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Special Issue: Polycarbonates and Green Chemistry

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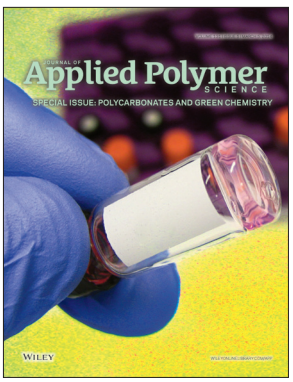

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Preparation and Properties of Polycarbonate Microspheres Containing Tetanus Toxoid Vaccine

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ABSTRACT: Polycarbonate microspheres TT-PC containing tetanus toxoid vaccine (TT) were prepared by the modified water-in-oil-in-water (W/O/W) solvent evaporation technique, using poly(trimethylene carbonate-*co*−5,5-dimethyl trimethylene carbonate) (P(TMC-*co*-DTC), or PC) as a polymeric carrier. TT release studies showed that the microspheres sustained a steady release rate of TT in the phosphate buffer saline solution (0.1 mol L^{-1}, pH 7.4) *in vitro* for 60 days. *In vivo* cytotoxicity assays demonstrated that the polycarbonate microspheres had low toxicity and high safety *in vivo* in guinea pigs. Immunity and inoculation tests indicated that the TT-PC microspheres acted as an immunological adjuvant *in vivo* in guinea pigs. In guinea pigs attacked with an active tetanus toxoid, an injection of the TT-PC microspheres with a single-dose and booster vaccination induced high levels of antitoxin efficiencies in the blood serum, showing good immunoprotection. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40048.

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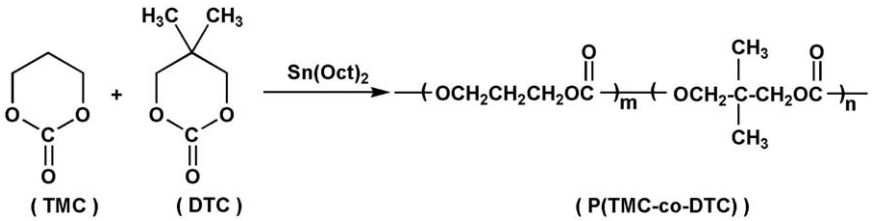
INTRODUCTION

Tetanus is a serious illness that causes convulsions (seizures) and severe muscle spasms, which can be strong enough to cause bone fractures of the spine, and can even result in death in 30–40% of cases. Tetanus toxoid (e.g., tetanus toxoid adsorbed) is a preferred vaccine for active tetanus immunization in wound management, and it is used to prevent tetanus (also known as lockjaw) by intramuscular use. Tetanus toxoid adsorbed is a sterile suspension of the alum-precipitated (aluminum potassium sulfate) toxoid in an isotonic sodium chloride solution containing a sodium phosphate buffer to control the pH. Booster injections may be required to protect (immunize) against a tetanus infection at the time of an injury. Immunosuppressive therapies, including radiation, corticosteroids, antimetabolites, alkylating agents, and cytotoxic drugs, are sometimes required to reduce the immune response to vaccines and other side effects.[1–4]

Microspheres and nanoparticles are being increasingly applied as treatment agents by exploiting their preferential accumulation in targeted structures for drug delivery or their response to external stimuli, magnetothermal therapy, laser activation, and so on. In designing microspheres and nanoparticles as delivery systems, the major goals are to control particle size, surface properties, and the release of pharmacologically active agents to achieve the site-specific action of the drug at the rational rate and dose. The polymeric microencapsulation and nanoencapsulation of biomacromolecular and water-insoluble drugs increase the drug efficacy, specificity, tolerability, water-solubility, stability, and therapeutic index of corresponding drugs.[5–14]

Polymeric microspheres based on biodegradable synthetic polymers, such as poly(lactide) (PLA) and poly(lactic-glycolic acid) (PLGA), have been designed as potential delivery systems of the tetanus toxoid vaccine against tetanus infection *in vivo*. These polymeric microspheres containing the vaccine were expected to improve the efficacy, reduce the toxicity and side effects, reduce the dosage and cost of tetanus toxoid vaccine, and simplify the inoculation process by replacing the booster vaccination with a single-dose vaccination. PLA and PLGA are both the biodegradable, thermoplastic, aliphatic polyesters. Because of good biocompatibility, good biodegradation, and being immunologically inert, PLA is used in many biomedical applications, including sutures, stents, dialysis media, tissue engineering matrices, and drug delivery devices. However, high crystallinity, bulk erosion properties, and high acidic conditions of the biodegradable residue of lactic acid are important obstacles that currently hinder medical applications of PLA *in vivo*. Copolymerization and



Scheme 1. The synthetic route of the carbonate copolymer PC.

chemical modification show tremendous promise for obtaining ideal properties of PLA copolymers for polymer scaffolds in the body. For example, PLA has been used as the hydrophobic block of amphiphilic synthetic block copolymers to form the vesicle membrane of drug delivery systems.[15–17]

Biodegradable aliphatic polycarbonates are attractive polymers for controlled drug release matrices due to their moderate biodegradability, low inherent toxicity, and weak inflammatory effect of the degraded products. Some homopolymers and copolymers of polycarbonates, such as poly(trimethylene carbonate) (PTMC) and poly(5,5-dimethyl trimethylene carbonate) (PDTC), have been widely used as biodegradable materials in drug delivery, surgical sutures, tissue engineering, and gene delivery because of their outstanding biocompatibility, good biodegradation of surface erosion, elasticity, low toxicity and good mechanical properties.[18–33] However, its high hydrophobicity, high crystallinity, slow biodegradation rate and lack of reactive chemical groups for modification have considerably limited its medical applications. Therefore, some amorphous polymers with flexible molecular chains and elasticity at room temperature, such as poly(ethylene glycol) (PEG), poly(D,L-lactic acid), poly (ε-caprolactone) (PCL) and aliphatic polycarbonates, are often selected to modify polycarbonate homopolymers in order to change their physical and chemical properties.[21,34–36]

The anionic polymerization of six-membered cyclic carbonates has been widely adopted to produce aliphatic polycarbonates (PCs) with Sn-based catalysts by a conventional heating reaction.[22] In this work, carbonate copolymers of poly(5,5-dimethyl trimethylene carbonate-*co*-trimethylene carbonate) P(TMC-*co*-DTC) were synthesized by the ring-opening bulk polymerization of 1,3-dioxan-2-one (trimethylene carbonate, or TMC) and 5,5-dimethyl-1,3-dioxan-2-one (5,5-dimethyl trimethylene carbonate, or DTC) (Scheme 1). Subsequently, P(TMC-*co*-DTC) was used to prepare the TT-PC microspheres containing the tetanus toxoid vaccine. *In vitro* and *in vivo* properties of microspheres were evaluated as potential tetanus toxoid vaccine systems for immunity and inoculation.

EXPERIMENTAL

Instruments and Reagents

The compounds were characterized using a Nicolet is10 Fourier transform-infrared (FT-IR) spectrophotometer (Thermo Fisher Scientific, Madison, WI), a UV–vis spectrophotometer (UV-2800 series, Unico, Shanghai, China), a Varian Mercury-VX300 NMR spectrometer (Varian, Palo Alto, CA), and an automatic contact angle meter (SL200A/B/D Series, Solon Tech, Shanghai, China). The molecular weight was measured by a gel permeation chromatography (GPC, Waters Corporation Milford, MA) (Waters 2965D separations module, Waters 2414 Refractive Index Detector, Shodex K802.5 & K805 with Shodex K-G Guard Column, Polystyrene Standard, DMF solvent, 1.0 mL min^{-1} flow rate, 323K column temperature, and 323K detector temperature). The glass transition temperature (T_g) of the copolymer was determined using a differential scanning calorimeter (DSC) (NETZSCH DSC 200 F3, Erich NETZSCH GmbH & Co. Holding KG, Gebrüder-Netzsch-Strasse, Selb, Germany). The SEM morphology of microspheres was studied using a scanning electron microscope (SEM, Hitachi-X650, Japan), and specimens were coated with gold in SEM coating equipment. The optical densities (OD_{570}) were measured using a DG-3022A ELISA-Reader (Hercules, CA). The male guinea pigs (6- to 8-weeks old, body weights: 350 ± 20 g) were provided by the Department of Pharmacy (Tongji Medical College, Huazhong University of Science and Technology, China) and raised according to the method described in the literature.[37] The tetanus toxoid (TT, 640 Lf mL^{-1}, 1455 Lf mg^{-1} PN) and aluminum-phosphate-adsorbed tetanus toxoid (TT-Al, 5 Lf mL^{-1}), were provided by the Chinese Academy of Preventive Medicine (Wuhan, China). The tetanus toxoid (TT, 20 mL, 640 Lf mL^{-1}) was sealed in a dialysis bag and concentrated using PEG (400) with the concentrated TT solution (3200 Lf mL^{-1}). The tetanus toxoid in the saline solution (TT-saline, 5 Lf mL^{-1}) was made by the dilution of TT with saline. A DG-3022A ELISA-Reader, a JEOL JSM-T300 Electron Microscope, a JY92-II Ultrasonic Homogenizer, a Pharmacia-Biotech Gene Quant DNA Calculator, and a MPIAS-500 Multimedia Color Histopathologic Image Pattern Analysis System (pixels 0.785 μm, metrical matrix 1.718E+05, color mode, Qing Ping, Wuhan, China) were also used. The ethical approval was obtained for the *in vivo* experiments in animals from the Department of Science and Technology of Hubei Province, China and the Animal Center of Tongji Medical College, Huazhong University of Science and Technology, China.

All chemicals and solvents were of analytical grade. Tin (II) 2-ethylhexanoate ($Sn(Oct)_2$) was purchased from Sigma–Aldrich (US). The growth medium was the RPMI-1640 media (10% fetal bovine serum (Gibco), 100 U mL^{-1} penicillium, 100 μg mL^{-1} streptomycin). DTC, TMC, and aliphatic carbonate copolymer PC were prepared according to the method described in the literature.[19–21] PC: ^1H NMR ($CDCl_3$, δ, ppm): 4.25 (s, $CH_2OC{=}O$), 3.95 (t, CH_2O), 2.04 (m, $C{-}CH_2{-}C$),1.0 (s, CH_3); IR (KBr, ν_{max}, cm^{-1}): 2982, 2877 (C—H), 1740 (C=O), 1465–1404 (CH_3) 1261 (C—O—C=O), 1111 (C—O); M_n: 7.615 × 10^4, M_w/M_n: 3.1, The repeat structure unit TMC/DTC in the copolymer (mol/mol) was 42.9/57.1; the T_g was 42.5°C; the

melting temperature (T_m) was 141°C; the water contact angle was 77°; the water absorption ratio was 0.67%.

Preparation of Microspheres

The PC (2.0 g) was dissolved in a 0.1% solution of Span-80 in dichloromethane (20 mL), and 2 mL of TT (3200 Lf mL^{-1}) and 5% solution of Span-80 in dichloromethane (5 mL) were added. The mixture solution was homogenized by sonication for 1 min (120 W) and then poured into a 2% solution of poly(vinyl alcohol) (PVA) in distilled water (100 mL). The mixture was vortexed for 3 min, stirred rapidly for a further 30 min, and then 270 mL of water was added dropwise under vigorous stirring at room temperature. Subsequently, the machine stirring was continued at room temperature in open air for a further 3 h to remove dichloromethane by evaporation. After centrifugation (10^5 rpm), the precipitate was collected, washed by distilled water, and lyophilized to produce a grey power of the microspheres containing TT (TT-PC). The empty P(TMC-*co*-DTC) microspheres (PC-Ms) were prepared by the same method. These microspheres were further given the cobalt-60 radiation sterilization. The encapsulation ratio and loading concentration of TT were tested.

Toxicology Study

The empty PC-Ms (35.7 mg) were suspended in 10 mL of PBS. Six guinea pigs (body weights: 350 ± 20 g) received an injection of 1 mL of this suspension solution via a subcutaneous injection at the abdomen. Each guinea pig was observed at time intervals of 7, 14, and 21 days after injection.

In Vitro TT Release Study

The microspheres TT-PC (5 mg) were suspended in 4 mL of phosphate buffer saline solution (PBS, 0.1 mol L^{-1}, pH 7.4). The mixture was slowly shaken in a thermostatically controlled water bath at 37°C. After centrifugation (8000 rpm, 5 min), the clear superstratum solution was taken. The concentration of TT was measured, and then the TT release content was calculated.

Immunity and Inoculation Tests

The microspheres TT-PC (35.7 mg) were suspended in 10 mL of PBS to make the solution of TT-PC (5 Lf mL^{-1}).

Single-Dose Vaccination. Eighteen guinea pigs (body weights: 350 ± 20 g) were divided into three groups, and each guinea pig was injected with 1 mL of TT-PC, TT-Al, or TT-saline solutions (5 Lf mL^{-1}), respectively, via a subcutaneous injection at the abdomen. Each guinea pig was anesthetized with Ketamine (30 mg kg^{-1}) injected by intramuscular injection and then 4-hydroxy-butyric acid (100 mg kg^{-1}) injected via the auricular vein, positioned supine, and fixed to a polystyrene cradle with adhesive tape to minimize respiratory motion. The fur on the obviously beating heart position was sheared, and the position was sterilized. A long, thin syringe needle was inserted directly into the heart at the lower right quarter in the chest wall. The blood of the heart was taken at time intervals of 14, 28, 42, 56, 70, and 84 days after injection. The blood serum was separated, and then the concentration of TT was measured. After 90 days, each guinea pig was infected with the same active tetanus, and the antitoxin efficiency was evaluated by the toxin neutralization tests.[38,39]

The toxin neutralization test was performed at L+/100 and L+/1000 levels by methods described in the literature.[38,39] The L+/100 and L+/1000 dose of tetanus toxin, which are the minimum amounts of tetanus toxin, when mixed respectively with 0.01 and 0.001 antitoxin unit (AU) of the standard tetanus antitoxin, kills 100% of guinea pigs in 4 days. The tetanus toxin was diluted to L+/100 or L+/1000 doses per mL. Various dilutions of standard tetanus antitoxin and blood serum samples were mixed with L+/100 or L+/1000 doses of toxin. The volume was made up to 1 mL with normal saline. The toxin–antitoxin or toxin–serum mixtures were incubated at room temperature for 1 h. Each mixture was assayed by injecting 0.5 mL subcutaneously into four guinea pigs. Each guinea pig was observed for 4 days for tetanic symptoms and deaths. The antitoxin efficiency of blood serum samples were calculated against the standards in terms of AU/mL.

Booster Vaccination. Eighteen guinea pigs (body weights: 350 ± 20 g) were divided into three groups, and each guinea pig was injected with 1 mL of TT-PC, TT-Al, or TT-saline solutions (5 Lf mL^{-1}), respectively, via subcutaneous injection at the abdomen. Each guinea pig received the same injection with 1 mL of TT-PC, TT-Al, or TT-saline solutions (5 Lf mL^{-1}) again after 7 days, respectively. The blood of the heart was taken at the different time intervals of 14, 28, 42, 56, 70, and 84 days after injection using the described method. The blood serum was separated, and then the concentration of TT was measured. After 90 days, each guinea pig was infected with the same active tetanus, and the antitoxin efficiency was evaluated by toxin neutralization tests.[38,39]

RESULTS AND DISCUSSION

Preparation and Characterization

Over past decades, passive and active targeted-drug delivery has been used to preferentially accumulate the drug at the site of interest and avoid nonspecific distribution. Polymeric drug delivery systems, such as nanoparticles, microspheres, and liposomes, can accumulate in the vicinity of the tumor mass. The polymeric nanoparticles and microspheres are usually prepared by the solvent evaporation technique, dialysis method, high-voltage electrostatic field-assisted atomization, and so forth.

Some magnetic materials, such as magnetite and Fe_3O_4 magnetic ultrafine powders, have been incorporated into microspheres to produce magnetic particles, which may act as combined targeted-drug delivery systems for specific cancer therapies. The delivery of the drug carrier magnetic polymeric microspheres may be described as passive when they rely on tumor-specific uptake and then metabolism or slow diffusion for the drug release mechanism. However, the delivery can be more active through the use of a magnet field applied outside the organ, which is used to drive the accumulation of drug-carrying magnetic microspheres into localized tumors.

The water-in-oil-in-water (W/O/W) solvent evaporation technique was adopted to produce anticancer magnetic microspheres containing tumor necrosis factor-α (TNF-α) genes and Fe_3O_4 magnetic ultrafine powder by using amphiphilic polycarbonate copolymers that include methoxy-terminated

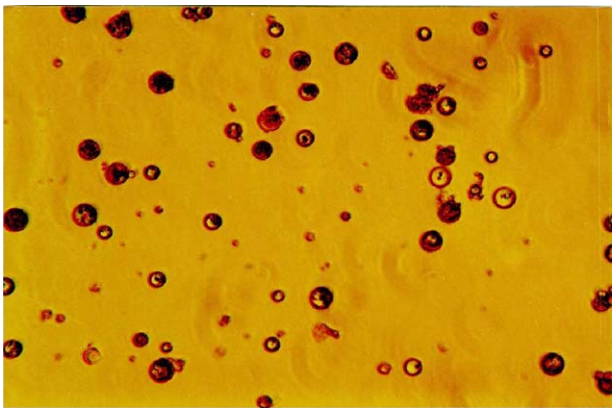


Figure 1. Micrograph of the TT-PC microspheres containing tetanus toxoid vaccine. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

poly(ethylene glycol)-*co*-poly(5,5-dimethyl trimethylene carbonate) and poly(ethylene glycol)-*co*-poly(trimethylene carbonate) as polymer carriers. These microspheres have a high inhibition and anti-tumor action to the human hepatocellular carcinoma (Bel-7204) cells *in vitro* and *in vivo*. Magnetic polymer microspheres generated from the PC and containing Fe_3O_4 magnetic ultrafine powders and an anticancer drug, mitomycin C (MMC), were prepared by a solvent evaporation technique. These microspheres provided sustained drug release and showed the steady release rates of MMC, strong magnetic responsiveness, high tumor inhibition, and high antitumor activity against Bel-7204 cells *in vitro* and *in vivo*.[40]

In this work, PC was synthesized by the polymerization of TMC and DTC using $Sn(Oct)_2$ as a catalyst by a conventional heating reaction. The ^1H NMR spectra showed the characteristic peaks (2.04 ppm) of the $-CH_2CH_2CH_2-$ group of the repeat unit TMC in the copolymer and the characteristic peaks (1.0 ppm) of the $-CH_3$ group repeat unit DTC in the copolymer. The data of ^{13}C NMR, IR, and DSC also showed evidence of the formation of PC.[23]

Subsequently, PC was chosen for use as an ideal polymer carrier because of its good biocompatibility, good biodegradation, surface erosion, and low toxicity of biodegradable residue as carbon dioxide. The TT-PC microspheres containing tetanus were prepared by the modified W/O/W solvent evaporation technique. These polymeric microspheres containing the vaccine were expected to improve the efficacy, reduce the toxicity and side effects, reduce the dosage and cost of tetanus toxoid vaccine, and simplify the inoculation process by replacing the booster vaccination with a single-dose vaccination.

The resultant homogenous, small TT-PC microspheres possessed high water-affinity, good dispersal, and mobility in PBS, in a 0.9% sodium chloride solution suiting the experimental purpose. Figure 1 shows the morphologies of the TT-PC microspheres. The average diameter of the TT-PC microspheres was 5.94 μm (ranging from 3.1 to 10.8 μm), and the average spherical surface acreage was 27.75 μm^2. In the microspheres, the encapsulation ratio and loading concentration of TT were 43.7% and 1.40 Lf mg^{-1}, respectively.

Toxicology Study

Six guinea pigs were alive and without symptoms of sickness at the time intervals of 7, 14, and 21 days after an injection of empty PC-M, and they gained more body weight than those guinea pigs that did not receive an injection. No swelling denaturation or necrosis existed at the local injection site, where the tissue microstructures appeared normal in photographs of tissue sections at the time intervals after injection. These results demonstrated that PC and polycarbonate microspheres possessed low toxicity and high safety *in vivo* in guinea pigs.

In Vitro TT Release Study

The overall process of the TT release from polymeric microspheres was mostly controlled by TT diffusion, dissolution, and polymeric degradation. Figure 2 shows the TT release profile of TT-PC microspheres in PBS. A substantial release rate from the TT-PC microspheres was sustained over 60 days of measurement. The microspheres had no obvious phenomenon of an abrupt release. The initial TT release was fast, but trended toward gentle release as time increased. The cumulative percent releases of TT-PC microspheres reached 30, 60, and 90%, respectively, after 3, 10, and 60 days of releases in PBS. The TT-PC microspheres released the TT slowly, presumably because of the low degradation rate, low water absorption rate, and high hydrophobicity of PC and the low diffusion coefficient of TT in TT-PC microspheres.

Immunity and Inoculation Tests

Tables I and II list the antitoxin efficiencies in the blood serum of guinea pigs after receiving an injection of different solutions of the tetanus toxoid vaccine. Table I shows the average data of three experiments per data point and lists the error bar. The antitoxin efficiencies in the blood serum of guinea pigs, after receiving an injection of TT-saline solutions (5.0 Lf) with a single-dose vaccination and a booster vaccination, showed a very low level. The antitoxin efficiencies, after an injection of aluminum-phosphate-adsorbed tetanus toxoid (TT-Al) solutions (5.0 Lf), showed high levels (>0.01 IU mL^{-1}), and TT-Al acted as an immunological adjuvant. The antitoxin efficiencies had

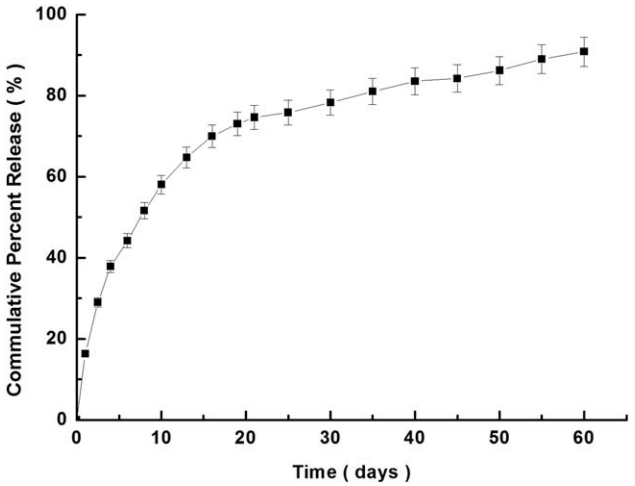


Figure 2. *In vitro* TT release profiles from the TT-PC microspheres containing tetanus toxoid vaccine. Each average data of three experiments per data point is calculated, and the error bar is listed.

Table I. Antitoxin Efficiencies (IU mL^{-1}) in the Blood Serum of Guinea-Pigs After Receiving Injection of Different Tetanus Toxoid Solutions

Tetanus toxoid vaccine	Status of TT	Time after receiving injection (days)[a]					
		14	28	42	56	70	84
TT-PC[b]	encapsulation	1.4 ± 0.056	1.4 ± 0.054	1.8 ± 0.072	1.4 ± 0.058	1.2 ± 0.048	1.2 ± 0.046
TT-PC[c]	encapsulation	1.6 ± .056	1.8 ± 0.073	2.1 ± 0.084	2.4 ± 0.096	2.0 ± 0.080	1.9 ± 0.076
TT-Al[b]	absorption	1.2 ± 0.050	1.2 ± 0.048	1.0 ± 0.040	0.8 ± 0.032	0.7 ± 0.028	0.6 ± 0.025
TT-Al[c]	absorption	1.5 ± 0.061	1.6 ± 0.064	1.9 ± 0.077	1.8 ± 0.075	1.8 ± 0.073	1.7 ± 0.069
TT-saline	dissolution	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[a] Each average data of three experiments per data point was calculated and the error bar was listed.
[b] Single dose vaccination.
[c] Booster vaccination.

peak values during the period of 14–28 days after receiving an injection of TT-Al solutions, and subsequently decreased during the period of 28–84 days after injection.

The antitoxin efficiencies, after an injection of TT-PC microspheres containing tetanus toxoid solutions (5.0 Lf), indicated a higher level than those after an injection of the TT-Al and TT-saline solutions (Table I). After injection of the TT-PC microsphere solutions (5.0 Lf), antitoxin efficiencies reached the peak value of 1.8 IU mL^{-1} with the single-dose vaccination at 42 days, and the peak value of 2.4 IU mL^{-1} with the booster vaccination at 56 days. The antitoxin efficiencies in these guinea pigs retained a high value of 1.2 IU mL^{-1} with a single-dose vaccination, and a high value of 1.9 IU mL^{-1} with a booster vaccination at 84 days after injection.

After tetanus infection, five guinea pigs receiving an injection of the TT-PC microsphere solutions with a single-dose vaccination survived, and all six guinea pigs receiving an injection of the TT-PC microsphere solutions with a booster vaccination survived, indicating the good immunoprotection of TT-PC microspheres. Meanwhile, half of guinea pigs receiving an injection of the TT-Al solution died with a single-dose vaccination, while all guinea pigs receiving an injection of the TT-Al solution with a booster vaccination survived. These results demonstrated that the TT-PC microspheres possessed better immunoprotection than that of TT-Al. However, six guinea pigs receiving an injection of TT-saline solutions with a single-dose vaccination and with a booster vaccination all died, showing no immunoprotection (Table II). These results indicated that the TT-PC microspheres acted as a good immunological adjuvant.

Table II. Survival Rates of Guinea Pigs Receiving an Injection of Different Tetanus Toxoid Solutions Attacked With the Same Active Tetanus

Tetanus toxoid vaccine	TT Dosage (Lf)	Number of guinea pigs (end/start)	
		Single dose vaccination	Booster vaccination
TT-Saline	5.0	0/6	0/6
TT-Al	5.0	3/6	6/6
TT-PC	5.0	5/6	6/6

CONCLUSIONS

PC was synthesized by the polymerization of TMC and DTC using $Sn(Oct)_2$ as a catalyst. The modified W/O/W solvent evaporation technique was adopted to produce TT-PC microspheres containing the tetanus toxoid vaccine. The experimental data showed that TT-PC microspheres sustained a steady release rate of TT in PBS *in vitro* for 60 days. The TT-PC microspheres demonstrated low toxicity and high safety, and acted as a good immune adjuvant *in vivo* in guinea pigs. After attacked with the live tetanus toxoid, the guinea pigs injected with TT-PC microspheres with single-dose and booster vaccinations showed high levels of antitoxin efficiencies in the blood serum. Therefore, TT-PC microspheres showed good immunoprotection and can be used as a potential tetanus toxoid vaccine system for immunity and inoculation.

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