmitin nanoparticles with different surface charge: formulation, characterization, radiolabelling, and biodistribution studies. AAPS 6: 55-64.

31. Verma AK, Chanchal A, Chutani K (2012) Augmentation of anti-tumour activity of cisplatin by pectin nano-conjugates in B-16 mouse model: pharmacokinetics and in-vivo biodistribution of radio-labelled, hydrophilic nano-conjugates. Int J Nanotechnology 9: 872-886.

32. Verma AK, Chanchal A, Panday RP, VOhra R, Islam N, et al. (2012) Pharmacokinetics and Biodistribution of Paclitaxel-Gelatin Nanoparticles. Int J Green Nanotech 4: 500-510.

33. Lee SJ, Koo H, Lee DE, Min S, Lee S, et al. (2011) Tumor-homing photosensitizer-conjugated glycol chitosan nanoparticles for synchronous photodynamic imaging and therapy based on cellular on/off system. Biomaterials 32: 4021-4029.

34. Jeong YI, Jin SG, Kim IY, Pei J, Wen M, et al. (2010) Doxorubicin-incorporated nanoparticles composed of poly(ethylene glycol)-grafted carboxymethyl chitosan and antitumor activity against glioma cells in vitro. Colloids Surf B 79: 149-155.

35. Bowman K, Leong KW (2006) Chitosan nanoparticles for oral drug and gene delivery. Int J Nanomedicine 1: 117-128.

36. Bae IH, Jeong BC, Kook MS, Kim SH (2013) Evaluation of a thiolated chitosan scaffold for local delivery of bmp-2 for osteogenic differentiation and ectopic bone formation. BioMed Research.

37. Lee CC, Gillies ER, Fox ME, Guillaudeu SJ, Fréchet JM, et al. (2006) A single dose of doxorubicin-functionalized bow-tie dendrimer cures mice bearing c-26 colon carcinomas. Proc Natl Acad Sci 103: 16649-16654.

38. Fox ME, Guillaudeu S, Fréchet JM, Jerger K, Macaraeg N, et al. (2009) Synthesis and in vivo antitumor efficacy of pegylated poly(l-lysine) dendrimer-camptothecin conjugates. Mol Pharm 6: 1562-1572.

39. Wang M, Thanou M (2010) Targeting nanoparticles to cancer. Pharmacol Res 62: 90-99.

40. Grenha A, Seijo B, Remuñán-López C (2005) Microencapsulated chitosan nanoparticles for lung protein delivery. Eur J Pharm Sci 25: 427-437.

41. Teijeiro-Osorio D, Remuñán-López C, Alonso MJ (2009) New generation of hybrid poly/oligosaccharide nanoparticles as carriers for the nasal delivery of macromolecules. Biomacromolecules 10: 243-249.

42. Tyagi A, Agarwal S, Leekha A, Verma AK (2014) Effect of mass and aspect heterogeneity of chitosan nanoparticles on bactericidal activity. Int JAdv Res 2: 357-367.

43. Verma AK, Kumar A (2013) Pharmacokinetics and biodistribution of negatively charged pectin nanoparticles encapsulating paclitaxel. Cancer Nanotechnology 4: 99-102.

44. ten Hagen TL, Seynhaeve AL, van Tiel ST, Ruiter DJ, Eggermont AM (2002) Pegylated liposomal tumor necrosis factor-α results in reduced toxicity and synergistic antitumor activity after systemic administration in combination with liposomal doxorubicin (Doxil) in soft tissue sarcoma-bearing rats. Int J Cancer 97: 115-120.

45. Udhrain A, Skubitz KM, Northfelt DW (2007) Pegylated liposomal doxorubicin in the treatment of AIDS-related Kaposi's sarcoma. Int J Nanomedicine 2: 345-352.

46. Gabizon A, Shmeeda H, Barenholz Y (2003) Pharmacokinetics of pegylated liposomal doxorubicin: review of animal and human studies. Clin Pharmacokinet 42: 419-436.

47. Gabizon AA (1992) Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long circulating liposomes. Cancer Research 52: 891-896.

48. Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS, et al. (2008) Nanoparticles in medicine: therapeutic applications and developments. Clin Pharmacol Ther 83: 761-769.

49. Maeda H (2001) The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. Adv Enzy Regul 41: 189-207.

50. Cai Z, Wang Y, Zhu LJ, Liu ZQ (2010) Nanocarriers: A general strategy for enhancement of oral bioavailability of poorly absorbed or pre-systemically metabolized drugs. Curr Drug Metab 11: 197-207.

51. Park H, Kwon S, Lee M, Chung H, Kim JH, et al. (2006) Self-assembled nanoparticles based on glycol chitosan bearing hydrophobic moieties as carriers for doxorubicin: in vivo biodistribution and anti-tumor activity. Biomaterials 27: 119-126.

52. Kratz F (2007) DOXO-EMCH (INNO-206): the first albumin-binding prodrug of doxorubicin to enter clinical trials. Expert Opin Invest Drugs 16: 855-866.

53. Kratz F (2008) Albumin as a drug carrier: design of prodrugs, drug conjugates and nanoparticles. J Control Release 132: 171-183.