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Targeted drug delivery system- Formulation and evaluation of chitosan nanospheres containing Doxorubicin hydrochloride

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Abstract

A chitosan molecule form self-assembled nanoparticles that can encapsulate a quantity of drugs and deliver them to a specific site. Chemical attachment of drug to chitosan throughout the functional linker has possibility to produce useful prodrugs, exhibiting biological activity at target site. In vivo residence time of the dosage form in the gastrointestinal tract and bioavailability of various drugs increases by mucoadhesive and absorption enhancement properties of chitosan. Antitumour activity of doxorubicin(DOX)-incorporated nanoparticles in vitro on DOX- resistant C6 glioma cells. Nanoparticles showed increased cytotoxicity compared to DOX alone. These results suggest that doxorubicin (DOX) was unable to penetrate into cells and did not effectively inhibit cell proliferation. In contrast, nanoparticles can penetrate into cells and effectively inhibit cell proliferation.

There are 3 batches of drug loaded nanospheres in which 2.5mg,5mg and 10mg of DOX were loaded into nanospheres where the concentration of chitosan is 1%w/v. Anticancer drugs without targeting a specific site cause side effects. The objective of this research is to reduce side effects. HPLC device was used to quantitatively analyze amount of doxorubicin loaded in nanospheres. The result had showed concentration of anticancer drug loaded in nanospheres is directly proportional to the drug payload capacity until saturation point. The in vitro drug release studies was carried out for 48 hours to obtain a more precise result by carrying out this studies in a medium resembling our body environment such as pH7.4, 37ºC with analytical grade water for this studies.

In vitro release of doxorubicin is of zero order kinetic. This shows that release is independent of the concentration of drug loaded in the nanospheres. Besides that, the graphs also show a sustained release manner, indicating these nanospheres formulation are suitable for targeting drug delivery system and for efficient treatment of cancerous cells.

Keywords: chitosan; naoparticles; doxorubicin; cytotoxicity; targeting

Introduction

Chemically, doxorubicin hydrochloride is (8S,10S)-10-[(3-amino-2,3,6-trideoxy - L-lyxo-hexopyranosyl)oxy]-8-glycolyl-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy5,12- naphthacenedione. Doxorubicin is a cytotoxic anthracycline antibiotic isolated from cultures of Streptomyces peucetius var. caesius. Doxorubicin consists of a naphthacenequinone nucleus linked through a glycosidic bond at a ring atom 7 to an amino sugar, daunosamine. The anthracycline ring is lipophilic, but the saturated end of the ring system contains abundant hydroxyl groups adjacent to the amino sugar, producing a hydrophilic center. Thus, the molecule is amphoteric which contains acidic functions in the ring phenolic groups and a basic function in the sugar amino group. It binds to the cell membrane as well as plasma protein. Usually, it is supplied in the hydrochloride form as a sterile red-orange lyophilized powder containing lactose and as a sterile parenteral, isotonic solution with sodium chloride for intravenous use only. [1]

Several mechanisms have been proposed to explain DOX antitumor activity. There are 2 major mechanisms: the intercalation into DNA, leading to inhibition of the DNA synthesis or poisoning of topoisomerase II (TOP2A); and generation of free radicals, leading to DNA and cell membrane damage. [2]

Targeted drug delivery is a form of delivery system where the drug is selectively targeted and delivered only to its site of action or absorption. The goal of a targeted drug delivery system is to concentrate the medication in the tissues of interest while reducing the relative concentration of the medication in the remaining tissues. This improves efficacy of the treatment while reducing side effects. [3]

The use of nanotechnology in drug delivery and imaging in vivo is a rapidly expanding field. Present nanoscale systems available including liposomes, micelles, emulsions, nanoparticulates, and dendrimer nanocomposites. [4]

Nanoparticles are sub-nanosized colloidal structures composed of synthetic or semi synthetic polymers. The major goals in designing nanoparticles as a delivery system are to control particle size,

surface properties and release of pharmacologically active agents in order to achieve the site-specific action of the drug at the therapeutically optimal rate and dose regimen.[5]

The primary goals of nanoparticles in drug delivery include more specific drug targeting and delivery,reduction in toxicity while maintaining therapeutic effects,greater safety and biocompatibility, and faster development of new safe medicines.[6]

Riddhi Dave et al [7] have prepared and evaluated chitosan nanoparticles containing doxorubicin by w/o emulsion method. They have prepared the nanoparticles using liquid paraffin 5%w/v and span 20. SEM indicated spherical structure of nanoparticle without agglomeration. In vitro drug release study suggests sustain drug release for longer period of time. The synthesized nanoparticles having diameter of 210nm are characterized by entrapment efficiency of 50-54%. Komal Patel et al [8] have prepared doxorubicin loaded Chitosan nanoparticles by ionic gelation method (IGM). An isocratic high-pressure liquid chromatography (HPLC) method was developed to quantify Doxorubicin Hydrochloride in rats plasma,vital organs. The Nanoparticles prepared by IGM show significantly increased the half life (T1/2) and mean residence time (MRT) of Doxorubicin in blood. [9]

Materials and Methods

Materials

Doxorubicin was purchased from SunPharma India. Chitosan (150cps) was obtained from Central Marine Fisheries Research Institute Cochin, India. Sodium chloride was purchased from Unilab. 3% Glacial Acetic Acid , Toluene and Span 80 were purchased from Quicklab Sdn. Bhd. 25% aqueous Glutaraldehyde solution was supplied from Plant Succeed. Linseed oil was from Medina Jaya Sdn. Bhd. Acetone was purchased from Merck.

Preparation of 1.0% w/v of 150cps Chitosan Gel

150cps Chitosan and sodium chloride were dissolved in 3% glacial acetic acid and stirred with slight warming. Then, the solution was kept overnight for air drying under room temperature to obtain a clear gel of 1.0% w/v of 150cps chitosan.

Preparation of Glutaraldehyde Saturated Toluene (GST)

Glutaraldehyde and 7mL Toluene were mixed well for 30 minutes and then it was shaken vigorously. The solution was left overnight for saturation and separation.

Preparation of Nanospheres without Drug Incorporation

The 1%w/v chitosan gel was added with acetone dropwise with stirring. Polymer gel was added dropwise into linseed oil and allowed to emulsify under magnetic stirring at room temperature for one hour. Polymer was precipitated to the evaporation of acetone with subsequent formation of smaller spheres suspended in the oil phase. GST with Span 80 was added dropwise to the emulsion and it was continued stirring for 4 hours. The emulsion was centrifuged at 4800rpm for 20 minutes. The supernatant was discarded and fresh toluene was added and centrifuged at 4800rpm for 20minutes. The centrifugation was repeated for 5 times. Acetone was added, mixed well and centrifuged at 4800rpm for 10minutes and repeated for 2 times. The content was dried in the incubator in glass petri plate. Powders were observed under 40X microscope.

Preparation of Drug Loaded Nanospheres with 1.0% w/v 150cps Chitosan

Three batches of 1.0% w/v of 150cps chitosan gel were prepared with different concentration of doxorubicin, which are 2.5mg, 5.0mg and 10.0mg. The emulsions were stirred stirrer for 1 hour then kept on standing for another 1 hour.

Preparation of Doxorubicin-loaded nanospheres

For the preparation of Doxorubicin-loaded nanospheres, firstly, acetone was added dropwise into beakers containing 1%w/v of 150cps chitosan gel prepared with different doxorubicin concentrations in the ratio of 1:1. Then the polymer gel was added in dropwise into linseed oil and was allowed to emulsify under magnetic stirring. Polymer was precipitated to the evaporation of acetone with subsequent formation of smaller spheres suspended in the oil phase. GST layer was separated and the Toluene layer which had prepared was mixed with Span 80. GST with Span 80 was added dropwise to the emulsion and it was continued stirring under magnetic stirrer. The emulsions in each beaker were centrifuged at 4800rpm and the upper oily supernatant was discarded.

Toluene was added into each tube and shaken vigorously. The tubes were centrifuged at 4800rpm and the supernatant was discarded. This step was repeated a few times and after that, acetone was added, mixed well and centrifuged at 4800rpm. After centrifugation, acetone was added into each test tube and the mixture was mixed well. Upon drying, brown coloured and free flowing of fine powder was obtained. Powders were observed under 40X microscope (Figure 1 & 2).

Figure 1: Chitosan Nanospheres under Microscope 40X

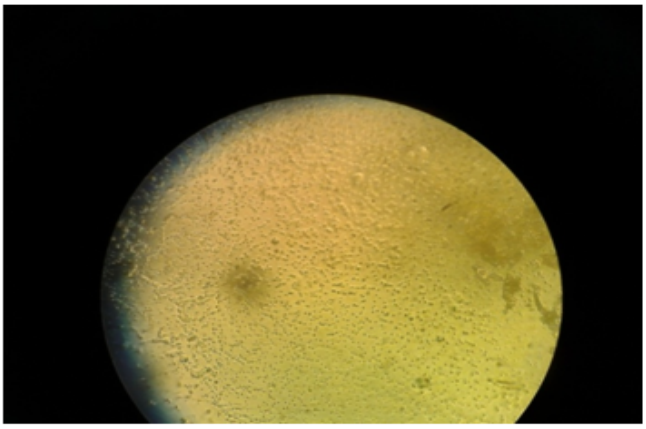
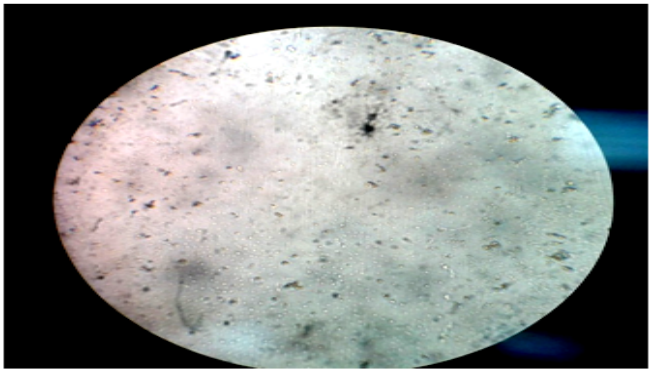


Figure 2: Doxorubicin-Chitosan Nanospheres under Microscope 40X



Preparation of calibration curve

For the preparation of calibration curve, 1mg/ml of Doxorubicin stock solution was prepared. Then the stock solution was used to prepare Doxorubicin concentrations of 100µg/ml, 75µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml and 6.25µg/ml by using serial dilution with deionized water. Standard solution was analyzed using HPLC and standard curve was plotted by taking the peak area at y-axis a concentration (µg/ml) at x-axis (Figure 3, Table 1).

Optimization Method of HPLC Analysis of Doxorubicin

Chromatographic separation was performed on a Shimadzu liquid chromatographic system equipped with a LC-10AT-vp solvent delivery system (pump),

Figure 3: Calibration Curve of Standard Doxorubicin HCl

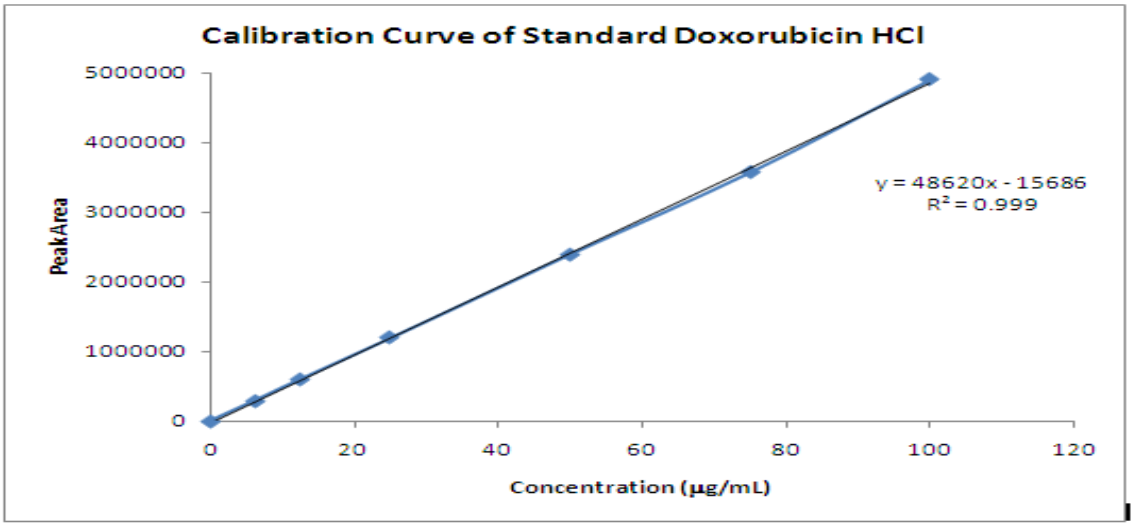
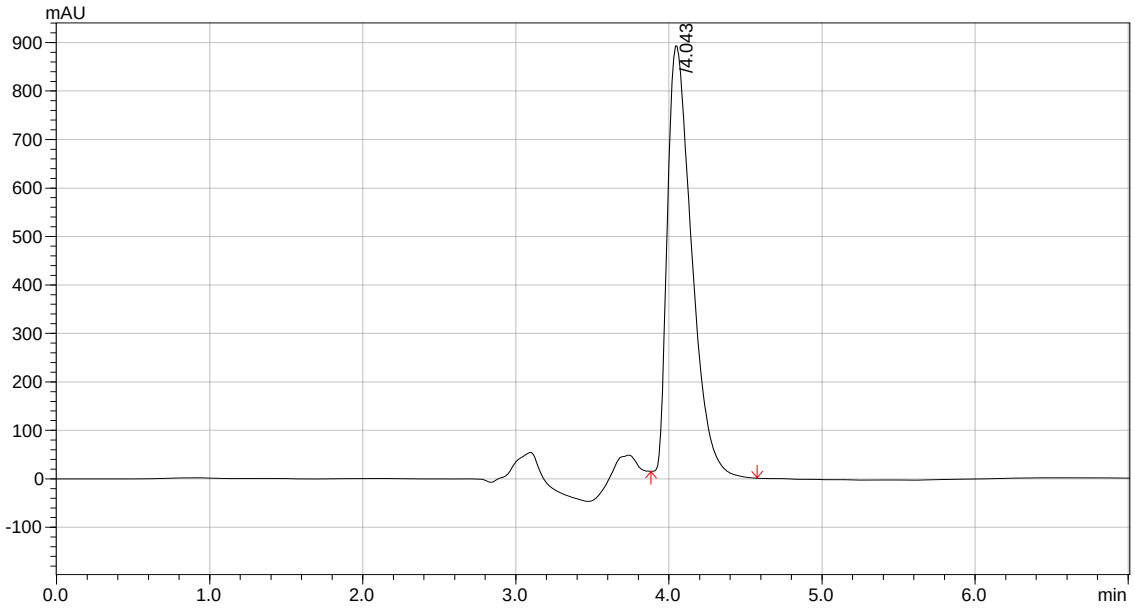


Table 1: The data for the preparation of calibration curve

Concentration (µg/mL)	Peak Area
0.00	0
6.25	293253
12.5	603127
25.0	1196055
50.0	2392110
75.0	3568164
100.0	4904219

SPD M 10AVP photo diode array detector, Rheodyne 7725i injector with 50 µl loop volume. Class-VP 6.01 data station was applied for data collecting and processing (Shimadzu, Japan).A Phenomenex Gemini C column (250 mmx 4.6 mm i.d., 5µ) was used for the separation, mobile phase of a mixture of acetonitrile (pH2.8) and 0.5% triethylamine (pH 3.5 adjusted with ortho phosphoric acid); (30:70 v/v) was delivered at a flow rate of 1.0 ml/min with detection at 480 nm. The mobile phase was filtered through a 0.2µ membrane filter and degassed. The injection volume was 50 µl and the analysis was performed at ambient temperature (Figure. 4).

Figure 4: Typical Chromatogram of Doxorubicin HCl Standard



Estimation of amount of drug incorporated

Estimation of amount of drug incorporated can be done by digesting 10mg Doxorubicin drug filled nanospheres (Batch A,B,C) in 1:1 ratio of 0.1N Hydrochloric acid and ethanol. The sample collected was centrifuged at 5300rpm for 20 minutes. After centrifugation, solution was analysed for the amount of the drug present using HPLC at 480nm with the help of standard curve prepared previously (Figure 5, Table 2).

Figure 5: Typical Chromatogram of Doxorubicin HCl Loaded Nanospheres

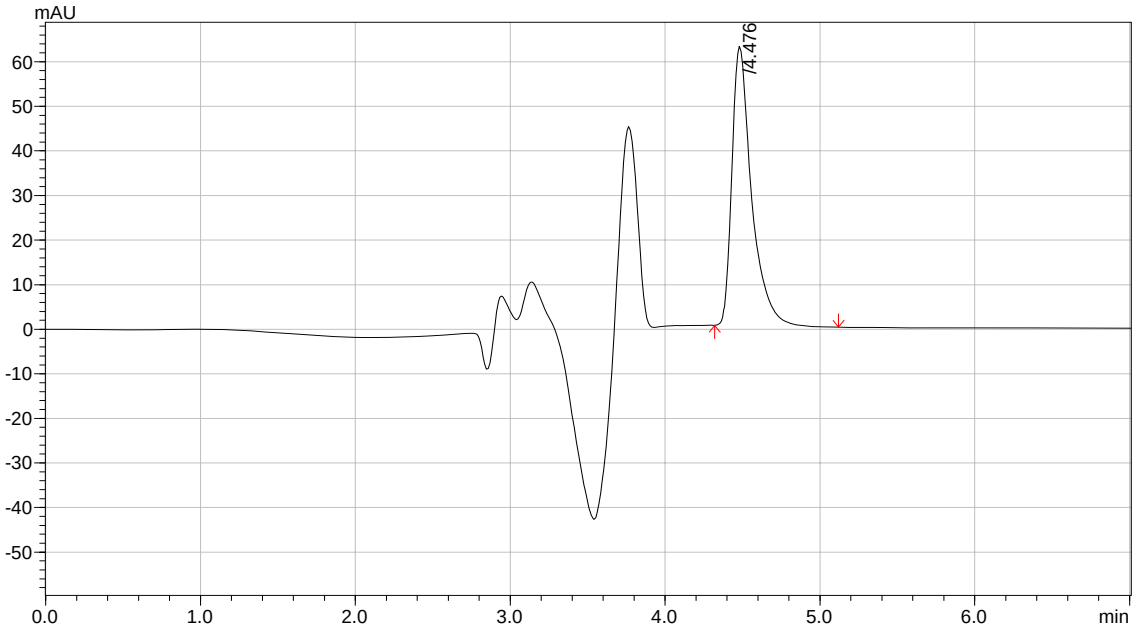


Table 2: Data for percentage drug loading in each batch of nanospheres

Sample	Total Weight (mg)	Peak Area	Concentration (µg/mL)	Practical Loading (mg)	Theoretical Loading (mg)	Percentage Drug Loading (%)
A	129.0	258645	5.6423	0.05642	0.1938	29.11
B	149.9	482216	10.2407	0.1024	0.3336	30.70
C	183.9	864419	18.1017	0.1810	0.5438	33.28

In-vitro Drug Release Studies

2.5mg/ml, 5mg/ml, 10mg/ml (Batch A,B,C) of drug loaded nanospheres were taken and 100ml of deionized water (pH7-7.4) was added into each of the conical flask. The flasks were kept in an incubator shaker at 37^{0}C ± 0.5 ^{0}C using 150 revolution/min for a span of 48 hours. 2ml of releasing solution was removed as samples at various time intervals and 1ml of releasing solution taken was then replaced with 2ml of deionized water immediately. The 2ml of releasing solution collected was passed through 0.45 µm nylon membrane filter to be filtered. Drug content was estimated spectrophotometrically using HPLC at 480nm (Table 3).

Table 3: *In-vitro* percentage release of doxorubicin from three batches of nanospheres (Sample A)

Time interval (hours)	Peak area	Concentration (µg/ml)	Amount of drug released (mg)	Label Claim(mg)	Percentage drug released (%)
0	0	0	0	0.1411	0.00
1	23534	0.8067	0.0807	0.1411	57.17
2	24890	0.8346	0.0835	0.1411	59.15
3	25013	0.8371	0.0837	0.1411	59.33
4	25780	0.8529	0.0853	0.1411	60.44
5	26580	0.8693	0.0869	0.1411	61.61
6	27188	0.8818	0.0882	0.1411	62.50
7	29275	0.9247	0.0925	0.1411	65.54
8	29859	0.9368	0.0937	0.1411	66.39
24	30179	0.9433	0.0943	0.1411	66.86
28	41785	1.1820	0.1182	0.1411	83.77
32	41955	1.1855	0.1186	0.1411	84.02
48	42410	1.1949	0.1195	0.1411	84.68

SEM Analysis

SEM is a powerful method for visualizing the structure of nanospheres (Figures 6 and 7). Recently, we were able to show that this special preparation technique can be successfully used for the detection of nanospheres.

Figure:6 SEM micrograph of the nanosphere

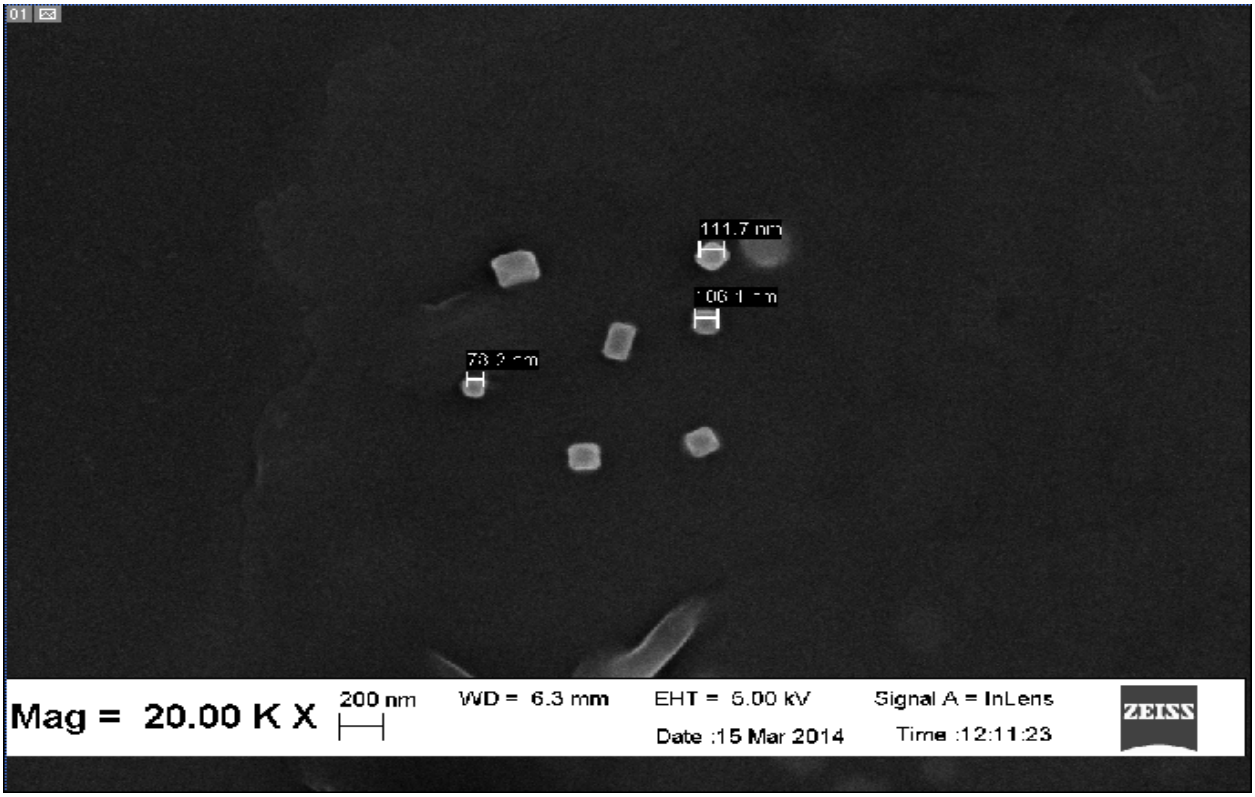
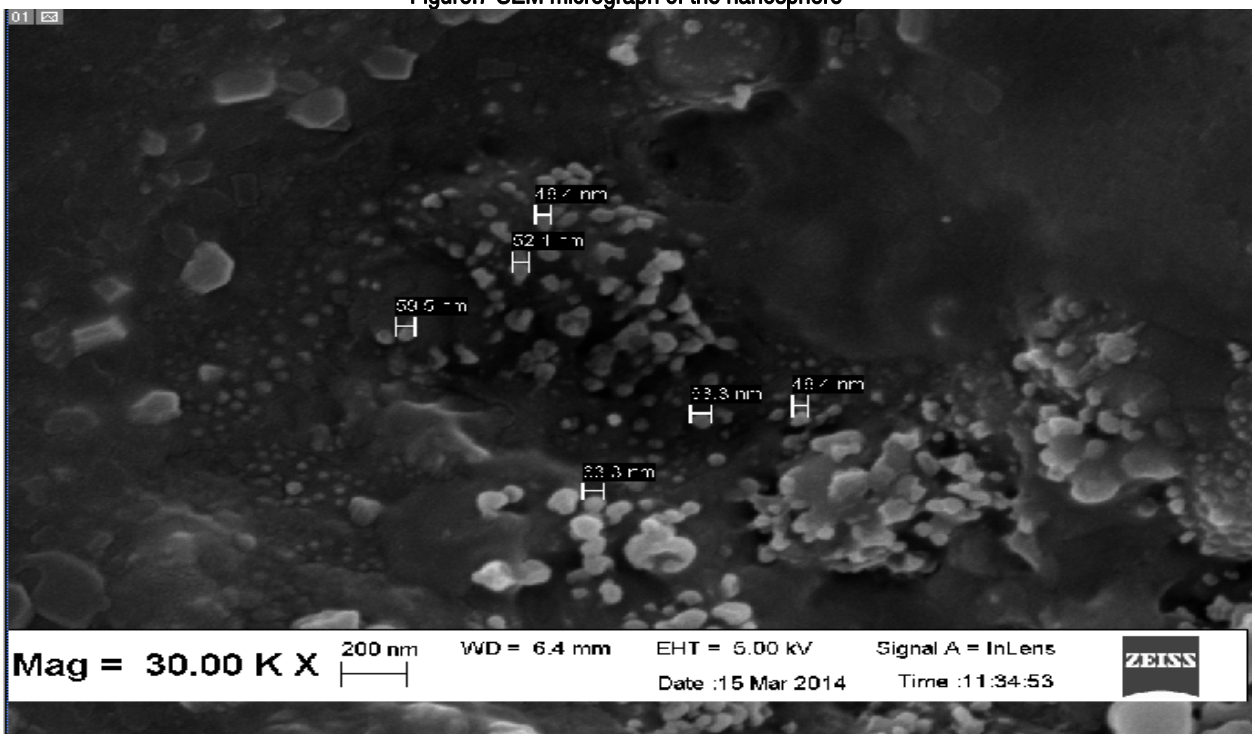


Figure:7 SEM micrograph of the nanosphere



Results and Discussion

Chitosan is a modified natural carbohydrate polymer which is soluble in most organic acidic solutions at pH less than 6.5 including formic, acetic, tartaric, and citric acid. By simple covalent modifications of the polymer, its physicochemical properties can be changed and can be made suitable for drug delivery purpose. Ionic interactions between positively charged amino groups in chitosan and the negatively charged mucus gel layer make it mucoadhesive. The favourable properties like biocompatibility, biodegradability, pH sensitiveness, and mucoadhesiveness have enabled chitosan to be chosen to be used as a polymer for nanoparticles.

The major goals in designing nanoparticles as a delivery system are to control particle size, surface properties and release of pharmacologically active agents in order to achieve the site-specific action of the drug at the therapeutically optimal rate and dose regimen. Nanospheres were prepared by modified spontaneous emulsification method which is water in oil emulsion type. First, chitosan was dissolved in glacial acetic acid to form an aqueous phase. After incorporating the drug into the system, linseed oil was added together with acetone into chitosan gel with drug mixture. It was allowed to emulsify under magnetic stirring.

Prepared GST with span 80 was added into the mixture and it was allowed to precipitate by the evaporation of acetone to form nanospheres. Toluene was then used to remove remaining oil substance in the emulsion. Then acetone was used to remove the toluene and to dry the resulting substance to prevent aggregation.

Some modifications were done in improving the formulation of nanospheres. 150cps chitosan was found to be better in the production of uniform and higher amount of nanospheres compared to 27 cps chitosan. 14mL glutaraldehyde instead of 7mL glutaraldehyde was used to form GST due to stronger cross linking and hardening of spherical particles. For better dispersion and less clumping of nanospheres, Span 80 in liquid form was utilized. In addition, the ingredients were added dropwise to ensure uniform mixing and further reduce the occurrence of aggregation. Frequency of washing with toluene was increased to maximize the removal of oil content before the washing of nanospheres with acetone. In the estimation of amount of drug incorporated, the three batches of drug filled nanospheres were digested with the ratio of 1:1 of 0.1N Hydrochloric acid and ethanol.

Three batches of nanospheres were prepared with the incorporation of 2.5mg(batch A), 5.0mg(batch B) and 10.0mg(batch C) of doxorubicin respectively. The concentration of drug incorporated was found to be 5.6423µg/mL, 10.2407µg/mL and 18.1017µg/mL for batch A, batch B and C respectively. Results show the drug loading capacity of 2.5mg batch was 29.11%, 5.0mg batch was 30.70% and 10mg batch was 33.28%. Based on the data in Table 3, when drug to polymer ratio was increased, the drug payload capacity was also increased.

In the *in-vitro* drug release study, deionised water with pH 7.4 was used at the temperature 37ºC which is similar to the internal environment of the body for the maximum drug release. From the figures 6, 7, and 8, it was observed that the *in-vitro* release of doxorubicin is zero order kinetics, which means that the release is independent of the concentration of the drug loaded in the nanospheres. It also showed a biphasic repsonse, whereby there was an initial burst release of drug from the nanospheres, followed by a plateau where the release of drug became constant and independent of drug concentration. 50% drug release in 2.5mg batch is at the 1st hour, whereas for the 5.0mg batch is at the 4th hour. The 50% drug release in 10mg batch on the other hand is at the 8th hour. Hence, it shows that the rate of drug release is inversely proportional to the drug loading. The highly drug-loaded nanospheres in 10mg batch shows slower onset action and prolonged release profile. This advantage enables the decrease in dosing frequency of anticancer drug. At the same time, there will be decrease in the occurrence of side effects. The release pattern and percentage drug release shown by the nanospheres was found satisfactory. However, the further optimization of the analytical method is required and the in vitro release study it is worthwhile to study the drug release in various mediums in order to thoroughly understand the behavior of drug release. be studied thoroughly to understand the behaviour of the drug release.

Conclusion

Three batches of nanospheres were formulated with varying amount of drug payload. The drug to polymer ratio is proportional to the drug payload capacity. It was shown that through our *in-vitro* release studies that the greater the amount of drug incorporated will result in slower rate of drug release. This indicates a sustained release of the drug from the nanospheres with higher drug pay load.

Initially, the graphs obtained from in vitro drug release studies shown a biphasic release, that is an initial burst release, followed by a plateau where the release of drug is constant and independent of drug concentration. This feature is one of the objectives of drug targeting delivery system. The small size of nanospheres enables rapid absorption in our circulation system and will accumulate especially in cancerous tissues with reduced doxorubicin systemic toxicity. This also facilitates the targeting drug delivery system.

This research has indeed achieved its main objective which is to provide the maximum therapeutic efficacy with the most reduced side effects. In a nut shell, there is a bright future for the development of doxorubicin-chitosan nanoparticles as one of the most efficient drug carrier for the treatment of cancer.

References

[1]. Drugs.com. Rubex® (doxorubicin hydrochloride for injection, USP).

[2]. Qin Tian et al. Glycyrrhetinic acid-modified chitosan/poly(ethylene glycol) nanoparticles for liver-targeted delivery. Biomaterials 31.2010; 4748-4756.

[3]. Gupta Manish and Sharma Vimukta. Targeted drug delivery system: A Review; Research Journal of Chemical Sciences, 2011; 1 (2):135-138

[4]. Otilia M. Koo, Israel Rubinstein, Hayat Onyuksei. Role of nanotechnology in targeted drug delivery and imaging: a concise review. Nanomedicine: Nanotechnology, Biology and Medicine, 2005;1(3): 193-212.

[5]. VJ Mohanraj and Y Chen: Nanoparticles- A Review. Tropical Journal of Pharmaceutical Research, 2006; 5 (1): 561-573.

[6]. Wim H De Jong and Paul JA Borm. Drug delivery and nanoparticles: Applications and hazards Int J Nanomedicine. 2008; 3(2): 133–149.

[7]. Riddhi Dave and Rakesh Patel. Preparation and characterization of doxorubicin HCl loaded chitosan nanoparticles by w/o emulsion method. Int. J. of Pharm. & Life Sci.2013; 4(2): 2407-2413.

[8]. Komal Patel et al. Preparation and in vivo study of Doxorubicin HCl loaded chitosan nanoparticles prepared by w/o emulsion method. International Journal of Current Research. 2012; 4(12): 438-440.

[9]. Parichart Naruphontjirakul et al. Development of Doxorubicin - Core Shell Chitosan Nanoparticles to Treat Cancer. 2011;11:18-40