The potential for production of freeze-dried oral vaccines using alginate hydrogel microspheres as protein carriers

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Oral administration of dry vaccine formulations is acknowledged to offer major clinical and logistical benefits by eliminating the cold chain required for liquid preparations. A model antigen, bovine serum albumin (BSA) was encapsulated in alginate microspheres using aerosolisation. Hydrated microspheres 25 to 65 µm in size with protein loading of 3.3 % w/w were obtained. Environmental scanning electron microscopy indicated a stabilizing effect of encapsulated protein on alginate hydrogels revealed by an increase in dehydration resistance. Freeze drying of alginate microspheres without use of a cryoprotectant resulted in fragmentation and subsequent rapid loss of the majority of the protein load in simulated intestinal fluid in 2 h, whereas intact microspheres were observed following freeze-drying of BSA-loaded microspheres in the presence of maltodextrin. BSA release from freeze-dried preparations was limited to less than 7 % in simulated gastric fluid over 2 h, while 90 % of the protein load was gradually released in simulated intestinal fluid over 10 h. SDS-PAGE analysis indicated that released BSA largely preserved its molecular weight. These findings demonstrate the potential for manufacturing freeze-dried oral vaccines using alginate microspheres.

Key words: Oral vaccine – Alginate hydrogel microspheres – Maltodextrin cryoprotectant – Protein – Bovine serum albumin – ESEM – In vitro release.

The advantages of dry oral vaccines over injected liquid preparations have stimulated numerous investigations of oral delivery systems aimed at avoiding the pain and discomfort of injection and reducing the need for trained medical personnel to administer the vaccine. In addition the logistical and cost benefits of eliminating the requirement for storage and distribution under refrigeration (the so called cold chain) to maintain the stability of injectable liquid preparations presents a further incentive for such studies. Oral vaccines are presently confined to protecting the individual against mucosally transmitted pathogens that enter and infect the intestinal, respiratory and genital tract. However oral vaccination is known to induce systemic humoral responses (involving antigen-specific serum IgG antibodies) and cell-mediated immune responses [1, 2] as well as local secretory IgA antibodies in the gastrointestinal (GI) tract and at distant mucosal sites via the common mucosal system. Thus oral vaccination also shows potential for protecting against pathogens that enter the body via the bloodstream resulting in infectious diseases such as malaria and hepatitis B, which are major public health concerns.

Although oral administration of vaccines has been reported to reduce the incidence of infection by *Escherichia coli* [3], *Vibrio cholera* [4], and *Chlamydia trachomatis* [5], their effectiveness is compromised by hydrolytic and proteolytic degradation of unprotected antigens in the GI tract. Accordingly, numerous delivery systems have been developed including nanoparticles, microparticles, liposomes, immune stimulating complexes (ISCOMs) and virus-like particles (VLPs) [6-8] to protect antigens from low gastric pH conditions and enhance uptake by gut associated lymphoid tissue for induction of the immune response. Encapsulation of Bordetella pertusis antigen in PLGA micro or nanoparticles has been reported to elicit specific mucosal IgA and systemic IgG responses following oral administration and conferred protection in a murine respiratory challenge model [9]. More recently, Hickey et al reported 50 % protection against Chlamydia muridarum infection following oral immunization using the chlamydial major outer membrane protein (MOMP) formulated in a lipid-based delivery system [5].

Natural polymers including alginate, chitosan [10] and denatured collagen (gelatin) [11] have also been widely employed in oral vaccine formulation. Alginate polysaccharide in particular is non toxic and biocompatible and moreover exhibits a unique property of forming gels in mild, aqueous conditions at room temperature using di- or trivalent metal ions [12]. Alginate processing, gel strength and drug release behaviour are greatly influenced by the ratio of manuronic acid (M) and guluronic acid (G) blocks within the alginate polymer since cross-linking selectively occurs between Ca^{2+} ions and the G acid sequences of the polymer [12, 13]. Since this behavior gives broad scope for modifying vaccine formulations to achieve controlled antigen release in the small intestine, a number of protein antigens, bacteria and viruses (which are susceptible to degradation by organic solvents) have been encapsulated in alginate microspheres and administered orally in a number of animal models [14, 15]. Cho *et al*. [16] found that serum IgG levels in mice increased after oral immunization of a conjugate of pneumococcal capsular polysaccharide type 19 (PS19)-cholera toxin B subunit (CTB) (PS19-CTB) in alginate microspheres. Suckow *et al*. reported protective immunity in rabbits after oral immunization of an extract of *Pasteurella multocida* entrapped in alginate microspheres [17]. However, these studies did not involve dried vaccine formulations.

In general dry powder formulations of active macromolecules such as proteins, vaccine antigens and DNA display enhanced stability relative to the liquid state due to reduced molecular mobility and the elimination of possible degradation pathways such as hydrolysis. Freeze-drying or lyophilisation in the presence of a stabilizer or cryoprotectant is the preferred drying method for biopharmaceuticals [24]. Although extensive research has confirmed the potential of alginate hydrogels to protect macromolecular actives from gastric fluid and to release them in the small intestine [23, 25], we have found only two

reports of protein encapsulation in freeze-dried alginate microspheres. This is probably explained by structural breakdown of the fragile, hydrogel structure during lyophilisation, resulting in exposure of the active. The aim of the present study, therefore, was to evaluate alginate microspheres as carriers for protein antigens in the production of freeze-dried (FD) oral vaccines. Apart from achieving needle-free delivery, a further objective was to align production with protein stabilization and storage practices prevalent in the biotechnology industry so as to eliminate the cold chain. BSA is often used as a model vaccine antigen due to its known structure and ease of quantitative assay [18, 19]. The capacity of the FD powders to protect and release antigen in the small intestine was investigated by sequential exposure of BSA-loaded samples to simulated gastric and intestinal fluids and assay of the release media to confirm conservation of protein molecular weight.

I. MATERIALS AND METHODS

1. Materials

Sodium alginate (Protanal LF 10/60, Mw 135kDa, guluronate/mannuronate ratio (G:M=2:1), (viscosity of a 1 % aqueous solution = 20-70 mPas) was provided by Swift and Company Limited, Mulgrave, Victoria, Australia. Bovine serum albumin (BSA) (Mw 66.5 kDa) was purchased from Sigma-Aldrich, Castle Hill, NSW, Australia. Calcium chloride (UNILAB) and hydrochloric acid (Univar) were obtained from Ajax Finechem Pty Ltd. (NSW, Australia). Phosphate buffered saline (PBS) was obtained from Amresco Inc. (Solon, Ohio). Maltodextrin (Fieldose 10C, dextrose equivalence (DE) 9.8 maximum) was obtained from Penford Australia Limited (NSW, Australia).

2. Production of BSA-loaded alginate gel microspheres by aerosolisation

BSA (30 mg) was dissolved in sodium alginate solution in distilled water (20 mL of 1.5 %w/v). The co-solution of BSA and alginate was pumped via a peristaltic pump (Watson Marlow Type NMRV/30, Motovario, Italy) at a flow rate of 10 mL/min using a speed controller (Telemecanique Altivar 31, Schneider Electric, France) to a spray nozzle (orifice diameter 0.1-1.5 mm). The solution was sprayed at a pressure of 60 psi using an air pressure regulator valve (AR series, 20-02H, China), into an aqueous $CaCl_2$ cross-linking solution (20 mL 0.1M or 0.5M) at an optimum distance of 20 cm between the surface of the $CaCl_2$ solution and nozzle orifice (*Figure 1*). The resulting BSA-loaded alginate gel microspheres were collected by low speed centrifugation (1000 rpm) for 3 min (Eppendorf centrifuge, model 5804R, Hamburg. Germany). The protein loading of dried microspheres was measured following freeze-drying of a sample of the microsphere suspension (Alpha 2-4 LD, Christ Freeze Dryer, Wertheim, Germany). Freeze-dried alginate microspheres encapsulating BSA were prepared by first adding 300 mg maltodextrin as a cryoprotectant to suspensions of hydrated gel microspheres (10 mL) to give a final maltodextrin concentration of 3 % (w/v). Samples were initially frozen by storing at -80 °C overnight and then freeze-dried at -50 °C for 48 h at a chamber pressure of 0.100 mbar. A secondary drying stage to remove non-frozen water was not employed since the resulting microsphere powder was re-suspended in distilled water (5 mL) for 15-30 min and washed by centrifugation at 1000 rpm for 3 min to remove the maltodextrin component prior to testing.

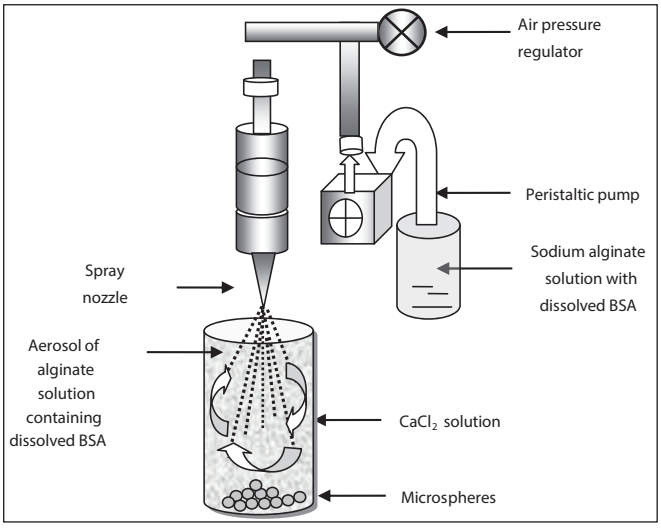

Figure 1 - Production of BSA-loaded alginate gel microspheres.

3. Size and morphology of protein-loaded alginate microspheres

Hydrated alginate microsphere suspensions were analyzed to provide data on particle size and size range using low angle laser light scattering (Mastersizer 2000, Malvern Instruments, United Kingdom). The mean diameter of hydrated microspheres was also determined by optical microscopy

The shape, size and dispersion of blank and protein-loaded alginate microspheres in distilled water were determined before and after freeze drying by optical microscopy (Olympus BH-2, Japan) with camera attachment (C-5050 Olympus) and scanning electron microscopy (SEM).

The morphology of BSA-loaded alginate microspheres freeze-dried in the presence or absence of maltodextrin cryoprotectant was examined using a Jeol JSM6380 scanning electron microscope (Jeol Datum, Tokyo, Japan). Specimens were mounted on SEM stubs using carbon tabs and sputter coated with platinum using a JFC1600 Autofine coater (Jeol, Tokyo, Japan) prior to examination in the SEM at a voltage of 15 kV. The morphology and dehydration behaviour of BSA-loaded alginate hydrogel microspheres was examined using environmental scanning electron microscopy (ESEM) (FEI Quanta 200, United States) in environmental mode using an accelerating voltage of 20kV and 8mm working distance. The sample chamber pressure was set to 5.0 Torr and the chamber gas was water vapour. Samples were examined over a range of hydration conditions by varying the pressure and temperature within the sample chamber. The output from a gaseous secondary electron detector (GSED) was used for image construction.

4. Measurement of BSA loading of alginate microspheres

The BSA loading of alginate microspheres was analyzed using the BCA total protein assay, which is based on the principle that Cu^{2+} reduction to Cu^{+} by protein in an alkaline environment is proportional to the concentration of protein. Bicinchoninic acid forms a purple complex with Cu^{+}, which strongly absorbs light at a wavelength of 562 nm, and provides a colorimetric assay of protein concentration. The BSA loading of alginate microspheres was determined following breakdown of 1mL aliquots of suspension over 24 h at room temperature in 0.5M sodium citrate solution (2 mL, pH 8.5). The BSA content was determined using the BCA total protein assay using UV-Vis spectrophotometry at an absorbance wavelength of 562 nm (Cary 50 Bio UV-vis spectrophotometer, Varian, Australia) and calculated by comparison with a calibration curve constructed using a series dilution of BSA in sodium citrate (concentrations of 5, 10, 20, 50 and 80 μg/mL). Protein loading was expressed as %w/w of the dried alginate microspheres.

5. Protein release behaviour in simulated intestinal fluid

Suspensions (1 mL) of hydrated BSA-loaded alginate microspheres (non-freeze-dried) in triplicate were incubated in 1 mL PBS (pH 7.4) in 10 mL polypropylene tubes at 37 °C for 24 h. Suspensions of freeze dried BSA-loaded alginate microspheres, following removal of maltodextrin