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Original Article

بررسی آزمایشگاهی بارگذاری بهینه توکسیدهای دیفتری و کزاز روی نانوذرات آلیژینات

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چکیده

سابقه و هدف: واکسن دیفتری و کزاز (DT) حاوی مقدار بالایی آلومینیوم به عنوان ادجونت است که پتانسیل تأثیر بر سیستم عصبی، به‌ویژه در نوزادان با بیماری‌های کلیوی را دارد. در اینجا هدف آماده‌سازی آزمایشگاهی و ارزیابی تحویل توکسوئیدهای دیفتری و کزاز با بارگذاری روی نانوذرات (NPs) بود.

مواد و روش‌ها: با استفاده از روش یونیزاسیون–ژل، نمونه‌های نانوذرات آلژینات سدیم تهیه و از نظر اندازه، پتانسیل زتا و شاخص پراکندگی (PDI) ارزیابی شدند. اثرات غلظت آلژینات، کلرید کلسیم و پلی‌ال–لایزین و سرعت و زمان همزدن به همراه بازده بارگیری، ظرفیت بارگیری و مشخصات آزادسازی در شرایط آزمایشگاهی بررسی گردید.

یافته‌ها: نانوذرات بهینه شده در غلظت آلژینات سدیم ۰/۲ w/v٪، کلرید کلسیم ۰/۱ w/v٪ و پلی‌ال –لایزین ۰/٤ w/v٪ طی ٤۵ دقیقه همزدن در ۱۳۰۰ دور در دقیقه تهیه شدند. آن‌ها همچنین دارای اندازه متوسط ذرات کوچک تر از ۱۵۰ نانومتر با PDI حدود ۰/۵ بودند. بازده بارگیری مناسب با غلظت‌های توکسوئیدهای بارگذاری شده مشابه واکسن DT رایج به‌دست آمد که منجر به آزادسازی طولانی مدت حدود ۸۵٪ سموم بارگذاری شده در طی ۱۲۰ ساعت شد. همچنین، نتایج SDS-PAGE و dot-blot فعالیت آنتی‌ژنی توکسوئیدهای آزاد شده را تأیید کردند.

نتیجه‌گیری: این نتایج می‌تواند به میزان قابل توجهی به توسعه بیشتر فناوری نانوذرات آلژینات حاوی توکسوئیدهای DT در شرایط بهینه in vitro کمک نماید تا به عنوان بستری برای ارزیابی بیشتر به صورت درون تن به منظور دستیابی به ابزاری نویدبخش برای ایمن‌سازی نوزادان و کودکان در برابر دیفتری و کزاز باشد.

واژگان کلیدی: توکسوئید دیفتری، هم–انتقالی، بازدهی بارگذاری، پتانسیل زتا، ادجونت.

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Original Article

In Vitro Evaluation of Optimized Diphtheria and Tetanus Toxoids Loaded Alginate Nanoparticles

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Abstract

Background and objective: Diphtheria and tetanus vaccine contains a high quantity of aluminum as an adjuvant potential to affect the nervous system, particularly in infants with kidney disease. Thus, the focus of this study was on in vitro preparation and evaluation to co-deliver DT toxoids by loading on alginate nanoparticles (NPs) as a non-toxic substance without antigenicity.

Materials and Methods: Using the gel-ionization method, alginate NPs samples were prepared and characterized in respect of size, zeta potential, and polydispersity index (PDI). The effects of alginate concentrations, calcium chloride, and Poly Lysine and the stirring time and speed in addition to the loading efficiency, loading capacity, and in vitro release profile were assessed.

Results: The optimized NPs were prepared at a concentration of 0.02 %w/v sodium alginate, 0.1 %w/v calcium chloride, and 0.04% w/v Poly L-Lysine during 45 minutes of stirring at 1300 rpm. They also had a mean particle size <150 nm with a mean PDI of around 0.5. The appropriate loading efficiency was obtained at a concentration of loaded toxoids similar to a conventional DT vaccine, which resulted in the prolonged release of about 85% of loaded toxoids over 120 hours. The SDS-PAGE and dot-blot confirmed the stability and antigenic activity of the released toxoids.

Conclusion: These results can significantly contribute to further developing alginate NPs containing DT toxoids in optimized in vitro conditions as a platform for in vivo evaluation to achieve a promising vehicle for immunization of infants and children against diphtheria and tetanus.

Key words: diphtheria toxoid, co-deliver, loading efficiency, zeta potential, adjuvant.

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Introduction

During the recent decades, nanoparticles have gained significant attention from scholars in the field of nanovaccinology. Their constant evaluation reflected numerous critical capabilities, indicating the possibility of effective improvement of the adjuvants commonly utilized in approved vaccines (1).

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Among the common adjuvants, aluminum salts (alum), have been the most widely used adjuvants in vaccine formulations for nearly a century due to their influences on the immune system (2). Adsorption of vaccine antigens to alum induces Th2 cell immune responses and enhances the production of specific antibodies against the vaccine antigens by directly stimulating the immune system through activating antigen presenting cells, complementary cascades, and inducing chemokine secretion (3). In addition, the alum adjuvant is identified by the NOD receptors (NLRs) to direct activating the NLRP3/NALP3 inflammasome complex or by releasing uric acid (2). Based on that, in developed countries, from infancy to preschool-age, many antigenic compounds formulated with alum get into the body through multiple injections of several vaccines and their booster doses recommended by the national immunization program (4). Typically, in a healthy state, more than 95% of aluminium, as a non-biodegradable toxin, is excreted through the kidneys (5). However, infants and children with chronic kidney disease cannot remove the toxic substances through their kidneys efficiently.

Consequently, during early life, that blood-brain barrier is more permeable to uremic toxins, infants' brain development could be affected by side effects of alum (2). Besides, patients with kidney disease show a significant decrease in their immune function, and it becomes worse for the children who had undergone transplantation or dialysis. Therefore, this group of patients may receive alum more than usual amounts. At the same time, they should be monitored periodically for their antibody titers against antigens such as diphtheria and tetanus toxoids to receive a vaccine booster shot if needed (6). Because of this, the formulation of vaccines for pediatric patients requires more attention and care. In this regard, the application of natural polymers in the development of nano-delivery systems indicates a promising approach to improving vaccine effectiveness and reducing the side effects of alum adjuvant (7). Natural polymers are remarkable due to valuable features such as non-toxicity, biocompatibility,biodegradability, cost-effectiveness, and the lack of antigenicity (8). Respecting these benefits, alginates derived from the brown algae (kelp) attract researchers' interest. Sodium alginate is a polysaccharide that confers the capability to be an antigen carrier. Alginate is made of two monomeric units: D-mannuronic acid (M) and L-guluronic acid (G) residues, and the ratio of them as well as their frequency in sequences affects the functional features and make different properties for alginate. The block combinations of alginate chains could be the repetition of 4 GG, MM, GM, and MG arrangements. The G-block enriched alginate results in a higher molecular weight of the gel and biological compatibility, less protein diffusion into the gel, and a more robust inflammatory reaction (9). Generally, using ionic gelation procedure promotes alginate gel particles lower than 1000 nm, defined as nanoparticles (NPs) (10) and proper for injection. In this procedure, the formation of alginate NPs can be achieved by replacing calcium ions instead of sodium ions of G blocks and ionic crosslinking with cations. Stable bonds are formed between cations and oxygen atoms from the carboxyl groups of G blocks, which lead to the formation of the egg box structure. Among the cations, calcium is widely used due to its non-toxicity (9).

Considering the susceptibility of pediatric patients with kidney disease to the alum adjuvant, the current study aims to develop

alginate NPs containing a combination of Diphtheria toxoid (DT) and Tetanus toxoid, and Tetanus toxoid (TT) in optimized conditions. Therefore, the impact of five variables, including sodium alginate concentration, calcium chloride concentration, PLL concentration, time, and speed of homogenization, on the main physical attributes of NPs were evaluated, in addition to loading efficiency, loading capacity, and *in vitro* release pattern of toxoids-loaded nanoparticles.

Materials and methods

In this research study, the materials and chemicals were purchased from Sigma–Aldrich in Germany, including: Low molecular and medium viscosity alginate sodium which is extracted from brown algae, Poly L-Lysine (PLL), Phosphate-buffered saline (PBS), ethanol 95%; phosphoric acid 85% (w/v), Bradford reagent and Coomassie brilliant blue G250; and calcium chloride dihydrate was obtained from Fluka Chemicals in Romania as well. Besides, the required (DT) and (TT) were acquired from the department of the human bacterial vaccine of Razi Vaccine and Serum Research Institute (Karaj, Iran) at the initial doses of 1500 and 2000 Lf/ml, respectively. Double distilled water was used for preparing all chemical solutions.

1. Diphtheria and Tetanus toxoids features: The purity and molecular weight of DT were estimated by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE). According to the procedure described by Matsumoto at al. (11), a mixture of each toxoid in buffer solution prepared separately, boiled for 10 minutes, and then subjected to SDS gels (12% polyacrylamide) under standard conditions. Followed by, the gels were stained using Coomassie brilliant blue 0.1% in an aqueous ethanol solution mixed with acetic acid 10%. The concentration of the toxoids was measured using the Bradford assay (12).

2. Preparation of the blank NPs: The ionic gelation method described by Rajaonarivony et al. (10), was employed to prepare alginate NPs. This step aimed to determine the optimal level of the variables that influence the gelification in blank NPs preparation. Therefore, the impacts of different factors, including concentrations of sodium alginate, calcium salt, and PLL solutions as well as stirring duration and agitation speed were estimated as below: Firstly, the effects of various concentrations of sodium alginate and calcium chloride solutions with a volume ratio of 1:1 were investigated in a way that 2.5 ml of various concentrations of calcium chloride solution (0.05, 0.1 and 0.2 % w/v) were added drop-wise to 2.5 ml of various concentrations of sodium alginate (0.1, 0.2 and 0.3 %w/v) while stirring to prepare 5 ml of pregel solution. At the next step, in order to complete the condensation of pregel samples, various concentrations of PLL (0.02, 0.04, 0.08, 0.1, 0.15 and 0.2 %w/v), were added to the pregel solutions. Finally, each sample was stirred at various speeds (1000, 1300, and 1600 rpm) and different time durations (32) (30, 45 & 60 min) at room temperature to induce NPs. The optimal amount of the examined factors was estimated by Dynamic Light Scattering (DLS) analysis.

3. Preparation of toxoids loaded NPs: This step is similar to the previous one, except that before adding various concentrations of calcium chloride and PLL solutions, TT and DT which combined at different concentrations (0.5X, 1X, 2X, 4X, 6X, and 8X) were added into sodium alginate solution. Then it was stirred for 5 minutes at room temperature (1X

represents the doses of toxoids equivalent to the toxoids in the conventional DT vaccine, which is 20 Lf of diphtheria toxoid and 6 Lf of tetanus toxoid per 0.5 ml of DT vaccine, produced by Razi Vaccine and Serum Research Institute of Iran). Toxoids were also loaded separately for preparing the control samples.

4. Characterization of NPs: To reach the optimal preparation parameters for the blank and toxoids-loaded NPs, particle size distribution, diameter, and zeta potential of samples were determined utilizing Dynamic Light Scattering (DLS) technique using Zetasizer (zen 3600 Laser Particle Size Analyser, Malvern Instruments, UK). To check the repeatability, these measurements were performed at least three times at 25°C with no dilution, centrifugation, sonication, and addition of any salts.

5. Loading capacity (LE) and loading efficiency (LC) of NPs: Determination of various parameters related to LE and LC of the prepared NPs was performed by NPs isolation from aqueous suspension through centrifugation of samples at 10000 rpm for 30 minutes at 4°C. Then the settled NPs were dried overnight at 80 ºC and, after that, weighed (13). The supernatants were collected and the contents of the proteins in them were determined by Bradford protein assay at 595 nm as free proteins. The amount of the residual toxoids within the NPs was calculated by subtracting the amount of free proteins from the total toxoids used to prepare the NPs. The LE and LC of prepared NPs were calculated using the following equations (14):

(Formula 1):

$$\% \text{ Loading Capacity} = \left(\frac{\text{Total amount of protein - Free protein}}{\text{Dried nanoparticles weight}} \right) \times 100$$

(Formula 2):

$$\% \text{ Loading Efficiency} = \left(\frac{\text{Total amount of protein - Free protein}}{\text{Total amount of protein}} \right) \times 100$$

6. In-Vitro release study: In-vitro release of toxoids from optimized DT, TT, and DT + TT NPs (loaded by 1X doses of the toxoids) was carried out by dispersing 100 mg of each sample of dried NPs in 10 ml of PBS, separately (0.01 M, pH 7.4).

Each suspension of toxoid-loaded NPs was equally divided into ten microtubes and incubated in a shaker incubator (60 cycles/min) at 37°. At some predetermined intervals (0, 2, 6, 12, 24, 36, 48, 72, 96, and 120 hours), one micro tube of each sample was removed and centrifuged at 10,000 rpm, for 30 minutes at 4°C. The supernatant of each microtube was stored to determine the concentration of the released toxoids using Bradford's method.

7. Stability and activity of the released toxoids: The integrity and stability of loaded toxoids were determined when released after two hours by SDS-PAGE under the non-reducing condition in a similar way explained above except for adding 2-mercaptoethanol.

The dot blot technique as a qualitative method for rapid screening of antigenic activity was performed on toxoid samples released after two hours of diluting the samples as Hoy and Sesardic (1994) described (15).

Results

1.*Characterization of DT and TT:* Before loading the toxoids to alginate NPs, the purity, molecular weights, and total concentrations of the toxoids were estimated. Based on the reducing SDS-PAGE pattern (Fig. 1); for DT, two bands of about 24 and 39 kDa were observed in concordance with the molecular weights of major fragments of Dt; for TT, two bands with a molecular weight of about 100 and 50 kDa were observed, which corresponded to its components and confirmed the purity of the toxoid samples.

2.Using the Bradford method, the total DT and TT concentrations were identified as 2.38±0.01 mg/ml and 2.84±0.01 mg/ml, respectively.

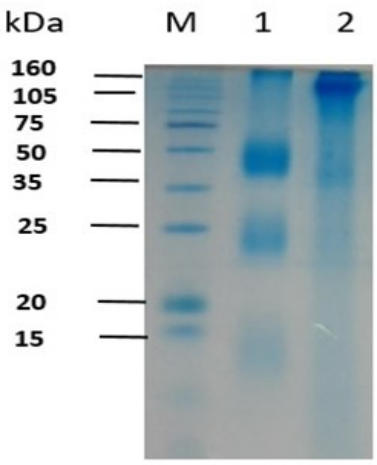

Fig 1: SDS–PAGE pattern of TT and DT under reducing condition (Lane M: Standard protein molecular weight marker; Lane 1: DT; Lane 2:TT.).

2. Characterization of blank NPs and DT + TT NPs: The optimal parameters for preparing suitable NPs were selected according to the appropriate particle size, PDI, and suitable zeta potential obtained by DLS reports.

Regarding the effects of the different concentrations of alginate and calcium chloride on the properties of NPs, it was observed that at a concentration higher than 0.2 %w/v of sodium alginate and 0.1 %w/v of calcium chloride, the hydrodynamic particle size of NPs was increased significantly (Fig 2A and 3A).

When the calcium chloride concentration is lower than 0.1 %w/v, the frequency of binding sites on alginate NPs is higher than that of $Ca2^{+}$ ions.

The alginate concentration deviated from 0.2 % w/v and the absolute values of zeta potential decreased (Fig 2B), which indicated the development of clusters and the lack of proper stability, while PDI of NPs was getting away from the generally recognized favorite range (Fig 2C).

Under the influence of different concentrations of calcium chloride from 0.1 %w/v, the zeta potential of blank and toxoids-loaded NPs tended to approach the levels closer to zero (Fig. 3B), while calcium chloride solution at 0.1 %w/v resulted in the most suitable PDI level around 0.5 (Fig 3C).

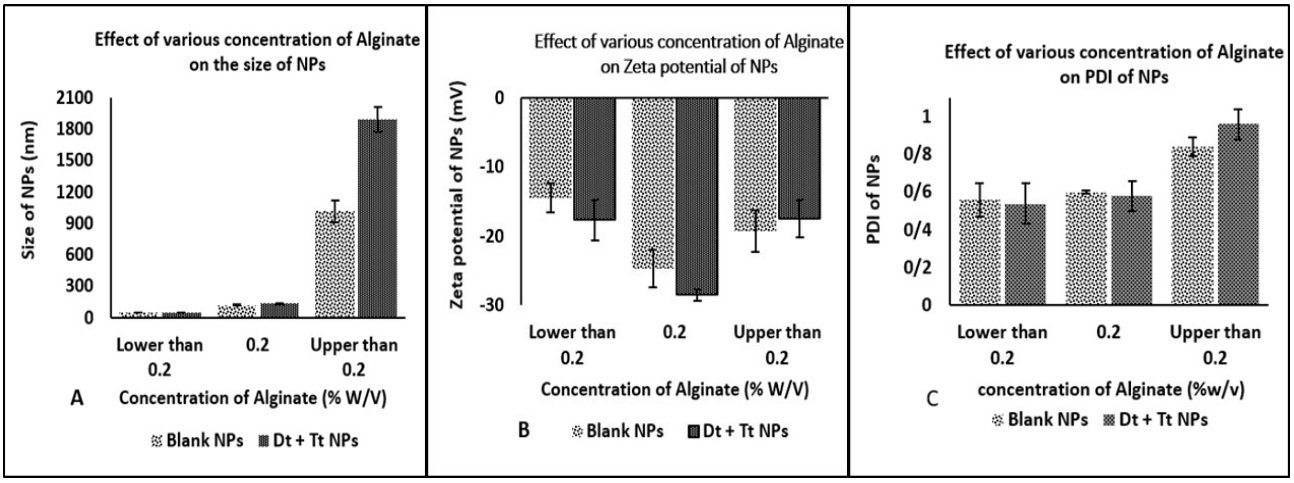

Fig. 2: Effect of different concentrations of alginate solution on DLS results; (A): size; (B): zeta potential; (C): PDI (n=3; mean ± standard deviation).

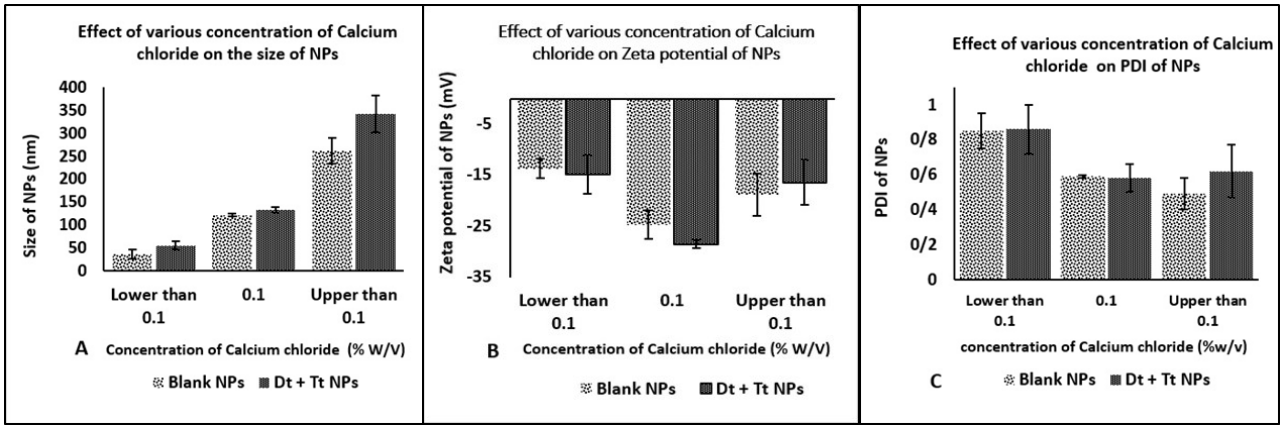

Fig 3: Effect of various concentrations of CaCl2 on DLS results; (A): size; (B): zeta potential; (C): PDI (n=3; mean ± standard deviation).

The effects of altering the PLL concentration on the size, zeta potential, and PDI of NPs were assessed while the other parameters were kept constant. NPs' size increased as the PLL concentration enhanced (Fig 4A).

Additionally, at PLL concentration differed from 0.04% w/v, the absolute zeta potential values tended to be lower than 20 mV away from the stable state observed for 0.04% m/v (Fig. 4B). At lower concentrations of PLL, the values of NPs' PDI were increased (Fig 4C). Based on DLS results, the most favorable concentration of PLL was 0.04% w/v.

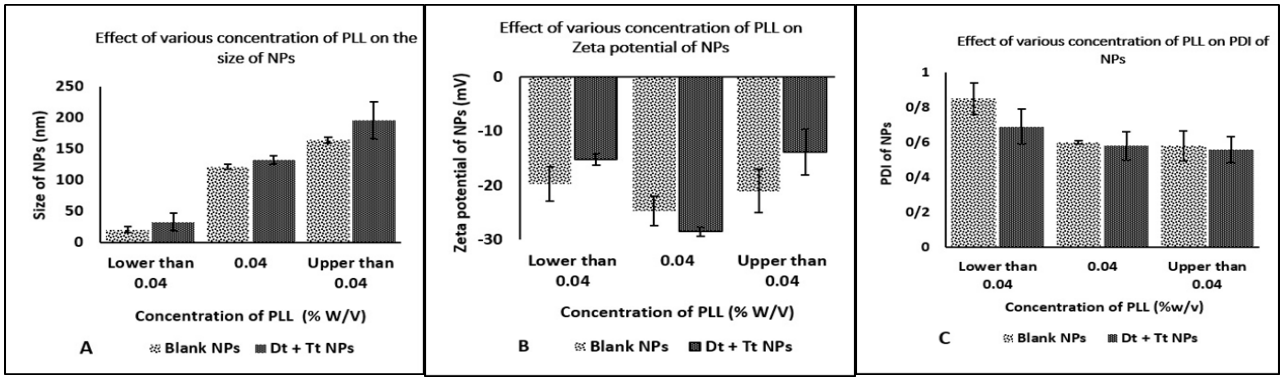

Fig 4: Effect of various concentrations of PLL solution on DLS results; (A): size; (B): zeta potential; (C): PDI (n=3; mean ± standard deviation).

The effects of various stirring speed that is often effective in controlling the particle size is shown in Fig 5. The results show that increasing stirring speed from 1000 rpm to 1600 rpm led the particle size of NPs to be drastically reduced from 300 to lower than 50 nm (Fig 5A). As shown in Fig. 5B, zeta potential responses to the stirring speed differently, and the absolute values of zeta potential moved away from 28 mV. They approached lower values up to 15 mV when deviating from 1300 rpm. It is also observed that the stirring speed has negligible impacts on the PDI of NPs (Fig 5C). Given this, the optimum speed for the preparation of blank and toxoids-loaded NPs, was found to be 1300 rpm. Stirring time has also been shown to influence the particle size and zeta potential of NPs substantially. To determine the effects of the stirring time, the blank and toxoids-loaded NPs were prepared at different stirring times (30, 45, and 60 minutes) while keeping the

other parameters constant at their optimum values. As shown in Fig. 6A to 6C, increasing homogenization time decreased the particle size of NPs. In contrast, the effects of the stirring time on the particle size and zeta potential were almost similar to the stirring speed. These clusters cause bimodality and approaching zeta potential close to zero, so these NPs had no stability.

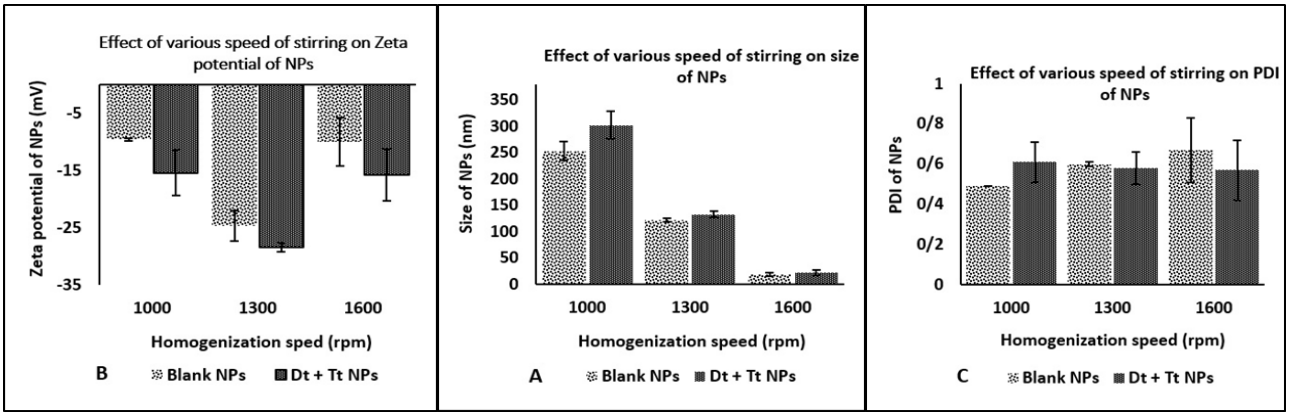

Fig 5: Effect of various speed of stirring on DLS results; (A): size; (B): zeta potential; (C): PDI (n=3; mean ± standard deviation).

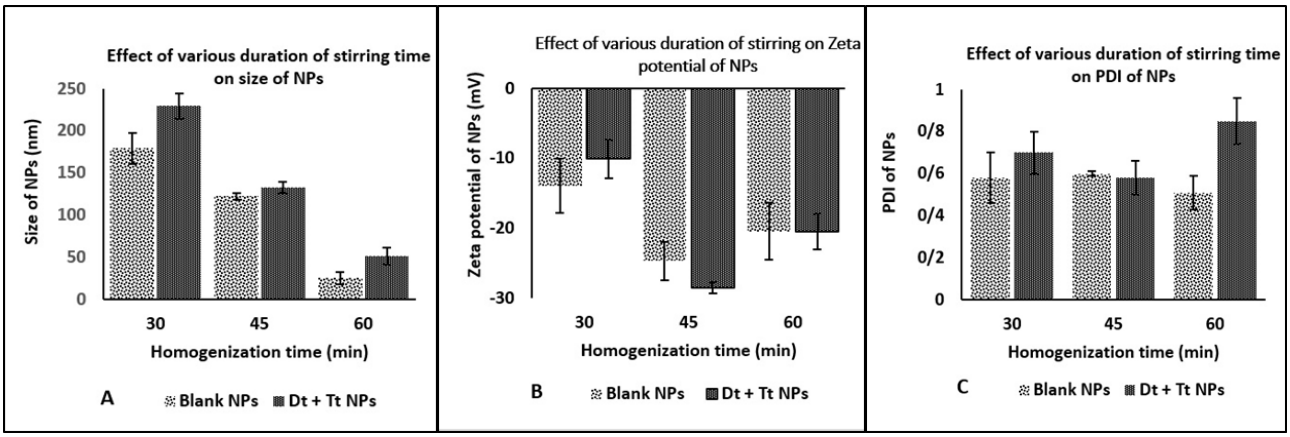

Fig 6: Effect of various durations of stirring time on DLS results; (A): size; (B): zeta potential; (C): PDI (n=3; mean ± standard deviation).

According to the above, the optimized NPs were prepared at the appropriate condition of 0.2 %w/v sodium alginate; 0.1 %w/v calcium chloride; 0.04 %w/v of PLL; and the stirring speed of 1300 rpm for 45 minutes. DLS results of the blank (Fig 7A and Fig 7B) and toxoid-loaded NPs (Fig 7C and Fig 7D) are reported as a sample under the optimal conditions. Besides, the average and standard deviation of DLS results of optimized blank and toxoids loaded NPs are demonstrated in Table 1. PDI of both blank NPs and toxoid-loaded NPs was 0.41 and 0.44, and their zeta potential of them –17.6 mV and –18.7 mV, respectively (Table 1).

3. Assessment of LE and LC and the effect of toxoids concentration on loading: Different doses of DT and TT were loaded individually and in combination to evaluate the ability of alginate NPs for entrapping dual toxoids and

the impacts of toxoids' concentrations on LE and LC efficiencies. It was found that the lower concentration of toxoids resulted in the maximum loaded efficiency (for DT + TT NPs higher than 93%). As the toxoids' concentration increased, LE dropped to the lower value around 75% and then remained almost constant (Fig 8A), while the LC percentage increased gradually (Fig 8B). Contrariwise, lower toxoid doses of 0.5X and 1X were the appropriate concentrations for loading toxoids consistent with quantities of the toxoids in conventional DT vaccine (1X represents the doses of toxoids equivalent to the toxoids in the conventional DT vaccine).

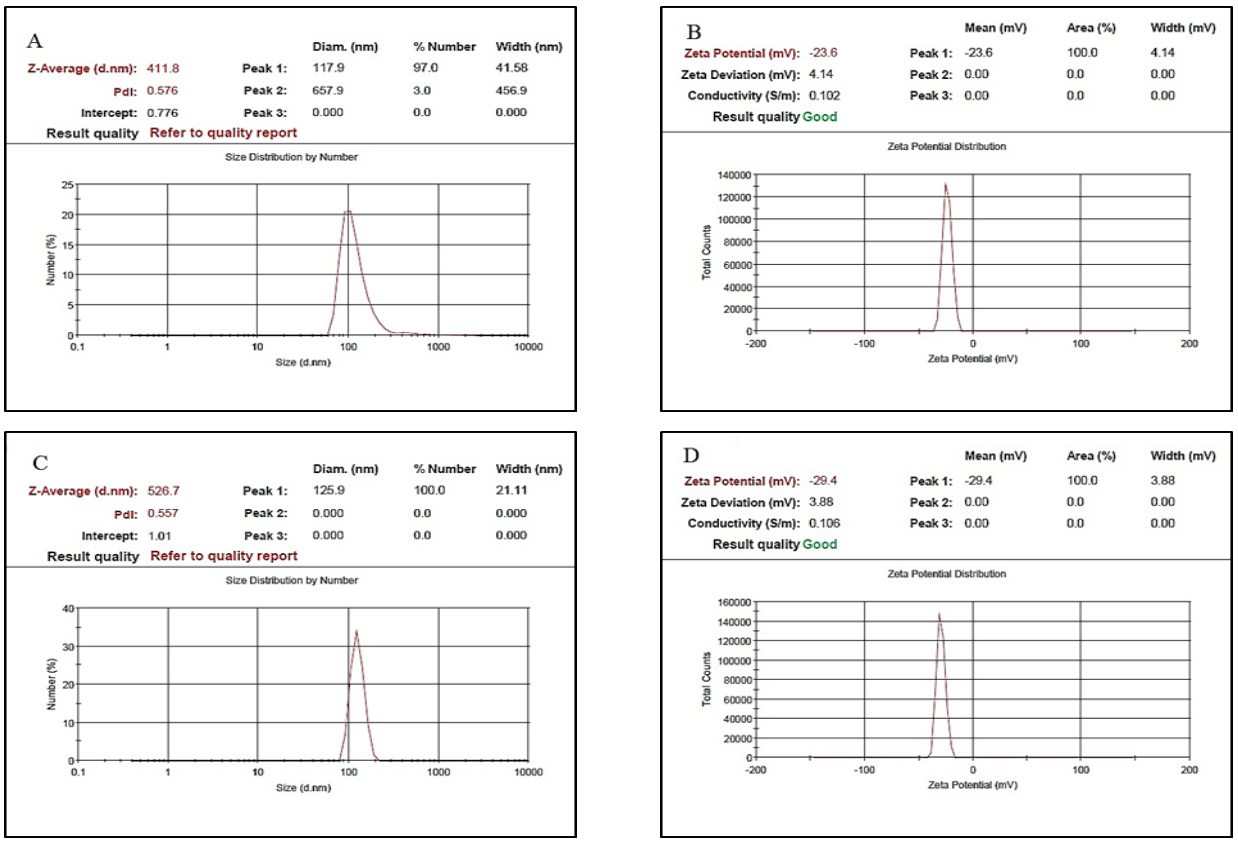

Fig 7: DLS reports for the optimized Blank NPs and Dt + Tt NPs; (A): Size and PDI of blank NPs; (B): Zeta potential of blank NPs; (C): Size and PDI of DT + TT NPs; (D): Zeta potential of DT + TT NPs.

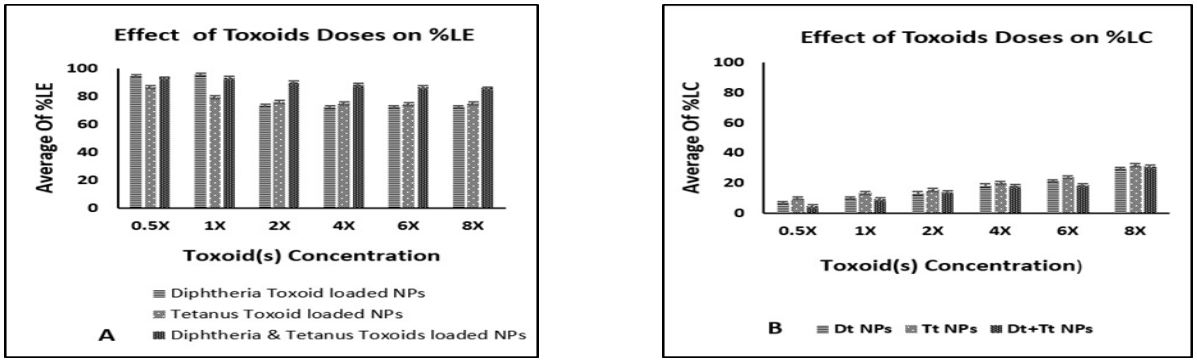

Fig. 8: Effect of various doses of toxoids on toxoids loading; (A): %LE of the optimized Dt, Tt, and Dt+Tt NPs and (B): %LC of the optimized Dt, Tt, and Dt+Tt NPs (n=3; mean ± standard deviation).

Table 1: Average and standard deviation of optimized DLS results (n=3; mean ± standard deviation).

Blank NPs			Dt + Tt NPs		
PDI	Size (nm)	Zeta potential(mV)	PDI	Size(nm)	Zeta potential(mV)
0.6±0.01	122.2±3.83	-24.7±2.72	0.58±0.08	132.8±6.33	-28.5±0.81

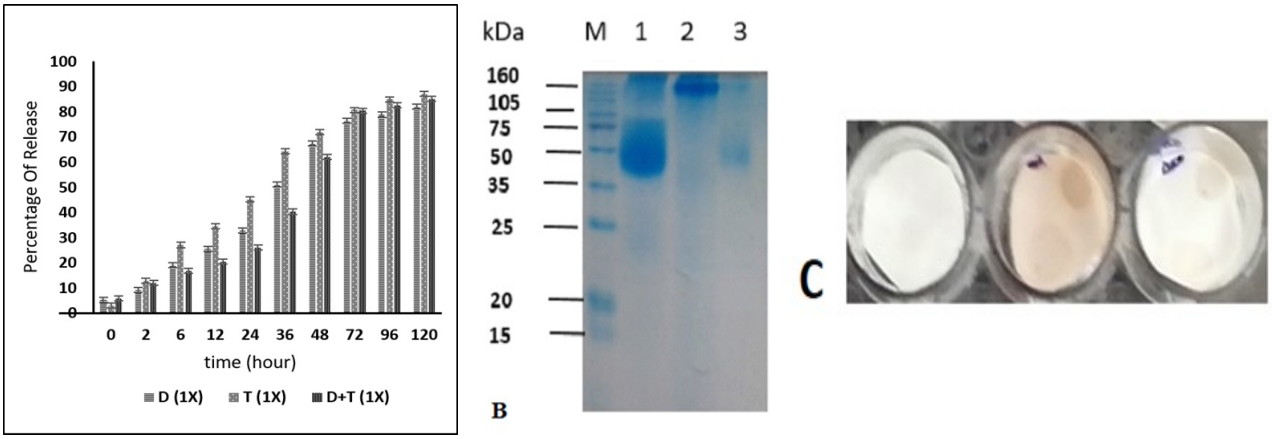

Fig 9: Release of toxoids at 37 ° C. (A): Release profile of toxoids from toxoids loaded NPs with 1X doses of toxoids under optimized condition; (B): SDS-PAGE of released toxoids after two hours under non-reducing condition. Lane M: Standard protein molecular weight marker; Lane 1: Dt; Lane 2: Tt; Lane 3: released toxoids. (C): From left to right: negative control sample (blank NPs), positive control sample (tetanus toxoid adjacent to the marking line and diphtheria toxoid below), a test sample containing released toxoids after 2 hours from Dt + Tt NPs.

4. In-vitro release behaviour: The in vitro release of toxoids was performed at 37 °C. A prolonged release of about 85% of loaded DT + TT toxoids was observed over 120 hours (Fig. 9A).

5. SDS-PAGE and dot blot: To evaluate the stability of toxoids from loading to release, the electrophoretic pattern of toxoids released after two hours was compared with the initial ones prepared by Razi Institute. As shown in Fig. 9B, the SDS-PAGE results confirmed the similarity of these patterns, and then it can be concluded that no damages accrued. Performing dot blot technique on the released samples revealed that the method used to preparing toxoids-loaded nanoparticles under optimized conditions could efficiently retain the antigenic activity of toxoids (Fig 9C).

Discussion

This study aimed to optimize the preparation of alginate nanoparticles loaded by diphtheria and tetanus toxoids and to evaluate the properties of such a platform.

It has been well-proven that polysaccharides micro/nanoparticles, particularly alginate as hydrophilic carrier prolongs the antigen release and enhance the immunogenicity greater than conventional vaccines due to their adjuvant properties (16).

Alginate is capable to improve the transportation or delivery of macromolecular proteins; to date, alginate microparticles have been utilized to transport insulin (17,18) and nasal delivery of antigens, i.e., tetanus (19) and diphtheria toxoid (20). Besides having the advantages of nanoparticles, alginate NPs can

enhance drug encapsulation, pharmacokinetics, bioavailability, therapeutic efficacy, and significant loading potential (20).

Therefore, in this investigation, attempts were made to co-deliver diphtheria and tetanus toxoids by loading on sodium alginate nanoparticles.

In studying the influence of various concentrations of alginate and calcium chloride on the properties of NPs, high levels of those two (>0.2 %w/v and >0.1 %w/v, respectively) enhanced the hydrodynamic particle size (Fig 2A and 3A). Similarly, Lin et al (2016) found that increasing the alginate concentration, resulting in the growing the particle size of astaxanthin-loaded alginate microspheres from 728.7µm to 1053.4µm (21). A vital source influencing the size of NPs is the alginate concentration, as Tsai and Ting (2019) reported (22). This increase could be explained by the possibility of the chaos in NPs formation, which results from the misbalancing between $Ca2^{+}$ and alginate binding sites (23). However, decreasing concentrations of the same variables lead to a minimal hydrodynamic particle size of the NPs, lower than 50 nm, which is not desirable (7). It was observed that in a low concentration of calcium chloride (<0.1 %w/v), the quantity of binding sites on alginate NPs is notably greater than that of $Ca2^{+}$ ions, which is in consistent with previous reports of González Ferreiro et al (2002) and Sarei et al (2013) and under these circumstances, induction of desirable NPs with proper density by $Ca2^{+}$ is not possible (24,25). Zeta potential values allow quantifying the charge on colloidal particles in liquid suspension where zeta potential of ±30 mV is often found to prohibit the accumulation and stabilization of particles in suspension (25). The absolute zeta potential values decreased at alginate concentrations higher or lower than 0.2 %w/v (Fig 2B), which could indicate the cluster formation and the absence of proper stability. In contrast, the PDI of NPs moved far beyond the commonly known suitable range (Fig 2C). In Nisaplinfi loaded alginate prepared by ionic gelation/complexation procedure, Zimet et al (2018) reported an increased particle size from 86 nm to 204 nm, zeta potential value from −33.2 mV to −38.7 mV, and reduction in overall encapsulation efficiency from 35.6% to 30.5% when the concentration of alginate went from 0.03% w/v to 0.07% w/v (26). NPs size enhanced from 512 nm to 4303 nm when alginate concentration incremented from 0.5% w/v to 1% w/v (27).

The calcium chloride concentration found to be effective on the zeta potential of blank and toxoids-loaded NPs where the calcium chloride solution at 0.1 %w/v was found to have the most suitable PDI level around 0.5 (Fig 3 B, C). Our observation is almost following the research results of Goudarzi et al (2016). They obtained optimized alginate nanoparticles containing pertussis toxin at a concentration of 0.1 %w/v calcium chloride, which results in a similar size range, zeta potential, and PDI of NPs (28). In formulating NPs integrating biomaterials for orally dosed insulin, similarly, Woitiski, Veiga (2009) observed a strong positive response in NPs size to calcium chloride (29). When calcium concentration is low, intramolecular crosslinking of individual alginate chains may cause a pregel state and concentrated coiled formation (10,30). However, as observed in this study, increasing calcium concentration lateral binding of alginate chains can lead to larger structures (10).

The addition of PLL as an additional crosslinking agent with the cationic feature

covers the negatively charged calcium alginate complex and is essential for NPs' stability (31). The stirring speed inversely correlated with the size of NPs (Fig 5A) as increasing stirring speed from 1000 rpm to 1600 rpm, NPs size dramatically lessened from 300 to less than 50 nm where the optimum stirring speed was found to be in 1300 rpm. This is because faster agitation can increase the diffusion rate, which leads to smaller particles (22,32).

Our observations for the stirring effects are similar to Moradi Bidhendi et al (2013) (33). As shown in Fig. 6A to 6C, similar to Ferreiro et al (2002), increasing homogenization time decreased the particle size of NPs (34). The effect of stirring time on the particle size and zeta potential was mainly analog to the stirring speed. This observation is due to growth mechanisms, including aggregation and accumulation, which are major determinative factors for creating large particle clusters (32). Accidental collision of NPs, due to Brownian motion and adhesion to each other is one reason for agglomeration that the entangling of slow settling particles could influence the more rapid ones (36). Settlement of aggregated particles at charges near-zero happens by gravitational force (37).

The fluctuation observed in LC and LE in response to the quantity of the loaded toxoid can be explained by the fact that the association of different toxoids with NPs can be due to further physio-chemical entrapment and strongly depends on the size and structure of the NPs and the number of the unsaturated sites to the formation of hydrogen and electrostatic bonds. Because of this, toxoids interact with hydroxyl groups of alginate chains via electrostatic and hydrogen bonds (38). Therefore, with increasing toxoids' concentration, the large amount of toxoids could be incorporated into NPs, leading to increased LC; despite this, LE did not increase due to the limited availability of saturated sites (13). On the contrary, the proper doses of toxoid were low (0.5X and 1X), which interestingly were following conventional DT vaccine concentration (1X). A stable and consistent release of DT toxoids was witnessed under *in vitro* assay, which the sustained release of toxoids without any burst effects is because they strongly bind to NPs (39).

Conclusion

In this study, alginate nanoparticles containing tetanus and diphtheria toxoids, individually and in combination, were prepared by ionic gelation method and optimized for the effects of five variables, including the concentration of sodium alginate calcium chloride, and PLL as well as homogenization time and speed. In vitro evaluation revealed that under optimized conditions, alginate NPs with a mean diameter range of 50 to 150 nm presented appropriate loading efficiency and capacity at a concentration of toxoids equal to doses in the conventional DT vaccine resulting from a sustained release profile. The outcomes of this study paved the way for further evaluation of the nano-formulation in an animal model, which could then promise an efficient vehicle for immunizing infants and children with kidney disease against diphtheria and tetanus toxoids.

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Declaration of interest

None declared.

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