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An overview of biosensor advancements for detecting botulinum neurotoxins: Addressing food safety and biowarfare risks

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ABSTRACT

Botulinum neurotoxins (BoNTs) are lethal toxins produced by bacteria *Clostridium botulinum*. Ingestion of BoNTs contaminated foods causes botulism which affects individual's nervous system by blocking the release of neurotransmitters causing flaccid paralysis. This review article deciphers the comprehensive account on mechanism of action of BoNTs, pathogenicity, and various innovative analytical detection techniques of BoNTs in foods. Potential misuse of BoNT as a biowarfare agent is also a major concern. Hence, for the detection of deadly BoNTs various conventional techniques like mouse lethality bioassay (MLB), SNAP-25 assay, mouse phrenic nerve hemidiaphragm (MPN) test, non-lethal mouse flaccid paralysis assay (NFPA) and modern techniques (immunoassays, cell-based assay, nucleic acid-based methods, endopeptidase mass spectrometry assays) have been discussed. This article also provides a detailed account on biosensing technology for detecting BoNTs in foods. Moreover, future research efforts should be focused on the development of advanced new-age biosensors for automated detection and real time monitoring of botulinum neurotoxin toxicity in food. Integration of biosensors with quantum technology and lab-on-chip platforms is required for increasing their versatility and robust detection. The insights presented in the review aim towards providing future research directions and increase the vigilance against potential future threats.

1. Introduction

Food safety and contamination is one of the major global concerns from the past decades. Improperly processed and contaminated foods have severe effect on the human as well as animal population. Consumption of contaminated foods can cause fatal diseases if not treated immediately and properly [1]. Food contaminants could be biological (bacteria, fungus, parasites, and biological toxins), physical (foreign objects like dirt, metals, glass etc.) or chemical [2]. Among the biological toxins, Botulinum neurotoxin (BoNT) is one of the most potent toxins known in nature. This neurotoxin is produced by a gram positive, spore forming anaerobic bacteria belonging to genus *Clostridium*. *C. botulinum* and several other related species are infamous for causing botulism disease [3].

The three most common forms of botulism include foodborne botulism, wound botulism and infant botulism [4]. The immature gut of an infant provides suitable environment for the germination of the spores of *C. botulinum* as the microbiota is not developed completely. It is usually caused by the ingestion of contaminated soil, food or packaged baby formula/powder [5]. The neurotoxin hence produced by bacteria further hampers the health of the baby with symptoms beginning around 18–36 h after ingestion [6]. Symptoms may include constipation, abdominal cramps, vomiting, descending paralysis, and, respiratory failure in some cases [7]. The wound botulism occurs on the exposure of the spores of *C. botulinum* to the open wounds or cuts and symptoms could take up to two weeks to appear [8]. It is commonly associated with substance abuse and increased subcutaneous injections, majorly with black tar heroin (BTH) [9]. Other less prevalent forms of botulism include adult intestinal toxaemia, inhalation botulism and, iatrogenic botulism due to the incorrect administration of BoNT for pharmaceutical and cosmetical purposes [10,11].

In humans the majority cases of botulism are reported to have originated from contaminated food. Food contaminated with BoNTs can cause huge outbreak of botulism disease, hence only one suspected case is enough to evoke public health emergency [12]. Considering the severity of disease, there is no specific permissible limit of BoNT

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contamination in the food. Various strategies are followed to prevent the disease occurrence through intentional or unintentional contamination at first place [13].

Botulinum is one of the deadliest exo-neurotoxin available in the nature with a lethal dose (LD_{50}) of 1 ng of toxin per kg of body mass [14]. BoNTs are found to be more lethal than hydrogen cyanide (LD_{50} of 100 mg/kg) [15] and Sarin gas (LD_{50} of 420 mg/kg) [16]. As for humans the mean lethal dose of BoNT/A is 0.09–0.15 μg per 70 kg if administered intravenously of intramuscularly, for inhalation route 0.7–0.9 μg and 70 μg for oral administration [3]. Theoretically, 1 g of pure crystalline BoNT is enough to kill more than 1 million of people if inhaled; hence it is kept in the Category- A of Centres for Disease Control and Prevention (CDC) (USA) and is also banned by Biological Weapon Conventions [3,17]. Pertaining to its high toxicity, ease of access, storage, and transmission, BoNTs have been used by terrorist organizations to fuel bioterrorism.

Also, the pharmaceutical validation of BoNTs can't be ignored as BoNT-based pharmaceuticals require stringent quality control, to check for the purity, quantity, and catalytic activity of toxin. The detection methods need to be quantitative and capable to provide the information regarding bioactivity of BoNT which could ensure the safety from batch to batch. Even, detection of BoNTs in clinical samples is challenging since these highly potent toxins are present in minute amounts. Moreover, BoNTs in clinical samples generally get mixed with biological components hence, the detection and isolation become even more harder.

The sensitivity and suitability of BoNT detection techniques is a crucial factor in determining the effectiveness of their diagnostic application. This required sensitivity for different contexts depend on the expected toxin concentration in each scenario. For instance, the BoNT concentration of as low as 70 μg in contaminated food products during botulism outbreak is considered lethal [18]. In the case of clinical samples BoNT concentration in serum or plasma is considerably low, in the range of 0.09–0.15 μg [19] and in biowarfare scenarios BoNTs concentrations can be highly variable depending upon the extent of the attack, or the use of dissemination method. Various conventional detection techniques have been used for the BoNTs detection. Although these techniques are still widely used and have good sensitivity but they majorly lack rapidity, real-time screening, portability, and cost-effectiveness. The conventional detection techniques however widely used for assessing clinical samples, faces limitations while dealing with the food samples due to the presence of complex matrix and are often not suitable in biowarfare scenarios where real-time and rapid detection is needed. Hence, there is an increasing demand for the development of more efficient, rapid, adaptable in-vitro platforms which could also be used as point-of-care devices. The new age biosensing technology offers a promising solution in the detection of BoNTs, which serve as a disease-related biomarkers indicative of botulism.

In this review article we would further discuss the pathogenicity associated with BoNTs, conventional detection and the critical analysis of different types of biosensors reported for the detection of BoNTs.

1.1. Pathogenicity of BoNTs

There exist seven serotypes of botulinum neurotoxins depending on their antigenicity and enzymatic substrate cleavage site. The seven serotypes are A, B, C, D, E, F, and G [3]. BoNTs producing *Clostridia* can be divided into six groups depending on their phenotypic characteristics, 16 rRNA gene sequences, and whole genome sequence comparison as depicted in Table 1.

BoNTs are zinc endopeptidases released during cell lysis of *C. botulinum* bacteria. BoNTs are encoded by *bont* genes and are initially produced as a single chain peptide with molecular weight of nearly 150 kDa. The detailed structure of BoNT is depicted in Fig. 1.

Botulinum neurotoxin is not produced in its active form directly by bacterial metabolism. Initially produced precursor, gains toxicity on interacting with the protein kinases and trypsin produced by the bacteria [24]. The neurotoxin hence absorbed through the blood reaches the neuro-muscular junction, inhibiting the release of acetylcholine. The process starts with the initial binding of BoNT to the presynaptic neurons at the neuromuscular junction [25]. Certain receptor proteins like gangliosides and synaptic vesicle protein SV2 further facilitates this binding. After binding, the BoNT is internalized and translocated into the cytoplasm of neurons by endocytosis. Inside the cytoplasm, BoNT acting as a zinc-dependent endopeptidase cleaves SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor) proteins that are involved in the neurotransmitter release as depicted in Fig. 2a [26]. Normal release of neurotransmitter and subsequent contraction of muscle fibre requires the formation of synaptic fusion complex as depicted in Fig. 2b.

2. Conventional techniques for detection of BoNTs

Botulinum neurotoxin contamination in the food is a growing concern and appropriate measures are needed to prevent it. Various efforts are being undertaken by the food industries to avoid the growth and production of toxin by *C. botulinum* [27]. Hence, it is of utmost important to detect the botulinum neurotoxin contamination in the food samples at early stages and to correctly determine the serotype of the toxin. This will not only allow the appropriate antitoxin detection but also will reduce the chances and proper management of a major

Table 1
Groups, subtypes and characteristics of Botulinum neurotoxins (BoNTs).

Groups	Subtypes	Neurotoxin-producing bacteria	Characteristics	Optimum temperature (°C)	Enzymatic substrate cleavage site	Causes infection in	Reference
Group I	BoNT/A, BoNT/B, BoNT/F, BoNT/H	*C. botulinum*	Proteolytic	Produces highly thermo resistant spores and grow between 10 °C and 37 °C	SNAP, VAMP	Human (Generally through canned food)	[20]
Group II	BoNT/E, BoNT/B4, BoNT/F6	*C. botulinum*	Non-proteolytic	Have moderate thermo resistant spores, and can grow at temperatures as low as 3.5 °C	SNAP, VAMP	Human (Generally less heated or freezed food)	[21]
Group III	BoNT/C, BoNT/D	*C. botulinum*	Non-proteolytic	Preferentially grow at higher temperatures (37–40 °C)	SNAP, VAMP, Syntaxin	Only in animals	[22]
Group IV	BoNT/G	*C. argentinense*	Proteolytic	Optimum growth at 37 °C	VAMP	No reported outbreaks	[23]
Group V	BoNT/F7	*C. baratii*	Non-proteolytic	Optimum growth occurs at 30–45 °C	VAMP	Human	[23]
Group VI	BoNT/E4, BoNT/E5	*C. butyricum*	Non-proteolytic	Optimum growth at 30–37 °C	SNAP	Human	[24]

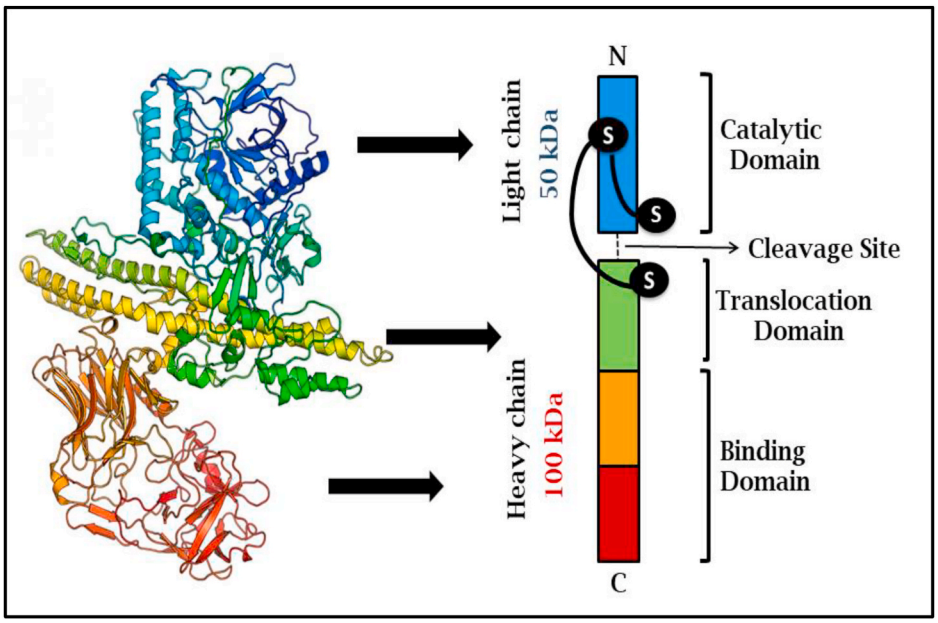

Fig. 1. Diagrammatic representation of Botulinum neurotoxin A structure.

outbreak of foodborne disease caused by BoNTs. Traditionally, the most accepted method for the detection of botulinum neurotoxin is mouse bioassay. Although it is widely used however; it has shortcomings along with the ethical concerns involving using animals for the detection [28]. To avoid this other *ex vivo* and *in vitro* techniques are used.

Aforementioned Table 2 provides a comparative analysis of the various conventional techniques being used for the botulinum neurotoxin detection. Although these techniques are widely used and reliable but they face significant drawbacks and limitations. Diagnostic techniques like the mouse bioassay, MPN test, NFPA are much time consuming hence, not suitable for urgent diagnostics in real-life situations. Immunoassays like enzyme-linked immunosorbent assay (ELISA) uses high-quality antibodies with limited dynamic range and potential for cross-reactivity hence, restricting its use for detection of very low toxin concentrations. Other advanced techniques such as, mass spectrometry and PCR require the use of sophisticated equipment and trained personnel, restricting their use in low-resource settings. Nucleic-acid based methods cannot confirm the presence of biologically active toxins as they can only detect specific nucleic acids. Hence, certain modern diagnostic techniques are required which could provide a promising alternative by addressing these limitations of conventional diagnostics.

3. Biosensors for the detection of BoNTs

The conventional methods used for the detection of Botulinum neurotoxin while effective, however, showed various limitations including potential for cross-reactivity, less-accuracy, ethical concerns, complicated sample procedure, delayed response time, and need of expensive instruments. These aforesaid limitations are overcome by biosensors which are simple, highly specific, sensitive, reliable, rapid response time and cost-effective [40]. They have the potential for targeting multiple analytes and can even provide real time monitoring [41]. The first biosensor was invented in 1956 by Prof. Leland C. Clark for the detection of oxygen in blood [42]. The historical development of biosensors could be traced through the development of three generations of biosensors [43]. The first-generation biosensors involve the diffusion of the normal product of the reaction to the transducer hence, generating an electrical response. The second-generation biosensors involve the use of specific mediators for improved response between the reaction and transducer. The third-generation biosensors are the newest in which the signal generation is accomplished by the reaction itself without the need of product or mediator diffusion [44].

Biosensors, with their high selectivity and sensitivity are designed for the detection of a specific target molecule called as analyte. These analytes could be biomarkers that serve as a measurable indicator of biological processes, disease conditions, or physiological states [45]. These biomarkers are crucial in diagnostics and can be categorized in two categories namely, health-related biomarkers, and disease-related biomarkers. Health-related biomarkers are indicative of normal physiological functions and critical for routine health monitoring. While, disease-related biomarkers are associated with infections, pathologies, or presence of harmful substances [46]. In the case of botulinum neurotoxin detection, BoNTs serve as disease-related biomarkers indicative of botulism. Biosensors specific for the detection of BoNTs as analyte could be designed to provide for a powerful tool for the early diagnosis of botulism.

A typical biosensor's principle is based on the detection of target analyte by a transducer consisting of a biological recognition element (BRE) or Bioreceptor [47]. Target BoNT in the sample mixture interacts and bind to the BoNT specific bioreceptor through molecular interactions. This binding is highly selective as well as specific. The bioreceptors can be enzymes, antibodies, proteins, aptamers, nucleic acids, or whole cells. The binding of analyte with specific bioreceptor generates a signal that is converted to a measurable output in the form of electrical, optical, thermal, or mass based stimulus by depending on type of transducer used. The output signal hence generated corresponds to the concentration of analyte in the sample which is amplified by the detector [48]. In this way the biosensor can provide both qualitative as well as quantitative analysis of contaminants or toxins in the food samples hence, ensuring food safety and quality [49]. The working and principle of biosensor is depicted in Fig. 3.

3.1. Classification of biosensors for the detection of BoNTs

The biosensors can be classified in various types depending on the type of transducer and bioreceptor used as depicted in Fig. 4. The biosensors can be developed by leveraging the enzymatic activity of the BoNTs this would ensure the enhancement of specificity which was lagging with the use of enzymatic assays alone. Although, the enzymatic

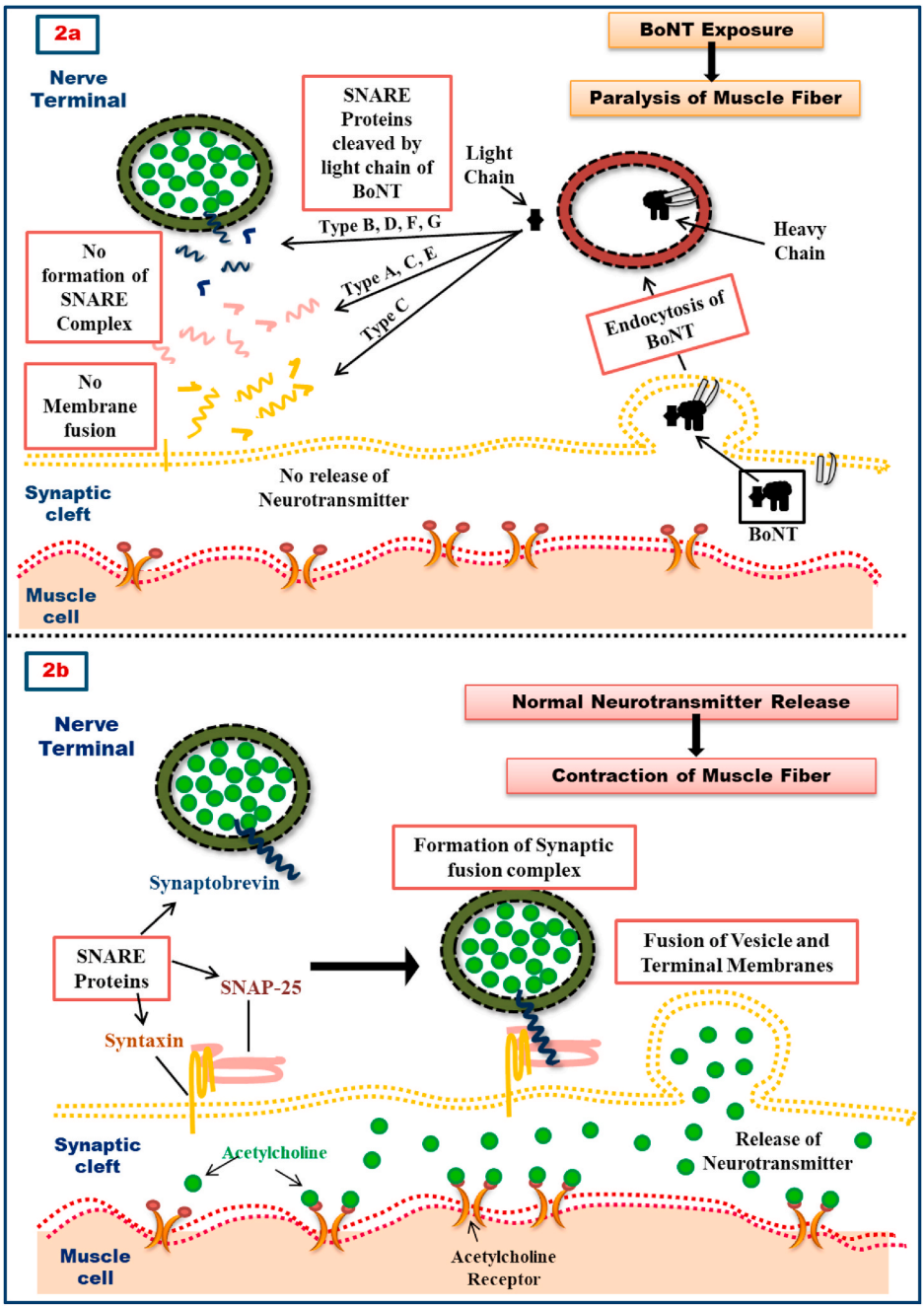

Fig. 2. (a): Diagram representing the mechanism of inhibition of neurotransmitters release by consumption of botulinum neurotoxins contaminated foods; (b): diagrammatic representation of normal neurotransmitter release at neuromuscular junction.

assays offer increased sensitivity and quantitative ability but the specificity of detection in the complex clinical or food matrices remains a challenge. These complex matrices may contain other cross-reactive enzymes, leading to false-positive results. Other interferences may arise due to the presence of food components like fats, proteins, carbohydrates, and cellular debris which could compete or inhibit binding sites hence, affecting the activity of BoNTs.

3.1.1. Electrochemical biosensor for the detection of BoNTs

Electrochemical biosensors are highly sensitive devices that can detect the presence of BoNTs by measuring the change in electrochemical properties of the system due to the binding of specific botulinum neurotoxin serotype with the recognition element [50]. The electrochemical biosensors are rapid, reliable, cost-effective, and self-contained [51]. The detection can be made through various electrochemical techniques such as voltammetric, chronoamperometric, potentiometric, impedimetric etc. [52]. Beside these, certain advanced forms of the electrochemical biosensors have also been developed in the recent times utilizing the Field-Effect Transistors (FET) [53]. FET based biosensors offers additional advantages from the traditional electrochemical biosensors by being more sensitive, providing ability of real-time monitoring as well as label-free detection. Ron et al. (2023) [54] developed a novel field-effect biosensor for the active detection of BoNT in test sample. This biosensor was based on FET electrode and its sensing bioreceptor was functionalized with SNAP-25 cleavage site containing peptide elements. The biosensor proved to be highly sensitive in detecting active BoNT/A from 0.5 μL drops of PBS with limit of detection (LOD) in fg/mL range and a linear range extending from 10

Table 2
A comparative analysis of different conventional techniques used for the detection of BoNTs in test samples.

Conventional Detection method	Principle	Experimental design	Limit of detection (LOD) (pg/mL)	Time required (including sample preparation) (hours)	Advantages	Disadvantages	References
Mouse lethality bioassay (MLB)	Sample is injected into the mice and their survival is observed. Sample with BoNT results in their paralysis and death.	*in vivo*	10	48	Assay is highly sensitive and specific. It also increasing the biological relevance of the detection technique as it is based on living cells.	It is strenuous, expensive, complicated and involves ethical concerns with the use of live animals for testing.	[29–31]
SNAP-25 assay	This assay provides direct measure of BoNT activity in sample by detecting and quantifying the cleavage of SNAP-25 protein by BoNTs.	*in vitro*	0.373	24	The assay exhibited specificity, sensitivity, accuracy,	It is highly complex, time consuming, and expensive.	[32]
Mouse phrenic nerve hemidiaphragm (MPN) test	It involves dissection and then electrical stimulation of phrenic nerve attached with the mouse hemidiaphragm. BoNTs if present in the sample will block the release of acetylcholine at neuromuscular junction leading to muscle paralysis. Extent could be measured through visual examination or EMG (electromyography).	*ex vivo*	30–50	24	It exhibits sensitivity, specificity and also mimics physiological response to BoNT	This method is highly complicated, expensive and raises ethical concerns as animals involved.	[33]
Non-lethal mouse flaccid paralysis assay (NFPA)	Suspected samples are intraperitoneally injected to mice and development of flaccid paralysis is observed.	*ex vivo*	10	48	It is non-lethal, relatively quick than MLB.	This method exhibits poor accuracy and cumbersome	[29,34]
Immunoassays	Detection involves usage of BoNT specific antibodies. Interaction could be detected using ELISA or lateral flow assay.	*in vitro*	3–17	24	These are highly specific, sensitive and give fast response time.	They can exhibit cross-reactivity and require specialized equipment and expertise.	[35]
Cell-based assay	This assay is based on the use of living cells like neuron or muscle cells for detection of the BoNT through cellular response.	*in vitro*	2–3	–	It provides biologically relevant results. A broad range of BoNT concentrations can be detected.	It requires specialized cell lines, culture conditions. Moreover, standardization of cell-based assays is difficult to achieve.	[30]
Endopeptidase mass spectrometry assays	These assays are based on endopeptidase activity of BoNT to cleave BoNT specific peptide substrate. The generated cleavage product are then analyzed by mass spectrometry.	*in vitro*	0.1–1000	24	They are highly specific in detecting unique cleavage products. Can detect low concentrations of BoNT. Multiple peptide substrates can be used in a single assay.	They are complex, time consuming and expensive.	[36,37]
Nucleic acid-based methods: Polymerase chain reaction (PCR)	These methods involve amplification and detection of BoNT specific nucleic acid sequences.	*in vitro*	1–5	24–48	It exhibits high specificity, sensitivity, rapidity and can be easily automated.	These methods are cumbersome, less reliable and require specialized equipment and trained personnel.	[38,39]

fg/mL to 100 ng/mL. The biosensor was able to overcome Debye challenge by using a short peptide bioreceptor and the detection mechanism involved label free sensing through *Meta*-nano-channel (MNC) FET platform. Although this biosensor performed well with the PBS buffer, its efficiency in dealing with complex real-world matrices have not been checked. These complex matrices could bind with FET electrode, generating background signals and even false positive results.

Another all-carbon nanotube based flexible and stretchable FET biosensor was developed by Lee et al. (2018) [55]. The developed sensor was highly sensitive and rapid as it was able to detect 52 fM of BoNT/E−light chain within a minute. The biosensor used layers of carbon nanotubes immobilized over an elastomer with FET comprised of p-type channel with electrodes. The device was able to detect cleavage of unique peptide on the specific binding of BoNT/E light chain with specific antibody. The biological signal is converted to measurable electrical signal by FET system. However, the performance of biosensor was not tested with complex matrices that could affect the interaction of antibody and peptide. Also, the detection principle relied on the ability of BoNT/E to cleave unique peptide sequence and the detection could be affected in the presence of partially degraded, misfolded, or inactive form which could lead to false negative results. But still, the developed biosensor has the potential to be used as a wearable and portable biosensor for real-time detection and monitoring of botulinum neurotoxins.

Moreover, graphene oxide-gold based electrochemical biosensor was developed by Chan et al. (2015) [56] for the detection of enzymatic activity of light chain of BoNT/A for early diagnosis and prevention. Principle of this biosensor involved detection of enzymatic activity of

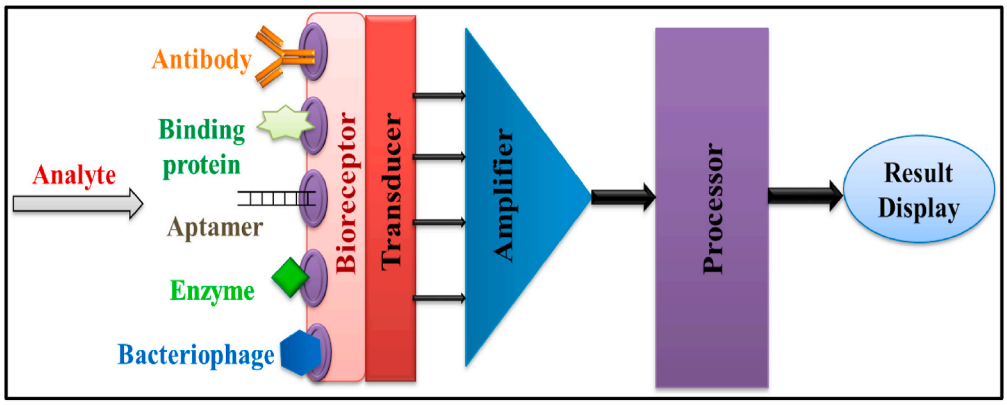

Fig. 3. Diagrammatic representation of working and principle of a typical biosensor.

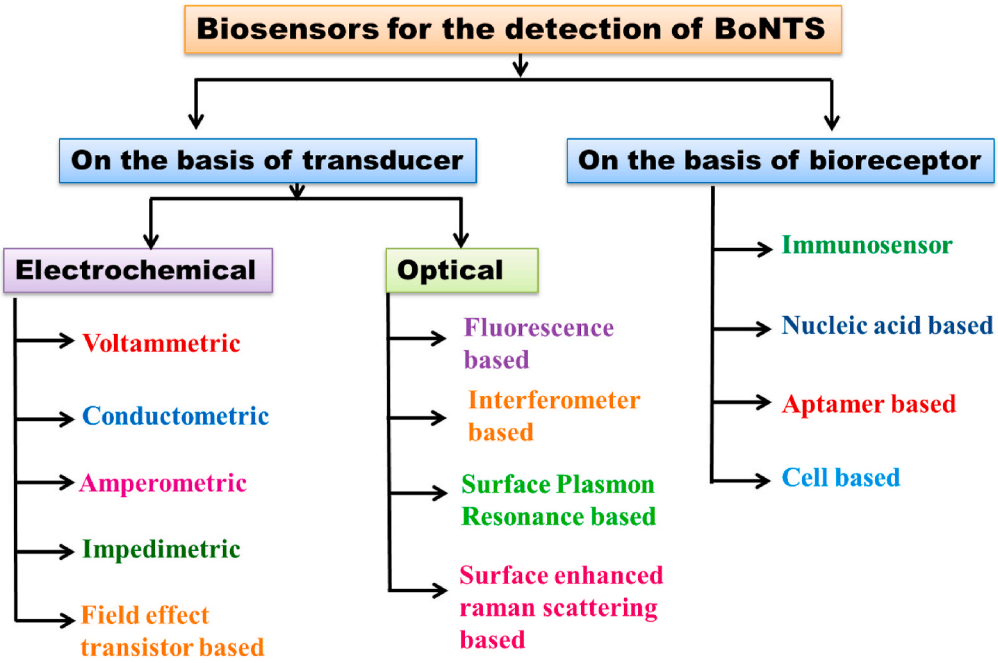

Fig. 4. Schematic depiction of different biosensors for the detection of BoNTs.

BoNT/A through its ability to cleave the immobilized SNAP-25-GFP peptide substrate at specific cleavage sites as illustrated in Fig. 5. SNAP-25-GFP was cross-linked with reduced graphene oxide/gold working electrode by using pyrenebutyric acid. The cleavage by BoNT/A caused release of a section of the peptide from the electrode surface which in turn resulted in the reduction of hindrance of redox probes. The reduced hindrance now generated increased electrochemical currents, which were detected through DPV. The biosensor was able to achieve a detection limit of 1 pg/mL and a linear range from 1 pg/mL to 1 ng/mL. They had also explored the feasibility of the designed biosensor in detection of BoNT/A light chain (BoNT LcA) in milk samples.

Electrochemical aptasensors are also gaining popularity nowadays. For instance, Yadav et al. (2024) developed an aptamer/rGO/SPGE aptasensor for the detection of BoNT/A in the food samples [57]. The basic detection mechanism relied on the hindrance of redox reactions due to the binding of aptamer with BoNT/A which led to a measurable decrease in the current. The biosensor was able to achieve a LOD of 1pM, linearity range of 1–100 pM with a response time of 6 s and stability for over 26 days. The high sensitivity was achieved due to high surface area and electron transfer properties of rGO and high specificity of aptamers used. The study was highly efficient and was tested in six different real food matrices. Also, the use of disposable screen-printed gold electrode (SPGE) made the study very economical and portable for use in low-resource settings. The sensor could further be tested in practical settings to ensure its robustness.

3.1.1.1. Voltammetric electrochemical biosensors for the detection of BoNTs. Biosensing through voltammetric biosensors involves detection of electrochemical reactions occurring at the electrode surface due to interaction with the target analyte. Based on this principle, a novel electrochemical immunosensor was developed by Parvin et al. (2022). The novel biosensor was fabricated for the simultaneous detection of BoNT/A and BoNT/E using a nanomagnetic immunosensing platform [58]. The biosensor used polystyrene-polydopamine microspheres to bind antibodies and redox probes (Cadmium (Cd^{2+}) and silver nanoparticles) and a screen-printed carbon electrode. The magnetic nanoparticles modified with boronic acid were used for immobilization of the polyclonal antibodies that increased the surface area and porosity, hence improved the sensitivity of immunosensor. The direct detection of metal ion labels was done by using square wave voltammetry and was able to detect BoNT/A and BoNT/E at low concentrations of 0.04 pg/mL and 0.16 pg/mL respectively. The dynamic range obtained lied between 0.1

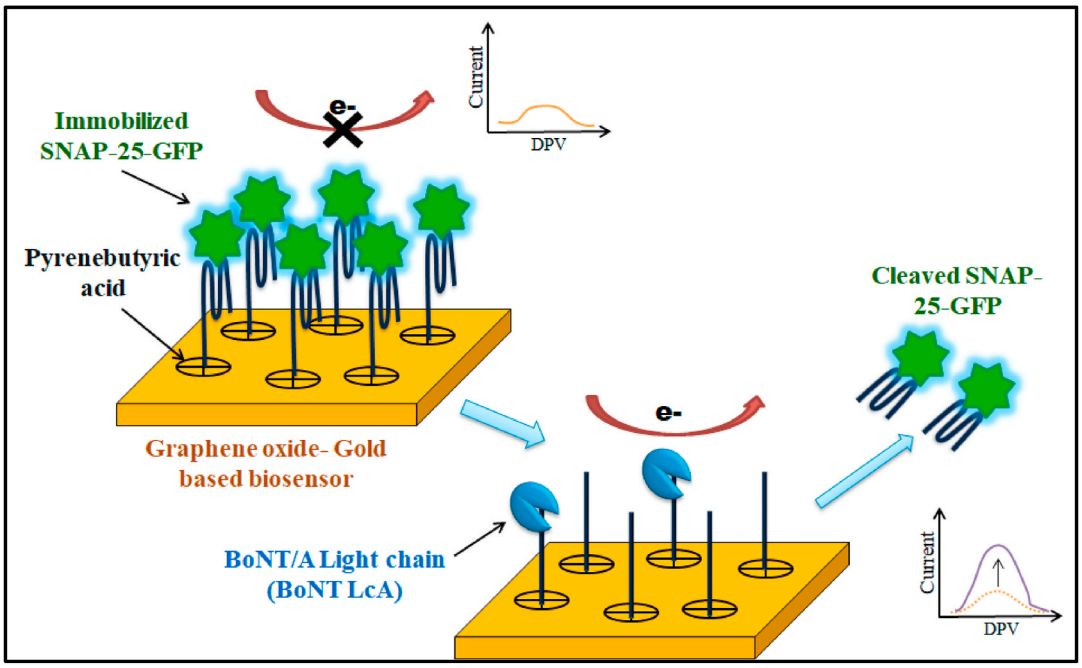

Fig. 5. Diagrammatic representation of graphene oxide-gold based biosensor depicting cleavage of SNAP-25-GFP peptide substrate by BoNT LcA generating electrochemical signal.

and 1000 pg/mL for BoNT/A and 0.5–1000 pg/mL for BoNT/E. The developed immunosensor represent a significant development in the biosensing field with enhanced sensitivity, specificity, stability and reproducibility. But the immunosensor need to be tested for long-term storage stability and environmental concerns with the use of cadmium nanoparticles should not be ignored.

In another study, Narayanan et al. (2015) developed an electrochemical biosensor for the detection of BoNT/E [59]. The GCE (glassy carbon electrode) used as the sensing platform was modified by electrografting a mixed monolayer of phenyl and aminophenyl (Ph-PhNH2/GCE) using diazotization reaction and graphene nanosheets were attached to the electrode surface. The signal amplifiers included enzyme-induced silver nanoparticles (AgNPs) deposited on gold nanoparticles (AuNPs). The biosensor utilized the immunoassay format as explained in Fig. 6 and for the detection 3-indoxyl phosphate (3-IP) was used as the substrate. 3-IP reduces silver ions and the deposited AgNPs were detected through linear sweep voltammetry. The biosensor was able to detect BoNT/E in spiked food samples (orange juice and milk) with a total analysis time of 65 min with a detection limit of 5 pg/mL and a linear range of 10 pg/mL to 10 ng/mL.

Park et al. (2015) exploited the proteolytic activity of BoNT/E along with an external peptidase L-leucine-aminopeptidase (LAP) to fabricate a novel electrochemical method for detecting the Botulinum neurotoxin type E (BoNT/E) [60]. The biosensor was based on two step proteolytic cleavage process in which firstly BoNT/E cleaved a peptide bond between arginine and isoleucine in the oligopeptide-4-amino-1-naphthol (oligopeptide-AN) and secondly LAP cleaved isoleucine residue, generating isoleucine-AN (Ile-AN). The AN thus generated undergoes electrochemical–chemical–chemical (ECC) redox cycling as shown in Fig. 7. The generation of naphthoquinone imine (NQI) on reaction with $Ru(NH_3)_6^{3+}$ and regeneration of AN results in the signal amplification. The signal was detected through measuring increased absorbance at 405 nm indicating cleavage of the isoleucine residue. The BoNT/E-LC was detected at concentrations ranges of 2.0 pg/mL, 0.2 ng/mL, and 3 ng/mL after 4 h, 2 h, and 15 min of incubation in phosphate buffered saline (PBS) buffer, respectively, indicating the highly sensitive and selective fast detection. The biosensor was able to detect the presence of BoNT/E by using unmodified ITO electrodes in PBS buffer and real-world samples of bottled water.

3.1.1.2. Amperometric electrochemical biosensors for the detection of BoNTs. Amperometric biosensors are based on the principle of amperometry which includes detection and measurement of current produced in an electrochemical reaction on the application of a constant potential [61]. Biorecognition element is immobilized on the working electrode where the electrochemical reaction takes place. In order to enhance the properties of electrodes, electrodes modified with nanomaterial are receiving considerable attention. Liu et al. (2014) in a study reported the use of multifunctionalized gold nanoparticles (AuNPs) in a glassy carbon electrode (GCE) based electrochemical immunosensor for the detection of BoNT/A [62]. AuNPs were modified with diazonium salt couplings with protruding functional groups served as electronic bridges and signal amplifiers as depicted in Fig. 8. Sensitivity of the designed biosensor was further enhanced using bioconjugate particles containing horseradish peroxidase (HRP) labels together with the detection antibodies (AbL) connected to AuNPs. The immunosensor was able to achieve LOD of 1 pg/mL and a linear range of 4–35 pg/mL. This sensing strategy was rapid and robust with a detection time of 10 min. The developed biosensor was found to be highly sensitive with good recoveries of BoNT/A for spiked milk samples ranging from 92 to 100 %.

3.1.1.3. Impedimetric electrochemical biosensors for the detection of BoNTs. Impedimetric biosensors measure the response of electrical impedance of a system by applying an alternating current (AC) signal [63]. Here the change in impedance in the form of resistance and capacitance indicates interaction between the analyte and the biorecognition element. Using this principle, Sorouri et al. (2017) fabricated an immunosensor to offer simple and sensitive detection of BoNT/A in real samples of milk and serum [64]. The LOD indicative of high sensitivity was found to be 0.15 pg/mL and a linear relationship between $\%\Delta RCT$ and the concentration logarithm of BoNT/A was observed in the range of 0.2–230 pg/mL. They used nanocomposites of electrosynthesized gold nanodendrites and chitosan nanoparticles on a screen-printed carbon electrode (SPCE). Over the SPCE BoNT/A polyclonal antibody was attached and the immuno-interactions between antibody and BoNT/A was detected through changes in charge transfer

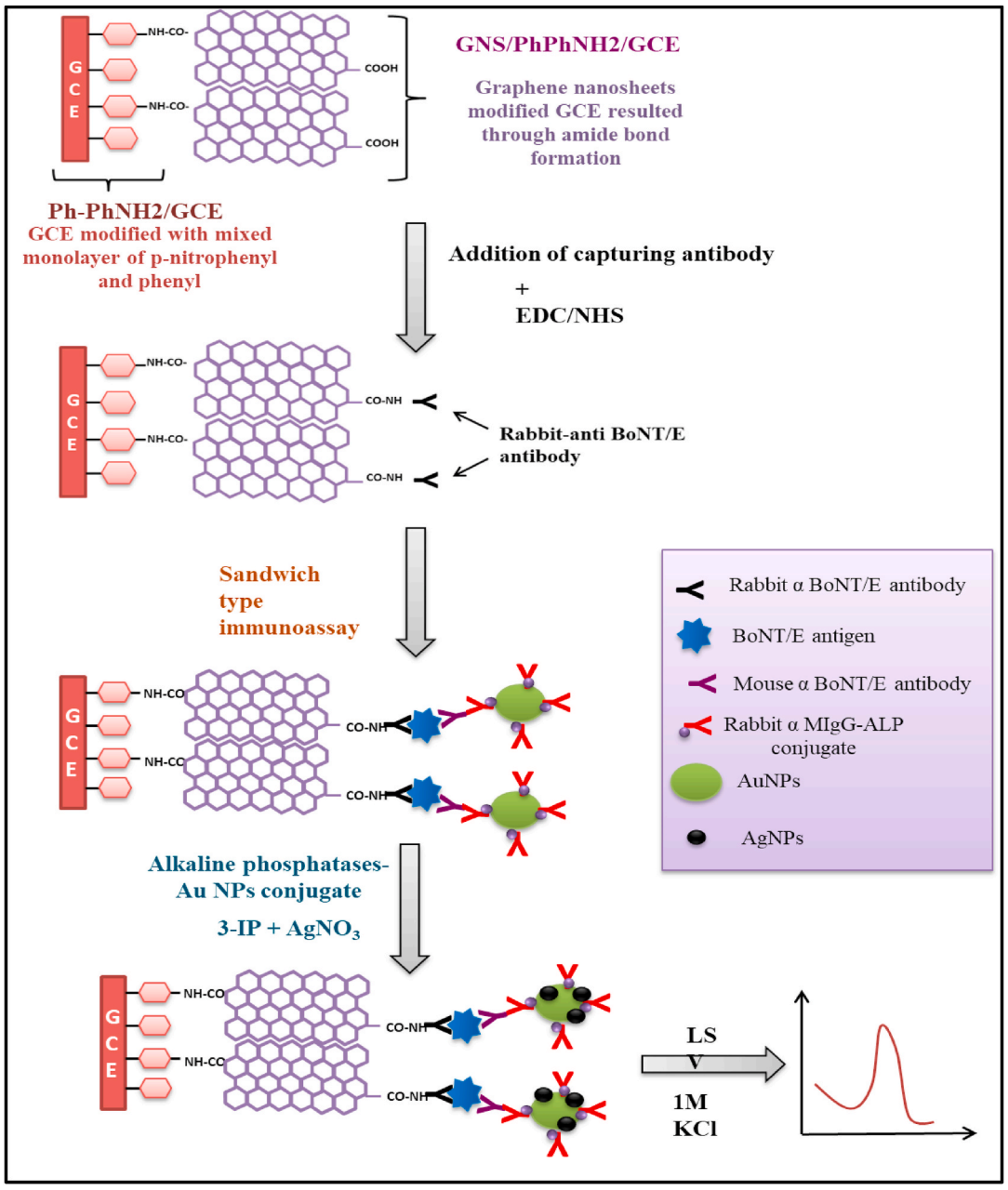

Fig. 6. Diagrammatic representation of voltammetric immunosensor for the detection of BoNT/E.

resistance (RCT) at the immunosensor surface. The immunosensor was characterized using cyclic voltammetry (CV) and Electrochemical impedance spectroscopy (EIS). The fabrication and working of biosensor are depicted in Fig. 9.

Savage et al. (2015) in a different study developed two electrochemical impedance-based biosensors. They used SNAP-25 and VAMP, types of SNARE proteins involved in neurotransmitter release [65]. The proteins were immobilized over gold electrodes to offer specific detection of different kinds of serotypes of Botulinum neurotoxins ranging from BoNT (A-E). The designed biosensors were able to detect active concentration of BoNTs at event low level of 25 fg/mL. Impedance-based electrochemical biosensors can offer a fast response for the detection of active botulinum toxin. However, for testing of other samples like body fluids or food samples certain purification steps like chromatography and centrifugation are to be included to remove any non-specific protease if present.

3.1.2. *Optical biosensors for the detection of BoNTs*

Optical biosensors are the advanced analytical devices that utilize optical transducers for the analysis and quantification of analyte [66]. Optical biosensors are being used for detection of target analytes including BoNTs in various products. They are highly specific, sensitive, small sized and cost-effective [67]. Optical biosensors on the basis of the transducer used can be classified as fluorescence-based, SERS-based, Surface Plasmon resonance-based biosensors, interferometers etc. [68]. Another way of classifying optical biosensors is by categorizing them as label-based and label-free. The label-based biosensors use colorimetric or fluorescent label for the detection of analyte and measure the change in intensity or colour. While no labels are required in case of label-free biosensors and the direct interaction of the target analyte with the transducer is detected in the form of a detectable signal [69]. Various scientists have detected BoNTs by using different optical biosensors. In this review article we have discussed biosensors based on different optical approaches such as interferometers, fluorescence-based, Surface

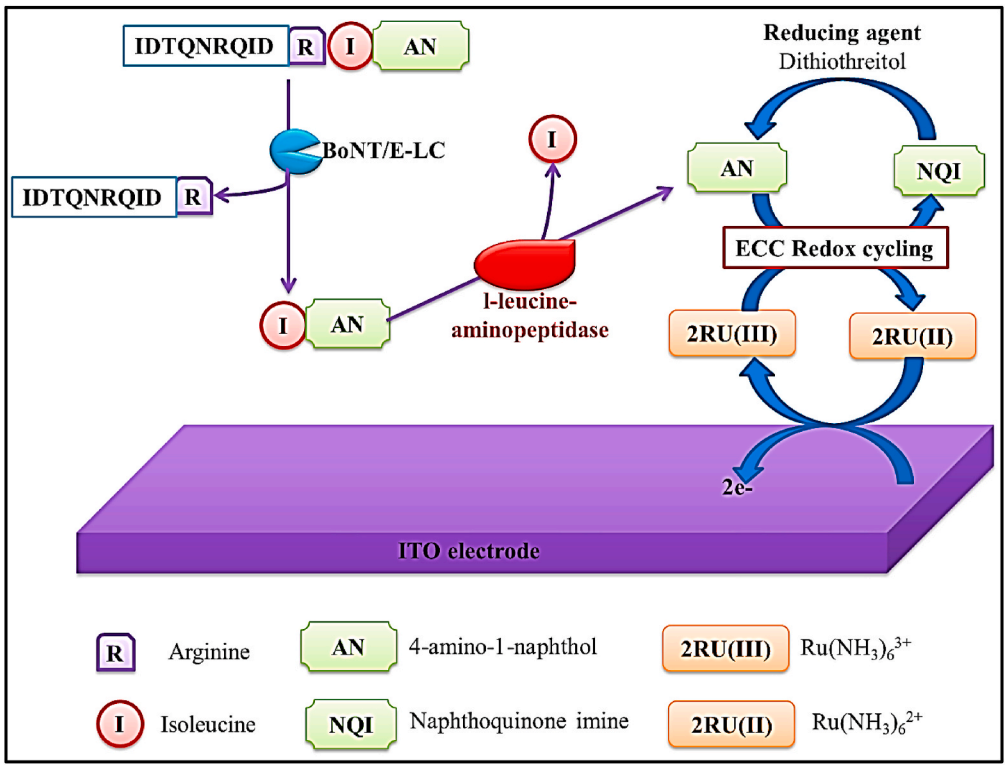

Fig. 7. Schematic representation of mechanism of detection of BoNT/E through enzymatic cleavage and electrochemical–chemical–chemical (ECC) redox cycling by voltammetric biosensor.

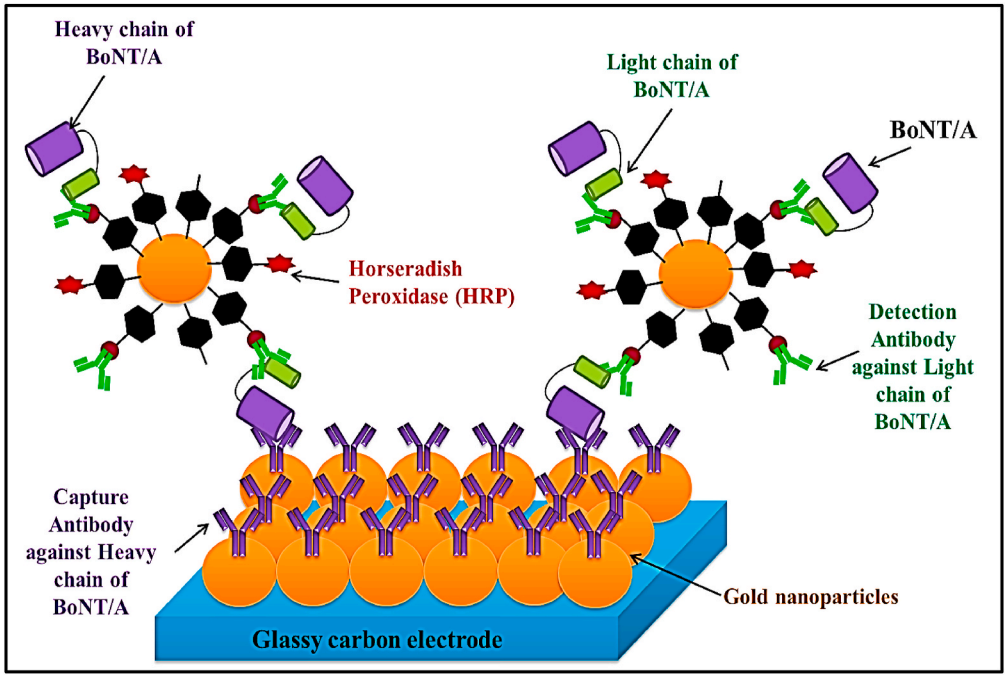

Fig. 8. Diagrammatic representation of mechanism of electrochemical immunosensor based on modified gold nanoparticles detecting BoNT/A in spiked milk sample.

Plasmon Resonance-based, Surface-enhanced Raman scattering (SERS)-based.

3.1.2.1. Interferometer-based optical Biosensor for the detection of BoNTs. An interferometer is a device that can split a beam of light and create interference pattern. Interferometer in optical biosensor is used to detect changes in the optical properties of the sensor caused by the presence of the target analyte [68]. A reflective-based biosensor for the detection as well as estimation of the activity of BoNT/C was developed by Kumar et al. (2022). This optical assay employed competitive immunoassay approach for the detection of BoNT/C and used two adjacent porous Si Fabry–Pérot interferometers [70]. This biosensor detected changes in reflectivity associated with the cleavage of specific peptides by BoNT/C.

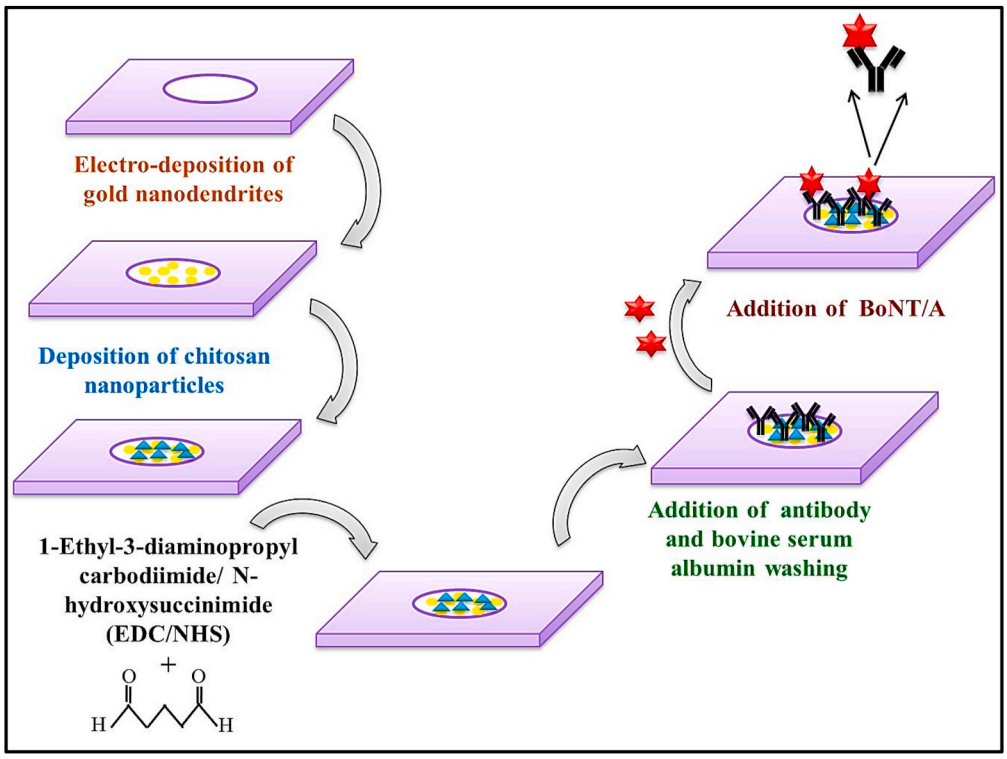

Fig. 9. Schematic illustration of mechanism of impedimetric immunosensor for the detection of BoNT/A.

The sensitivity of biosensor was further enhanced by amplifying reflectivity signals from each interferometer, hence achieving a detection limit of 4.24 pg/mL. This designed biosensor addressed the problems of *in vivo* assays and provided a promising on-site assessment of botulinum outbreaks.

Prior to this Kumar et al. (2021) had also fabricated a label-free biosensor based on a Fabry–Pérot interferometer that was fabricated with porous silicon based electrode for the detection of BoNT/C [71]. The detection mechanism here involved the use of competitive immunoassay and a biochemical cascade reaction which led to the formation of insoluble precipitates within the silicon nanostructure. The insoluble precipitates altered the reflectivity spectra allowing for the minute detection of BoNT/C. The reflectivity spectrum was obtained within visible and near infra-red range. The analysis of the obtained reflectance

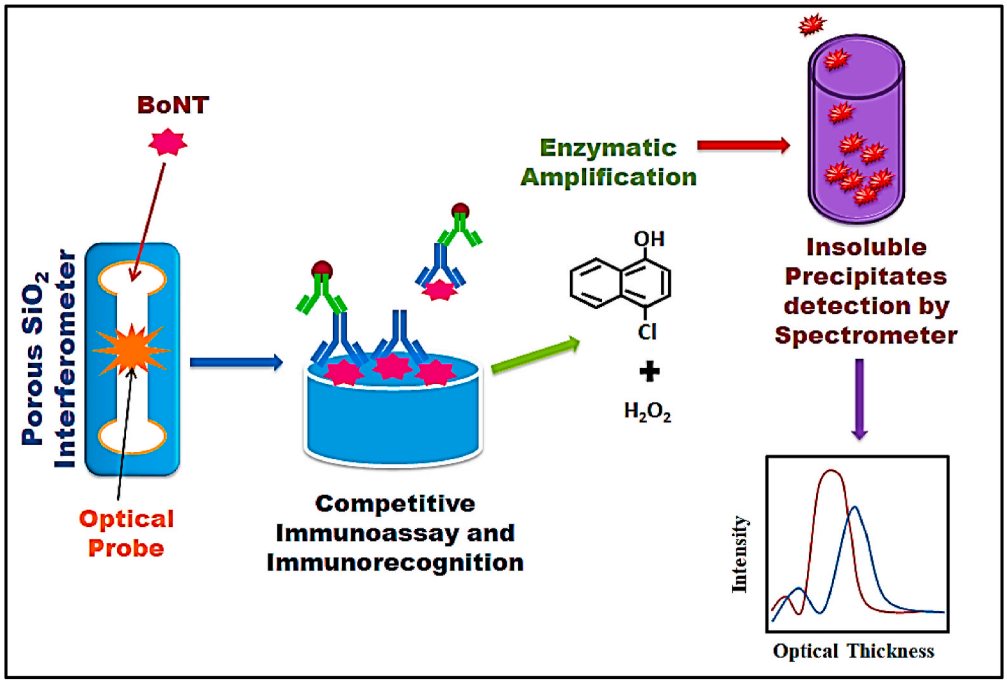

Fig. 10. Diagram illustrating detection mechanism involving competitive immunoassay and subsequent enzymatic amplification using silicon interferometer based optical biosensor.

spectra was done by using fast fourier transformation (FFT). Detection mechanism is further explained through Fig. 10. This biosensor demonstrated high sensitivity with a detection limit of 4.8 pg/mL and a linear response of 10–10000 pg/mL. They had also validated the cross-reactivity, specifically with BoNT/D and had established a foundation for using a miniaturized interferometer for routine on-site botulism detection. Both the samples of BoNT/C and BoNT/D were obtained from the routinely diagnosed animals for botulism from the National Reference Laboratory for Botulism at the Department of Bacteriology in the Kimron Veterinary Institute.

3.1.2.2. Fluorescence optical biosensors for the detection of BoNTs. Fluorescence optical biosensors use fluorophores as labels. The binding of target analyte with recognition element, brings fluorophore into close proximity, resulting in change in the fluorescence signal which could be detected using an optical detection system [72]. In the recent times, aptamer-based biosensors integrated with nanomaterials are being developed as highly sensitive and portable tool for on-site detection of Botulinum neurotoxin. A similar fluorescent aptasensor was developed by Ren et al. (2017). They utilized a novel porous silicon-sol-gel SELEX (Systemic Evolution of Ligands using Exponential Enrichment) and designed ssDNA aptamer specific to BoNT/E [73]. The detection platform was designed by using graphene-oxide matrix. This matrix was immobilized with fluorescently labelled aptamer specific for BoNT/E with low nanomolar binding affinity as shown in Fig. 11. The emitted fluorescence signals were detected at 535 nm. The fabricated aptasensor was able to detect BoNT/E at concentrations even low as 125 ng/mL and functional linear range was obtained up to 5 μg/mL. This aptamer-based biosensor overcame the limitations associated with antibody-based immunosensors that required long incubation time and costly instrumentation.

While addressing ethical concerns as well as to provide a replacement for animal experimentation in the detection of Botulinum neurotoxin, Weingart et al. (2016)developed a nerve cell-mimicking liposome based biosensor for BoNT/B detection [74]. The sample was obtained from a commercial manufacturer and diluted in (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES buffer. The biosensor comprised of a cell-free *in vitro* system to measure the cleavage activity of the BoNT/B light chain in short time as depicted in Fig. 12. Here the operationalized liposomes served as a nanoscaled reaction chambers that allowed BoNT/B detection. Cleavage of PL 150 peptide substrate by BoNT/B light chain resulted in increased fluorescence intensity. This cleavage activity was then recorded by using a FRET-based reporter assay and analyzed by a spectrofluorometer for every 30 s. It was able to measure fluorescence signals at even low concentration of 3.3 pM of BoNT/B.

Similarly, Weingart et al. (2019) in another study developed a nerve cell-mimicking nanoreactors (NMN) biosensor which was integrated into a microfluidic chip. The analyte detected was BoNT/A from the pharmaceutical preparations and detection mechanism involved similar mechanism as mentioned in earlier study that is cleavage of FRET peptide reporter by BoNT/A light chain in the lumen of liposome [75]. The integration of biosensor with microfluidic chip resulted in the reduction time to less than 10 h and enhanced sensitivity of 16.7 ± 1 pM. The limit of detection obtained was nearly 29 pg (dissolved in 10 μL, 2.9 ng/mL).

A simplified fluorescent based biosensor detection platform fabricated by Sapsford et al. (2008) demonstrated novel use of phosphor-based electroluminescence (EL) strips for the spatial excitation of a surface along with charge-coupled device (CCD) as a point of care device for BoNT/A detection [76]. This FRET activity assay used FITC/DABCYL-SNAP-25 peptide substrate which remained quenched due to the nearby placed 4-(dimethylaminoazo) benzene-4-carboxylic acid (DAB-CYL). The peptide when cleaved by BoNT/A emits an increased fluorescent signal as now the quenching of fluorescein thiocarbamoyl (FITC) fluorescence is removed. This emitted fluorescence was detected by EL-CCD detector. Detailed mechanism is explained in Fig. 13. The limit of detection ranged between 0.625 and 1.25 nM for light chain A and 0.313 nM for full toxin, BoNT/A. The use of EL semiconductor strip simplified the need of complex optical systems and provided with a sensitive and user-friendly point of care diagnostic device.

3.1.2.3. Surface Plasmon resonance biosensors for the detection of BoNTs. Surface Plasmon resonance (SPR) is an optical sensing technique that detects the alterations in the intensity and strength of reflected light hence assessing the binding of a molecule at the metal surface [77]. SPR biosensors enable label-free detection of BoNTs. Based on this principle, Huong & Nhiem (2023) developed gold nanoparticle (AuNP) Langmuir–Blodgett (LB) film biosensor, which was capable of detecting Botulinum neurotoxin at even low concentrations of 1 pg/mL [78]. They utilized UV–Visible spectroscopy to record the shifts in the Localized

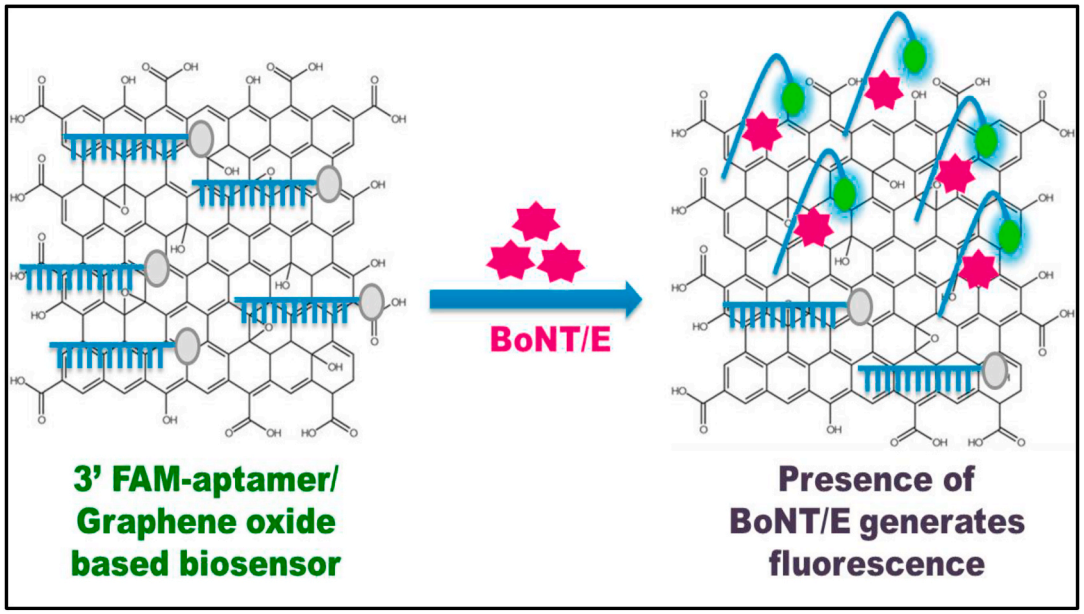

Fig. 11. Diagrammatic representation of BoNT/E detection by using fluorescent tagged aptamers immobilized on graphene oxide matrix.

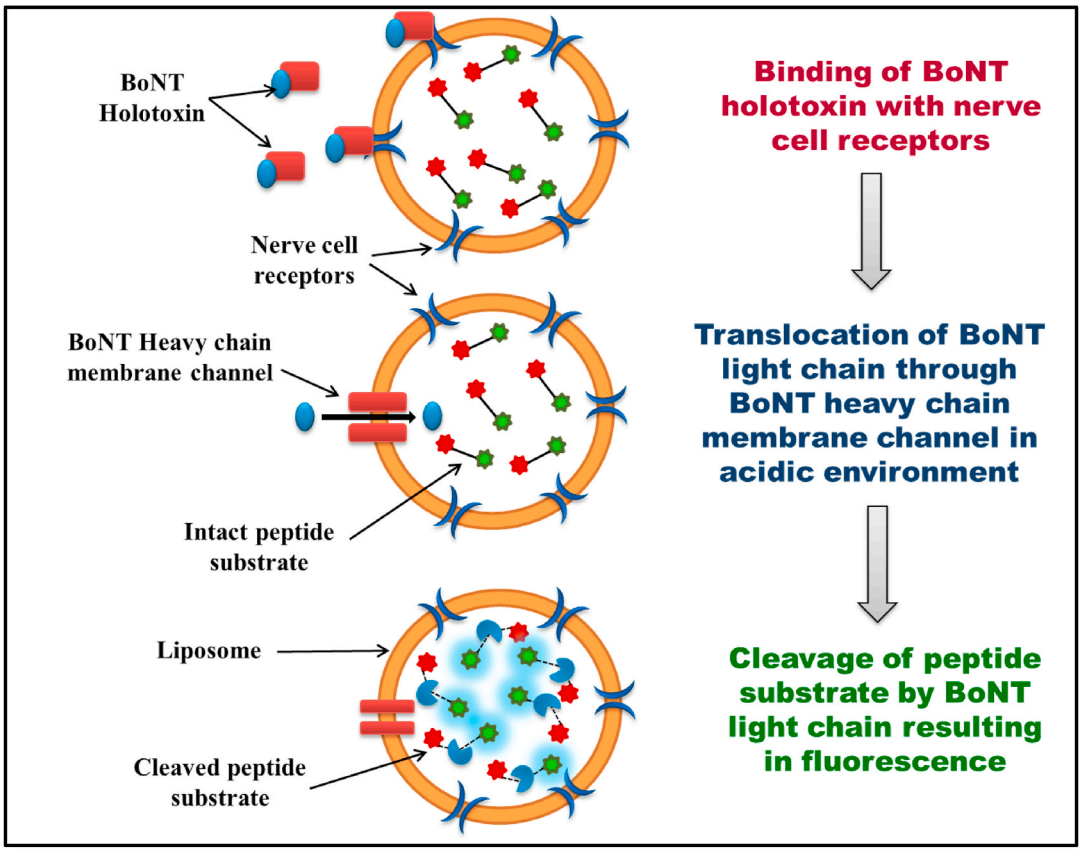

Fig. 12. Schematic representation of nerve cell-mimicking liposome-based biosensor for BoNT/B detection.

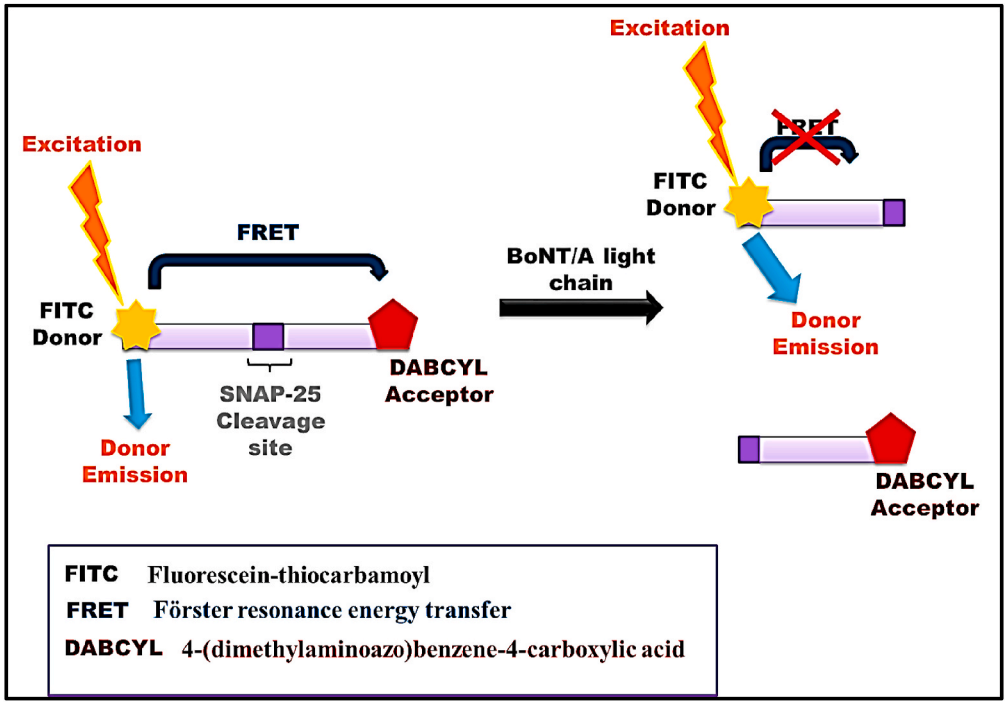

Fig. 13. Diagrammatic illustration of mechanism of BoNT/A detection using FRET activity assay by EL/CCD based biosensor.

Surface Plasmon Resonance (LSPR) signals due to the binding of BoNT molecules to the AuNP LB film surface as depicted in Fig. 14. This biosensor held numerous advantages including easy fabrication, quick detection as well as high sensitivity. This research suggested the importance of controlling nanoparticle distribution and density on the substrate as with the formation of AuNP clusters sensing capacity of LB films tend to reduce.

A novel and highly sensitive method for the detection of BoNT/A

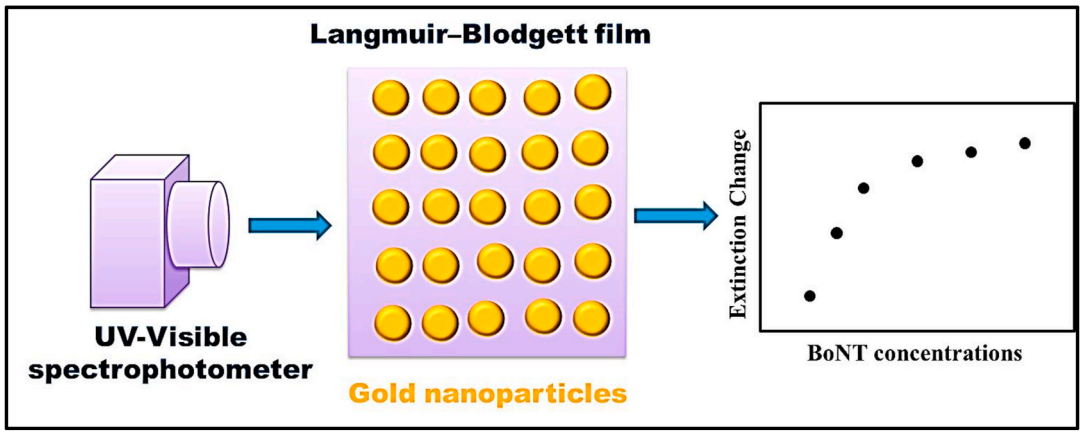

Fig. 14. Diagrammatic representation of Localized Surface Plasmon Resonance based optical biosensor fabricated with gold nanoparticles/Langmuir–Blodgett film.

based on a glycolipid chip with double layer of gold nanoparticles was developed by Uzawa et al. (2023). The designed biosensor was able to detect BoNT/A in the range of 5–50 ng/Ml by using principles of LSPR [79]. The biosensor was highly specific and sensitive because of the immobilized ganglioside GT1b specific to BoNT/A.

Another noteworthy SPR assay for BoNT detection was developed by Patel et al. (2017). The fabricated waveguide SPR biosensor was based on gold electrode and used nanoparticle waveguide SPR system for sensing BoNT/A [80]. The activity of light chain of BoNT/A was separately confirmed using FRET peptide substrate. The detection was carried out by using BoNT specific gold-labelled cleavage substrate. The change in the mass of cleaved and uncleaved peptides substrate caused shifts in the refractive index that led to differences in SPR signal. The SPR signals were then recorded by using Newton Photonics (NP) proprietary waveguide-SPR sensor in the form of sensograms. The biosensor was able to detect BoNT/A samples at even low concentrations of 6.76 pg/mL with a detection time of less than 20 min and the detection sensitivity similar to the traditionally employed mouse LD50 bioassay.

Specific immunosensors are also being developed for providing sensitive and specific detection of BoNTs. The use of antibodies is very critical in the development of such biosensors. Polyclonal antibodies (pAbs) produced from different B-cell clones have multiple epitopes with strong affinity to the target analytes. They are relatively faster and cheaper to produce but can lack uniformity and have less specificity due to the potential cross-reactivity with non-target proteins [81]. On the other hand, monoclonal antibodies (mAbs) produced by single B-cell clone offers exceptional specificity by reducing the chances of cross-reactivity [82]. Hence, the choice between pAbs and mAbs or their combination depend on the specific requirement of biosensor. For example, pAbs in particular are useful in detecting minute levels of BoNTs due to their broad epitope recognition and mAbs are advantageous in detecting specific BoNTs serotype. In a study, Lévêque et al. (2015) had also described an integrated optical on-chip method for the simultaneous detection of BoNT/A and BoNT/E with high accuracy. They developed a monoclonal antibody, mAb11C3 specific to the cleaved product of SNAP-25 [83]. The His-SNAP25 was adhered by

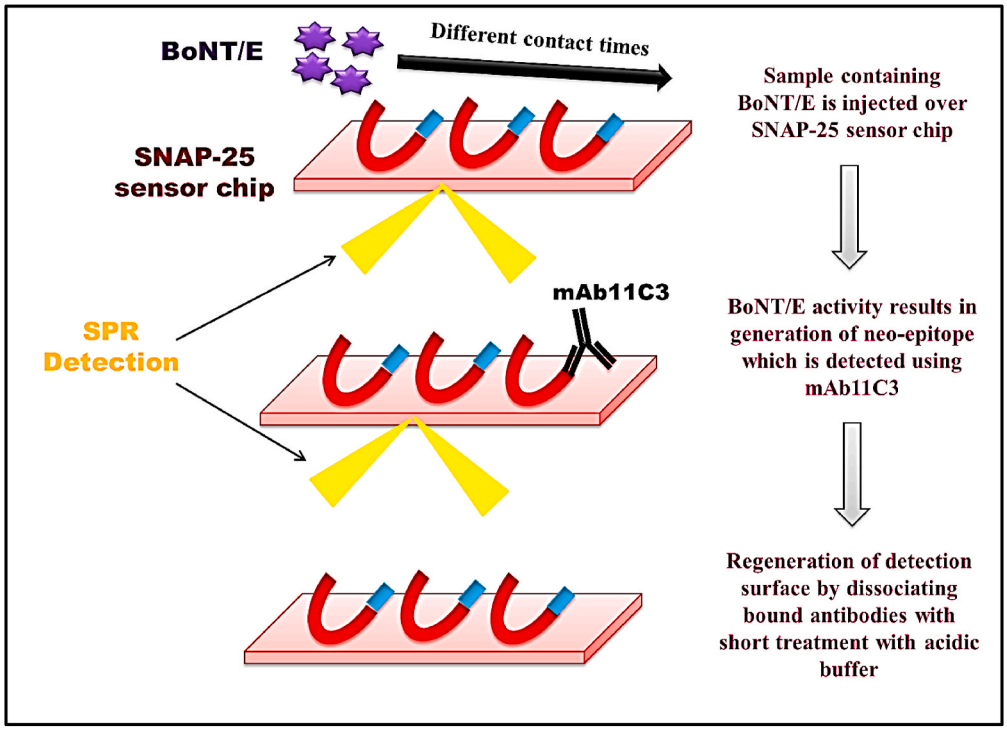

Fig. 15. Schematic depiction of the principle of detection of BoNT/E and BoNT/A through SPR based biosensor.

amination on ProteOn GLC (BioRad) sensor chips. The antibody was further used to detect the appearance of the new epitope created by the cleavage of His-SNAP25 by BoNT/E as depicted in Fig. 15. The binding of antibody was further confirmed through increased resonance units (RU) by SPR. The biosensor was able to detect BoNT/E activity in serum samples at 1 LD50/ml in few minutes. This assay was found to be nearly 10 times more sensitized than the existing conventional *in vivo* detection methods. However, the use of antibodies come with their own set of challenges, including chances of disruption of 3D structure of antibodies by certain chemical and physical conditions of biosensor which could in turn reduce the specificity and sensitivity of biosensor.

3.1.2.4. Surface-enhanced Raman scattering (SERS) based biosensors for detecting BoNTs. Raman scattering is a spectroscopic phenomenon that gives the information regarding the vibrational modes of molecules [84]. SERS in turn enhances the Raman signal of the adsorbed molecule on certain metallic nanostructures. SERS-based biosensors have the potential of detection of a single target molecule with great precision and high sensitivity [85]. However, the detection of a mix of analyte is usually challenging through the SERS technique. Recently, SERS-based biosensor was developed by Ambartsumyan et al. (2024) for the detection of BoNT/A. The biosensor involved the use of an aptasensor specific to BoNT/A for the rapid and precise detection in short time of 1 h. The limit of detection was obtained to be 2.4 ng/mL [86].

In another study, Subekin et al. (2023) fabricated an aptamer-based SERS biosensing device for detecting BoNT/A to overcome the shortcomings of existing SERS based biosensor. The sensor utilized a silver nanoisland substrate operationalized with an aptamer conjugated with a Raman label [87]. The binding of BoNT/A with the aptamer disrupted the orientation of the Raman label that caused changes in the intensity of SERS. The SERS spectra were measured using optical scanning microscope based on the RamanLife RL532 spectrometer. The intensity changes were then detected in a one-stage manner with a LOD of 2.4 ng/mL. The sensor was highly sensitive and provides for a quick and easy detection technique by removing the additional staining or amplification steps. Table 3 depicts the merits and demerits of different types of biosensors used for detection of BoNTs in foods. The use of a specific biosensor for the detection of BoNTs is influenced by these merits and demerits.

3.1.3. Miscellaneous biosensors for the detection of BoNTs

3.1.3.1. Cell based biosensors for detecting BoNTs. These biosensors are innovative analytical tools utilizing living cells as the sensing element to recognize and respond to specific analytes through different cellular processes [91]. The traditional biosensors offer limitations in form of long experiment times, high-priced equipment and the incompatibility to be used with complex food materials. Tam et al. (2018) reported the use of Cellular Analysis and Notification of Antigen Risks and Yields (CANARY®) Zephyr, a B-cell based biosensing device for detecting BoNT/A [89]. The assay utilizes the principles of immunoprecipitation and subsequent influx of intracellular calcium to activate the aequorin molecules. The luminescence is detected by a luminometer confirming the presence of neurotoxin. The mechanism of action is depicted in Fig. 16. The assay demonstrated a detection limit of 10.0 ± 2.5 ng/mL for BoNT/A holotoxin. Sensitivity varied across different sample matrices, with LODs ranged for assay buffer: 10.0 ± 2.5 ng/mL; milk matrices: 7.4–7.9 ng/mL; carrot, orange, and apple juices: 32.5–75.0 ng/mL; solid complex foods (ground beef, smoked salmon, green bean baby puree): 14.8–62.5 ng/mL; viscous liquid egg matrix: 171.9 ± 64.7 ng/mL. However, challenges are noted in detection in complex matrices like liquid egg, which required further dilution and optimization. The future improvements in sample processing and the protocols of biosensor operation could further enhance its sensitivity and pave the way for making it more field-deployable and user-friendly.

Table 3
A comparative analysis of merits and demerits of different kind of biosensors utilized for the detection of BoNTs in food samples.

Types of biosensors	Merits	Demerits	References
Interferometer-based optical biosensor	Not requirement of animal experiments, hence preventing ethical issues. Simple and rapid, offering real-time detection. Can quantify hazardous BoNT with high sensitivity. Can be utilized for on-site analysis as based on reflective-based approach.	Could not discriminate different BoNT serotypes. Involves the use of complex instrumentation. Interferences from background noise or non-specific binding could lead to false positive results.	[71]
Fluorescence optical biosensors	Can provide high sensitivity and specificity for accurate detection of BoNT. Can be adapted for various assay platforms like aptamer-based, and liposome mediated.	May be susceptible to background fluorescence. Photobleaching of fluorescent labels always remains a concern. Sample preparation steps are generally complex and time consuming.	[73]
Surface Plasmon resonance biosensors	SPR enables real-time monitoring and detection of BoNT analytes injected over the chip. Allows label-free detection of molecular interactions between immobilized ligand on sensor surface.	Requirement of specialized equipment is there. Complex sample matrix could interfere with the measurements of SPR.	[88]
Surface-enhanced Raman scattering (SERS)	No additional staining procedures required. High sensitivity and specificity. SERS signals remain relatively stable.	Insufficient adaptation for analysis of real samples. On-site monitoring is limited. Requirement of specialized equipments.	[87]
Impedimetric electrochemical biosensors	Rapid and label free detection Real time monitoring Potential to be miniaturized	May lack specificity when dealing with complex food matrices. Optimization of instrument is often time consuming.	[65]
Voltammetric electrochemical biosensors	Highly sensitive High specificity as provided by voltammetric techniques like CV, DPV.	Requirement of specialized equipment. Optimization of experimental conditions is often laborious.	[59]
Amperometric electrochemical biosensors	Real time monitoring Potential to be miniaturized High sensitivity	Interference from electroactive species could disturb electrochemical signals.	[62]
Cell based biosensors	Provides for a biologically relevant detection method. Living cells are highly sensitive for botulinum detection. Could also utilize complex food	These biosensors could be complex to develop and optimize. Sample preparation steps are generally too tedious and extensive.	[89]

(*continued on next page*)

Table 3 (*continued*)

Types of biosensors	Merits	Demerits	References
	matrices after purification.		
Paper based electrochemical biosensors	Low cost Are easy to carry and portable Require minimal sample volume Environmentally friendly	Limited shelf life Are susceptible to environmental factors like humidity.	[90]

3.1.3.2. Paper based electrochemical biosensors for the detection of BoNTs. Paper based electrochemical biosensing devices work on the same principles as of the conventional electrochemical biosensors. The biosensor utilizes paper as a substrate for the immobilization of bio-recognition elements and facilitates capillary action to transport fluids. A conductive material is placed over the surface of paper to serve as working electrode. The paper based analytical devices are easy to use, cheap and provide environmentally friendly analysis. Caratelli et al. (2021) proposed first of its kind electrochemical peptide sensor that utilized a paper-based platform for the detection of botulinum neurotoxin types A as well as C [90]. This biosensor overcame the limitations of traditional antibodies-based immunosensor which were complex and time consuming to produce and provided a sensitive and user-friendly platform with a limit of detection of 10 pM and the linearity range up to 1 nM. The detection mechanism as represented by Fig. 17 involved use of a synthetic peptide (mimicking SNAP-25) labelled with electroactive molecule methylene blue. The labelled peptide is immobilized over paper-based electrode modified with AuNPs. The botulinum neurotoxins present in the sample cleave the peptide, hence removing methylene blue. This led to the reduction in the signal which could be detected through a smartphone assisted potentiostat. The fabricated biosensor was also checked for its on-site applicability and had given a recovery value close to 100 % when used for detecting BoNT in spiked orange juice sample. This developed sensor is a potential tool for on-site and point-of-care detection device in food safety as well as in medical diagnostics.

In another study by Hu et al. (2024), a new cost-effective paper-based biosensor was fabricated for the detection of BoNT/A in complex matrices such as, bottled water, milk, and human serum. The biosensor was based on a two-step enzymatic amplification strategy and electrochemical stripping analysis. SNAP-25 peptide was immobilized over a paper-based electrode for specific binding with BoNT/A [92]. The first enzymatic reaction was triggered with the cleavage of peptide by BoNT/A. The second enzymatic reaction involved catalysis of silver deposition on the gold nanoparticles and graphdiyne composite on electrode by the alkaline phosphatase. This resulted in enhanced signal amplification and sensitivity. The LOD of this biosensor was found out to be 1 pg/mL and a linear range of 5–100 pg/mL. The biosensor also had some limitations for instance, the use of enzymes could hamper its long-term stability and testing of biosensor in highly complex samples containing fatty or acidic components still remains unexplored.

3.1.3.3. Nano-immunosensors for the detection of BoNTs. A nano-immunosensor is a type of biosensor that uses both the principles of nanotechnology and immunology for the precise and specific detection of target analyte or biological molecule. One such nano-immunosensor based on a bead-based diffusometric technique was developed by Cheng et al. (2019) for the detection of BoNT/A. A sandwiched immunocomplex was generated when the fluorescent probe containing secondary antibody was introduced over the bound sample bound to the primary antibodies conjugated with gold nanoparticles [93]. This binding resulted in the change of diffusivity by reducing the Brownian movement, which was detected by the change in fluorescence pattern. The sensitivity of the fabricated immunosensor was of 10 pg/mL and it was able to detect even low concentrations in the range of 0.01–500 ng/mL of the BoNT/A in the samples of whole milk, bovine serum and PBS. The specificity of immunosensors was found to be comparable to that of standard technique of ELISA and was further enhanced by the use of monoclonal and polyclonal antibodies specific to the targeted BoNT/A and SNAP-25 proteins. The nano-immunosensor was able to generate result within 2 min and could be suitable for rapid on-site

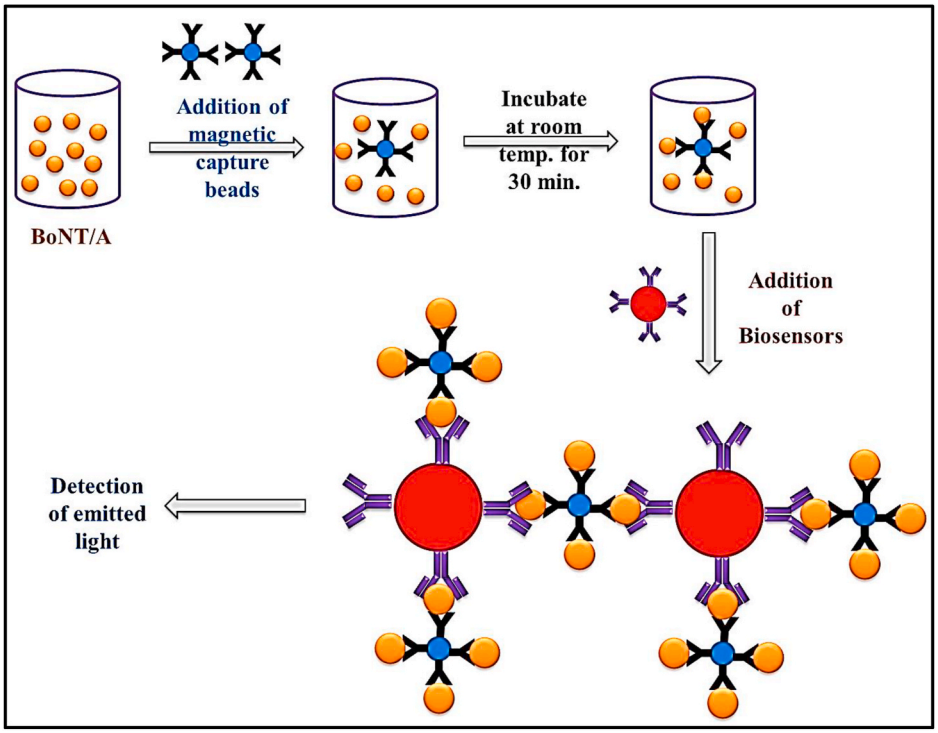

Fig. 16. Diagrammatic illustration of B-cell based biosensor for detecting BoNT/A.

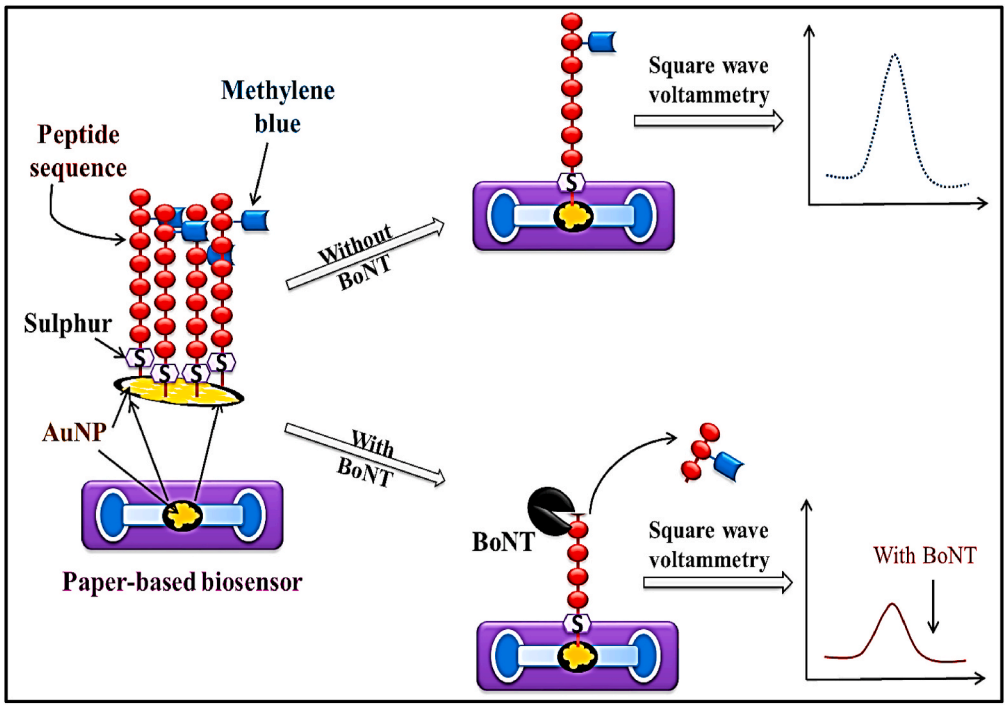

Fig. 17. Schematic representation of principle of paper-based biosensor for the detection of botulinum neurotoxin type A and C.

food-screening and testing for medical instrument hygiene.

Grabka et al. (2023) fabricated a love-type acoustic wave based immunosensor for the detection of BoNT/A light chain in liquid samples. The mechanism involved change in the properties of acoustic waves on the binding of BoNT/A light chain to the immobilized antibodies [94]. The sensor was able to detect even the minute concentration of 500 ng/mL of analyte within 19 min. This study provides a promising approach of using surface acoustic wave-based biosensors for the detection of botulinum neurotoxins. These biosensors would also have potential of regeneration, miniaturization and field applications.

3.1.3.4. Lateral flow assay biosensors for the detection of BoNTs. A portable diagnostic device with multiplexing capability in the form of lateral flow assay biosensor was developed by Wang et al. (2019). The lateral flow assay device was able to detect BoNT/A and staphylococcal enterotoxin B (SEB) from the test samples of spiked milk and grape juice [95]. The fabricated biosensor used magnetic quantum dot nanoparticles-based core satellite structure with Fe_3O_4 core and polyethyleneimine shell and multiple carboxylated quantum dots over the surface. This core structure was responsible for capturing and enrichment of toxins from the sample. The magnetic quantum dots conjugated with BoNT/A and SEB specific antibodies were introduced with toxin sample solution. The formed magnetic quantum dot-toxin complexes were separated with external magnet and suspended in a running buffer. The buffer was then introduced over LFA strip, where the complexes were captured by corresponding secondary antibodies over the test lines, where fluorescent signal was detected. The biosensor was highly sensitive with a LOD of 2.52 pg/mL for BoNT/A. No cross-reactivity was observed with other common food toxins and recovery rates were obtained between 78.8 % and 98.0 %. The entire detection process was cost-effective and time saving with only 30 min of preparation and detection time. The magnetic quantum dot-based lateral flow assay-based biosensor developed in this study have the potential to be used as a portable, POC device with multiplexing ability for rapid on-site detection of BoNT/A and hence ensuring food safety and security.

In a different study, Jia et al. (2022) fabricated a highly sensitive lateral flow immunoassay-based biosensor. The biosensor was based on SiO_2 @ Au nanoparticles and had multiplexing capabilities. It was used for simultaneous detection of three analytes namely ricin, Staphylococcal endotoxin B (SEB), and BoNT/A [96]. The LFA biosensor showed high sensitivity and high specificity for all the three samples and limit of detection was found to be 0.1 ng/mL, 0.05 ng/mL, and 0.1 ng/mL for ricin, SEB, and BoNT/A respectively. The detection was based on the principle of SERS and the detection was completed within 15 min. The developed SERS-LFIA strips based on SiO_2@Au nanoparticles-based biosensor represents significant advancement in the field of food safety and toxin detection. It also has a potential to be used in food safety, and mitigating bioterrorism risks. A comparison of distinct analytical parameters of biosensors employed for BoNTs are depicted in Table 4.

3.2. Cost estimation of existing techniques for BoNT detection

A diverse number of detection strategies are deployed for the detection of BoNTs. These techniques differ in terms of sensitivity, specificity, practicality of use, and affordability. The conventional techniques like MBA were of high cost and involved ethical concerns. ELISA is widely used for food safety assessment as its sensitivity and cost is generally moderate but it would fall short while dealing with clinical samples where the toxin concentration is very low. The other techniques like PCR although shows high sensitivity, is also not cost-effective with the use of specialized reagents and equipment. In contrast to them, biosensors strike a balance between cost and sensitivity, making them ideal for routine and on-site usage.

The cost of generally employed CV and EIS in biosensing is $20 and $24 respectively [16]. Cost considerations also influence the selection of biosensors for example, the paper-based biosensor would be much cost-effective as compared to the one using fluorescence.

Table 4 depicts a comparative analysis of major biosensors reported for the detection of BoNTs. Out of these reported biosensors, the electrochemical biosensor fabricated by Savage et al. (2015) was found to detect minimum concentration of BoNT/A and BoNT/C that is 0.025 pg/mL the biosensor developed by Cheng et al. (2019) took minimum time of only 2 min for detecting BoNT/A. These different biosensors have

Table 4
A comparative analysis of major biosensors reported for the detection of BoNTs.

Types of biosensors	Serotype detected	Detection Principle	Matrix used	LOD (pg/mL)	Working/linear range (pg/mL)	Detection time (minutes)	Stability (days)	Analytical recovery (%)	Types of samples for investigation	Reference
Interferometer-based Optical Biosensor	BoNT/C	Immunoassay	Porous SiO_2	4.24	–	–	–	–	–	[70]
Interferometer-based Optical Biosensor	BoNT/C, BoNT/D	Immunoassay	Porous SiO_2	4.8	10- 10×10^3	90	–	–	Animal samples positive for BoNT/C and BoNT/D	[71]
Fluorescence optical biosensor	BoNT/E	Aptamer based	Graphene-oxide	1.25×10^5	1.25×10^5–5.0×10^6	–	–	–	–	[73]
Fluorescence Resonance Energy Transfer (FRET)-based biosensor	BoNT/A	Förster Resonance Energy Transfer (FRET) activity detection	Nerve cell-mimicking nanoreactors (NMN) integrated into a microfluidic chip	29	–	600	–	–	Pharmaceutical BoNT preparations	[75]
Fluorescence Resonance Energy Transfer (FRET)-based biosensor	BoNT/B	Motoneuronal membrane receptors	Liposome mediate	3.3	–	–	–	–	Pharmaceutical BoNT preparations	[74]
Fluorescence optical biosensor	BoNT/A	Förster Resonance Energy Transfer (FRET) activity assay	Phosphor-based electroluminescence (EL) strips	3.1×10^4	3.1×10^4–6.2×10^4	120–1440	–	–	Pharmaceutical BoNT preparations	[76]
Localized Surface Plasmon Resonance (LSPR)-based biosensor	BoNT/A	Interaction between BoNT and Au NP-capping ligands on the LB films	Langmuir–Blodgett film	1	1–80	–	–	–	Botulinum neurotoxin type A toxoid pharmaceutical sample	[78]
Localized Surface Plasmon Resonance (LSPR)-based biosensor	BoNT/A	Interaction between immobilized ganglioside GT1b and BoNT/A	Glycolipid chip with double layer of gold nanoparticles	5.0×10^3	5.0×10^3 - 5.0×10^4	–	–	–	Pharmaceutical sample	[79]
Surface Plasmon Resonance (SPR)-based biosensor	BoNT/A	Interaction between the toxin and the SPR sensor surface	Gold electrode	6.76	6.0– 6.0×10^6	60	–	–	BoNT/A light chain pharmaceutical sample	[80]
Chip assay based on Surface Plasmon Resonance (SPR)	BoNT/A and E	Monoclonal antibody specifically recognizing SNAP25 cleaved by BoNT/E.	SNAP25 immobilized on a chip	–	–	15–30	–	–	Serum samples containing Botulinum neurotoxins	[83]
Surface-enhanced Raman scattering (SERS)-based biosensor	BoNT/A	Aptamer based	–	2.4×10^3	–	60	–	–	–	[97]
Surface-enhanced Raman scattering (SERS)-based biosensor	BoNT/A	Aptamer conjugated with a Raman label	Silver nanoisland SERS substrate	2.4×10^3	–	60	180	–	Serum samples	[87]
Field-Effect Transistor (FET) biosensor	BoNT/A	Specific and label-free sensing	*Meta*-Nano-Channel (MNC) FET biosensor	–	0.01–1.0×10^6	–	–	–	Active BoNT/A from 0.5 μL drops of PBS	[54]
Electrochemical biosensor	BoNT/A	BoNT-LcA protease activity through the enzymatic cleavage of SNAP-25-GFP peptide substrate.	Reduced graphene oxide (rGO)/Au electrode	1	1.0– 1.0×10^3	–	–	–	Milk samples	[56]
Impedimetric immunosensor	BoNT/A	Immunoassay	Electrosynthesized gold nanodendrites and chitosan nanoparticles (AuNDs/CSNPs) Nanocomposite	0.15	0.2–230	60	4	97.4–103.1	samples of milk and serum	[64]

(*continued on next page*)

Table 4 (*continued*)

Types of biosensors	Serotype detected	Detection Principle	Matrix used	LOD (pg/mL)	Working/linear range (pg/mL)	Detection time (minutes)	Stability (days)	Analytical recovery (%)	Types of samples for investigation	Reference
Electrochemical impedance biosensor	BoNT/A, C, and E for the SNAP-25 sensor, and BoNT/B and D for the VAMP sensor.	Immobilized SNARE proteins SNAP-25 and VAMP	Gold electrodes	0.025	0.025–0.125	–	–	–	Pharmaceutical samples	[65]
Voltammetric electrochemical immunosensor	BoNT/A and BoNT/E	Immunoassay	Screen-printed carbon electrode	BoNT/A- 0.04 & BoNT/E– 0.16	BoNT/A- 0.1-1.0 × 10^3 & BoNT/E– 0.5-1.0 × 10^3	–	–	–	–	[98]
Voltammetric electrochemical immunosensor	BoNT/E	Immunoassay	Glassy carbon electrodes (GCE) modified with graphene nanosheets-aryldiazonium salt	5.0	10- 1.0 × 10^4	65	–	96	Spiked milk and orange juice samples	[59]
Voltammetric electrochemical biosensor	BoNT/E	Cleavage of a peptide bond by BoNT/E-LC and l-leucine-aminopeptidase (LAP)	Indium-tin oxide (ITO) electrodes	2.0	2.0–3.0 × 10^3	15–240	–	–	PBS buffer and bottled water.	[60]
Amperometric electrochemical biosensor	BoNT/A	Horseradish peroxidase (HRP)-labelled detection antibodies (AbL) attached to AuNPs	Gold nanoparticles (AuNPs) modified with multifunctionalized protruding functional groups	1	4–35	10	40	Spiked full milk-92 and Spiked skim milk samples - 95, Phosphate buffer 100	Spiked full milk and skim milk samples	[62]
Paper-based electrochemical peptide sensor	BoNT/A and C	Synthetic peptide mimicking the natural substrate SNAP-25, labelled with methylene blue	Paper-based electrode modified with gold nanoparticles	10	10–1.0 × 10^3	–	21	1 nM BoNT/A = 104 ± 6 and 0.5 nM BoNT/A = 98 ± 9	Spiked samples of orange juice	[90]
Cell-based biosensor	–	Immortal B-cell lines that express antibodies specific to the target analyte	B-cell based biosensor system	7.4 × 10^3	Milk matrices: 7.4 × 10^3- 7.9 × 10^3; Juices: 3.25 × 10^4-7.5 × 10^4; Complex foods: 1.48 × 10^4-6.25 × 10^4	40	–	–	Various complex matrices including milk, juices, ground meat, eggs, and smoked fish	[89]
Nanoimmunosensor	BoNT/A	Fluorescence based	Gold nanoparticles	10	10–5.0 × 10^5	2	–	–	Whole milk, bovine serum and PBS	[93]
Immunosensor	BoNT/A	Detects changes in love-type acoustic wave	Immomibilized antibodies	5.0 × 10^5	–	19	–	–	–	[94]
Lateral flow assay biosensor	BoNT/A	SERS	SiO2 @ Au nanoparticles	Ricin-100, SEB-50, and BoNT/A-100	–	15	–	–	–	[99]
Lateral flow assay biosensor	BoNT/A	Immunoassay	Magnetic quantum dot nanoparticles	2.52	–	30	–	78.8–98	Spiked milk and grape juice	[95]

their own sets of merits and demerits as earlier mentioned in the article. The choice of use of a specific biosensor depends on the specific use case. Overall, integrating advanced molecular technologies with cost-effective biosensors could offer a promising direction for the development of next-generation BoNT diagnostics.

4. Future prospects

Food contaminated with botulinum neurotoxin poses serious threat to human well-being. There is an increasing consumption of commercially produced foods and along with that the risk of botulinum contamination is also increased [100,101]. The biological toxins like botulinum neurotoxin have the potency to cause severe harm and could be easily misused due to their easy availability [102]. Protection of foods from contamination and development of ultra-sensitive biosensing devices is the need of hour. Future research should be undertaken to provide for automated nanomaterial-based devices which could simultaneously detect BoNTs in multiple contaminated food samples. The point of care devices for rapid on-site detection is also needed with the changing lifestyle. Moreover, the use of botox, a neurotoxin drug derived from *Clostridium botulinum* is increasing in therapeutic and cosmetology however, the misuse potential always remains [103]. If the misuse is done by terrorists or military in the form of bioterrorism or biowarfare, it could cause a huge number of causalities [102]. Hence, there is an urgent need of on-site rapid detection of this lethal toxin. Paper-based electrochemical biosensing devices, point-of-care detection, and the development of sensitive diagnostic kits could solve this purpose. The fabricated devices could be linked with mobile phones and utilize artificial intelligence for ultra-selective and sensitive detection of BoNT. The biosensors also have the potential of incorporating quantum dots, lab-on-chip platforms for increasing their versatility. Future research is also needed to make these biosensing devices both economical as well as environmentally friendly.

5. Conclusion

Botulism is one of the lethal and life-threatening disease encountered by mankind. Owing to the potential of BoNT to be used as a weapon of mass destruction, early detection with high sensitivity and accuracy becomes the urgent need of an hour. In this review we had discussed the conventional diagnostic strategies as well as the importance of new age biosensing techniques in botulinum neurotoxin detection and surveillance. The conventional methods involving use of animals though had good sensitivity but were hindered by ethical considerations. While other methods like immunoassays, nucleic acid-based analysis, cell-based analysis, and catalytic activity-based assay were time consuming, complex with limited sensitivity, and specificity. The advent of biosensors paved the way for rapid, sensitive, specific, portable, and cost-effective measure for the detection of BoNT. Biosensors developed on optical, electrochemical and biological principles to detect botulinum neurotoxins with improved sensitivity facilitates real time detection, multiplexing and label free detection. They hold importance in clinical diagnostics, environmental monitoring, food safety, and biodefense. Though optical biosensors offer sensitive and specific detection approach for BoNT detection however, they also exhibit many limitations. They are costly, complicated, require labeling, and can show false positive results. On the other hand, electrochemical biosensors often exhibit higher sensitivity and selectivity by utilizing specific recognition elements like immobilized enzymes and antibodies. Electrochemical biosensors could also be used for real time on-site testing as they could be miniaturized and integrated into compact and portable devices. They require minimal sample preparation and could also be adapted for multiplexed detection of various analytes simultaneously. Overall, electrochemical biosensors are highly attractive in BoNTs detection by exhibiting high sensitivity, selectivity, portability, cost-effectiveness, and multiplexing capabilities.

CRediT authorship contribution statement

Arzoo Saini: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Neelam Yadav:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **Bijender Singh:** Writing – review & editing, Validation. **Jogender Singh Rana:** Writing – review & editing, Validation.

Ethical issues

Not required.

Declaration of competing interest

This work has not been carried out previously and not submitted to any other journal for its publication. All the authors of this manuscript declare no conflict of interest after its publication.

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Data availability

Data will be made available on request.

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