Journal of Drug Delivery Science and Technology 44 (2018) 19–26

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

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

Novel approach to improve vaccine immunogenicity: Mannosylated chitosan nanoparticles loaded with recombinant hepatitis B antigen as a targeted vaccine delivery system

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ARTICLE INFO

Keywords:
Mannosylated chitosan nanoparticles
rHBsAg
Cytotoxicity
Immune system

ABSTRACT

The design of effective vaccine delivery system is opening up new feasibilities for making immunization more safe and efficient. In this study, recombinant hepatitis B virus surface antigen (rHBsAg) was loaded in mannosylated chitosan (MC) nanoparticles to be used as a targeted vaccine delivery vehicles. The nanoparticles were prepared by ionic gelation method and characterized for physicochemical properties, cytotoxicity and antigenicity. The rHBsAg-loaded MC nanoparticles showed spherical shape with mean particle size of 246 ± 33 nm, zeta potential of 25.6 ± 1.7 mV, loading capacity of 12.2 ± 1.4%, and encapsulation efficiency of 90 ± 1.6%. *In vitro* release profile of rHBsAg-loaded MC nanoparticles exhibited an initial burst release of about 26% in the first 7 days followed by a slow release of 25% for 49 days, with release kinetic similar to Higuchi model. SDS-PAGE analysis confirmed integrity of released rHBsAg and structural stability of the antigen during entrapment process. The rHBsAg-loaded MC nanoparticles indicated time-and concentration-dependent cytotoxicity using MTT assay. It can be concluded that entrapment of rHBsAg in MC nanoparticles appears to be a suitable approach for targeting this antigen into body immune system.

1. Introduction

Hepatitis B virus (HBV) infection is still a major public health concern affecting 2 billion people around the world. There are still 350 million chronic carriers of HBV worldwide, of which many are high risk for development of liver cirrhosis, liver failure, and hepatocellular carcinoma. As a result, hepatitis B and its complications account for approximately 1.2 million deaths per year [1–3]. One of the best strategies for prevention of hepatitis B is vaccination. Current vaccination techniques exhibit relatively low immune response, therefore, to induce a full protective antibody response against the HB antigen, a multiple-dose vaccination schedule is required [4,5]. In order to improve the vaccine efficacy, various methods have been investigated. The recent strategies for improvement of vaccines immunogenicity include use of ligands which are recognized by immune cells, resulting in improved processing of antigen by presenting cells (APCs). Incorporation of mannosylated nanosized materials with mannose receptors on immune cell surface can significantly increase antigen processing and presentation. In this regards, attachment of mannose into liposomes was shown so strongly increase internalization by human dendritic cells (DCs) compared to native liposomes [6]. Chitosan (CS) has emerged as a promising biomaterial with valuable properties such as biocompatibility, biodegradability, mucoadhesivity, modifiable, non-toxic, and ability to provide sustained release of loaded therapeutics [7–9]. Its unique properties make it an attractive candidate for many industrial and biomedical applications. Its amine groups serve for covalent attachment of biomolecules [10].

CS nanoparticles can be synthesized by ionic gelation technique

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https://doi.org/10.1016/j.jddst.2017.11.022
Received 14 May 2017; Received in revised form 18 November 2017; Accepted 24 November 2017

utilizing triphosphate (TPP) as a cross-linking agent. This method employs simple and mild preparation conditions without the use of high shear forces and organic solvents [11,12]. CS nanoparticles have a high positive zeta potential, large surface area, and they can provide high intensity activities [13]. The particles with different physicochemical and biological characteristics can be prepared using different synthesizing conditions [14,15].

Among the different types of ligand-conjugated CS, MC has gained many attentions due to specific interactions between mannose receptor on antigen presenting cells and mannose ligand [16–18]. In the present study, MC nanoparticles containing recombinant hepatitis B antigen (rHBsAg) were prepared and *in vitro* antigenicity and cytotoxicity of this novel targeted vaccine delivery vehicle were evaluated.

2. Materials and methods

2.1. Materials

Low molecular weight CS (with degree of deacetylation ≥95%) was purchased from Primex (Iceland). TPP, Coomassie Blue G250, mannopyranosylphenyl isothiocyanate, dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (USA). The recombinant hepatitis B antigen (rHBsAg) and HEK293 cell line were supplied by Pasteur Institute of Iran. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco (USA). All other materials and reagents were obtained commercially as reagent grade products.

2.2. Methods

2.2.1. Preparation of MC

MC was prepared by as previously reported with some modification [19,20]. 100 mg CS was dissolved in 2 ml of double distilled water, pH adjusted to 5.5 and mixed with solution of mannopyranosylphenyl isothiocyanate in 1 ml of DMSO. After stirring for 24 h at room temperature, the MC was precipitated by adding 10 vol of isopropanol and centrifuged at 15000 RCF for 20 min. After repeating this process four times, obtained pellets were dried in a vacuum oven. Then, MC powder was analyzed by fourier transform infrared spectroscopy (FTIR) and proton nuclear magnetic resonance (^{1}H NMR). FTIR spectra of MC were recorded and analyzed in the wave number range 400–4000 cm^{-1} (Jasco FTIR-410, Japan), using KBr pellets (95 mg KBr + 5 mg CS or MC nanoparticles).^{1}H NMR spectrum of MC (2 mg/ml) was measured in 0.5 ml D_2O containing (2%) trifluoroacetic acid (TFA)/D2O, using a 500 MHz spectrometer (Bruker, Germany).

2.2.2. Preparation of MC nanoparticles loaded with rHBsAg

MC nanoparticles were prepared by the ionic gelation method [21–24]. Low molecular weight CS was dissolved in deionized water (1.85 mg/ml), then, pH of solution was adjusted to 3.43. TPP solution (1 mg/ml) was added dropwise to the MC solution under magnetic stirring (1300 rpm) at room temperature. MC nanoparticles were formed via interactions between positively charged amino groups of CS and negatively charged of TPP. Antigen loaded nanoparticles were prepared with the incorporation of TPP solution containing rHBsAg (108 μg/ml) to MC solution. MC nanoparticles were separated from the suspension medium by centrifugation at 17000 RCF at 8 °C for 30 min and supernatants were analyzed for free antigen.

2.2.3. Characterization, shape and surface morphology of nanoparticles

The morphology, size and zeta potential of the nanoparticles were studied by scanning electron microscope (SEM) (Hitachi H-600, Japan) and dynamic light scattering (DLS) (Malvern zetasizer Nano ZS, UK). The samples were air dried and sputter coated with gold for SEM studies and suspended in acetic acid 0.25 %w/v solution (pH 3.43) with sonication for DLS measurements.

2.2.4. Evaluation of rHBsAg loading capacity (LC) and encapsulation efficiency (EE)

To determine the LC and EE, the nanoparticles were centrifuged at 17000 RCF and 8 °C for 30 min. The amount of free rHBsAg in the supernatant was determined by Bradford protein assay. Bradford reagent contained Coomassie Brilliant Blue G-250 and absorbance was measured at 595 nm after 10 min [2,25]. LC and EE of the nanoparticles were calculated from equations (1) and (2) indicated below [26]:

$$EE = \frac{\text{Total amount of rHBsAg} - \text{amount of Free rHBsAg}}{\text{Total amount of rHBsAg}} \times 100 \quad (1)$$

$$LC = \frac{\text{Total amount of rHBsAg} - \text{amount of Free rHBsAg}}{\text{Weight of Nanoparticles}} \times 100 \quad (2)$$

2.2.5. In vitro release study

Antigen release profile from MC nanoparticles was determined as follows: 1 mg of freeze-dried MC nanoparticles and 1 ml of phosphate buffer (pH = 7.4) containing thimerosal (0.01%) were transferred to tubes and shaker incubator (37 °C and 100 rpm). One sample at predetermined times of 1, 3, 7, 14, 21, 28, 35, 42 and 49 days was picked up and centrifuged at 18000 RCF for 20 min. The amount of rHBsAg released was determined using Bradford assay.

2.2.6. Cell viability assay

The MC nanoparticles containing rHBsAg with various concentrations were assayed for cytotoxicity over 24 and 48 h on HEK293 cell line (human embryonic kidney cells 293) [27,28]. Cells were cultured for 48 h in DMEM medium, at humidified atmosphere of 5% CO2/air, 37°Cbefore treatment and supplemented with 10% FBS and 1% Pen/Strep. Cell viability of the samples was evaluated by MTT (3-(4, 5-dimethylthiazol-2yl)-2, 5- diphenyltetrazolium bromide) assay. 24 h before treatments, cells 2×10^4 cells/well were cultured in a 96 well plate. After discarding the old medium, the cells were immediately exposed with a fresh medium containing various concentrations of rHBsAg loaded MC nanoparticles (12.5, 25, 50, 100, 200, 400, 800 and 1600 μg/ml). After 24 and 48 h incubation times, 20 μL MTT (5 mg/ml) was added to each well and the cells were incubated for another 4 h. Then, the medium was replaced with 150 μL of DMSO and plate was incubated for 30 min at room temperature. Subsequently, the viability was estimated by measurement of absorbance of each well at 570 nm using equation (3). Untreated cells were used as control. IC_{50} values were calculated using SigmaPlot 12.0 software [29,30].

$$viability(\%) = \left(\frac{Optical\ density\ of\ the treated\ cells}{Optical\ density\ of\ the\ untreated\ cells}\right) \times 100 \quad (3)$$

2.2.7. Characterization of structural integrity of rHBsAg

In process stability and integrity of the entrapped antigen in nanoparticles was assessed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE containing 4% stacking gel and 12% separation gel was used in order to check effects of nanoparticles preparation condition on structural of the antigen. Three samples were run including free rHBsAg, markers and released antigen (overnight in PBS at 37°c) and examined and the gel was stained with silver staining method [5].

2.2.8. Antigenicity

The qualitative antigenic properties of rHBsAg were evaluated by Ouchterlony double immunodiffusion assay as well as quantitative values were estimated by determination of antigen/total protein ratio measured by ELISA and Bradford assays. The quantitative results were compared together using student t-test.