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A biotechnological approach to immunotherapy: Antivenom against *Crotalus durissus cascavella* snake venom produced from biodegradable nanoparticles

Fiamma Gláucia-Silva [a], Manoela Torres-Rêgo [a], Karla Samara Rocha Soares [b], Igor Zumba Damasceno [c], Denise Vilarinho Tambourgi [d], Arnóbio Antônio da Silva-Júnior [a,*], Matheus de Freitas Fernandes-Pedrosa [a,b,*]

[a] Graduate Program on Pharmaceutical Sciences, Faculty of Pharmacy, Federal University of Rio Grande do Norte, Rua General Gustavo Cordeiro de Farias, S/N, Petrópolis, Natal 59012-570, Brazil
[b] Graduate Program on Biochemistry, Bioscience Center, University Campus, Federal University of Rio Grande do Norte, Avenida Senador Salgado Filho, 3000, Lagoa Nova, 59072-970 Natal, Brazil
[c] Department of Materials Engineering, Technology Center, University Campus, Federal University of Rio Grande do Norte, Avenida Senador Salgado Filho, 3000, Lagoa Nova, 59078-970 Natal, Brazil
[d] Laboratory of Immunochemistry, Instituto Butantan, Avenida Vital Brasil, 1500, Instituto Butantan, São Paulo 05503-000, Brazil

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ABSTRACT

Snakebite envenoming is a tropical disease neglected worldwide. In Brazil, the *Crotalus durissus cascavella* (CDC) snake belongs to a genus with venom of highest lethality. A search for new immunoadjuvants aimed to expand the therapeutic alternatives to improve vaccines and antivenom. This approach proposed to produce small and narrow-sized cationic CDC venom-loaded chitosan nanoparticles (CHNP) able to induce antibody response against the CDC venom. The ionic gelation method induced the formation of stable and slightly smooth spherical nanoparticles (<160 nm) with protein loading efficiency superior to 90%. The interactions between venom proteins and CHNP assessed using FT-IR spectroscopy corroborated with the *in vitro* release behavior of proteins from nanoparticles. Finally, the immunization animal model using BALB/c mice demonstrated the higher effectiveness of CDC venom-loaded CHNP compared to aluminum hydroxide, a conventional immunoadjuvant. Thus, CHNPs loaded with CDC venom exhibited a promising biotechnological approach to immunotherapy.

1. Introduction

Envenoming by snakebites is an occupational and environmental disease that affects mainly impoverished rural communities of tropical and subtropical countries in Africa, Asia, Oceania, and Latin America. Snakebite envenoming is an important but neglected tropical disease, with 1.8–2.7 million human victims of snake accidents worldwide each year and fatal cases ranging from 81,000 to 138,000 [1,2]. In Brazil, according to data acquired in the System of Information of Notification Aggravations (SINAN), snakebite envenoming has been increasing over the years, with approximately 30,000 snake accidents occurring in 2017 [3].

Although the *Bothrops* genus is responsible for approximately 90% of the accidents reported annually in Brazil, the highest lethality is caused by the *Crotalus* genus [4]. In Brazil, the *Crotalus* genus is represented by the unique species *Crotalus durissus*, which is subdivided into seven subspecies: *C. d. dryinas*, *C. d. terrificus*, *C. d. collilineatus*, *C. d. trigonicus*, *C. d. ruruima*, *C. d. marajoensis,* and *C. d. cascavella*, which are distributed throughout Brazil, with *C.d. cascavella* being prevalent in the Northeast region (Caatinga) [4–6].

Neurotoxic, myotoxic, nephrotoxic, and antithrombotic effects characterize crotalic envenoming. Neurotoxic effect induces unilateral or bilateral palpebral ptosis, facial muscle paresthesia (myasthenic face), blurred or double vision, and progressive respiratory muscle paralysis. Myotoxic effect implies systemic tissue injury of skeletal muscles resulting in diffuse muscular pain and rhabdomyolysis followed by myoglobinuria that causes acute renal failure, which is the most important systemic symptom because it is one of the main causes of victims' death [4–6]. In addition, antithrombotic effect occurs due to the activity of thrombin-like serine proteases, but when present, hemorrhagic manifestations are discrete [7–9].

Serotherapy, the most effective and scientifically-validated approach in the cases of snakebite envenoming, may have limited efficiency due to the delay of its administration, as well as the occurrence of problems

* Corresponding authors at: Laboratory of Technology and Pharmaceutical Biotechnology (Tecbiofar), Department of Pharmaceutical Sciences, Faculty of Pharmacy, Federal University of Rio Grande do Norte, Avenue General Gustavo Cordeiro de Farias, Petrópolis, Natal, RN, Brazil.
E-mail addresses: fiammaglaucia@gmail.com (F. Gláucia-Silva), manoelatorres@ufrn.edu.br (M. Torres-Rêgo), karllasamara@yahoo.com.br (K.S. Rocha Soares), igorzumba@ufrn.edu.br (I.Z. Damasceno), denise.tambourgi@butantan.gov.br (D.V. Tambourgi), arnobiosilva@gmail.com (A.A. Silva-Júnior), mffpedrosa@gmail.com (M.F. Fernandes-Pedrosa).

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associated with the traditional adjuvant [10]. The antivenom production requires hyper-immunizations of large animals, usually horses, using formulations (vaccine) containing adjuvant associated with the venom of one or more snake species, producing monovalent or polyvalent sera [11]. Adjuvants are substances that enhance the immunogenicity of the determined antigen. Aluminum salts, aluminum hydroxide, and phosphate are the only adjuvants approved for human use (FDA). However, some problems are reported due to these immunoadjuvants for vaccines and sera [12]. Recent studies reported problems such as pruritic subcutaneous nodules, hypersensitivity, and severe granulomatous inflammation, as well as an increase in the immunoglobulin E (IgE) titers, allergenicity, and potential neurotoxicity [11–16].

Currently, new technologies have been developed to search for new adjuvants able to enhance the efficacy of sera and vaccines, decreasing side effects related to traditional adjuvants. In this way, nanotechnology has been advancing and attracting scientists and engineers to investigate nanoparticles to improve vaccine technology, leading to the birth of "nanovacinology" [18].

Previous studies have shown polymeric nanocarriers to be a great alternative for protein controlled-delivery applied in the production of antivenom sera against snake venom and scorpion venom [17–19]. In addition, these nanocarriers have advantages such as the ability to stimulate the immune responses, protect the antigen from degradation, preserve toxin antigenicity, display controlled delivery, and require a small amount of antigen [20,21]. In this way, chitosan is a natural copolymer, sustainable, with excellent biocompatibility and biodegradability, along with properties of mucoadhesion and controlled drug delivery [22–25]. The purpose of this study was to evaluate the potential immunoadjuvant effect of biodegradable and biocompatible chitosan nanoparticles in the production of new and safe serum against *C. d. cascavella* venom.

2. Materials and methods

2.1. Preparation of cross-linked chitosan nanoparticles

The cross-linked chitosan nanoparticles (CHNP) were prepared by the ionic gelation method as previously described by Rocha-Soares et al. [26] with few adjustments. A 0.1% (w/v) tripolyphosphate (TPP, Sigma-Aldrich®, Saint Louis, Missouri, USA) aqueous solution was dripped in a 0.1% (w/v) chitosan (85% deacetylated, molecular weight: 90–190 kDa, Sigma-Aldrich®) in a 0.175% (w/v) acetic acid solution, under magnetic stirring of 300 rpm at 25 °C for 30 min. The self-assembling of compounds occurred spontaneously and an opalescent suspension was observed, which were characterized after 24 h [26].

2.2. Preparation of Crotalus durissus cascavella venom-loaded cross-linked chitosan nanoparticles

The *C. d. cascavella* venom (Cdc) (Butantan Institute, São Paulo, Brazil) was added in the cross-linked chitosan nanoparticles (opalescent suspension) under magnetic stirring of 300 rpm at 25 °C for 60 min. Distinct protein ratios of 0.5% (CHNP-CDC-0.5%) and 1.0% (w/w) (CHNP-CDC-1.0%) relative to the used chitosan concentration were tested in this experiment. After 24 h, the CDC venom-loaded CHNP were characterized.

2.3. Venom incorporation efficiency assay

The amount of incorporated proteins in the CHNP (CHNP-CDC-0.5% and CHNP-CDC-1.0%) was estimated by calculating the difference between the amount of total protein loaded into the nanoparticles and the amount of non-incorporated venom remaining in the supernatant after centrifugation at 20,000× *g* at 4 °C for 30 min. Samples were quantified by BCA Protein Assay Kit according to the manufacturer's recommendations (Thermo Fisher Scientific, Waltham, Massachusetts, United States of America). The calculations of incorporation efficiency (IE) were possible according to Eq. 1 and expressed in percentage [26–28].

$$\mathrm{IE}\ (\%) = \frac{(totalprotein - protein\ determined\ in\ the\ supernatant)}{total\ protein}\ x\ 100 \quad (1)$$

2.4. Physicochemical characterization

2.4.1. Particle size and zeta potential measurements

The zeta potential, particle size, and polydispersity index (PdI) of nanoparticles were determined using the cumulative method according to the intensity of the dynamic light scattering (DLS) in a particle size analyzer (Zeta Plus-Brookhaven Instruments, New York, USA). The analyses were realized at 25 °C at 659 nm wavelength with 90° detection angle. The Zeta Plus® Particle Sizing version 3.95 software was used for data analysis. All analyses were carried out in triplicate and data expressed as mean ± standard deviation.

2.4.2. Atomic force microscopy and scanning electron microscopy

The shape and surface aspect of nanoparticles were evaluated by Field Emission Gun Scanning Electronic Microscopy (FEGSEM, Zeiss Microscopy, Auriga, Jena, Germany) and Atomic Force Microscopy (AFM, SPM- 9700, Shimadzu, Tokyo, Japan) images. To obtain AFM images, one drop of nanoparticle dispersion was placed on a microscope slide and dried under desiccator for 24 h before observation. For SEM analysis, the samples were prepared by applying one drop of the nanoparticle dispersion on the carbon tape slide and kept in a desiccator for 24 h.

2.4.3. Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) analyses of venom-free and venom-loaded cross-linked chitosan nanoparticles were performed in a FT-IR spectrophotometer, Prestige 21, (Shimadzu, Tokyo, Japan). The samples were dried by speed vacuum concentrator, Centrivap Labconco (Kansas City, Missouri, USA), and then mixed uniformly with potassium bromide (KBr) in 1:200 (w/w) proportion and compressed by a hydraulic press.

2.4.4. Stability assay

CHNP, CHNP-CDC-0.5%, and CHNP-CDC-1.0% samples were stored at 4 ± 2 °C. The measurements of particle size and polydispersity index were performed once a week for eight weeks using the Zetasizer (ZetaPlus - Brookhaven Instruments Corporation, USA). All analyses were carried out in triplicate and data expressed as mean ± standard deviation. The measurements were performed using the parameters described in Section 2.4.1.

2.5. In vitro venom release assay

The venom release profile from the venom-loaded cross-linked nanoparticles was measured in 1 mL of buffered solution (pH = 7.4, KH_2PO_4 0.05 mol L^{-1}), in a thermostatic bath (model SL-150/22, Solab, Piracicaba, Brazil) at 37 °C ± 0.2 °C. At specific intervals, the samples were centrifuged at 16000× *g* for 30 min, and the venom protein content in the supernatant was determined using BCA Protein Assay Kit according to the manufacturer's recommendations (Thermo Fisher Scientific, Waltham, Massachusetts, United States of America). The cumulative percentage of released protein was plotted *versus* time. All analyses were carried out in triplicate and data expressed as mean ± standard deviation [29].

2.6. In vivo efficacy studies

2.6.1. Animals

Male and female BALB/c mice (25–35 g), 6–8 weeks of age, were maintained at a temperature of 22 ± 2 °C and at a 12/12 h light/dark