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Short communication

A long acting biodegradable controlled delivery of chitosan microspheres loaded with tetanus toxoide as model antigen

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ABSTRACT

The chitosan microspheres formulated by emulsion cross-linking method were found to be smooth and spherical without aggregation. The particle size range was between 1 and 90 μm. The particle sizes were found to be influenced by the concentration of the chitosan gel. Tetanus toxoide (TT) vaccine was loaded by passive adsorption from an aqueous solution into the preformed chitosan microspheres cross-linked with glutaraldehyde. The loaded TT on to microspheres was estimated by ELISA method. The loading capacity was found to be 40% with microspheres prepared with 1% chitosan gel, 43% for 2% and 46% for the mixed batch of microspheres prepared from 1% and 2% chitosan gel. The loading efficiency was found to decrease with increase in the concentration of chitosan gel. The in vitro release of the antigenic TT into the phosphate buffer at 37 °C from different batch of microspheres was studied and release had a remarkable dependence on the size of micropsheres. The percentage release of TT from chitosan microspheres prepared from 1% chitosan gel was 2.7% in 120 days and that from 2% chitosan gel was only 2%. The mixed batch of microspheres could release 2.3% in 120 days. The antigen integrity was investigated by SDS–PAGE with brilliant blue staining. The SDS–PAGE analysis confirmed that the antigen integrity was not affected by passive adsorption of protein antigen to preformed chitosan microspheres. The study revealed that the cross-linked chitosan microspheres would be an interesting system for long-term delivery of macromolecules drugs.

1. Introduction

To decrease the mortality and morbidity due to infectious diseases, there is an urgent need for an improved vaccine [1]. For a vaccine to be effective, two or three booster doses are required after primary immunization. The factors affecting the efficacy of orally administered vaccines are the degradation of the antigen in the GIT and the low uptake by the gut associated lymphoid tissue. The dropout rates of individuals receiving the first dose of immunization are high in developing countries. The critical factor in achieving the appropriate levels of immunity against an infectious organism depends on the number of doses. The conversion of multiple dose vaccines to a single dose vaccine containing the priming and successive booster doses will help to reduce the dropout rate in vaccination and also help to reduce the cost of vaccination, making it affordable and popular [2,3].

The WHO/UNDP has focused on developing new vaccine for parenteral administration with the specific target on neonatal tetanus. The vaccine delivery systems using polymeric particle provide a viable alternative to multi-dose immunization schedule, which is capable of inducing neutralizing antibody titers to provide protective immunity. The microsphere technology is the core technology used for delivering the protein antigen and for protecting it from the acidic environment. The main polymers used for microencapsulation of vaccines are aliphatic polyesters, poly (lactides) and poly (lactide-co-glycolides) [4]. A number of biodegradable polymeric carriers have been investigated to the sustained release of macromolecular drugs; these include poly (lactic acid), poly (lactic acid-co-glycolic acid), poly (ortho esters), poly (anhydrides) and poly (E-caprolactone) [5–11]. The PLGA polymers are having regulatory approval for using them in humans. Many more bioerodible polymeric carriers were investigated for prolonging the delivery of macromolecular drugs. The drugs coming under this category include polypeptides, hormones, polysaccharides, antigens, antibodies and other biologically active agents. The natural polymers, like proteins and polysaccharides were also investigated as potential drug carrier for the low molecular weight drugs [12–14]. The usages of natural polymers for sustaining the delivery of macromolecular drugs

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were not given much importance and may be due to the rapid degradability, thereby limiting the delivery for a short period. The major limitations of formulating controlled delivery of vaccine and protein using microencapsulation are its instability during formulation, lyophilization, storage and polymer degradation [15–18]. So, here we have tried to incorporate tetanus toxoide (TT) on a preformed chitosan microspheres formulated by emulsion cross-linking procedure, subsequently avoiding the exposure of the protein antigen to the harsh environment, like organic solvents causing the denaturation, which otherwise is seen with microencapsulation process [19–21]. Chitosan, a natural polysaccaride obtained by deacetylation of chitin, are suitable for many biomedical applications [22,23]. The cross-linking of chitosan matrix using dialdehyde, like glutaraldehyde, makes them less susceptible to the degradation by lyzozyme.

2. Materials and methods

2.1. Materials

Chitosan was obtained from Central Institute of Fisheries and Technology, Cochin, India and was used without further purification. The glacial acetic acid, toluene, acetone, sulphuric acid, span 80 (all analytical grade) was procured from E Merck, Mumbai. The glutaraldehyde (biological grade), BSA was purchased from Sigma Chemical Company, USA, the tetanus toxoide (TB0207) from Shantha Biotechnics Ltd, Hyderabad), tetanus antitoxin from Central Institute Kasauli. The ELISA kit was obtained from diagnostic Automation, INC, Calabasas, CA. The anti-TT antibody was procured from Pro Sci INC, USA. The anti-TT HRPO conjugate was obtained from KPL Inc, USA. The orthophenydiamine was obtained from Geni, Bangalore.

2.2. Preparation of chitosan microspheres

The chitosan microspheres were prepared by emulsion cross-linking method as reported in the earlier work [24–28]. Chitosan gel was prepared by dissolving 1 g of chitosan in 3% (w/v) acetic acid containing 2% (w/v) sodium chloride and mixed in a mechanical stirrer at 3000 rpm for 1 h. The gel was kept overnight for stabilization to get 1% chitosan gel. For preparing 2% chitosan gel, 2 g of chitosan was used. Chitosan microspheres were prepared by dissolving 1 mL of chitosan gel in equal volume of 0.01 N hydrochloric acid and 50 mL of toluene that contained 5 mL of span 80 was added. The emulsion mixture was stirred for 1 h at 2000 rpm. To the above W/O emulsion, 10 mL of GST was added dropwise and stirred at 2000 rpm on a mechanical stirrer for 4 h. The product was then centrifuged to separate microspheres and washed thrice with toluene and acetone. The microspheres were then dried at 37 °C and collected as free flowing powder. The same procedure was adopted to prepare microspheres with 2% chitosan gel and a mixed batch comprising of microspheres from both 1% and 2% chitosan gel, which were used for the study.

2.3. Swelling behavior of chitosan microspheres

The preformed chitosan microspheres were studied for the swelling behavior in order to incorporate the protein antigen. About 100 mg of chitosan micropshere were weighed and added to 100 mL of phosphate buffer maintained at pH 7.4 in a conical flask and incubated at 37 °C for 24 h. The microspheres was later separated by centrifugation and dried. The microspheres were analyzed under SEM for change in surface characteristic [29].

2.4. Sterilization effect on chitosan microspheres

The microparticle used as adjuvant in vaccine delivery via parental route was sterilized by dry heat sterilization at 80 °C for 16 h. The sterilized microspheres were then evaluated for the change in surface morphology under SEM [25].

2.5. Particle size distribution analysis

The release kinetics of microparticle depends on the particle size distribution. The preformed chitosan microspheres prepared from both 1% and 2% chitosan gel were evaluated for the particle size distribution analysis.

2.6. Loading of tetanus toxoide on to chitosan microspheres

About 400 mg of empty chitosan microspheres were added to 100 mL conical flask containing phosphate buffer of pH 7.4 and stirred for 24 h using a magnetic stirrer. The conical flask was kept in an incubator maintained at 37 °C overnight to increase the pore size for incorporation of diluted tetanus toxoide vaccine. The swelled microspheres were later separated by centrifugation, and washed thrice with distilled water and dried. Diluted tetanus toxoide measuring 10 mL was mixed with the swelled microspheres and rotated on a hematology mixer for 24 h at 37 °C for proper adsorption of TT. The microspheres were separated by centrifugation and washed thrice with distilled water and dried. The loading capacity of TT onto the chitosan microspheres were determined by dispersing 10 mg of the TT incorporated chitosan microspheres in 10 mL of 5% HCl and kept for 24 h at 4 °C. The microspheres were later separated by centrifugation and the free TT present in the supernatant was estimated by ELISA.

2.6.1. ELISA

An amount of 100 μL of anti-TT antibody (10 mg/mL) was loaded in 96-well flat bottom ELISA plates and kept at 37 °C for 1 h. The plate was washed once with phosphate buffered saline tween-20 (PBS-T) and then, non-specific binding site was blocked by filling the wells with 300 μL of 1% BSA solution in PBS-T and was incubated for 1 h at 37 °C. The content was later removed and washed again with PBS-T. The TT from chitosan microparticle and standard TT (pure form) in PBS-T were loaded in the wells and incubated for 1 h at 37 °C. The content was discarded later and washed the wells thrice with PBS-T. Then, 100 μL of anti-TT HRPO conjugate was added to the well and incubated at 37 °C for 1 h. The content was discarded and washed thrice with PBS-T. An amount of 50 μL of orthophenyldiamine dissolved in citrate phosphate buffer with H_2O_2 was added and kept for 20 min for the colour development. The reaction was stopped by adding 50 μL of 5 N H_2SO_4 and absorbance was read at 495 nm [30].

2.7. Antigen integrity estimation of TT from preformed chitosan microspheres [15]

An SDS–PAGE analysis was carried out using the extracted TT antigen (Bio–Rad, USA) with stacking and separating gels of 4 and 8% polyacrylamide, respectively. The samples of equal concentrations were loaded carefully into the gel and made to run at a constant voltage (150 V) until the dye band reached the gel bottom. Finally, the gel was stained with a 0.1% coomassie blue and then, destained by an aqueous solution containing 40% (v/v) methanol and 10% (v/v) acetic acid.

2.8. In vitro release of TT antigen from chitosan microspheres

The in vitro release of the protein antigen from the chitosan microspheres was studied in PBS (pH 7.4) containing 0.2% sodium