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Paper-based electrochemical peptide sensor for on-site detection of botulinum neurotoxin serotype A and C

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ABSTRACT

Botulinum neurotoxins (BoNTs) produced by soil bacterium *Clostridium botulinum* are cause of botulism and listed as biohazard agents, thus rapid screening assays are needed for taking the correct countermeasures in a timely fashion. The gold standard method relies on the mouse lethality assay with a lengthy analysis time, i.e., 2–5 days, hindering the prompt management of food safety and medical diagnosis. Herein, we propose the first paper-based antibody-free sensor for reliable and rapid detection of BoNT/A and BoNT/C, exploiting their cleavage capability toward a synthetic peptide able to mimic the natural substrate SNAP-25. The peptide is labelled with the electroactive molecule methylene blue and immobilized on the paper-based electrode modified with gold nanoparticles. Because BoNT/A and BoNT/C can cleave the peptide with the removal of methylene blue from electrode surface, the presence of these neurotoxins in the sample leads to a signal decrease proportional to BoNT amount. The biosensor developed with the selected peptide and combined with smartphone assisted potentiostat is able to detect both BoNT/A and BoNT/C with a linearity up to 1 nM and a detection limit equal to 10 pM. The applicability of this biosensor was evaluated with spiked samples of orange juice, obtaining recovery values equal to 104 ± 6% and 98 ± 9% for 1 nM and 0.5 nM of BoNT/A, respectively.

1. Introduction

Botulinum neurotoxins (BoNTs) encompass seven toxin types or serotypes (A to G) and several toxin subtypes, as well (Rossetto et al., 2014). BoNTs are produced mainly by the Gram-positive neurotoxigenic anaerobic bacterium *Clostridium botulinum* as single 150 kDa inactive proteins and they are constituted of a 50 kDa light chain and a 100 kDa heavy chain, linked by a conserved disulfide bond with two functional domains, namely C- and N- terminal ones (Peck et al., 2017; Tehran and Pirazzini, 2018). The carboxy-terminal domain mediates the selective binding to specific receptors on the presynaptic membrane of peripheral nerve terminals (Rummel, 2016), while the N-terminal domain chaperones the membrane translocation of the light chain endowed with endopeptidase activity into the nerve cytoplasm (Montal, 2010; Pirazzini et al., 2016; Montecucco and Schiavo, 1994).

BoNTs act with a mechanism that embraces different sequential steps, including the rapid and specific binding to peripheral nerve terminals, followed by the internalization through vesicles thanks to ATPase proton pump. After, the membrane translocation is triggered by vesicular lumen acidification, with a structural rearrangement of the toxin and the translocation of the light chain into the cytosol. Finally, the reduction of the interchain disulphide bond in the nerve cytosol allows for the releasing of the catalytic light chain with Zn-dependent proteolytic activity against SNARE complex proteins, including synaptic Vesicle-Associated Membrane Protein (VAMP), Synaptosomal-Associated Protein (SNAP-25), and Syntaxin-1A/1B (Pirazzini et al., 2017; Schiavo et al., 2000).

Notably, BoNT/B,/D,/F and/G cleave VAMP at selected peptide bonds, BoNT/A and/E cleave at specific sites of SNAP-25 C-terminal (Schiavo et al., 2000; Simpson, 2004), while BoNT/C cleaves both

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SNAP-25 and Syntaxin-1A/1B. The cleavage of SNARE proteins hinders the release of neurotransmitter, mainly acetylcholine from peripheral motor nerve terminals, thus causing an ensuing neuromuscular paralysis that is the primary symptom of botulism.

Since BoNTs are characterized by toxicity in the low ng/kg range (Rossetto and Montecucco, 2019), the Centers for Disease Control and Prevention (CDC), USA, have listed BoNTs as the priority agents and catalogued them (category 'A') as highest risk threat agents for bioterrorism (Arnon et al., 2001).

BoNT serotypes A, B, E, and F are generally associated with human foodborne botulism (Rasetti-Escargueil et al., 2018), while BoNT/C and/D cause botulism in animals (Thirunavukkarasu et al., 2018). Foodborne botulism, reported since 17th century (Erbguth and Naumann, 1999), is due to the contamination of foodstuffs by spores of *Clostridium botulinum,* which yields the vegetative bacterial cells with production of BoNTs under suitable anaerobic conditions (Rossetto et al., 2014). Innovations in the foodstuff processes and the supply chain modernization have increased the threat due to the contamination of centralized sources with the risk of botulism widespread, thus rapid and reliable detection methods are necessary for promptly taking suitable countermeasures (Maslanka et al., 2016).

The lethality mouse bioassay is considered the gold standard method for the detection of BoNTs in clinical, food, and environmental samples. It monitors the occurrence of botulism symptoms after intraperitoneal injection of the sample into mice for several days, evaluating the minimum lethal dose (1 MLD), which is the minimum amount of toxin that kills all the inoculated mice roughly corresponding to 10 pg for BoNT/A (Ferreira, 2001). However, this method has significant drawbacks, including the need for mice, expensive animal facilities, and skilled personnel. Furthermore, this method requires a minimum of four days to determine the serotype, making this assay not suitable for routine toxin quantification and for its use in biodefense scenarios, where rapid detection is necessary.

Electrochemical biosensor technology represents an attractive and highly diverse area of research, with the advantages to provide ready to use analytical tools for a rapid, cost-effective, and on-site measure of toxins. Fetter et al. developed an electrochemical DNA (E-DNA) biosensor consisting of a selected aptamer immobilized onto a gold electrode surface functionalized with methylene blue as a redox label. Interactions between the oligonucleotide scaffold and BoNTs allow for DNA conformation changes, bringing methylene blue in close contact with the electrode surface and generating a higher electrical current output. This device was characterized by an apparent dissociation constant (K_D) values of 0.3 ± 0.4 nM for BoNT/A, and a linear range comprised between 10^{-11} and 10^{-7} M (Fetter et al., 2015). Another example of biosensor for the detection of BoNTs was reported by Savage et al. (2015) using electrochemical impedance spectroscopy as a technique to exploit the ability of BoNTs to cleave SNARE proteins. This device was able to detect a concentration of botulinum toxins as low as 25 fg/mL by following the steric hindrance on the working electrode surface. Notably, at increasing concentration of toxins there is an increasing proteolytic activity, which allows for the decrease of SNARE protein on the gold electrode surface, with the simultaneous decreasing of resistance to charge transfer (R_{ct}) value.

In the sensing field, paper has been used as support to design cost-effective and easy to use analytical tools for fast, cheap, and environmentally friendly analyses. Indeed, paper-based devices have proved to be valid analytical tools that match the sustainable strategy of European Commission (Circular Economy Action Plan of the European Green Deal) and the 11th principle of Green Chemistry (Anastas and Warner, 1998). In the last decade several paper-based (bio)sensors have been developed for the detection of analytes in samples with application in several fields including agri-food sector, being the quality control of food products and process monitoring hot topics. To this regard, our group developed an office paper-based electrode modified by drop-casting with a dispersion of carbon black/Prussian Blue nanoparticles and alcohol oxidase for the detection of ethanol in commercial beers. In detail, ethanol was quantified by measuring the amount of hydrogen peroxide generated by the enzymatic reaction between alcohol oxidase and ethanol at low applied potential, - 0.1 V vs Ag/AgCl. The suitability of this paper-based device was tested by analyzing different samples of beer, comparing the results obtained with the amount of ethanol reported on label (Cinti et al., 2017). Henry's group developed an interesting paper-based colorimetric sandwich immunoassay for Salmonella detection. In detail, they designed a 3D-printed rotational manifold with disposable paper-based layers for a semi-automated reagent delivery, washing, and detection of Salmonella in milk in 65 min (Carrell et al., 2019). A paper-based dual-mode aptasensor for the quantification of Ochratoxin A (OTA) was reported by Zhang et al. The authors developed an origami paper-based device using a sandwich approach with two different aptamers "$apta_1$-OTA-$apta_2$" giving both electrochemical/colorimetric signal for the quantification of OTA in corn samples (Zhang et al., 2021).

Herein, we propose the first peptide-based paper electrochemical sensor for the detection of BoNT/A and BoNT/C by using an immobilized short peptide labelled with methylene blue which mimics SNAP-25. The presence of BoNT/A and BoNT/C was evaluated by monitoring their proteolytic activity through the measurement of methylene blue electrochemical response. BoNT/A and BoNT/C can cleave the portion of the peptide bound to methylene blue, leading to the decrease of the signal due to the removal of electrochemical probe from the working electrode surface. This device is the first one that detects BoNT/A and BoNT/C by using a paper-based sensor and a smartphone-assisted device, delivering an easy to use and cost-effective analytical tool for on-site monitoring.

2. Materials and methods

2.1. Reagents and equipment

Sodium chloride, sodium dihydrogen phosphate hydrate (NaH_2PO_4 H_2O), sodium hydrogen phosphate (Na_2HPO_4), 6-mercapto1-hexanol (C6–OH), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The amino acids used for the peptide synthesis Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys (Mtt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Ahx-OH, Fmoc-PAL-PEG Resin (0.16 mmol/g) and the activators NHydroxybenzotriazole (HOBT) and O-Benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluorophosphate (HBTU) were purchased from Novabiochem (Gibbstown, NJ, USA). Monocarboxymethylene Blue was purchased from EMP BIOTECH. Acetonitrile (ACN) was from Reidel-deHaën (Seelze, Germany) and dry N,N-dimethylformamide (DMF) from LabScan (Dublin, Ireland). All other reagents were purchased from Fluka (Milan, Italy).

The gold nanoparticles were synthesized according to our previous work (Moccia et al., 2020). The paper-based sensors (S4M-OP03DW) were supplied by Sense4Med (Rome, Italy) and they were characterized by graphite working and counter electrodes and Ag/AgCl pseudo-reference electrode (geometric working electrode surface area equal to 12.56 mm^2). A portable potentiostat, Sensit Smart (PalmSens, Netherlands) connected to a smartphone was used to carry out the electrochemical measurements. The dedicated app PStouch allows for displaying real time voltammetric measurements in the video of smartphone.

2.2. Synthesis of BoNT-substrate peptide

To obtain the branched MB-BoNT-substrate peptide conjugate, the BoNT-substrate peptide was first synthesized on solid phase and acetylated at the N-terminus. The synthetic approaches (Ahx e cysteine) were in agreement with our previous work (Moccia et al., 2020) and the

reported peptide strategies (Le Chevalier et al., 2009). The MB-BoNT-substrate peptide conjugate was purified by RP-HPLC and analyzed by LC-MS, and its sequence is: Cys-Ahx-KTRIDEANQ-RATK (MB)M. Mass Calculated: 2259.20; $[M+2H]^{2+}$: 1130.60; $[M+3H]^{3+}$: 754.06; $[M+4H]^{4+}$: 565.80. Found: 2259.13; $[M+2H]^{2+}$: 1130.57; $[M+3H]^{3+}$: 754.05; $[M+4H]^{4+}$: 565.79.

2.3. Construction of the peptide-based paper sensor

The preparation of the peptide-based paper sensor started with the reduction of 20 μM of methylene blue-modified peptide (prepared in phosphate buffer 10 mM with NaCl 100 mM, pH = 7.4) in the presence of 10 mM TCEP (prepared in phosphate buffer 10 mM with NaCl 100 mM, pH = 7.4) for 1 h. The solution was then diluted to obtain the selected concentration (nanomolar range) to be immobilized onto the working electrode surface, where 8 μL of AuNPs had been previously drop-cast. AuNPs nanoparticles were drop-cast onto the working electrode surface for anchoring the peptide to the paper-based SPE thanks to the introduction of thiol group at the extremity of the peptide sequences, employing a cysteine residue. Then, 10 μL of the probe were added onto the working electrode area and left for 1.5 h at RT in a humid chamber. SPEs were rinsed with distilled water, then incubated overnight in a humid chamber with 2 mM C6–OH (prepared in phosphate buffer 10 mM with NaCl 100 mM, pH = 7.4) to fill in the empty spaces on the working electrode surface. SPEs modified with AuNPs, labelled peptide, and C6–OH were rinsed again with distilled water to wash away the unbound C6–OH from the electrode surface, thus the peptide-based sensors were ready to use for the electrochemical measurement.

2.4. Measurement of botulinum neurotoxin proteolytic activity

The measurement of the toxin target encompasses two steps in a signal-off method, as follows:

i) First step (Fig. 1B, signal before the exposure of the sensor to the toxin): 100 μL of 10 mM phosphate buffer (with NaCl 100 mM, pH = 7.4) were added on the peptide modified-SPE for the measurement of the initial methylene blue (MB) signal using Square Wave Voltammetry (SWV) as electrochemical technique (E begin = −0.6 V, E end = 0.0 V, E step = 0.001 V, Amplitude = 0.05 V, Frequency = 100 Hz).

ii) Second step (Fig. 1C, signal after the exposure of the sensor to the toxin): 100 μL of BoNT solution were added on the same peptide modified-SPE peptide and left to react for 4 h. After that, the sensor was washed and 100 μL of 10 mM phosphate buffer (with NaCl 100 mM, pH = 7.4) were added on the peptide modified-SPE for the measurement of MB signal using SWV as electrochemical technique (E begin = −0.6 V, E end = 0.0 V, E step = 0.001 V, Amplitude = 0.05 V, Frequency = 100 Hz).

The current signal decrease observed in the second voltametric measurement is due to the loss of peptide portion containing the electrochemical probe, removed by the proteolytic activity of the toxin, and directly proportional to the concentration of BoNT/A and BoNT/C present in the sample analyzed. The percentage of signal decrease was then plotted against the BoNT concentrations for the construction of the calibration curve.

3. Results and discussion

Botulinum neurotoxins are zinc-dependent metalloproteases able to cleave the SNARE proteins, which play an essential role in the release of the neurotransmitters from neuronal cells. BoNT/A, BoNT/C and BoNT/E cleave the SNAP-25 protein, which comprises 206 amino acids. BoNT/A cleaves SNAP-25 in position Gln_{197}–Arg_{198}, BoNT/C between Arg_{198} and Ala_{199}, while BoNT/E cleaves the peptide bond Arg_{180}–Ile_{181} (Schiavo et al., 1993). Herein, we developed a synthetic peptide carrying the cleavage sites for BoNT/A and BoNT/C with a selected length to accomplish the methylene blue redox reaction at the electrode surface. For this reason, the synthetic peptide was chosen with a length of 14 amino acids (Cys-Ahx-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys(Methylene blue)-Met) containing the cleavage sites of the toxins between Gln and Arg for BoNT/A, and between Arg and Ala for BoNT/C. The peptide labelled with the electrochemical probe was immobilized on AuNP-modified electrode surface for the detection of BoNT/A and BoNT/C.

3.1. Optimization of the peptide amount immobilized onto the working electrode surface

For delivering an effective paper-based electrochemical device, a crucial step is the optimization of the parameters that affect the measurement of BoNTs, including the amount of the peptide immobilized onto the working electrode surface, the blocking agent, and the time of the reaction between the peptide and BoNTs. We started the optimization study evaluating the effect of the amount of MB-modified peptide to be immobilized onto the working electrode. The presence of the peptide

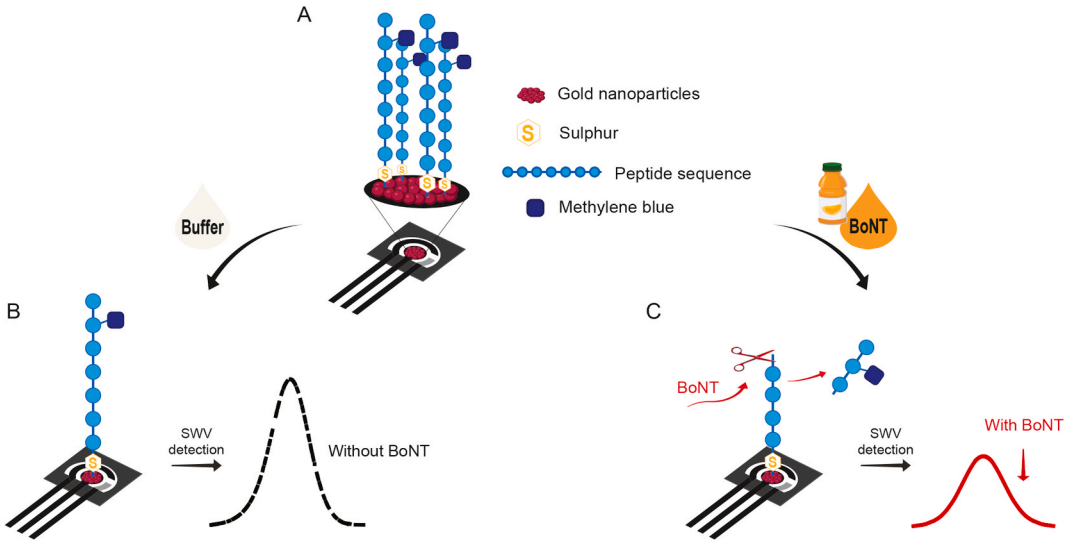

Fig. 1. BoNT detection using the signal-off method. **A)** Schematic representation of the peptide-modified paper-based sensor. **B)** Signal obtained before the exposure of the sensor to the toxin. **C)** Signal obtained after the exposure of the sensor to the toxin.

on the surface of the working electrode allows for the electrochemical signal due to the presence of the redox label MB in each peptide sequence. As expected, varying the concentration of peptide at a fixed immobilization time, we observed an increase of signal due the larger number of the labelled peptides immobilized onto the working electrode surface. In detail, this behaviour was observed increasing the concentration up to 0.5 μM, after that a reduced repeatability was observed, probably due to the presence of double layers, thus 0.5 μM was the concentration selected as compromise between current intensity and repeatability (RSD % equal to 3%) (Fig. 2A). The time used for the peptide immobilization was also evaluated confirming 1.5 h as the best condition between sensitivity and repeatability: lower time allowed for lower amount of the labelled peptide immobilized onto the working electrode surface, while longer immobilization time can probably produce double layers with the reduction of both repeatability and sensitivity. In details, the best RSD % of 2.5% was achieved at 1.5 h, thus 1.5 h was confirmed as the immobilization time selected (Fig. 2B).

3.2. Optimization of 6-mercaptohexanol (C6–OH) incubation time

As reported in literature, 6-mercaptohexanol has been frequently utilized for the passivation of Au species on 2D surfaces and large nanostructures (Park et al., 2004). Moreover, it has been applied for the development of electrochemical DNA sensors because it hinders the nonspecific adsorption of thiolated DNA, enhancing the hybridization to complementary strands (Ricci et al., 2007). In this work, we used 2 mM C6–OH to passivate the working electrode surface and to line up the attached modified peptide sequences in a more accessible orientation. We tested three different incubation times (1 h, 3 h and overnight). Fig. 2C showed that it was necessary to incubate the SPE with C6–OH overnight to obtain a stable current signal together with good repeatability (RSD % equal to 1.5%).

3.3. Optimization of BoNT/A incubation time

Before evaluating the suitability of the paper-based sensor to measure different concentrations of the selected BoNT/A, we investigated the reaction time between the immobilized labelled peptide and BoNT/A. This optimization is needed because the toxin requires some time to cleave the peptide and to generate a detectable signal change. We tested 1 h, 2 h, 4 h and overnight as incubation time. The experimental results reported in Fig. 2D showed how the signal changes in function of time. After 1 h we observed a decrease of the initial signal around of 50%, demonstrating a good sensitivity; however 4 h as incubation time gave a better repeatability (RSD % = 3%), thus it was selected as the time of reaction between the immobilized peptide and the target analyte.

3.4. Analytical features

Once optimized all the main experimental parameters affecting the sensitivity of the device, the analytical performance of the platform was investigated measuring the signal change obtained with different concentrations of BoNT/A and BoNT/C in standard solution, evaluating the capability of these two toxins to recognise their own cleavage site on the peptide sequence.

Fig. 3A showed the optimized sensor with 500 nM of MB-modified immobilized peptide in presence of BoNT/A concentrations ranging from 0.01 to 10 nM. The calibration curve in buffer solution was linear up to 1 nM ($y = (31.7 \pm 1.1) + (29 \pm 2)$ x, $R^2 = 0.954$). The plateau at concentration higher than 1 nM is ascribed to the amount of BoNT/A that exceeds the amount of peptide immobilized on the working electrode surface. Indeed, the number of the cleavage sites present on the peptide sequences is saturated at BoNT/A concentration around 1 nM, thus even increasing the amount of BoNT/A, the signal change does not increase.

The detection limit obtained was found equal to 10 pM (as the concentration of BoNT/A that gave a signal change equal to 30%). The platform was also suitable for the detection of different concentrations of BoNT/C in the same range described above, as shown in Fig. 3B. The response curve obtained was described by the equation $y = (23.1 \pm 1.3) + (24.2 \pm 2.4)$ x, $R^2 = 0.912$ with LOD equal to 10 pM, calculated as the concentration of BoNT/C which gave a signal difference of 20%.

3.5. Selectivity study and shelf life study

To evaluate the selectivity of the biosensor, BoNT/E was tested.

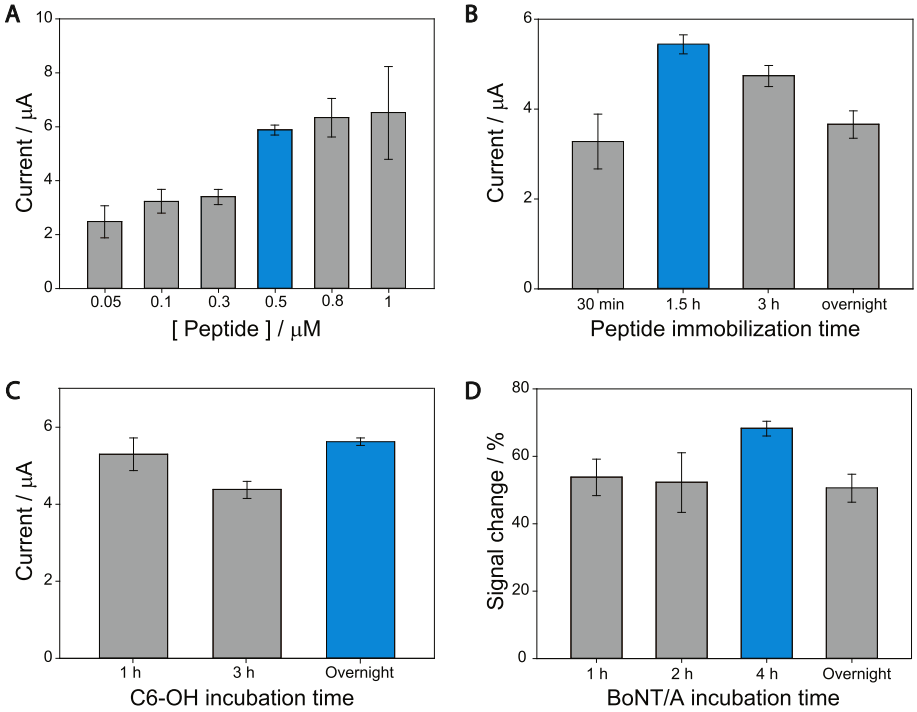

Fig. 2. A) Optimization of the peptide concentration. Concentrations tested: 0.05, 0.1, 0.3, 0.5, 0.8 and 1 μM prepared in phosphate buffer 10 mM with NaCl 100 mM, pH = 7.4. Selected peptide immobilization time = 1.5 h and C6–OH incubation time = 1 h (n = 3). **B)** Optimization of the peptide immobilization time. Times tested: 30 min, 1.5 h, 3 h and overnight. [Peptide] = 500 nM prepared in phosphate buffer 10 mM with NaCl 100 mM (pH = 7.4), selected C6–OH incubation time = 1 h (n = 3). **C)** Optimization of C6–OH incubation time. Times tested: 1 h, 3 h and overnight. [Peptide] = 500 nM prepared in phosphate buffer 10 mM with NaCl 100 mM (pH = 7.4), selected peptide immobilization time = 1.5 h. **D)** Optimization of the BoNT/A incubation time. Time tested: 1 h, 2 h, 4 h and overnight. [Peptide] = 500 nM prepared in phosphate buffer 10 mM with NaCl 100 mM (pH = 7.4) and [BoNT/A] = 1 nM. Selected peptide immobilization time = 1.5 h and C6–OH incubation time = overnight (n = 3).

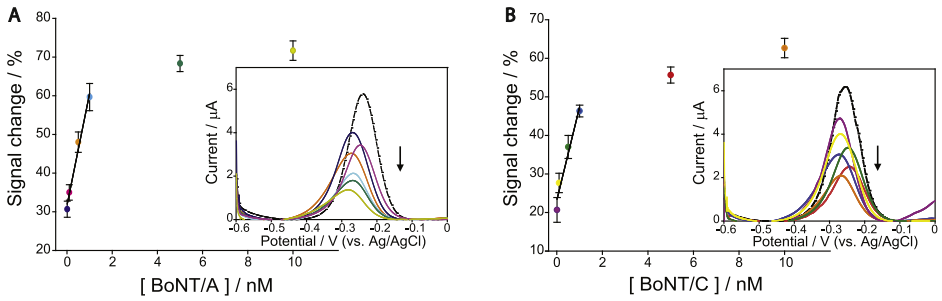

Fig. 3. A) Calibration curve of BoNT/A. Concentrations tested: 0.01, 0.1, 0.5, 1, 5 and 10 nM prepared in phosphate buffer 10 mM with NaCl 100 mM, pH = 7.4. [Peptide] = 500 nM prepared in phosphate buffer 10 mM with NaCl 100 mM (pH = 7.4), selected BoNT/A incubation time = 4 h. Inset: voltammograms obtained in absence (dashed line) and in presence (solid lines) of different concentrations of BoNT/A. **B)** Calibration curve of BoNT/C. Concentrations tested: 0.01, 0.1, 0.5, 1, 5 and 10 nM prepared in phosphate buffer 10 mM with NaCl 100 mM, pH = 7.4. [Peptide] = 500 nM prepared in phosphate buffer 10 mM with NaCl 100 mM (pH = 7.4), BoNT/C incubation time = 4 h. Inset: voltammograms obtained in absence (dashed line) and in presence (solid lines) of different concentrations of BoNT/C.

BoNT/A, BoNT/C, and BoNT/E cleave SNAP-25, however BoNT/E is able to selectively cleave SNAP-25 in the position Arg_{180}–Ile_{181}, 17 amino acids before the cleavage site of BoNT/A (Gln_{197}–Arg_{198}) and 18 amino acids before the cleavage site of BoNT/C (Arg_{198}–Ala_{199}). Since the selected peptide mimics the SNAP-25 sequence from the amino acid Thr_{190} to Met_{202}, BoNT/E should not cleave the MB-modified peptide immobilized on the electrode surface. Fig. 4A showed that the presence of 10 nM BoNT/E (red line) produced only a slight variation of the signal. Considering that the same concentration of BoNT/A and BoNT/C gave a signal decrease equal to 65–70%, this result indicated the high selectivity of the paper-based device developed.

To evaluate the stability of the biosensor in storage condition, the response of paper-based biosensors was tested by storing the peptide-modified SPEs wet with the working buffer solution (phosphate buffer 10 mM with NaCl 100 mM, pH = 7.4) at 4 °C in humid chamber up to 21 days and evaluating its ability to detect BoNT/A. The results are shown in Fig. 4B and they highlighted no significant difference in the signal change until 7 days. However, a slight decrease of the signal change was observed after 14 (around 10% of decrease) and 21 days (around 20% of decrease).

3.6. BoNT/A detection in real samples

The bacterium *Clostridium botulinum* can contaminate different common foods not properly handled, including home-canned, bottled and vacuum-packed foods, honey, fermented, salted and smoked meat and seafood. Here we evaluated the efficacy of the paper-based biosensor to detect BoNT/A in a real matrix such as orange juice, because this commercial product was already linked to foodborne botulism in human beings (Thirunavukkarasu et al., 2018). Orange juice sample was spiked with different BoNT/A concentrations in the range between 0.01 and 10 nM and the calibration curve obtained was characterized by the following equation $y = (33.8 \pm 1.5) + (21.7 \pm 2.8)\,x$, $R^2 = 0.860$ (Fig. 4C).

The accuracy of the sensor was estimated by recovery studies, spiking orange juice samples with 1 nM and 0.5 nM BoNT/A. Percentage recoveries of 104 ± 6% and 98 ± 9% were obtained respectively, demonstrating the suitability of the paper-based platform to measure botulinum neurotoxin in real matrix.

4. Conclusion

Herein we reported the first paper-based antibody-free electrochemical sensor for the detection of botulinum neurotoxin serotypes A and C. We exploited the proteolytic activity of these neurotoxins towards SNAP-25, a component of the SNARE complex. The experimental results obtained showed a high selectivity of the developed biosensor, with a detection limit equal to 10 pM for both BoNT/A and BoNT/C. Its selectivity was demonstrated when the sensor was tested in presence of another serotype, BoNT/E. Although BoNT/E cleaves SNAP-25, the sensor was not affected by the presence of this toxin, because of the absence of its specific cleavage site on the synthetic peptide sequence immobilized on the electrode surface. These results give a promising prospect for its practical use, being: i) the first electrochemical paper-based sensor easy to use combined with a smartphone-assisted device; ii) suitable to be incinerated after the measure, reducing the waste; and iii) able to carry out the measure without the requirement of animals during the test or for the production of biocomponents needed for analysis. The demonstration of suitability of peptide and paper-based device paves the way for the development of an array of peptide-based sensors able to simultaneously measure all the botulinum neurotoxins by selecting different synthesized peptides, each containing the specific cleavage site for the particular serotype, combined with a smartphone assisted potentiostat for reliable, easy and on-site sustainable

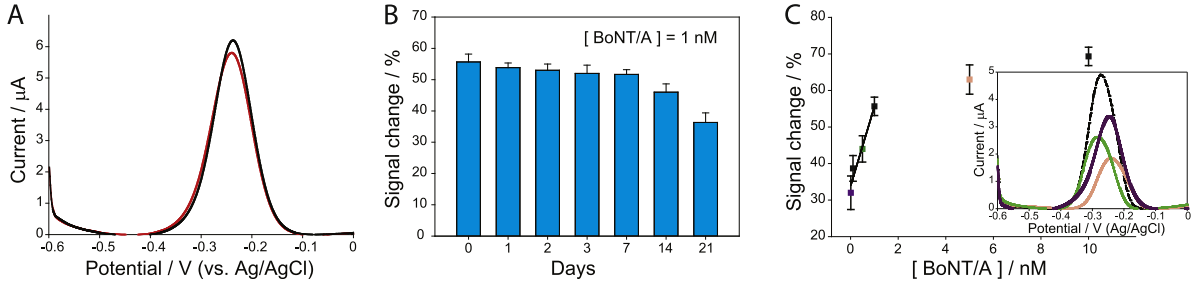

Fig. 4. A) Selectivity study. Voltammetric measurements in absence (black line) and in presence (red line) of 10 nM BoNT/E (phosphate buffer 10 mM with NaCl 100 mM, pH = 7.4). [Peptide] = 500 nM prepared in phosphate buffer 10 mM with NaCl 100 mM (pH = 7.4), BoNT/E incubation time = 4 h. **B)** Stability study. The paper-based electrodes were tested after several days of storage at 4 °C in humid chamber. [Peptide] = 500 nM prepared in phosphate buffer 10 mM with NaCl 100 mM, pH = 7.4. [BoNT/A] = 100 nM. BoNT/A incubation time = 4 h (n = 3). **C)** Calibration curve of BoNT/A in orange juice samples. Concentrations tested: 0.01, 0.1, 0.5, 1, 5 and 10 nM. [Peptide] = 500 nM prepared in phosphate buffer 10 mM with NaCl 100 mM, pH = 7.4. BoNT/A incubation time = 4 h (n = 3). Inset: voltammograms obtained in absence (dashed line) and in presence (solid lines) of different concentrations of BoNT/A in orange juice sample. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

measure of BoNTs.

CRediT authorship contribution statement

Veronica Caratelli: Experimental investigation, paper-based. **Silvia Fillo:** peptide design, Methodology, Formal analysis, Writing – review & editing, Experimental investigation, Writing – review & editing. **Nino D'Amore:** Writing – review & editing. **Ornella Rossetto:** Experimental investigation, Writing – review & editing. **Marco Pirazzini:** Experimental investigation, Writing – review & editing. **Maria Moccia:** Peptide design and synthesis, Writing – review & editing. **Concetta Avitabile:** Peptide synthesis, Writing – review & editing. **Danila Moscone:** Review & editing. **Florigio Lista:** Conceptualisation of the biosensor, Funding acquisition, Project administration. **Fabiana Arduini:** Conceptualisation of the biosensor, Supervision, of experiments, Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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