

Research Article**Preparation and evaluation of alginate nanoparticles containing pertussis toxin as a particulate delivery system**

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ABSTRACT

During the last decades, research on different kinds of nanoparticles (NPs) has been increased to prepare various medical applications, for instance in vaccine and gene delivery and as new generation of adjuvant candidates. The aim of the present study was to prepare sodium alginate nanoparticles (Alg-NPs) containing pertussis toxin (PTX) as an candidate acellular vaccine. Formulation of antigen loaded Alg-NPs were assessed for immunological activities and their role as potential immunological adjuvant. Alg-Nps were prepared using mild ionic gelation method. Optimal formulation was obtained by concentration of 0.2% w/v sodium alginate, 0.1% w/v CaCl₂ solution and magnetically homogenization condition of 45 min and rate of 2000 rpm. Obtained Alg-NPs showed average size of 88 nm and zeta potential of -32mV in blank and 72 nm and -29 mV in PTX entrapped nanoparticles, respectively. A Loading efficiency of more than 90% was determined for PTX. The antigen loaded nanoparticles showed 75.3% of release within 144 h in *in vitro* release studies. The immunological evaluation in female Balb/c mice groups revealed that the Alg-NPs formulation induced significantly higher serum antibody titers ($p < 0.01$) as compared with commercial acellular pertussis vaccine and conventional alum-adjuvanted antigen administered by subcutaneous route. The results showed the potential of Alg-NPS to be a simplex and efficient delivery system. This study also indicated the potential of Alg-NPs as new generation of immunostimulant adjuvant to boost the antigenicity of the antigens in a cellular pertussis vaccines.

Keywords: Alginate Nanoparticles; Pertussis toxin; Vaccine delivery; Adjuvants

1. INTRODUCTION

Pertussis toxin (PTX) is the main virulence factor of the *Bordetella pertussis* bacterium, which is the causative agent of whooping cough [1]. Since 1940, vaccination against whooping cough is based on the use of a whole-cell pertussis vaccine (wP) composed of *Bordetella pertussis* strains in most countries [2]. Recent few decades, acellular pertussis vaccines (aP) containing different virulence factors of *B. pertussis* with a focus on pertussis toxin have been developed to replace wP [3]. Based on recent studies concerning the lack of protection against colonization or transmission by acellular

pertussis vaccination, maximizing the efficacy of current vaccines is imperative [4]. Over the last decades, research on different kinds of nanoparticles (NPs) to improve vaccine delivery system and as new generation adjuvant has been considered[5]. Biocompatible and biodegradable polymers such as alginate salts due to individual characteristics are considered as desirable source to develop mentioned above goals [6]. The aim of this study was to develop and optimize the preparation of alginate nanoparticles as new carrier of pertussis toxin as well as evaluation of the probably influences of nanoparticles

properties compared with aluminum phosphate as conventional adjuvant.

2. MATERIALS AND METHODS

Sodium alginate (low molecular, medium viscosity), calcium chloride dehydrate, pertussis toxin of *Bordetella pertussis* were purchased from Sigma - Aldrich (USA), Merck, and European Pharmacia Reference Standard, respectively. Commercial acellular pertussis vaccine (manufactured by Sanofi Pasteur) and Alum-adjuvanted PTX were prepared from human bacterial vaccine department of Razi vaccine and serum research institute. Aqueous solutions were prepared with double distilled water. All of the other materials used in this study were analytical grade.

2.1. NPs preparation

Preparation of alginate nanoparticles performed based on ionic gelation method [7]. CaCl_2 (0.1% w/v) as cross-linker [8] was added drop by drop to different concentrations of sodium alginate under homogenizing magnetically at room temperature. In order to prepare antigen entrapped NPs, 10 μg of pertussis toxin were added to 2 ml alginate solution. Alg-NPs were isolated by centrifugation at 10000 rpm on a glycerol bed, for 30 min at 5 °C.

2.2. NPs characterization

Prepared nanoparticles were characterized for their morphology by TEM (Em 900 Ziess, Germany). Size distribution and zeta potential were determined by zeta sizer (Malvern, Denmark).

2.3. Loading efficiency and *in vitro* release of protein

Loading efficiency (%LE) of entrapped pertussis toxin was calculated based on the following formula.

$$\text{Loading Efficiency} = \frac{\text{Total amount of protein} - \text{Free Protein}}{\text{Total amount Protein}} \times 100$$

In vitro release behavior of PTX from alginate NPs was determined as follows: The PTX entrapped NPs was divided in several test tubes

equally. Afterwards, 1 ml of phosphate buffer saline (PBS, 0.05 M, pH=7.4) was added to each test tube. Then, the tubes were placed in shaker-incubator with shaking rate of 60 rpm at 37 °C [9]. At scheduled time intervals, samples were taken and centrifuged at 10000 rpm for 20 min. The amount of released PTX in the supernatant was detected by reverse phase HPLC on an Agilent system (USA) using a 0.46×25 cm analytical column [10]. Solvents A and B contained 0.1% TFA in HPLC-grade water and HPLC-grade acetonitrile, respectively. The column was initially equilibrated with 40% acetonitrile at room temperature. Samples in 200 μl of supernatant were injected to determine released PT by applying a linear gradient of 40-95% acetonitrile over 50 min at 1 mL/min flow. The effluent was monitored by UV absorption at 230 nm and quantification of peak areas from chromatogram was estimated automatically by Chem Station software.

2.4. Immunization of Mice

Five groups of five female Balb/c mice were used in immunization program. The groups 1-5 were injected (days of the 1st, 15th and 30th) subcutaneously as follows: group1: PBS 0.05 M, group2: PTX free alginate NPs group3: commercial acellular pertussis vaccine, group4: aluminum phosphate adjuvanted PTX, group5: PT loaded alginate NPs, respectively. Bleeding from caudal vein of mice were performed three time individually of each mouse subsequent the injections (days of the 14th, 29th and 45th). Blood samples were centrifuged for 10 min, 5000 rpm at 4 °C; serum (supernatant) were collected and stored at -20 °C.

2.5. ELISA

The raised antibody to PT was checked by means of enzyme-linked immunosorbent assay (ELISA). The standard active PT was used through the study as positive control. Polysorp 96-well flat plates were coated with 100 ng standard pertussis toxin (overnight at 4°C). All other parts of the analysis were performed at room temperature. Blocking was performed with 300 μl per well 1.5% powdered skimmed milk in PBS, Sera were diluted in PBS. Secondary

antibody, anti-mouse IgG labeled with HRP 1:2000 (art. no. A9044; Sigma-Aldrich, USA), diluted in PBS was used 100 μ L per well. One hundred microliters per well of 3, 3', 5, 5'-tetramethylbenzidine (TMB) were used as the enzyme substrate. Incubation times were 30 min of blocking, 1 h of incubation with serum, 30 min of incubation with secondary antibodies, and 15 min of incubation with TMB. All wash cycles were performed with 3 times 300 μ l per well of PBS with 0.05% Tween 20 (at pH 7.4). The color reaction was terminated with 100 μ L of 1 mol/L sulfuric acid per well. The optical density was read with an ELISA plate reader at 450 nm with a reference wavelength of 650 nm.

2.6. Statistical analysis

Data analysis of the study was performed based on pair T-test, ANOVA and repeated measure test. Software of SPSS v.22 was used for mentioned statistical analysis.

3. RESULTS

3.1. NPs preparation

Preparation of nanoparticles was optimized as follows: 1. Alginate concentration of 0.2% w/v, 2. CaCl₂ (cross-linker) 0.1% w/v, and 3. Magnetically homogenization in 2000 rpm for 45 min at room temperature. Mentioned conditions leads to suitable weight efficiency of NPs (Table 1).

3.2. NPs characterization

The morphological characteristics of nanoparticles were examined using TEM. The

image of nanoparticles is shown in fig. 1, which revealed relatively smooth and spherical nanoparticle structures.

Protein free nanoparticles were obtained with average size of 88 nm and zeta potential of -32 mV having polydispersity index (PDI) 0.285 (Figures 2 and 3). Also the average size of nanoparticles was estimated 72 nm and zeta potential changed to -29 mV after loading with pertussis toxin. Recent nanoparticles showed PDI of 0.293 (Figures 4 and 5).

3.3. Loading efficiency and release detection

A Loading efficiency of more than 90% was determined for pertussis toxin based on following calculation.

$$LE = \frac{10 \mu\text{g} - 0.32 \mu\text{g}}{10 \mu\text{g}} \times 100 = 96.8 \%$$

The antigen loaded nanoparticles showed 21.4% of release within 24 h in *in vitro* release studies, table 2 and fig. 6.

3.4. Result of Immunization

The immunological evaluation in female Balb/c mice groups revealed that the Alg-NPs formulation induced significantly higher serum antibody titers ($p < 0.01$) as compared with commercial acellular pertussis vaccine and conventional alum-adjuvanted antigen (Figure 7).

Table 1: 0.2% w/v concentration was optimum condition to produce desirable NPs (≤ 100 nm), each procedure of NPs preparation was performed five time

No.	Cross linker 0.1 % w/v	Concentration of polymer % w/v (2 ml)	Mean of Dried gel Weight (mg \pm SD)	Output Dried gel weight% \pm SD	Average Size
1	1.2 ml	0.05	-	-	-
2	1.4 ml	0.1	1.5 \pm 0.4 \pm	44.1 \pm 1.2	<5 nm Nanoclusters
3	1.7 ml	0.2	5.1 \pm 0.2	88.8 \pm 3.6	\leq 100 nm Nanoparticle
4	1.8 ml	0.3	7.1 \pm 0.3	91 \pm 3.7	> 1000 nm Micro particle

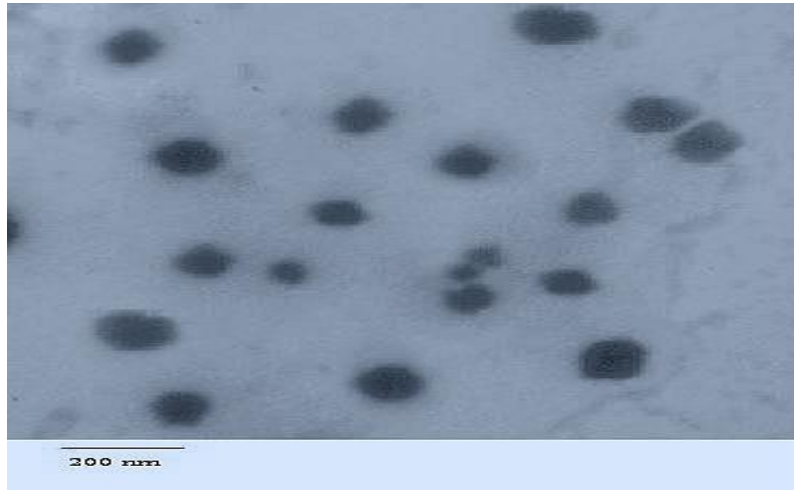


Fig 1: TEM image of nanoparticles

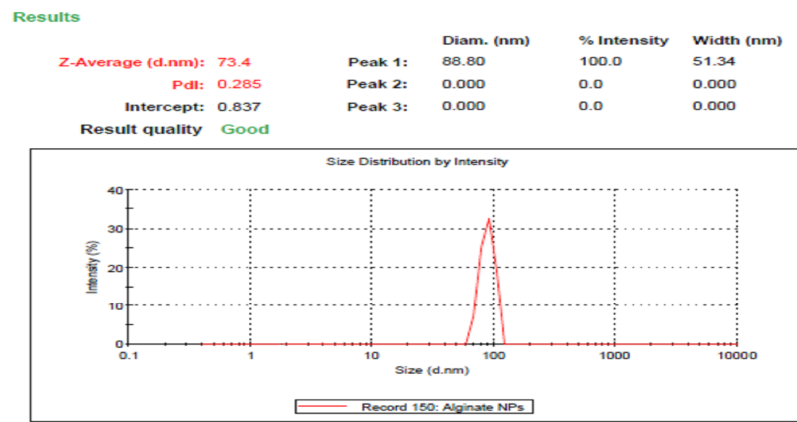


Fig 2: Size distribution record of antigen free Alg NPs (blank NPs) by intensity

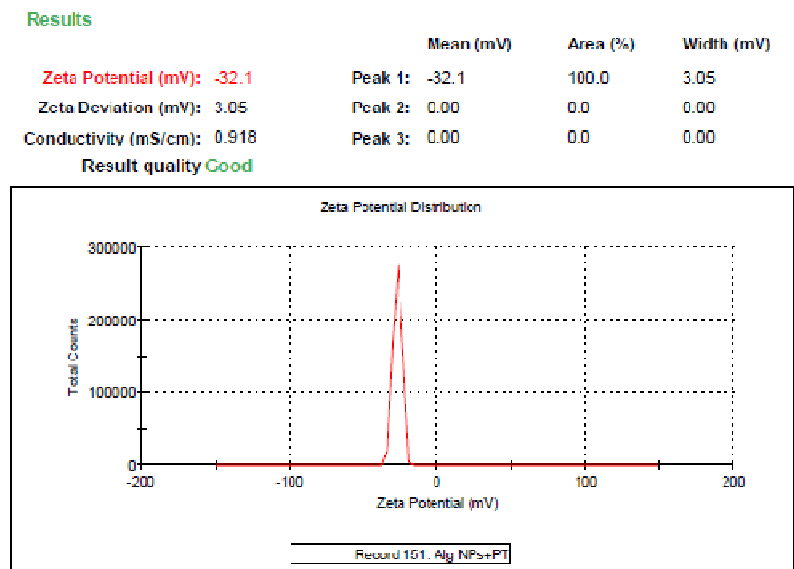


Fig 3: Zeta potential record of antigen free Alg NPs (blank NPs)

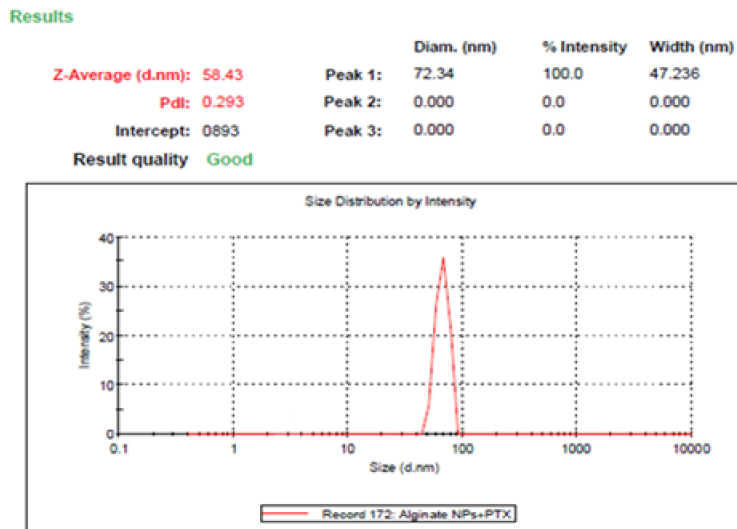


Fig 4: Size distribution record of antigen loaded Alg NPs by intensity

Fig 5: Zeta potential record of antigen Loaded Alg NPs

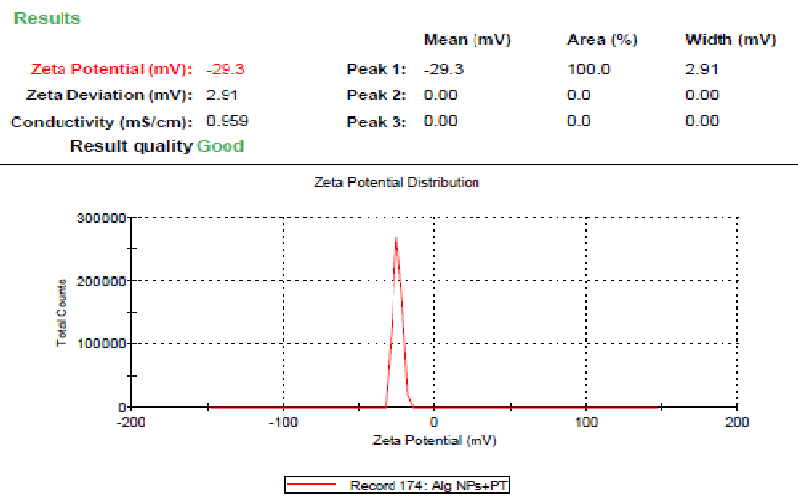


Table 2: Result of *in vitro* release study

Released protein (μg) $\mu\text{protein}$ (μg)	% Cumulative released protein	Time (hour)
0.32	3.2	1
0.48	8	6
0.61	14.1	12
0.73	21.4	24
0.85	29.9	36
0.96	39.5	48
1.05	50	72
1.2	62	96

0.93	71.3	120
0.4	75.3	144
0	75.3	170

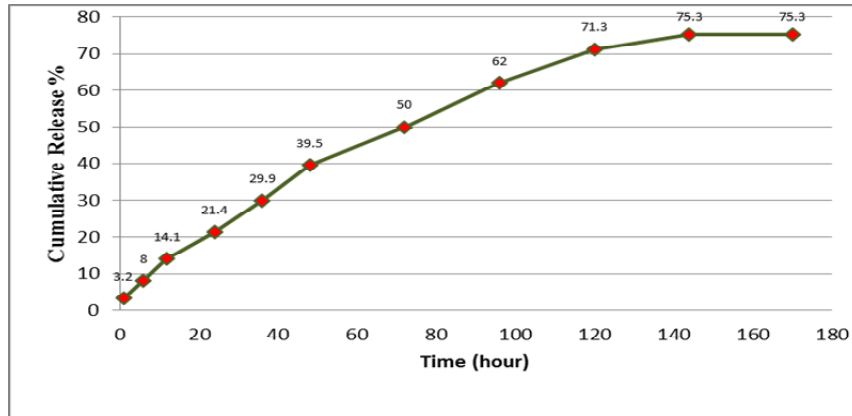


Figure 6: 6% Cumulative Release of protein

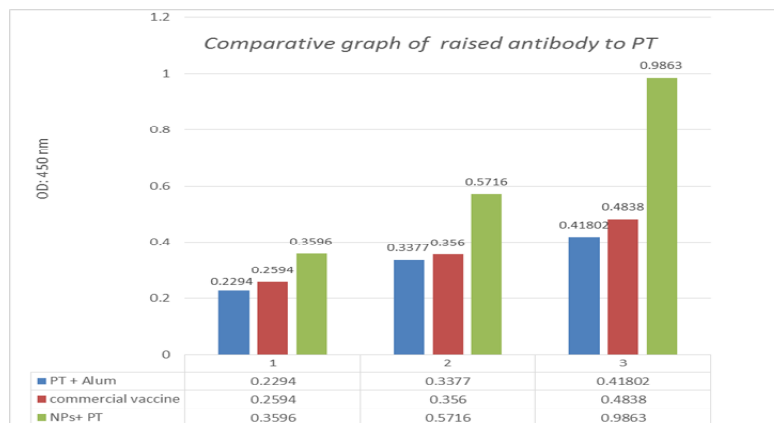


Figure 7: Raised antibody to PT in different test groups. Raised antibody to PT was measured by means of enzyme-linked immunosorbent assay (ELISA) for three times blood sampling 1. Alum-adjuvanted PTX (blue bars), 2. Commercial acellular pertussis vaccine (red bars) and 3. PTX loaded alginate nanoparticles (green bars)

4. DISCUSSION

Previous studies have shown that the concentration of alginate solution as well as calcium chloride as cross linker agent are effective on particle size[11]. However, specific conditions in the laboratory and production scale will change somewhat in the amount of raw materials. Ionic gelation method was applied with alginate which jellify with calcium ions. Stableness of nanogels formed with calcium can be achieved by addition of a positively charged polyelectrolyte [12]. Alginate nanoparticles produced by controlled gelation can easily be loaded with proteins [13]. This system was also highly investigated for the delivery of oral

formulation of peptides including insulin [14,15]. In some works, Poly-L lysine (PLL) have used in order to create more consolidation of nanoparticles. Because of the plan of present study based on *in vivo* investigations, due to toxic and immunogenic aspects of PLL, it was not used in preparing NPs.

In this study, alginate nanoparticles at different concentrations were prepared by the mild ionic gelation method. Evaluation of different concentrations of alginate on the characteristics of nanoparticles was investigated. Finally preparing of nanoparticles was optimized as follows: 1. Alginate concentration of 0.2% w/v, 2. Cross-linker 0.1% w/v, and 3. magnetically

homogenization at 2000 rpm for 45 min at room temperature. Whereas Saraei et al (2013) reported 0.3% w/v as optimal amount of alginate solution. Also they used PLL to prepare NPs, off ours their study was limited to *in vitro* assay. The results indicated that the use of sodium alginate with the concentrations of 0.3% w/v leads in formation of particles with average size of more than 1000 nm.

5. ACKNOWLEDGMENT

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