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Divalent toxoids loaded stable chitosan–glucomannan nanoassemblies for efficient systemic, mucosal and cellular immunostimulatory response following oral administration

Harshad Harde, Krupa Siddhapura, Ashish Kumar Agrawal, Sanyog Jain *

Centre for Pharmaceutical Nanotechnology, Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research, SAS Nagar, Punjab 160062, India

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ABSTRACT

The present study reports dual tetanus and diphtheria toxoids loaded stable chitosan–glucomannan nanoassemblies (sCh–GM-NAs) formulated using tandem ionic gelation technique for oral mucosal immunization. The stable, lyophilized sCh–GM-NAs exhibited ~152 nm particle size and ~85% EE of both the toxoids. The lyophilized sCh–GM-NAs displayed excellent stability in biomimetic media and preserved chemical, conformation and biological stability of encapsulated toxoids. The higher intracellular APCs uptake of sCh–GM-NAs was concentration and time dependent which may be attributed to the receptor mediated endocytosis via mannose and glucose receptor. The higher Caco-2 uptake of sCh–GM-NAs was further confirmed by *ex vivo* intestinal uptake studies. The *in vivo* evaluation revealed that sCh–GM-NAs posed significantly ($p < 0.001$) higher humoral, mucosal and cellular immune response than other counterparts by eliciting complete protective levels of anti-TT and anti-DT (~0.1 IU/mL) antibodies. Importantly, commercial 'Dual antigen' vaccine administered through oral or intramuscular route was unable to elicit all type of immune response. Conclusively, sCh–GM-NAs could be considered as promising vaccine adjuvant for oral mucosal immunization.

1. Introduction

Vaccines have been evolved as a revolutionary invention to transform the 'killer' perspectives of infectious diseases; however their limited access, to the remote areas, is still a big challenge. Conventional alum adsorbed vaccines *viz.* monovalent vaccine in form of TT (Tetanus toxoid) and DT (Diphtheria Toxoid) or combination vaccines such as DT, DTaP, DTaP- Hib DTP-Hib) are unable to produce complete immunostimulatory response and need cold storage facility. Furthermore, autoimmunity, long-term brain inflammation and neurological complications are the associated drawbacks of conventional vaccines which trigger the exploration of alternative affordable, accessible and acceptable route for immunization (Arora et al., 2013; Tomljenovic and Shaw, 2011). Among the different alternative routes, oral route is associated with high patient compliance, avoidance of pain and trauma, less stringent manufacturing conditions, and elimination of risk of needle borne infections (Kersten and Hirschberg, 2007; Silin et al., 2007). Additionally, induction of mucosal immune response to combat pathogen at entry site is another advantage which make the oral immunization as route of choice (Jain et al., 2014a). However, the degradation of antigen in the harsh gastric milieu and poor permeability through GIT membrane are the major hurdles which need to be addressed for peroral delivery of antigens (Jain et al., 2011b).

A variety of nanotechnology based delivery approaches have been proven competent for oral immunization. Nano-carriers not only preserve the antigen integrity and immunogenicity by protecting the entrapped antigen from harsh environment, but also result into the enhanced activity due to enhanced permeation through M cells of Peyer's patches and presentation of antigen to the antigen presenting cells (APCs) via endocytic uptake (Harde et al., 2011). Nanoparticles (NPs) as immunopotentiator, capable of effective elicitation of broad immunostimulatory response such as humoral (IgG), cellular (interleukin and interferon) and mucosal (sIgA) in comparison with conventional aluminium based adjuvants, is also a well reported fact (Peek et al., 2008). However presence of residual organic solvent, poor shelf life and scalability are the major concern (Jung et al., 2001). Among the different nano-carrier based approaches chitosan nanoparticles (Ch-NPs) have been explored as a potential vaccine adjuvant. Although low cost, ease of preparation, and avoidance of organic solvents

* Corresponding author. Tel.: +91 172 2292055, fax: +91 172 2214692.
E-mail addresses: sanyogjain@niper.ac.in, sanyogjain@rediffmail.com (S. Jain).

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(Bowman and Leong, 2006) are the advantages of chitosan nanoparticles yet, instability in biological milieu, burst release, and poor storage stability are the drawbacks which need to be addressed (Jonassen et al., 2012; Lopez Leon et al., 2005).

In present technology, chitosan nanoparticles were prepared by tripolyphosphate (TPP) as cross linking agent. As TPP alone is not able to stabilize nanoparticles sufficient enough, glutaraldehyde was added as surface cross-linker to have more robust and stabilized nanoparticles suitable for oral delivery. Furthermore, glucomannan (GM) along with chitosan was selected as matrix as well as ligand forming polymer. It was assumed that, non-hydrolysis of GM by digestive enzymes may be helpful in formation of stable and robust system. Additionally, polymeric nature of GM may be helpful in increasing the mannose molecules density over the surface of NPs which ultimately may result into more precise targeted delivery to antigen presenting machinery (Jain et al., 2014b; Wang and He, 2002).

2. Experimental

2.1. Materials and reagents

Tetanus toxoid (TT) and diphtheria toxoids (DT) were obtained as generous gift from Panacea Biotec Ltd., Punjab, India. Commercial 'Dual antigen' vaccine, Serum Institute, Pune, India was obtained from local pharmacy. Chitosan (medium molecular weight, 190–300 kDa; deacetylation degree, 87%), pentasodium tripolyphosphate (TPP), bicinchoninic acid (BCA), sucrose, Sephadex G-100, bovine serum albumin – fluorescein isothiocyanate conjugate (BSA-FITC), 4′,6-diamidino-2-phenylindole (DAPI), Concanavalin A (Con-A), sucrose, sodium dodecyl sulphate (SDS), acrylamide, bisacrylamide, ammonium persulpahte (APS), TEMED, Tween 20, acetic acid pilocarpine, anti-mouse IgA (α-chain specific) peroxidase conjugate, anti-mouse IgG (γ-chain specific) peroxidase conjugate, 3,3′,5,5′-tetramethylbenzidine (TMB) and Nunc Immuno™ Maxisorb F96 well solid plates were procured from Sigma, Missouri, USA. Copper sulphate (pentavalent), panacreatin, pepsin, and glutaraldehyde (25% w/v) were purchased from Loba chemie, Mumbai, India. Sodium dihydrogen phosphate, and dipotassium hydrogen phosphate were acquired from Central Drug House, New Delhi, India. Novex Sharp pre-stained protein standard (3.5–260 kD) was obtained from Invitrogen, California, USA. Bromophenol Blue, Coomassie Brilliant Blue G, Glycine, β-mercaptoethanol, and Tris Buffer were procured from Himedia, India. Konjac Glucomannan (GM) was procured from Megazyme, Wicklow, Ireland. Cryomatrix was obtained from Thermo Shandon, USA. Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium 1640 (RPMI-1640), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from PAA laboratories, Pasching, Austria. Mouse IL-2 and IFN-γ Legend ELISA MAX™ Deluxe kits were obtained from Biolegend Inc., California, USA. All other chemicals and reagents used were of HPLC or analytical grade. Ultrapure water (Labostar TWF UV 7, Siemens, USA) was prepared in house and used throughout the experimentation.

2.2. Preparation of stable chitosan–glucomannan nanoassemblies (sCh–GM-NAs)

The conventional chitosan nanoparticles (Ch-NPs) were prepared by ionic gelation technique using TPP as crosslinking agent (Csaba et al., 2009; Harde et al., 2014a; Ma et al., 2002). The chitosan was dissolved in acetic acid (0.1% w/v; pH 3.2) and pH was adjusted to 6.0 with the help of 1N NaOH. The crosslinking solution (1 mL) comprising of 0.1% w/v TPP and 420 Lf/mL toxoids (1:5 ratio of TT and DT) in distilled water was added dropwise to chitosan solution (4 mL) with continuous stirring at 1500 rpm and kept for 15 min to finally obtain Ch-NPs.

For glucomannosylation, 1 mL GM (0.1% w/v) solution was added to 4 mL chitosan, keeping all other process and formulation parameters constant to obtain Ch–GM-NAs. The glucomannosylation of Ch-NPs (Ch–GM-NAs) was confirmed by Concanavalin A agglutination assay, while amount of GM associated with Ch-NPs was evaluated by sulphuric acid – phenol (SAP) colorimetric method (see Supporting information) (Jain et al., 2014b).

To further obtain the stable chitosan-glucomannan nanoassemblies (sCh–GM-NAs), glutaraldehyde (1 mL, 0.05% w/v) was added to Ch-GM-NPs dispersion (6 mL) at 500 rpm and kept on stirring for 15 min. All operations were carried out at room temperature. All other formulation and process parameters were kept constant.

2.3. Lyophilization

The formulations were lyophilized using 5% w/v sucrose to remove off unreacted glutaraldehyde and to obtain final stable dispensable form. A previously optimized and patented lyophilization cycle was used for this study (Vir Tis Wizard 2.0, USA) (Agrawal et al., 2014; Jain et al., 2012a). Any trace of residual glutaraldehyde in formulations was determined by gas chromatography (see Supporting information). The lyophilized products were also examined for appearance of the cake, change in particle size, redispersibility index (ratio of size obtained after reconstitution of lyophilized product to the initial size before lyophilization), and reconstitution time.

2.4. Characterization of toxoids loaded formulations

The particle size, polydispersity index (PDI), and zeta potential were measured by photon correlation spectroscopy (Zetasizer, Nano ZS, Malvern Instruments Corp, Worcestershire, UK) at 25 °C. All measurements were performed after proper dilution of formulation with ultrapure water.

The entrapment efficiency (%EE) and lime of flocculation (Lf) of formulation was calculated by direct method in supernatant and by flocculation test, respectively. The formulation was centrifuged at 41,000 g for 30 min (High speed centrifuge, 3K30, Sigma, USA) and pellet was dispersed in 1% acetic acid solution followed by mild sonication. Then dispersion was incubated for 48 h at 37 °C. The supernatant containing toxoids was collected, separated by Sephadex G-100 column (25 cm column and 33 cm^2 active surface area) and amount of each toxoid was calculated using validated microBCA colorimetric assay at 561 nm by UV spectrophotometer (PowerWave XS2, Biotek Instruments Inc., Vermont, USA) (see Supporting information). For flocculation test, varying amount of antitoxin in transparent glass tube was incubated with resolved toxoids by Sephadex G-100 at 37 °C. The lime of flocculation was recorded visually on the basis of time required for immunoprecipitation (Lyng and Bentzon, 1987).

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were accomplished to analyze the shape and morphology of the toxoids loaded formulations. A drop of formulation was positioned on piece of glass cover slip, air dried, and gold coating was implemented using gold sputter. The processed samples were observed under SEM (S-3400N, Hitachi, Japan). A drop of formulation was placed on formvar coated grid, stained with 1% w/v phosphotungstic acid and observed under TEM (FEI, Technai G2 F20, USA).

2.5. Chemical integrity and conformational stability of antigen

The sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and far UV circular dichroism (CD) spectroscopy

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