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Preparation and in-vitro evaluation of sodium alginate microspheres containing diphtheria toxoid as new vaccine delivery

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ABSTRACT

During last two decades, polysaccharides such as alginate (Alg) alone and in combination with other biopolymers are widely used in vaccine and drug delivery systems. The aim of the present work was to investigate the potential utility of microparticles made of alginate (Alg) as new vehicles for improving nasal vaccine delivery. For this purpose, diphtheria toxoid (DT) was chosen as a model antigen. DT was entrapped within microparticles made of Alg of different molecular weight cross-linked using 1M $CaCl_2$ or 3.75 %w/w $CaCl_2$ in n-octanol. DT-loaded microparticles were characterized for their size, loading efficiency and *in vitro* release of toxoid. The resulting microparticles had a size, which varied depending on formulation conditions and Alg Mw. The results of the *in vitro* release studies displayed a biphasic release of toxoid, the intensity of the first phase being less pronounced for microparticles cross linked with aqueous $CaCl_2$ than octanolic $CaCl_2$.

Keywords: Alginate microspheres, Alginate molecular weight, $CaCl_2$ cross linking agent, Diphtheria Toxoid

INTRODUCTION

Vaccination has become one of the most successfully employed therapies to achieve protection against environmental potentially harmful organisms. Unfortunately, the most human pathogens initiate their infection processes at mucosal surfaces. However, traditional vaccines have been developed in order to achieve systemic immunity by means of a parenteral injection (Sah *et al* 1996). Mucosal immunization is an attractive alternative to parenteral immunization as with the appropriate delivery system it is possible to stimulate both humoral and cell-mediated responses and to induce mucosal and systemic immunity simultaneously, especially if the antigen is adjuvanted using an immunostimulator or a delivery system (Saraf *et al* 2006). Nowadays, several mucosal surfaces such as the nasal, pulmonary and

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per-oral mucosa are being extensively explored as alternative routs for the systemic administration of macromolecular drugs and vaccines. Among mucosal sites, the nasal route of administration has gained in interest. The large absorptive surface and high vascularity of the nasal mucosa ensure a rapid absorption of compounds under circumvention of the hepatic first pass elimination (Merkus *et al* 1994). The easy accessibility of the nasal route facilitates self-medication thus improving patient compliance when compared to parenteral routes (Pontiroli *et al* 1994). Another advantage of mucosal immunization is to induce disseminated immune response at distant mucosal sites due to the existence of common mucosal immune system (Partidos 2000, De Magistris 2006). In recent years, there has been an increasing interest in the development of novel vaccine systems for prophylactic and therapeutics purposes. To prevent degradation and increase the absorption of administered antigen, novel strategies including enclosure of antigen in biodegradable microspheres or liposomes or their expression in viral or bacterial vectors and in plants are currently being considered (Partidos 2000)

Among the delivery systems investigated until now, the use of microparticles made of hydrophobic/philic polymers such as the poly(lactic-co-glycolic acid) (PLGA) copolymers (Almeida *et al* 1993, Ray *et al* 1993, Moor *et al* 1995, Shanin 1995, Eyles *et al* 1998) chitosan (Prego *et al* 2005) and alginate (Koch *et al* 2003) offer great promise for nasal vaccination. More recent work carried out by researchers have revealed that the size and surface composition play a significant role on the ability of these biodegradable particles to transport the associated antigen across the nasal mucosa and elicit an immune response.

During this last decade many of biodegradable polymers such as polysaccharides (such as alginate, chitosan), polyesters (poly lactic acid, poly glycolic acid and poly caprolacton) have been used as devices for protein and drug delivery. Among them the polysaccharides and their derivatives have more attractive attentions. Alginate (Alg) is an anionic linear polysaccharide consisting of β-(1→4)-D-mannuronic acid (M-block) and α-(1→4)-L-guluronic acid (G-block). Sodium alginate is gelled when contacted with calcium ions in solution by cross linking between the carboxylate anions of alginate guluronate units and the calcium ions (Kikuchi *et al* 1997). Alginate microspheres are produced by two major techniques, extrusion (Murata *et al* 1993, Lee *et al* 1996, Thu *et al* 1996) and emulsification (Wan *et al* 1992, Chan *et al* 1997, Gürsoy *et al* 1998, Rebeiro *et al* 1999). A variety of core materials such as drugs (Gürsoy *et al* 1998 a, Bodmeier *et al* 1992, Acartürk *et al* 1999, Gürsoy *et al* 2000 b), proteins (Leonard *et al* 2004, Burak 2007), DNA (Jiang *et al* 2007) and microorganisms (Quong *et al* 1999) have been encapsulated in an alginate matrix. These microspheres are stable in acidic media but easily swell and disintegrate in alkaline media (Acartürk *et al* 1999). As calcium ions are being released by the ion exchange with sodium ions in the medium, electrostatic repulsion between the carboxylate anions further accelerates the swelling and erosion of alginate gels (Kikuchi *et al* 1997). Alginate has several applications in food and pharmaceutical technologies and its gelification by divalent cations is well known and frequently used for several applications (Cui *et al* 2000). Based on this information, the aim of the present study was to explore further the potential of Alg microspheres as a vehicle for the nasal administration of vaccines.

MATERIALS AND METHODS

Low and high molecular weight sodium Alg samples with apparent viscosities (a 2% w/v solution) of 1500 and 14000 cP respectively purchase from Sigma-Aldrich Chemical (Germany), Purified Diphtheria Toxoid (DT, 1500 Lf/ml) in

phosphate buffer saline pH 7.4, purified equine DT anti-toxin (100 Lf/ml) and purified guinea pig anti-DT IgG gifted kindly from Razi Vaccine and Serum Research Institute (Tehran-Iran). Purified guinea pig immunoglobulin (reagent grade), rabbit anti-guinea pig immunoglobulin G (whole molecule) peroxidase conjugate, o-phenylenediamine dihydrochloride (OPD) and trehalose were supplied by Sigma-Alderich Chemical (USA). Calcium chloride, n-Octanol and mineral oil were purchased from Merk Co. (Germany) and castor oil was gifted by Baharak chemical Co. (Tehran-Iran)

Alg microspheres preparation. Alg microspheres were prepared by water-in-oil (w/o) emulsion-solidification technique (Heng *et al* 2003). Briefly, aqueous Alg 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 Tween® 80: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 (1 M aqueous or 3.75 %w/v octanolic $CaCl_2$) 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 2 h at room temperature. Microspheres loaded with DT were prepared by adding 312-8790 µg of DT to the polymer (Alg) solutions. Prepared microspheres were then collected by centrifugation at 2000 ×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 Alg 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, Alg 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 and in-vitro release. Encapsulation efficiency and the yield of microspheres prepared were determined by centrifugation of the samples at 10000 ×g for 30 min. Microspheres were then dried at 80 ºC overnight and weighed. The amount of DT associated within the microspheres was then calculated by dissolving the microspheres completely in the sodium citrate buffer, pH 7.4 for 24 h with magnetic stirring. The total protein loaded within microspheres was determined using the micro-BCA protein assay against blank microspheres (Heng *et al* 2003, Di Toro *et al* 2004). The in-vitro release carried out in PBS solution, pH 7.4. A known amount (40 mg) of microspheres were accurately weighed and suspended within enclosed 1.5 ml eppendorf™ tubes containing 1 ml PBS solution, pH 7.4 and incubated at 37 °C. At pre-determined time intervals, the samples were centrifuged at 10000 ×g for 40 min at 5°C and supernatant was removed and replaced by fresh release medium. Samples removed were then concentrated (if it was necessary) by being centrifuged using a centrifugal ultra-filter (Ultrafree-Cl, ultra-4 10000 NMWL, Millipore, Sigma, Germany) at 5000 ×g for 40 min at 5 °C and

the amount of DT released was determined using the micro-BCA assay method.

Antigen integrity. The antigen integrity before and following microencapsulation was evaluated by the SDS-PAGE and 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 integrity of DT released in-vitro (Goraltchouk *et al* 2004). Briefly, flat-bottom micro-titration plates (DYNEX, immulon®, USA) was coated with equine DT antitoxin. After washing the plates, purified DT (as the reference) and the test samples diluted serially in two-fold steps were added to the wells. Plates were then incubated at 37 ºC for 2 h and washed. Next hyper-immune serum sample, raised in guinea pig (1:500) was added to the wells and allowed to react for 2 h at 37 ºC. Following this stage rabbit anti-guinea pig IgG peroixdase conjugate (1:2500) was added to the wells and incubated for another 2 h at 37 ºC. The plates were then washed and the substrate 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 micro-plate reader (Spectra Rainbow V A 88, Austria).

Statistical analysis. For the purpose of statistical comparison of data obtained from this study, General Linear Model test ($p<0.05$) was used to determine between groups difference. Unpaired student's t-test ($p<0.05$) was also used in some cases as would be described later in the text.

RESULTS

Preparation and characterization of Alg microspheres. Alg microspheres were prepared base on the water-in-oil (w/o) emulsion-solidification technique. Stability and homogenously distributed globules of primary emulsion prepared was evaluated using light microscope (Figure 1).

In order to find the appropriate conditions for the association of DT to Alg microspheres it was first evaluated the influence of two critical formulation parameters, such as the oily phase type and the Alg Mw and their concentrations. The SEM micrographs and particle size distribution of these microspheres are shown in Figure 2 and 3. Efficiency of Alg microspheres for encapsulation of DT based on amount of toxoid added to the primary emulsion is shown in Figure 4.

In vitro release behavior. The in vitro release of microspheres was carried out at 37 ºC for 50 h. in PBS pH 7.2. Because of aggregation of microspheres during centrifugation/re-suspension cycles, the study was discontinued after this time. The results of the in vitro release experiments performed for the two selected formulations are shown in Figure 5.

DISCUSSION

As indicated in the introductory section, the investigators have previously shown that Alg, chitosan and many of polysaccharides micro-nanoparticles, administered as an aqueous suspension or dry powder, are able to enhance the transport of macromolecular proteins, i.e. insulin (Naik *et al* 2005) and some antigens such as tetanus (Fernández-Urrusuno *et al* 1999) and diphtheria toxoid across the nasal mucosa (Vila *et al* 2004, Mortazavi *et al* 2004). Alg 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 the epithelial cells, and

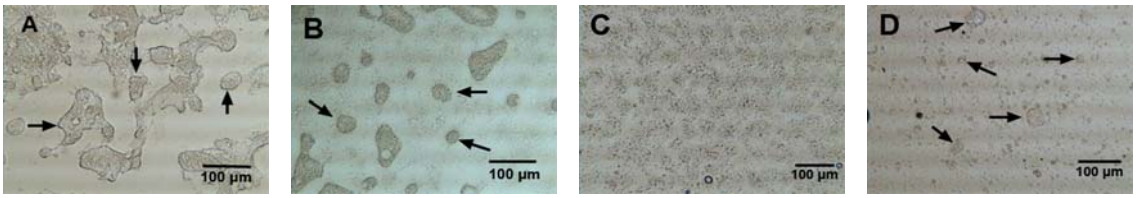

Figure 1. Light microscopic photographs of primary emulsion of Alg solutions in: A) n-Octanol, B) liquid paraffin, C & D) Alg 1 %w/v and Alg 2%w/v in castor oil, respectively. (Arrows show Alg globules).

relatively high loading capacity for proteins (Jiang *et al* 2007, Rezaei Mokarram *et al* 2006, Schneider *et al* 2003, Xing *et al* 2003). Hence, in this study, steps were taken to load DT (as an antigen model)within Alg microspheres, based on the desirable properties of Alg, as a carrier for intranasal vaccine delivery system.

Preparation and characterization of Alg microspheres. In order to find the appropriate conditions for the association of DT within Alg microspheres, primarily, the influence of two critical formulation parameters, such as the oily phase type and the Alg Mw and their concentrations were evaluated. During the study, we found that Alg solutions emulsified by a 50:50 ratio of Tween® 80: Span® 80 mixture within a high viscosity medium (i.e. castor oil, which has viscosity around 250 cP) than the low viscosity oily phases (n-octanol and mineral oil with viscosity of 45 and 110 cP respectively), could produce more stable emulsions and therefore homogenously distributed microspheres (Figure 1). Then, the effect of Alg Mw and polymeric solutions concentration on the characteristics of the resulting microspheres were investigated. The formation of microspheres was closely monitored under a light microscope as well as SEM, as described earlier. Based on this study, microspheres prepared from high Mw Alg solutions with a concentration around 1 %w/v produce a regular and homogenous microspheres, but at lower or higher than 1 %w/v the microspheres prepared were rough, irregular and agglomerated together (Figure 2). Evaluation of particle size distribution of these microparticles show that the microparticles prepared with lower or higher concentrations have a bimodal dispersion and wide particle size distributions, which prepared with 1 %w/v high Mw Alg, were monodispersed with a mean diameter of

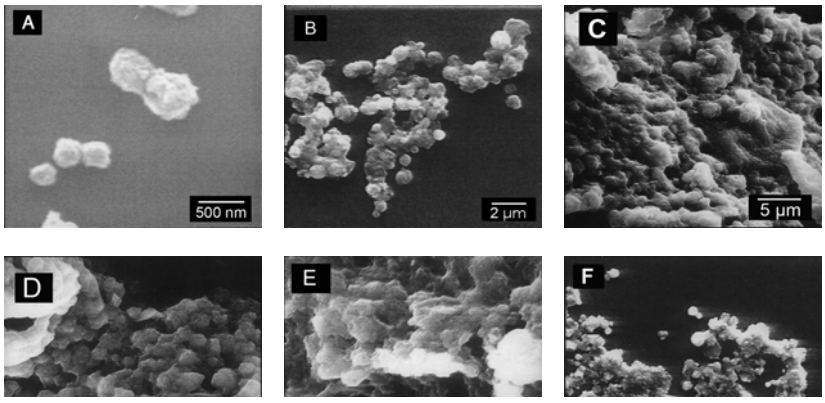

Figure 2. SEM photographs of Alg microspheres prepared with high Mw Alg in castor oil. A) 1 %w/v, cross linked with 1 M $CaCl_2$, B & C) 1 & 2 %w/v cross linked with 3.75 %w/v octanolic $CaCl_2$, D) 2 %w/v cross linked with 1 M $CaCl_2$, E &F) 0.5 %w/v cross linked with 1 M $CaCl_2$ and 3.75 % w/v octanolic $CaCl_2$.

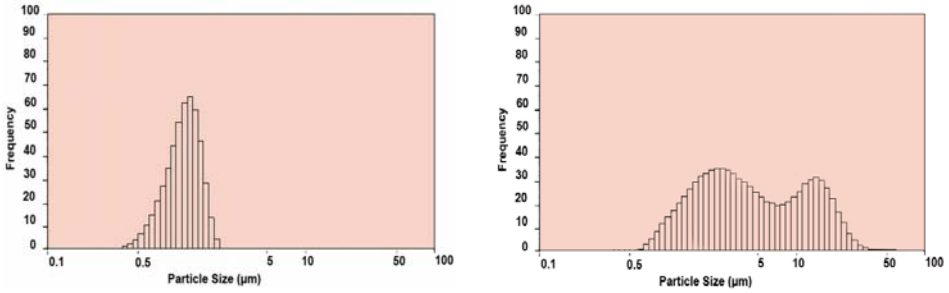

Figure 3. Particle size distribution of microparticles prepared by high Mw Alg cross-linked with octanolic $CaCl_2$ A) 1% w/v and B) 2% w/v

1.09±0.5 µm (Figure 3). Therefore, based on these initial studies and for the purpose of comparison, among the various concentrations of Alg investigated, a 1 %w/v Alg solution was found to be most appropriate and hence used for the preparation of Alg microspheres throughout this study.

Microspheres loading level. In order to investigate the influence of the amount of DT added to the primary Alg-containing emulsion on the toxoid loading level and encapsulation efficiency within the microspheres the high Mw Alg was used. As it could be seen (Figure 4) the amount of DT encapsulated within microparticles depends on two parameters: the original amount (theoretical loading) of DT added to primary emulsion for preparation of microspheres and the type of cross-linker (Table 1). By increasing the theoretical loading level, the actual amount of toxoid loaded initially increases and then reaches a plateau in the presence of 1367 µg and higher amounts of DT. In fact, the encapsulation efficiency of DT was found to decrease significantly ($p<0.05$) as a result of an increase in the original amount of toxoid used. Previously, investigators showed that the encapsulation efficiency of proteins within hydrophilic biopolymers depends on their abilities and numbers of sites to formation of hydrogen and/or electrostatic bounds (Vila *et al* 2004). Hence, it seems that DT interacts with hydroxyl groups of Alg chains via electrostatic and hydrogen bounds. So, the amount of DT at concentrations less than 1367 µg coiled with polymer chains by electrostatic and hydrogen bounds; but by increasing the DT concentration in the emulsion, all binding sites within polymer chains saturated with DT and appear a plateau at concentration more than 1367 µg.

Effect of cross linking agents on amount of DT encapsulated within Alg microspheres showed that aqueous cross linker has lower potential for entrapment of DT than the octanolic cross linking agent. These results are very close to results reported by Mortazavi *et al* 2004 and Heng *et al* 2003.

Table 1. Effect of polymer Mw, polymer concentration and cross linking agent on loading efficiency of alginate microspheres loaded with DT (the theoretical loading level for all microspheres were 1367 µg).

Polymer Mw	Polymer concen.	Cross linker	Actual loading (µg)	Encapsulation efficiency (%)
High Mw	1.5	Oct.	451.04±27.36*	32.99
	1	Oct.	434.87±35.96	31.80
	1.5	Aqu.	380.34±32.06	27.2
	1	Aqu.	309.93±31.50	22.68
Low Mw	2	Oct.	48.43±30.99	32.07
	1.5	Oct.	361.43±45.23	26.3
	1	Oct.	283.83±20.93	20.75
	2	Aqu.	286.28±13.48	20.94
	1.5	Aqu.	234.59±18.35	17.16
	1	Aqu.	220.33±24.71	16.12

Oct. 3.75 %w/v $CaCl_2$ in n-Octanol, Aqu. 1 M $CaCl_2$ solution, *(n=3)

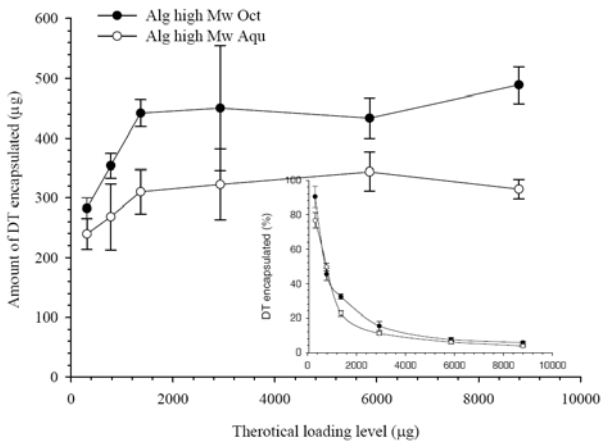

Figure 4. Effect of theoretical loading level on the amount of DT encapsulated within Alg microspheres. (The inner plate shows the encapsulation efficacy of DT). (n=3)

burst release of DT for microparticles cross linked with 1M $CaCl_2$ should be less than octanolic cross linker (Figure 4). At the second step some parameters affecting on DT loading levels were evaluated. These studies showed that the encapsulation efficiency of DT within microspheres depends on Alg Mw and the amount of DT added to the initial emulsion. Table 1 shows the results obtained from assessing the loading level and encapsulation efficiency of DT within microspheres prepared from Alg solutions of different Mw. by the Mw of Results show that the actual amount of DT loaded within Alg microspheres, and as a result, their encapsulation efficiencies are significantly affected the Alg polymer.

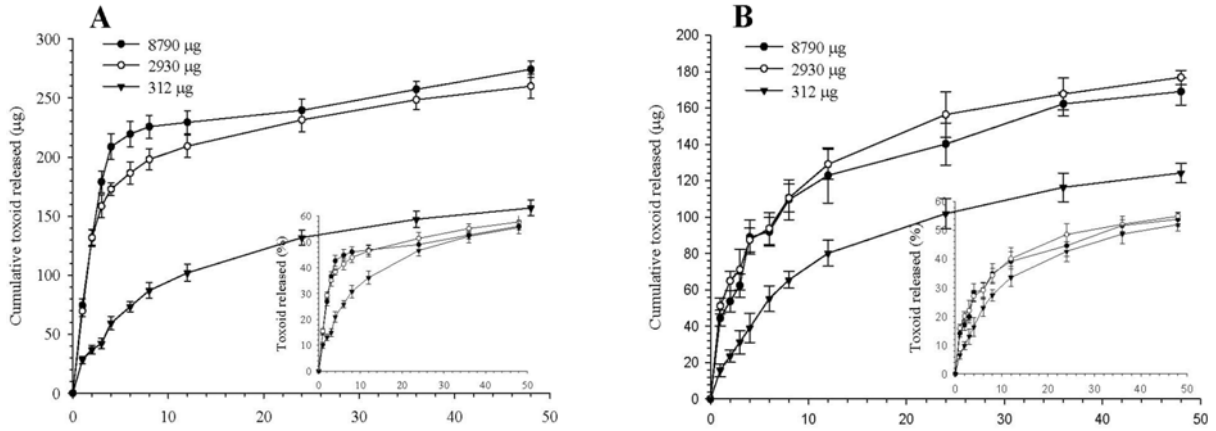

Figure 5. Profile of DT released from Alg microspheres prepared by high Mw Alg and cross linked with A) octanolic $CaCl_2$ and B) 1M $CaCl_2$, theoretical DT loading (µg): (▼) 312, (○) 2930 and (●) 8790 (inner plots show percentage of DT released) (n= at least 3)

Alg is an anionic biopolymer and at pH values 5.5-6.0 which were used for the preparation of microspheres, is expected to be negatively charged (Cui *et al* 2000). Accumulation of negative charge on the surface of microparticles carry out to repulsion a part of DT which adsorbed on the surface and leakage to aqueous phase of cross linking agent. Therefore, one could expect that, the

By increasing the Mw; and therefore polymeric chain length, 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.

Evaluation of In-vitro DT release. The results show that release of DT depends on the loading levels of DT within microspheres and type of cross

linking agents. Moreover, under the present experimental conditions, the DT release was not affected by the molecular weight of the polymer; however, it was clearly dependent on the loading level and cross linker type. These results provide some insights about the mechanism of release. Under the present experimental conditions, the polymer is not expected to degrade and, thus, the release of the associated protein will be a result of two simultaneous processes: (i) the disassociation of the Alg chains, which will may lead to the fragmentation of the microspheres and, consequently, to the release of the entrapped protein (ii) the disassociation of the protein from the Alg polymer chains. Therefore, in agreement with this hypothetical mechanism, the disassociation process of the protein is not affected by the Alg chain length but, it is importantly influenced by the cross linking intensity. The microspheres loaded with high level of DT have clearly a biphasic linear release. The first phase has a very fast release, which fallowed with a slow release phase which continued to about 50 h. As it illustrated in Figure 5, however, the different between DT released from microspheres loaded with 2930 and 8790 µg is not significant ($p<0.05$). Accordingly, one could speculate that, either the cross linking intensity of polymer chains using octanolic $CaCl_2$ is more than aqueous $CaCl_2$, or as it mentioned above some of DT adsorbed on particles surface, leakage to aqueous phase. However, both possibilities agree well with the more important protein association efficiency observed for the chitosan-PEG nanoparticles. It is note worthy to mention that these results are similar to those previously reported for bovine serum albumin (BSA) (Coppi *et al* 2002, Calvo et al 1997). The similarity may come from the fact that both DT and BSA have a comparable size and isoelectric point, and, consequently, the interaction forces between the protein and the polymer might also be comparable. A very different profile was observed for the release of tetanus toxoid from chitosan nanoparticles (Fernández-Urrusuno *et al* 1999). In this case, the release was very slow; a result that may be related to the large size of this protein (150 kDa) and its interaction forces with chitosan.

Conclusions. Alg microspheres were able to efficiently associate diphtheria toxoid and deliver it adequately for long lasting time. So, they could be considered as promising new nasal vaccine delivery systems.

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