Original Article

Hirudo medicinalis Saliva Extraction and Ag-NPs and Particles Mediated Saliva Preparation

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Abstract

Background: Since the beginning of time, people have employed a variety of plants and animals to prevent and treat illnesses. Leeches produce physiologically and pharmacologically active tools into the wound when they are feeding. Bloodsuckers and infections becoming resistant to synthetic medications have increased the need for new, environmentally safe, and active control methods. **Objectives:** The aim of present study is to extract, synthesize, and characterize *Hirudo medicinalis* saliva. **Materials and Methods:** Twelve weeks starvation of *Hirudo Medicinalis*, leech saliva extraction done by feeding with phagostimulatory solution, leech then freezing for 15 min and remove from ice bag vomited saliva then squeezed to collect saliva, centrifuged at 4°C, 9000 rpm for 10 min. For synthesis Ag-NPs saliva, 0.1 g $AgNo_3$ was mixed with the 1 mL crude leech saliva at controlled conditions of darkness, absence of oxygen, and 25°C, Ag-NPs were kept in a tube covered with aluminum foil, and stored at 4°C. Ag-NPs characterization was done using a dynamic light scattering test and field emission scanning electron microscopy. **Results:** The starved leeches give saliva. Leech Ag-NPs size is 694.1 and zeta potential is −0.060 by Dynamic light scattering, square shape about 20–720 nm with an average value of 600 by field emission scanning electron microscopy. **Conclusion:** Saliva collection from starved and feeding leech with phagostimulatory solution and Ag-NPs saliva synthesized.

Keywords: Dynamic light scattering, leech saliva extraction, leech saliva mediated silver nitrate

Introduction

According to the study of Yadav and Zhang,[1] leeches (Hirudinea) are segmented worms that belong to the phylum Annelida. The word “leech” is derived from the Anglo-Saxon verb “to heal”, that is, “loece”. In Egyptian paintings dating back to 1500 BC, leeches are frequently used. Since ancient times, bloodletting has been utilized as a form of treatment for “local reduction.”[2,3] It was also used in ancient Greece, Rome, and Arabia.

Depending on how they eat, leeches are divided into two groups. Several invertebrates are preyed upon by ferocious leeches, for instance. Another group of leeches are sanguivorous leeches, which are ectoparasites that eat vertebrate blood, including human blood. Leeches use their suckers and biting teeth to take blood from their prey, then naturally detach themselves once they are fully engorged, leaving no vital organs behind the desire to keep eating.[4] Both wet terrestrial environments and aquatic habitats (ponds, streams, lakes, and the ocean) are home to them.[5] Although they can be found everywhere, bloodsucking leeches are most prevalent in North America, Europe, and Southeast Asia.[6]

The components of leech saliva demonstrate a wide range of pharmacologically beneficial potentials in addition to being necessary for the existence and feeding activities of leeches. In addition to being essential for leech existence and feeding activities, leech saliva contains a complex mixture of several physiologically active substrates that have demonstrated numerous useful pharmacological potentials.[7]

In June 2004, the Food and Drug Administration approved the submission of a medicinal leech as a treatment

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method.[8] The site biting, blood suction, and most crucially, the injection of leech saliva—which contains a variety of bioactive substances—into the area are all associated with the therapeutic qualities of leeches.[9].In addition to having anti-inflammatory, anticoagulant, platelet inhibitory, thrombin regulating, analgesic, extracellular matrix degradative, and antibacterial capabilities, leech salivary contents also contain a complex mixture of diverse biologically active substrates.[10]

One of the most exciting and promising fields of inquiry in contemporary medicinal science is nanotechnology. According to the studies,[11-14] nanoparticles are typically made up of clusters of atoms that range in size from 1 to 100 nm. They display new and improved properties based on size, distribution, and morphology compared to larger particles of the bulk ingredients used to make them.

Wide-ranging uses for silver nanoparticles include catalysis, optoelectronics, imaging and diagnostics, antimicrobials, and therapies.[15-18] Silver has a long history of being used as a powerful antibacterial agent with little human toxicity and a variety of *in vitro* and *in vivo* uses.[19] To prepare Ag-NPs-mediated leech saliva, the study will collect leech saliva from *Hirudo medicinalis*.

Materials and Methods

The period of our present study is extended from September 2022 to May 2023 and this study is performed in the Malaria and Vector Research Group, Biotechnology Research Center, Pasteur Institute of Iran which included the following steps:

Leeches sampling and maintenance

Leeches were prepared from the rice farm of Bandar-e-Anzali which located in the northern city of Iran, and then maintained in well-non-chlorinated tap water in aerated plastic containers that are maintained at room temperature in a separate room. Every 2 days, the water was changed routinely.[20]

Leech feeding and saliva extraction

For 12 weeks, leeches were hungry. Saliva was obtained using a little modification of a technique that has been described in the literature.[20-22] In a nutshell, a parafilm membrane (PARAFILM “M”; Bemis) was spread across a funnel holding 0.001 M arginine (0.02 g) (Sigma-Aldrich; CAS Number: 74-79-3) and 0.15 M (0.08 g) (Sigma-Aldrich; CAS 7647-14-5NaCl) saline solution. Placed the solution in the newly developed apparatus and added 10 mL of distilled water. Leeches were left to drag the solution through the membrane while the solution was held at a temperature of 37°C. Leeches were allowed to suckle till they became full. Leeches that had already descended from the membrane were prevented by placing them in a plastic bowl with ice surrounding it for 5–10 min. The leeches use this method to spit out whatever they have consumed. Leeches were hugged from the posterior area near the anterior (mouth) sucker without difficulty as a result of the saliva collecting. Bloody fluids were discarded, collected liquid was centrifuged at 4°C, 9000 rpm for 10 min. Supernatant obtained after centrifugation was referred to as crude leech saliva extract. All gagged bodily fluids were collected and refrigerated at 4°C in clean Eppendorf pipe test tubes.

Synthesis of leech salivary extract-silver nanoparticles conjugate

Materials and methods

Silver nitrate ($AgNO_3$); Reagent Grade, Carolina Biological 887788, Crude extracted leech saliva.

Equipment

A 200-mL beaker, digital scale, thermometer, a 50-mL plastic tube, a 1000- and 200-µL pipette, aluminum foil, dynamic light scattering (DLS) (Malvern Instruments Ltd., England, UK), TESCAN MIRA3 (Iran), Desk Sputter Coater [DSR1] Nanostructured Coatings Co. (Iran).

Ag-NPs synthesis

Synthesis was done via a chemical reduction process. The production of silver nanoparticles from LSE was done using the method described by Jaganathan *et al*.[23] Necessary calculations were done to determine the appropriate values according to the $AgNO_3$ molecular weight. So Nanoparticles were prepared in different final concentrations as follows:

1—0.1 g $AgNo_3$; 2—0.02 g; 3—0.001 g; and 4—0.0006 g.

Briefly, 0.1 g $AgNo_3$ was mixed with the 1 mL crude leech saliva at controlled conditions such as darkness, absence of oxygen, and 25°C, formation of the nanoparticle was associated with a change in colorless solution to a brown which lasted 72 h. Finally, the Ag-NPs were moved to a tiny tube that was covered with aluminum foil to protect it from light, and then stored at 4°C[23] $AgNO_3$-leech saliva nanoparticle size produced which was done using DLS test (Malvern Instruments Ltd., England, UK) and report two is zeta potential determination.[24] The analysis condition is as bellow:

Dispersant: water; temperature: 25°C; viscosity: 0.887; and measurement position (mm): 5.50.

Field emission scanning electron microscopy (FE-SEM) is high-tech used to study the topography of the nanomaterials such as the shape and size of nanoparticles. During electron microscope imaging, it is necessary that the surface of the samples is conductive. For this purpose, a layer of conductive metals should be created on the samples. To characterize the produced nanoparticles in this study, in the first step, we dripped a drop of nanoparticle onto the slide to dry and this sample was transferred to the DSR1 instruments. DSR1 is made by Nano-Structured Coating Co., Iran. In this step, a thin layer of gold (Au) was placed on the sample using the Desk Sputter Coater (DSR1). Subsequently, TESCAN MIRA3-15.0 kV was used to take images from the surface of the samples. Images were taken at a magnification of 10K× (10,000×) and 35K×. The test was performed with a confidence level of 95%.[23]

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients' verbal and analytical approval before the sample was taken. The study protocol and the subject information and consent form were reviewed and approved by University of Babylon ethical committee according to the document number M221204 (December 6, 2022) to get this approval.

Results

Leeches feeding and saliva collection

The results of the current study demonstrate that when brought in close proximity to the parafilm membrane, starved leeches began drinking the phagostimulatory solution after 12 weeks. Leeches tolerated the solution very well. Leeches were first placed in an ice container for 15 min after they descended from the membrane to collect saliva extract. Leeches were gently squeezed to complete the extraction, moving from the back toward the anterior (mouth) sucker. Seventy leeches were used to gather the saliva. These leeches typically weigh between 0.08 and 3.07 g in body weight. Between 3 and 8 mL is the volume of the leech-sucked fluid.

Silver nanoparticles produced by leech salivary extract have the following characteristics

Silver nanoparticles created by leech salivary extract are linked to a shift in colorless solution to brown that lasts for 72 h. $AgNo_3$-Leech saliva nanoparticle average size done using the DLS test (Malvern Instruments Ltd., England, UK), and report two is zeta potential determination is 649.1 nm [Figure 1] and their zeta potential is −0.060 [Figure 2].

Our FE-SEM results showed that samples include particles that are evenly scattered and nearly square in shape. The Nanoparticles ranged in size from about 20–720 nm [Figures 3 and 4] with an average value of 600 nm. The test was performed with a confidence level of 95%. FE-SEM images of the synthesized nanoparticles using Leech salivary extract.

Discussion

Due to the production of chemicals in its saliva as a result of its innate immune defense system, which is a result of its feeding process and surviving techniques in its habitat, *H. medicinalis* is one of the species that is most commonly used as a model in medicine. Although this organism secretes more than 100 different substances, only a small number of them exhibit potent anti-inflammatory, antibacterial, analgesic, and anticoagulant activities.[25]

There are numerous ways to examine the saliva of various members of the leech family. According to the procedure described by Abdualkader *et al.*,[20] leech saliva extraction is accomplished in the current study. The freezing temperature causes the animal to regurgitate (vomit) the desired sucked solution and causes the arrest of its movement, which in turn helps the squeezing to maximize the total amount of saliva obtained. We discovered that it

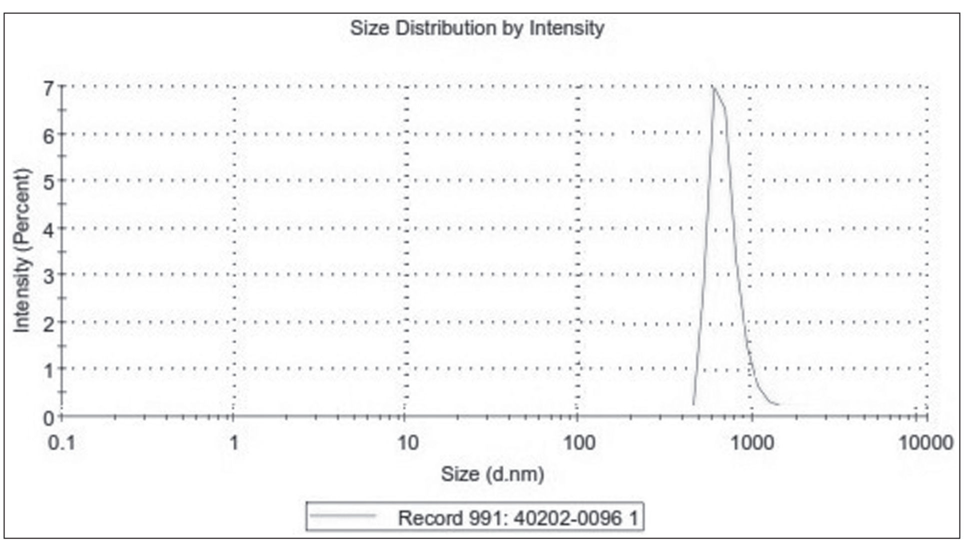

Figure 1: Zeta nano size distribution pattern of leech salivary extract-mediated silver nanoparticles

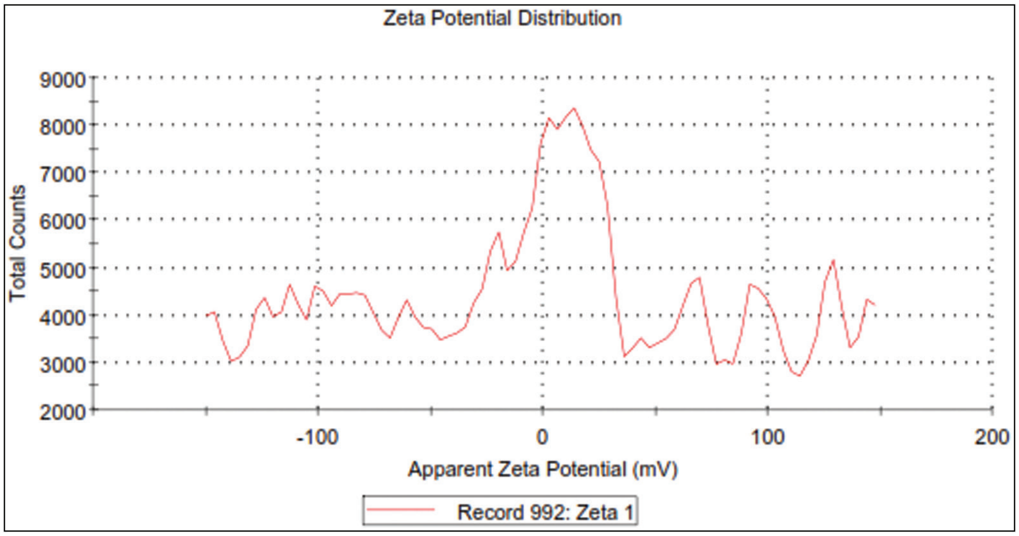

Figure 2: Zeta nano potential pattern of leech salivary extract-mediated silver nanoparticles

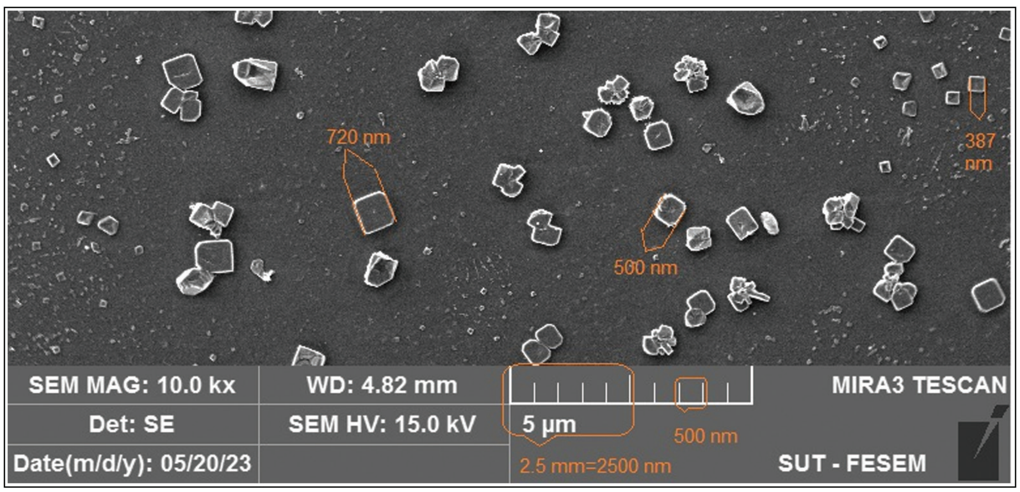

Figure 3: FE-SEM images of the synthesized particles using leech salivary extract

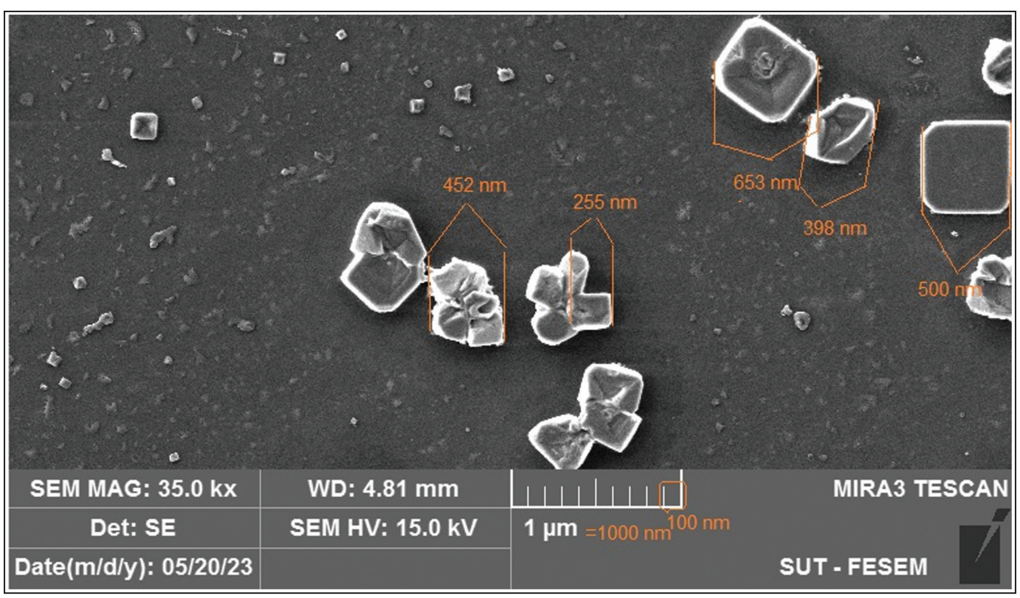

Figure 4: FE-SEM images of the synthesized particles using leech salivary extract

is much simpler to collect the saliva by feeding the hungry leeches with phagostimulatory solution then placing the leeches in ice immersion inside a plastic test tube. Last but not least, the animal is not killed by the ice, and it is necessary to re-immerse him in water for him to resume all of his activities and remain alive to depart normally for an arbitrary amount of time.

Leeches must be starved for a while to produce a leech saliva extract with a high concentration of proteins and peptides.[26] This result agreed with Alaama *et al*.,[26] who revealed that after a 12-week period of starvation, the concentration was peaked.[27] In our case, leeches began sucking the phagostimulatory solution after 12 weeks of fasting, and a maximum concentration was reached at that time.[28]

This study observed a change in color from colorless to dark brown as a result of the creation of silver nanoparticles using leech salivary extract. Similar to this, Saravana *et al*.[29] reported that surface plasmon resonance excitation caused silver nanoparticles to exhibit dramatic color variation in aqueous solution, ranging from colorless to dark brown.

It was determined that the size of the unit synthesized in this investigation is 649.1 nm and their zeta potential is −0.060. We know that a nanoparticle that has smaller a size and strong zeta (high negative or positive electrical charge) potential characteristic is more suitable. Many factors such as purity can interfere with the nanoparticle features. Extracted leech saliva is not pure and contains many factors such as different enzymes.[5] During the formation of nanoparticles, these impurity compounds may be placed on the nanoparticle surface and can cause large size and weak zeta potential which has happened in this study. If we want to extend our project, we can try to increase leech saliva purity and prepare much more pure nanoparticles. However, our $AgNo_3$-leech saliva nanoparticle is bioactive and has suitable antibacterial activity. The high-tech method used to examine the topography of the field is called FE-SEM. Our results revealed that samples contain the particles well dispersed and almost have square shapes. The average size of the nanoparticles and particles, which ranged from 20 to 720 nm, was 600 nm. The test was performed with a confidence level of 95%. FE-SEM images of the synthesized nanoparticles using Leech salivary extract. The leech saliva is abundant in protein, vitamins, amino acids, antioxidants, and other nutrients that have an important effect in lowering the amount of leech saliva-Ag-NPs.[5] EW-AgNP with a size range of 4–10 nm was consistently dispersed, according to FE-SEM. The presence of a high concentration of bioactive chemicals in the colloids may be the cause of the spherical cluster formation.[30]

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Nil.

Conflicts of interest

There are no conflicts of interest.

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