Asian Journal of Chemistry; Vol. 23, No. 7 (2011), 3279-3282

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Optimization and *In Vitro* Evaluation of Alginate: Hydroxypropyl Methylcellulose Microspheres Loaded Tetanus Toxoid Vaccine

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(*Received*: 10 January 2011; *Accepted*: 26 March 2011) AJC-9779

Tetanus toxoid loaded alginate microspheres has been developed and investigated for their feasibility in release by *in vitro* release. Tetanus toxoid was stabilized, encapsulated in microspheres prepared from alginate by using protein stabilizer (sucrose). Alginate microspheres were prepared by W/O/W double emulsion method. The developed materials were characterized for their size, surface morphology, protein loading efficiency, release of tetanus toxoid-loaded alginate microspheres (TTAL). The morphology of TTAL was smooth and spherical in shape with a diameter of around 10 µm. The TTAL were evaluated for vaccine entrapment and the *in vitro* release study was carried out by differing stabilizer sucrose concentration (5, 7, 10 and 12 % w/v) for the period of 70 days. Antigen release from microspheres was determined by ELISA. Based on these findings, 10 % w/v of sucrose shows good sustained antigen delivery with polymer degradation and the release of tetanus toxoid was increased. This approach should have potential application in the field of vaccine delivery.

Key Words: Tetanus toxoid, Adjuvant, Alginate microspheres, Biodegradable polymers.

INTRODUCTION

Neonatal tetanus is a rare disease in most industrialized countries, but it is still an important public health problem in many parts of the developing world and it kills 4,00,000 annually[1,2]. It is caused by *Clostridium tetani*, a gram positive, anaerobic bacillus that forms spores found in soil and faeces. *Clostridium tetani* is an non-invasive opportunist that relies on spore introduction through damaged skin. Clinical symptoms of tetanus are mediated by the tetanospasmin neurotoxin. The toxin blocks inhibition of spinal cord reflex arcs, causing muscle rigidity and contraction and also interferes with release of transmitters in autonomic nervous. Autonomic dysfunction may manifest in the form of labile hypertension, tachycardia and other cardiac arrhythmias, pyrexia, peripheral vascular constriction and sudden cardiac death.

The requirement for multiple injections of the currently licensed tetanus vaccine dictates that there is often comparatively poor coverage in countries where economic or logistical factors preclude this. For this reason, the WHO has indicated that the development of improved immunization strategies for diseases is a priority[3]. Aluminium phosphate and aluminium hydroxide are the adjuvants currently approved for human vaccination[4]. Controlled drug delivery technology using biodegradable polymers as carriers represents one of the most rapidly developing areas of science. It consists of biodegradable microspheres can potentially delivery either the antigens or adjuvants to the desired location at predetermined rates and durations to generate an optimum immune response[5].

Polysaccharides, a class of naturally available carbohydrate polymers, have been used extensively in food industry as gelling agents and for encapsulation of living cells[6-8]. Sodium alginate (NaAlg), a natural polysaccharide, composed of D-mannuronic acid and D-guluronic acid, is derived from the brown seaweeds. Sodium alginate is a biodegradable polymer used extensively in drug delivery applications[9-11]. Alginate salts are known to form a reticulated structure in contact with calcium ions or glutaraldehyde and this characteristic has been used to prepare the sustained release particulate systems for a variety of drugs, proteins and cells[12-14].

Hydroxypropyl methylcellulose (HPMC) is also a carbohydrate polymer, is soluble in water. It forms aqueous solutions and demonstrates a unique property to form reversible physical gels due to hydrophobic interactions when heated above a particular temperature[15]. Hydroxypropyl methylcellulose finds applications as a binder or thickener in pharmaceutical, food and ceramic processing industries and can undergo thermoreversible gelation in aqueous solution upon heating[16-18].

On the basis of this, the development of single dose controlled release tetanus toxoid vaccine that can be administered soon after birth would eliminate the need for booster immunization is the greatest development towards the human health care.

EXPERIMENTAL

Sodium alginate, bicinchoninic acid (BCA), bovine serum albumin (BSA), Tween 80 and Span 80 were purchased from Fluka (Buchs, Switzerland). Tetanus toxoid (MW-150KDa) having concentration 1250 Lf/mL and the standard tetanus antitoxin were received as gifts from Central Research Institute, Kasauli, H.P., India. Sucrose, calcium chloride and was purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade and were used as received.

Preparation of tetanus toxoid encapsulated alginate microspheres: Alginate microspheres were prepared by W/O/W double emulsion method, followed by the method of Wan *et al*.[19], with some minor modifications. In order to prepare alginate microspheres having a diameter of 10 mm or less, the effect of stabilizer concentration was investigated. 4 batches of microspheres were prepared by altering stabilizer (sucrose) 5, 7, 10 and 12 % w/v. In brief, 1-2 mL of aqueous solution of 9:1 of sodium alginate and HPMC was mixed separately to the dispersion phase containing *n*-octanol with 3 % (w/v) of lipophilic surfactant Span-80 at 8000 rpm. To this, 2 mL of alum free tetanus toxoid (1250 Lf/mL) which is previously added with various concentrations of stabilizer sucrose and it was transferred to the above emulsion and the stirring was continued at 8000 rpm for 1 h. The calcium chloride solution was added and the dispersion was mixed for another 10-15 min. Isopropyl alcohol was then used to further harden the formed microspheres. The microspheres were collected by filtration, washed 5 times with isopropyl alcohol and finally dried 2 h at 37 ºC. The microspheres thus obtained were stored in sealed glass vial in a vacuum desiccator.

Estimation of tetanus toxoid content in alginate microspheres: For the estimation of tetanus toxoid content of microspheres a known weight of (5 mg) microspheres were dissolved in 750 µL sodium citrate (0.1 M, pH 7.4) by shaking at room temperature for 3 h[20]. Bicinchoninic acid assay (BCA) was used to determine the tetanus toxoid concentration in the degraded microsphere solution. Bovine serum albumin (BSA) was used as the standard protein. The tetanus toxoid loading and the encapsulation efficiency were determined for each batch of microspheres in triplicate.

***In vitro* release study:** *In vitro* release studies of the encapsulated tetanus toxoid from alginate microspheres stabilized with sucrose at different concentrations (5, 7, 10 and 12 %, w/v) were carried out separately in conical flasks by taking 50 mg of microspheres and 50 mL of PBST (pH 7.4) were incubated at 37 ºC on a constant shaking mixer[21]. The content of the vial was withdrawn and centrifuged 5000 rpm for 5 min at predetermined time intervals (days 0, 2, 4, 8, 12, 16, 21, 28, 35, 42, 49, 56, 63 and 70). The tetanus toxoid concentrations in supernatants were determined by ELISA method. Placebo microspheres without antigen were used as control.

ELISA for tetanus toxoid: Tetanus toxoid antigenicity was measured by ELISA as described by Johansen *et al*., with slight modifications[22]. Briefly, flat-bottom 96 well NUNC immuno microtitre plates were filled with 100 µL of 2 IU/mL of horse anti-tetanus IgG in 0.05 M carbonate buffer of pH 9.6 for overnight. The plates were washed three times with 300 µL of PBS containing 0.05 % Tween 20. After washing and blocking, a twofold dilution series of sample and reference tetanus toxoid samples were prepared using PBS with 0.5 % BSA. The plates were held at room temperature for 24 h followed by addition of peroxidase labelled sheep antitetanus toxoid serum (in PBS with 0.5 % BSA) at room temperature for 2 h. Finally, 100 µL of 0.2 mg/mL peroxidase substrate 2,2'- azino-*bis*(3-ethyl benz-thiazoline-6-sulphonate) in 100 mM NaH_2PO_4 solution was added to the plates. The reaction was stopped by the addition of 50 µL/well of 5N H_2SO_4. The plates were read on an ELISA reader after 0.5 h at wavelength of 405 nm.

RESULTS AND DISCUSSION

Physical characterization of stabilized tetanus toxoid loaded microspheres: The most important requirement for the successful development of single-dose tetanus vaccine is that protein encapsulated and released remains competent as an immunogen to invoke immune response. Four batches of microspheres (B1 to B4) of alginate were prepared and the effect of addition of stabilizer (sucrose) on the morphological and size characteristics of microspheres was evaluated. All the formulations of alginate microspheres entrapping tetanus toxoid were prepared having the size ranges between 1-50 µm. These size ranges were selected to delineate the role of macrophage uptake as an essential requirement for single point immunization. The large size particles (50-100 µm) will not be taken by macrophages where as the particle having size ranges 10-70 µm will have very little chance to be taken up as 5 µm has been reported to be the upper limit for phagocytosis by macrophages[23,24]. In the case of 2-8 µm, more than 90 % of the particles have diameter less than 5 µm so they will be taken up by antigen presenting cells[25] where as in the size range of < 2 µm there will be further enhanced cellular uptake due to submicron size ranges of polymer particles[26].

***In vitro* release study of alginate microspheres encapsulated tetanus toxoid:** The *in vitro* release of tetanus toxoid from alginate microspheres stabilized with sucrose at different concentrations (5, 7, 10 and 12 % w/v) was compared with microspheres without sucrose. In alginate microspheres, 10 % of sucrose showed good release rate and 7-10 % of antigen was released within first 2 days. This may be due to various factors including surface morphology, particle size, stirrer speed, polymer composition and cross linking agent concentration. Among all the batches of alginate microspheres encapsulated tetanus toxoid, 10 % of sucrose concentration showed the lowest release rate which could be considered as an appropriate release pattern for mucosal immunization, respectively.

Currently, alum is the only adjuvant that is approved for clinical use. The use of alum-type adjuvants for immunization has several disadvantages. They induce inflammation and

stimulate the local production of granulomas. In addition, alum is not a universal solvent as it is not suitable for small peptides or recombinant proteins and cannot be frozen or lyophilized. Conventional alum-type vaccines require multiple recall injections at appropriately timed intervals in order to achieve long-lasting and optimal immune responses. However, it is very difficult, especially in developing countries, to maintain high re-immunization programs. Therefore, the development of more efficient and safe adjuvant/vaccine delivery systems, requiring only a single dose to obtain high and long-lasting immune responses, is of primary importance[4].

In present study, the main objective is to evaluate the suitability and potential of alginate polymeric systems as adjuvants for tetanus toxoid vaccine that are easy to deliver and elicit a long-lasting immune response. The actual size of the individual microspheres was in the range of 1-50 μm and few of them are more than 50 μm (Fig. 1). In alginate microspheres, 9:1 ratio of alginate: HPMC, 8000 rpm stirrer speed and 8 % of $CaCl_2$ was selected for small sized microspheres (> 10 μm).

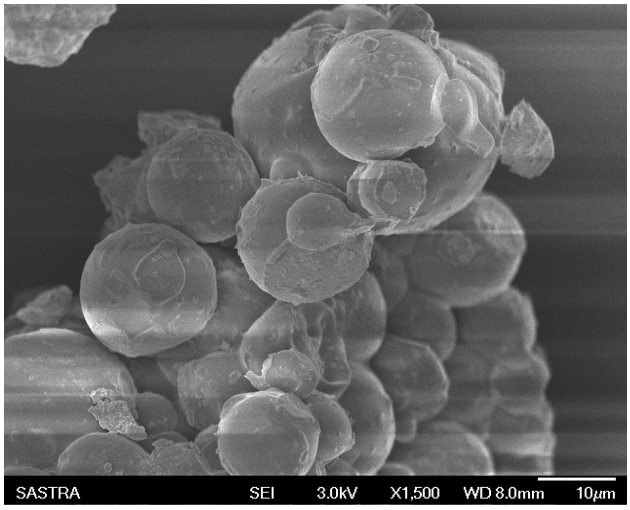

Fig. 1. Scanning electron microscope of the tetanus toxoid encapsulated microspheres prepared with 9:1 of alginate: HPMC

During the preparation of alginate microspheres needs the use of discordant solvents that may degrade proteins. The microspheres when exposed to physiological environments may also destabilize the protein molecules[27-31]. It is thus necessary to stabilize the protein during both encapsulation process and release from the microspheres. Therefore, the microspheres were co-encapsulated with cheap potential protein stabilizer, sucrose. It was also hypothesized that protein stabilizer can shield the antigen from the organic solvent *via* preferential hydration of their surface, thus preventing protein-interface exposure to deleterious solvent effects. The loading efficiency of both chitosan and alginate microspheres containing 10 % of sucrose was increased from 80-90 %, whereas lowest tetanus toxoid payload was observed without sucrose. The tetanus toxoid release pattern from the alginate microspheres stabilized with sucrose at different concentrations (5, 7, 10 and 12 %, w/v) was compared with microspheres without sucrose. In alginate microspheres without sucrose releases only 5 % of the loaded tetanus toxoid and no further

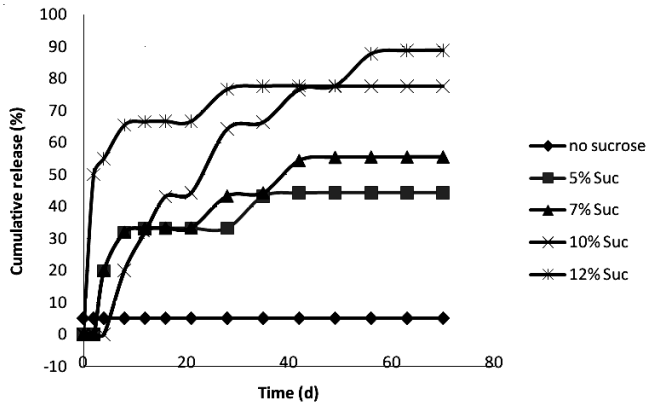

Fig. 2. Release profiles of tetanus toxoid-loaded alginate microspheres prepared with different concentrations of sucrose

release was observed up to 70 days (Fig. 2). This may be due to protein unfolding, inactivation and irreversible aggregation in the first emulsification step[32]. Mechanical forces employed in the first emulsion might also cause protein structural perturbations, which often result in irreversible aggregation[33]. The potential approach to increase the tetanus toxoid concentration and loading during emulsification, a protein stabilizer (sucrose), which resulted in good stabilization upon encapsulation in and release from microspheres made using a W/O/W method[34]. The protein stabilizer (sucrose) prevents the denaturation at W/O interface, which is reflected in augmented cumulative percentage release. In addition, sucrose is having good solubility in aqueous media. The protein stabilizer (sucrose) concentration is directly proportional to release of tetanus toxoid from microspheres. To this end, the higher concentration of sucrose (12 %, w/v) increased the initial burst of release *i.e.*, > 75 % of tetanus toxoid was released within 5 days. But in the case of 10 % sucrose increased the sustained release of tetanus toxoid up to 70 days as compared to 12 % of sucrose. So, 10 % sucrose was suitable and it will help to improve the stability during encapsulation and release system.

ACKNOWLEDGEMENTS

The authors thank Sourabh Sharma and Tejpal Kashyap in DTP section, Central Research Institute, Kasauli for offering the gift sample of plain tetanus toxoid.

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