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Original Article

Preparation and Evaluation of Diphtheria Toxoid-Containing Microspheres

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Abstract

Preparation of chitosan (CS) microspheres as a novel drug delivery vehicle for intranasal immunization using high, medium and low CS molecular weight (MW) was investigated in this study. Diphtheria toxoid (DT) was used as a model antigen. The emulsion-solidification method was adopted for microencapsulation of DT. In the first step, following the purification of semi-crude DT by the ion-exchange column chromatography technique, the antigenicity and biological characteristics of DT were investigated by the bicinchoninic acid protein assay, ELISA and western blot techniques. Results showed that the purification process was successful and the purified toxoid gave an activity of 1500 Lf/ml; which was three times more than that of the semi-crude toxoid. Next, DT-loaded microspheres were prepared and characterized for their surface morphology, particle size distribution, loading efficiency and in-vitro antigenically active DT release. This study showed that the loading efficiency of CS microspheres depends on the MW, as well as the type of cross-linker used, such that, microspheres prepared by high MW CS and glutaraldehyde (cross-linking agent) had the highest DT loading level (95.61±3.57 percent). Size distribution studies showed that the particle size of microspheres prepared by low and medium MW CS solutions with a concentration of 1 %*w/v* was below 10 μm. These microspheres also had a smoother surface morphology than those prepared using high MW CS solutions with concentrations above 1 %*w/v*. In addition, by investigating the antigenicity of the prepared CS microsphere, no significant reduction in the activity of DT before and after microencapsulation was noted. Finally, in-vitro release studies showed an initial burst effect followed by an extended release of antigenically active DT over a period of 15 days.

Keywords: Diphtheria toxoid; Diphtheria toxoid purification and characterization; Chitosan microspheres; Emulsion-solidification method; In-vitro release.

Introduction

Most of the human pathogens causing diseases such as measles, pertussis, diphtheria and influenza enter body through the mucosal surfaces. Nevertheless, the majority of conventional vaccines employed against these agents are used parenterally, suffering from an undesirable patient compliance and comfort. Hence, it seems that mucosal immunization is a good solution to overcome this problem. The mucosal surfaces of human body compose an area over 400 m^2 (1). There are several mucosal routes for immunization including nasal, oral, vaginal and rectal. It is now well established that nasally administered vaccines can provide effective immuno-stimulation both in term of humoral and cell-mediated responses (2-4). In recent years, the nasal route has received a great deal of attention as a convenient and reliable method for systemic administration of drugs and vaccines (3, 4). The major reasons put forward for using nasal cavity for the delivery of vaccines are: a) nasal mucosa is the first site of contact with inhaled antigen, b) nasal passages are rich in nasal associated lymphoid tissue (NALT), c) achieving of both mucosal (sIgA) and systemic (IgG) immune responses, d) an

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age-related decline in systemic immune responsiveness in human and e) a possible increase in effectiveness in the elderly patients (5-8).

Application of microencapsulation technology to immunization has shown that polymeric delivery systems are indeed capable of continuous antigen release and stimulation of immune system (9-11). On the other hand, intranasal immunization in mice using microencapsulated antigens obtained from Bordetella Pertussis or ricin toxoid has been shown to induce protective immunity against aerosol challenge with the bacteria or toxin (12-14), and optimal protection was achieved when more than one antigen administered simultaneously (13).

During the past two decades, a wide range of biodegradable polymers such as polylactide-co-glycolide (PLGA), starch, chitosan, sodium alginate and dextran have been used to prepare nano- or microspheres as carriers for nasal or oral drug delivery (15-20). A significant problem associated with PLGA microencapsulation is the harsh condition used for encapsulation, such as the possibility of antigen degradation as a consequence of exposure to organic solvents. In addition, during encapsulation process it may also be exposed to high shear, solvent-aqueous interphase, cavitation and localized elevated temperature.

Chitosan (CS) is a polysaccharide comprising copolymer of glucosamine and N-acetylglucosamine and could be obtained from the partial deacetylation of chitin; a material found in exoskeleton of crustaceans such as lobster, prawn and crab and also found in some micro-organisms and in yeast and fungi (21). CS is insoluble in alkaline and neutral pH, but forms salts with organic and inorganic acids. In addition, the amino functional groups of CS are protonated and the resultant soluble polysaccharide is positively charged. A number of properties of CS render it a potentially useful carrier for delivery of vaccines and proteins. It is non-toxic, biocompatible (22, 23) and degrades in body by lysozymes into a common amino sugar, N-acetylglucosamine, which is then incorporated into the synthetic pathway of glycoprotein (24). CS is also known to be a mucoadhesive material (25-27). It could interact with the negatively charged sialic acid residues present within the mucin (28). Hence, in term of a nasal or oral drug carrier, the mucoadhesive properties of CS potentially permit a sustained interaction with the mucosal surfaces, promoting a more effective drug delivery and uptake. Based on our literature review, no previous study has focused on the use of CS as a carrier for the preparation of microspheres containing DT, as a potential system for nasal vaccination. Hence, in this study attempts were made to prepare and evaluate these microspheres in-vitro.

Experimental

Materials

Low, medium and high molecular weight chitosan (CS) (85% deacetylated) samples with viscosities (a 2% *w/v* solution) of 670, 1500 and 15000 cP respectively; bicinchoninic acid (BCA) micro-kit; O-phenylenediamine dihydrocholride (OPD) and conjugated rabbit anti-guinea pig immunoglobulin (IgG) peroixdase were all purchased from Sigma-Aldrich Chemical Co. (USA). Diphtheria toxoid (DT); purified equine DT antitoxin (100 Lf/ml); and purified guinea pig anti-DT IgG were gifted kindly from Razi institute for serums and vaccines (Tehran, Iran). Castor oil was obtained from Baharak Co. (Iran). Protein marker (14-96 kDa) was purchased from Bio-rad Co (USA). Calcium chloride, n-octanol, glutaraldehyde, sodium tripolyphosphate, mineral oil, Tween 80 and Span 80 (reagent grades), and all the other reagents and materials used were obtained from Merck Chemical Co. (Germany).

Methods

Purification and characterization of DT

The semi-cured (semi-crude) DT was purified in two steps by column chromatography. In the first step a sephadex G-25 gel column, eluted with phosphate saline buffer (PBS, 100 mM, pH 7.4) at a rate of 0.5 ml/min was used. In the second step an ion-exchange gel column [sephadex diethylamino ethyl A-50 (sephadex DEAE A-50)]; eluted with tris buffer (0.015 M, pH 7.2) at a rate of 0.4

ml/min was employed. The amount of protein present in different fractions was determined spectrophotometrically (Shimadzu model 210A uv-visible spectrophotometer, Japan) at a wavelength of 280 nm. Fractions containing a high level of protein were then pooled together. Finally, 0.001 %*w/v* Thiomersal (as preservative) was added to the purified sample and stored at 4 ºC until use.

Potency and the total amount of protein present within the purified antigen (DT) were determined by the flocculation test method (Dean-Webb method), using equine DT anti-toxin (29), and the micro-BCA assay (30) respectively. The MW of the DT was also estimated by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the standard pertained markers, using a slightly modified laemmli's method (31). Briefly, the native (unpurified) as well as the purified DT were separately mixed with the PBS solution, boiled for 5 min and subjected to SDS-PAGE under standard operating conditions (a constant current of 25 mA overnight, at room temperature). Protein bands were visualized by staining with Coomassie blue.

Preparation and characterization of CS microspheres

CS microspheres were prepared by a water-in-oil (w/o) emulsion-solidification technique (32, 33). Briefly, aqueous acidic (0.5 %*w/v* acetic acid) CS solutions at various concentrations of 0.5, 1, 1.5 and 2 % *w/v* were prepared. 4 ml samples of these solutions were individually emulsified in 20 ml of an oily phase (n-octanol, mineral oil or castor oil) containing a 1:1 ratio of a mixture of Tween80:Span 80 (as surfactant) at a 1 %*v/v* concentration, using a homogenizer (Heidolf Diax 910, Germany) set at 20000 rpm in an ice bath at 4 ºC for 30 sec. In addition, 100 ml of a second emulsion containing the oily phases mentioned above was prepared using 3 ml of a cross-linker (glutaraldehyde or a 5 %*w/v* Tpp Na solution) along with a 1 % *v/v* mixture (1:1) of Tween 80:Span 80, using a homogenizer set at 20000 rpm for 30 sec. Next, the first emulsion was added to the second emulsion under continuous stirring at 1000 rpm for at least 5 h at room temperature. Microspheres loaded with DT were prepared by adding 312-8790 µg of DT to the polymer (CS) solutions. Prepared microspheres were then collected by centrifugation at 3500 rpm (approximately 1750 ×g) for 30 min. They were then washed with isopropyl alcohol, freeze dried and stored at -20 ºC.

Formation of microspheres was monitored during preparation by the use of an optical microscope (Carl Zeiss, Germany) set at ×500 magnification.

The particle size distribution of microsphere was determined using a particle size analyzer (Analysette 22, Fritsch, Germany). 40 mg microspheres were suspended in a 0.5 %*w/v* particle free sodium dodecyl sulfate solution and sonicated for 2 min in order to prevent any aggregation between CS microspheres present. Data obtained were collected, and particle size distribution of the microspheres was expressed as the geometric mean diameter ± standard deviation.

The surface morphology of microspheres was determined by the use of a scanning electron microscope (SEM). For this purpose, CS microspheres were mounted and coated for 2-3 min with a mixture of gold and palladium, and then examined for morphology using a SEM (DSM 960 A, Zeiss, Germany).

Evaluation of DT encapsulation

Encapsulation efficiency and the yield of microspheres prepared were determined by centrifugation of the samples at ~1750 ×g for 30 min. Microspheres were then incubated at 80ºC overnight and weighed. The amount of DT associated within the microspheres was then calculated by the difference between the total amount (µg) of DT used to prepare the particles and the amount (µg) of DT present in the aqueous phase, determined using the micro-BCA protein assay against a supernatant of blank microspheres (16). Encapsulation efficiency was then calculated as follows:

$$DT\ encapsulation\ efficiency\ (\%) = \frac{total\ DT - free\ DT}{total\ DT}$$

Evaluation of the in-vitro DT release

40 mg microspheres were accurately

Table 1. Statistical parameters of microspheres prepared from 1%w/v chitosan solutions, using glut araldehyde as the cross-linking agent (n=3, mean ± standard deviation).

Chitosan Mw	Geometric diameter (□m)	Mode (□m)	Specific surface area (m^2)
High	6.37 ± 3.51	10.09	1.23 ± 0.54
Medium	4.56 ± 2.68	2.45	2.00 ± 0.98
Low	3.85 ± 2.11	4.92	1.84 ± 0.90

weighed and suspended within enclosed 2 ml eppendorf™ tubes containing 1 ml pH 7.4 PBS solution and incubated at 37°C. At predetermined time intervals, the samples were centrifuged at 10000 ×g for 40 min at 5°C and 0.5 ml supernatant was removed and replaced by fresh release medium. Samples removed were then concentrated by being centrifuged using a centrifugal ultrafilter (Ultrafree-Cl, ultra-4 10000 NMWL, Millipore, Sigma, Germany) at 10000 ×g for 30 min. The amount of released DT was determined using the micro-BCA assay method.

Antigen Activity

The antigen activity before and following microencapsulation was evaluated by the SDS-PAGE Western-blot analysis. For this purpose a hyper-immune serum sample (against DT), raised in guinea pig, was used for binding to the antigen. Antigen samples were then analysed before microencapsulation and after being released from the microspheres, using a 10% SDS-PAGE gel electrophoresis. The blots were visualized with OPD.

In addition, an indirect ELISA method was used for the determination of antigenic activity of DT released in-vitro (34). Equine DT antitoxin (100 □l), present within the pH 7.4 PBS solution, was added to flat-bottom microtitration plates (DYNEX, immulon®, USA) and allowed to incubate overnight. The plates were then washed three times between each step with the pH 7.4 PBS solution containing 0.2 %*v/v* Tween 20 (PBST). To minimize the non-specific interactions, 100 □l PBST solution containing 5 %*w/v* dried skimmed milk powder (PBSTM) was added to the wells and incubated for 2 hr at 37ºC. After washing the plates three times with PBST, purified DT (as the reference) and the test samples were diluted serially in two-fold steps in PBSTM and added to the wells. Plates were then incubated at 37ºC for 2 hr and washed. Next 100 □l hyper-immune serum sample, raised in guinea pig (1:500) and present within PBSTM, was added to the wells and allowed to react for 2 hr at 37ºC. Following this stage 100 □l rabbit anti-guinea pig IgG peroixdase conjugate (1:2500) in PBSTM was added to the wells and incubated for another 2 hr at 37ºC. The plates were then washed and the substrate (0.4 mg/ml OPD), present within 0.05 M citro-phosphate buffer (pH 5), was added to each well. Following color development (30 min) the reaction was stopped by the addition of 1 M sulfuric acid and absorption measured at 492 nm on a microplate reader (Spectra Rainbow V A 88, Austria).

Statistical analysis

For the purpose of statistical comparison of data obtained from this study, the one way analysis of variance (ANOVA) was employed. Bonferroni post-Hoc test was used for further analysis at a significancy level of $p<0.05$. Unpaired student's t-test was also used in some cases as would be described later in the text.

Results and Discussion

As mentioned earlier, CS has been recognized as a suitable biopolymer for protein drug delivery, due to possessing various desirable characteristics. These include suitable biodegradability, ease of ability to prepare micro- and nanospheres, mucoadhesive nature and adhesion to tight junctions of the epithelial cells, and relatively high loading capacity for proteins (25, 35, 36). Hence, in this study, steps were taken to load DT within CS microspheres, based on the desirable properties of CS, as a novel nasal drug delivery system for vaccination.

Prior to loading the DT within CS microspheres, the available semi-crude toxoid was purified. The total concentration and potency of purified DT were found to increase three folds compared with the semi-crude toxoid, reaching values of 5.86± 0.16 mg/ml and

1500 Lime flocculation/ml (Lf/ml), respectively. In addition, based on the SDS-PAGE studies (data not shown), and in agreement with the literature (37), the presence of two distinct bands relating to two major structural components with MWs of 20 and 43 KDa was confirmed. No extra bands were observed, confirming the purity of the purified DT prepared. Hence, it seems that the purification process was successful, producing a DT with increasing potency and greater toxoid concentration, as well as high purity and therefore good immunogenicity. Moreover, characterization of the purified DT, using the Western blot analysis (data not shown) as well as the ELISA method, before and after encapsulation within the CS microspheres, also confirmed that the purity, integrity, and immunogenicity of the encapsulated DT remains intact and unaltered. This finding suggests that the process of encapsulation has no adverse effect on the nature, integrity, activity and immunogenic properties of DT.

In this study, the W/O emulsion-solidification method was used for the incorporation of DT within the CS

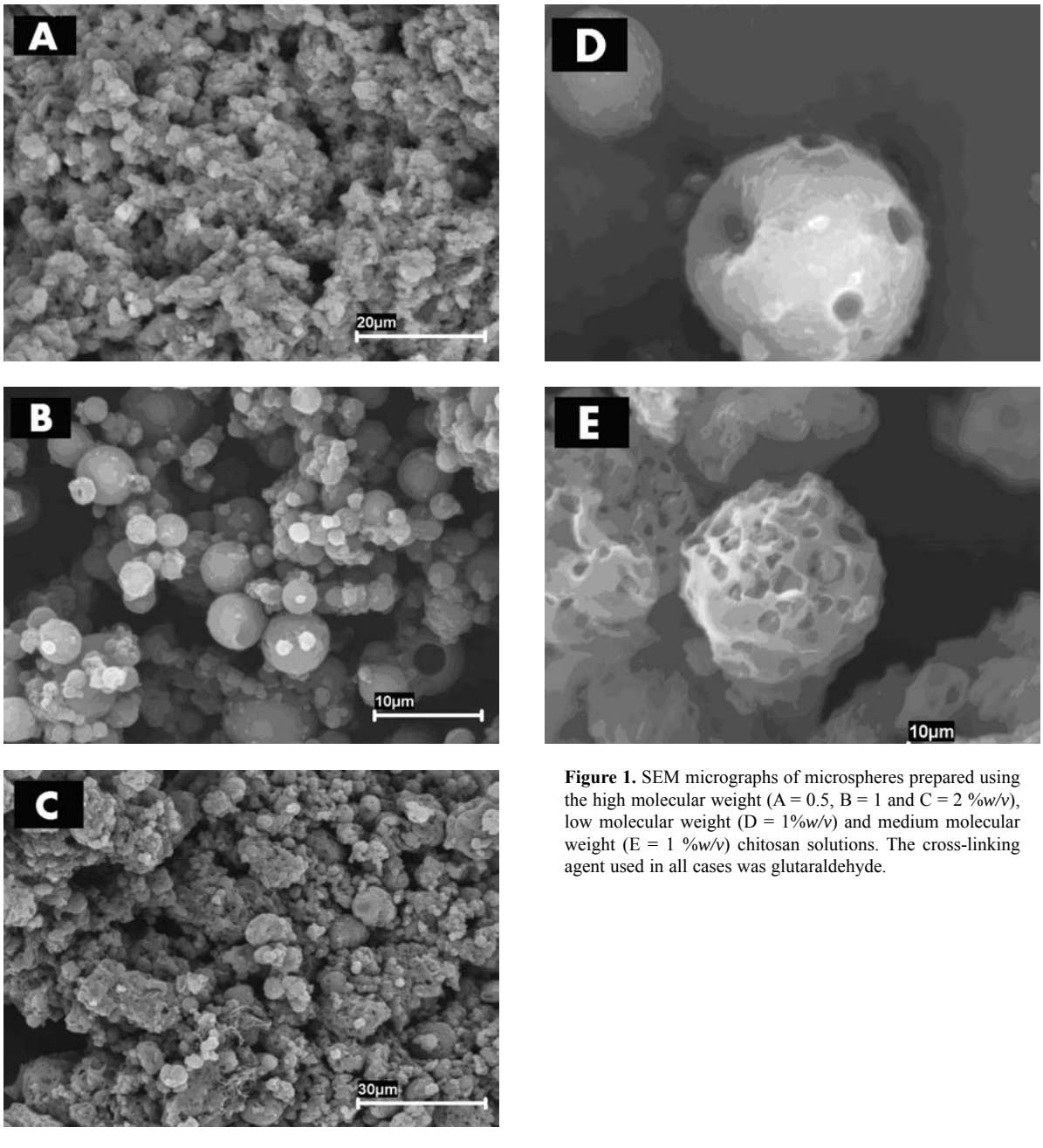

Figure 1. SEM micrographs of microspheres prepared using the high molecular weight (A = 0.5, B = 1 and C = 2 %*w/v*), low molecular weight (D = 1%*w/v*) and medium molecular weight (E = 1 %*w/v*) chitosan solutions. The cross-linking agent used in all cases was glutaraldehyde.

microspheres. Previous studies have shown that CS-containing emulsion could only remain stable if a mixture of Tween and Span are used (38). The use of these emulsifiers individually results in the instability of the emulsion. Hence, a mixture of Tween 80:Span 80 (1:1) was used in this study. Furthermore, by investigating various oily phases (n-octanol, mineral oil and castor oil), it was found that castor oil, which has a greater viscosity (around 250 cP) than the other two oily phases, could produce more stable emulsions (investigated by visualization under a light microscope) and therefore homogenously distributed microspheres.

In the first part of this study, the effect of CS MW on the characteristics of the resulting microspheres was investigated. The formation of microspheres was closely monitored under a light microscope as well as SEM, as described earlier. Figures 1 and 2 show the SEM micrographs of some of CS microspheres, made using various MW CS solutions with different concentrations. Based on this study, microspheres prepared from low and medium MW CS solutions with polymer concentrations around 1-1.5 %*w/v* (viscosities around 300-450 cP) as well as a high MW CS solution with a concentration of 1 %*w/v* (viscosity around 1500 cP) were found to be smaller, more regularly shaped, and had a smoother and more uniform surface (specially those prepared with low and medium MW CS solution) than those produced with solutions containing higher CS concentrations. Therefore, based on these preliminary studies and for the purpose of comparison, among the various concentrations of CS investigated, a 1 % *w/v* CS solution (of all MW) was found to be most appropriate and hence used for the preparation of CS microspheres throughout this study.

Table 1 shows the particle size distribution of CS microspheres prepared, using 1 %*w/v* CS solutions with varying MWs. All the microspheres prepared had a mean diameter below 7 □m. This could aid their uptake by NALT system (39). Figure 3 shows the particle size distribution of a 1 % *w/v* high MW CS solution. It is clear that the prepared microspheres have a monomodal distribution. This study also shows that as a result of an

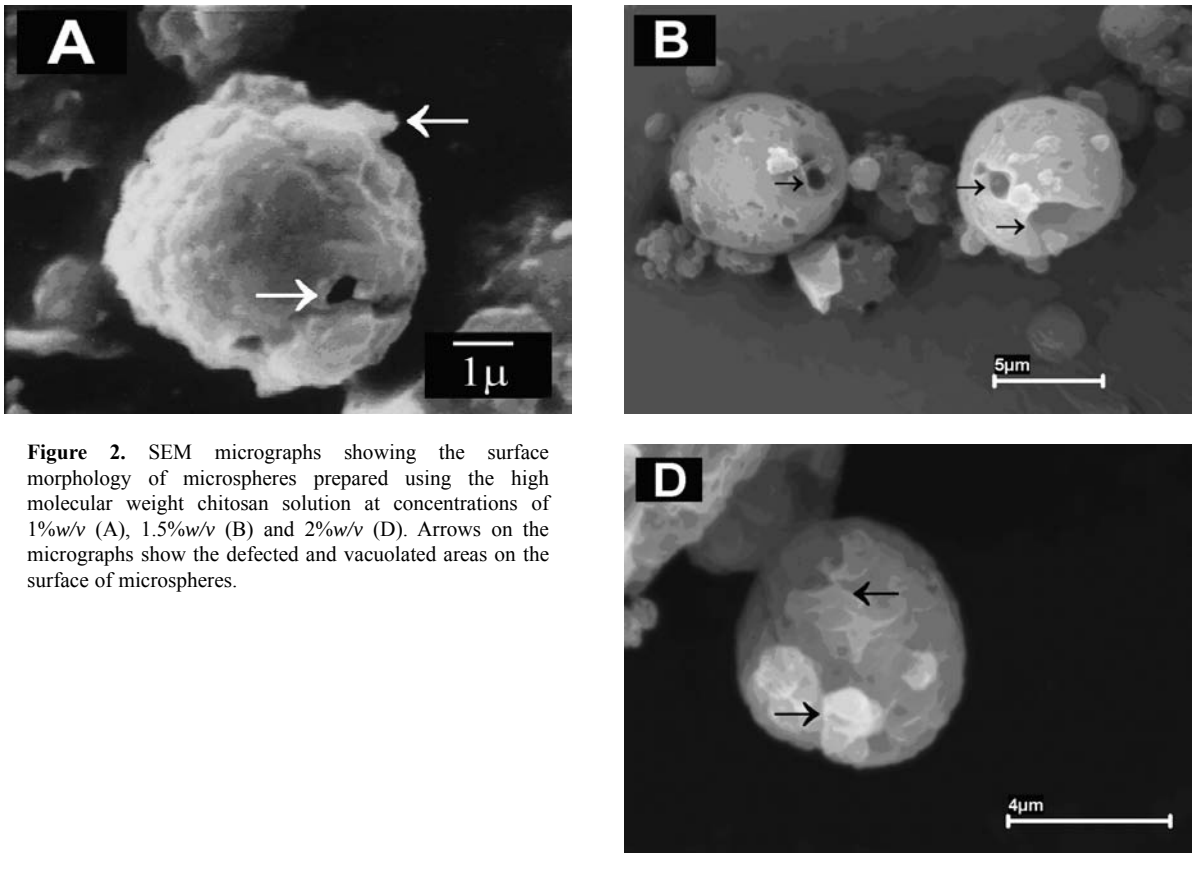

Figure 2. SEM micrographs showing the surface morphology of microspheres prepared using the high molecular weight chitosan solution at concentrations of 1%*w/v* (A), 1.5%*w/v* (B) and 2%*w/v* (D). Arrows on the micrographs show the defected and vacuolated areas on the surface of microspheres.

Table 2. Effect of the molecular weight of 1%w/v chitosan solutions as well as the type of the cross -linking agent used, on the loading level and encapsulation efficiency of diphtheria toxoid within the prepared microspheres (n=3, mean ± standard deviation).

Chitosan Mw	Cross-linker	Theoretical loading (ì g)	Actual loading (□g)	Encapsulation efficiency (%)
Low	Glu	1367	270.85 ± 12.19	19.81 ± 0.89
Medium		1367	470.75 ± 24.14	34.42 ± 1.77
High		1367	671.20 ± 55.16	49.09 ± 4.03
Low	Na Tpp	1367	254.36 ± 29.81	18.60 ± 2.18
Medium		1367	431.92 ± 8.56	31.59 ± 0.63
High		1367	641.44 ± 66.06	49.40 ± 4.83

increase in the MW, as well as viscosity of CS solution (especially with high MW CS), the surface of microspheres becomes slightly rough and somewhat vacuolated (as shown in Figure 2). This is speculated to be due to the slower permeability rate of the cross-linking agent into the microspheres, as a result of an increase in the viscosity of the CS solution. This could in turn result in a delayed cross-linking of the polymer chains. Hence, a contraction or shrinkge of the superficial polymer chains could be observed, and the surface to volume ratio would be reduced. This could lead to the appearance of void spaces and eventually pits on the surface of microspheres. On the other hand, the free (uncross-linked) cross-linking agent could leak out of the interior parts of the microspheres, and form cross-links on the surface of microspheres, resulting in the formation of a slightly rough and irregular surface.

Table 2 shows the results obtained from assessing the loading level and encapsulation efficiency of DT within microspheres prepared from 1 %*w/v* CS solutions of different MWs. As could be seen, the actual amount of DT loaded within CS microspheres, and as a result, their encapsulation efficiencies are significantly (ANOVA, $p<0.05$) affected by the MW of the CS solution. By increasing the MW and therefore polymer chain length of CS, the amount of DT loaded within the microspheres increases. Therefore, it seems that longer polymer chains could slightly coil up and entangle better than the smaller chains, enhancing the absorption and entrapment of DT molecules.

In continuation of this study, in order to investigate the influence of the amount of DT added to the original CS-containing emulsion on the toxoid loading level and encapsulation efficiency within the microspheres, the high MW CS (1 %*w/v* solution), which showed the greatest loading levels of toxoid and had a reasonably small size and acceptable morphology, was used. Results obtained are presented in Table 3. It could be seen that by increasing the original amount (theoretical loading) of DT during the preparation of microspheres, the actual amount of toxoid loaded also initially increases significantly (ANOVA, $p<0.05$) and then reaches a plateau in the presence of 1367 □g and higher amounts of DT. In fact the encapsulation efficiency of DT was found to decrease significantly (ANOVA, $p<0.05$) as a result of an increase in the original amount of toxoid used. CS is a cationic biopolymer and at pH values between 5.5-6.0, which were used for the preparation of microspheres, is expected to be positively charged (36). On the other hand, DT has a pI of around 4.5-5.5 and hence at the pH values mentioned above is expected to be negatively charged. Hence, the polymeric chains of CS could bind to the negatively charged DT chains via electrostatic and hydrogen bonds. However, at a fixed CS concentration (in here 1 %*w/v* solution), its binding capacity would also be specific. Hence, the presence of excess amounts of DT could not be encapsulated within the microspheres. As a result, the loading level of

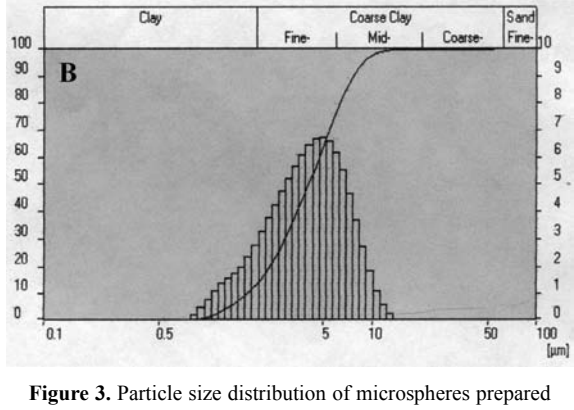

Figure 3. Particle size distribution of microspheres prepared using a 1%*w/v* high molecular weight chitosan solution (n=3).

Table 3. Effect of the amount of diphtheria toxoid added (theoretical loading) as well as the type of cross -linking agent used, on the loading level and encapsulation efficiency of toxoid within microspheres prepared from a 1%w/v high molecular weight chito san solution (n=3, mean ± standard deviation).

Cross-linker	Theoretical loading (ì g)	Actual loading (ì g)	Encapsulation efficiency (%)
Glu	312	298.82 ± 11.13	95.61 ± 3.57
	781	602.49 ± 24.89	77.11 ± 3.19
	1367	671.20 ± 55.16	49.09 ± 4.03
	2930	718.01 ± 68.91	24.51 ± 2.35
	5860	700.11 ± 41.95	11.95 ± 0.72
	8790	724.37 ± 45.89	8.24 ± 0.52
Na Tpp	312	286.12 ± 17.62	91.55 ± 5.65
	781	589.58 ± 22.84	75.42 ± 2.92
	1367	610.44 ± 40.21	44.66 ± 2.94
	2930	615.35 ± 44.58	21.00 ± 1.52
	5860	619.94 ± 85.52	10.58 ± 1.46
	8790	622.49 ± 35.57	7.08 ± 0.41

toxoid within the microspheres reaches a plateau and overall the encapsulation efficiency would be reduced. This study also shows that the nature of the cross-linking agent could influence the loading level and encapsulation efficiency of DT within microspheres (Table 3). As could be seen, the presence of neutrally charged glutaraldehyde has resulted in a greater loading of DT within the prepared microspheres, compared with the anionic Na Tpp solution. This finding could be due to the fact that the negatively charged cross-linker, Na Tpp, might counteract and partially bind to the positively charged polymeric chains of CS. Hence, neutralizing them results in a less expanded CS polymeric network and a reduced uptake and loading of the negatively charged DT.

The total and antigenically active DT profile of in-vitro release from CS microspheres in the pH 7.4 PBS solution over a period of 15 days is depicted in Figure 4 and Table 4. All formulations exhibited a biphasic release profile. A rapid DT release over the first 24 hr followed by a slow release for up to 15 days was noted. The first release phase could be attributed to the release of DT molecules which were loosely associated with the CS molecules and located near the surface of the microspheres. In this phase a sudden burst effect was observed in all formulations regardless of the type of cross-linker and MW of CS used for the preparation of microspheres, whereas, the second release phase could correspond to those DT molecules that are more efficiently entrapped and tightly bound to the CS molecules. Further release could be expected from these microspheres, but release studies were not continued after this time because of the particle aggregation, which took place after a number of centrifugation/resuspension cycles during the release studies. With respect to the influence of CS MW on the release process, results showed a slower release rate of DT at the second release phase, as the CS MW increased. This could be attributed to an easier detachment of the low MW CS and DT molecules within the microspheres, and/or due to a decrease in the porosity and hence diffusion of DT out of the CS microspheres. Previous studies have also shown that the major factors affecting the release of proteins from CS nano- or microspheres are the chemical structure of the protein, and its interaction with CS molecules in the release medium (16). Furthermore, by comparing the type of cross-linker used for the preparation of DT microspheres, it was found that regardless of the CS MW used, the effect of Na Tpp on increasing the DT release rate was significantly

Table 4. The percentage of distribution of antigenically active DT released in -vitro with respect to the amount of DT released from CS microspheres made of different MWs and cross-linkers (n=3, mean ± standard deviation).

Cross-linker	Polymer Mw	Time (hr) 1	10	24	48	96	192
Glu	high	66.79±4.45	37.37±7.28	13.81±1.70	4.98±2.31	5.31±1.29	n.d
	medium	58.25±2.76	21.09±1.29	10.08±1.37	2.91±1.74	n.d	n.d
	low	14.84±2.47	10.88±1.52	7.16±1.97	1.65±1.14	0.86±0.52	n.d
Na Tpp	High	50.88±4.20	30.47±3.07	12.37±2.22	3.33±0.89	0.26±0.3	n.d
	medium	45.11±4.31	26.18±3.36	6.83±1.63	1.28±0.87	1.38±0.37	n.d
	low	17.65±1.84	9.57±1.66	4.35±0.68	1.90±0.97	n.d	n.d

n.d =not detectable

greater than that of glutaraldehyde (t-test, $p<0.05$). However, under the experimental condition used in this study, the important point to emphasize is that DT is released from the microspheres in its antigenically active form. This is a crucial issue since DT is known to be easily inactivated during the course of the release studies, when associated with hydrophobic polymers such as PLGA (40, 41). Moreover, it is important to emphasize that these data can not be extrapolated to the in-vivo scenario, since following nasal administration the particles do not necessarily undergo a dilution process. Furthermore, the physiological conditions under which DT release may occur are expected to be quite different from those of in-vitro. Therefore, the in-vitro release data only indicate that DT is released in an active form for an extended period of time.

Conclusion

In conclusion, based on the results obtained from this study it seems that the use of high MW CS microspheres, prepared using Glu and Na Tpp as the cross-linking agent, appears to provide a successful vehicle for the delivery of DT. The microspheres prepared maintained their antigenicity following encapsulation and their size distribution were found to be acceptable. In addition, they managed to produce an extended profile of DT release over a period of 15 days. Hence, in-vitro studies suggest their effectiveness as a suitable basis for the delivery of DT.

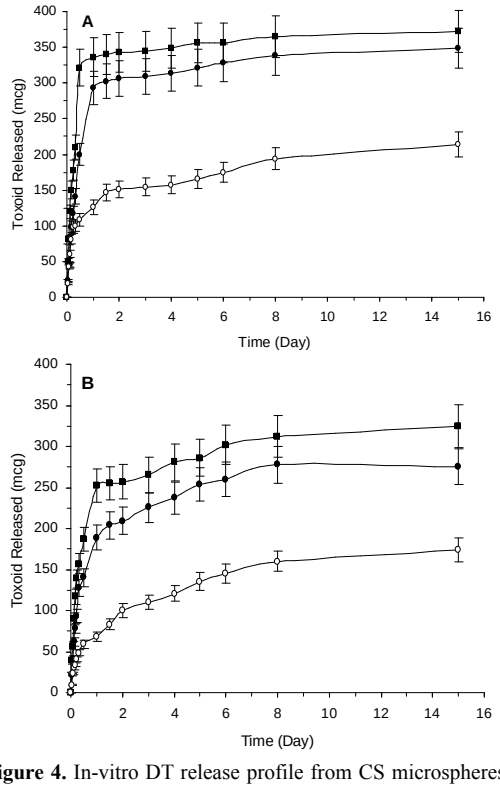

Figure 4. In-vitro DT release profile from CS microspheres prepared using (■) high, (●) medium and (o) low MW CS, cross-linked with: A) Glu and B) Na Tpp (n=3, mean ± standard deviation).

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