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Mini Review

Clostridium Neurotoxins Detection Systems Based on Biosensors

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Abstract

Clostridium species can produce a wide variety of toxins. They block acetylcholine (ACh) release from presynaptic nerve terminals at the neuromuscular junctions. Botulinum neurotoxin is the deadliest toxin ever known to human knowledge. One of the most serious consequences of these toxins (BoNTA specifically) is flaccid muscle due to defective acetylcholine (ACh) release at the neuromuscular junction (NMJ) as a noticeable clinical symptom, resulting in irreversible botulinum toxicity. Interestingly, cosmetic and therapeutic applications have also been described for this agent. Thus, an urgent need is felt for rapid, precise, and cost-effective detection and real-time monitoring of these toxins. Using biosensors such as optical biosensors, electrochemical biosensors, feedback control systems (FSC) and aptamers is a method for detecting BoNTs. Optical biosensors use optical transducers for detection, which consist of fluorescence biosensors and surface plasmon resonance (SPR) biosensors. Electrochemical biosensors are based on the principle of straight transduction of the reaction rate into a current. They consist of impedimetric, voltammetric, amperometric, and impedance spectroscopy biosensors. Aptamers are oligonucleotide derivatives isolated by the SELEX approach. Aptamers are highly specific, very cost-effective, and quick. In this review study, the most common and effective detection methods, including novel methods, have been described and discussed briefly. In this review, our focus was on the main concepts and applications that have been achieved up to this date to diagnose the in-field botulism.

Keywords: Biosensor, *Clostridium*, Neurotoxin, Application

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Introduction

Botulinum neurotoxins (BoNTs) are produced from various strains of *Clostridium* such as *Clostridium botulinum*.[1] *Clostridium botulinum* is a gram-positive, spore-forming, anaerobic and rod-shaped bacterium.[1,2] BoNTs are the most lethal toxins known to human knowledge and at a dose of as little as 62 ng can kill a human.[3] Defective acetylcholine (ACh) release at the neuromuscular junction (NMJ) is the significant clinical symptom of botulism.[1] BoNTs have eight serotypes (A-H) produced by different strains, each of which cleaves different proteins (SNARE complex) that are necessary for the release of ACh from axon endings, leading to flaccid paralysis.[4] There is no treatment for the reversal of BoNT muscle paralysis so far.[5] Very low doses of BoNTA and BoNTB can cause paralysis, migraine, cerebral palsy, and hyperhidrosis.[6-9]

Despite their hazardous nature, BoNTA and B have cosmetic and therapeutic applications. Their very low LD_{50} dose and ease of production have made them a bioterrorist agent and their detection must be precise and quick.[10,11] Despite ethical issues, the common method for BoNT detection is based on mouse bioassay.[12] The sensitivity for this assay is 1 MLD or 20-30 pg of BoNTA.[13]

Several methods have been proposed to detect BoNT including enzyme-linked immunosorbent assay (ELISA), electro-chemiluminescence, endopeptidase-mass spectrometry (Endopep-MS), immune-polymerase chain reaction (immune -PCR), and protease activity assay, which is the latest introduced method.[14-17] Many authors experimented these methods and found none of them to be the standard assay; so there has been a growing demand to detect them precisely and quickly.[18]

High specific, accurate, and sensitive biosensors have been introduced to determine very low analyte concentrations of toxins in biological samples.[19,20] Biosensor, by definition, is an analytical device, which is made up of biological elements that produce signals (electrical, thermal, or optical)

based on the interaction with a tested element and a transducer that converts these signals into a measurable electrical parameter.[19,21] Since they utilize specific biological elements, biosensors have unique selective properties.[22]

Aptamers are fragments of oligonucleotides that can bind to target proteins in the presence of appropriate conformational change.[23-25] They are simpler to use, reusable, and more stable than antibodies. Recently, through an approach called SELEX (Systemic Evolution of Ligands by exponential enrichment), high affinity single-stranded DNA (ssDNA) aptamers have been generated against aldehyde-inactivated BoNTA.[26] Based on different aptamer designs, the dissociation constant (K_ds) was in the range from 3 nM to 50 nM.[27] In this study, we reviewed the applications of different biosensors for the detection of *Clostridium botulinum* neurotoxins, and then their benefits and limits were discussed.

Detection of Botulinum Neurotoxins Using Biosensors

This technology encompasses a wide spectrum of BoNT detection methods and normally consists of optical biosensors, which involve fluorescence biosensors and surface plasmon resonance (SPR) biosensors, and electrochemical biosensors, which involve impedimetric, voltammetric, amperometric electrochemical biosensors and impedance spectroscopy.[3] A brief review will be given regarding these methods

Aptamer-based Biosensors

Aptamers are not limited to one category of biosensors and can be used in combination with different biosensors. For example, Wei and Ho (2009) combined an electrochemical method with enzymatic amplification and an aptamer probe for the detection of BoNTA.[27] The aptamer is dual-labeled with biotin and fluorescein, which is a reporting and anchoring tag. Furthermore, an anti-fluorescein antibody conjugated to horseradish peroxidase (HRP) was added to bind the fluorescein label to the aptamer. This method can generate an amplified current much better than just using BONTA. Shu Ren et al. used the strategy of novel porous silicon-sol gel (PS-SG) and SELEX to detect aptamers against BoNTE toxoid.[18] They used 10^{15} ssDNA sequences against BoNTE. Then, the sequences were amplified by PCR and potential aptamers were selected. They obtained a linear response in the range of 0.125-5 μg/ml.[18]

Optical Biosensors

Optical biosensors, by definition, are compact analytical devices containing a biorecognition sensing element integrated with an optical transducer system. The basic objective of an optical biosensor is to produce a signal, which is proportionate to the concentration of a measured analyte.[28] The use of these sensors has experienced exponential growth over the last decade. Optical biosensors consist of fluorescent and SPR biosensors, which have been used to detect BoNTs, as described below.

Fluorescence Biosensors

The use of this type of immunoassay dates back to 1975, when Kronick and Little introduced a new technique based on fluorescence excitation.[29] Their method uses internally reflected light to excite the fluorescence of fluorescein-labeled antibody bound to a hapten–protein conjugate absorbed on a quartz plate in the antibody solution. They used it for the detection of morphine which was achieved at the concentration of 2×10^{-7}. Since then, studies have grown around this type of detecting technique, especially in the last decade. Their properties, including high sensitivity and selectivity, have made them a good toxin detector. In addition, due to their high speed, they provide real-time monitoring of analytes,[18] which is a serious issue in current mouse bioassay considering the very low LD50 and high fatality of BoNTs. Besides, researchers have used this biosensor to detect BoNTs.

Sapsford et al. introduced a new method of spatial illumination based on electroluminescence (EL) that combines EL excitation with charge-coupled device (CCD)-based detection of fluorescence for detecting BoNTA. Moreover, they used a Forster Resonance Energy Transfer (FRET) activity assay to characterize and optimize the EL-CCD detector for BoTN-A detection. Toxin detection by this method needed a 24 h incubation and had a LOD of 0.313 nM. They marked the SNAP-25 peptide substrate for this toxin with a FITC donor/DABCYL acceptor FRET pair. The combination of this substrate with the light chain LcA derivative or the full toxin resulted in the isolation of peptide sequence, interrupting the FRET, and providing high FITC donor emission (Figure 1).[30]

Surface Plasmon Resonance (SPR) Biosensors

Surface plasmon resonance (SPR) is a very common technique in the analysis of toxins and microorganisms since the 90's.[31] The most significant feature of this aptasensor is to efficiently differentiate the natively folded toxin from denatured, inactive toxin, which is important for surveillance and threat assessment, hence providing a convenient and easy-to-use biosensor that does not need any marked protein nor fluorescent tags.[32] The mechanism of this biosensor is based on the addition of analyte mass onto the surface of the sensor and modifying the angle of resonance.[22] There are a variety of SPR assays that have been employed for the detection of Botulinum neurotoxin.[33]

Leveque et al. identified a new C-terminal end of SNAP25 resulting from BoNTE with a designed monoclonal antibody, that did not interfere with generated products by other BoNT subtypes. This was a simple and very fast method; it provided BoNTE activity detection at 1 LD50/ml

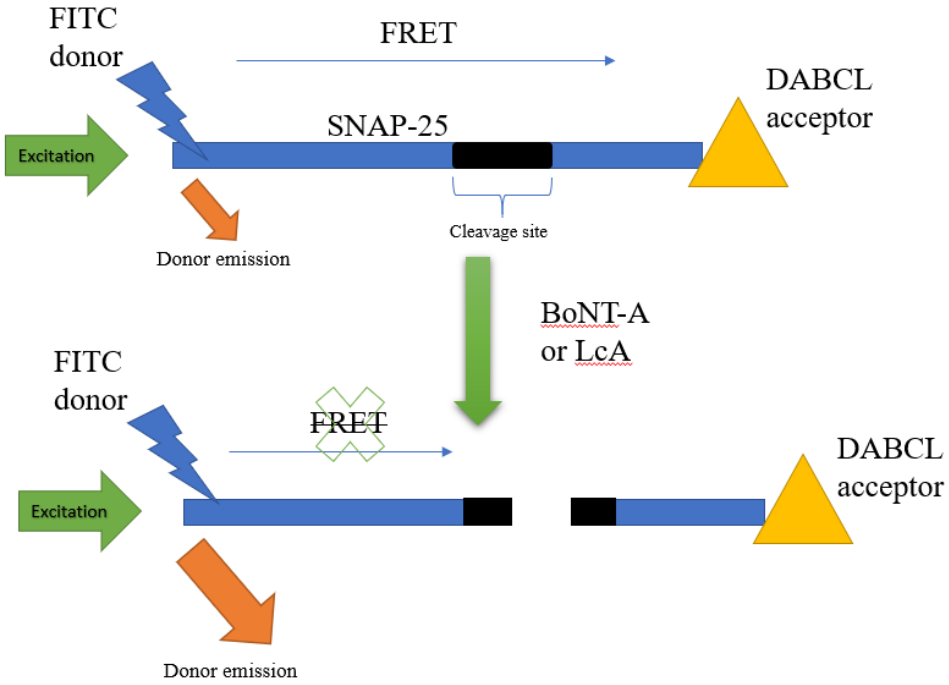

Figure 1. FRET Biosensor Using EL-CCD Detector to Determine BoNT-A or LcA.

in less than 15 min. In addition, the "on-chip" assay could detect BoNTE and BoNTA at the same time.[34]

Ladd et al. introduced a custom-built SPR biosensor for types A, B, and F botulinum toxins. Their biosensor was based on four independent detection channels based on wavelength modulation. A gold-coated prism was used to stimulate a surface plasmon upon incidence with the metal-dielectric interface once the collimated light beam was transmitted through the prism. Types A, B, and F were detected at 1, 1, and 0.5 ng/ml, respectively.[35]

Janardhanan et al. used a SPR-based RNA aptasensor for the fast detection of natively folded BoNTA. By using the DR BoNTA surrogate, they reported the detection limit of the aptasensor in buffered saline, carrot juice, fat-free milk, and 5-fold diluted human serum to be 5.8 ng/ml, 20.3 ng/ml, 23.4 ng/ml, and 22.5 ng/ml respectively. Their work developed the first sensor system for the rapid detection of natively folded toxin.[36]

Electrochemical Biosensors

These sensors are based on the principle of straight transduction of the reaction rate into a current.[37] They give real-time information about the arrangement of a system by joining the recognition element to an electrochemical transducer.[38] The chemical energy of the selective interaction between the target analytes and the sensor is transduced into an analytically useful signal. Electrochemical biosensors are the oldest and biggest group in chemical sensors, which is due to the straightforward nature of their procedure. The most important feature in this type of sensors is that they are easy to downsize and integrate into systems without any change.[18] There are four types of electrochemical biosensors that will be introduced here.

Impedimetric Electrochemical Biosensors

This type of sensor coating with gold nanoparticles/ graphene-chitosan composite is one of the most efficient BoNTA detectors.[32] In a study, Sorouri et al. employed Aunanodendrites/chitosan nanoparticles (AuNDs/CSNPs) to develop a screen-printed carbon electrode (SPCE) based impedimetric immunosensor to detect BoNTA. The system based on a novel nanocomposite offered a linear relationship in the range of 0.2-230 pg/ml^{-1} with a detection limit of 0.15 pg/ml.[39] In another study, Afkhami et al. used this immune-sensor for rapid and precise detection of BoNTA. They accumulated the BoNTA on a glassy carbon electrode, modified with AuNDs/CSNPs, for the amplification of the signal. Besides, antigen-antibody immobilization increased the ability of this assay.[32] The most important part of the electrochemical electrodes is the immobilization of antibodies onto the surface of the electrodes. CS or chitosan is a substance that has high film-forming capability, high adhesion, and high biocompatibility that facilitate the biomolecule immobilization.[40]

Voltammetric Electrochemical Biosensors

Due to their characteristics, these immunosensors are employed in the biosensor field. Narayanan et al. invented an electro-chemical immunosensor for the detection of BoNTE.[41] This type of assay provides a specific and sensitive platform to

detect BoNTE and has the advantages of low cost, simple use, and low sample volume. The recognition platform incorporated graphene nanosheets (GNS) aryldiazonium salt with modified glassy carbon electrodes (GCE). Enzyme-induced AgNPs (silver nanoparticles) were then deposited on AuNPs (gold nanoparticles) to emit the signal. Finally, GNS were joined on the electrode surface by a covalent bound. This immunoassay detected BoNTE in 65 min in a linear range from 10 pg/ml to 10 ng/ml within a detection limit of 5.0 pg/ml.[41]

Another way to detect the neurotoxins of botulinum is to measure protease. In a study by Park et al., a toxic protease, i.e., BoNTE, was detected via an electrochemical method. In their procedure, a target BoNTE and an exopeptidase, L-leucine-aminopeptidase (LAP) were used based on their capability of the cleaving peptide bonds between isoleucine and arginine in IDTQNRQIDRI-4-amino-1-naphthol (oligo peptide-A-N). Amino-naphthol (AN) particles were produced due to this interaction that resulted in an amplified signal through a redox cycling, involving a reducing agent and $Ru(NH_3)_6^{3+}$. The LOD after 4 h, 2 h, and 15 min was 2, 0.2, and 3 ng/ml, respectively. The mechanism of this system is based on the enzymatic activity of protease, which is fast, sensitive, and selective.[42]

Amperometric Electrochemical Biosensors

The main cause of AuNP interactions is the affinity between thiols and gold to form S-Au and gold-amine affinity to form NH-Au bond. This interaction paves the way for the fabrication of the AuNP-modified electrodes.

In a study, Liu et al. employed gold particles as electronic bridges and signal amplifiers toward an electrochemical immunosensor for BoNTA. In this immunoassay, piles of biomolecules were attached to a simple bioconjugation platform.[43] Since AuNPs are attached to the platform quite simply, this method provides high selectivity and sensitivity. Among the most important physicochemical properties of AuNP are good biocompatibility, high surface-to-volume ratio, and ease of transferring electron between biomolecules and electrode. The unparalleled properties of Au-C as well as its high stability have made it suitable for sensing application. A strict control over the components of aryl films on AuNP surfaces is required to provide a well-organized and precise biosensor. The biosensor used by Liu et al. was very stable with good anti-fouling properties with high affinity to BoNTA. The linear range was 4-35 pg/ml^{-1} with the lowest detection limit of 1 pg/ml^{-1} and assay duration time of 10 min.[43]

Impedance Spectroscopy

In case of applying a current, impedance, which is shown with the letter Z, is the resistance that depends on frequency. It has a straightforward and rapid operation and can be downsized without bargaining any features. These characteristics make impedance spectroscopy a very popular choice as a sensing system. The impedance at a provided angular frequency consists of a real and an imaginary part. To explain the process of impedimetric detection, it is necessary to use the Nyquist plot. When the imaginary part is plotted on the Y-axis and the real part of the impedance on the X-axis, the spectroscopy is performed.[44]

Various studies have been conducted on impedimetric determination recently. In a study by Halliwell et al., the best biosensor system for the active form of the toxin was the impedimetric technique.[45] For the detection of active botulinum neurotoxin, two electrochemical immunoassays were employed. For the modification of related electrodes, self-assembled monolayers of the SNARE protein SNAPE-25 were used. This protein is selectively cleaved by active BoNTA. With this impedimetric bioassay, it was possible to detect active toxins at a low concentration of 25 fg/ml. The main advantage of this biosensor over other methods such as ELISA is that the detection of active toxin is conducted by assessing the proteolytic activity rather than just by the presence of the molecules. A significant result of this study was that the lack of correlation in the EIS assay makes them inappropriate for analysis, but when only the detection of the related toxin is needed, it is widely used in various clinical and security environments.[45]

As mentioned before, in the cases of bio-terrorism and food poisoning, a rapid detection of various neurotoxins is critical for both ascertaining the presence of toxin and its analysis. Savage et al. used a VAMP and a SNAP-25 biosensor assay coupled with EIS to detect the performance of five BoNTs (A-E).[46] The reason that made this method so dominant is its quick response compared to other methods. This study also detected toxins at a low concentration of 25 fg/ml (i.e., much lower than mouse immunoassay limit of detection). To this date, this type of assay is not used for diagnostic and detection purposes and has remained just as an experiment.

Feedback System Control (FSC)

This type of assay has been developed to promptly ascertain optimum combinations for therapeutic goals. The main reasons for using the feedback system are: 1) establishing an optimized small molecule inhibitor cocktail that can support a long-lasting maintenance of human embryonic stem cells),[47] 2) combinatory systems control method for reactivating Kapsoi's sarcoma-associated herpesvirus (KSHV) with drugs[48], and 3) closed-loop control of cellular functions via combinatory drugs directed by a stochastic search algorithm.[49]

In a study by Wei et al., the detection of BoNTA was achieved by prompt detection of optimized ionic mixtures for the aptamer-based electrochemical biosensor.[50] The sensitivity and recognition time of an aptasensor for the

detection of BoNT is determined by the proper tertiary architecture of aptamer, which depends on combinatorial effects of manifold types of ions and their present concentrations in the buffer. This optimizing process was done for 4 different ions (K^+, Na^+, Mg^{2+}, and Ca^{2+}) at 12 different concentrations. In each of them, only 10 repetitions with about 50 experiments were suitable to detect optimum ionic concentration. Unlike other methods, which could take hours or days in some cases, this test only took a 5 min of the detection time with the LOD of 40 pg/ml. When used in combination with other multiparameter chemical systems, this method can improve the rate of parameter optimization. Table 1 summarizes the biosensors for better access and differentiation.

Table 1. Biosensors at a Glance

Detection Method		Mechanism of action	LOD	Detected BoNTs	Reference
Optical	Fluorescent	fluorescence excitation	0.4 ng/ml	A, E	[47]
Electrochemical	SPR	Addition of analyte	5.8 ng/ml	A, E, F	[53]
	Impedimetric	Modification with AuNDs/CSNPs,	-	A	[49]
	Voltammetric	GNS with modified GCE	0.4 ± 0.2 nM	E	[42]
	Amperometric	Forming NH-Au bond	-	A	[60]
	Impedance spectroscopy	Real and imaginary angles using Nyquist plot	25 fg/ml	A	[62]
Feedback Control Systems (FSC)		Combination of different systems	40 pg/ml	A	[50]

Conclusion

Botulinum neurotoxins (BoNTs) are the most lethal toxins that human beings have ever discovered. They have seven serotypes (A-H), and because of their ubiquitous nature, they can be found in almost every place. Regarding the high toxicity and omnipresent nature of this toxin and due to the fact that even 62 ng of this toxin can cause human mortality (LD50), several methods have been introduced to detect BoNT toxins. In this view, the most common approach is to use the enzyme-linked immunosorbent assay (ELISA) along with monoclonal antibodies. Using fluorescence sandwich immunoassay has improved the detection sensitivity. Mouse immunoassay is another widely used method worldwide. Evidently, all these methods are time-consuming and either have ethical issues or lack a cost-benefit ratio. To solve these problems, biosensors were introduced, which were cost-effective, rapid, sensitive, and highly specific. Biosensors consist of optical, electrochemical, and FSC biosensors, each with further subdivisions. Furthermore, aptamers can be used in combination with the aforementioned biosensors. This technology has been rapidly growing, especially in the last decade. Research has also been conducted to improve or introduce methods. The revolution in this field of study has led to the development of many commercial biosensors that are able to detect biomolecules on-site. These commercial BoNT biosensors are small in size, have simple construction, and are ideal for the real-time monitoring and in-field detection of the toxins. Over the last few decades, much attention has been paid to the study of BoNT biosensors and advances in sensor systems to detect BoNT are reported in the literature. As shown in this review study, most of the previous studies were just kept as a survey and have not been implemented in any biosensor systems in real time despite their high potential to be used at the highest level of toxin detection.

Authors' Contributions

All authors equally contributed to the current study.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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References

1. Rossetto O. Botulinum toxins: molecular structures and synaptic physiology. In: Jabbari, B. (eds) Botulinum Toxin Treatment in Clinical Medicine. Springer, Cham. 2018:1-12. doi:10.1007/978-3-319-56038-0_1
2. Tareq FK, Fayzunnesa M, Kabir MS, Nuzat M. Mechanism of bio molecule stabilized selenium nanoparticles against oxidation process and *Clostridium Botulinum*. Microb Pathog. 2017;115:68-73. doi:10.1016/j.micpath.2017.12.042
3. Hobbs RJ, Thomas CA, Halliwell J, Gwenin CD. Rapid detection of botulinum neurotoxins—A review. Toxins. 2019;11(7):418. doi:10.3390/toxins11070418
4. Ambrin G, Kumar R, Singh BR. Differential endopeptidase activity of different forms of type A botulinum neurotoxin: A unique relationship between the size of the substrate and activity of the enzyme. Toxicon. 2018;144:34-41. doi:10.1016/j.toxicon.2017.12.055
5. Lauridsen LH, Veedu RN. Nucleic acid aptamers against biotoxins: a new paradigm toward the treatment and diagnostic approach. Nucleic Acid Ther. 2012;22(6):371-9. doi:10.1089/nat.2012.0377
6. Chung YH, Lee HS. Non-diffusive botulinum toxin causing local muscle paralysis, and purification method thereof. U.S. Patent 9,598,683. 2017.
7. Sandrini G, De Icco R, Tassorelli C, Smania N, Tamburin S. Botulinum neurotoxin type A for the treatment of pain: not just in migraine and trigeminal neuralgia. J Headache

Pain. 2017;18(1):38. doi:10.1186/s10194-017-0744-z
8. Read FA, Boyd RN, Barber LA. Longitudinal assessment of gait quality in children with bilateral cerebral palsy following repeated lower limb intramuscular Botulinum toxin-A injections. Res Dev Disabil. 2017;68:35-41. doi:10.1016/j.ridd.2017.07.002
9. Girard B, Piaton JM, Keller P, Abadie C, Nguyen TH. Botulinum neurotoxin injection for the treatment of epiphora in nasolacrimal duct obstruction. J Fr Ophtalmol. 2017;40(8):661-5. doi:10.1016/j.jfo.2017.03.006
10. Benedetto AV. The cosmetic uses of botulinum toxin type A. Int J Dermatol. 1999;38(9):641-55. doi:10.1046/j.1365-4362.1999.00722.x
11. Arnon SS, Schechter R, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, et al. Botulinum toxin as a biological weapon: medical and public health management. JAMA. 2001;285(8):1059-70. doi:10.1001/jama.285.8.1059
12. Hanna PA, Jankovic J, Vincent A. Comparison of mouse bioassay and immunoprecipitation assay for botulinum toxin antibodies. J Neurol Neurosurg Psychiatry. 1999;66(5):612-6. doi:10.1136/jnnp.66.5.612
13. Lindstrom M, Korkeala H. Laboratory diagnostics of botulism. Clin Microbiol Rev. 2006;19(2):298-314. doi:10.1128/cmr.19.2.298-314.2006
14. Sharma SK, Ferreira JL, Eblen BS, Whiting RC. Detection of type A, B, E, and F *Clostridium botulinum* neurotoxins in foods by using an amplified enzyme-linked immunosorbent assay with digoxigenin-labeled antibodies. Appl Environ Microbiol. 2006;72(2):1231-8. doi:10.1128/AEM.72.2.1231-1238.2006
15. Rivera VR, Gamez FJ, Keener WK, White JA, Poli MA. Rapid detection of *Clostridium botulinum* toxins A, B, E, and F in clinical samples, selected food matrices, and buffer using paramagnetic bead-based electrochemilumin escence detection. Anal Biochem. 2006;353(2):248-56. doi:10.1016/j.ab.2006.02.030
16. Rosen O, Feldberg L, Gura S, Zichel R. Improved detection of botulinum type E by rational design of a new peptide substrate for endopeptidase–mass spectrometry assay. Anal Biochem. 2014;456:50-2. doi:10.1016/j.ab.2014.03.024
17. Chao HY, Wang YC, Tang SS, Liu HW. A highly sensitive immuno-polymerase chain reaction assay for *Clostridium botulinum* neurotoxin type A. Toxicon. 2004;43(1):27-34. doi:10.1016/j.toxicon.2003.10.013
18. Eivazzadeh-Keihan R, Pashazadeh-Panahi P, Baradaran B, de la Guardia M, Hejazi M, Sohrabi H, et al. Recent progress in optical and electrochemical biosensors for sensing of *Clostridium botulinum* neurotoxin. TrAC Trends Anal Chem. 2018;103:184-97. doi:10.1016/j.trac.2018.03.019
19. Mokhtarzadeh A, Eivazzadeh-Keihan R, Pashazadeh P, Hejazi M, Gharaatifar N, Hasanzadeh M, et al. Nanomaterial-based biosensors for detection of pathogenic virus. TrAC Trends Anal Chem. 2017;97:445-57. doi:10.1016/j.trac.2017.10.005
20. Eivazzadeh-Keihan R, Pashazadeh-Panahi P, Baradaran B, Maleki A, Hejazi M, Mokhtarzadeh A, de la Guardia M. Recent advances on nanomaterial based electrochemical and optical aptasensors for detection of cancer biomarkers. TrAC Trends Anal Chem. 2018;100:103-15. doi:10.1016/j.trac.2017.12.019
21. Pashazadeh P, Mokhtarzadeh A, Hasanzadeh M, Hejazi M, Hashemi M, de la Guardia M. Nano-materials for use in sensing of salmonella infections: recent advances. Biosens Bioelectron. 2017;87:1050-64. doi:10.1016/j.bios.2016.08.012
22. Arora P, Sindhu A, Dilbaghi N, Chaudhury A. Biosensors as innovative tools for the detection of food borne pathogens. Biosens Bioelectron. 2011;28(1):1-12. doi:10.1016/j.bios.2011.06.002
23. Liao W, Randall BA, Alba NA, Cui XT. Conducting polymer-based impedimetric aptamer biosensor for *in situ* detection. Anal Bioanal Chem. 2008;392:861-4. doi:10.1007/s00216-008-2354-8
24. Song S, Wang L, Li J, Fan C, Zhao J. Aptamer-based biosensors. TrAC Trends Anal Chem. 2008;27(2):108-17. doi:10.1016/j.trac.2007.12.004
25. Liao W, Cui XT. Reagentless aptamer based impedance biosensor for monitoring a neuro-inflammatory cytokine PDGF. Biosens Bioelectron. 2007;23(2):218-24. doi:10.1016/j.bios.2007.04.004
26. Tok JB, Fischer NO. Single microbead SELEX for efficient ssDNA aptamer generation against botulinum neurotoxin. Chemical Communications. 2008(16):1883-5. doi:10.1039/B717936G
27. Wei F, Ho CM. Aptamer-based electrochemical biosensor for Botulinum neurotoxin. Anal Bioanal Chem. 2009;393:1943-8. doi:10.1007/s00216-009-2687-y
28. Damborskэ P, vitel J, Katrlнk J. Optical biosensors. Essays Biochem. 2016;60(1):91-100. doi:10.1042/EBC20150010
29. Kronick MN, Little WA. A new immunoassay based on fluorescence excitation by internal reflection spectroscopy. J Immunol Methods. 1975;8(3):235-40. doi:10.1016/0022-1759(75)90116-7
30. Sapsford KE, Sun S, Francis J, Sharma S, Kostov Y, Rasooly A. A fluorescence detection platform using spatial electroluminescent excitation for measuring botulinum neurotoxin A activity. Biosens Bioelectron. 2008;24(4):618-25. doi:10.1016/j.bios.2008.06.018
31. Wu B, Mathews N, Sum TC. Plasmonic Organic Solar Cells: Charge Generation and Recombination. Springer; 2016.
32. Afkhami A, Hashemi P, Bagheri H, Salimian J, Ahmadi A, Madrakian T. Impedimetric immunosensor for the label-free and direct detection of botulinum neurotoxin serotype A using Au nanoparticles/graphene-chitosan composite. Biosens Bioelectron. 2017;93:124-31. doi:10.1016/j.bios.2016.09.059
33. Ben Haddada M, Hu D, Salmain M, Zhang L, Peng C, Wang Y, et al. Gold nanoparticle-based localized surface plasmon immunosensor for staphylococcal enterotoxin A (SEA) detection. Anal Bioanal Chem. 2017;409:6227-34. doi:10.1007/s00216-017-0563-8
34. Lévêque C, Ferracci G, Maulet Y, Mazuet C, Popoff MR, Blanchard MP, et al. An optical biosensor assay for rapid dual detection of Botulinum neurotoxins A and E. Sci Rep. 2015;5(1):17953. doi:10.1038/srep17953
35. Ladd J, Taylor AD, Homola J, Jiang S. Detection of botulinum neurotoxins in buffer and honey using a surface plasmon resonance (SPR) sensor. Sens Actuators B: Chem. 2008;130(1):129-34. doi:10.1016/j.snb.2007.07.140
36. Janardhanan P, Mello CM, Singh BR, Lou J, Marks JD, Cai S. RNA aptasensor for rapid detection of natively folded type A botulinum neurotoxin. Talanta. 2013;117:273-80. doi:10.1016/j.talanta.2013.09.012
37. Wilson MS. Electrochemical immunosensors for the simultaneous detection of two tumor markers. Anal Chem. 2005;77(5):1496-502. doi:10.1021/ac0485278
38. Turner A, Karube I, Wilson GS. Biosensors: fundamentals and applications. Oxford University Press; 1987.
39. Sorouri R, Bagheri H, Afkhami A, Salimian J. Fabrication of a novel highly sensitive and selective immunosensor for botulinum neurotoxin serotype A based on an

effective platform of electrosynthesized gold nanodendrites /chitosan nanoparticles. Sensors. 2017;17(5):1074. doi:10.3390/s17051074

40. Racoviţă S, Vasiliu S, Popa M, Luca C. Polysaccharides based on micro-and nanoparticles obtained by ionic gelation and their applications as drug delivery systems. Rev Roum Chim. 2009;54(9):709-18.
41. Narayanan J, Sharma MK, Ponmariappan S, Shaik M, Upadhyay S. Electrochemical immunosensor for botulinum neurotoxin type-E using covalently ordered graphene nanosheets modified electrodes and gold nanoparticles-enzyme conjugate. Biosens Bioelectron. 2015;69:249-56. doi:10.1016/j.bios.2015.02.039
42. Park S, Shin YM, Song JJ, Yang H. Facile electrochemical detection of botulinum neurotoxin type E using a two-step proteolytic cleavage. Biosens Bioelectron. 2015; 72:211-7. doi:10.1016/j.bios.2015.05.016
43. Liu G, Zhang Y, Guo W. Covalent functionalization of gold nanoparticles as electronic bridges and signal amplifiers towards an electrochemical immunosensor for botulinum neurotoxin type A. Biosens Bioelectron. 2014; 61:547-53. doi:10.1016/j.bios.2014.06.001
44. Lvovich VF, Smiechowski MF. Impedance characterization of industrial lubricants. Electrochimica Acta. 2006;51(8-9):1487-96. doi:10.1016/j.electacta.2005.02.135
45. Halliwell J, Savage AC, Buckley N, Gwenin C. Electrochemical impedance spectroscopy biosensor for detection of active botulinum neurotoxin. Sens Bio-Sens. 2014;2:12-5. doi:10.1016/j.sbsr.2014.08.002
46. Savage AC, Buckley N, Halliwell J, Gwenin C. Botulinum neurotoxin serotypes detected by electrochemical impedance spectroscopy. Toxins. 2015;7(5):1544-55. doi:10.3390/toxins7051544
47. Tsutsui H, Valamehr B, Hindoyan A, Qiao R, Ding X, Guo S, et al. An optimized small molecule inhibitor cocktail supports long-term maintenance of human embryonic stem cells. Nat Commun. 2011;2(1):167. doi:10.1038/ncomms1165
48. Sun CP, Usui T, Yu F, Al-Shyoukh I, Shamma J, Sun R, et al. Integrative systems control approach for reactivating Kaposi's sarcoma-associated herpesvirus (KSHV) with combinatory drugs. Integr Biol. 2009;1(1):123-30. doi:10.1039/b815225j
49. Wong PK, Yu F, Shahangian A, Cheng G, Sun R, Ho CM. Closed-loop control of cellular functions using combinatory drugs guided by a stochastic search algorithm. Proc Natl Acad Sci USA. 2008;105(13):5105-10. doi:10.1073/pnas.0800823105
50. Wei F, Bai B, Ho CM. Rapidly optimizing an aptamer based BoNT sensor by feedback system control (FSC) scheme. Biosens Bioelectron. 2011;30(1):174-9. doi:10.1016/j.bios.2011.09.014