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# In vivo efficacy and toxicity evaluation of polycaprolactone nanoparticles and aluminum based admixture formulation as vaccine delivery system

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ABSTRACT

Delivery of antigen through admixture formulation containing poly caprolactone (PCL) and aluminum phosphate was studied as a promising strategy to generate antigen specific immune response. The present study demonstrates the synergistic effect of admixture formulation of PCL with reduced aluminum (PCL-Al 0.2 mg-TT and PCL-PEG-Al 0.2 mg-TT) as a potential adjuvant system using tetanus toxoid (TT) as a model antigen. On evaluation of the magnitude of efficacy for the proposed formulation by ELISA as well as challenge method, persistent and strong antibody response was obtained throughout the 180 day study period on storage at $5 \pm 3\,^{\circ}C$. In comparison to the aluminum phosphate based conventional tetanus vaccine, higher levels of IFN-γ and IL-4 were obtained with PCL-Al 0.2 mg-TT and PCL-PEG-Al 0.2 mg-TT, indicating the presence of cell mediated as well as humoral immune responses. Histopathology and serum biochemistry profile in mice further indicated the suitability of the proposed formulation. Percent adsorption/encapsulation of the antigen also increased to nearly 95% in the admixture formulation compared to 55% adsorption in the conventional tetanus vaccine. The present study established a useful baseline for designing biocompatible and effective delivery system for toxoid vaccines through judicious use of PCL based biodegradable nanoparticles in combination with aluminum phosphate.


## 1. Introduction

Aluminum based adjuvants are being used in human vaccines since last eight decades and have contributed significantly in increasing the life expectancy, especially among children [1]. At present aluminum based adjuvants are being primarily used in Diphtheria, Tetanus and Pertussis group of vaccines [2], and form major part of the vaccines used in the universal programme of immunisation (UIP). In addition, aluminum based adjuvants are also being used in Hepatitis B, Pneumococcal and *Haemophilus influenzae* type b vaccines. But, reported incidences of swelling, erythema, indurations, cutaneous nodules, delayed onset of diffuse myalgia, neurodegenerative and autoimmune diseases etc., have led to some concerns regarding their use in childhood vaccines [3,4]. The advent of aluminum containing newer vaccines may further increase the chances of these side effects. In this direction, controlled release micro and nanoparticulate formulations based on biodegradable polymers such as poly (lactic acid), poly (lactic/glycolic) acid (PLGA) and poly (ε-caprolactone) (PCL) etc. have been investigated as alternative adjuvants [5–9]. In our previous work with PLA based polymeric nanoparticle and admixture formulations, the later elicited improved immune response as compared to the conventional tetanus vaccine available in the market [10]. Slow degrading and hydrophobic polymer is expected to be more effective due to better processing by antigen presenting cells [11], therefore, the present study was designed with the aim to evaluate the effectiveness of the biodegradable PCL polymeric NPs and aluminum based admixture formulation as a potential antigen delivery system. In our previous work with PLA, the ELISA test revealed the presence of antibodies, but the formulations proved ineffective on evaluation of the efficacy by challenge method. Therefore, it was envisaged to evaluate the immunogenicity of the proposed adjuvant system by ELISA as well as single dilution challenge method.

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The quantum of generation of cell mediated ($T_H1$) and humoral ($T_H2$) type immune response was also envisaged. Further, histopathology and serum biochemical profile in mice were also studied to demonstrate biocompatibility of the admixture formulation.

## 2. Materials and methods

### 2.1. Materials

Tetanus toxoid (TT) (3300 Lf/ml) was received as a gift from Human Biological Institute, Hyderabad. Tetanus antitoxin serum (TATS) was obtained from Central Research Institute, Kasauli, India. Polycaprolactone (PCL, Mn 80 kDa), poloxamer F-127, bovine serum albumin (BSA) and anti-goat HRP conjugate were procured from Sigma–Aldrich, USA. Polyethylene glycol (mol. wt. 4 kDa) was supplied by CDH, New Delhi. Dichloromethane and acetonitrile were obtained from Merck, Mumbai. 4-Dimethyl aminopyridine (DMAP) and N,N-dicyclohexylcarbodiimide (DCC) were obtained from Spectrochem, Mumbai. 3,3′,5,5′-Tetramethylbenzidine (TMB) was purchased from Biorad, USA. IFN-γ and IL-4 kits were purchased from Thermo Scientific, USA. MilliQ water with a conductivity of 18.2 $m\Omega\,cm^{-1}$ was used in all experiments. Tetanus vaccine manufactured by Serum Institute of India Limited (SIIL), Pune, India (Al 0.45 mg/0.5 ml) was used as conventional tetanus vaccine in the study.

### 2.2. Synthesis of PCL-PEG block copolymer

Polycaprolactone (Mn 80 kDa) and polyethylene glycol (Mw 4 kDa) were used to synthesize PCL-PEG block co-polymer using Steglich esterification reaction, using DCC and DMAP as coupling agent and catalyst respectively. Briefly, 1 g of PCL and 47 mg of PEG were dissolved in 100 ml of dichloromethane and allowed to stir at $0 \pm 2\,^{\circ}C$. To this solution, 50 mg of DCC was added slowly followed by the addition of 20 mg of DMAP. The reaction mixture was stirred for 18–24 h. The resulting PCL-PEG block copolymer was precipitated with a 50:50 mixture of methanol and diethyl ether, to remove any unreacted PEG. Synthesized PCL-PEG block copolymer was dried under vacuum and stored at $-20 \pm 5\,^{\circ}C$ till further use.

### 2.3. Preparation of vaccine formulations

#### 2.3.1. Preparation of tetanus toxoid loaded nanoparticles (NPs)

PCL and PCL-PEG nanoparticles were prepared by double emulsion solvent evaporation method as described elsewhere. Briefly, 300 mg of the desired polymer was dissolved in 20 ml acetonitrile, followed by addition of tetanus toxoid (3300 Lf/ml) in quantity sufficient to get the concentration of 10 Lf/ml in the final formulation. The resulting w/o emulsion was sonicated for 30 min and added drop wise into a 75 ml aqueous solution comprised of Poloxamer F127 (480 mg) in distilled water and stirred at room temperature for overnight. The suspension was centrifuged at 13,000 rpm/60 min to get the pellet. After removing the supernatant, the pellet was re-suspended in 5 ml of milliQ water and freeze dried.

#### 2.3.2. Preparation of tetanus vaccine containing 0.20 mg/dose aluminum

Since our aim was to evaluate the efficacy of the tetanus vaccine at reduced concentration therefore Al at 0.2 mg/dose concentration was used in admixture formulations. To prepare the vaccine, tetanus toxoid (3300 Lf/ml) was added in 75 ml of normal saline to get the final concentration of tetanus antigen as 10 Lf/ml and mixed with $AlPO_4$ to get 0.2 mg of aluminum/dose in the final formulation. The pH was adjusted to $6.2 \pm 0.2$, and the mixture was homogenised on a shaker at 250–350 rpm for 3–4 h. After keeping the mixture for overnight at RT, the formulation was stored at $5 \pm 3\,^{\circ}C$ till further use.

#### 2.3.3. Preparation of admixture of polymeric nanoparticles and aluminum phosphate

For preparation of admixture formulation, the tetanus vaccine containing 0.20 mg/dose aluminum was prepared as mentioned in Section 2.3.2. Thereafter, the formulation was centrifuged at 3000 rpm and supernatant was collected. The pellet was dissolved in milliQ water to make up the volume and labeled as Part-I. To the supernatant having un-adsorbed toxoid, poloxamer F-127 (480 mg) was added and the resulting emulsion was stirred for 1 h, followed by the slow addition of PCL/PCL-PEG solution (300 mg in 15 ml acetonitrile). The reaction was run for overnight at RT. Next day, the emulsion was centrifuged at 13,000 rpm/60 min to get the pellet. After drying in vacuum, pellet was re-suspended in the required volume of milliQ water (Part-II). Part-I and Part-II were mixed in equal proportion and stored at $5 \pm 3\,^{\circ}C$ till further use.

### 2.4. Determination of size, surface charge of nanoparticles and surface morphology of admixture formulation

The particle size distribution and zeta potential ($\zeta$) of the tetanus toxoid encapsulated NPs were determined by Malvern nano zetasizer (Malvern instruments, UK). Surface morphology was observed by Stereoscan 360 (Cambridge Scientific Industries, USA) scanning electron microscope.

### 2.5. Nuclear magnetic resonance (NMR) and gel permeation chromatography (GPC) analysis

Proton ($^1H$) NMR spectra of the synthesized block copolymer sample was recorded on a Bruker AC 300 MHz spectrometer in $CDCl_3$ solvent at room temperature. GPC analysis was performed at room temperature with a Malvaren Viscoteck GPC system (USA). Tetrahydrofuran (THF) was used as the mobile phase at a flow rate of 1 ml/min.

### 2.6. Determination of encapsulation efficiency

The amount of actual tetanus toxoid loaded per unit weight of nanoparticles was determined using the bicinchoninic acid (BCA) assay and absorbance was measured at 570 nm. Briefly, 10 mg of particles were dispersed in 1 M NaOH and incubated at $37 \pm 1\,^{\circ}C$ overnight until all the polymer was degraded and completely dissolved. BCA reagent was added to the solution after neutralizing it to pH 7 with 1 N HCl [11]. Values were obtained from the standard curves plotted with BSA as reference protein. Encapsulation efficiency of tetanus toxoid was calculated as follows:

$$\text{Encapsulation efficiency}\,(\%) = \frac{[TT_{\text{total protein}} - TT_{\text{protein in supernantant}}] \times 100}{[TT]_{\text{total protein}}}$$

### 2.7. In vitro release studies

In vitro release studies of tetanus antigen from polymeric nanoparticles were performed by the method described elsewhere with slight modification [12]. Briefly, known amount of particles were suspended in 1 ml of PBS (pH $7.2 \pm 0.2$) and kept in incubator shakers maintained at $37 \pm 1.0\,^{\circ}C$. Sampling was done at predetermined time points during 30 day study period. For sampling, eppendorf tubes were spun at 13,000 rpm/15 min to pellet the

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