International Journal of Pharmaceutics 495 (2015) 220–233

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Alginate coated chitosan microparticles mediated oral delivery of diphtheria toxoid. Part A: Systematic optimization, development and characterization

Anshuman Shukla [a,b,*], Vijay Mishra [c], Bhupinder Singh Bhoop [a], Om Prakash Katare [a]

[a] Drug Delivery Research Group, University Institute of Pharmaceutical Sciences, Punjab University, Chandigarh 160014, India
[b] Novel Drug Delivery Research Laboratory, Adina Institute of Pharmaceutical Sciences, Sagar 470002, India
[c] Pharmaceutical Nanotechnology Division, Adina Institute of Pharmaceutical Sciences, Sagar 470002, India

ARTICLE INFO

Article history:
Received 5 June 2015
Received in revised form 1 August 2015
Accepted 8 August 2015
Available online 28 August 2015

Chemical compounds studied in this article:
Chitosan (PubChem CID: 21896651)
Rhodamine 123 (PubChem CID: 65218)
Acrylamide (PubChem CID: 6579)
Sodium dodecyl sulfate (PubChem CID: 3423265)

Keywords:
Diphtheria toxoid
Microparticles
Oral immunization
Chitosan
Mucosal delivery
Formulation-by-design

ABSTRACT

The current study was embarked upon to develop "optimized" alginate coated chitosan microparticles (ACMs) loaded with diphtheria toxoid (DTx) employing formulation by design approach. The developed system was characterized for particle size, zeta potential, surface morphology, acidic degradation protection studies, in process stability studies, storage stability studies and in vivo uptake studies. Microparticles with minimum of average size of 5 μm (PDI, 0.184) were chosen after optimizing the composition and process conditions. The optimized chitosan microparticles were subjected to alginate coating for better protection of loaded antigen till it reached to uptake site i.e. M cells in the Peyer's patches (PPs) and transport of higher amount antigen to the PPs. The zeta-potential values for uncoated chitosan microparticles and ACMs were found to be $+29 \pm 3.3$ mV and -32.6 ± 4.2 mV, respectively. This change of zeta potential, for uncoated to coated, can be explained by the fact that the coating of alginate on chitosan microparticles led to negative side of the zeta potential by virtue of its predominance on the surface. The developed ACMs were able to transport the antigen effectively to the M cell as revealed by confocal laser scanning microscopy. Further, DTx-loaded ACMs demonstrated significant immune responses at serum IgG as well as mucosal sIgA level.

1. Introduction

Oral vaccination at the level of the gut mucosa can elicit production of secretory antibodies at distant mucosal surfaces, which increases its potential for immunization against infectious diseases. Further, oral vaccination offers several advantages like simplicity, ease of administration, improved patient compliance, minimum side effects and greater opportunity of frequent boosting over the parenteral vaccination. However, the major constrain of oral immunization is the denaturation and degradation in the gastrointestinal tract of host. This consequently requires larger and more frequent dosing of antigen, which leads to oral tolerance. However, this can be overcome by utilizing appropriate delivery system (Shukla et al., 2008, 2010).

Chitosan, a natural polymer, has demonstrated enhancement of the immunogenicity of poorly immunogenic vaccine antigens in the form of micro/nanoparticles (Amidi et al., 2010; Zaharoff et al., 2007). Chitosan, a cationic polysaccharide, due to its several favorable biological properties such as non-toxicity, biodegradability and mucoadhesivenss has gained increasing attention (Gan et al., 2005; George and Abraham, 2006). However, on incubation in physiological environment, chitosan loses its mucoadhesive properties and permeation enhancing effect due to its deprotonation. Meanwhile, because of its hydrophilic nature and solubility in acidic medium chitosan has limited ability for controlling the release of encapsulated macromolecule compounds (George and Abraham, 2006; Kotze et al., 1999).

Coating of sodium alginate, an acid-resistant polymer, onto the surface of chitosan microparticles may be an interesting method to overcome these obstacles. Alginate, as an anionic polysaccharide,

* Corresponding author at: Novel Drug Delivery Research Laboratory, Adina Institute of Pharmaceutical Sciences, Sagar 470002, India.
E-mail addresses: anshumanshukla12@gmail.com (A. Shukla), vijaymishra2@gmail.com (V. Mishra).

http://dx.doi.org/10.1016/j.ijpharm.2015.08.028
0378-5173/

Table 1
Formulation and process variables with their respective high and low levels, investigated using Taguchi screening design.

Variables	Levels	
	Low (−1)	High (+1)
Polymer concentration	100 mg	400 mg
Cross linking agent concentration	5%	10%
Stirring speed (rpm)	1000	3000
Concentration of glacial acetic acid	2%	4%
Stirring time (min)	30	60
Rate of cross linker addition	At intervals	At once
Volume of glacial acetic acid	50 mL	100 mL

Amount of antigen was kept constant (200 μg/mL).

can easily interact with cationic chitosan microparticles to form the polyelectrolyte complex via electrostatic interactions (Kim et al., 2002; Lee and Min, 1996; Okada et al., 1997; Severian and Esteban, 1998). Moreover, this coating procedure was performed without using any organic solvent. This comparatively mild process has enabled proteins as well as cells and DNA to be incorporated into the chitosan/alginate matrices with retention of biological activity (Gombotz and Wee, 1998).

The current study was aimed to develop the systematically optimized DTx-loaded ACMs and investigate their possibility as an effective vaccine delivery system.

2. Materials and methods

2.1. Materials

Gift sample of Diphtheria toxoid was received from M/s Panacea Biotech Ltd., Panjab, India. Rhodamine 123 (R123) was purchased from Sigma Chemical Co., St. Louis, MO, USA. Sorbitan tristearate, cholesterol and BCA protein estimation kit were purchased from Central Drug House (P) Ltd., New Delhi, India, Loba Chemie Pvt. Ltd, Mumbai, India and Genei, Bangalore, India, respectively. Reagents used in SDS-PAGE were procured from Bio-Rad, India. All other chemicals and reagents were of analytical grade and purchased from the local suppliers.

2.2. Pre-optimization of DTx-loaded microparticles employing Taguchi screening design

2.2.1. Screening of influential variables

The Taguchi design was employed for factor screening among various formulation and process variables involved in the development of microparticles. The high and low levels of various variables screened for their influence in the development of DTx-loaded ACM were described in Table 1. The L8 array layout for 7 factors like polymer concentration, cross linking agent concentration, stirring speed, glacial acetic acid concentration, stirring time, rate of cross linker addition and volume of glacial acetic acid in two-level Taguchi design was adopted for their pre-optimization screening (Table 2). Percent antigen loading efficiency and particle size were the key response variables investigated thoroughly for selecting the significant formulation and process variables.

Eight formulations of DTx-loaded microparticle as per design matrix were prepared by precipitation/coacervation method as described earlier (Berthold et al., 1996). Briefly, chitosan was dissolved at different concentrations in a solution of acetic acid and 1% (w/v) of Tween® 80. The addition of sodium sulphate was made at a rate of 1 mL/min under mild agitation (<50 rpm) and continuous sonication. After the addition of sodium sulfate solution to the chitosan solution the formation of the particles was achieved. Sonication was maintained for additional 15 min and the agitation for 60 min at room temperature (RT). The suspension was centrifuged for 30 min at 3500 rpm (4590 × *g*) and the supernatant was discarded. The particles were re-suspended twice in water, centrifuged again for 30 min and the supernatants were discarded.

The pre-optimized microparticles were then subjected to coating of sodium alginate. In order to prepare coated microparticles, suspension of microparticles was prepared and added under agitation to sodium alginate coating solution in various concentrations (i.e., 0.1%, 0.5%, 1.0%, 1.5%, 2.0% and 2.5%, w/v). For 30 min, on a magnetic stirrer, the suspension of the microparticles was maintained under agitation. The supernatant was discarded after the centrifugation of suspension at 1500 rpm (2050 × *g*) for 15 min. Finally the microparticles were re-suspended in 0.5 mM calcium chloride solution and kept under agitation for 10 min to chemically cross link the alginate at the particle's surface.

2.2.2. Percent antigen loading capacity (PALC) and loading efficiency (PALE)

In order to investigate the DTx loading of microparticles, the chitosan microparticles (1%, w/v) and DTx (0.2%, w/v) were incubated in phosphate buffer saline (PBS; pH 7.4) under shaking at 25 °C. Subsequent to 2 h of incubation the suspension was centrifuged 1500 rpm (2050 × *g*) for 30 min to remove unloaded, free DTx. The loading degree was calculated by estimating the unloaded DTx in the supernatant using BCA protein estimation kit (Genei, India). The values of PALC and PALE were determined as follows:

$$\text{PALC} = \frac{(\text{Total amount of DTx} - \text{free DTx})\,\text{mg}}{(\text{Weight of microparticles})}$$

$$\text{PALE} = \frac{(\text{Total amount of DTx} - \text{free DTx})\,\text{mg}}{(\text{Total amount of DTx})\,\text{mg}}$$

2.2.3. Particle size analysis

The mean vesicle size and size distribution of the samples were determined using a laser-diffraction based particle size analyzer (Zetasizer Nano ZS 90, Malvern Instruments Co., U. K.).

Table 2
L_8 array layout as per six factors, two level Taguchi screening design.

Trials	Polymer concentration	Cross linking agent concentration	Stirring speed	Glacial acetic acid concentration	Stirring time	Rate of cross linker addition	Volume of glacial acetic acid
1.	−1	−1	−1	−1	−1	−1	−1
2.	−1	+1	−1	+1	+1	+1	−1
3.	+1	+1	−1	−1	−1	+1	+1
4.	+1	−1	−1	+1	+1	−1	+1
5.	+1	−1	+1	+1	−1	+1	−1
6.	+1	+1	+1	−1	+1	−1	−1
7.	−1	+1	+1	+1	−1	−1	+1
8.	−1	−1	+1	−1	+1	+1	+1

Amount of antigen was kept constant (20 Lf/mL).