



Electrochemical impedance spectroscopy biosensor for detection of active botulinum neurotoxin



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ABSTRACT

The standard method for the detection of botulinum neurotoxin is currently the mouse bioassay which is considered to be the most reliable method for the detection of the active form of this toxin. Despite this it is a time-consuming and expensive assay to run and as such many alternative assays have recently been proposed. Herein we report the development of two electrochemical assays for the detection of active botulinum neurotoxin in a pharmaceutical sample. Gold electrodes were modified with self-assembled monolayers of the SNARE protein SNAP-25 which is selectively cleaved by active botulinum neurotoxin A. Cyclic voltammetry and electrochemical impedance spectroscopy were performed on the modified working electrodes to observe changes to the layer on addition of the toxin. Both methods were able to distinguish the difference between the presence of the active toxin and a placebo containing the excipients of the pharmaceutical product. The electrochemical impedance spectroscopy assay also allowed for detection of the active toxin at concentrations as low as 25 fg/ml, with results being obtained in under an hour outperforming the mouse bioassay.

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1. Introduction

Botulinum neurotoxins are zinc-dependant endopeptidases known to selectively cleave the soluble N-ethylmaleimide sensitive attachment protein receptor (SNARE) group of proteins [2]. Until recently the only approved method of determining the activity of a botulinum neurotoxin sample was the mouse bioassay. This assay involves injected mice intraperitoneally with a sample of the toxin and observing them for symptoms of the disease such as a wasp-waist and paralysis over 4 days [16]. The mouse bioassay is capable of detecting 10 pg/ml of active toxin; however, it is very lengthy, expensive and has ethical problems due to the use of live animals [14]. In 2011 a cell-based assay developed by Allergan Inc., was granted approval by the FDA to replace the mouse bioassay for the potency testing of BOTOX® [10]. In brief this assay uses neuronal cells on a 96 well plate to which the toxin sample is added. After incubating with the sample the cells are lysed and ELISA performed using antibodies for whole and cleaved SNAP-25. This assay is reported to equal the sensitivity of the mouse bioassay but is still lengthy taking 3 days to perform [4]. The major benefit of the mouse bioassay and this cell based assay is the ability to assess the toxins full activity from cell uptake through to

proteolytic activity which is vital for the pharmaceutical industry. The development of an assay which can detect and quantify the presence of the toxin at similar or lower limits of detection in a shorter period of time is desirable to improve on the existing methods whilst eliminating the need for live animals. A point of care sensor for suspected botulinum poisoning cases would not need to assess all modes of action just the presence of toxin in a blood sample. This study reports the development of a biosensor for the detection of active botulinum neurotoxin A using gold electrodes modified with the SNARE protein SNAP-25.

We previously reported a colourimetric assay based on a SNAP-25 monolayer on colloidal gold [8]. This assay has been developed into an electrochemical assay presented in this paper.

Cyclic voltammetry and electrochemical impedance spectroscopy were employed to monitor the interaction between the active toxin and SNAP-25 at the gold surface. A gold electrode can be easily modified with the SNAP-25 protein due to its four cysteines that are able to form strong sulfur-gold bonds to give a monolayer on the surface. Cleavage of nine amino acids from the SNAP-25 protein upon incubation with botulinum neurotoxin A causes a significant change at the surface which is detected using these techniques.

Electrochemical techniques, such as cyclic voltammetry and electrochemical impedance spectroscopy, are label-free techniques that are highly sensitive to changes and interactions at a surface. Over the past few years a number of advances have been made

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in this area with the development of sensors specific for various toxins, pathogens and biomarkers [3,12,17] due to their high sensitivity and rapid detection times. Cyclic voltammetry provides information on the amount of oxidisable species on a working electrode and the number of electrons involved in the oxidation, which is given in terms of charge [13]. Variations in the charge indicate binding events and changes to the self-assembled monolayer. Impedance is a measure of the complex resistance met when current flows through a circuit made up of resistors, capacitors and inductors. Electrochemical impedance spectroscopy utilises redox probes such as $\text{Fe}(\text{CN})_6^{3-/4-}$ or $\text{Ru}(\text{NH}_3)_6^{2+/3+}$ measuring the ability of these ions to become oxidised and reduced at the working electrode [1]. At unmodified working electrodes the route to the electrode surface is not blocked and these chemicals easily undergo the reactions. If the electrode surface is modified by an alkanethiol or a protein then the ions are more blocked increasing the charge transfer resistance (R_{ct}) of the circuit [11].

2. Materials and methods

Gold working electrodes were purchased from Winkler GmbH Germany, SNAP-25 was from Abcam (ab155885) and all other chemicals were purchased from Sigma Aldrich. Dysport® samples containing lyophilised *Clostridium botulinum* Type A toxin-haemagglutinin complex (4.35 ng), human serum albumin (125 µg) and lactose (2.5 mg) and placebos containing the excipients from Dysport® were kindly supplied by IPSEN Biopharm. Water was purified and had a nominal resistivity of 18 MΩ cm at 25 °C.

All measurements were performed using an Autolab PGSTAT 30 computer-controlled electrochemical measurement system (Eco Chemie, Holland) with a home-made three electrode cell with a SNAP-25 modified Au(111) working electrode, a platinum counter electrode and a saturated calomel reference electrode.

2.1. Self-assembled monolayers of SNAP-25

Gold working electrodes were flame annealed to produce Au(111) terraces [7]. After cooling they were placed in a solution of SNAP-25 (125 µg/ml) in water and left to incubate for 48 h at 20 °C.

2.2. Stripping voltammetry of SNAP-25 monolayer

Gold electrodes were removed from the SNAP-25 solution, rinsed thoroughly with ultra-pure water, dried under nitrogen and sealed in the electrochemical cell. NaOH (100 mM, 15 ml) was added and degassed for 20 min with nitrogen. Two cyclic voltammograms were measured between 0 and –1100 mV at a scan rate of 50 mV s⁻¹.

2.3. Pre-treatment of samples

Samples were prepared as described previously [8].

2.4. Cyclic voltammetry assay

The sample (200 µl) was added to the SNAP-25 modified gold electrode and incubated for 10 min at 37 °C. The sample was then removed and the surface rinsed thoroughly with ultra-pure water. Phosphate buffer (100 mM, pH 7) was added to the cell and degassed for 20 min with nitrogen before the voltammogram was performed with a start potential of 0 mV to a first vertex potential of 1100 mV at a scan rate of 50 mV s⁻¹. The resulting scan was compared to an average scan of whole SNAP-25.

2.5. Electrochemical impedance spectroscopy using ferri/ferrocyanide as a redox probe

Electrochemical impedance spectroscopy (EIS) was performed using ferri/ferrocyanide (500 µM) as a redox probe in potassium chloride (100 mM). A potential of 147 mV was applied with a perturbation amplitude of 10 mV s⁻¹ between the frequencies of 25 kHz and 0.1 Hz.

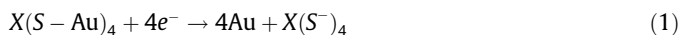
The impedance of the SNAP-25 monolayer was measured as above then the redox probe solution removed and the cell thoroughly rinsed with ultrapure water. Samples (200 µl) were added to the electrode surface and incubated for 25 min at 37 °C then removed and the surface again rinsed with ultrapure water. Redox probe was then added to the cell and the impedance re-measured.

3. Results and discussion

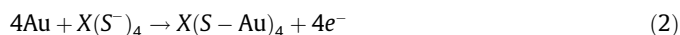
3.1. Stripping voltammogram of SNAP-25 monolayer

Binding of the SNAP-25 monolayer was analysed using cyclic voltammetry. Fig. 1 shows the voltammogram of a SNAP-25 modified Au(111) electrode compared with a bare Au(111) electrode.

The characteristic peak for the reduction of a gold sulfur bond is shown by Peak 1, at –970 mV. The reaction is outlined in Eq. (1) where X represents each SNAP-25 molecule presuming all four cysteines bind to the electrode.



Peak 2, at –850 mV, indicates some of the thiols re-adsorb onto the gold surface as the scan returns to more positive potentials as in Eq. (2).



3.2. Cyclic voltammetry assay for detection of botulinum neurotoxin

A cyclic voltammetry assay was developed that could detect the toxin by measuring changes to the charge of a SNAP-25 monolayer after incubating with the toxin. A typical voltammogram is shown in Fig. 2.

The anodic peak at 950 mV occurs on the oxidation of the gold and protein, the charge was calculated from the area of this peak with the expected decrease in charge on addition of the toxin as shown.

Fig. 3 shows the correlation graph of change in charge against a range of known concentrations of Botulinum neurotoxin in triplicate.

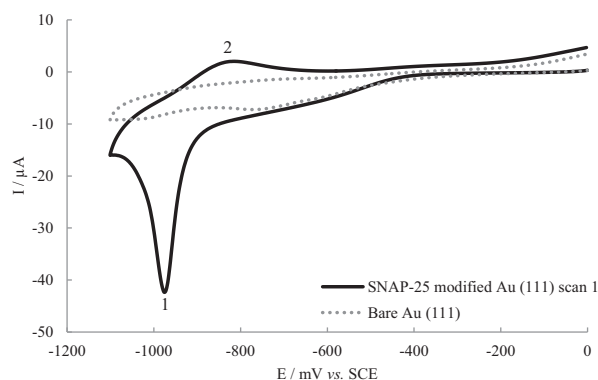


Fig. 1. Voltammograms at a sweep rate of 50 mV s⁻¹ in 0.1 M NaOH; the working electrodes were SNAP-25 modified Au(111) and a bare Au(111) slide.

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