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Original Article

Green Synthesis of Silver Nanoparticles and their Application as Antigen Delivery System

Synthèse par Chimie Verte de Nanoparticules d'Argent et leur Utilisation comme Système de Délivrance d'Antigènes

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ABSTRACT

Introduction: Scorpion envenomation is a major health problem in tropical and subtropical regions, especially in North Africa where *Androctonus australis* is one of the most deadly species, with a mean of 115000 stung patients each year. New therapies are now studied to prevent the pathophysiology of scorpion envenomation to improve immunotherapy, so far the only specific treatment to treat envenomed patients. For this purpose, the aim of the study is to develop a silver nanovector adsorbing *Androctonus australis hector (Aah)* venom to be used as a nanoformulation stimulating the host immune system. **Materials and Methods:** Silver nanoparticles (AgNps) were synthesized by green synthesis, using a leave extract of *Eucalyptus globulus* as reducing agent. AgNps were characterized by Scanning Electron Microscopy for the size, X-Ray Diffractometry and FTIR for structural characterization. AgNps were then used as a vaccine formulation adsorbing *Aah* venom and tested in mice in a single injection protocol. Specific IgG titers, myeloperoxidase (MPO) and eosinophil peroxidase (EPO) activities have been evaluated during the immunization protocol. **Results:** Physic-chemical characterization of AgNps revealed a relatively spherical shape of Nps, not exceeding 200 nm.Evaluation of MPO and EPO activities showed a low pro-inflammatory profile of AgNps while the nanovaccine adsorbing *Aah* venom shown an increase in MPO activity, marker of neutrophil polynuclear infiltration. Evaluation of specific antivenom IgG titers revealed the activation of the humoral response resulting in an antibody titer of 1/16000, 15 days after AgNps-*Aah* injection.**Conclusion:** AgNps adsorbing *Aah* venom is an easy and promising nanovector for the antigen delivery and activation of immune cells after a single immunization.

KEYWORDS: Silver nanoparticles, Green synthesis, *Androctonus australis hector*, scorpion venom, immunogenicity.

RESUME

Introduction: L'envenimation scorpionique est un problème de santé publique dans les régions tropicales et subtropicales, surtout en Afrique du Nord où l'espèce la plus dangereuse, *Androctonus australis*, est responsable de presque 115'000 cas de piqures annuelles. A ce jour, de nouvelles thérapies sont étudiées pour améliorer la prise en charge et le traitement des patients envenimées. L'objectif de cette étude est de développer un nanovecteur d'argent t le venin d'*Androctonusaustralis hector* (*Aah*) à utiliser comme nanoformulation capable de stimuler le système immunitaire de l'hôte. **Matériels et Méthodes :** Les nanoparticules d'argent (AgNps) ont été

synthétisées par synthèse verte, en utilisant un extrait aqueux de feuilles d'*Eucalyptus globulus* comme agent réducteur. Les nanoparticules AgNps ont été caractérisées par microscopie électronique à balayage (MEB) pour évaluer leur taille et forme et par diffractométrie aux rayons-X et FTIR afin de procéder à une caractérisation structurale. Les nanoparticules AgNps ont ensuite été utiliséesdans une formulation vaccinale adsorbant le venin d'*Aah* et testées chez la souris dans un protocole vaccinal à injection unique. Les titres d'IgG spécifiques antivenin, les activités myelopéroxidase (MPO) et éosinophile peroxydase (EPO) ont été évalués durant le protocole vaccinale. **Résultats :** La caractérisation physico-chimique des nanoparticules synthétisées par chimie verte a permis de confirmer la formation de nanoparticules de taille inférieure à 200 nm et de nature d'argent. L'évaluation des activités MPO et EPO a permis de montrer un profil pro-inflammatoire faible concernant les animaux ayant recu les nanoparticules AgNps alors que le nanovaccin adsorbant le venin d'Aah a montré une relative augmentation de ces activités sériques, marqueur d'infiltration polynucléaire neutrophile et éosinophile. L'évaluation des titres d'IgG spécifique antivenin d'*Aah* a révélé une activation de la réponse immunitaire humorale aboutissant à un titre d'anticorps de 1/16000, 15 jours après l'injection d'AgNps-*Aah*. **Conclusion :** Les nanoparticules AgNps-*Aah*constituent un système de délivrance antigénique de synthèse facile capable d'activer le système immunitaire après une seule immunisation.

MOTS CLÉS : Nanoparticules d'argent, synthèse verte, *Androctonus australis hector*, venin, immunogénicité

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1. Introduction

Scorpion envenomation is a major public health problem observed in five continents [1]. Each year millions of scorpion stings occur with minor problems resulting inlocalized pain and minimal systemic involvement, however some patients can present severe envenomation leading to death [2]. Scorpions are spread especially in rural and peri-urban areas of tropical and subtropical regions of Maghreb, Middle East and Central/South America. Among the most deadly species of scorpions, *Androctonus australis hector (Aah), Androctonus amoreuxi (Aam) and Buthusoccitanustunetanus (Bot)* arefound in Algeria. These species are responsible for the majority of envenomations and lethal accidents in humans [3, 4]. Immunotherapy is the only specific treatment for envenomation [5]. Antivenoms are obtained by hyperimmunization of horses and sheeps with repeated venom injections. They can be monovalent, when animals are immunized against a single venom, or polyvalent against a mixture of venom of different species [5-7]. Several improvements of the actual immunotherapy have been done so far. Fab/F(ab')2 fragment mixture, experimental monoclonal antibodies, nanobodies and IgY antibodies have been developed and tested in animals, but not yet in humans for lack of safety studies [8-13]. For this purpose, preventive therapies using adjuvants and nanoparticles as antigen delivery systems for scorpion venom are undertaken in order to boost the immune system against scorpion envenomation. Formulations using MF-59 and Alum adjuvants with irradiated *Aah* venom have been tested in a vaccine protocol in rabbits, reach a protection of all injected animals up to 4 LD 50 of *Androctonus australis hector* venom, three months after the last injection [14]. A formulation of encapsulated irradiated *Aah* venom in calcium-alginate nanoparticles performed in rabbit and mice is able to induce an effective immunogenicity, a safety and immunoprotection of all injected animals of up-to 6 LD50 of *Androctonus australis hector* venom [15]. Recently, the neurotoxin *Aah II*have been encapsulated in chitosan-TPP nanoparticles, reducing its toxicity and eliciting a specific anti-toxin immunity in vaccinated mice [16]. In the present study, we aimed to develop a new nanocarrier based on silver nanoparticles adsorbing *Androctonus australishector* venom. These nanoparticles synthesized by an easy green synthesis are used in a one-shot immunization protocol, to study the preliminary activation of the host immune system.

2. Materials and methods

1. Venom and chemical products

Lyophilized *Androctonus australis hector* venom and chemical products were provided by the Laboratory of Cellular and Molecular Biology, Faculty of Biological Sciences, USTHB, Algiers, Algeria.

2. Animals

NMRI animals (20 +/- 2 g) were provided by the Faculty of Biological Sciences animal facility and housed in a controlled room temperature, with food and water *ad-libitum.* Experiments were carried out according to the Federation for Laboratory Animal Science Associations (FELASA) rules of the ethics for animal welfare.

3. Green synthesis of silver nanoparticles

Leaves of *Eucalyptus globulus*, previously dried at room temperature, were powdered using a mortar. The powder (50 g) was dissolved in 500 ml boiled deionized water for 2 hours, then filtered. Leave extract (6 ml) was then added to 100 ml of 0.01 mM aqueous $AgNO_3$ solution (EMD Millipore, Billerica, MA, USA), gently stirred and incubated at room temperature. The obtained solution was then centrifuged at 13'000*g* for 20 minutes and the AgNp pellets were washed three times with distilled water. AgNps were resuspended in ethanol 70% (EMD Millipore), dried at 75°C for 120 minutes and stocked for physic-chemical analysis.

4. Adsorption of AgNps with Aah venom

Adsorption of *Aah* venom on AgNps have been done in accordance to Asgary et al., 2016 [17]. Briefly, AgNps (0.3 mg) were added to 10 µl of *Aah* venom for each injection.

5. Scanning Electron Microscopy

The morphology of AgNps was studied by scanning electron microscopy (SEM) at Sonatrach Research and Development Center, Boumerdes, Algeria.Images were captured at 100000 x magnification.

6. X-Ray Diffractometry

X-ray diffractometry (XRD) measurement of AgNps was carried out using an X-ray diffractometer (Rigaku D/max 2500V) atSonatrach Research and Development Center,Boumerdes, Algeria.

7. Fourier-Transform Infrared Spectroscopy

Chemical interactions of silver nanoparticles were analyzed by an infrared spectrometer with Fourier transmission IR700. All spectra were recorded in the range 600-4000 cm^{-1}.

8. Experimental procedure

In this study, four groups of mice were used. The control group of animals was injected with saline solution (NaCI0.9%) by subcutaneous (s.c.) route. The second group of mice was injected with AgNps alone by s.c. route. The third group received an injection of AgNps-*Aah* and the fourth group was injected with a sublethal dose of *Aah* venom antigen alone. All mice received the injection at day 1 and were humanely sacrificed at day 7 and 15 after injection, for plasma recovery.

9. Evaluation of myeloperoxidase activity (MPO)

Myeloperoxidase activity was evaluated as a biomarker of inflammation. It was measuredaccording to the intensity of the oxidation color of theortho-diasidine product. The reaction used chromogenicsubstrate H_2O_2 with O-dianisidine diluted in aphosphate buffer (pH 5.8). The enzymatic activitywas evaluated at 460 nm.

10. Evaluation of eosinophil peroxidase activity (EPO)

The eosinophil peroxidase was released by activatedeosinophils after interaction with different mediators of the inflammatorycascade.It wasmeasuredusingamethodbasedontheoxidation of the substrate, the hydrogen peroxide by the extracellular EPOin media with a chromogen, O-Phenylenediamine (OPD)(Sigma Germany). The enzymatic activity was evaluated at 490 nm.

11. Evaluation of specific anti-Aah venom IgG titers by ELISA

ELISA was used to evaluate the specific IgG antibodies titer against *Aah* venom. A preparation of 5 µg/ml *Aah*venom in 0.1 M carbonate buffer pH 9.5 (100 µl) wasincubated in 96-well plates overnight at 4°C. Non-specific siteswere blocked with 100 µl of 0.1 M PBS, pH 7.4 containing 5% skimmilk for 1 h at 37°C and washed five times with PBS-Tween 20.Sera were diluted in PBS 0.1 M and added to the plates and thenincubated for 1 h at 37°C. After a washing step, anti-mouse IgG conjugated with peroxidase (Sigma) (diluted at 1/500) was added tothe plate and incubated for another hour at 37°C. After a last washing,wells were filled with 100 µl of ortho-Phenylenediamine dihydrochloride solution (10mg/ml in phosphate buffer, pH 7.4, $H_2O_2$0.03%). After blocking the reaction by adding 50 µl of H_2SO_4 (2 N),absorbance values was read at 490 nm.

12. *Statistical analysis*

All the data were analyzed withone-way ANOVA statistical test and *P*-value<0.05 wasconsidered a significant difference between groups.

3. Results

3.1 Morphological analysis of AgNps by SEM

The morphological analysis of silver nanoparticles by scanning electron microscopy shows a spherical shape and a size between 50 and 110 nm (**Figure 1**).

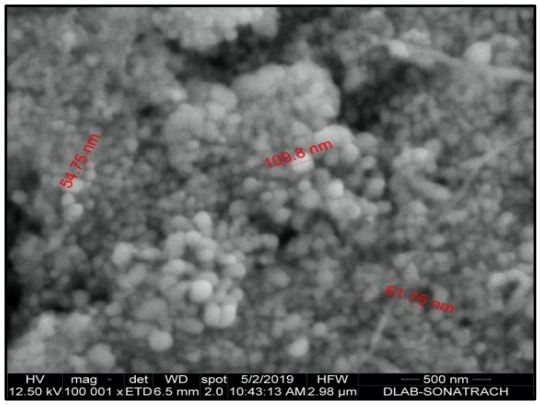

Figure 1: Scanning Electron microscopy of AgNps

3.2 XRD analysis of AgNps

X-ray diffraction patterns of AgNps synthesized from an extract of *E. globulus* leaves was observed (Figure 2). The obtained diffraction peaks are characteristic of silver nanoparticles and were found at 2θvalues corresponding to 20, 32.2, 46.1, 54.9, 57.5 and 67.5respectively attributed to the crystallographic planes 4000, 3100, 4500, 2900, 1500 and 1000. Other peaks have been observed corresponding to impurities, probably due to the residues of *Eucalyptus* extracts after green synthesis (**Figure 2**).

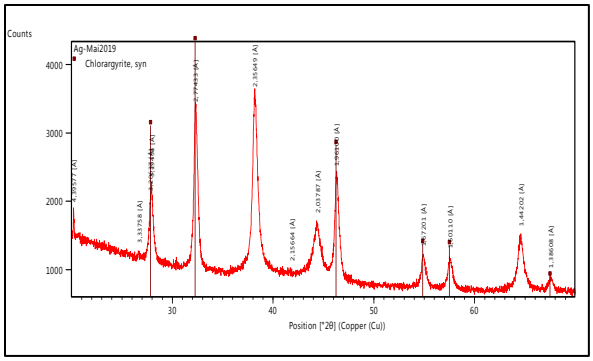

Figure 2: XRD analysis of AgNps

3.3 FTIR analysis of AgNps

FTIR spectroscopy was used to identify functional groups existing in silver nanoparticles, after green synthesis. Two characteristic peaks were observed. The first peak at 1640 cm^{-1} absorbance is the silver nanoparticles-OH bond bending, which gives them a spherical form. The second characteristic peak is found at 3462 cm^{-1} absorbance, attributed to the stretching of hydroxyl groups (**Figure 3**).

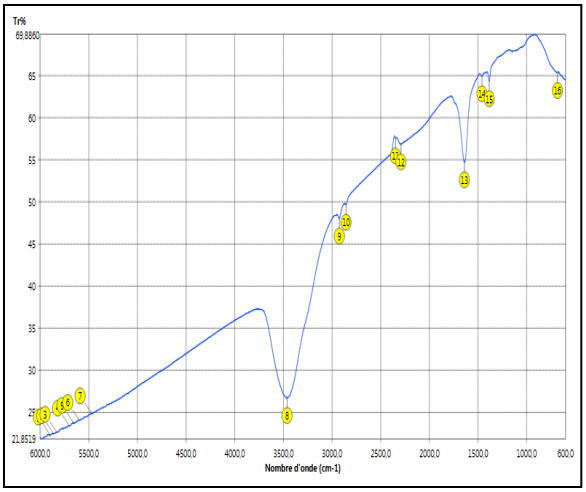

Figure 3: FTIR analysis of AgNps

3.4 Evaluation of myeloperoxidase activity

After injections (d1) a significant difference (*P*=0.0075) on the myeloperoxidase activity was found in sera of mice receiving AgNps-*Aah* and *Aah* alone compared to those of animals receiving free Nps (AgNps) and saline (0.1231 vs 0.069 vs 0.033 mM H_2O_2/min/ml). A decrease of MPO activity is observed at day 7 and day 15 in control groups, less important in sera of mice receiving the nanovaccine injection (AgNps-*Aah*) (0.033 and 0.039 vs 0.0886 mM H_2O_2/min/ml) (**Figure 4**).

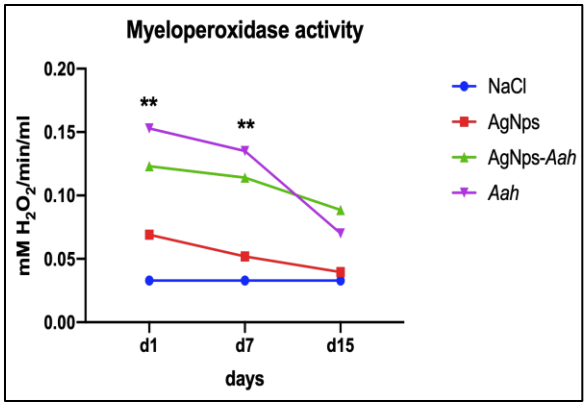

Figure 4: Evaluation of MPO activities in the sera of mice, 1, 7 and 15 days after the nanovector injection.
Activities are expressed as mM of used H_2O_2/min/ml of sera

3.5 Evaluation of eosinophil peroxidase activity

During the immunization protocol, there are no differences of levels of EPO activities in sera of groups receiving saline solution and AgNps injection (0.0180 vs 0.0181 mM H_2O_2/min/ml) (*P*=0.0069). Group receiving the nanoformulation adsorbing *Aah* venom shows a EPO activity at day1 (0.0379 mM H_2O_2/min/ml) lesser than the venom control group (0.061 mM H_2O_2/min/ml), decreasing almost at control values at day7 and day15 (0.0250 mM H_2O_2/min/ml) (**Figure 5**).

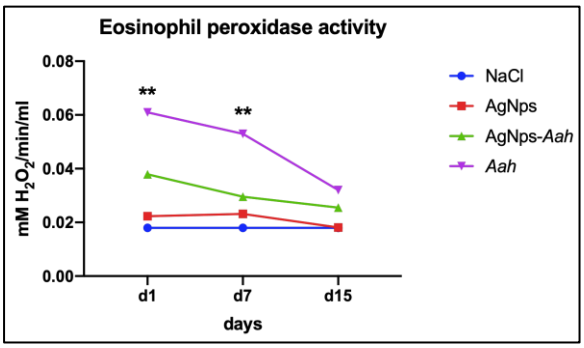

Figure 5: Evaluation of EPO activities in the sera of mice, 1, 7 and 15 days after the nanovector injection. Activities are expressed as mM of used H_2O_2/min/ml of sera

3.6 Evaluation of specific antivenom IgG titers

Specific IgG titers obtained against *Aah* scorpion venom were evaluated at d7 and d15 after the injection of AgNps-*Aah* on mice. Results showed a specific anti-*Aah* IgG titer of 1/16000 reached after 15 days post-injection, resulting on the activation of humoral immune cells secreting specific IgG after antigen presentation by APCs (**Figure 6**).

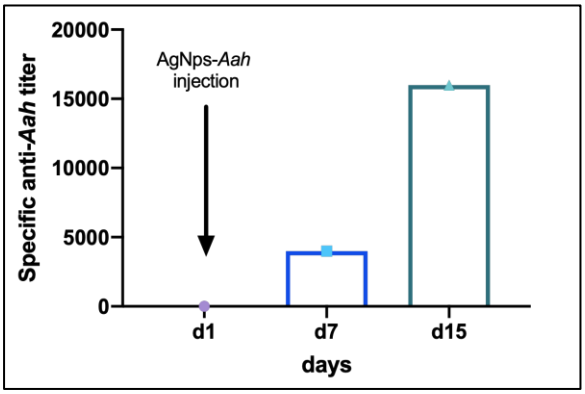

Figure 6: Specific IgG titers obtained after 7 and 15 days of AgNps-*Aah* injection

4. Discussion

In this study, we evaluated the preliminary safety and immunogenicity of a nanoparticulate vector for *Aah* venom delivery using silver nanoparticles synthesized by green chemistry. The use of nanoparticles as a vector in animmunization protocol can lead to a better antigen presentation,extended injection intervals and reduced doses of antigens [18]. The method of AgNps synthesis used in this study was chosen based on a previous study on green synthesis of silver nanoparticles as adjuvant in the veterinary rabies vaccine [17].

The physic-chemical characterization of silver nanoparticles synthesized revealed their spherical shape and a sizes between 60 and 150nm, with an obtained structural characterization by XRDshowing 2θvalues corresponding to 20, 32.2, 46.1, 54.9, 57.5 and 67.5similar to Sandjenbam et al., 2013, where theyfound crystalline peaks at 2θ values of 38.158,44.358, 64.528 and 77.498 on silver nanoparticles synthesized with five leave extracts (*Pinus desiflora, Diopyros kaki, Ginko biloba, Magnolia Kobus and Platanus orientalis*) [19]. Resultswith *Capsicum annuum L*. extract showed that amine groups of proteins played astabilization and reducing role during the synthesis of silver nanoparticles [20]. These properties positively influence their interaction with immune cellsbecause of the spherical shape and size of nanoparticles that can be easily endocytosed by antigen presenting cells, starting a specific immune response [21,22]. For this purpose, AgNps adsorbing *Androctonus australis hector* venom were used in an experimental protocol of a single injection in mice as an antigen delivery system. The evaluation of the systemic inflammation showed that a few hours after the first injection, an increase of MPO and EPO activitiesin sera of mice receiving AgNps-*Aah* was recorded resulting from a degranulation of azurophilic endosomes of polynuclear neutrophils and eosinophils in the extracellular medium. This could be explained by the spherical shape of AgNps as well as their small size which favors their phagocytosis by antigen presenting cells (APCs) but also because of nanoparticles adsorbed *Aah* venom, with possible residual toxicity [23]. Once internalized, AgNps induced not only the antigen presentation by APCs but also the synthesis of pro-inflammatory cytokines such as IL-1β and IFN-γ [24,25]. More interestingly, mice receiving AgNps alone did not display any important MPO or EPO activity, demonstrating a safety profile of these nanoparticles, largely used for their known antibacterial activity [26]. The single injection of AgNps-*Aah* induce specific IgG titer of 1/16000 at 15 days after the injection. This can be

explained by of the activation of humoral B-cells secreting specific antibodies, that probably need an injection boost to reach more important IgG titers and a long term protection against *Androctonus australis hector* envenomation [15].

Conclusion

Taken together,our preliminaryresults revealed that green synthesis using *E. globulus* leaves extract as reducing agent lead to the development of a safe antigen delivery system based on silver nanoparticles, promising for the design of an immunization protocol that can be further used against scorpion envenomation.

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Conflicts of interest

Authors do not declare any conflict of interest.

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