Carbohydrate Polymers 121 (2015) 403–410

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Development and characterization of alginate coated low molecular weight chitosan nanoparticles as new carriers for oral vaccine delivery in mice

Subrata Biswas [a], Mainak Chattopadhyay [b], Kalyan Kumar Sen [c,*], Malay Kumar Saha [a]

[a] National Institute of Cholera and Enteric Diseases, Indian Council of Medical Research, Kolkata 700010, India
[b] Department of Pharmacology, Bengal School of Technology, Hooghly 712102, West Bengal, India
[c] Department of Pharmaceutics, Gupta College of Technological Sciences, Ashram More, G.T. Road, Asansol 713301, West Bengal, India

ARTICLE INFO

Article history:
Received 31 January 2014
Received in revised form 9 December 2014
Accepted 10 December 2014
Available online 2 January 2015

Keywords:
Adjuvant
Cytotoxicity
Mucosal immunity
Vaccine

ABSTRACT

In the present study, nanoparticles of low MW chitosan (CS) were formulated in which measles antigen was entrapped and subsequently coated with sodium alginate. The size and surface properties of the nanoparticle can be tuned with different MW of CS. *In vitro* release studies showed initial burst release followed by extended release, best fitted in the Makoid–Banakar model ($R^2 > 0.98$). SDS-PAGE assay revealed that alginate coating could effectively protect antigen in acidic condition for at least 2 h. Cell viability was assessed using MTT assay into HT 29 cell line. Formulations were orally administered to mice and immunological responses were evaluated using ELISA method. Obtained results showed that measles antigen-loaded CS nanoparticles induced strong immune response and significant correlation was observed between the immune response with CS MW. Protecting ability of antigen in gastric environment, sustained release kinetics, systemic and mucosal immune responses and low cytotoxicity observed for the alginate coated nanoparticles demonstrated that LMW CS could be promising platform for oral vaccine delivery.

1. Introduction

It is well established that protection against pathogenic organisms occurs better with the presence of mucosal antibody than with serum antibody (Baumann, 2008). Compared to traditional routes of administration, oral vaccine delivery systems offer several attractive advantages such as lower cost, ease of administration, higher patient compliance, reducing the need for trained personnel, averting vaccine-related infections correlated to the disposal and reuse of needles in systemic delivery as well as higher capacity for mass immunizations (Fooks, 2004; Kim, Lee, & Jang, 2012; Kostrzak et al., 2009; Zhu & Berzofsky, 2013). It has also been demonstrated that orally administered antigens can induce both local and systemic immune responses, providing a complete immune response (Neutra & Kozlowski, 2006). However, immune responses following oral administration of vaccines are usually poor due to rapid degradation of antigen in the harsh gut environment and their poor uptake by appropriate target sites, namely M cells located in the Peyer's patches (Czerkinsky & Holmgren, 2009). Keeping this in mind, several studies have been performed to show that by associating the vaccine with a number of particulate delivery systems, the degradation of the antigen in the harsh environment of the GI tract is prevented and the uptake by M-cells is enhanced by several times (Delie & Blanco-Prieto, 2005; Eyles, Sharp, Williamson, Spiers, & Alpar, 1998; van der Merwe, Verhoef, Verheijden, Kotze, & Junginger, 2004). In addition, biodegradable particles allow a sustained release of the antigen, increasing the duration of the contact between antigen and immune cells thus favouring an effective immune response.

In the past decades, chitosan (CS) has been extensively used as a carrier candidate because of its excellent stability, mucoadhesive property, enhanced penetration capacity across the mucosal barriers and good compatibility with vaccine antigen (Borges et al., 2007; Lee et al., 2011; Yoo et al., 2010; Zaharoff, Rogers, Hance, Schlom, & Greiner, 2007). The formation of CS nanoparticles is a process based on the complexation of oppositely charged molecule

Abbreviations: PAGE, polyacrylamide gel electrophoresis; GALT, gut associated lymphoid tissue.

* Corresponding author. Tel.: +91 341 2312130; fax: +91 341 2314604.
E-mail addresses: sai_brata@yahoo.com (S. Biswas), mainakcool@yahoo.co.in (M. Chattopadhyay), kks_kpa@yahoo.co.in (K.K. Sen), sahamk@yahoo.com (M.K. Saha).

http://dx.doi.org/10.1016/j.carbpol.2014.12.044
0144-8617/

by electrostatic interactions (Csaba, Koping-Hoggard, & Alonso, 2009). Nanoparticles can be easily constructed using an ionic gelation process that involves sodium tripolyphosphate as a negatively charged molecule and CS as polycation (Calvo, Remunan-Lopez, Vila-Jato, & Alonso, 1997; Oliveira et al., 2012). The mild technique involves the mixing of two aqueous solutions at ambient temperature while stirring without using sonication or organic solvents. Proteins with low isoelectric point, such as BSA and measles antigen, were better associated with the nanoparticles when dissolved in the alkaline sodium TPP solution (Li et al., 2008). Although CS nanoparticles have numerous advantages as delivery carriers for oral vaccination, their limited ability to control the release of encapsulated antigens and easy solubility in acidic medium largely hamper their development (Li et al., 2008). These obstacles may be overcome by coating those antigen loaded particles with an acid-resistant polymer, like sodium alginate (Borges et al., 2006).

Sodium alginate, an anionic polysaccharide with favourable biological properties, can be easily interacted with cationic CS nanoparticles to form an electrolyte complex *via* electrostatic interactions (Du et al., 2013; Kim et al., 2002). Alginate-CS polyionic complexes form through ionic gelation *via* interactions between the cationic amine groups of CS and the anionic carboxyl groups of alginate (Gazori et al., 2009). This coating procedure was performed at relatively mild conditions without using any organic solvent at room temperature, enabling antigens to be incorporated into the CS/alginate matrices with retention of biological activity (Wee & Gombotz, 1998). Calcium ion is used as crosslinking agent to strengthen and stabilize the particles (Borges et al., 2006). Recently, Liu et al. (2013) demonstrated that alginate-coated CS nanoparticles containing DNA are effectively taken up and expressed by the Payer's patches. Release profiles of vaccines from these delivery systems showed that burst release of loaded BSA in pH 1.2 (simulated gastric fluid) was largely prevented by its entrapment in alginate-coated CS particles (Anal, Bhopatkar, Tokura, Tamura, & Stevens, 2003). Moreover, Borges et al. (2006) demonstrated that sodium-alginate coated nanoparticles were readily able to be taken up by rat Peyer's patches which is one of the essential features to internalize, deliver and target the intact antigen to specialized immune cells from gut associated lymphoid tissue (GALT). *In vivo* studies have shown higher antibody titre for alginate coated CS nanoparticles compared to plain CS nanoparticles (Malik, Goyal, Zakir, & Vyas, 2011). This delivery system also acted as an effective adjuvant for hepatitis B surface antigen when subcutaneously administered in a mouse model (Borges et al., 2008). It has been demonstrated that transfection efficiency is closely related to the MW of polymer. CS of 10–150 kDa with a specific degree of deacetylation allows maximum transgenic expression *in vitro* and CS nanoparticles less than 500 nm in diameter are transported through an endocytotic mechanism (Godbey, Wu, & Mikos, 1999; Lavertu, Methot, Tran-Khanh, & Buschmann, 2006; Liu et al., 2013).

Recently, there has been growing interest in low molecular weight CS (CS-LMW) as a new mucosal delivery vehicle due to its greater solubility in neutral aqueous solvents with low viscosity and faster degradation than higher molecular weight CS counterparts (Amoozgar, Park, Lin, & Yeo, 2012; Fernandes et al., 2012; Liu et al., 2013). Vila et al. (2004) reported that nanoparticles made of CS-LMW with tetanus toxoid (TT) as a model protein could be produced by an ionic-cross linking technique and proving to be promising carrier for nasal vaccine delivery. Following intranasal administration, TT-loaded nanoparticles elicited an increasing and long-lasting humoral response as compared to the soluble antigen. Plasma or serum samples have traditionally been used for generation of serological data due to the difficulties associated with handling whole blood. However, this approach requires relatively large volumes (200–1000 μl) of blood in order to produce the required volume of matrix for bioanalysis, which makes it difficult to generate serial profiles in mice test models. Composite profiles are therefore necessary, which can result in lower quality data and requires the use of a greater number of animals. Conventional ELISA are formatted to be used only for determining whether a given sample contains antibody at a concentration above or below a set threshold value, although quantification of antibody response is prerequisite to monitor the progress after immunization. In recent years, dried blood spot (DBS) assays have received increasing attention as an alternative to venous blood sampling and can be adopted in preclinical serological studies.

To the best of our knowledge, there is no published data whereby alginate coated CS-LMW nanoparticles are explored as vehicles for the oral administration of vaccine. In this study, we have tried to develop a novel oral vaccine carrier based on alginate coated CS-LMW nanoparticles to meet the requirement of oral vaccine. So the objectives of the studies are to develop the nanoparticle based oral vaccine delivery system and the delivery system will be adopted to evaluate the physicochemical properties, loading efficiency of the particles and the stability of antigen loaded nanoparticles against acidic condition. Cytotoxicity will be evaluated using HT 29 human epithelial cell lines for the different formulation of nanoparticles. Finally, the effects of molecular weight of CS (42, 74 and 106 kDa) on the ability of these nanoparticles will be studied in a mouse model to elicit significant immune response as measured using DBS ELISA method.

2. Experimental

2.1. Materials

CS-LMW of three different molecular weight (42, 74 and 106 kDa) was obtained through oxidation–reduction reaction of high molecular weight CS (MW 375 kDa) (Sigma Aldrich, USA) using $NaNO_2$ as described by Mao et al. (2004). Briefly, varying quantities of 0.1 M $NaNO_2$ were added to the CS solution at 25 °C under magnetic stirring, and the reaction was left overnight to assure completion of the degradation. The depolymerisation process of CS has been previously simulated based on Box–Benkhen design experiments. With this approach, the required amount of $NaNO_2$ could be predicted in order to obtain approximate molecular weight of 42, 76 and 106 kDa. The molecular weight of the CS was determined using high pressure size exclusion chromatography (HPSEC).

Sodium tripolyphosphate (TPP), sodium alginate, $CaCl_2$, DMEM (Dulbecco's Modified Eagle's Medium), HEPES [N-(2-hydroxyethyl piperazine-N-(2-ethanosulphonic acid)], MTT (methylthiazolyl diphenyl-tetrazolium bromide) reagents, goat anti-mouse IgG peroxidise conjugate and TMB/H_2O_2 substrate for ELISA were procured from Sigma (USA). Anti measles monoclonal antibody was purchased from Millipore. BCA kit was purchased from Pierce (USA). Measles antigen was kindly donated by Serum Institute of India Ltd (Pune, India). 96-well flat bottom polystyrene plate was purchased from Nunc (Denmark). All other reagents were of analytical grade. Ultrapure water (MilliQ, Waters, USA) was used to prepare all solutions and freshly prepared solutions were used in all experiments.

Human intestinal epithelial cells (HT 29) were cultured and maintained in DMEM with supplemented with 10% foetal bovine serum and 1% antibiotic–antimycotic solution (Invitrogen, Carlsbad, CA). Cells were maintained in 5% CO_2 at 37 °C humidified incubator for 10 days.

Immunogenicity of the antigen was evaluated in 6 weeks old specific pathogen-free male BALB/c mice. Animal care and handling were maintained according to the guidelines established by the Institutional Animal Ethical Committee (IAEC) of Gupta College of Technological Sciences, India. The experiment protocols were