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## Original Article

# Fabrication of Polymethyl Methacrylate Microspheres for Improving Immunogenicity of Tetanus Toxoid

Sellappan Mohan* and Arumugam Nagarajan

*Department of Pharmaceutics, Karpagam College of Pharmacy, TN Dr MGR Medical University, Ottakkalmandapam, Coimbatore, Tamil Nadu, India*

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## Abstract

**Background and objectives:** The polymer's slow hydrolysis facilitates the sustained release of the immunogen, stabilizing the antigen encapsulated within the microspheres. As a result, microspheres ranging from 40 µm to 70 µm in diameter can be formed. This innovative microsphere formulation allows for efficient uptake by macrophages and other antigen-presenting cells. This study aimed to use biocompatible polymethyl methacrylate microspheres for the controlled delivery of antigens.

**Methods:** The potency of various formulations containing encapsulated tetanus toxoid (TT) with polymethyl methacrylate polymer microspheres was assessed using the toxin neutralization and challenge methods. The neutralization test was conducted on pooled sera two weeks after the initial immunization and weekly for four weeks following the booster dose administration. Scanning electron micrographs of the microspheres revealed drug leaching from spherical granular matrices.

**Results:** The injection site showed a higher distribution of smaller microparticles, resulting in depot release. The polymer coating's thickness was significantly lower compared to the 25% polymer microspheres. Concentrations ranging from 0.00024 mL to 0.00030 mL caused significant tetanic paralysis. Two weeks after the initial immunization, the antigenic activity of TT was below the minimum threshold, possibly due to insufficient levels of antigenic TT within the system within seven days post-immunization. The polymethacrylate microsphere elicited a notable immune response, but only the polymer concentration of 25% w/v met the I.P. requirements; lower polymer concentrations were ineffective.

**Conclusions:** The polymer's slow hydrolysis facilitates the sustained release of the immunogen, stabilizing the antigen encapsulated within microspheres. Consequently, microspheres ranging from 40 µm to 70 µm in diameter can be assembled. This innovative microsphere formulation allows for efficient uptake by macrophages and other antigen-presenting cells.

## Introduction

Clostridium tetani toxin is responsible for causing tetanus infection, which damages the nervous system.[1] Tetanus toxoid (TT) vaccination has helped reduce tetanus infections by about 89% worldwide.[2] Nowadays, approximately 80% of the vaccination cost is incurred due to cold chain requirements for protecting the vaccines, which poses a significant challenge.[3,4] The application of controlled-release methodology to vaccines is highly beneficial as it reduces or even eliminates the need for spaced administration of antigen doses, using stabilizers and preservative compounds.[5–8] Controlled delivery of bioactive macromolecules has become increasingly important with the use of polymers to control the release of antigens, thereby stimulating the immune response.[9,10] The potential of biodegradable microspheres to enhance the immunogenicity of poorly immunogenic molecules and specific vaccines has been reported.[10–13] The utility of Poly(*D,L*-lactic/glycolic acid) (PLGA) co-polymer is advantageous due to its tissue-compatible and biodegradable nature.[14] TT entrapped within Poly(*L*-lactic acid) (PLA) and PLGA microspheres was significantly more immunogenic in mice than fluid toxoid.[15–17] The polymeric delivery systems were indeed capable of continuous antigen release and stimulation of antibody formation.[18–20] A single injection of bovine serum albumin-loaded poly (Є-carprolactone) microspheres was shown to provide an immune response comparable to that of a conventional three-injection schedule of the antigen in a rat model.[21]

TT in Poly(*DL*-lactic-co-glycolic acid) microspheres with gela-

**Keywords:** Vaccine delivery; PLGA; Microsphere; Immunogenicity; Tetanus toxoid; PMMA; Controlled delivery.

***Correspondence to**: Mohan Sellappan, Department of Pharmaceutics, Karpagam College of Pharmacy, TN Dr MGR Medical University, Ottakkalmandapam, Coimbatore, Tamil Nadu 641032, India. ORCID: https://orcid.org/0000-0002-4026-9405. Tel: +91-7397769755. E-mail: mohan@kcp.edu.in

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tin and human serum albumin showed stability at 37°C for four weeks.[22] Serum IgG antibody response to ovalbumin entrapped in microparticles was significantly greater than the response to soluble ovalbumin.[18,21] Aluminium hydroxide and calcium phosphate in diphtheria-tetanus vaccines revealed that the adjuvanticity of calcium phosphate was lower than or equal to aluminium hydroxide.[18] Microparticles of size 1.5 µm were significantly more immunogenic than microparticles of size 72.6 µm in the immunogenicity of PLGA with entrapped ovalbumin.[19] The immunogenic potential of TT was improved with entrapped microparticles consisting of PLGA polymers.[21] Charles *et al*. have reported the preparation and preclinical evaluation of bioresorbable hydroxyethyl starch microspheres for transient arterial embolization.[23] Mark *et al*. investigated the immunoglobulin E responses to diphtheria and TTs after a booster with aluminium-adsorbed and fluid diphtheria-tetanus vaccines.[24–26] Chitosan nanospheres encapsulated with TT, biocompatible poly(methyl methacrylate) (PMMA), in combination with the cationic lipid dioctadecyl dimethyl ammonium bromide, have shown high immunogenicity.[27–31]

PMMA polymer as a nanoparticulate vaccine adjuvant displayed immune responses when used with whole inactivated influenza virus.[32–34] PMMA microspheres have been applied for vaccine delivery, exemplified by the preparation of HIV Tat protein adsorbed on anionic core-shell NPs.[35–37] There are no reports yet available on the use of biodegradable methylacrylate or PMMA in TT preparation, as searched through PubMed until September 2023. Crosslinked copolymers of methacrylic acid are ion exchangers and are able to bind drug cations.[22,38] These complexes are used for peroral application to achieve sustained-release effects. They are also applicable as suspensions.[39] Neutral poly(meth)acrylic esters are pharmacologically inactive, even when applied parenterally. They were used for encapsulation of vaccines in the form of nanocapsules.[40] Additionally, the more biodegradable polycyanoacrylates were used for this purpose.[41]

Microparticles consisting of biodegradable polymers that release their antigen content in a controlled manner may provide an ideal delivery system for a new tetanus vaccine.[23] According to the World Health Organization (WHO), the prevention of neonatal tetanus by maternal immunization requires at least two doses of alum-adsorbed TT in previously non-immunized women. These controlled-release vaccines would be designed to release entrapped antigens at predetermined intervals following a single immunization, thus potentially eliminating the need for booster doses. A literature review showed that microencapsulated TT was significantly more immunogenic in mice than a similar dose of fluid toxoid but did not differ much from a similar dose of aluminium phosphate-adsorbed toxoid. Only two polymers, PLA and PLGA, have so far been used in such studies, indicating the novelty of the choice of PMMA, which could significantly improve the immunogenicity.

## Materials and methods

### *Materials*

Tetanus toxin was acquired from the Pasteur Institute of India, Coonoor (Batch No. 40/95). Tetanus antitoxin was obtained from the National Reference, Central Research Institute, Kasauli (Batch No. 64/96). PMMA was provided as a gift sample. Adsorbed TT—International Reference (WHO, Geneva) (340 I.U), Adsorbed TT [Batch No. IA-0760, Pasteur Institute of India, Coonoor (PIIC)]. Carboxymethyl cellulose (LR-Grade), sorbitol (LR-Grade), Tween 80 (LR-Grade), peptone water, and normal saline were procured from Sigma-Aldrich.

### *Characterization of PMMA*

#### Preparation of PMMA

Methyl methacrylate was used for the preparation of PMMA by first washing the precursor with NaOH solution (10 g/100 mL water), followed by washing with deionized water and drying over anhydrous sodium sulfate. The purification of the monomer was achieved by distillation under reduced pressure. Sodium lauryl sulfate and cetyltrimethylammonium bromide were employed as surfactants. PMMA was prepared using an ultrasonication procedure under a uniform nitrogen atmosphere for an hour, with the temperature maintained at 28°C. The emulsion obtained after the process was poured into a 1:1 ice-cold water-methanol mixture, after which the precipitate was collected through centrifugation at 30,000 RPM. It was further purified by washing with cold water three times. The characterization of the polymer thus obtained was performed using a scanning electron microscope (SEM), and the particle size was determined through this analysis.

The pure PMMA absorption peak starts at 300 nm with an absorbance of around 0.05, slowly rising to 0.16 at 250 nm. Additionally, there was a low-intensity peak around 275 nm and a high-intensity peak from 230 to 200 nm, as reported in the literature.[42]

### *Animals*

Guinea pigs (weighing 250–350 g) and Swiss albino mice (weighing 17–22 g) from the Pasteur Institute of India, Coonoor, were used and maintained on a normal diet throughout the experiment.

### *Methods*

*In vivo* studies: Evaluation of TT polymer microspheres was performed using a potency test to estimate the immunizing capacity of the vaccine. There are specific potency requirements that must be met for the vaccine to be accepted as suitable for immunization purposes. Two methods prescribed in the Indian Pharmacopoeia were used.[43]

Challenge method

Toxin neutralization method

#### Determination of potency of the TT

Polymer microspheres by toxin neutralization method

In this method, the potency of the tetanus vaccine was determined by assessing the vaccine's efficacy in stimulating the production of tetanus antitoxin in guinea pigs. The sera of the guinea pigs were examined for antitoxin by comparing their ability to protect mice from the paralytic effects of a fixed dose of tetanus toxin (LP1200 dose) with that of the standard preparation of tetanus antitoxin, which provides the same level of protection.

Test toxin

Tetanus toxin (Batch No. 40/95), supplied by the Pasteur Institute of India, Coonoor, was used as the test toxin.

Standard preparation

Standard tetanus antitoxin - National Reference (Batch No. 64196 - CRI, Kasauli), obtained from the Pasteur Institute of India, Coonoor, was used as the standard preparation.

#### Determination of the Limes paralyticum/200 (LP/200) dose of the test toxin

The LP/200 dose is defined as the smallest quantity of toxin that, when mixed with 0.005 units of the standard antitoxin and injected into mice, causes tetanus paralysis within four days.

**Table 1. Tetanus toxoid loaded within polymethyl methacrylate**

| | | | | | |
|---|---|---|---|---|---|
| Polymer concentration (%) | 5 | 10 | 15 | 20 | 25 |
| microsphere Size (µm) | 4.263 | 5.299 | 8.127 | 9.205 | 13.454 |
| Quantity of TT added (mL) | 8 | 9 | 10 | 11 | 12 |
| Quantity of TT washed off (mg) | 17.71 | 16.72 | 17.98 | 15.96 | 11.60 |
| Theoretical amount of protein loaded (mg) | 27.09 | 28.08 | 26.82 | 28.84 | 33.20 |
| Total weight of yield (g) | 0.7947 | 1.7272 | 2.7395 | 3.8117 | 4.6384 |
| LF content of yield/g | 3,340 | 1,593 | 959 | 742 | 702 |
| 10 LF equivalent of the coated polymer (mg) | 2.99 | 6.27 | 10.43 | 13.48 | 14.25 |

LF content of TT used: 550/mL; protein content of TT (average of eight values): 5.60 mg/mL; LF equivalent of 1 mg of protein: 98 LF; quantity of TT added in terms of total protein: 44.80 mg. LF, flocculation value; TT, tetanus toxoid.

Standard antitoxin dilution

0.1 mL of the standard preparation (10 I.U./mL) was diluted (1/200) with 19.9 mL of normal saline so that each 0.5 mL of the diluted standard preparation contained 0.025 units of tetanus antitoxin.

Test toxin dilution

0.05 mL of test toxin was diluted (1/1,000) with 49.95 mL of peptone water so that each 1.0 mL of diluted test toxin contained 0.0010 mL of undiluted tetanus toxin.

Mixtures preparation

Mixtures were prepared by adding 0.5 mL of the diluted solution of the standard preparation into six graded volumes of diluted test toxin, corresponding to 0.4 mL, 0.5 mL, 0.6 mL, 0.7 mL, 0.8 mL, and 0.9 mL. Their volumes were then made up to 2.5 mL with peptone water so that 0.5 mL of each mixture contained 0.005 units of tetanus antitoxin. The mixtures were incubated for one and a half hours at room temperature, protected from light.

Inoculation

After the incubation period, 0.5 mL of each mixture was injected subcutaneously into six groups of mice. Mice weighing 17–22 g were used, with each group consisting of four animals. The injected animals were observed for four days.

**Inoculation experiment**

TT loaded within PMMA

In this experiment, none of the mixtures produced tetanus paralysis within four days. The test was repeated with increased test toxin volumes corresponding to 1.0 mL, 1.1 mL, 1.2 mL, 1.3 mL, 1.4 mL, and 1.5 mL to determine the correct endpoint. The results are shown in Table 1.

*Procedure for toxin neutralization test*

Animals

This study was carried out in accordance with the recommendations and was approved by JSS College of Pharmacy, Ooty, Tamil Nadu, India (Protocol Number: JSS_1996). Animals received humane care in accordance with guidelines and regulations from the Animal Welfare Act: https://www.nal.usda.gov/animal-health-and-welfare/animal-welfare-act] (1990).

Immunization of guinea pigs

Six groups, each consisting of ten guinea pigs weighing 250 g–350 g, were selected and quarantined with a regular diet. 2.99 mg, 6.27 mg, 10.43 mg, 13.48 mg and 14.20 mg of TT polymer microspheres corresponding to batch numbers 1, 2, 3, 4, and 5—i.e., 5%, 10%, 15%, 20%, and 25% *w/v* of PMMA, equivalent to 10 LF of TT (Table 2)—were each dispersed in 20 mL of an aqueous vehicle (0.5% sorbitol, 0.1% carboxy methyl cellulose, and 0.02% Tween 80) to achieve a final concentration of 0.5 LF/mL.

The control sample, sorbed TT (Batch No. 1A0760, 10 LF/mL), obtained from Pitc, was diluted 20-fold with normal saline to obtain 1/10 of a single human dose in 1 mL (0.5 LF/mL). 1.0 mL from each batch was injected subcutaneously into the six corresponding groups of guinea pigs. Four weeks after the first immunization, the same dose of booster was given to all the groups. Two weeks after the first immunization, blood was collected from all the guinea pigs via cardiac puncture. After the booster dose, blood

**Table 2. Determination of the LP/200 dose of the test toxin**

| S.No | Toxin dose (µL) | Antitoxin dose (TNR LU) | Mixtures | | | | Observations | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Diluted toxin (mL) | Diluted antitoxin (TNR) mL | Peptone water (mL) | Total volume (mL) | 1 day | 2 day | 3 day | 4 day |
| 1 | 0.08 | 0.005 | 0.4 | 0.5 | 1.6 | 2.5 | S S S S | S S S S | S S S S | S S S S |
| 2 | 0.1 | 0.005 | 0.5 | 0.5 | 1.5 | 2.5 | S S S S | S S S S | S S S S | S S S S |
| 3 | 0.12 | 0.005 | 0.6 | 0.5 | 1.4 | 2.5 | S S S S | S S S S | S S S S | S S S S |
| 4 | 0.14 | 0.005 | 0.7 | 0.5 | 1.3 | 2.5 | S S S S | S S S S | S S S S | S S S S |
| 5 | 0.16 | 0.005 | 0.8 | 0.5 | 1.2 | 2.5 | S S S S | S S S S | S S S S | S S S S |
| 6 | 0.18 | 0.005 | 0.9 | 0.5 | 1.1 | 2.5 | S S S S | S S S S | S S S S | S S S S |

S, survival; TNR, Tetanus antitoxin-national reference.

was collected at the end of every week for four weeks. All blood samples were incubated at 37°C for one hour and stored at 4°C in a refrigerator. The sera were separated by centrifugation at 7,000 rpm for 10 m. The upper portion was collected, labeled, and stored at −20°C before testing for neutralization.

Neutralization tests with guinea pigs' sera

The neutralization test was carried out on pooled sera two weeks after the first immunization and every week for four weeks after the administration of the booster dose. Pooled sera were subjected to the neutralization test. To obtain the endpoint of the international units per mL, the pooled sera were further diluted accordingly so that the endpoint value could be determined. Sera were diluted, and the volume was made up to 0.5 mL with normal saline, followed by the addition of 1.0 mL of peptone water.

Dilution of standard preparation (antitoxin 10 I.U/mL)

0.1 mL of standard antitoxin was diluted (1/200) with 19.9 mL of normal saline so that each 1.0 mL of diluted standard antitoxin contained 0.05 I.U.

Dilution of the test toxin

0.05 mL of test toxin was diluted with 41.55 mL of peptone water so that each 1.0 mL of diluted test toxin contained five times the LP/200 dose of test toxin.

**Preparation of control**

Control titrations were carried out alongside the neutralization test to check the validity of the test according to the I.P. ′85. From the diluted standard antitoxin solution, five different concentrations were prepared by adding correspondingly 0.3 mL, 0.4 mL, 0.5 mL, 0.6 mL, and 0.7 mL of diluted standard antitoxin, and the volume was made up to 1.5 mL with peptone water.

Preparation of neutralization mixtures

1.0 mL of diluted test toxin was added to 1.5 mL of each dilution of the six groups of test sera and five concentrations of the control preparation so that 0.5 mL of each mixture contained 0.00024 mL toxin (LP/200 dose). The mixtures were incubated for one and a half hours at room temperature, protected from light.

Inoculation

After the incubation period of the neutralization mixtures, 0.5 mL of each mixture was injected subcutaneously into mice from the corresponding groups and observed for four days. Each group consisted of four mice weighing 17 g–22 g. All tests were valid because the mice injected with mixtures containing 0.5 mL of the diluted standard antitoxin having 0.005 I.U. per dose developed paralysis. The mice injected with mixtures containing 0.3 mL and 0.4 mL of the diluted standard antitoxin developed paralysis earlier in the observation period, while the mice injected with mixtures containing 0.6 mL and 0.7 mL of the diluted standard antitoxin did not develop paralysis. The survival of the mice injected with mixtures made with 0.5 mL of undiluted serum, even on the fourth day after injection, indicates a concentration of antitoxin in the undiluted serum of not less than 0.05 I.U./mL. For all other dilutions of the sera, the dilution factor multiplied by 0.05 would give the concentration of antitoxin in undiluted serum in terms of international units.

Determination of potency of the TT polymer

Microspheres by challenge method: In this method, the potency of the tetanus vaccine was determined by comparing the dose of the vaccine required to protect mice from the lethal effect of a subcutaneous injection of tetanus toxin with the dose of the standard preparation needed to provide the same protection. For this comparison, the standard preparation of adsorbed tetanus vaccine and a suitable preparation of tetanus toxin for use as a challenge toxin were required.

Challenge toxin preparation of the tetanus toxin containing not less than 100 times the fifty percent lethal dose (LD50) per 1.0 mL was determined and used as the challenge toxin.

**Estimation of LD50 of the test tetanus toxin**

Procedure

*Dilution of the tetanus toxin*

Tetanus toxin (Batch No. 40/95) obtained from the Pasteur Institute of India, Coonoor, was used. Four dilutions of the toxin corresponding to 1/50,000, 1/100,000, 1/200,000, and 1/400,000 were prepared in peptone water. Each 0.5 mL of the diluted toxin contained 0.00001 mL, 0.000005 mL, 0.0000025 mL, and 0.00000125 mL of undiluted tetanus toxin, respectively.

*Test animals*

Four groups of six mice, each weighing 20–22 g, were grouped and quarantined with a regular diet.

*Inoculation*

0.5 mL of each diluted toxin was injected subcutaneously into the corresponding group of mice, which were then observed for five days. After five days, the LD50 was calculated using the method suggested by Reed and Muench (Reed *et al*., 1938). LD50 calculation was performed using the Reed & Muench Method.

P.D = 0.0750

$$\begin{aligned}\text{Fifty percent endpoint} &= \text{Antilog (log of dilution just above 50\% + proportional distance)}\\ &= \text{Antilog}\,(5.3+0.0750)\\ &= \text{Antilog}\,5.3750\\ &= 10^{5.3750}\\ &= 1/237137\end{aligned}$$

$$\begin{aligned}\text{Fifty percent endpoint} &= \text{Antilog (log of dose just above 50\} - \text{proportional distance)}\\ &= \text{Antilog (log of } 0.0000025 - 0.0750)\\ &= \text{Antilog}\,(-5.6020-0.0750)\\ &= \text{Antilog}\,(-5.6770)\\ &= 0.0000021\ \text{mL}\end{aligned}$$

i. 0.5 mL of the toxin, which has been diluted 237,137 times, is equal to one LD50, i.e., it would theoretically kill 50% of the animals in a group (mice). (or)
ii. 0.0000021 mL of toxin is equal to one LD50

**Procedure for the challenge test**

Test animals

Healthy mice with a body weight ranging from 20 g to 22 g were used. The animals were maintained on a normal diet throughout the experiment. Twenty-one groups were made with twenty animals in each group, and four groups were made with ten animals in each group. The males and females were distributed equally between the twenty-one groups.

Preparation of the challenge toxin solution

The challenge toxin solution was prepared from the test toxin

**Table 3. Potency test of tetanus toxoid polymer microspheres-challenge method (on mice) details of samples and immunization**

| | | |
|---|---|---|
| Weight of mice | 20–22 g | Microspheres, B. No. 1 (5% PMMA) |
| No. in each group | 20 | Microspheres, B. No. 2 (10% PMMA) |
| Dosage | 0.5 mL | Microspheres, B. No. 3 (15% PMMA) |
| Route | Subcutaneous | Microspheres, B. No. 4 (20% PMMA) |
| Adsorbed TT, PIIC B. No. 1A-0760 | | Microspheres, B. No. 5 (25% PMMA) |
| Reference TT vaccine-adsorbed, WHO | | |

PIIC, Pasteur Institute of India, Coonoor; PMMA, polymethyl methacrylate; TT, tetanus toxoid.

(Batch No. 40/95) obtained from PIIC, where one LD50 was 0.0000021 mL. The test toxin was diluted with peptone water, i.e., 1 in 4761, so that each 1 mL of diluted toxin contained 0.00021 mL of toxin (100 times the 50% lethal dose). This diluted toxin was used as the challenge toxin solution.

Immunization of mice

14.95 mg, 31.35 mg, 52.15 mg, 67.40 mg, and 71.25 mg of TT polymer microspheres corresponding to batch numbers 1, 2, 3, 4, and 5 (i.e., 5%, 10%, 15%, 20%, and 25% w/v of PMMA) were equivalent to 50 LF of TT (Table 2). These were each dispersed in 5 mL of 10 LF/mL solution. For standard controls, adsorbed TTs obtained from PIIC and WHO were used.

From the solution of each polymer microsphere sample and control preparation (PIIC), three dilutions were made, i.e., 1/40, 1/100, and 1/250. From the WHO standard (80 I.U./mL), three dilutions were made, i.e., 1/60, 1/150, and 1/375. Afterward, 0.5 mL of each dilution from the test samples and standard (WHO) was injected subcutaneously into the corresponding groups of mice.

### *Challenge*

Twenty-eight days after the immunization, 0.5 mL of the challenge toxin preparation (containing fifty times the 50% lethal dose) was injected subcutaneously into the mice of the twenty-one groups of twenty animals each. For control, three dilutions were made from the challenge toxin preparation, i.e., 1/25, 1/50, and 1/100, as well as undiluted challenge toxin. 0.5 mL of each dilution of the control preparations was injected subcutaneously into mice of four groups consisting of ten animals each. All the injected mice were observed for five days.

The number of protected mice was counted five days later, and the potency of the vaccine being examined was calculated relative to the potency of the standard preparation based on the number of animals that survived in each of the twenty-one groups. Standard statistical methods were used. In this experiment, all the animals died on the second day, rendering the entire experiment invalid. This might have been due to the lower doses of toxoid samples chosen for immunization.

## Results

### *PMMA microsphere preparation*

TT-loaded PMMA microspheres were prepared with different concentrations of polymer, specifically 5%, 10%, 15%, 20%, and 25% w/v, and were subjected to various *in vivo* evaluation tests.

- Determination of the potency of different formulations of TT-loaded polymer microspheres by toxin neutralization method according to the protocol described in "Materials and methods".
- For the estimation of potency by the toxin neutralization method, the LP/200 dose of the tetanus test toxin was first determined using the procedure described in "Materials and methods". The calculated LP/200 dose, i.e., 0.00024 mL of test toxin, was used for the toxin neutralization test. Various concentrations of test toxin dose per mouse, ranging from 0.00008 mL to 0.00018 mL, were initially tested for the estimation of the LP/200 dose of tetanus toxin. The results shown in Tables 2 and 3 indicate that the concentrations used failed to produce tetanus paralysis within four days.Therefore, a higher toxin dose per mouse, ranging from 0.00020 mL to 0.00030 mL, was selected and injected into the mice. Within four days, the concentrations from 0.00024 mL to 0.00030 mL produced significant tetanic paralysis. The results are shown in Table 3. Based on this observation, 0.00024 mL of test toxin per mouse was selected as the LP/200, which was the smallest quantity of the toxin that, when mixed with 0.005 units of the standard antitoxin preparation, caused tetanus paralysis within four days.

### *Determination of potency of TT-loaded polymer microspheres*

2.99 mg, 6.27 mg, 10.43 mg, 13.48 mg, and 14.20 mg of TT polymer microspheres corresponding to batch numbers 1, 2, 3, 4, and 5 (i.e., 5%, 10%, 15%, 20%, and 25% PMMA) were equivalent to 10 LF of TT (Table 4). These were each dispersed in 20 mL of aqueous vehicle and used for the immunization of guinea pigs, and the potency was calculated as per the described procedure. Two weeks after the first immunization, all the animals were bled, and the sera were separated by centrifugation. Undiluted serum and diluted sera (1/2 and 1/4) were tested for neutralization capacity. All five batches of microspheres containing TT produced an antitoxin level below the I.P. standard of 0.05 I.U. per mL. Our control group, i.e., adsorbed TT (PIIC), showed an antitoxin level of 0.1 > 0.05 I.U. per mL.

During all our sera titration tests, the control titrations were carried out in parallel to confirm the validity of our neutralization test (Table 5).

*In vivo* release data revealed that there was a 98.8% release with 25% PMMA loading, whereas in all lower PMMA loads, a significantly lower release was observed (Table 6).[44] One month after the first immunization, a booster dose was given to all groups of animals. After the booster dose, the animals were bled at the end of every week for four weeks. The pooled sera titrations after the first week of the booster dose were subjected to a neutralization test. The results are shown in Table 6. The undiluted serum and diluted sera (1/2 and 1/4) of the first four batches of microspheres (5%, 10%, 15%, and 20%) indicated the production of low-level antitoxin that was below 0.05 I.U./mL. The fifth batch, i.e., 25% polymer concentration of microspheres, showed a significant increase in the antitoxin level of more than 0.2 I.U./mL, which, when compared with the level of pooled sera prepared after the second

**Table 4. Determination of LD50 of the tetanus toxin**

| Toxin dilution | Observation of mice | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Observations | | | | | | | | | |
| | I day | | II day | | III day | | IV Day | | V Day | |
| 1/50,000 | S S T | T T T | T2 T2 T2 | T2 T2 T2 | D D D | D D D | | | | |
| 1/100,000 | S S S | S S S | T1 T1 T1 | T1 T1 T1 | D D T2 | T2 T2 T2 | D | D D D | | |
| 1/200,000 | S S S | S S S | S S S | T T T | T T T1 | T1 T1 T1 | T1 T1 T2 | T3 T3 T3 | T1 T1 D | D D D |
| 1/400,000 | S S S | S S S | S S S | S S S | S T T | T T T | T T T1 | T2 T2 T2 | T2 T2 T2 | T2 T2 T3 |

D, death due to tetanus; S, survival; TNR, Tetanus antitoxin-national reference; T, beginning of tetanic paralysis; T1, tetanic paralysis on one limb; T2, tetanic paralysis of two limbs; T3, tetanic paralysis of three limbs.

**Table 5. Potency test of tetanus toxoid polymer microspheres-challenge method (on mice) immunization**

| Details of Samples | | Challenge | |
|---|---|---|---|
| 1 | Microspheres, B. No. 1 (5 % PMMA) | | |
| 2 | Microspheres, B. No. 2 (10 % PMMA) | volume | 0.5 ml |
| 3 | Microspheres, B. No. 3 (15 % PMMA) | route | subcutaneous |
| 4 | Microspheres, B. No. 4 (20 % PMMA) | toxin distribution | 1/476l in peptone water |
| 5 | Microspheres, B. No. 5 (25 % PMMA) | dose | 50 LD50's in 0.5 ml |
| 6 | Adsorbed TT, PIIC, B. No. 1A-0760 | reference used/unitage | WHO-TEXA 340 IU, diluted to 80 IU/ml |
| 7 | Reference TT vaccine-adsorbed, WHO | | |

Weight of mice: 20–22 g; No. in each group: 20; Dosage: 0.5 mL; Route: subcutaneous. PMMA, polymethyl methacrylate; TT, tetanus toxoid.

week of the first immunization, was around a four-fold increase. However, when the I.U. per mL of the 25% batch was compared with our control group (PIIC), it was significantly lower.

The pooled sera titrations after two weeks of the booster dose also produced similar results to the first week's sera. It was unexpected to find that the first four batches of TT-loaded polymer microspheres were inefficient in raising the first four batches of TT level, even though all contained the same 10 LF dose of active TT. This may be due to the size of the tetanus toxoid microspheres, which ranged from 4.26 µm to 9.20 µm, corresponding to the increasing polymer concentration of 5% to 20%.

The microparticles of lower size had more distribution at the injection site, and depot release, as in the case of adjuvant site vaccines, was not possible. Additionally, the polymer coating thickness was significantly less than that of the 25% polymer microspheres, where the size was around 13 µm, and the coating thickness was much greater, as evident from Plate Nos. 1, 2, 3, 4, 5, and 5A. Since the release from the microspheres can be much faster, and the concentration of the microspheres at the injection site was also lower than that of the 25% batch and control group, the activity of the antitoxin (plain TT) may have been lost within a short time.

All other batches of microspheres from our study did not show any significant antigenic TT activity after the second week of the first immunization. The *in vitro* release data is shown in Figure 1 [Release of plain TT (% Release and concentration of protein in µg/mL)] and Table 6. The maximum cumulative percentage release was observed on the fourth day for lower concentrations, i.e., between 5% and 15%, while the maximum percentage release was observed on the third day for higher polymer concentrations, including the 25% batch. After this, the release of TT gradually diminished within seven days. When these results were correlated with the *in vivo* findings, we observed that the antigenic activity of TT was lower than the minimum level after two weeks of the first immunization. This may be due to the poor level of antigenic

**Table 6. *In vivo* release of Tetanus in terms of protein from PMMA microspheres**

| S.No. | No. of batches | Release of Plain TT | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 day | 1 day | 2 day | 3 day | 4 day | 5 day | 6 day | 7 day | 8 day | 9 day | 10 day | cumulative release |
| 1 | 5% PMMA | 0 | 47.2 | 53.24 | 54.95 | 58.76 | 57.45 | 56.5 | 53.58 | 51.08 | 50.33 | 50.29 | 58.76 |
| 2 | 10% PMMA | 0 | 38.84 | 50.32 | 56.69 | 60.33 | 56.34 | 51.57 | 47.03 | 43.89 | 40.31 | 40.08 | 60.33 |
| 3 | 15% PMMA | 0 | 44.83 | 50.42 | 52.55 | 59.58 | 56.27 | 49.5 | 42.59 | 42.71 | 41.34 | 41.34 | 59.58 |
| 4 | 20% PMMA | 0 | 44.48 | 55.09 | 63.94 | 75.95 | 65.54 | 59.55 | 50.67 | 50.87 | 47.8 | 47.8 | 75.95 |
| 5 | 25% PMMA | 0 | 83.33 | 93.93 | 98.8 | 93.75 | 90.25 | 75.17 | 69.95 | 66.69 | 67.21 | 67.26 | 98.8 |

SD = 3.4, 95% CI [2.04, 9.77]. SEM image of different ratios of PMMA loading (units in µM) for 5 % PMMA, 15 % PMMA and 20 % PMMA are from M. Pharm dissertation, S. Mohan, JSS College of Pharmacy, Ooty.[44]

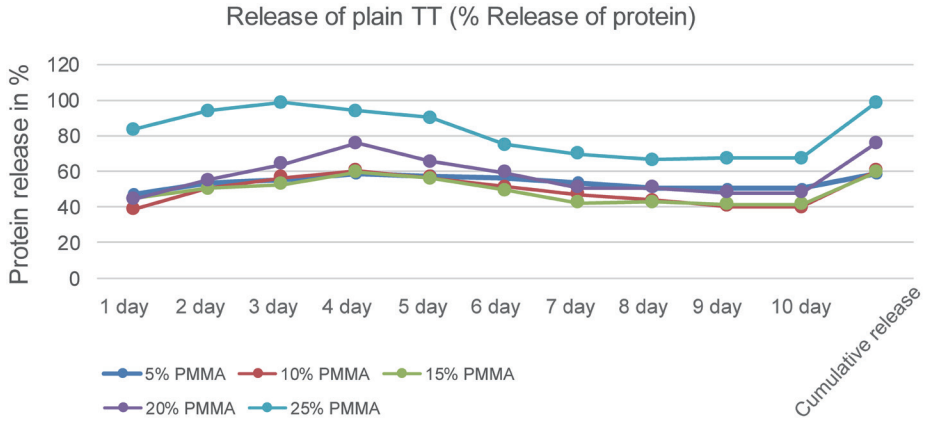


**Fig. 1. Release of plain TT (% Release of protein).** PMMA, polymethyl methacrylate; TT, tetanus toxoid.

TT in the system within seven days following immunization. An observation by the toxin neutralization test after the first week of the first immunization might have shown a better antitoxin level. This reasoning is supported by the increased antigenic TT activity observed after the booster dose, where measurements were taken weekly following the immunization.

The four batches with 5%, 10%, 15%, and 20% PMMA were excluded from antitoxin level estimation after the first week of the booster dose, as the antitoxin level was below 0.05 I.U./mL.

To calculate the endpoint of the antitoxin level produced after the booster dose of the sample toxoids [25% PMMA batch and adsorbed TT (PIIC)], the pooled sera from the first, second, and third weeks after the booster dose were further diluted. The fifth batch was diluted to 1/10, 1/20, 1/40, and 1/80, while the control batch was diluted to 1/80, 1/160, 1/240, and 1/320. These dilutions were titrated to calculate the antitoxin level.

The antitoxin levels indicated by these dilutions were less than 0.5 I.U./mL in the case of the 25% batch and more than 16.0 I.U./mL in the case of the control batch. To estimate the exact antitoxin level, intermediate concentrations of 1/4, 1/6, and 1/8 diluted pooled sera from the first and second weeks, and 1/2, 1/4, and 1/6 diluted pooled sera from the third and fourth weeks after the booster dose were titrated for batch No. 5. Similarly, 1/320, 1/360, and 1/400 diluted pooled sera from the first and second weeks, and 1/240, 1/280, and 1/320 diluted pooled sera from the third and fourth weeks after the booster dose were titrated for the control batch using a similar method. The results for the fifth batch and the control batch indicate that the antitoxin levels for pooled sera from the first, second, third, and fourth weeks after the booster immunization were approximately 0.35, 0.20, 0.15, and 0.10 I.U./mL, respectively, for the fifth batch. The antitoxin levels for pooled sera from the first, second, and third weeks after the booster immunization were around 18, 20, and 14 I.U./mL, respectively, for the control group. The antigenic activity of the 25% polymer microsphere sample batch met the I.P. requirements for the production of antitoxin levels in the range of 0.3 to 0.1 I.U./mL over the weeks following the booster dose.

## Discussion

The SEM images were compared for 5%, 10%, 15%, 20%, and 25% loading of PMMA, and the results indicate that the release of the vaccine from the microspheres is significantly better at higher PMMA percentages (Fig. 2). A similar study by Gupta *et al*. describes that larger microspheres can increase antitoxin levels more effectively than smaller microspheres.[45,46] This information, specifically the increase in microsphere size to more than 40 µm to 70 µm to produce sufficient antigenic T.T. activity, unfortunately became available to us only after the formulation stage of our work. The antigenic activity observed was significantly lower than that of the antitoxin level in the adsorbed TT control. Similar observations have been reported,[46] indicating that none of the controlled-release preparations induced higher levels of antitoxin production compared to aluminum-adsorbed TT. According to I.P. requirements, more than 50% of the animals in the first dilution group should be protected for five days after being challenged with the test toxin. However, the data from the protection against lethal effects, evaluated by observing the animals for five days after challenging, showed that all the animals in all groups died by the second day, likely because we used lower concentrations of the samples. It has been reported that PMMA enhances the efficacy of a DNA vaccine encoding TSA, thereby improving the immunogenicity of the antigen.[47] The SEM images of the microspheres comprising PMMA confirm the formation of uniformly sized microsphere particles, which is crucial for the controlled release of the vaccine. The primary objective was to develop a cost-effective polymer coating system using PMMA instead of PLA and PLGA polymers. Although PMMA has been used in vaccine delivery systems as early as 1976, a thorough investigation of the controlled-release characteristics of PMMA polymers was still necessary. The release characteristics of PMMA were found to be unsatisfactory and did not follow a zero-order release. Twenty-eight days after immunization, all groups were challenged with a toxin dose previously calculated using the Reed and Muench method, as described in "Materials and methods". It was observed that the release of TT was better with 25% PMMA loading, which may be attributed to the increased level of hydrophobic polymer, enabling a network of polymer-vaccine interactions that facilitate sustained vaccine release. Our findings are consistent with recent advances reported on PMMA polymers as carriers in the delivery of cancer vaccines, where they have shown the ability to enhance immune effects.[48,49] PMMA characteristics sufficiently satisfy the requirements for denture base polymers due to their biodegradability and biocompatibility.[50] *In vivo* studies using mannosylated micelle formulations have shown activation of dendritic cells with superior immunogenicity.[51] The role of methacrylic acid polymers in cancer growth processes underscores the utility of PMMA polymers as adjuvants.[52]

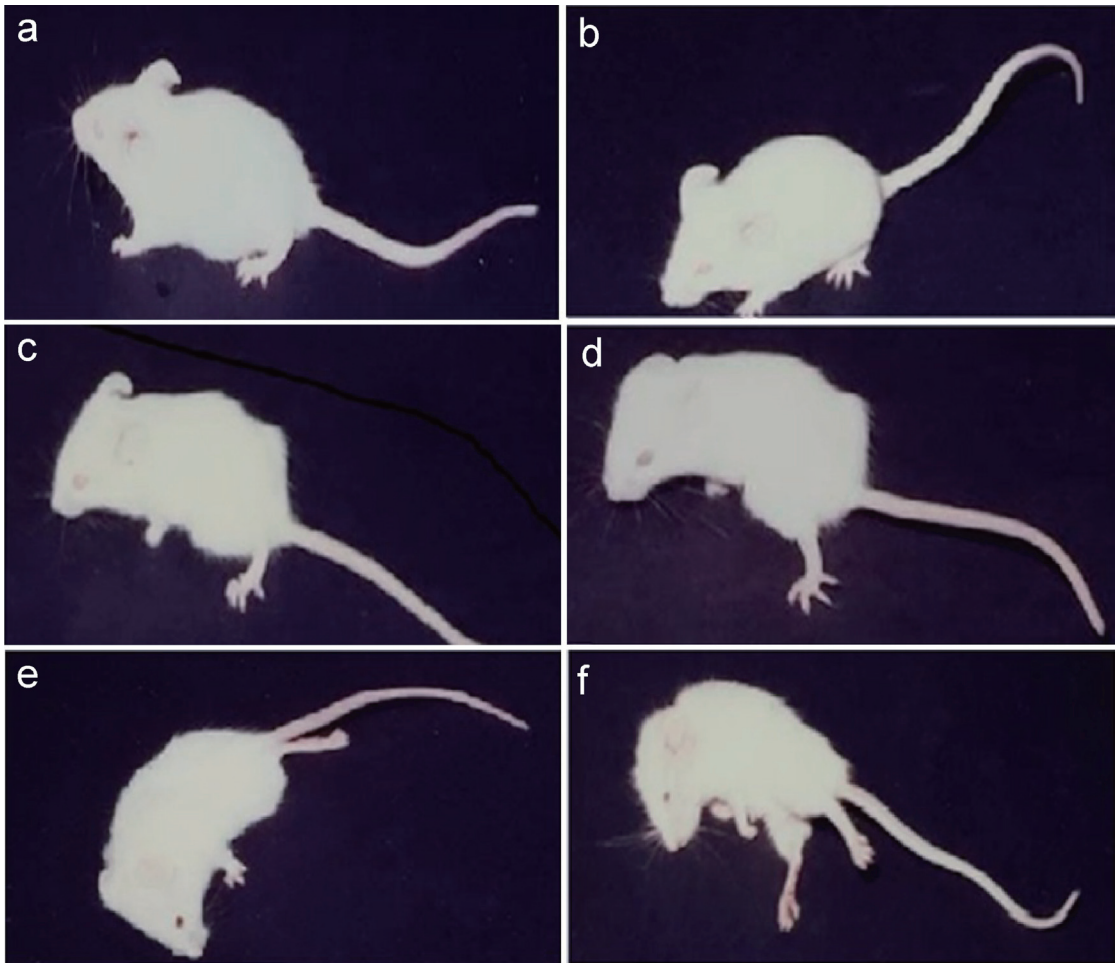


**Fig. 2. Mice treated with PMMA-loaded tetanus toxoid.** PMMA, polymethyl methacrylate. (a) Normal mice; (b) Beginning of paralysis; (c) Tetanic paralysis 1 limb; (d) Tetanic paralysis 2 limbs; (e) Tetanic paralysis 3 limbs; (f) Death due to tetanic paralysis.

## Future directions

The utility of biodegradable PMMA polymers as carriers for TT release and improved immunogenicity offers significant advantages. Microspheres in the µm range provide benefits over other adjuvants. Storage-stable vaccines that avoid loss of activity in extreme weather conditions and in remote areas with limited refrigeration facilities are a boon for vaccine formulation. Future exploration of PMMA polymers in the delivery of vaccines for COVID-19 or other diseases could be promising with this adjuvant.

## Conclusions

The potency of different formulations of PMMA polymer microspheres encapsulating TT demonstrated significant immune response and stability. An optimal polymer concentration of 25% w/v was found to meet I.P. requirements and showed the maximum particle size of 13 µm among those evaluated. Antitoxin levels increased gradually from 5% to 25% PMMA loading, with the faster release at smaller sizes—even with 25% loading—accounting for the lower concentration of microspheres at the site of action. The described method can be used to prepare microspheres with the desired diameters, ideally in the range of 40 µm to 70 µm. Evaluating the potency of the formulations described using this method in future work is expected to be rewarding.

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## Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

## Author contributions

Preparing the materials, collecting and analyzing the data (SM), writing the first draft of the manuscript (AN), contributing to the

study's conception and design, and commenting on previous versions of the manuscript (SM, AN). All authors read and approved the final manuscript.

## Ethical statement

This study was conducted in accordance with the recommendations and was approved by JSS College of Pharmacy, Ooty, Tamil Nadu, India (Protocol Number: JSS_1996). Animals received humane care following guidelines and regulations from the Animal Welfare Act: https://www.nal.usda.gov/animal-health-and-welfare/animal-welfare-act (1990).

## Data sharing statement

No data available for sharing.

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