Original Article

Design, Synthesis, Physicochemical and Immunological Characterization of Dendrimer-HBsAg Conjugate

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ABSTRACT

Introduction: Manufacturing new Hepatitis B virus vaccines, specifically by the use of nanoparticles, is of high global interest. In this paper, a new structure of nano-sized hepatitis B virus' surface antigen (HBsAg) was generated by conjugation with dendrimers. **Methods:** The physicochemical properties of the conjugate were characterized by zeta potential and size distribution analyses and FT-IR spectrometry. **Results:** The results confirmed the conjugation between HBsAg and the dendrimers. Immunological assays indicated that the immunogenicity of the conjugated HBsAg is more than HBsAg alone. **Conclusion:** In the current study, a cost-effective, biodegradable and biocompatible polymer was used to enhance the immunogenicity of HBsAg. Also, further investigations are required to explore the mechanisms of action of this nanocomplex vaccine candidate.

KEYWORDS: HBsAg, Dendrimers, Vaccine, Conjugate

INTRODUCTION

Hepatitis B virus (HBV) infection is a major global health problem. More than 240 million people are chronically infected with HBV and an estimated 600'000 die each year from the consequences of this infection, worldwide [1, 2]. Among the infected patients, 15%–40% of them will succumb to liver fibrosis, cirrhosis and finally hepatocellular carcinoma [3]. Vaccination against hepatitis B is one of the success stories in modern medicine [4] and the mainstay control of HBV is by preventing the infection, following acute and chronic liver diseases. Since more than 20 years ago, several clinical trials exploited the conventional prophylactic means (i.e. protein vaccine, T-cell vaccine and combination therapy), based on hepatitis B surface antigen (HBsAg) for therapeutic vaccination [5-22]. Immunogenic complexes activate T cell responses by accumulating uptake of HBsAg through Fc receptors on antigen-presenting cells and then, enhance HBsAg processing and presentation. It has been demonstrated that this vaccine administered to HBsAg-positive patients could lead to decrease of HBV DNA in serum and HBsAg seroconversion in some patients [23]. The vaccines do not frequently present good immunogenic properties as seen by native microorganisms and in many cases, adjuvants are required to induce more powerful immune responses.

Nanoparticles are recently assessed for their capacity to increase the immune responses as adjuvants [24]. However, more investigations on humans are required to establish their potential as vaccines against hepatitis B infection [25, 26]. Dendrimers, a family of nanoscale three-dimensional polymers are defined as compact globular structures which have advantageous properties for application in drug delivery systems, compared to other polymers. Due to their limited polydispersity and nanometric scale, they can easily pass easily through biological barriers. In addition, during the process of dendrimer making, their size and molecular weight can be controlled precisely. The unique features of dendrimers such as their controllable size, monodispersity and variable surfaces make them desirable for biomedical applications. Furthermore, the end groups of dendrimers can be functionalized for therapeutic and imaging purposes as well as targeted drug delivery [27]. In this paper for the first time, a new biocompatible and biodegradable structure composed of a protein moiety (HBsAg) and a chemical segment (dendrimers) was generated in order to investigate the adjuvanticity effect of this nanocomplex.

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MATERIALS and METHODS

Materials

Polyethylene glycol 600 (PEG-600), citric acid, dicyclohexylcarbodiimide (DCC), dimethyl sulfoxide (DMSO), methanol, chloroform, iodine, toluene, calcium chloride anhydrous, acetone, curcumin and TLC Silica gel 60 were purchased from Merck (USA). Dialysis membranes (100-500 Da and 500-1000 Da cutoffs) and HBsAg were obtained from Spectrum Co. (USA) and Production and Research Complex of Pasteur Institute of Iran, respectively.

Dendrimer synthesis and antigen conjugation

For dendrimer synthesis, 3.72 mmol DCC was dissolved in 10 ml dry DMSO and added to a balloon containing 2 ml PEG-600 (3.7 mmol). The mixture was then stirred for about 15 minutes. Subsequently, 3.72 mmol citricacid was added and stirred for 1 h. Reaction was stopped by adding 10 ml deionized distilled water (ddH2O) and filtered by a filter paper. The clear filtrate was collected and subjected into dialysis bag (cut off 100–500Da). The bag was transferred into a flask containing 700 ml ddH2O at room temperature under stirring condition. The ddH2O was continuously refreshed with 1 h intervals for 16 h. The product was removed from the dialysis bag and dried using freeze-drying. Dendrimers (1 mmole) were conjugated to HBsAg (5mg/ml) in an EDC mediated reaction for 24 h at 4°C. The conjugated HBsAg was purified by dialysis (Cutoff 10 kDa) as previously described and freeze-dried for further analysis.

Characterization of physico-chemical properties of conjugated HBsAg

Zeta potential and particle size were measured to determine the charge and size distribution of the conjugated HBsAg (Malvern Nano-ZS, UK). Water was used as a dispersant and the zeta potential values of the samples were measured at 24°C. FT-IR spectra was also measured on a Bruker Model Tensor-27 spectrometer (Kyoto, Japan).

Immunization Protocols

For immunization, pathogen-free, female BALB/c mice (20 g average weight) were used and handled according to the international animal care ethics. Groups of six mice were immunized at weeks 0, 3 and 6 either subcutaneously (s.c.) in the tail base with 5 µg of HBsAg or the nano-sized Ag resuspended in 100 µl of PBS. After 2-weeks post–immunizations, mouse blood samples were collected by retro-orbital bleeding and the sera were stored at −70°C before testing.

Immunoassays by ELISA

Humoral response of the immunized mice was analyzed by ELISA method. Briefly, purified recombinant HBsAg (1µg/ml, Pasteur Institute of Iran) was used as capture molecules to coat ELISA plates (96-well polyvinyl chloride plate-Nunc, Denmark) overnight at 4°C. This dilution had been optimized to give the highest readings with positive control samples and the lowest background readings with naive serum samples. After washing and blocking steps, the wells were probed with serial dilutions of serum of each mouse (1/100 – 1/3200), incubated for 1 h, washed and further incubated with HRP-labeled goat anti-mouse IgG-γ chain (Sigma, Aldrich) as secondary antibody. Finally, by addition of TMB (tetramethylbenzidine; Sigma, Aldrich) and color development, the absorbance was measured at 450 nm. [20].

RESULTS

Zeta potential and size distribution analysis

The zeta potential and size distribution analyses of HBsAg and conjugated HBsAg were confirmed the conjugation reaction. Size distributions were 91.37 and 145.3 nm for HBsAg and conjugated HBsAg, respectively (Fig. 1).

Mean zeta potentials were obtained as -23.1 and -33.7 mV for HBsAg and conjugated HBsAg, respectively (Fig. 2).

FT-IR spectrometry analysis

FT-IR spectra demonstrated peaks regarding dendrimer were suppressed by HBsAg peaks. Peaks of 1639 and 2900-3000 cm-1 were the significant signs of conjugation (Fig. 3).

Evaluation of HBs-specific total IgG

As shown in Fig. 4, both groups vaccinated with either the purified antigen or the conjugated antigen induced HBs-specific total IgG, albeit in different intensities. Accordingly, conjugated HBsAg was capable of inducing higher levels of total IgG. These data showed the efficiency of nano-conjugated form of the antigen regimen in comparison with the antigen alone vaccination. These results showed that the conjugated form of the antigen was more immunogenic than the non-conjugated form.

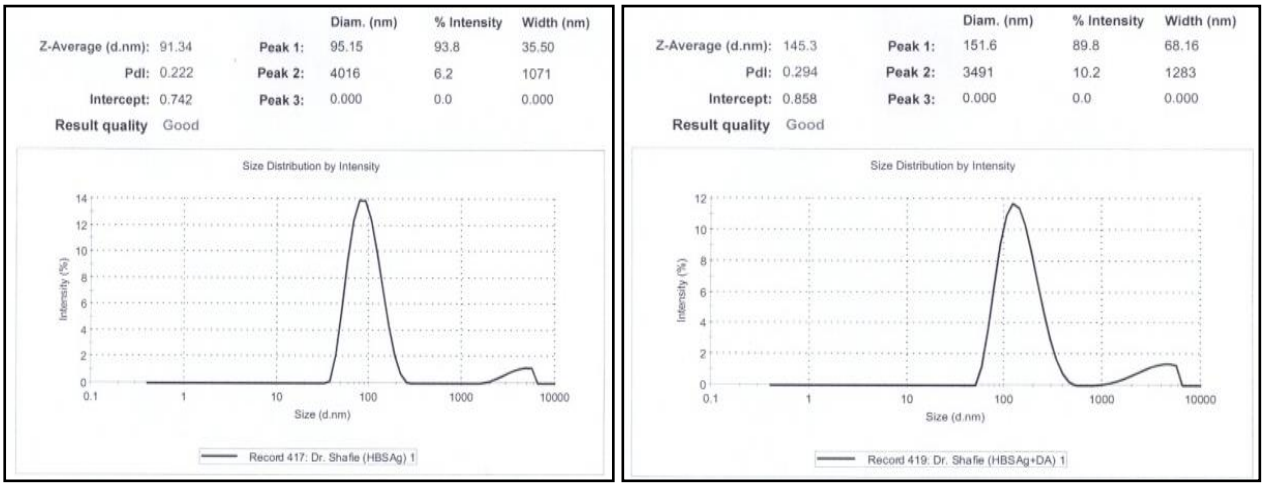

Fig. 1. Size distribution of HBsAg (Left) and conjugated HBsAg (Right).

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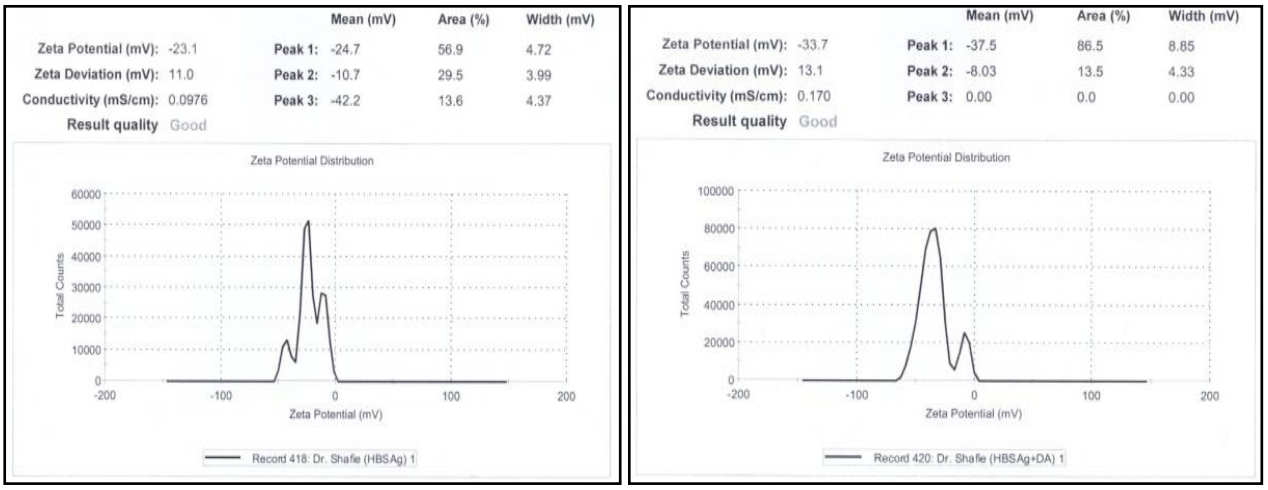

Fig. 2. Zeta potential of HBsAg (Left) and conjugated HBsAg (Right).

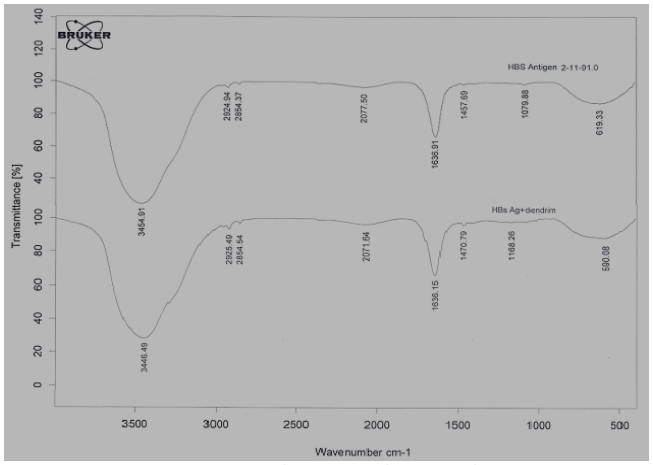

Fig. 3. FT-IR spectra analysis of HBsAg before and after conjugation with dendrime

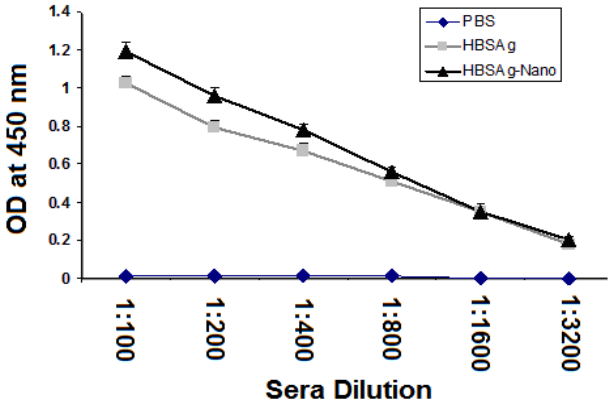

Fig. 4. Analysis of total IgG induced by different forms of the antigen. Each formulation is abbreviated on the diagrams (see the text for detailed materials and methods). Total IgG were determined at serial dilution of mice sera. Bars indicate Standard Deviation.

DISCUSSION

Many studies have pursued suitable potential adjuvants to be used in commercial HBV surface antigen vaccine formulations [28-32]. Hepatitis B vaccine, when managed in conjunction with alum adjuvants, induces Th2 immunity that confers protection against HBV [32]. Mishra *et al*. have shown that Mannosylated solid lipid nanoparticles appear to be capable of functioning as carriers for vaccine delivery against hepatitis B as demonstrated by in vitro and in vivo experiments [27]. In 2013, the new nano-complex, Hep-c, was shown to improve the immunogenicity of the hepatitis B vaccine [33]. Lugade and colleagues have suggested that chitosan nanoparticle vaccines represent a promising un-adjuvanted platform to generate robust and durable immunity to HBsAg and other subunit antigens following a single low-dose administration [34]. However, based on the results of these studies, the currently available vaccine formulations and adjuvants do not provoke suitable Th1 and CTL responses that are important for inhibition of maternal transmission of the virus.

Many studies have been investigated the application of nanoparticles in vaccine development area [24, 34-37]. In the current study for the first time, a cost-effective, biodegradable and biocompatible polymer was used to enhance the immunogenicity of HBsAg. In contrast to linear polymers, anionic globular dendrimers are composed of a polyethylene glycolic core and citric acid branches with a precisely controllable size and molecular weight. The presence of a large number of terminal citric acid ends increases the solubility and reactivity of the dendrimer. Water-soluble dendrimers, like anionic globular dendrimers, are able to connect to hydrophobic molecules with antifungal or antibacterial properties for medicinal goals [38]. In addition, they have potential properties like sustained or targeted delivery of compounds or proteins to living cells for tumor-targeted drug and gene delivery systems [39]. The emergence of armed dendrimers with polyethylene glycolic core has led to generation of dendrimers with higher solubility in water and higher drug loading capacity as we used in this investigation [40].

In this study for the first time, a new biocompatible and biodegradable structure of HBsAg was generated by chemical conjugation between HBsAg and dendrimers and its immunogenicity was then characterized by immunoassays. Further biological assays are required for better characterization of such nano-sized version of HBsAg as a component of hepatitis B vaccine in future.

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CONFLICT of INTEREST

The authors declare that they have no conflict of interest.

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