**ABSTRACT**

**Introduction:** In this study, Poly-(ε-caprolactone) (PCL) and Poly-(lactic-co-glycolic acid) (PLGA) microparticles encapsulating diphtheria toxoid (DT) were investigated for their potential as a mucosal vaccine delivery system.

**Materials and Methods:** The antigen-containing microparticles were prepared using double emulsion (w/o/w) solvent evaporation method.

**Results:** The average geometric diameter of the particles were found between 7 and 24 µm which is suitable for uptake by the antigen presenting cells in the nasal mucosa. Although the differences were not significant, PLGA polymer containing formulations exhibited the highest encapsulation efficiency. The microparticle formulations, prepared with both PLGA and PCL polymers, was successfully produced at high production yields. The in vitro release profile was presented as a biexponential process with an initial burst effect due to the release of the protein adsorbed on the microsphere surface and the subsequent sustained release profile is the result of protein diffusion through the channels or pores formed in the polymer matrix. DT loaded microparticles, DT solution in phosphate buffered saline and empty microparticles (as control) were administered via nasal route and subcutaneously to guinea pigs. The antibody content of each serum sample was determined by an enzyme-linked immunosorbent assay.

**Conclusion:** Absorbance values of ELISA test showed that PLGA and PCL bearing microparticles were able to stimulate adequate systemic immune response with intranasal vaccination. Additionally, PLGA and PCL microparticles resulted in significantly increased IgG titers with intranasal administration as a booster dose following subcutaneous administration. PCL polymer elicited a high immune response compared to PLGA polymer (p<0.05).

**Key Words:** Diphtheria toxoid, Immunity, Intranasal, Microparticulate formulations

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1. Introduction

The effects of emerging technologies have shown not only the success of correlation between vaccination and immunogenic components of vaccine, but also the delivery systems. For this reason, the studies on an effective vaccine formulation or a delivery system is of great importance for the development of modern vaccines. Most important viral and bacterial infections occur through the mucosal membranes such as respiratory, intestinal, tear or urogenital tract.1 Adjuvants have been developed to facilitate and improve the immune response obtained after mucosal immunization.2 Particulate adjuvants or antigen delivery systems are considered as an alternative to other immune stimulating adjuvants.3 The concept of polymeric delivery system has emerged for targeting specific regions of proteins or antigens. This approach for the development of mucosal vaccine delivery systems has become mandatory especially for protecting the protein against fragmentation of antigens in mucosal
environment and to increase the uptake by the immune system. Polymeric particles are adsorbed at higher efficiency rates compared to soluble molecules in mucosal epithelial tissues and thus, high antigen concentrations are provided in the area required an immune response. Biodegradable and biologically compatible polyesters and poly-lactic-co-glycolics (PLG) are one of the most preferred polymeric particulate systems because they have been used in humans for many years on surgical suture materials, as well as the controlled-release drug systems.

Diphtheria is a bacterial disease caused by a toxin produced by Corynebacterium diphtheriae and casuses clinical symptoms. Because the ability to create long-term immunological memory of the vaccine applied is parenterally weak, booster doses should be applied intermittently. Booster vaccination is recommended as a strategy to reduce both health and economic burden of the disease in these populations. The reason of not providing a formation of permnenant immunological memory is thought to arise from the inability to stimulate the local humoral immunity in the respiratory tract. Because the mucosal immunization stimulates both systemic and mucosal immunity, it is considered to be an alternative route to the parenteral administration. In addition, the side effects are minimized with the application of mucosal vaccines which is an easier route than the parenteral route. Therefore, mucosal immunization holds notable importance when the vaccination needs to be repeated in certain intervals as in diphtheria. Nasal mucosa is an important part of the mucosal immune system. It is the first point of contact for antigens inhaled into the organism, and thus, pathogen neutralization occurs at the first point of the pathogens enter the organism with nasal vaccination. As a result, both systemic and mucosal immune response could be accomplished. Recent studies have shown that nasal route is more sufficient for a systemic transport of low molecular weight polar drugs, peptides and proteins other than mucosal delivery routes. On the other hand, traditional drugs applied nasally are used in the treatment of local diseases such as nasal allergy, nasal congestion and nasal infection.

The purpose of this study was to create microparticulate vaccine systems against diphtheria with long-term immunological memory. One of our main purposes was to eliminate repetitive doses to improve patient comfort. Also, alternative intranasal administration was studied instead of parenteral root which is more difficult and painful.

2. Materials and methods

2.1. Materials

Poly(lactide-co-glycolide) (PLGA, 50:50) was obtained from Boehringer Ingelheim. Poli-(ε-caprolactone) (PCL, M.A. 14Kda) was purchased from Sigma. Poly vinyl alcohol (PVA; %87-89 hydrolyzed, molecular weight 13000-23000) was purchased from Aldrich-Chemical Company (USA). Diphtheria toxoid (800 Lf/ampoule, Japanese Lot) was provided from Refik Saydam National Public Health Agency (Turkey) as a standart. Dichlorometane (DCM) was purchased from Sigma-Aldrich (Germany). Other agents were all of special reagent grade.

2.2. Preparation of DT-loaded microparticles

Microparticles were prepared by the double emulsion-solvent evaporation technique as follows. 250µl of diphtheria toxoid in ultrapure water was emulsified with 2,5ml of 5% (wt/vol) PLGA or 5% (wt/vol) PCL in dichloromethane, using an Ultraturrax model T 25 (IKA Laboratory Technology, Staufen, Germany) at 8,000 rpm for 5 min or Sonicator model Sonopuls HD 2070 (Bandelin Electronics GmbH&Co, Germany) at 60 W for 30 s. The resulting water-in-oil emulsion (2.5 ml) was then emulsified at 8,000 rpm for 5 min in a mechanical stirrer model Eurostar (IKA Laboratory Technology, Staufen, Germany) with 50 ml of 5% (wt/vol) polyvinyl alcohol to produce a water-in-oil-in-water emulsion. This emulsion was stirred magnetically overnight under pressure at room temperature to allow evaporation of the organic solvent and formation of microparticles. Microparticles were isolated by centrifugation (10 min at 4,000×g), washed three times in 10 ml of ultrapure water, and freeze dried. Prepared formulations were given in Table 1.

2.3. Characterization and Quantification of the DT-loaded microparticles

2.3.1. Particle appearance and particle size analysis

Photomicrographs of microparticles were taken with Lieca DM 4000B microscope. Lyophilized microparticles were dispersed in purified water and then analyzed using (Sympatec Helos H0728) particle size analyzer.

The surface morphologies of microspheres were also observed using environmental scanning electron microscope (ESEM) (FEI Quanta Model 200FEG, Tokyo, Japan). For the sample preparation, a small aliquot of
the microspheres was mounted onto metal stubs using double-sided adhesive tape. After being vacuum coated with a thin layer (100–150 Å) of gold, the microspheres were examined by ESEM operated at 5 kV accelerating voltage. The photomicrographs were then taken at a magnification of 10000.

2.3.2. In Vitro Release of Protein from PLGA and PCL Microparticles

In vitro drug release was determined by statical method.\textsuperscript{16} Microparticles were suspended in pH 7.4 PBS containing 0.01% NaN\textsubscript{3}. The samples were retained in a water bath at 37°C and shaked at 40 rpm. At appropriate time intervals, the release medium was completely withdrawn after centrifugation at 7500 rpm and replaced with fresh buffer. Diphtheria toxoid concentration in the supernatant was determined by mBCA technique. Also, all release data (n=3) were calculated (with SPSS) according to the % released amount of diphtheria toxoid to determine the release kinetics.

2.3.3. Diphtheria Toxoid Loading in Microparticles

The protein contents of the microparticles were assayed by digestion technique. For this technique, a NaOH/SDS solution (1 N NaOH, 3% w/v SDS) was added to microparticles and dissolved.\textsuperscript{3} The mixture was neutralized with 1N HCl and centrifugated. The diphtheria toxoid content was determined using microbicinchoninic acid (mBCA) (Molecular Devices, SPECTRAmax 190 pc) total protein assay at 560 nm.

2.3.4. Stability studies

D1 and D5 formulations were stored in a refrigerator at 2-8°C and in a climate cabinet at 25±2°C, 60±5% relative humidity for 3 months. The diphtheria toxoid content was determined by mBCA assay. At the end of 3 months, nonreduced-PAGE analysis were performed to understand whether the protein integrity of diphtheria toxoid was preserved.

2.3.5. Polyacrylamide Gel Electrophoresis

Diphtheria toxoid calibration samples and formulations remained at 2-8°C for three months were carried out to PAGE gel under nonreducing condition using an electrophoresis system. Samples and reference materials for diphtheria toxoid were run on discontinuous gel prepared with stacking and separating gel of polyacrylamide in sample buffer.

2.4. Immunization studies

The immunization program we followed includes intranasal vaccination and triple repeat blood sample collection from animals; thus, guinea pig was selected as the most suitable experimental animal and the ethical approval had been obtained from Refik Saydam Hıfızıssıhha Center Presidency Scientific Committee and the Ethics Committee (06.01.2011/14886). For \textit{in vivo} studies, weights ranging from 300 grams to 350 grams of 54 male albino guinea pigs (7-8 years old) were used. The morphology of D1, D2, D3, D4, D5 and D6 coded formulations, the sizes and distribution of particles, the encapsulation and production efficiency and \textit{in vitro} release results were taken into consideration. For the application of D1 and D5 coded formulations, \textit{in vivo} studies were conducted. In the control group, diphtheria toxoid with PBS (pH 7.4) was preferred.

Many formulations containing diphtheria toxoid is 25 to 30 Lf in the first vaccination and booster dose application for the pediatric population whereas it contains 2 Lf and above diphtheria toxoid for adults.\textsuperscript{17} The formulation of 25 Lf/50 μl (25 μl/nostril) was applied to the nostrils of male guinea pigs with the help of a micropipette without using anesthetics for intranasal vaccination.\textsuperscript{18} With the application to both nostrils, the membrane area and thus the absorptions of formulations were aimed to increase. Guinea pigs were kept still for about two minutes in order to avoid any loss after administration of the vaccine into the nose. For subcutaneous vaccination, 25 Lf/500 μl of formulation was injected to the male guinea pigs without using anesthetics.

For \textit{in vivo} studies, formulations containing diphtheria toxoid were applied in certain ways and days to nine different groups (n = 6) of male guinea pigs (Table 2). In order to obtain information about whether the experimental animals had ever experienced diphtheria toxoid or not, blood samples were taken from each animal a week before the first vaccination. In this way, the exposure to stress conditions of experimental animals were prevented and the best response after immunization was aimed to be obtained. To determine the changes in
immune responses of guinea pigs, 1 ml of blood samples from each one were taken from their hearts when IgA and IgG antibody formation takes place on 20th and 42nd days. During the blood-collection, guinea pigs were anesthetized with ketamine (35mg/kg) and xylazine (5mg/kg). The collected blood samples were centrifuged at 10,000 rpm for 10 minutes after waiting 1 hour at room temperature and then another 1 hour in the refrigerator (2-8 °C). The serum samples collected from supernatant and were kept at -20°C before the analysis. Immune responses in collected serum samples were evaluated with Elisa kit (total IgG guinea pig).

2.5. Anti-DT antibody assays

DT-specific IgG antibodies were measured by enzyme-linked immunosorbent assay (ELISA). The immunization chart was given in Table 2. In this assay, the IgG present in the samples reacts with the anti-IgG antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound proteins by washing, anti-IgG antibodies conjugated with horseradish peroxidase (HRP) were added. These enzyme-labeled antibodies form complexes with the previously bound IgG. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of IgG in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of IgG in the test sample. The quantity of IgG in the test sample can be interpolated from the standard curve constructed from the standards and corrected for sample dilution.

3. Results

3.1. Preparation and characterization of the DT-loaded PCL and PLGA microparticles

The microparticles prepared by w/o/w solvent evaporation method had regular morphology and smooth surface. The particle size of the formulations lay between 7 and 34 µm (Figure 1). D5 formulation is also observed in scanning electron microscope (SEM) with a smooth spherical shape (Figure 2).

3.2. DT loading

DT loaded microparticles were prepared using different polymers (PCL and PLGA) by variation in the weight of polymer dissolved in dichloromethane to investigate the eventual modifications of the particle size, protein loading and efficiency of entrapment (Table 3). It was indicated that the type of polymer used also affected the encapsulation efficiency. It was determined that formulations prepared with PLGA polymer showed higher encapsulation efficiency than formulations prepared with PCL polymer.

3.3. Stability Studies

D1 and D5 formulations were stored for three months at 2-8°C and 25±2°C (60±5 % RH). Bands of digestion samples and calibration samples were obtained as seen in Figure 3. According to the bands of integrity we can conclude that diphtheria toxoids could kept their stability during the formulation process. The amount of changes during stability studies at different conditions on active substance and encapsulated DT were given in Table 4.

To examine the stability of diphtheria toxoid, it was stayed at refrigerated conditions for multiple months. It was stated that DT remains stable for years when stored at 2-8°C.

3.4. PAGE Electrophoresis analysis

In our previous study the structural integrity of the diphtheria toxin was analysed by SDS-PAGE. Our standart, diphtheria toxin has a broad band around 66 kDa, with two fragments (A fragment around 45 kDa, and B fragment around 29 kDa). Preservation of the integrity and the stability of the diphtheria toxin in three months at both conditions couldn't find any significant differences at non-denaturing environment (p>0.05). Samples extracted from microparticles in both stability conditions were applied to the polyacrylamide gel under non-denaturing conditions (Figure 3). There was only one band around 66kDa. This shows us that the antigen encapsulated in the formulations still remains intact.

3.5 In-vitro release studies

The rapid drug release from polymeric particles, called “burst release” were examined at all formulations (Figure 4). This rapid initial release is attributed to the fraction of the drug which is adsorbed or weakly bound to the surface of the microspheres. Similar to previous investigation, we have obtained triphasic release profiles
generally found in formulations prepared with PLGA and PCL polymers. It was observed that D1 and D2 coded formulations, prepared with the PLGA polymer had a faster release than prepared with PCL polymers. At the end of five weeks PLGA polymer used formulation released all active ingredient. On the other hand, PCL formulations (coded as D3,D4,D5 and D6) couldn’t release all of the active ingredient in five weeks. Also, previous studies have shown that the release rates of particulate systems prepared with PLGA are higher than the release rates of particulate systems prepared with PCL. Furthermore, all of the prepared formulations were compatible with the higuchi kinetics which is expected outcome in matrix systems. Also, the silver staining electrophoresis technique was applied to in vitro release samples and no extra band formation was observed (data not shown). Obtained single band indicates that the structural integrity of diphtheria toxoid is preserved during microparticle formation.

3.6. Immunization studies

The first vaccination experiment was performed to investigate if DT loaded microparticles are able to induce a systemic immune response following nasal administration to guinea pig. Results of guinea pig serum antibody (IU/ml) titres are summarized in Figure 5.

By comparing the groups of F2 and F4, the formulation coded D1 was found to increase the IgG titer on the 42nd day when administered intranasally. When the IgG titers were examined of group F6, increased concentrations were observed after intranasal application. Although this group firstly applied subcutaneously, continuous intranasal administration showed increased titers. According to these results we examined that formulation D1 can be a good candidate for a booster (rappel) formulation.

When the groups of F3 and F5 are compared, the D5 formulation administered intranasally was found to have an increment effect on the IgG titer at the 42nd day. When the blood concentrations of the group F7 were examined, similar increasing titers observed as formulation D1’s.

When D1 and D5 coded formulations are compared with each other, D5-coded formulation was observed to stimulate antibody formation stronger than D1-coded formulation and the difference was statistically evaluated by T test and it was determined to be significant (p < 0.05). The reason is resulted from the PCL polymer. On the formulation that PCL polymer alone is applied intranasally (group F3), there is a significant increase in immune response obtained by F1 group. This increase in IgG is thought to play an active role on the stimulation of systems forming the immune response of PCL polymer. Murillo et al observed the same adjuvant effect when they applied the PCL polymer to animals in vivo. However, we concluded that PLGA polymer did not show an adjuvant effect with nasal application.

The immune response of F4 and F5 groups in which only intranasal application was performed has found to be higher than the immune response of F6 and F7 groups in which the subcutaneous injection follows the intranasal application. In the first and second immunization, the reason why the immune response obtained after intranasal application is higher than the others is thought to be from a disease that keeps the upper respiratory tract. This finding supports the request of WHO to encourage the mucosal immunization with the diseases that holds upper respiratory tract like diphtheria.

4. Discussion

The purpose of this study is to develop microparticulate systems containing diphtheria toxoid as the model antigen and also to evaluate their intranasal administration. New approaches to diphtheria vaccination with equal or surpass generating immune responses according to the conventional vaccine must have an increased safety profile. In this study, our reason for the selection of mucosal vaccination is the capacity to stimulate both systemic and mucosal immune responses. It has been examined that by using nasal route most of soluble antigens can induce immune responses. Conversely, it should not be ignored that these antigens can induce poor immunogenicity with immunological tolerance. In our previous study, we found that nasal administration of diphtheria toxoid alone induces very weak immune responses. In contrast, using of the polymeric-DT microparticulate formulations can considerably enhanced their immunogenicity for intranasal immunization of the guinea pigs. These microparticulated formulations which contain diphtheria toxoid were prepared with double emulsion (w/o/w) solvent evaporation method. Poly(lactic-co-glycolic acid) (PLGA) and poly-ε-caprolactone (PCL) polymers were used in microparticulate formulations because of their biodegradable and biocompatible properties. Furthermore, recent studies have shown that PLGA copolymers significantly affect the stability and biological activities of active substances, especially depending on the hydrophobicity of the polymer and the presence of acidic degradation products.
Sayin et al., established that chitosan can enhance immune responses in nasal route. They studied on MMW chitosan which could successfully enhance the absorption of diphtheria toxoid nasally in rat models. In the nasal cavity, the biodegradable chitosan increases the residence of the antigen and can open tight junctions between the cells, thereby promoting absorption into the nasal cells.19 On the other hand organic solvent residues in the formulation while preparing chitosan solutions, can be caused the lower stability of these molecules against proteolysis and lower absorption when administered intranasally.28 We also used similar organic solvents while preparing microsphere formulations. However in order to get rid of the organic solvent residue, during the preparation step, the solvent was allowed to evaporate overnight under pressure and the washing procedure was applied under high speed centrifugation for three times. The encapsulation yields and the immune response of the formulations show that we eliminated this negative condition.

Isaka et al.7 previously demonstrated that an immunization of mice with alum-adsorbed DT were considerably enhancing by a booster immunization by the same route. Immunization of the mice by this way generated similar IgG and neutralizing antibody titre in parenterally immunized mice, furthermore generated high levels of local IgA.7 The development of the alternative microparticulate formulation for mucosally delivered diphtheria toxoid booster vaccines may eliminate some of the side-effects associated with the conventional vaccine with aluminium salts and may also be more acceptable for frequent boosting against diphtheria. Similar results were obtained with our previous in-situ gel formulations.27 When the release kinetics were compared, it was showed that the microsphere formulation was more effective than in-situ gel formulations. In addition, it is one step ahead of other formulation designs due to the adjuvant effect of the PCL polymer, which are used for microsphere formulations.

The morphological properties, particle size, encapsulation efficiencies and production yields of microparticles were investigated during pre-formulation studies. According to the obtained results, formulations were optimized and certain amounts of diphtheria toxoid were added into the formulations (D1, D2, D3, D4, D5 and D6). For these formulations; morphological properties, particle size and distribution, encapsulation efficiencies, production yields, drug release profiles and stability of microparticles were investigated. Formulations coded D1 and D5 were considered to be superior to other formulations given in the in vitro tests (eg., encapsulation efficiencies, particle size and distribution, particle size and distribution). Therefore, these formulations were tested in guinea pigs in order to determine immune responses that would produce following intranasal and subcutaneous administration. Absorbance values of ELISA test showed that formulations coded D1 and D5 were able to stimulate adequate systemic immune response with intranasal vaccination. Additionally, D1 and D5 formulations exhibited a significant increment on IgG titers with intranasal administration as a booster dose following subcutaneous administration. PCL polymer elicited a high immune response compared to PLGA polymer (p<0,05). However, the PCL polymer when used alone, was also found to have an adjuvant effect.

In this study, we developed intranasal vaccines to be an alternative to parenteral formulations. So, it was investigated for any inflammation, edema, or other side effects that occurred in the injection and nasal area for all guinea pigs, within 42 days. The absence of adverse effects is also positive for our formulations for mucosal administration.

In our previous study, in-situ gel formulations improve the residence time of the diphtheria toxoid in the mucosa for its therapeutic efficacy.27 Although this gel formulations can be applied easily by droplet into intranasal way, both Kruskal–Wallis and Wilcoxon tests showed that microparticulated formulation (D1 and D5) resulted in an increased systemic immune response according to in-situ gel formulation (F3 and F7). This might have been caused by the late migration of DT in gel formulations.

**Conclusion**

This study demonstrates that microparticles of poly(ε-caprolactone) and Poly-(lactic-co-glycolic acid) encapsulated with diphtheria toxoid can be produced by a modified double emulsion (w/o/w) technique. The morphology, size and distribution of particles, the encapsulation, production efficiency and in vitro release of the formulations were investigated and two candidate formulations were applied to the nostrils of male guinea pigs. The immune response of formulations applied through intranasal route was higher to groups in which the formulations were investigated and two candidate formulations were applied to the nostrils of male guinea pigs. This study demonstrates that microparticles of poly(ε-caprolactone) and Poly-(lactic-co-glycolic acid) encapsulated with diphtheria toxoid can be produced by a modified double emulsion (w/o/w) technique. The morphology, size and distribution of particles, the encapsulation, production efficiency and in vitro release of the formulations were investigated and two candidate formulations were applied to the nostrils of male guinea pigs. The immune response of formulations applied through intranasal route was higher to groups in which the formulations were investigated and two candidate formulations were applied to the nostrils of male guinea pigs. In this study, we developed intranasal vaccines to be an alternative to parenteral formulations. So, it was investigated for any inflammation, edema, or other side effects that occurred in the injection and nasal area for all guinea pigs, within 42 days. The absence of adverse effects is also positive for our formulations for mucosal administration.

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Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

5. References

Table 1. Description of formulations

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Diphtheria toxoid (mg)</th>
<th>Route of administration</th>
<th>Polymer used</th>
<th>1st step of homogenization</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>75.8</td>
<td>Intransal</td>
<td>PLGA</td>
<td>Ultraturrax</td>
</tr>
<tr>
<td>D2</td>
<td>75.8</td>
<td>Intransal</td>
<td>PLGA</td>
<td>Sonicator</td>
</tr>
<tr>
<td>D3</td>
<td>75.8</td>
<td>Intransal</td>
<td>PCL (70-90kDA)</td>
<td>Ultraturrax</td>
</tr>
<tr>
<td>D4</td>
<td>75.8</td>
<td>Intransal</td>
<td>PCL (70-90kDA)</td>
<td>Sonicator</td>
</tr>
<tr>
<td>D5</td>
<td>75.8</td>
<td>Intransal</td>
<td>PCL (14kDA)</td>
<td>Ultraturrax</td>
</tr>
<tr>
<td>D6</td>
<td>75.8</td>
<td>Intransal</td>
<td>PCL (14kDA)</td>
<td>Sonicator</td>
</tr>
</tbody>
</table>
Table 2. Groups of guinea pigs immunised with adjuvant-free, microencapsulated diphtheria toxoid (Dtxd) and control (n=6).

<table>
<thead>
<tr>
<th>Group code</th>
<th>Formulation</th>
<th>Polymer used</th>
<th>Administrati on route</th>
<th>Dose (Lf)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>I. Immunisation 0. day</td>
<td>II. Immunisation 7. day</td>
</tr>
<tr>
<td>Control 1</td>
<td>Dtxd-Solution</td>
<td>-</td>
<td>i.n.</td>
<td>25 Lf/50 µL (i.n.)</td>
</tr>
<tr>
<td>Control 2</td>
<td>Placebo MP</td>
<td>PLGA</td>
<td>i.n.</td>
<td>50 µL (i.n.)</td>
</tr>
<tr>
<td>Control 3</td>
<td>Placebo MP</td>
<td>PCL</td>
<td>i.n.</td>
<td>50 µL (i.n.)</td>
</tr>
<tr>
<td>MP-D1 (F4)</td>
<td>Dtxd-MP</td>
<td>PLGA</td>
<td>i.n.</td>
<td>25 Lf/50 µL (i.n.)</td>
</tr>
<tr>
<td>MP-D5 (F5)</td>
<td>Dtxd-MP</td>
<td>PCL</td>
<td>i.n.</td>
<td>25 Lf/50 µL (i.n.)</td>
</tr>
<tr>
<td>MP-D1 (F6)</td>
<td>Dtxd-MP</td>
<td>PLGA</td>
<td>s.c. + i.n.</td>
<td>25 Lf/500 µL (s.c.)</td>
</tr>
<tr>
<td>MP-D5 (F7)</td>
<td>Dtxd-MP</td>
<td>PCL</td>
<td>s.c. + i.n.</td>
<td>25 Lf/500 µL (s.c.)</td>
</tr>
<tr>
<td>Control 4</td>
<td>Dtxd-Solution</td>
<td>-</td>
<td>s.c. + i.n.</td>
<td>25 Lf/500 µL (s.c.)</td>
</tr>
</tbody>
</table>

Dtxd: Diphtheria toxoid, MP: Microparticle, s.c.: subcutan, i.n.: intranasal

Table 3. Characterization and physicochemical properties of DT-loaded microparticles

<table>
<thead>
<tr>
<th>Code</th>
<th>Type of Polymer</th>
<th>1st step of homogenization</th>
<th>Antigen (mg)</th>
<th>Encapsulation eff. (%) ± SS*</th>
<th>Yield (%)</th>
<th>Geometric diameter (µm) ± SS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>PLGA</td>
<td>Ultraturrax</td>
<td>75.8 (144 µg protein)</td>
<td>60.6 ± 0.39</td>
<td>88.38</td>
<td>6.93 ± 0.45</td>
</tr>
<tr>
<td>D2</td>
<td>PLGA</td>
<td>Sonicator</td>
<td>75.8 (144 µg protein)</td>
<td>53.7 ± 1.53</td>
<td>87.23</td>
<td>7.98 ± 0.83</td>
</tr>
<tr>
<td>D3</td>
<td>PCL (70-90 kDa)</td>
<td>Ultraturrax</td>
<td>75.8 (144 µg protein)</td>
<td>46.8 ± 1.97</td>
<td>73.57</td>
<td>22.47 ± 0.31</td>
</tr>
<tr>
<td>D4</td>
<td>PCL (70-90 kDa)</td>
<td>Sonicator</td>
<td>75.8 (144 µg protein)</td>
<td>52.6 ± 1.08</td>
<td>74.00</td>
<td>34.4 ± 1.22</td>
</tr>
<tr>
<td>D5</td>
<td>PCL</td>
<td>Ultraturrax</td>
<td>75.8</td>
<td>55.7 ± 0.86</td>
<td>88.30</td>
<td>8.88 ± 0.02</td>
</tr>
</tbody>
</table>
Table 4: The diphtheria toxoid integrity during stability studies (n=3)

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Condition</th>
<th>Remaining amount of diphtheria toxoid (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>1st month</td>
</tr>
<tr>
<td>DT alone</td>
<td>5±3°C</td>
<td>99,85 ± 0,27</td>
</tr>
<tr>
<td>D1</td>
<td>5±3°C</td>
<td>60,6 ± 0,39</td>
</tr>
<tr>
<td>D5</td>
<td>5±3°C</td>
<td>55,7 ± 0,86</td>
</tr>
<tr>
<td>D1</td>
<td>25±2°C</td>
<td>60,6 ± 0,39</td>
</tr>
<tr>
<td>D5</td>
<td>25±2°C</td>
<td>55,7 ± 0,86</td>
</tr>
</tbody>
</table>

* n=6

Figure 1. Optical micrograph of (a) PLGA and (b) PCL (90kDa) microparticles entrapping BSA produced by the standard double emulsion solvent evaporation method
Figure 2. Environmental scanning electron microscopy images of D5 microparticles

Figure 3. Bands which obtained by Polyacrylamide Gel Electrophoresis for D1 and D5 coded formulations A) 2-8°C B) 25±2°C 1) initial 2) 1st month 3) 2nd month 4) 3nd month
Figure 4. In vitro release profiles of diphtheria toxoid from the formulations

Figure 5. Ig G titres at specified intervals