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Incorporation of a model protein into chitosan–bile salt microparticles

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Abstract

In order to develop a mucosal delivery system based on biocompatible polymers, a new methodology for production of protein-loaded microparticles is developed. Chitosan anionic precipitation/coacervation is accomplished by the addition of sodium deoxycholate (DCA). These microparticles were prepared under mild conditions, where bovine serum albumin (BSA) and DCA were simply dipped into a chitosan solution under stirring. Platelet-like and/or spherical microparticles, having high protein loading efficiency and relatively low protein external exposure, are obtained. To achieve a better compaction of the microparticle matrix, block copolymers and other non-ionic surfactants are added to the formulation. BCA analysis and fluorescence quenching were used to assess the degree of protein exposure. BSA release profiles for chitosan–DCA formulations in PBS pH 7.4 and HCl 0.1N revealed, in most cases, an initial burst release, but more than 55% of the BSA remains protected inside the microparticles. It is also observed that in acidic environment (HCl 0.1N) the protein is better shielded from the environment. Some of the formulations show good properties for mucosal protein delivery, and one of those here developed is now being tested in vivo, for mucosal administration of an adenovirus vaccine.

Keywords: Microparticles; Chitosan; Protein delivery; Mucosal drug delivery; Bile salts

1. Introduction

Recombinant oligopeptides and proteins have been increasingly used in applications in the pharmaceutical field along the last decade. This development constitutes one of the critical trends in present-day pharmaceutical technology with particular impact in the field of vaccine formulation. However, protein formulations present serious stability problems and are classically administrated by parenteral route. The alternative mucosal administration, although convenient, is prone to bioactive degradation, and its efficiency may be compromised due to deficient transport across the epithelial barrier (Chen, 2000). To overcome these problems, some authors have developed methods of encapsulation based on colloidal polymeric matrices, which are adequate for different routes of mucosal administration, protect the protein and enhance its absorption (Merkus et al., 1993; Chen, 2000).

In the case of vaccines, the oral and nasal routes are very attractive, leading to both, mucosal and systemic immune response stimulation (Merkus et al., 1993; Janes et al., 2001). This response can be modulated by the size of the particle loaded with the immunologic agent. For instance, particles smaller than 200 nm are said to be taken up by the M cells, while larger ones remain at the surface of the epithelium, releasing the macromolecule by enzymatic digestion (Thanou et al., 2001; van der Lubben et al., 2001). However, when particles are larger than 20 μm, they are prone to be washed out, being inefficient for mucosal delivery.

We are interested in developing a method suitable for the microencapsulation of an adenovirus for cattle vaccination by either nasal or oral delivery. In the present work we develop a methodology for encapsulation of a model protein, bovine serum albumin (BSA, 69 kDa), under mild conditions, as a preliminary step for future application to adenoviral delivery. Attending to the objectives, microencapsulation should involve cheap materials and methodology, and result in an effective protection against adverse temperature and humidity conditions.

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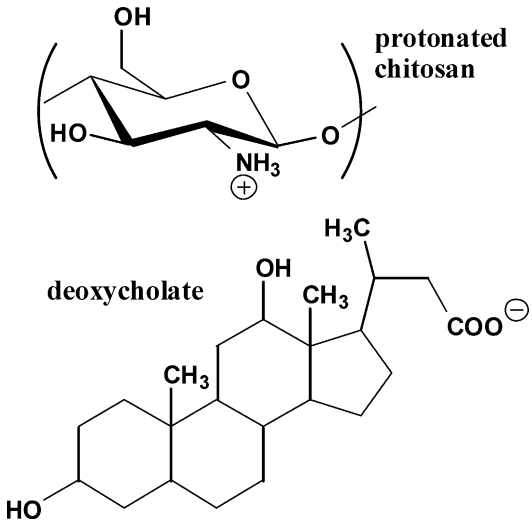
Several polymers were until now used to encapsulate proteins. In this work we will focus on chitosan matrixes whose advantages are already well described in the literature (Illum, 1998). Chitosan is a natural hydrophilic polymer obtained by partial deacetylation of chitin, an abundant polysaccharide present in crustacean shells (Illum, 1998) and is one of the polymers that were proposed for preparing protein-stabilizing matrixes (Carrara and Rubiolo, 1994). Chitosan promotes tight junction permeability (Thanou et al., 2001; Ranaldi et al., 2002), has strong mucoadhesive properties (Lehr et al., 1992; Bernkop-Schurch et al., 1998) and is used as mucosal immunoadjuvant and immunopromoter (van der Lubben et al., 2001). The positively charged amino groups on chitosan position C2 interact with the negatively charged cell surface, facilitating paracellular penetration of hydrophilic macromolecules due to tight junction opening (van der Lubben et al., 2001). Also, mucoadhesion of chitosan microparticles to Peyer's patches has been related to the enhancement of bioactive uptake (van der Lubben et al., 2002). Being biocompatible and biodegradable (its biodegradation is catalyzed by some human enzymes as lysozymes and chitinases produced by macrophages; Muzzarelli, 1997), it is an ideal carrier for bioactive molecules. Additionally, chitosan as a polycationic polyelectrolyte, has already been applied for DNA (polyanionic) complexation, and proved efficiency as a non-viral transfection agent (Mao et al., 2001; Liu and Yao, 2002).

A great advantage of chitosan relative to other encapsulation polymers is its high hydrosolubility at pH lower than 6.4 due to the protonation of the glucosamine groups. These properties enable interaction with negatively charged polymers, macromolecules and polyanions in aqueous environment (Janes et al., 2001). This is particularly interesting, since organic solvents and phase separation processes are not needed in chitosan formulations for encapsulation of macromolecules, avoiding problems related with protein denaturation during processing and regulatory specifications approvals (Chen, 2000).

Different colloidal devices for protein delivery using chitosan as a polymer have been described, as for example, spray-dried microparticles (Illum, 1998; Xu and Du, 2003), covalently cross-linked particles (Janes et al., 2001; Sinha et al., 2004), ionically coacervated particles (Aydin and Akbuga, 1996; Calvo et al., 1997; Dumitriu and Chornet, 1998; de Kruif and Tuinier, 2001; Janes et al., 2001; Ozbas-Turan et al., 2002; Shu and Zhu, 2002), chitosan-coated anionic core systems (Janes et al., 2001), thermal cross-linking, emulsification methodologies, among others (Agnihotri et al., 2004; Sinha et al., 2004).

Coacervation of chitosan can be done with several anions, namely sulfate, citrate, alginate and tri-polyphosphate as described in the literature (Calvo et al., 1997; Shu and Zhu, 2002). In this work we study the ability of bile salts to coacervate chitosan. Although not directly comparable with our study, it has been observed that chitosan derivatized with bile salts, forms hydrophobic (micelle-like) microdomains in aqueous solution (Lee et al., 1998a,b), and some authors have proposed the use of these hydrophobically modified polymers for DNA delivery (Lee et al., 1998b). Based on these data, it is predictable that bile salts may induce the aggregation of chitosan chains. Furthermore, bile salts are known to interact with lipid membranes by intercalation, increasing the permeability (Merkus et al., 1993). Therefore, it may be interesting for mucosal administration of proteins to associate bile salt to chitosan, since both are known to be good absorption enhancers.

In this study the model protein, BSA, is encapsulated into chitosan microparticles coacervated with bile salt, sulfate and citrate (Shu and Zhu, 2002; van der Lubben et al., 2002). Characterization and comparison of those microparticles, in which concerns the efficiency of coating and the release of the protein from the matrix are presented. Criteria followed for the comparative evaluation will focus on the following points: microparticle morphology, protein protection, incorporation yield, protein integrity during the process and inside the matrix and suitable drug release profile for mucosal administration (van der Lubben et al., 2001).



2. Materials and methods

2.1. Materials

Chitosan medium molecular weight (86.2% deacetylation degree, 400,000 Da average molecular weight) and the fluorescence-labeling reagent, fluorescein isothiocyanate isomer I (FITC), were purchased from Aldrich (Steinheim, Germany). Deoxycholic acid sodium salt (for microbiology), trehalose (for biochemistry), BSA (Albumin Fraction V from bovine serum, 69,000 Da), polysorbate 80 (Tween® 80), tri-sodium citrate dihydrate (GR), sodium sulfate dihydrate (GR), potassium iodide and all PBS buffer salts (GR) were purchased from Merck (Darmstadt, Germany). Pluronic® F68 and Pluronic® F127 (PEO–PPO–PEO triblock copolymers) were from BASF (Mount Olive, NJ, USA). All cellular media and supplements were purchased to GIBCO, Invitrogen (Scotland, UK). All reagents were used as acquired.

2.2. Protein-fluorescein conjugation

For detection and quantification purposes, BSA was labeled with FITC. Fluorescence probe conjugation of BSA was achieved according to a previously described methodology