Advanced Biosensor-based Strategy for Specific and Rapid Detection of Snake Venom for Better Treatment

Guduru KVVNSK Aditya Teja, Namdev More and Govinda Kapusetti*

Department of Medical Devices, National Institute of Pharmaceutical Education and Research (NIPER), Ahmedabad, Palaj, Gandhinagar, India

Abstract

Specific and rapid detection of snake venom type is a complex practice, even with the contemporary medical technology. Generally, in cases for which the species are not identified, the nonspecific polymeric antivenom is injected into the patient. Thus, the effectiveness of treatment is limited, as it acts arbitrarily on the target. Since most snakes are nonpoisonous and treatment is applied with a cautionary approach, the patient can experience severe side effects of a nonspecific agent and in some cases mortality. Therefore, there is an immediate need to develop a suitable medical methodology to avoid this arbitrary practice. The proposed hypothesis may be the best practice for rapid and specific determination of snake venom type by biosensor intervention.

Introduction and background

Snakes are a versatile species, made up of elongated, legless, carnivorous reptiles of the suborder Serpentes.[1] The most distinctive feature of the snakes are its fangs and, in some, venomous glands.[2] The venom produced in venomous glands reaches the fangs through an anatomical tubing that is known as venomous ducts. The ducts open into the fangs, which are sharp and pointed tooth-like structures that help to inject the venom into a prey's body upon biting (Fig. 1).

Venom is a clear, viscous fluid of amber or poisonous straw-colored fluid, comprised of many biologically active agents, such as proteases and hyaluronidase, metal ions, biogenic amines, lipids and free amino acids, *etc*. However, only 80 large and small proteins and polypeptides have been identified to date.[3] Interestingly, most of the snake species are not venomous; although, for those that are, the venom is generally used for self-protection and obtaining food. The snake venoms are broadly classified into three types: 1) neurotoxic; 2) cytotoxic; and, 3) hemotoxic. The neurotoxins affect the central nervous system,[4] while the cytotoxins kill the cells in a particular area, where the bite occurs[5] and the hemotoxic attacks the cardiovascular system.[6]

Chemically, the toxins are composed of four main categories, including enzymes, glycoproteins, polypeptides and low molecular weight molecules. The enzymes, in particular, are represented by amino acid oxidase, thrombin-like procoagulant, kallikrein-like serine proteases metalloproteinases and phospholipase A2. Different types of toxins are present in the venom and the profile varies from species to species, primarily for α-bungarotoxin, α-cobratoxin, α-toxin, erabutoxin, notexin, ammodytoxin, cardiotoxin, cytotoxin, myotoxin-a, crotamine and peptides like pyroglutamylpeptide.[7–10] The toxins primarily participate in immunogenic reactions when the venom is injected into the host body. In the case of snake bites, antivenom or antiserum immunoglobulins are employed to treat the patient. Antivenom includes a monovalent antibody or commonly used polyvalent antibody against the venom.[9]

The biosensor is a tiny analytical device, capable of converting biological information into a detectable signal.[11] As such, the device is able to determine the concentration of substances and other parameters of biological interest. This noninvasive technique is highly advantageous for its high accurately and sensitivity.[12] In modern-day medicine, the biosensor is widely used, for various applications, to determine a broad range of factors, such as blood glucose, cholesterol, catechol and bilirubin, *etc*.[13] Examples include the amperometric biosensor PDMS/glass capillary electrophoresis biosensor microchip developed by Schoning *et al*.[14] for the detection of catechol and dopamine, the biosensors employed in forensic science for the detection of DNA, and the microbial biosensors utilized for the detection of pathogenic microorganisms.[15]

Most significantly, any biosensor is very specific and accurate, and requires the smallest amount of analyte for detection. Basically, the device is comprised of sensing material (bioreceptor), a transducer and a detector. The receptor may be an enzyme, antibody, microorganism or a cell, which senses the presence of the

Keywords: Snake venom; Electrochemical biosensor; Antibody and Quartz Crystal Microbalance.

Abbreviations: ASV, Antisnake venom; DNA, Deoxyribonucleic acid; ELISA, Enzyme-Linked Immunosorbent Assay; PDMS, Polydimethylsiloxane; QCM, Quartz Crystal Microbalance.

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***Correspondence to:** Govinda Kapusetti, Department of Medical Devices, National Institute of Pharmaceutical Education and Research (NIPER), Ahmedabad, Palaj, Gandhinagar, India 382355. E-mail: govindphysics@gmail.com

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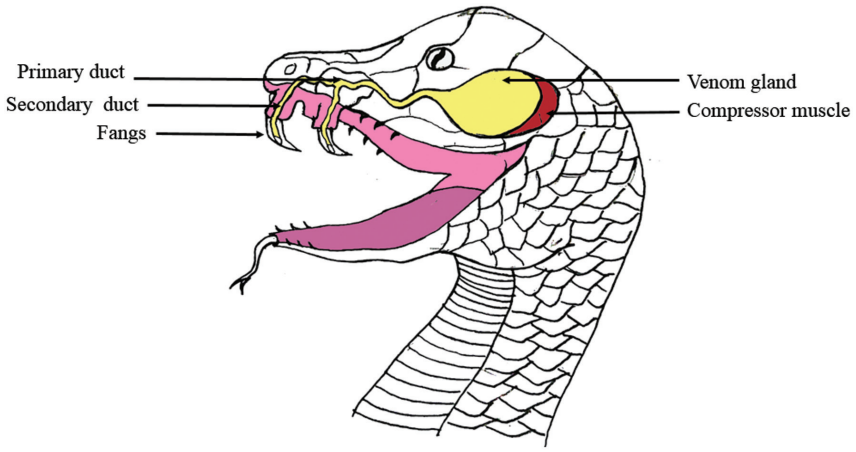

Fig. 1. The anatomical presence of the venom gland with fangs, primary duct, secondary duct and compressive muscle in the snakehead.

desired substance in the analyte, resulting in a chemical, physical or electrical change in the transducer. The transduced signal is then subjected to amplification and subsequent display by the detector of the analyte in a quantified manner.[12,15,16]

Biosensors have been used as an integrated part of many systems for regular monitoring, primarily in food processing to determine quality and safety.[17,18] Various sensors are well designated for different kinds of applications, like *E. coli* detection in vegetables by the detection of change in pH,[19] enzymatic detection of aging of beer,[20] and contamination of food.[21] Even more, the sensors are utilized for continuous monitoring of food substances in transit and during processing.[22,23]

According to recent statistics, around 50,000 deaths due to snake bites occur annually in India,[24] and time is one of the crucial factors for treatment. Injection of antisnake venom (ASV) is the best practice for snake bite treatment. The maximum number of deaths occur from the bite of the most abundant venomous snakes, including the Indian cobra, Indian krait, Russell's viper, Saw-scaled viper and Indian pit viper. Table 1 presents the different venomous snakes and their nature of toxicity.[25]

Mostly, the antivenom consists of antibodies collected from immunized animals. The antibodies are collected from the animal serum, which is subjected to a specific venom type by a number of doses for specific time intervals.[26] Owing to the advantages of the treatment, it benefits outweigh the side effects in many cases, but sometimes it leads to mortality of the patient.[27] Also, in many cases, the injection of ASV leads to anaphylactic shock.[28] Besides antibodies, molecules like melatonin, are reported to underlie the antivenom effect. The study of such was established in Egyptian cobra (*Naja haje*) venom using a rat model; the vital organs, like kidney, liver and heart, of the rat was protected from the venomous effect.[29]

Table 1. Most commonly found venomous snakes in the Indian subcontinent and toxicity type[25]

Snake name	Venom type
Indian cobra, spectacled cobra	Neurotoxic
Indian krait	Neurotoxic
Russell's viper	Hemotoxic
Saw-scaled viper	Hemotoxic and cytotoxic
Indian pit vipers	Cytotoxic

Usually, the antivenom is synthesized using two methodologies: 1) monomeric and 2) polymeric.[30] The monomeric antivenom is produced against single species venom, while for polymeric, a venom mixture of different species is injected into the target animal.[31] The polymeric antivenom is nonspecific and less effective than the monomeric. Even in the 21st century, nonspecific polymeric antivenom is commonly administrated for snake bites and achieves chaotic results, when the snake is unknown.[32,33] Due to the lack of proper diagnosis technology for rapid identification of the venom type/snake, the dicey therapy leads to death in many cases.

There is a critical need to develop a technology to detect the venom type accurately and rapidly, to facilitate administration of the precise ASV. It is well documented that the general random practice of ASV creates serious complications, which may appear immediately or over the long-term. Predominantly, adverse effects are observed immediately in 20% of cases (within a few hours)[34] and in extended time death occurs due to envenomation of the ASV. The gravity of the situation is further complicated by the lack of knowledge regarding the diagnosis and management of such conditions.[35] One vital study reported by Deshpande *et al*.[36] concluded that 92 patients out of 164 who were treated with ASV (>50%) suffered from antivenom reactions. So, there is an urgent need for a device/kit that is capable of identifying the venomous snake for better ASV.

A handfull of techniques have been developed for detection of the specific snake venom for better treatment, but the modalities have achieved limited success. Dong *et al*.[37] developed a silicon-based optical biosensor chip with specific binding affinity. In it, once the optical source is illuminated, the chip changes its color from purple to blue if antibody binding has taken place. The kit is able to semi-quantitatively detect venom from blood, urine, feces and bile. Zahani *et al*.[38] developed an impedometric biosensor for the detection of phospholipase A2 activity in snake venom, which is responsible for inflammation and pain at the site of injection. Similarly, Pawade *et al*.[39] developed a lateral flow-based immunochromatographic assay with application of gold nanoparticles for detection of the Indian Cobra venom and Russell's viper venom. In addition, Shaikh *et al*.[40] developed a dot ELISA-based specific snake venom detection technique for Indian snakes; however, the techniques are intended for specific venom detection and their applications are limited by high time-consumption. Furthermore, the aforementioned techniques have employed antibodies of rat and rabbit origin. Hence, the best possible method may be a simple

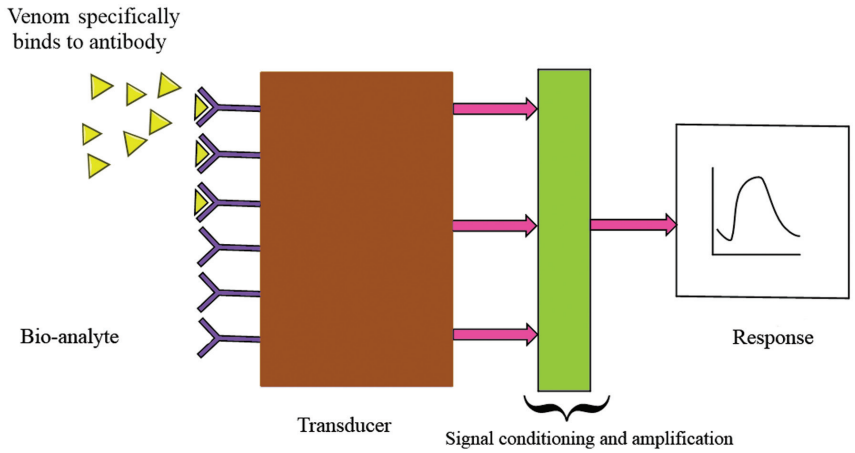

Fig. 2. Block diagram of the biosensor showing the immobilization of different antivenom antibodies on the transducer surface. Specific interaction of the venom to its counterpart will generate a detectable specific signal to identify the snake species.

blood test for a rapid detection technique.

One commercial product is available for specific detection of venom of five different kinds of Australia- and Papua New Guinea-originated snakes. The colorimetric enzyme immunoassay assists in selection of the monovalent antivenom to neutralize the snake venom involved in the bite.[41] While the method provides high sensitivity to determine the venom type, it has some constraints. As per the manufacturer's information, the assay method is highly time-consuming, needing at least 35–45 min to get the result; more significantly, the assay provides equivocal reactions in case of high concentration sample testing, involves multiple complicated sample processing steps and stringent storage conditions, and needs a trained person with good laboratory practices for proper results. Therefore, there is a need for a simple methodology to identify the snake venom for administering an antidote with minimal time. Most importantly, the South Asian countries like India need a highly specific and simple kit for diagnosing venom type, since there is a lack of specialized labs and persons to process the analysis.

Hypothesis

The basic idea behind this hypothesis is to develop a device or kit which can detect the type of snake by analyzing its venom. Moreover, the device can be designed in such a way that it can detect the venom type from a blood sample, which can be collected from either the bite site or the bloodstream. Even more, it will help to identify whether the bite is venomous or nonvenomous, so as to avoid unnecessary ASV administration and the subsequent trauma. The hypothesis is based on a chip-based biosensor to detect venom type for better treatment. The sensor will give the information by the formation of an immune complex by agglutination of the specific antivenom antibodies with the venom. Initially, the selected antibodies of different antivenom types will be immobilized on the transducer surface of the sensor. Once the sample is collected from the patient, it is immediately analyzed by the sensor, which generates a signal that will suggest the venom type by its specific binding with immobilized antivenom antibody. The result will be obtained from the transducer (possibly a quartz crystal microbalance) in the form of electric signal generation by mass variation on the sensor surface through aggregation of the antigen of venom binding with the immobilized antibody (Fig. 2).

Evaluation of the hypothesis

Isolation of specific antibodies

Antibodies against specific venom are obtained by immunization of hens with the particular venom collected from the selected snakes. Following the immunization, the antibodies will be isolated from the egg yolk.[42,43]

Immunization procedure

A handful of literature is available for the immunization of different animal models with various methodologies. Conventionally, the antivenom antibodies are isolated from an immunized horse, goat or rabbit. Nevertheless, it exhibits the major constraints of an anti-compliment reaction,[44,45] serum sickness[46] and anaphylactic shock.[47,48] Moreover, the isolation and standardization of the purified antibodies is a tedious process.

As per the literature, the hen's egg procedure is the best possible, safe and easy isolation method. Briefly, the laying hens will be immunized with the interested (Indian-origin snake venoms in the present proposal) snake venoms in different groups for specific periods and the specific procedure shown in Figure 3. Initially, the chickens of a specific breed free-from-pathogens (fed and bred in a clean environment) are selected for the procedure. After specific growth, the hen is subjected to the administration of small doses of venom by injection into the pectoralis muscle.[49] Before injection, the venom is exposed to radiation to reduce its toxicity.[50,51] Further, the eggs of the immunized hens will be collected and the isolated yolk will be frozden at −20 °C for the subsequent procedure. The supernatant collected by centrifugation and filtered by various stages[52] will be used to obtain the antibodies upon precipitation by addition of ammonium sulfate.[53,54]

The antibodies will be immobilized onto the transducer which detects the change in physical or chemical changes.[55] The important factor to be optimized is the concentration of the antibodies and their orientation. Antibody orientation will be achieved by slight modification through adding bifunctional thiol containing reagents.[56] Different antivenom antibodies will be collected from the eggs of different hens, which have been immunized with a unique venom type. The isolated antibodies are immobilized on

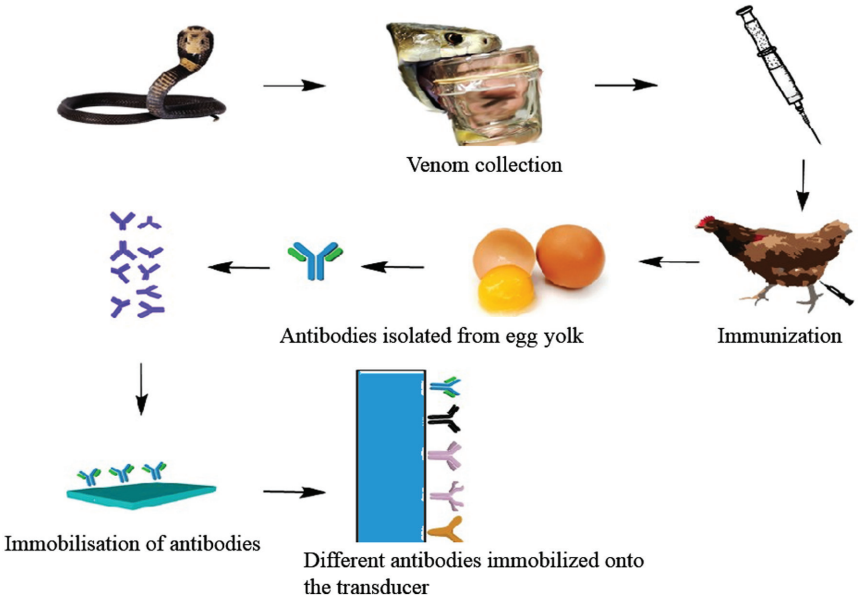

Fig. 3. The detailed procedure of antibody generation in the hen's egg model, followed by the development of a venom-specific biosensor.

the discrete receptor surfaces of the biosensors. The generated signal during antigen-antibody interaction is amplified using amplifiers circuits, for better scrutiny.[57]

Bruce *et al*.[58] isolated rattlesnake and scorpion antibodies from the aforementioned method. Similarly, Mayadevi *et al*.[50] employed the same method, with slight modification in the lipid removal method to isolate antiviper antibodies. Paul *et al*.[59] isolated and purified antibodies of anti-*Echis carinatus* venom from egg yolk by the water dilution method.

Detection principle

The collected blood sample from the wound site interacts with the biosensor. The antibodies present on the biosensor specifically bind with the venom as a result of agglutination, changing the physical or chemical state at the receptor site. The change can be detected by various technologies; the highly sensitive quartz crystal microbalance (QCM) is the best recommend transducer and recognizes frequency change with mass variance.[60] QCM possesses very high accuracy rate and detects mass densities of 1µg/cm^2.[61] It can perform even under vacuum. A wide variety of immobilization techniques can be employed for fabrication of a biosensor in QCM. It can also detect the difference in nano-gram/unit area by measuring the change in resonant frequency.[62] Structurally, the QCM quartz crystal is sandwiched between two "T" shaped electrodes and connected to electric terminals to supply voltage. The immobilized antibody on QCM selectively interacts with its counterpart and causes the mass change. The result of mass change can cause the resonance frequency shift, to help in quantification of the analyte. The frequency change depends on various factors, like mass, shape, structure and thickness of the analyte.[62,63]

In recent days, QCM has shown massive advancement in biosensor application. Park *et al*.[60] fabricated a hemoglobin QCM biosensor with high sensitivity of detection (limit of 0.147%) for HbA1c (hemoglobin A1c) against hemoglobin. Similarly, Şerife *et al*.[64] developed a QCM-based highly sensitive biosensor for detection of ochratoxin A, with a reported detection limit of 17.2–200 ng/mL. Interestingly, the sensitivity of QCM can differentiate the normal to physiological conditions, like for C-reactive protein, which is a key biomarker for inflamed liver and related disorders.

The intended biosensor will exhibit high sensitivity, with linearity detection ranging from 0.04–100 µg/mL and lower detection limit of 0.02 µg/mL.[65] Similarly, Lourdes *et al*.[66] developed the High Fundamental Frequency QCM-based Immunosensor for detection of pesticides in honey. The sensor exhibits a limit of detection of 0.035 µg/mL in a diluted honey sample. Furthermore, the QCM demonstrates high sensitivity, and is cost-effective, fast and reliable compared to the conventional techniques.

Rapidity

In case of life-threatening snake bites with narrow antidose periods to save the patient's life, rapid detection time is an essential parameter for sensor development. The response generation must be immediate for when the antibody interacts with a specific antigen in the sample. Ajeet *et al*.[67] developed a biosensor with the aid of antibodies to detect the presence of ochratoxin, which is produced by *Aspergillus* species and found in foodstuffs. For this, the IgG antibodies are isolated and immobilized onto an indium tin oxide layer, with the help of chitosan and iron oxide composite. The electrodes have a very fast response time (18 s), with greater sensitivity and a minimum detection limit of 0.5 ng dL^{-1}. An attempt has been made to measure the concentrations of cortisol and corticotrophin-releasing hormone by immobilizing the polyclonal antibodies on a platinum electrode. These probes are capable of giving a response within 30 s, with high sensitivity.[68]

Specificity

Although blood samples from bite regions have been exposed to a range of biosensors, accurate and specific results have only been obtained upon specific antibody-antigen interaction.[69,70] The rest have shown negative results for suggesting proper treatment. The

antibody-antigen reactions are very specific, even at very minute concentrations, and have been proven by various research groups to underlie the specificity of antibody-antigen reactions.[71–73] The mouse immune globulin IgG is detected by using fluorophore-modified antibodies. This approach has been demonstrated as very useful for the detection of various antigens in different disease conditions.[74] A rapid detection-capable amperometric biosensor has been developed for *Streptococcus agalactiae* detection, and a biotinylated antibody is utilized for the application. The antibody is conjugated with horseradish peroxidase-labeled streptavidin and the complex is immobilized on the carbon electrode.[75]

Perspectives

The proposed hypothesis is a potential methodology for the rapid and accurate identification of a snake that has bitten a victim. The proposed design offers greater specificity, selectivity and accuracy, in comparison to the current existing conventional techniques. The proposed device will be reusable and cost-effective. The main advantage of this analysis will be bypassing sample preparation for analysis. The sensor will directly detect the analyte (venom) from the sample (blood collected from injury region or bloodstream). The device will be able to save the precious lives of many people and to prevent the occurrence of adverse effects of nonspecific ASV administration.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Guduru KVVNSK Aditya Teja and Namdev More have collected the necessary literature and prepared the manuscript to fulfill the hypothesis. Dr. Govinda Kapusetti has developed the hypothesis and review the final draft.

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