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# Venom conjugated polylactide applied as biocompatible material for passive and active immunotherapy against scorpion envenomation

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ABSTRACT

Scorpion envenoming represents a public health issue in subtropical regions of the world. Treatment and prevention needs to promote antitoxin immunity. Preserving antigenic presentation while removing toxin effect remains a major challenge in toxin vaccine development. Among particulate adjuvant, particles prepared with poly (D,L-lactide) polymer are the most extensively investigated due to their excellent biocompatibility and biodegradability. The aim of this study is to develop surfactant-free PLA nanoparticles that safely deliver venom toxic fraction to enhance specific immune response. PLA nanoparticles are coated with AahG50 (AahG50/PLA) and BotG50 (BotG50/PLA): a toxic fraction purified from *Androctonus australis hector* and *Buthus occitanus tunetanus* venoms, respectively. Residual toxicities are evaluated following injections of PLA-containing high doses of AahG50 (or BotG50). Immunization trials are performed with the detoxified fraction administered alone without adjuvant. A comparative study of the effect of Freund is also included. The neutralizing capacity of sera is determined in naive mice. Six months later, immunized mice are challenged subcutaneously, with increased doses of AahG50.

Subcutaneous lethal dose 50 (LD50) of AahG50 and BotG50 is of 575 μg/kg and 1300 μg/kg respectively. By comparison, BotG50/PLA is totally innocuous while 50% of tested mice survive 2875 μg AahG50/kg. Alhydrogel and Freund are not able to detoxify such a high dose. Cross-antigenicity between particulate and soluble fraction is also, ensured. AahG50/PLA and BotG50/PLA induce high antibody levels in mice serum. The neutralizing capacity per mL of anti-venom was 258 μg/mL and 186 μg/mL calculated for anti-AahG50/PLA and anti-BotG50/PLA sera, respectively. Animals immunized with AahG50/PLA are protected against AahG50 injected dose of 3162 μg/kg as opposed all non-immunized mice died at this dose. We find that the detoxification approach based PLA nanoparticles, benefit the immunogenicity and protective efficacy of venom immunogen.



## 1. Introduction

Scorpion envenomation represents a public health issue [1]. Close to five million scorpion stings are recorded each year in the world (mostly in Africa, Asia and Latin America subtropical regions). The impact is the loss in human potential and economic productivity. *Androctonus australis hector* (Aah) and *Buthus occitanus tunetanus* (Bot) scorpions are the most prevalent in Tunisia, especially among children and elderly persons with 21,000 cases recorded annually (Direction des soins et santé de Base Report, Tunisia 2008) [2,3]. The toxicity of Aah and Bot venoms is due to toxins (~2% solid content) that bind with high affinity to the ionic channels of excitable cells causing severe cardiac failure and pulmonary edema. These molecules are low molecular weight and cationized monomer [4,5]. Previous study demonstrated their fast resorption, distribution and elimination through animal body [6]. Currently, serotherapy is the most common approach to protect populations from lethal outcome. However, it has limited efficiency due to the delay of its administration. Preventive treatment could be for interest to promote antitoxin

*Abbreviations:* Aah-G50 and Bot-G50, toxic fraction of *Androctonus australis hector* and *Buthus occitanus tunetanus* scorpion venoms; LD50, 50% lethal dose; *i.c.v.*, intracerebroventricular; *s.c.*, subcutaneous; PLA, polylactide.

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immunity. Following chemicals, radiation or heat temperature, detoxified toxins can be administered to mount toxin specific immune responses. Preserving toxin antigenicity while removing virulence remains a major challenge in vaccine development. Thus appropriate toxin/adjuvant formulation will influence the magnitude and the specificity of the immune response elicited against antigens. Among particulate adjuvant, microparticles and nanoparticles prepared with poly (D,L-lactide) polymer have been the most extensively investigated due to their excellent biocompatibility and biodegradability [7]. These particles have been used in the preparation of various vaccines including tetanus toxin (TT), diphtheria (DT) and HIV proteins vaccines [8–10]. Generally, nanoparticles having a comparable size to pathogens can be easily recognized and are consequently taken up efficiently by antigen presenting cells (APCs) for induction of immune response. The coating of antigens onto particles can allow presentation of the immunogen to the immune system in much the same way that it would be presented by native antigen, thereby provoking a similar response. In this study, nanoparticles based on poly (D,L-lactide) were formulated with toxic fraction from Aah and Bot scorpions venoms in order to safely vaccinate animals for producing sera with neutralizing activity and the immunoprotection outcome.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Antigens

AahG50 and BotG50 are toxic fractions that correspond to the major toxic peak after Sephadex G50 chromatography of crude venoms obtained from Aah and Bot scorpions, respectively [4].

#### 2.1.2. Adjuvants

Alhydrogel® (2%) and Freund (complete (FCA) and incomplete (FIA)) adjuvants were purchased respectively, from Superfos Biosector (Denmark) and Sigma-Aldrich co. (Germany).

#### 2.1.3. Mice

Female *Swiss* mice (18–22 g) were hosted in the Animal Unities Facility of Pasteur Institute of Tunis (I.P.T.). Experimental protocol (registry number: 2015/13/I/LR11IPT08) was approved by the Institutional Ethical Bio Medical Committee of I.P.T. (US registry number IRB00005445, FWA00010074).

#### 2.1.4. Chemicals and reagents

Bovine serum albumin (BSA), QuantiPro™ BCA Assay Kit, acetone, OPD Sigma Fast™ and Anti-Mouse IgG (A9917) were from Sigma-Aldrich Co. (Germany). Syringe 29Gx 0.5 (insulin) and Hamilton syringe (Model 1701 SN SYR, point style 4) were used.

### 2.2. Methods

#### 2.2.1. Preparation of poly (D,L-lactide) nanoparticles

Poly (D,L-lactide) polymer (Mw = 16,000 g/mol) was purchased from PURAC Biomaterials (The Netherlands). Poly (D,L-lactide) nanoparticles (PLA) were prepared by the nanoprecipitation method with modifications [11]. Briefly, 0.2 g of poly (D,L-lactide) polymer was dissolved in 10 mL of acetone then added drop wise to 30 mL of MilliQ water under moderate stirring. The solvent was removed by evaporation under reduced pressure. Nanoparticles size distribution was determined by quasi-elastic light scattering at 25 °C, *using a Zeta Sizer (Malvern 88 Instruments, UK) and a highly diluted colloidal sample (1 mM NaCl).* Zeta potentials were measured by laser Doppler with a Zeta Sizer (Malvern Instruments, UK).

#### 2.2.2. Formulation of AahG50/PLA and BotG50/PLA conjugates

Adsorption of AahG50 and BotG50 onto PLA was carried out separately. One volume of PLA particles at 1.2 mg/mL in water was mixed to one volume of fraction at various concentrations (0–100 μg/mL) in 10 mM NaCl solution for 15 min at room temperature (RT). After adsorption, samples were centrifuged at 2600 g for 20 min. Unadsorbed fraction in supernatant was quantified using QuantiPro™ BCA assay to calculate the adsorption yield as the weight ratio $100 \times (\text{input fraction} - \text{unadsorbed fraction})/(\text{input fraction})$. The AahG50/PLA (or BotG50/PLA) pellet was resuspended in 10 mM NaCl and washed one more time by centrifugation at 2600 g for 20 min. For in vivo experiments, Pellets were resuspended saline solution (0.15 M) prior to injections. Particle size and net surface charge were determined using a Zetasizer (Malvern Instruments, UK).

#### 2.2.3. In vitro release study

The AahG50/PLA (or BotG50/PLA) pellet was resuspended in phosphate buffer solution (PBS 150 mM, pH 7.4) then incubated at 37 °C under moderate stirring using 1 mL of medium. Samples were withdrawn at predetermined time intervals (12 h, 1, 2, 3, 7, 14, 21 and 30 days), centrifuged at 2600 g for 20 min. Released AahG50 (or BotG50) collected in supernatant was quantified using QuantiPro™ BCA assay. Pellets were resuspended in fresh buffer.

#### 2.2.4. Toxicity assessment of AahG50/PLA and BotG50/PLA

In vivo toxicities were evaluated for the following formulations: AahG50 (or BotG50) either adsorbed onto PLA nanoparticles (AahG50/PLA and BotG50/PLA), or mixed with Alhydrogel (AahG50 (or BotG50) + Alhydrogel) or emulsified in Freund adjuvants (AahG50 (or BotG50) + Freund). As control, the LD50 mean values of AahG50 and BotG50 were assessed either by the subcutaneous or intra-cerebro-ventricular routes. In fraction formulation, Doses equal to or higher than the LD50 were used prior injection to mice ($n = 10$/group). Survivals were recorded 24 h later.

#### 2.2.5. Immunization protocols

The first protocol (protocol 1) was performed based on BotG50 formulated with adjuvants. Mice ($n = 10$/group) were immunized by *s.c.* injections into the flank region on days 0, 7, 14, 28 and 35 with 200 μL of one of the following immunogens: BotG50 either adsorbed to PLA nanoparticles (BotG50/PLA) or mixed with Alhydrogel (BotG50 + Alhydrogel v/v) or Freund's (FCA for the first injection and FIA for the followings) (BotG50 + Freund) adjuvants. As negative control, bovine serum albumin adsorbed onto PLA nanoparticles (BSA/PLA) was injected. Immunizations were performed with 20 μg (day 0) and 40 μg (days 7, 14, 28 and 35) of BotG50 dose. At the end, each mouse has had a total dose equal to 180 μg.

The second protocol (protocol 2) was performed based on AahG50 formulated with adjuvants. Mice ($n = 10$/group) were immunized by *s.c.* injections into the flank region on days 0, 7, 14, 28 and 35 with 200 μL of one of the following immunogens: AahG50 either adsorbed to PLA nanoparticles (AahG50/PLA) or mixed with Alhydrogel (AahG50 + Alhydrogel v/v) or Freund's adjuvants (AahG50 + Freund). Another mice group was used to evaluate the co-adjuvant effect of Alhydrogel mixed to PLA-coated with AahG50 (AahG50/PLA + Alhydrogel (v/v)). Immunizations were performed with doses of 40 μg (days 0 and 7) and 80 μg (days 14, 28 and 35). Thus, a total amount injected per mouse was 320 μg after the last boost. Due to BotG50 and AahG50 toxicities, their use alone was excluded. Controls corresponded to adjuvant solutions given to mice groups.



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