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FORMULATION AND EVALUATION OF ATORVASTATIN CALCIUM LOADED CHITOSAN NANOPARTICLES

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ABSTRACT

Atorvastatin happens to be a potential inhibitor of HMG-CoA reductase involved in the synthesis of cholesterol and is being used to decrease the low-density lipoproteins in blood. The drug has profound effect, but the dosage is high compared to other statins. Therefore, it was thought to minimize the frequency of doses to avoid the possibility of drug resistance by atorvastatin and deliver using nanocarriers. The aim of this research was to characterize and evaluate the atorvastatin-loaded chitosan nanoparticles before delivery. The nanoparticles were prepared by ionic gelation. The sizes were visualized by dynamic laser light scattering method. The size of the nanoparticles ranged between 220 and 314 nm. Upon considering the encapsulation efficiency, 50% were found to be the least and 75% the most. The formulation exhibited a slow and sustained release (~76%) of atorvastatin from the nanoparticles in <12 hours. The atorvastatin-loaded chitosan nanoparticles were stable, hemocompatible and found to be fit for drug delivery.

KEYWORDS: **Atorvastatin, Chitosan, Ionic gelation, Encapsulation, Nanoparticles.**

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INTRODUCTION

Biodegradable and hydrophilic nanocarriers have important potential application to deliver a variety of therapeutic molecules like peptide, antigen, protein, oligonucleotides and genes by oral, intravenous, and mucosal administration[1–4]. Naturally occurring polymers are used extensively in the field of food and pharmaceutical research[5,6]. Chitosan is also a naturally occurring polymer obtained from the deacetylation of chitin as found to be nontoxic, biocompatible, biodegradable, inexpensive and cationic polysaccharide[7,8]. It has an unequivocal mucus adhesive property and ability to enhance the penetration of large molecule across the mucosal surface. Chitosan polymer can easily cross link with counter poly anion like sodium tripolyphosphate to control the release of drug[9]. Chitosan nanoparticles are prepared by the process of ionic gelation method based on the interaction between the negatively charged groups of sodium tripolyphosphate and the positively charged amino groups of chitosan polymer[10]. Generally, atorvastatin is used as secondary prevention for the treatment of already elevated cholesterol, low density lipoprotein and triglycerides[11]. On the other hand, atorvastatin is being used to elevate the high density lipoprotein cholesterol. Although the other drug candidates of statins group exhibit different oral absorption rates, the candidate atorvastatin is found to show 30% of oral absorption and 12% of bioavailability[12,13]. A number of clinical and experimental studies have demonstrated the effect of atorvastatin on atrial fibrillation, but the results are equivocal. Due to technological innovations, today it is possible to enhance the bioavailability and improve the efficacy and safety of atrial fibrillation by developing the biodegradable atorvastatin-loaded chitosan nanoparticles for oral delivery[14,15].

MATERIALS AND METHODS

Chitosan (MW of 30 kDa) was a gift sample from Laxmish I P, Cochin Central Marine Fisheries, Cochin, India. Atorvastatin calcium salt procured from Indswift Laboratories, Mumbai. Pentasodium tripolyphosphate (TPP) was purchased from Sigma (St. Louis, MO, USA), Tween 80 and Acetic acid were purchased from Himedia Chemical Co. (Mumbai, India). Double distilled water was used throughout the study. All other reagents were of analytical grade unless otherwise stated.

(i) Preparation of Atorvastatin-loaded Chitosan Formulations by Ionic Gelation Method

Atorvastatin-loaded chitosan nanoparticles were prepared by ionic gelation method as reported by Calvo et al with slight modification[23]. Atorvastatin-loaded chitosan nanoparticles can easily be prepared upon addition of TPP to a chitosan solution containing atorvastatin and mixing by using magnetic stirrer. The ionic interaction of chitosan with TPP finally establishes an equilibrium leading to the reduction of the aqueous solubility of chitosan. In the process of doing so, it traps the atorvastatin into the core to make nanoformulations. During the formation of nanoparticles the ratio between chitosan and TPP is critical and that controls the size and nanoparticles distribution. Encapsulating polymer solution was prepared by dissolving chitosan in acetic aqueous solution to achieve 1.0, 2.0 and 3.0 (mg/ml) different formulations. Drug solution was prepared separately by dissolving atorvastatin (5mg) in methanol (200 µl) and added with 1% (w/v) tween 80. The chitosan and drug solutions were mixed together using magnetic stirrer (25°C, 30 min) and 0.2% (w/v) TPP was added dropwise while stirring. The resultant nanoparticles suspension was centrifuged (10,000 rpm, 30 min), the particles were washed thrice with distilled water and freeze dried. The procedure was repeated for all the three formulations ACN1, ACN2 and ACN3 (Table.1)[16-20].

Table 1
Preparation of Atorvastatin-loaded Chitosan Formulations

Sl. No	Formulations code	Atorvastatin (mg)	Chitosan (mg)	0.2% TPP solution (ml)	Atorvastatin : Chitosan Ratio
1	ACN1	5	10	3	1:2
2	ACN2	5	20	3	1:4
3	ACN3	5	30	3	1:6

(ii) Particle Size Distribution

The Photon correlation spectroscopy is routinely used method to determine the mean hydrodynamic diameter and the particle size distribution to study the polydispersity index (PDI = 22/Γ2) of the nanoparticles. The size of formulations ACN1, ACN2 and ACN3 was determined using Zetasizer 4000, Malvern Instruments Ltd., Malvern, UK. The dynamic light-scattering measurements were done with a wavelength of 532 nm at 25°C with an angle detection of 90º.

(iii) Evaluation of Atorvastatin Encapsulation Efficiency (%)

The amount of atorvastatin in each formulations ACN1, ACN2 and ACN3 was calculated by the difference between the total amount of atorvastatin added initially into the preparation medium and the amount of atorvastatin remained in the supernatant after centrifugation. The atorvastatin present in the supernatant was determined spectrophotometrically by measuring the absorbance at 246 nm using UV–VIS spectrophotometer (Shimadzu 1650, Kyoto, Japan) [21].

Table 2
Particle Size and Encapsulation Efficiency (%) of Atorvastatin-loaded Chitosan Formulations.

Sl. No	Formulation code	Atorvastatin : Chitosan ratio	Particle Size (nm)	PDI	Encapsulation efficiency (%)
1	ACN1	1:2	220.0	0.383	77.48
2	ACN2	1:4	256.0	0.401	68.55
3	ACN3	1:6	314.3	0.336	65.23

(iv) In Vitro Release Study of Atorvastatin

The *in vitro* release[19] of atorvastatin from atorvastatin-loaded chitosan formulations ACN1, ACN2 and ACN3 were carried out by dialysis method. Atorvastatin-loaded chitosan nanoparticles were redispersed in freshly prepared in PBS (5 ml, pH 7.4) and dialyzed using membrane with 12 kDa cut-off. During dialysis, the bag was placed in a jar containing PBS (150 ml) and incubated at 37°C in a shaking water bath (50 rpm). The amount of atorvastatin released from the atorvastatin-loaded chitosan nanoparticles was measured by sampling out 1 ml each time at predetermined time intervals (0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 36 and 48 hour). From an aliquot, the amount of atorvastatin released was determined spectrophotometrically at 246 nm. A standard calibration curve was drawn using atorvastatin calcium as reference standard[21-22].

Table 3
In Vitro Release of Atorvastatin from ACN1, ACN2 and ACN3 Formulations at pH 7.4 (37 °C).

Time Interval (Hours)	Atorvastatin-loaded Chitosan Formulations		
	ACN1 (%)	ACN2 (%)	ACN3 (%)
30min	19.20	22.52	20.29
1	24.41	26.71	24.26
2	28.24	27.32	29.34
3	34.57	31.15	32.51
4	46.42	38.07	39.09
5	52.08	46.09	44.71
6	61.44	56.61	52.22
8	70.02	64.24	59.13
12	76.35	67.47	63.97

(v) Stability Studies

The ACN1, ACN2 and ACN3 formulations were evaluated to assess their stability at three different temperature levels as described by Vyjayanthimala *et al*[22]. The formulations were incubated at 4°C, room temperature and 45°C for a period of 1 month. The samples were collected at every week and measured the amount of atorvastatin release using UV-Spectrophotometer at 246 nm (Table 4)[22].

Table 4

Stability Study of Atorvastatin-loaded Chitosan Formulations.

Sl. No	Time in Week	ACN1 Formulation			ACN2 Formulation			ACN3 Formulation		
		4°C	Room Temp	45°C	4°C	Room Temp	45°C	4°C	Room Temp	45°C
1	0	100	100	100	100	100	100	100	100	100
2	1	99.22	99.04	93.22	97.60	98.00	94.10	96.99	95.00	94.88
3	2	98.00	98.12	91.36	96.00	97.20	95.30	97.00	96.30	95.08
4	3	97.66	97.69	90.78	98.20	97.40	96.60	96.49	97.78	96.11
5	4	96.44	96.06	89.99	95.99	94.78	89.98	93.99	95.14	91.44

(vi) Hemocompatibility Study of Atorvastatin-loaded Chitosan Formulations

Fresh blood from a healthy volunteer was collected in a blood collection tube containing anticoagulant (EDTA, 0.5 mg). The blood was centrifuged (2000 rpm, 20 min) at room temperature using REMI 24C centrifuge. The concentrated leukocyte band (a Buffy-coat) and a small portion of the plasma was removed. Later, the concentrated RBCs in the packed cells were separately collected and washed thrice with normal saline (0.9% NaCl). The RBC cells and saline were taken in 1:1 ratio and centrifuged (2000 rpm, 10 min). The supernatant was discarded and washings were repeated thrice. Washed RBCs were further diluted to a 50% hematocrit by adding normal saline. Hemolysis experiments were followed in accordance with a method used previously in our laboratory with slight modifications. 100 µl cell suspension taken into a clean dry test tube was added with an appropriate negative control in normotonic condition. The positive control was prepared with 100 µl cell suspension by diluting with double distilled deionized water (3ml) and the RBCs lysis was compared. An aliquot of 100 µl of ACN1, ACN2 and ACN3 formulations were taken, where drug-free chitosan nanoparticles formulation (chitosan polymer-alone) served as blank nanoparticles. Then, each formulation was added with 100 µl of RBC suspension and made to 3 ml by adding normal saline. The experiment was carried out in the triplicate. All the samples were incubated at 37°C for 1 hour in a water bath (ILE instrument, Bangalore). The reaction was terminated using 50 µl of gluteraldehyde (2.5%). The samples were then centrifuged at 1000 rpm for 15 min and the absorbance of the supernatant was measured at 246 nm using UV-VIS spectrophotometer (Optizen 2120UV Plus, Mecasys co., Ltd, Korea)[21].

$$\text{Hemolysis (\%)} = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the positive control}} \times 100$$

RESULTS AND DISCUSSION

As the size matters in delivering the cargo in drug delivery systems, it necessitates the appropriate formulations by achieving the better drug and polymer ratio for potential therapeutic effects. The three formulations ACN1, ACN2 and ACN3 were measured by dynamic light scattering at 27ºC, which exhibited differences in their size characteristics ranging between 220 nm and 314 nm (Table. 1). The encapsulation efficiency of the formulations was determined by centrifugation method (Table 2). Of three different formulations, ACN1 (77.48%) exhibited the highest encapsulation than the rest ACN2 (68.55%) and ACN3 (65.23%). The variation between the size and percent encapsulation formulations were due to the concentration of drug (5mg) with respect to and polymer ratio (1:2, 1:4 and 1:6) in the presence of tween 80 used as detergent (1ml).

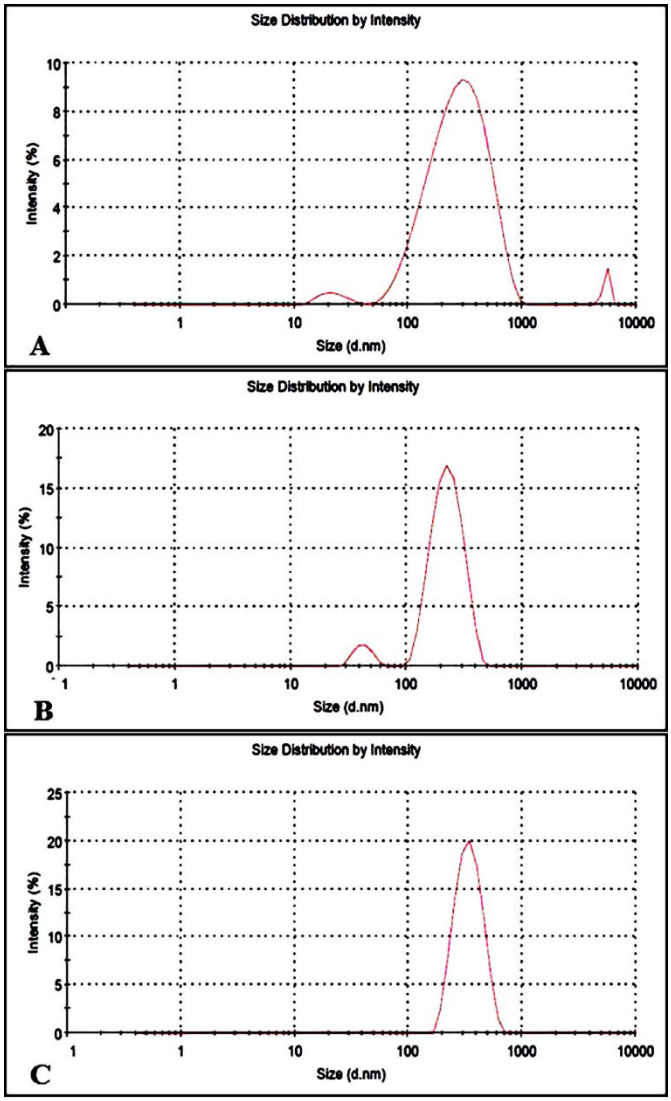

Figure 1
Size Intensity of Atorvastatin-loaded Chitosan Formulation (A. ACN1, B. ACN2, C. ACN3)

The *in vitro* release of Atorvastatin (Table 3) from ACN1, ACN2 and ACN3 formulations exhibited burst release (19.20-22.52 %) at first 30 min and later on showed a kind of monophasic release pattern with a gradual increase in releasing drug until 12 hours period (63.97-76.35%). This sudden burst effect was may be due to the drug present on the surface of the particles. The instantaneous release from the initial burst effect would allow the drug to gain an opportunity to enter into plasma *in vivo* to exert the pharmacological activity at the nanoscale level. In the subsequent continued phase of release drug showed a kind of gradual retardation and the remaining releases accounted in the range 63.97–76.35 (%). This was due to drug and polymer concentration in the formulations. The increased concentration of the polymer increased the size of the particles. This would allow better diffusion of the dissolution medium in to the particles, otherwise which will slow down the dissolution due to the thickened wall of the particles wrapping around the polymer over and over again resulting in multi-layered encapsulation. It is evident from the formulations that as the particle size increases from ACN1 - ACN3 with respect to increased polymer concentration, the drug release gets retarded during 12 hours.

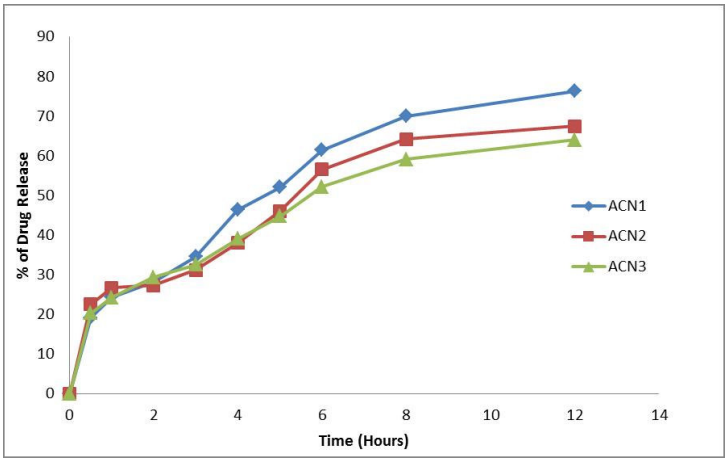

Figure 2
In Vitro Release of Atorvastatin from ACN1, ACN2 and ACN3 Formulations

In general, the ACN1, ACN2 and ACN3 formulations exhibited a steady and slow sustained release of drug. Of three formulations, ACN1 formulation was more stable that of ACN2 and ACN3 with respect to time and temperature (Tables. 3 and Fig 2 & 3). *In vitro* blood compatibility tests of nanoparticles formulations (ACN1, ACN2 and ACN3) were carried out to assess the percent hemolysis. In this compatibility study, all the three formulations were found to be non-toxic to the RBCs and that could be considered for *in vivo* studies to further the investigations. The formulations had appropriate amount of atorvastatin (5 mg) and the drug released from the nanoparticles would not cause any hemolysis at that concentration (Figure 4). In comparison, atorvastatin-loaded chitosan nanoparticles (ACN1, ACN2 and ACN3) were found to be potential formulations for sustained release of drug.

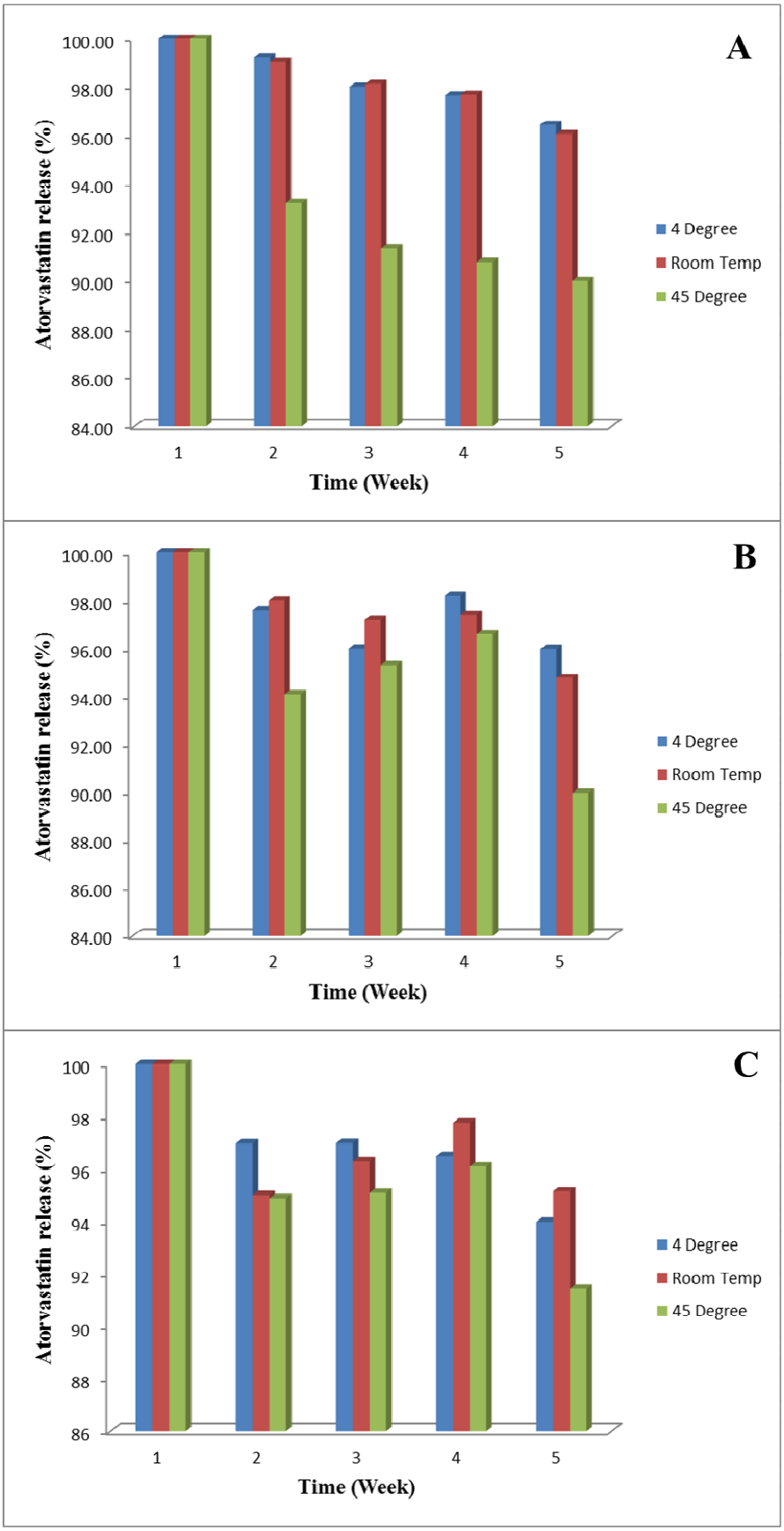

Figure 3
Stability Studies of Atorvastatin-loaded Chitosan Formulation (A. ACN1, B. ACN2, C. ACN3)

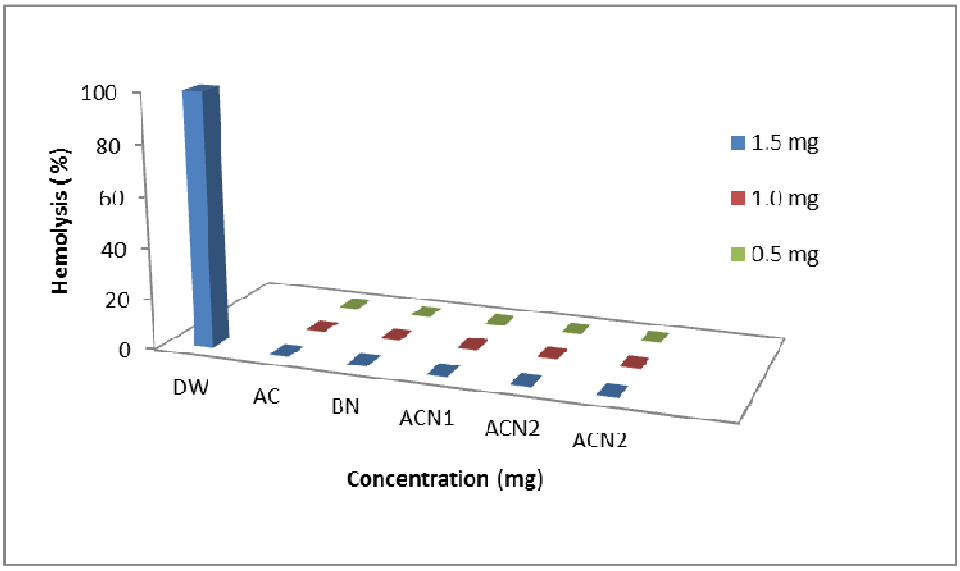

Figure 4
Hemolysis (%) of Atorvastatin-loaded Chitosan Formulations

CONCLUSION

Atorvastatin-loaded chitosan formulations ACN1, ACN2 and ACN3 were prepared by ionic gelation method, characterized and the hemocompatibility was investigated. The formulations were found to have excellent encapsulation efficiency and better stability. It may be suggested from the information that drug-loaded nanoparticles are pharmacologically active and sustained release of atorvastatin from the nanoparticles failed to produce hemolysis. All the formulations exhibited retarded release of atorvastatin in 12 hours study duration. The hemolysis studies showed negligible amount of toxicity. Based on their hemocompatibility characteristics all the three formulations of atorvastatin-loaded chitosan nanoparticles could be considered as effective for chemotherapeutic applications.

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