

METHODS AND SYSTEMS FOR ELECTROCHEMICAL DETECTION

FIELD

The present invention concerns methods, systems and kits of parts suitable for detecting
5 the presence of optionally substituted nitrobenzenes. The described methods, systems
and kits may find particular application in the detection of a target in a sample.

BACKGROUND

High sensitivity assays are crucial tools in the point of care diagnostics industry. The
10 need for high sensitivity assays is increasing as new and clinically important biomarkers
are identified, many of which are present in very low concentrations. Lab based high
sensitivity assays exist but typically require numerous assay steps which need to be
carried out by trained laboratory technicians. Such assays can take up to an hour to
generate results. Standardised methods may take longer, such as about 30 minutes to
15 prepare samples, calibration standards and spiked samples and about 90 minutes to
generate results.

Endotoxin is a biological toxin produced by gram negative pathogens that can cause
deleterious effects to human health, such as septic shock, when introduced to the body
20 at significant concentrations. The nature of endotoxin production means that it is
persistently present in the everyday environment. As such, pharmaceutical and food
manufacturers are required by law to regularly test their products for endotoxin and
ensure that endotoxin concentrations adhere to regulatory guidelines and limits. This is
particularly important for the manufacture of medication that is administered via high-risk
25 routes e.g., intravenous, intrathecal, and oral.

Current common endotoxin testing are based on chromogenic, turbidimetric and gel-clot
measurements of an amebocyte lysate, most commonly *Limulus* amebocyte lysate (LAL)
based on the Atlantic horseshoe crab (*Limulus polyphemus*), on interaction with a
30 sample suspected of containing endotoxins. A similar test exists, in which *Tachypleus*
amoebocyte lysate (TAL) based on *Tachypleus gigas* or *Tachypleus tridentatus* horseshoe
crabs is used in place of amebocyte lysate. For a review of methods used to detect
endotoxins, see Su and Ding. Methods of Endotoxin Detection. Journal of Laboratory
Automation. 2015;20(4):354-364 or Tamura, Reich, Nagaoka. Outstanding Contributions
35 of LAL Technology to Pharmaceutical and Medical Science: Review of Methods,

Progress, Challenges, and Future Perspectives in Early Detection and Management of Bacterial Infections and Invasive Fungal Diseases. *Biomedicines*. 2021 May 11;9(5):536.

5 The chromogenic and turbidimetric measurements are photometric techniques that require the production of a chromophore (such as para-nitroaniline, pNA) via an enzyme cascade reaction between an amebocyte lysate, such as LAL, and endotoxin. The presence of endotoxin results in activation of Factor C, which in turn causes activation of Factor B, which causes conversion of proclotting enzyme to clotting enzyme. Once generated, the clotted enzyme acts to cleave a peptidyl-pNA synthetic substrate to
10 release peptide and pNA (yellow chromogen). (See overview of process in Figure 13). The concentration of the chromophore is calculated by measuring the absorbance of the solution in which the cascade reaction took place, and comparing the results to a standard curve to correlate endotoxin concentration against signal intensity. These assays require lengthy reaction times, elevated temperatures, expensive proprietary
15 analysis technology and trained staff for sample preparation, measurement, and analysis.

Electrochemical detection of endotoxin is described by Inoue et al. in *Electrochemical detection of endotoxin using recombinant factor C zymogen*, *Electrochemistry Communications*, 12 (2010) 1066-1069. A recombinant Factor C proenzyme is used, to
20 which endotoxin binds and activates, the resultant activated complex hydrolyzing a synthetic substrate of Boc-Val-Pro-Arg-*p*-nitroaniline to generate pNA. This was detected using differential pulse voltammetry.

25 There remains a need for low cost, high sensitivity assays allowing for quicker detection of targets such as endotoxins without the need for numerous assay steps or trained laboratory technicians. Ideally, the assays are easy to use and reliably produced at low cost and high volume. The present invention provides alternative methods, systems and kits to address these needs.

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SUMMARY

The present inventors have found that optionally substituted nitrobenzenes on or in proximity to a working electrode can be electrochemically detected with unexpectedly enhanced sensitivity by applying a pre-conditioning voltage to the working electrode such
35 that the optionally substituted nitrobenzene is converted to a first state, and then applying

a testing voltage, different from the pre-conditioning voltage, such that the first state is converted to a second state, and an electrical response is sensed. Where the optionally substituted nitrobenzene is part of a first compound and is conjugated to a cleavable group, the inventors have found that the electrical response may be reduced or negligible, i.e. there may be no electrical response, but on cleaving the cleavable group, for example with a cleaving agent, the resultant (cleaved) optionally substituted nitrobenzene generates or increases the electrical response. Where the cleaving agent is, or is generated by, a target, the method may be used to determine the presence or absence of target in a sample: generation of or increase of an electrical response may indicate the presence of a target in a sample.

Without being bound by theory, the inventors have recognised that applying a specific pre-conditioning voltage to optionally substituted nitrobenzene reduces the nitro group to a hydroxylamine group, which may be a first state. This is believed to be an irreversible four electron reduction reaction. The hydroxylamine group can then be detected by applying a different voltage (a specific testing voltage and/or a specific potential window), which reversibly oxidises the hydroxylamine group to a nitroso group, which may be a second state, and thus generates an electrical response which can be sensed. The inventors have found that the electrochemical signal generated by reduction of the nitroso group to the hydroxylamine group, i.e. generated on applying the testing voltage, is unexpectedly greater than that generated on reduction of the nitro group to the hydroxylamine group. The electrical response detected is proportional to the concentration of optionally substituted nitrobenzene present on or in proximity to the working electrode. Thus, these new findings have also led the inventors to unexpectedly identify that electrochemically detecting the hydroxylamine group, i.e. the first state, rather than the nitro group of the optionally substituted nitrobenzene, gives rise to surprisingly more sensitive detection of the optionally substituted nitrobenzene.

Accordingly, viewed from a first aspect, the invention provides a method of detecting an optionally substituted nitrobenzene, the method comprising:

(i) providing a first compound comprising an optionally substituted nitrobenzene conjugated to a cleavable group and contacting the first compound with a cleaving agent such that the cleaving agent cleaves the cleavable group from the first compound to provide the optionally substituted nitrobenzene; and then:

(ii) applying a pre-conditioning voltage to a working electrode such that an optionally substituted nitrobenzene on or in proximity to the working electrode is converted to a first state;

5 (iii) applying a testing voltage to the working electrode such that the first state is converted to a second state; and

(iv) sensing an electrical response.

10 The inventors have recognised that the described methods of detecting an optionally substituted nitrobenzene may have wider applicability in the detection of other groups and targets. In particular, the electrical response sensed via the method of the first aspect is proportional to the concentration of optionally substituted nitrobenzene cleaved by the cleaving agent, which may itself be proportional to the concentration of the cleaving agent. The cleaving agent may be, or may be generated by, a target. Thus, the method of the first aspect may be used to detect a target in a sample.

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Viewed from a second aspect, therefore, the invention provides a method of detecting a target in a sample, wherein the target is a cleaving agent, or the presence of the target generates a cleaving agent, the method comprising:

20 (i) contacting a first compound, comprising an optionally substituted nitrobenzene conjugated to a cleavable group, with a sample such that when the target is present, the cleaving agent (e.g. which may be the target itself or may be generated by the presence of the target) cleaves the cleavable group from the first compound to provide the optionally substituted nitrobenzene;

25 (ii) applying a pre-conditioning voltage to the working electrode such that cleaved optionally substituted nitrobenzene, when present, is converted to a first state;

(iii) applying a testing voltage to the working electrode such that the first state, when present, is converted to a second state; and

(iv) sensing an electrical response.

30 The method may further comprise providing the first compound on or in proximity to the working electrode. Thus, any cleaved optionally substituted nitrobenzene is also on or in proximity to the working electrode once cleaved from the cleavable group.

35 The target may be detected indirectly: the electrical response may be proportional to the concentration of optionally substituted nitrobenzene present on or in proximity to the

working electrode, which is itself proportional to the concentration of the cleaving agent, wherein the cleaving agent is or is generated by the target. In some embodiments, the target generates the cleaving agent. For example, the target may interact with, e.g. react with, one or more reagents within, e.g. added to, the sample to generate the cleaving agent. The cleaving agent may be the product of a number of reaction pathways initiated by the presence of the target. Alternatively, the cleaving agent may be the target. In both cases, when the target is not present in the sample, no cleaving agent is generated or present in the sample, thus no optionally substituted nitrobenzene (without a cleavable group) is present, and minimal, or substantially no, electrical response is generated at the testing voltage.

Where the target is present in the sample, and it is or it generates a cleaving agent which cleaves the cleavable group from the first compound, cleaved optionally substituted nitrobenzene (i.e. optionally substituted nitrobenzene without a cleavable group) is generated in the sample. Detecting the hydroxylamine group, i.e. the first state, of the cleaved optionally substituted nitrobenzene, rather than the nitro group of the cleaved optionally substituted nitrobenzene, gives rise to surprisingly more sensitive detection of the optionally substituted nitrobenzene. Thus, the method of the invention is unexpectedly sensitive, and is capable of detecting low concentrations of target, such as about 1 pM, about 500 fM or about 1 fM of target in a sample, or about 5 ng/mL, about 500 pg/mL, about 50 pg/mL, about 5 pg/mL, about 500 fg/mL or about 100 fg/mL.

The inventors have further recognised that the electrical response for optionally substituted nitrobenzene may be significantly amplified when square wave voltammetry is used to apply the testing voltage and sense the electrical response. Thus, in some embodiments, the testing voltage is applied and the electrical response is sensed by square wave voltammetry. However, the electrochemical method need not be limited to square wave voltammetry: differential pulse voltammetry, cyclic voltammetry, electrical impedance spectroscopy, chronoamperometry, coulometry and potentiometry may be used in addition or alternative to square wave voltammetry.

Viewed from a third aspect, the invention provides a system comprising:

- (a) a test-strip comprising:
 - (i) a working electrode; and

(ii) a first compound comprising an optionally substituted nitrobenzene conjugated to a cleavable group; and

(b) a potentiostat,

wherein the first compound is on or in proximity to the working electrode;

5 the potentiostat is configured to apply a pre-conditioning voltage to the working electrode such that the optionally substituted nitrobenzene is converted to a first state; and

the potentiostat is configured to apply a testing voltage to the working electrode such that the first state is converted to a second state; and

10 wherein the pre-conditioning voltage is different to the testing voltage.

Viewed from a fourth aspect, the invention provides a kit of parts comprising, as separate components:

(a) a test-strip comprising:

15 (i) a working electrode; and

(ii) a first compound comprising an optionally substituted nitrobenzene conjugated to a cleavable group;

(b) a potentiostat; and

20 (c) one or more reagents capable of reacting with a target to generate a cleaving agent capable of cleaving the cleavable group from the first compound;

wherein the first compound is on or in proximity to the working electrode;

the potentiostat is configured to apply a pre-conditioning voltage to the working electrode such that the optionally substituted nitrobenzene is converted to a first state; and

25 the potentiostat is configured to apply a testing voltage to the working electrode such that the first state is converted to a second state; and

wherein the pre-conditioning voltage is different to the testing voltage.

BRIEF DESCRIPTION OF FIGURES

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The present disclosure will now be further described, by way of example only, with reference to the following figures:

35 Figure 1: Electrochemical characterisation of pNA. The plot shows the cyclic voltammagram (CV) trace for a -1.2V to 0.3 V scan. The CV results for the -1.2 to 0.3 V

scan (blue) identified four peaks, three of which are pNA reactions. These occurred at -0.8 V, -0.4 V, -0.1 V and 0 V. The -0.1 V and 0 V peaks were dependent on the -0.8 V peak occurring beforehand. This was justified by observing the lack of peaks at -0.1 V and 0 V (red CV trace) which did not include the -0.8 V reaction.

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Figure 2: Square-wave voltammetry (SWV) scan showing dose response of the -0.8 V and -0.1 to 0V pNA reactions. Dark green line shows 10mM pNA, -1.2 to 0.3 V; red line shows 1mM pNA, -1.2 to 0.3V; blue line shows 0.1 mM pNA, -1.2 to 0.3V; light green line shows 10 mM pNA, -0.6 V to 0.3V; light red line shows 1mM pNA, -0.6 to 0.3V; light blue line shows 0.1 mM pNA, -0.6 to 0.3V.

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Figure 3: Electrochemical characterisation of pNA: Showing the results obtained using different frequencies for SWV.

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Figure 4: Electrochemical characterisation of pNA: Showing the results obtained using different amplitudes for SWV.

Figure 5: Electrochemical characterisation of pNA: -0.8 V preconditioning tests for SWV, showing no preconditioning, KCl background (-0.8V preconditioning, 20 seconds), pNA (-0.8 V preconditioning for (i) 20 seconds; (ii) 10 seconds; and (iii) 2 seconds).

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Figure 6: Electrochemical characterisation of pNA: -1 V preconditioning tests for SWV, showing no preconditioning, KCl background (-1.0V preconditioning, 20 seconds), pNA (-1.0 V preconditioning for (i) 20 seconds; (ii) 10 seconds; and (iii) 2 seconds).

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Figure 7: Electrochemical characterisation of pNA. Showing results obtained with different parameters (blue – top line on trace): 50 Hz frequency, 0.1 V amplitude, -1 V for 20 seconds preconditioning and a potential window of -0.4 to 0.3 V. Original parameters (red – bottom line on trace).

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Figure 8: Electrochemical characterisation of pNA: Showing the results obtained using different frequencies for SWV of pNA.

Figure 9: Electrochemical characterisation of pNA: Showing the results obtained using different Estep (step potential increments) for SWV of pNA.

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Figure 10: Electrochemical characterisation of pNA: Showing further optimised parameters vs original parameters.

5 Figure 11: Electrochemical characterisation of pNA generated in an endotoxin assay: Showing further optimised settings (blue – top line on trace) vs original settings (red – bottom line on trace) in an endotoxin assay using 5 EU/mL Lonza kinetic-QCL.

Figure 12: Showing the results of three additional cyclic voltammogram (CV) studies
10 were carried out on the following mixtures: (i) pNA peptide only; (ii) 0 EU/mL endotoxin and pNA peptide; and
(iii) 5 EU/mL endotoxin and pNA peptide.

Figure 13: Overview of LAL (limulus ameobocyte lysate) reaction cascade that can be
15 used to detect bacterial endotoxins.

Figure 14: Endotoxin dose response curve for an electrochemical sensor assay using rFC reagents.

20 **DETAILED DESCRIPTION**

In the discussion that follows, reference is made to a number of terms, which have the meanings provided below, unless a context indicates to the contrary. The nomenclature used herein for defining compounds, in particular the compounds according to the invention, is in general based on the rules of the IUPAC organisation for chemical
25 compounds, specifically the “IUPAC Compendium of Chemical Terminology (Gold Book)”.

It should be understood that throughout this specification, the terms “comprise”, “comprising” and/or “comprises” is/are used to denote that aspects, embodiments and
30 examples of this disclosure “comprise” a particular feature or features. It should be understood that this/these terms may also encompass aspects, embodiments and/or examples which “consist essentially of” or “consist of” the relevant feature or features.

The term “comprising” or variants thereof will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

- 5 The term “consisting” or variants thereof will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, and the exclusion of any other element, integer or step or group of elements, integers or steps.

- 10 The term “about” herein, when qualifying a number or value, is used to refer to values that lie within $\pm 5\%$ of the value specified. For example, where a voltage is described as being 1 V, a range of 0.95 V to 1.05 V is included.

- 15 Herein, where a linking moiety, such as a bivalent moiety, is described as being “capable of donating or withdrawing electrons”, this is relevant to the ability of methylene ($-\text{CH}_2-$) to donate or withdraw electrons. In other words, a linking moiety that is capable of donating electrons is one that is capable of donating electrons more strongly than methylene and a linking moiety that is capable of withdrawing electrons is one that is capable of withdrawing electrons more strongly than methylene. Electron donation and electron withdrawal may be by resonance or induction.

- 20 As used herein, the term substituent may include, but is not limited to, hydroxy, thiol, carboxyl, cyano (CN), nitro (NO_2), halo, haloalkyl (e.g. a C_1 to C_6 haloalkyl or a C_1 to C_4 haloalkyl), an alkyl group (e.g. C_1 to C_{10} or C_1 to C_6), an alkenyl group (e.g. C_2 to C_6), an alkynyl group (e.g. C_2 to C_6), aryl (e.g. phenyl and substituted phenyl for example benzyl or benzoyl), morpholino, N- C_{1-6} alkylenylmorpholine, alkoxy group (e.g. C_1 to C_6 alkoxy or C_1 to C_4 alkoxy), haloalkoxy (e.g. C_1 to C_4 haloalkoxy), aryloxy (e.g. phenoxy and substituted phenoxy), hydroxyalkynyl (e.g. C_2 to C_6), thioether (e.g. C_1 to C_6 alkyl or aryl thioether), alkylthio (e.g. C_1 to C_6 alkylthio), cyanoalkyl (e.g. C_1 to C_6), oxo, keto (e.g. C_1 to C_6 keto), ester (e.g. C_1 to C_6 alkyl or aryl ester, which may be present as an oxyester or carbonylester on the substituted moiety), thioester (e.g. C_1 to C_6 alkyl or aryl thioester), alkylene ester (such that attachment is on the alkylene group, rather than at the ester function which is optionally substituted with a C_1 to C_6 alkyl or aryl group), amine (including monoalkylamino, dialkylamino, a five- or six-membered cyclic alkylene amine optionally substituted with one or more halo, further including a C_1 to C_6 alkyl amine or a
- 35 C_1 to C_6 dialkyl amine which alkyl groups may be substituted with one or two hydroxyl

groups, and also including alkylphenylamino or alkylphenyl(alkyl)amino groups), amido (including $-\text{C}(\text{O})\text{NH}_2$, $-\text{C}(\text{O})\text{NH}(\text{alkyl})$ such as $-\text{C}(\text{O})\text{NH}(\text{C}_{1-4}\text{alkyl})$, $-\text{C}(\text{O})\text{N}(\text{alkyl})_2$ such as $-\text{C}(\text{O})\text{N}(\text{C}_{1-4}\text{alkyl})_2$, $-\text{NHC}(\text{O})\text{alkyl}$ such as $-\text{NHC}(\text{O})\text{C}_{1-4}\text{alkyl}$, $-\text{NHC}(\text{O})(\text{phenyl})$, $-\text{N}(\text{alkyl})\text{C}(\text{O})(\text{alkyl})$ such as $-\text{N}(\text{C}_{1-4}\text{alkyl})\text{C}(\text{O})(\text{C}_{1-4}\text{alkyl})$, $-\text{N}(\text{alkyl})\text{C}(\text{O})(\text{phenyl})$ such as $-\text{N}(\text{C}_{1-4}\text{alkyl})\text{C}(\text{O})(\text{phenyl})$, $\text{N}-\text{C}_{1-6}\text{alkylenylamino}$, amido (e.g. which may be substituted with one or two C_1 to C_6 alkyl groups (including a carboxamide which is optionally substituted with one or two C_1 to C_6 alkyl groups), aminoalkyl (e.g. C_1 to C_4 aminoalkyl), alkanol (e.g. C_1 to C_6 alkyl, C_1 to C_4 alkyl or aryl alkanol), or carboxylic acid (e.g. C_1 to C_6 alkyl or aryl carboxylic acid), sulfoxide, sulfone, sulfinimide, sulfonamide, and urethane (such as $-\text{O}-\text{C}(\text{O})-\text{NR}_2$ or $-\text{N}(\text{R})-\text{C}(\text{O})-\text{O}-\text{R}$, wherein each R in this context is independently selected from C_1 to C_6 alkyl or aryl), a heteroaryl, arylalkyl (such as an aryl $\text{C}_{1-4}\text{alkyl}$), heteroarylalkyl (such as a heteroaryl $\text{C}_{1-4}\text{alkyl}$), $-\text{OC}_{1-4}\text{alkylphenyl}$, $-\text{C}(\text{O})\text{alkyl}$ such as $-\text{C}(\text{O})(\text{C}_{1-4}\text{alkyl})$, $-\text{C}(\text{O})\text{alkylphenyl}$ such as $\text{C}(\text{O})(\text{C}_{1-4}\text{alkylphenyl})$, $-\text{C}(\text{O})\text{haloalkyl}$ such as $-\text{C}(\text{O})(\text{C}_{1-4}\text{haloalkyl})$, $-\text{SO}_2(\text{alkyl})$ such as $-\text{SO}_2(\text{C}_{1-4}\text{alkyl})$, $-\text{SO}_2(\text{phenyl})$, $-\text{SO}_2\text{haloalkyl}$ such as $\text{OSO}_2(\text{C}_{1-4}\text{haloalkyl})$, $-\text{SO}_2\text{NH}_2$, $-\text{SO}_2\text{NH}(\text{alkyl})$ such as $-\text{SO}_2\text{NH}(\text{C}_{1-4}\text{alkyl})$, $-\text{SO}_2\text{NH}(\text{phenyl})$, $-\text{NHSO}_2(\text{alkyl})$ such as $-\text{NHSO}_2(\text{C}_{1-4}\text{alkyl})$, $-\text{NHSO}_2(\text{phenyl})$, $-\text{NHSO}_2(\text{haloalkyl})$ such as $-\text{NHSO}_2(\text{C}_{1-4}\text{haloalkyl})$, $-\text{S}-\text{C}_{1-3}\text{haloalkyl}$, $-\text{CH}_2\text{C}(\text{O})\text{N}(\text{R}^C)_2$, $-\text{C}_{3-4}\text{alkynyl}(\text{NR}^C)_2$, $\text{deuteroC}_{2-4}\text{alkynyl}$, $(\text{C}_{1-3}\text{alkoxy})\text{haloC}_{1-3}\text{alkyl}$, $\text{C}_{3-6}\text{cycloalkyl}$ (wherein said $\text{C}_{3-6}\text{cycloalkyl}$ is optionally substituted with halo or $\text{C}_{1-3}\text{alkyl}$), $\text{HC}(\text{O})-$, $-\text{CO}_2\text{R}^C$, or $-\text{CO}_2\text{N}(\text{R}^C)_2$, wherein R^C is hydrogen or $\text{C}_{1-3}\text{alkyl}$.

In some examples, and unless the context indicates otherwise, a "substituent" may include, but is not limited to, halo, C_1 to C_6 alkyl, C_1 to C_6 haloalkyl, C_1 to C_6 alkoxy, hydroxyl, oxo, amino (such as NR^1R^2), amido (such as $-(\text{C}=\text{O})\text{NR}^1\text{R}^2$ or $\text{NR}^1(\text{C}=\text{O})\text{C}_{1-6}\text{alkyl}$), keto (such as $-(\text{C}=\text{O})\text{C}_{1-6}\text{alkyl}$), and ester (such as $-\text{O}(\text{C}=\text{O})\text{C}_{1-6}\text{alkyl}$ or $-(\text{C}=\text{O})\text{OC}_{1-6}\text{alkyl}$); and wherein R^1 and R^2 are each independently selected from H and C_1 to C_6 alkyl.

As used herein, "alkyl" defines univalent groups derived from alkanes by removal of a hydrogen atom from any carbon atom, wherein the term "alkane" is intended to define acyclic branched or unbranched hydrocarbons having the general formula $\text{C}_n\text{H}_{2n+2}$, wherein n is an integer ≥ 1 . Alkyl groups may be $\text{C}_{1-6}\text{alkyl}$ groups. In some cases, alkyl groups are $\text{C}_{1-4}\text{alkyl}$ groups. $\text{C}_{1-4}\text{alkyl}$ refers to any selected from the group consisting of methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *sec*-butyl, *iso*-butyl and *tert*-butyl.

As used herein, the term “alkenyl” defines monovalent groups derived from alkenes by removal of a hydrogen atom from any carbon atom, wherein the term “alkene” is intended to define acyclic branched or unbranched hydrocarbons having the general formula C_nH_{2n} , wherein n is an integer ≥ 2 . Examples of alkenyl groups include ethenyl, n-propylenyl, iso-propylenyl, n-butylenyl, sec-butylenyl, iso-butylenyl and tert-butylenyl.

As used herein, the term “alkynyl” defines monovalent groups derived from alkynes by removal of a hydrogen atom from any carbon atom, wherein the term “alkyne” is intended to define acyclic branched or unbranched hydrocarbons having the general formula C_nH_{2n-2} , wherein n is an integer ≥ 2 . Examples of alkynyl groups include ethynyl, n-propylnyl, iso-propylnyl, n-butylnyl, sec-butylnyl, iso-butylnyl and tert-butylnyl.

The term “aryl” is well known in the art and defines univalent groups derived from a mono- or polycyclic aromatic hydrocarbon system having 6 to 14 carbon atoms, in some cases having 6 to 10 carbon atoms. Representative examples of suitable “aryl” groups include, but are not limited to, phenyl, biphenyl, naphthyl, 1-naphthyl, 2-naphthyl and anthracenyl.

The term “organyl” refers herein to a univalent organic substituent (any substituent comprising carbon) with one free valence at a carbon atom. Typically, the organic substituent comprises only non-metallic elements. Non-metallic elements include reactive non-metals and metalloids.

The term “halo” is well known and defines univalent halogen ions. Typically, the halo includes fluoro, chloro, bromo and iodo. In some cases, halo is fluoro. The term halide may be used to refer to the halogen anion having a single negative charge (and may encompass the anions of each halogen described above).

The term “haloalkyl” is also well known and defines univalent groups derived from alkyl groups by replacement of one or more hydrogen atoms from one or more carbon atoms with a halo group. Haloalkyl groups may comprise one or more different types of halo. For example, one or more independently selected from fluoro, chloro, bromo and iodo. In some cases, the haloalkyl is a fluoroalkyl.

The term “alkoxy” defines univalent groups derived from alcohols by removal of a hydrogen atom from an OH group, wherein the term “alcohol” is intended to define groups derived from alkanes by the replacement of a hydrogen atom with a hydroxy group. Often, alkoxy groups are C₁₋₆alkoxy groups or C₁₋₄alkoxy groups. The term
5 alkoxide may be used to refer to an anion group derived from an alcohol, wherein a proton has been lost from the OH group, leaving an oxygen having a single negative charge (and may encompass the respective anions of each alkoxy group described above).

10 The term “aryloxy” defines univalent groups derived from an aromatic alcohols by removal of a hydrogen atom from an OH group. Often, aryloxy groups are phenoxy groups. The term aryloxide may be used to refer to an anion group derived from an aryl alcohol, wherein a proton has been lost from the OH group, leaving an oxygen having a single negative charge (and may encompass the respective anions of each aryloxy group
15 described above).

The term “alkylol” defines univalent groups derived from alcohols by removal of a hydrogen atom from a carbon atom. Often, alkylol groups are C₁₋₆alkylol groups or C₁₋₄alkylol groups.

20 A “peptide nucleic acid” (PNA) monomer is a synthetic mimic of a nucleotide, in which the deoxyribose phosphate is replaced by a pseudo-peptide polymer to which the nucleobases are linked. PNAs are able to hybridize with complementary DNAs or RNAs. For a review of peptide nucleic acids, see Shakeel, Karim and Ali, Peptide nucleic acid
25 (PNA) – a review, Journal of Chemical Technology and Biotechnology, 2006, 81, 6, 892-899.

A “locked nucleic acid” (LNA) monomer, also known as a “bridged nucleic acid” (BNA), is a modified RNA nucleotide in which the ribose moiety is modified to comprise a bridge
30 (typically a methylene bridge) connecting the 2' oxygen and 4' carbon. The bridge “locks” the ribose in the 3'-endo conformation, which provides for increased stability against enzymatic degradation. LNAs are able to hybridize with complementary DNAs or RNAs. For a review of LNAs, see Vester and Wengel, LNA (Locked Nucleic Acid): High-Affinity Targeting of Complementary RNA and DNA, Biochemistry, 2004, 43, 42, 13233-13241.

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5 A “glycol nucleic acid” (GNA) monomer is a nucleotide differing from DNA or RNA nucleotides by the composition of the sugar-phosphodiester backbone. Propylene glycol is used in place of ribose or deoxyribose (see, for example, Zhang, Peritz and Meggers, A simple Glycol Nucleic Acid, Journal of American Chemical Society, 2005, 127, 12, 4174-4175).

10 A “xeno nucleic acid” (XNA) monomer is a nucleotide differing from DNA or RNA nucleotides by chemical modification of the sugar moiety. Typically, the XNA monomer comprises xylose (i.e. is a xylo-nucleic acid, XyNA) or deoxyxylose (i.e. is a deoxyxylo-nucleic acid, dXyNA) rather than ribose or deoxyribose (see, for example, Sharpe, Röder and Wales, Energy Landscapes of Deoxyxylo- and Xylo-Nucleic Acid Octamers, Journal of Physical Chemistry B, 2020, 124, 20, 4062-4068).

15 A “phosphorodiamidate morpholino oligomer” (PMO) monomer is a nucleotide differing from DNA or RNA nucleotides by the composition of the sugar-phosphodiester backbone. The phosphate and deoxyribose or ribose are replaced with phosphorodiamate and morpholine, respectively (see, for example, Nan and Zhang, Antisense Phosphorodiamidate Morpholino Oligomers as Novel Antiviral Compounds, Front. Microbiol., 2018, 9, 750).

20 A “molecularly imprinted polymer” (MIP) is a polymer that has been processed using the molecular imprinting technique which leaves cavities in the polymer matrix with an affinity for a desired template molecule.

25 In a further aspect, there is provided a method of detecting an optionally substituted nitrobenzene, the method comprising:

- (i) applying a pre-conditioning voltage to a working electrode such that an optionally substituted nitrobenzene on or in proximity to the working electrode is converted to a first state;
- 30 (ii) applying a testing voltage to the working electrode such that the first state is converted to a second state; and
- (iii) sensing an electrical response.

35 The method of detecting an optionally substituted nitrobenzene described herein, such as in the first aspect, comprises:

(i) applying a pre-conditioning voltage to a working electrode such that an optionally substituted nitrobenzene on or in proximity to the working electrode is converted to a first state;

5 (ii) applying a testing voltage such that the first state is converted to a second state, wherein the testing voltage is different to the pre-conditioning voltage; and

(iii) sensing an electrical response.

10 As stated above, the first state may be the hydroxylamine derivative of the optionally substituted nitrobenzene (i.e. the optionally substituted nitrobenzene may be irreversibly reduced to the hydroxylamine to provide the first state). The second state may be the nitroso derivative of the optionally substituted nitrobenzene (i.e. the hydroxylamine may be reversibly oxidised to the nitroso compound to provide the second state).

15 The optionally substituted nitrobenzene may be positioned on or in proximity to the working electrode by any suitable means. For example, by depositing on or in proximity to the electrode as part of a solution or suspension, for example by spraying or dripping. The optionally substituted nitrobenzene may be positioned on the working electrode, or may overlap with the working electrode such that some of the optionally substituted nitrobenzene is on the working electrode and some is not. However, at least some of
20 the optionally substituted nitrobenzene is in proximity to the working electrode. The optionally substituted nitrobenzene may be positioned on or in proximity to the working electrode such that it is converted to a first state upon application of a pre-conditioning voltage and a second state upon application of the testing voltage.

25 The pre-conditioning voltage acts to convert the optionally substituted nitrobenzene on or in proximity to the working electrode to a first state. As described above, and without being bound by theory, the inventors have recognised that applying a specific pre-conditioning voltage to optionally substituted nitrobenzene reduces the nitro group to a hydroxylamine group. This hydroxylamine group is detectable by applying a different
30 voltage (a specific testing voltage applied to the working electrode), which oxidises the hydroxylamine group to a nitroso group, and thus generates an electrical response which can be sensed.

35 The precise pre-conditioning voltage and testing voltage applied to the working electrode and/or used in the described methods may vary, for example, in accordance with the

nature of the optionally substituted nitrobenzene (e.g. the nature of any further substituents on the nitrobenzene), the type of electrodes used and the desired level of sensitivity.

5 In some examples, a pre-conditioning voltage is selected such that the optionally substituted nitrobenzene is reduced to the first state, wherein the first state is a hydroxylamine group. By way of example only, the pre-conditioning voltage may be any voltage between about -0.3 V to about -2 V, about -0.65 V to about -1.1 V, or about -0.8 V to about -1.0 V. The pre-conditioning voltage may be about -0.3 V to about -2 V, about
10 -0.65 V to about -1.1 V, or about -0.8 V to about -1.0 V, such as about -1.0 V.

The pre-conditioning voltage may be applied for any duration of time such that the optionally substituted nitrobenzene (or a sufficient amount thereof) is reduced to the first state. By way of example, the pre-conditioning voltage may be applied for a duration of
15 about 0.01 to about 40 seconds, about 0.1 to about 30 seconds, or about 1 second to about 20 seconds. In particular embodiments, the pre-conditioning voltage is applied for a duration of about 20 seconds.

In some examples, the pre-conditioning voltage may be between about -0.8 and about -
20 1 V and/or may be applied for a duration between about 5 and about 30 seconds. In yet further examples, the pre-conditioning voltage may be about -1 V and/or may be applied for a duration of about 20 seconds.

As stated above, the testing voltage acts to oxidise the first state to the second state. In
25 other words, and without being bound by theory, the testing voltage acts to oxidise the optionally substituted hydroxylaminobenzene to an optionally substituted nitrosobenzene. The testing voltage may be any one voltage or a potential window selected to ensure that the first state is oxidised to the second state. A potential window may refer to a scan between a first and second voltage, e.g. any of the upper and lower
30 limits of voltage described herein.

In some examples, the testing voltage is any one voltage or a potential window between about -1 and about 1 V, between about -0.5 V and about 0.5 V, between about -0.4 V and about 0.3 V, or between about -0.1 and about 0.1 V. In particular examples, the
35 testing voltage is any one voltage or a potential window of about -1 to about 1 V, about -

0.5 V to about 0.5 V, about - 0.4 V to about 0.3 V, or about -0.1 to about 0.1 V. For example, the testing voltage may be a potential window or scan of about -0.1 V to about 0.2 V. In particular examples, the testing voltage may be a potential scan between about -0.4 V and about 0.3 V.

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In particular embodiments, the testing voltage is a potential scan of about -0.1 V to about 0.2 V. The inventors have found that scans across such voltages unexpectedly lead to an improved reproducibility of electrical responses and thus provided more sensitive assays that could be used to detect lower concentrations of target.

10

The testing voltage may be applied immediately (about 0 s) after the pre-conditioning voltage. In alternative examples, the testing voltage may be applied a short period after the pre-conditioning voltage, such as about 0.01 to about 10 seconds, or about 0 to about 10 seconds after the pre-conditioning voltage.

15

The described methods involve applying a pre-conditioning and testing voltages to a working electrode. The working electrode is typically part of an electrode system comprising at least one further electrode, i.e. the methods of the disclosure typically comprises an electrode system comprising two or more electrodes.

20

In some examples, the electrode system is electronically connected to a sensor such as a potentiostat. In some examples, the electrical response that is sensed in the described methods is current, voltage, resistance and/or impedance. In particular examples, the electrical response that is sensed in the described methods is a current.

25

In some examples, the electrode system may be any system suitable for the application of a potential difference to the working electrode. To supply a potential difference, the electrode system comprises a minimum of two electrodes. For example, the working electrode may apply the pre-conditioning voltage to the optionally substituted nitrobenzene and transport charge to reduce the optionally substituted nitrobenzene. A second electrode is required with a known potential, with which to use as a reference, and to complete the circuit and balance the charge, for example if the working electrode acts as an anode and reduces the optionally substituted nitrobenzene, then the second electrode balances the charge by oxidising a component of the system, thereby acting

30

as a cathode. The second electrode may act as both a reference and a counter electrode.

Typically, the electrode system comprises 2 to 4 electrodes, often 3 or 4. Commonly,
5 the electrode system comprises 3 electrodes. When the electrode system comprises 3 electrodes, it comprises a working electrode, a reference electrode (of a known potential and with which to use as a reference) and a counter electrode (to complete the circuit and balance the charge). In such an electrode system, changes in the potential difference at the working electrode are measured independently to the changes in the
10 potential difference at the counter electrode. This set-up is advantageous over a 2 electrode system because analysis of the electrical response is simpler.

When the electrode system comprises 4 electrodes, it may comprise a working electrode, a reference electrode, a counter electrode and a working sense electrode. In this set-
15 up, the potential difference is measured at the working sense electrode (relative to the reference electrode), and is independent to the electrochemical reaction occurring at the working electrode, i.e. the effect of an applied current on, the system itself is being measured. Such a set-up is useful for the measure of impedance across the system.

20 The electrodes of the electrode system may be made of any material suitable for conducting electrons. It is preferable that the material is resistant to corrosion, and is able to conduct a suitable current load. Sometimes, the current load required is in the range of ± 1 nA to ± 1 mA; ± 10 nA to ± 0.1 mA; or ± 100 nA to ± 0.01 mA (with an error of $\pm 1\%$). Suitable materials include any one or a selection from the group consisting of
25 gold, silver, platinum, palladium, titanium, graphite, carbon, brass, tungsten, ruthenium, iridium, titanium, nickel, aluminium, tin, or one or a selection of their oxides. One or a selection of chlorides of the listed materials may also be suitable electrodes. Often, the electrodes of the system are made from any one or a selection from the group consisting of gold, silver, platinum, palladium, titanium, graphite and carbon. Typically, the
30 electrodes are made from any one or a selection from the group consisting of gold, silver, platinum and palladium, preferably gold and silver. In particular embodiments, the electrodes are made from silver, silver chloride or carbon. In even more particular embodiments, the working electrode and counter electrode are each made from carbon and the reference electrode is made from silver and silver chloride. Commonly, the
35 electrodes are made of one type of material, i.e. they are not made of a selection of

materials, by which is meant that each electrode is made of one type of material: two electrodes may each be made of one type of material, which one type of material may be different for each electrode. In some embodiments, the working electrode and counter electrode are each made of one type of material and the reference electrode is made of a selection of materials.

The electrode system may be produced via additive printing processes, such as 3D-printing or screen-printing. These are suitable approaches for the production of cost-effective electrode systems and sensors (see, for example, Tan, C., Nasir, M. Z. M., Ambrosi, A., Pumera, M., 2017. *Anal. Chem.* 89, 8995-9001, the contents of which are herein incorporated by reference). The electrode system may be microfabricated and may be produced by depositing the desired material, patterning the material with the desired micro features (e.g. by UV photolithography), and if necessary, removing or etching material. Preferably, the electrode system is screen-printed. Screen printed electrodes (SPEs) feature many advantages over more traditional electrodes such as ease of fabrication and cleaning procedures, reliability, low-cost, repeatability and provide rapid time to result. SPEs are amenable to mass production, whereby a large volume of electrodes can be produced at relatively low-cost compared to traditional macro or microelectrodes (see, for example, Hayat, A., Marty, J. L., 2014. *Sensors*. 14, 10432-10453, the contents of which are herein incorporated by reference). Due to these advantages, SPEs lend themselves nicely to prototyping and for the development of novel sensing technologies, as reported here.

The working electrode (and any other electrodes present) may be electronically connected to the potentiostat. The electronic connections may be any suitable connections to carry an electronic signal between the potentiostat and the electrode system. Typically, such electronic connections are electric wires. Each electrode of the electrode system is electronically connected to the potentiostat to allow the application and detection of electrical signals to and from each electrode.

A multitude of electrochemical techniques (square wave voltammetry (SWV), cyclic voltammetry (CV), differential pulsed voltammetry (DPV), chronoamperometry (CA), electrochemical impedance spectroscopy (EIS) chronoamperometry, coulometry, and potentiometry) may be used for measurement and analysis. Thus, the testing voltage may be applied and the electrical response may be sensed by any one electrochemical

technique selected from square wave voltammetry, differential pulse voltammetry, cyclic voltammetry, electrochemical impedance spectroscopy, chronoamperometry, coulometry, and potentiometry. In particular examples, the testing voltage may be applied and the electrical response may be sensed by square wave voltammetry.

5

In square wave voltammetry, typically the current at the working electrode is measured while the potential between the working electrode and a reference electrode is pulsed forward and backward at a constant frequency. The potential waveform can be viewed as a superposition of a regular squarewave onto an underlying staircase. Where square wave voltammetry is used in the methods of this disclosure, the frequency, amplitude and/or step potential increments of the square wave pulse may be selected to further enhance the sensitivity and/or reliability of the detection method.

In some examples, the frequency of the square wave pulse may be less than or equal to about 200 Hz, about 150 Hz, or about 125 Hz. In yet further examples, the frequency of the square wave pulse may be between about 10 and 125 Hz, or between about 25 and 100 Hz, such as about 70 Hz, about 80 Hz, or about 90 Hz. In some embodiments, the frequency of the square wave pulse is less than or equal to about 200 Hz, about 150 Hz, or about 125 Hz, or about 10 to about 125 Hz, or about 25 to about 100 Hz, such as about 25 Hz, about 70 Hz, about 80 Hz, or about 90 Hz. In particular embodiments, the frequency of the square wave pulse is about 25 Hz.

In some examples, the amplitude of the square wave pulse may be between about 0.001 V and about 5V, between about 0.01 V and about 2.5 V, or between about 0.05 V and 0.15 V, such as about 0.1 V. In some embodiments, the amplitude of the square wave pulse is about 0.001 V to about 5 V, about 0.01 V to about 2.5 V, or about 0.05 V to about 0.15 V, such as about 0.05 V or about 0.1 V. In particular embodiments, the amplitude of the square wave pulse is about 0.05 V.

In some examples, the step potential increments of the square wave pulse may be between about 0.001 V and about 0.1 V, or between about 0.01 V and 0.025 V. In some embodiments, the step potential increments of the square wave pulse are about 0.001 V to about 0.1 V, or about 0.01 V to about 0.025 V, such as about 0.01 V.

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For the avoidance of doubt, the scan rate is equal to the frequency of the square wave pulse multiplied by the step potential increments of the square wave pulse. In particular embodiments, the scan rate is about 0.1 to about 0.4 V/s, such as about 0.15 to about 0.35 V/s or about 0.2 to about 0.3 V/s. In more particular embodiments, the scan rate is about 0.25 V/s.

The optionally substituted nitrobenzene detected in the methods of the disclosure may have been cleaved from a first compound comprising an optionally substituted nitrobenzene conjugated to a cleavable group. In particular, the method may comprise providing a first compound comprising an optionally substituted nitrobenzene conjugated to a cleavable group; and then contacting the first compound with a cleaving agent such that the cleaving agent cleaves the cleavable group from the first compound to provide the optionally substituted nitrobenzene (to which the pre-conditioning voltage may be applied according to the methods of the disclosure).

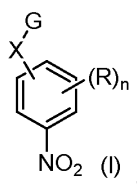
As stated previously, the methods of the disclosure may find particular application in the detection of a target in a sample. In particular, in certain examples, the target is itself a cleaving agent that can cleave and release the optionally substituted nitrobenzene from the first compound. Alternatively, the presence of the target may generate and/or activate the cleaving agent.

Thus, as stated above, there is provided a method of detecting a target in a sample, wherein the target is a cleaving agent, or the presence of the target generates a cleaving agent, the method comprising:

- (i) contacting a first compound, comprising an optionally substituted nitrobenzene conjugated to a cleavable group, with a sample such that when the target is present, the cleaving agent cleaves the cleavable group from the first compound to provide the optionally substituted nitrobenzene;
- (ii) applying a pre-conditioning voltage to the working electrode such that cleaved optionally substituted nitrobenzene, when present, is converted to a first state;
- (iii) applying a testing voltage to the working electrode such that the first state, when present, is converted to a second state; and
- (iv) sensing an electrical response.

As stated above, the first compound comprises an optionally substituted nitrobenzene conjugated to a cleavable group. In other words, the first compound may comprise a nitrobenzene core that is conjugated to a cleavable group and that may optionally comprise one or more (e.g. up to four) additional substituents as defined herein. In some examples, the first compound may comprise a nitrobenzene core that is covalently bonded to a cleavable group (such that the bond to the cleavable group replaces a hydrogen atom on the nitrobenzene ring). In some particular examples, the cleavable group is covalently bonded to the nitrobenzene ring by way of a linking moiety. The linking moiety may be a moiety capable of donating or withdrawing electrons. The linking moiety may be an inductively withdrawing moiety and/or a resonance donor. The optional one or more additional substituents may be covalently bonded to the nitrobenzene ring (again, when present, these groups replacing one or more hydrogen atoms on the nitrobenzene ring).

In some examples, the first compound may be of formula (I):



wherein:

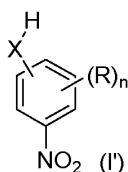
X is NH, O, S -C(CR¹₂)-, -NR¹-, -SO₂-, -C(O)-, -C(O)O-, -OC(O)-, -C(O)NR¹-, or NR¹C(O)-, wherein R¹ is H or an organyl group (such as C₁₋₆ alkyl);

each R is independently selected from the group consisting of C₁₋₆alkyl, halo, C₁₋₆haloalkyl, hydroxy, C₁₋₆alkoxy, C₁₋₆alkylol and phenyl;

n is 0 to 4; and

G is the cleavable group.

Following cleavage of the cleavable group, the cleaved optionally substituted nitrobenzene may be represented by formula (I'):



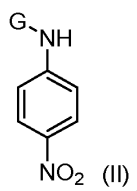
wherein X, R and n are as defined for formula (I).

In some examples of formulae (I) and (I'), X is selected from NH, O and S. In particular examples, X is NH. In some examples, n is 0.

- 5 In some examples of formula (I'), the cleaved optionally substituted nitrobenzene may be nitroaniline, e.g. ortho-, meta-, or para-nitroaniline.

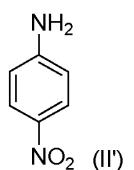
10 In formulae (I) and (I'), the X-G group is shown as being appended to the benzene ring at any chemically suitable position. In other words, it may be any one of an ortho, meta or para-substitution pattern in relation to the nitro group. In some particular examples of the present disclosure, the X-G group may be appended to the benzene core in a para-substitution pattern in relation to the nitro group i.e. by way of covalent bond between the X group and a carbon atom on the benzene ring.

- 15 Accordingly, the first compound may comprise a structure according to formula (II):



wherein G is the cleavable group.

- 20 Following cleavage of the cleavable group from formula (II), the cleaved optionally substituted nitrobenzene may be represented by formula (II'):



In other words, the cleaved optionally substituted nitrobenzene may, in some examples, be para-nitroaniline.

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As stated above, and illustrated in formulae (I) and (II), the first compound comprises a cleavable group. The cleavable group may be any group that is cleaved from the optionally substituted nitrobenzene in the presence of a cleaving agent. Representative

examples of suitable cleavable groups include biological molecules, or any chemical molecule that acts as a leaving group.

5 In some examples, the cleavable group may be a biological molecule comprising any one or more selected from the group consisting of an amino acid, a nucleotide, a saccharide, a phospholipid and a triglyceride. In some yet further examples, the cleavable group may be a biological molecule comprising any one or more selected from the group consisting of a peptide, an oligonucleotide, DNA, RNA, a peptide nucleic acid (PNA), a locked nucleic acid (LNA), a glycol nucleic acid (GNA), a xeno nucleic acid
10 (XNA), a phosphorodiamidate morpholino oligomer (PMO), and a molecularly imprinted polymer (MIP). In a particular example of the disclosure, the biological molecule is a peptide or an oligonucleotide. In particular embodiments, the biological molecule is a peptide, such as a peptide comprising no more than 4 amino acids. In particular embodiments, the peptide comprises no fewer than 3 and no more than 4 amino acids.

15 In some examples, the cleavable group may be a chemical moiety, such as a leaving group. Suitable leaving groups, include, but are not limited to phosphate, sulfonate, halide, alkoxide, aryloxy (such as phenoxide), an amine-based leaving group or a carbonyl-based leaving group (e.g. carboxylate). In some cases, the leaving group (G) will depend upon the nature of the linker moiety (X). One of skill in the art will be able to select an appropriate X and G combination that will cleave in the presence of the cleaving agent, and/or wherein in the presence of a cleaving agent, G acts as a leaving group from X. For example, where X is NH, G may be a carbonyl-based leaving group, such that X and G together form an amide bond, which may be cleaved in a hydrolysis reaction
20 following the addition of an appropriate cleaving agent. In other examples, X may be a carbonyl group and G may be an amino group, such that X and G together form an amide bond, which again may be cleaved in a hydrolysis reaction following the addition of an appropriate cleaving agent.

30 The cleaving agent may be any agent that promotes or facilitates the cleaving of the cleavable group and the optionally substituted nitrobenzene in the first compound. The nature of the cleaving agent may determine the selection of the cleavable group that is conjugated to the optionally substituted nitrobenzene.

Suitable cleaving agents may include, but are not limited to, a catalyst such as an enzyme, an organic catalyst, or an inorganic catalyst. In some examples, the cleaving agent may be an enzyme such as a restriction enzyme, an endonuclease, or an exonuclease. In other examples, the cleaving agent may be irradiation (e.g. visible light, UV light or heat irradiation).

By way of further example, where the cleaving agent is irradiation, the cleavable group is one which will be cleaved under irradiation. In some examples, the cleaving agent may be light or UV light irradiation and the cleavable group may be a photocleavable group.

In some particular examples of the disclosure, the cleaving agent may be a catalyst (such as an enzyme) and the cleavable group is one which is cleaved (e.g. selectively cleaved) by the catalyst. In a yet further example, the cleaving agent may be an enzyme and the cleavable group may comprise a biological molecule, such as a peptide or an oligonucleotide. By way of example, where the cleaving agent is an enzyme, the cleavable group may be any biological molecule that is a substrate for the enzyme and/or that is selectively cleaved by the enzyme. For example, a peptide containing an amino acid sequence that is selectively cleaved by the enzyme, or an oligonucleotide containing a nucleic acid sequence that is selectively cleaved by the enzyme.

As stated previously, the cleaving agent may be a target, or may be generated by a target. Thus, the methods described herein may be useful for detecting the presence and/or levels of a target in a sample. In particular, the amount of cleaving agent (which itself corresponds to, or is proportional to, the amount of target) determines the amount of optionally substituted nitrobenzene cleaved from the first compound.

In those examples where the cleaving agent is generated by a target, the cleaving agent may be the product of a reaction or a series of reactions (such as a cascade reaction) initiated by the presence of a target. In particular, the first compound may be contacted with, positioned at the same position as, or in proximity to, one or more reagents that are capable of reacting with the target to generate a cleaving agent capable of cleaving the cleavable group from the first compound.

In some examples, the cleaving agent is clotting enzyme and/or the target may be endotoxin. The clotting enzyme may be the product of a cascade reaction initiated by an

endotoxin. In particular, the one or more reagents comprise limulus clotting factor C, limulus clotting factor B, and proclotting enzyme. In such an example, the presence of endotoxin in the sample activates factor C, which in turn activates factor B, which in turn converts proclotting enzyme to clotting enzyme (as illustrated in Figure 13). The clotting enzyme then acts as the cleaving agent in the methods of the disclosure to cleave the optionally substituted nitrobenzene from the first compound. In particular, in this example, the first compound is an optionally substituted nitrobenzene conjugated to a peptide by way of a peptide linkage that is specific to the clotting enzyme and/or that is selectively cleaved and/or hydrolysed by the clotting enzyme. Thus, in the presence of endotoxin, clotting enzyme is produced which cleaves the optionally substituted nitrobenzene from the peptide. In such embodiments, the reagents may be Limulus Amebocyte Lysate (LAL) reagents, derived from amebocytes, which are specialized immune cells in the horseshoe crab. However, the reagents are more typically recombinant Factor C (rFC) reagents.

In particular embodiments, the first compound is an optionally substituted nitrobenzene bonded to a peptide, such as a peptide comprising no fewer than 3 and no more than 4 amino acids. The peptide may comprise at least one positively charged amino acid, such as a basic positively charged amino acid, e.g. arginine, histidine or lysine (typically arginine). This amino acid may be bonded to the optionally substituted nitrobenzene. In some embodiments, the peptide comprises just one positively charged amino acid, such as a basic positively charged amino acid, e.g. arginine, histidine or lysine (typically arginine), and the rest of the amino acids are selected from aliphatic, non-polar amino acids, or negatively charged amino acids. Examples of aliphatic, non-polar amino acids are glycine, alanine, valine, isoleucine, leucine and phenylalanine, e.g. glycine, alanine, valine, isoleucine, and leucine. Examples of negatively charged amino acids are glutamic acid and aspartic acid, e.g. glutamic acid. In some embodiments, the peptide comprises just one positively charged amino acid, such as arginine, no more than one negatively charged amino acid, such as aspartic acid, and the rest of the amino acids are selected from aliphatic, non-polar amino acids, such as glycine, alanine, valine, isoleucine, and leucine. In even more particular embodiments, the peptide is any one of the following: Ac-Ile-Glu-Ala-Arg (SEQ ID NO: 1), Boc-Leu-Gly-Arg and Boc-Val-Pro-Arg (where Ac is acetyl and Boc is tert-butyloxycarbonyl). Ac-Ile-Glu-Ala-Arg-pNA (where pNA is para-nitroaniliny) (SEQ ID NO:2) is used in Lonza's commercially available LAL

assays. Boc-Leu-Gly-Arg-pNA is commercially available from various vendors, including BioChemPartner. Boc-Val-Pro-Arg-pNA is used by Inoue et al (supra).

5 In some examples of the disclosure, the strength of the electrical response may be proportional to the amount of target present in the sample. Alternatively or additionally, the strength of the electrical response may be proportional to the amount of cleaving agent generated in a reaction, or a series of reactions, such as a cascade reaction, initiated by the target.

10 As stated previously, the methods of the disclosure have been found to be particularly sensitive. In some examples, the methods described herein may detect the presence of a target in a sample at concentrations less than or equal to about 100 pM, about 50 pM, about 10 pM, about 1 pM, about 100 fM, about 50 fM, or about 1fM, or less than or equal to about 5 ng/mL, about 500 pg/mL, about 50 pg/mL, about 5 pg/mL, about 500 fg/mL or
15 about 100 fg/mL.

In some examples of the methods of this disclosure, a background electrical response may be determined. Typically, the background electrical response may be determined before a sample and/or cleaving agent is contacted with the first compound. In other
20 examples, the background electrical response may be determined at the same time as the sample and/or cleaving agent is first contacted with the first compound (e.g. at time = 0). Accordingly, in some examples, the background electrical response may be measured by:

(i) applying a pre-conditioning voltage to the working electrode such that the
25 optionally substituted nitrobenzene is converted to a first state;

(ii) applying a testing voltage such that the first state is converted to a second state; and

(iii) sensing an electrical response,
before, or at the same time as, the sample and/or cleaving agent is contacted with the
30 first compound.

As stated previously, the first compound may be positioned on or in proximity to the working electrode. In some examples, the first compound may be deposited on or in proximity to a working electrode prior to the addition of the cleaving agent and/or sample.
35 The positioning of the first compound may be by depositing the first compound, for

example by any suitable means of contacting. In some cases, the first compound may be deposited onto the test strip as a solution or suspension, for example by spraying or dripping, and the strip is allowed to dry. In some embodiments, the first compound is printed onto the test strip.

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The step of contacting the first compound with a cleaving agent may be carried out in a liquid.

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In some examples, the cleaving agent may be deposited on or in proximity to the first compound as a solution or suspension.

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In some examples, the first compound may be mixed and/or incubated with a cleaving agent in a liquid (e.g. an aqueous liquid). The mixture may then be deposited on the test strip on or in proximity to the working electrode. In some examples, the first compound may be mixed and/or incubated with a sample and one or more reagents that are capable of reacting with a target to generate the cleaving agent prior to deposition on the test strip. In some embodiments, the first compound may be mixed and/or incubated with a cleaving agent on the test strip on or in proximity to the working electrode. In some examples, the first compound may be mixed and/or incubated with a sample and one or more reagents that are capable of reacting with a target to generate the cleaving agent whilst on the test strip.

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In some embodiments, the first compound and cleaving agent or sample and one or more reagents capable of reacting with a target to generate the cleaving agent are incubated on the test strip on or in proximity to the working electrode. In particular embodiments, incubation is at an elevated temperature, such as at a temperature best suited or optimised for cleavage of the cleavable group. For example, where the cleaving agent or one or more reagents capable of generating the cleaving agent are biological molecules, such as enzymes, incubation may be at a temperature optimal for a particular enzyme. In some examples, the temperature may be about 37 °C.

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As stated previously, the present disclosure further provides a system for performing the methods described herein. The system may comprise:

(a) a test-strip comprising:

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(i) a working electrode; and

(ii) a first compound comprising an optionally substituted nitrobenzene conjugated to a cleavable group; and

(b) a potentiostat,

wherein the first compound is on or in proximity to the working electrode;

5 the potentiostat is configured to apply a pre-conditioning voltage to the working electrode such that the optionally substituted nitrobenzene is converted to a first state; and

the potentiostat is configured to apply a testing voltage to the working electrode such that the first state is converted to a second state; and

10 wherein the pre-conditioning voltage is different to the testing voltage.

Additionally, the disclosure further provides a kit of parts for performing the methods described herein. The kit of parts may comprise, as separate components:

(a) a test-strip comprising:

15 (i) a working electrode; and

(ii) a first compound comprising an optionally substituted nitrobenzene conjugated to a cleavable group;

(b) a potentiostat; and

20 (c) one or more reagents capable of reacting with a target to generate a cleaving agent capable of cleaving the cleavable group from the first compound;

wherein the first compound is on or in proximity to the working electrode;

the potentiostat is configured to apply a pre-conditioning voltage to the working electrode such that the optionally substituted nitrobenzene is converted to a first state; and

25 the potentiostat is configured to apply a testing voltage to the working electrode such that the first state is converted to a second state; and

wherein the pre-conditioning voltage is different to the testing voltage.

30 The test strip may comprise a test substrate and a lid. In some examples, and as stated previously, the optionally substituted nitrobenzene may be positioned on or in proximity to the working electrode. For the avoidance of doubt, the optionally substituted nitrobenzene may be positioned and/or deposited anywhere on the substrate or the lid provided that it is on or in proximity to the working electrode. In some embodiments the optionally substituted nitrobenzene is within the test strip, for example the optionally substituted nitrobenzene may be enclosed by the positioning of the substrate with

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respect to the lid. In some embodiments, the optionally substituted nitrobenzene is on the substrate and within the test strip, e.g. on the substrate and enclosed by the lid of the strip.

5 In some embodiments, the system or kit of parts further comprises a heater capable of controllably heating the first compound and cleaving agent or sample and one or more reagents capable of reacting with a target to generate the cleaving agent whilst they are positioned on the test strip on or in proximity to the working electrode. In particular
10 °C.

The various features of the system and kit of parts have been described in detail in the context of the methods of the disclosure. For the avoidance of any doubt, the various features described in the context of the methods (e.g. features of the working electrode,
15 first compound, potentiostat, pre-conditioning voltage and testing voltage, first state and second states) are equally applicable to the system and kit of parts for use in such methods as described above.

Experimental - pNA electrochemical characterisation and development

20 The present inventors carried out investigations to determine if pNA is electrochemically active and if so, establish the behavior of this activity using cyclic voltammetry (CV). The inventors then looked at a response to pNA concentration response using high purity pNA spiked into a salt buffer to minimize potential interference from contaminants
25 present in more complex and applicable solutions. Following from this the electrochemical parameters were investigated for optimization to produce a maximal signal. Finally, these optimized settings were tested on an endotoxin assay using the Lonza Kinetic-QCL kit. The detailed methods used in these investigations and the results are described below.

30

1. Reagents

Reagent	Supplier product code	Supplier
---------	-----------------------	----------

4-nitroaniline (para-nitroaniline, pNA) ≥ 99%,	185310	Merck
Dimethyl sulfoxide (DMSO) ≥99.9 %	276855	Sigma-Aldrich
Potassium chloride (KCL) ≥99.0 %	P9541	Sigma-Aldrich
Limulus Amebocyte kinetic-QCL	K50-643L	Lonza
Endotoxin free water	SH30529.03	Cytiva
Endotoxin E. coli (0111.B4)	A39552	Thermofisher

Table 1 Reagents used, supplier product codes and supplier details.

2. Materials

Equipment	Supplier product code	Supplier
Multi-channel potentiostat PalmSens4	MPS42211017908	PalmSens
Electrodes used Working - Gold Counter - Gold Reference - Ag/AgCl	DEV-1067-1811	FlexMedical Solutions

5 Table 2 shows materials used, supplier product codes and supplier details.

3. Methods

3.1. Electrochemical characterisation of pNA

0.1 M KCL was made by adding 37.25 g of KCL powder (as described in Table 1) into 500 mL of deionised water (DI water). 10 mM pNA was made by adding 13.8 mg of pNA powder into 1 mL DMSO (as described in Table 1). 1 mM pNA was made by diluting 50 μL of the 100 mM pNA solution into 4.95 mL 0.1 M KCL. 0.1 mM pNA solution was made by diluting 5 μL of 100 mM pNA solution into 4995 μL 0.1 M KCL.

10

0.1 mM pNA was tested electrochemically using CV to first establish the electrochemical behaviour of the solutions. Then 10, 1 and 0.1 mM pNA solutions were tested using SWV to establish any dose dependent behaviour. 50 µL of solution was deposited on the electrodes then the measured. The electrochemical parameters used for both methods is outlined in Table 3.

Electrochemical parameter	CV settings	SWV settings
Ebegin	0 V	NA
Potential window	-1.2 to 0.3 V and -0.5 to 0.3 V	-1.2 to 0.3 V and -0.6 to 0.3 V
Estep	0.01 V	0.01 V
Scan rate	0.1 V/s	NA
# of scans	2	NA
Frequency	NA	25 Hz
Amplitude	NA	0.1 V/s

Table 3 Electrochemical parameter settings for CV and SWV experiments for pNA characterisation and dose response.

10 **3.2. Electrochemical optimisation of pNA signal**

The electrochemical parameters were then optimised to maximise the current signal measured. This was achieved by adjusting the frequency, amplitude, and preconditioning settings of the applied SWV measurement. Table 4 shows the parameters tested.

Parameter	Settings
Frequency	25 default, 50, 100, 500 and 1000 Hz
Amplitude	0.01, 0.05, 0.1 default and 0.25 V
Potential window	-1.2 to 0.3 V, -0.4 to 0.3 V and -0.6 to 0.3 V

32

Precondition	None
	-0.8 V for 2, 10 and 20
	secs
	- 1 V for 2, 10 and 20
	secs

Table 4 Parameters used in the optimisation of pNA signal.

50 µL of solution was deposited on the electrode and then measurements performed. Each parameter tested had an n = 5 population. Each parameter had a corresponding blank also n = 5. The blanks consisted of the same background solution KCL but excluded pNA. During the preconditioning testing there were two controls. One, the KCL with no pNA solution the other, the pNA containing solution but not preconditioned.

3.3. Further optimisation

The optimisation from previous experiments was continued and refined to further boost the pNA signal. The settings tested for optimisation are shown in Table 5.

Parameter	Settings
Frequency	60, 70, 80 and 90 Hz
Amplitude	0.1 V default
Potential window	-0.4 to 0.3 V
Estep	0.01 default, 0.015,0.02 and 0.025 V
Precondition	- 1 V for 2, 10 and 20 secs

Table 5 Further optimisation settings: frequency and Estep size

3.4. Endotoxin assay Lonza kinetic-QCL

5 EU/mL endotoxin was made by reconstituting the lyophilised endotoxin *E. coli* (0111.B4) with 2 mL of endotoxin free water. This was then mixed in a vortexer for 15 minutes to fully incorporate the endotoxin. This equated to 10 EU/mL endotoxin. 1 mL of this was added to 1 mL endotoxin free water to produce 5 EU/mL endotoxin sample.

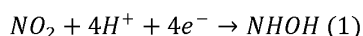
Reconstituted Lonza kinetic-QCL LAL by adding 2.6 mL of endotoxin free water to the lyophilised LAL. Inverted 6x to allow for material in the lid to dissolve. Agitated in a circular motion on a flat surface until solution become clear and free of bubbles. Mixed

the 360 μL of 5 EU/mL endotoxin with 360 μL of LAL and incubated at 37 $^{\circ}\text{C}$ for 30 minutes. Added 60 μL to electrode surface and measured using SWV.

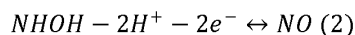
4. Results

5 4.1. Electrochemical characterisation of pNA

The CV results for the -1.2 to 0.3 V scan identified 4 peaks (see Figure 1). These occurred at -0.8 V, -0.4 V, -0.1 V and 0 V. It was hypothesized that the peak at -0.4 V represented, was most likely a KCL, DMSO interaction with gold. It was however evident it did not play a role in the pNA signals of interest. Without being bound by theory, the shape and behavior of this CV (blue profile in Figure 1) agreed with literature (Li-qiang Luo, 2008) (M.A. El Mhammedi, 2008) (Mohammad Hossein Arbab Zavar, 2012) and so it was believed that the -0.8 V peak was a four-electron irreversible reduction of the nitro group to hydroxylamine (1).



15 And the peaks at -0.1 and 0 V were a reversible redox reaction (red profile in Figure 1) cycling between hydroxylamine and a nitroso group in a two-electron transfer process (2).



20 The results from the shortened potential window -0.6 to 0.3 V produced no peaks at 0.1 or 0 V demonstrating the dependence of the -0.8 V reaction (1) on the 0.1 and 0 V reactions (2).

A dose response was observed using SWV for both the -0.8 V reaction and the reactions about 0 V. The current decreased for both peaks as the concentration of pNA decreased as expected (see Figure 2). The peaks about 0 V were larger and more sensitive than those of the prerequisite -0.8 V peaks. This suggested that this would be a more ideal peak to measure for the detection of pNA. The SWV behaviour with the reduced potential window again demonstrated the requirement of the -0.8 V reaction on the production of peak response about 0 V.

30

4.2. Electrochemical optimisation of pNA signal

4.2.1. Frequency optimisation

25 Hz produced a small signal peak at -0.05 V but was a clean signal with little noise, artifacts and was different to the KCL baseline (see Figure 3). 50 Hz increased the signal peak over the baseline and had little noise and artifacts. 100 Hz produced an even larger signal however there were the beginnings of a second peak to the right of the pNA peak at 0.2 V. 500 and 1000 Hz further amplified this second peak. A potential drift was also observed between the two peaks. Although the signals were larger, the specificity of these were poor. The reason for these secondary peaks was likely due to KCL breakdown where the difference between baseline and sample become non-existent. In this initial experiment 50 Hz was determined to be the most optimal frequency due to its difference over the baseline without producing non-specific peaks.

4.2.2. Amplitude optimisation

There was not much difference between 0.01, 0.05 and 0.1 V amplitude (see Figure 4). The baseline current increased with increasing amplitude however the peak heights at -0.05 V were similar, with 0.1 V being the largest. There were no artifacts in the region of interest (-0.05 V). 0.25 V amplitude produced a peak potential shift to 0.05 V as well as a peak signal decrease. It was determined that the default 0.1 V amplitude was the optimal value due to its maximised peak signal while maintaining minimal artifacts and potential shifts.

4.2.3. Preconditioning and potential window optimisation

It was evident that without any preconditioning and using the reduced potential window there was no pNA signal produced at -0.1 V (see Figure 5; no preconditioning is bottom line on trace shown on Figure 5). Introduction of a -0.8 V precondition on the baseline (0.1 M KCL only; second line from the bottom on trace on Figure 5) also resulted in no peak signal as expected. The pNA containing solutions all demonstrated a peak signal response with 2 second pre-treatment (third line from bottom on trace) producing the smallest signal and the 20 producing the largest (top line on trace). 10 seconds was slightly smaller than 20 seconds. The optimal precondition at this potential was 20 seconds producing a 47 μ A signal.

The - 1 V precondition experiment is shown in Figure 6. Again, it was shown that no preconditioning (bottom line on Figure 6) and KCL only precondition (second bottom line

on Figure 6) produced no peak signal response and that pNA containing solutions did. The results were similar to -0.8 V with 20 seconds being the optimal time for preconditioning producing a $59\text{ }\mu\text{A}$ peak signal response. The full precondition optimal settings were discovered to be -1 V for 20 seconds.

5

Combining all optimisation parameters produced an initial optimised signal that had a reduced potential window thus a reduced chance for solution component interference and easier analysis. The peak signal height was also boosted 3.5 times (blue) over the original parameter settings (red) as shown in Figure 7.

10

4.3. Further optimisation

The further optimised frequency data is shown in Figure 8. All frequencies tested produced suitable current peaks over their respective baselines and the optimal result came down to which had the largest signal with suitable variation. 90 Hz produced the largest signal and although the variation was not as small as 70 Hz it was still more than suitable. The final optimal frequency was determined to be 90 HZ.

15

All Estep sizes tested produced good peak signals different from the baseline with no artifacts and no potential shifting (see results shown in Figure 9). 0.01 V had the smallest peak signal response with the other three values similar in size. 0.025 V produced the smallest variation and so was determined to be the optimal value.

20

With all the optimised parameters investigated thus far. Combining all the optimised parameters showed a 4.7 times larger signal while reducing the potential window from -1.2 to 0.3 V to -0.4 to 0.3 V (see Figure 10).

25

4.4. Endotoxin assay Lonza kinetic-QCL

The optimised parameters were compared to the original parameters on an endotoxin assay using the Lonza kinetic-QCL kit. The data is shown in Figure 11.

30

The optimised settings were shown to be transferable to a full endotoxin assay producing a peak at a similar potential to the original settings. The peak height was about 6.4 times larger confirming the pretreatment and optimised settings make a significant effect on endotoxin detection.

35

5. Conclusions

The behaviour of pNA in a simple salt solution was established. This was able to be correlated to results in literature allowing for the determination of chemical reactions for each Signal peak. The dependence of the redox reaction about 0 V on a preceding reaction at -0.8 V was established. Using SWV, a dose response of pNA was achieved and demonstrated that the redox peak about 0 V was larger and more sensitive than the prerequisite -0.8 V signal leading to the conclusion that the signal around 0 V would be a preferable potential to use in the detection of pNA. Since SWV scanned the entire potential range in about 1 second, the time the potential spent around -0.8 V was miniscule. Thus, the amount of hydroxylamine produced to undergo the redox reaction about 0 V was relatively low. The idea of applying a pretreatment potential to generate larger amounts of hydroxylamine that would therefore result in a larger signal about 0 V (signal boost) was theorised.

This theorised signal boost was investigated by applying various pretreatments to the measured solutions. The optimal pretreatment was -1 V for 20 seconds. Other electrochemical parameters were optimised along with the addition of this preconditioning step. It was shown that this precondition step was able to increase the signal response significantly over the non-preconditioned step. The optimisations of frequency, amplitude, potential window also further increased the signal boost in a KCL and pNA solution by about 3.5 times.

Further optimisation of the frequency was investigated as well as the Estep size, further boosting the signal response by 4.7 times.

This was then transferred to an endotoxin assay using 5 EU/mL endotoxin and the Lonza Kinetic-QCL kit. A full preconditioned endotoxin assay was able to produce a significant signal boost (6.4 times) over the non-optimised settings.

These discoveries open the possibility to more sensitive, faster, and efficient endotoxin assays.

35 Additional investigations

Three additional cyclic voltammogram (CV) studies were carried out on the following mixtures:

- (i) pNA peptide only;
- 5 (ii) 0 EU/mL endotoxin and pNA peptide; and
- (iii) 5 EU/mL endotoxin and pNA peptide.

Further details on these mixtures and the measurement methods are shown in Table 6.

Reagent	Preparation	Measurement details
pNA peptide	2.6 mL endo free water added to lyophilised powder	Immediately measured
5 EU/mL	50 EU/mL endotoxin stock diluted 9:1 in endo free water and vortexed	NA
5 EU/mL + pNA peptide	5 EU/mL endotoxin mixed 1:1 with pNA peptide	Incubated for 30 mins and then measured
0 EU/mL + pNA peptide	endotoxin free water mixed 1:1 with pNA peptide	Incubated 30 mins and then measured

Table 6 providing details on reagents, preparation and measurement details for the mixtures used in the subsequent CV studies.

As noted previously, the known gel clot Limulus Amebocyte Lysate (LAL) assay was used in these studies. The Limulus Amebocyte kinetic-QCL from Lonza.

The results are shown in Figure 12.

The blue trace is the pNA peptide and endotoxin. In this mixture, the known cascade reaction can take place and cleaves the pNA from the peptide. The trace in yellow is the pNA peptide and endotoxin free water (0 EU/mL). The red trace is pNA peptide only. The red and yellow traces show minimal cleaved pNA signal i.e. mostly uncleaved peptide – under the conditions of the LAL assay the pNA will only be cleaved from the pNA peptide in the presence of endotoxin. The difference between the 0 EU/mL and peptide only, is

the 0 EU/mL has been 1:1 diluted further with endo free water of the peptide in endotoxin free water. Note: The peptide is reconstituted initially with endo free water.

In trace resulting from the test on pNA peptide only there is no the reduction peak at -0.85 V. Consequently that the redox peaks at 0.1 V and -0.1 V are not observed. Similar results are observed for 0 EU/mL endotoxin and pNA peptide. In contrast, in the presence of 5 EU/mL endotoxin, the LAL cascade is initiated and the pNA is cleaved from the peptide. In this case, the CV trace shows the same behaviour as has been observed for pNA in the earlier studies described above (see, for example, Figure 1).

References:

Li-qiang Luo, X.-l. Z.-p.-s. (2008). Derivative voltammetric direct simultaneous determination of nitrophenol. Sensors and Actuators B: Chemical, 61-65.

M.A. El Mhammedi, M. A. (2008). Electrochemical determination of para-nitrophenol at apatite-modified carbon. Journal of Hazardous Materials, 323-328.

Mohammad Hossein Arbab Zavar, *. S. (2012). Electrochemical behavior of para-nitroaniline at a new synthetic crown. Analytical Methods, 953 - 958.

Further Optimisation

The reagents used for further optimisation are set out in the Tables 7 to 9, below.

Reagent	Supplier product code	Supplier
Endosafe Trillium Recombinant Cascade Reagent, 6x vials, 32-Test Vial	RCRV3032PK	Charles River Microbial Solutions
PyroSmart NextGen Multi- test	PNG050-2	Associates of Cape Cod
PYROSTAR Neo+ Recombinant Endotoxin Specific Testing	WAKF294-36731	Avantor
PYROSTAR Neo+ Recombinant Endotoxin Specific Testing	283-43111	Alpha Labs

Control Endotoxin Standard (CSE), Escherichia coli 055:B5	E110	Charles River Microbial Solutions
Control Endotoxin E.coli (0111:B4)	EC010-5	Associates of Cape Cod
Endotoxin Tests, Control standard endotoxin, 500 ng vial, lyophilised, Endotoxin derived from E. coli UKT-B	WAKFCSE4037-5006	Avantor
Endotoxin Tests, Control standard endotoxin, 500 ng vial, lyophilised, Endotoxin derived from E. coli UKT-B	CSE4037-5006	Alpha Labs

Table 7 Reagents used, supplier product codes and supplier details.

Equipment	Supplier product code	Supplier
Electrodes used – Working – Carbon Counter – Carbon Reference – Ag/AgCl	Gii-Sens Carbon Circular	Integrated Graphene

Table 8 shows materials used, supplier product codes and supplier details.

Reagent	Preparation	Measurement details
5 EU/mL	50 EU/mL endotoxin stock diluted 1:9 with endotoxin free water	Following application of endotoxin standard and enzyme cascade reagent to the strip, electrochemical pre-treatment and square wave voltammetry (SWV) were performed every minute for 60 to 90 minutes as required.
0.5 EU/mL	5 EU/mL endotoxin standard diluted 1:9 with endotoxin free water	As above

0.05 EU/mL	0.5 EU/mL endotoxin standard diluted 1:9 with endotoxin free water	As above
0.005 EU/mL	0.05 EU/mL endotoxin standard diluted 1:9 with endotoxin free water	As above
0 EU/mL	endotoxin free water	As above
Recombinant Cascade Reagent	1.7 to 2.7 mL endotoxin free water added to lyophilised powder as per pack insert	

Table 9 Providing details on reagents, preparation and measurement details for the mixtures used in the subsequent SWV studies.

5 The optimised settings are set out in the Tables below. A temperature controlled electrochemical kinetic strip assay was performed on a strip at 37°C to optimize the performance of the enzymes involved in the cascade. Following application of Controlled Standard Endotoxin (CSE) and enzyme cascade reagent to the strip, electrochemical pre-treatment and square wave voltammetry (SWV) were performed every minute for 60 to 90 minutes as required. The kinetic nature of the assay enables measurement of the reaction rate of the enzymatic cascade, which is inversely related to the concentration of endotoxin present. As free pNA is released via the cascade, electrochemical monitoring of the change in pNA concentration over time was used to determine ‘onset time’.

10

ETX – GiiSens Pretreatment Settings	
Electrode Setup	Carbon.Carbon.Ag/AgCl (working.counter.reference)
t condition	20 s
E condition	-1 V
ETX – GiiSens SWV Settings	
Electrode Setup	Carbon.Carbon.Ag/AgCl (working.counter.reference)

t equilibrium	0 s
E begin	-0.1 V
E end	0.2 V
E step	0.01 V
Amplitude	0.05 V
Frequency	25 Hz
Scan rate	0.25 V/s
(Frequency x E Step)	

Table 10 Further optimisation settings

Optimised Results

5 The endotoxin dose response curve for the electrochemical assays carried out under the optimised conditions is shown in Figure 14. The results are set out in the Table below.

Endotoxin Concentration (EU/mL)	0	0.005	0.05	0.5	5
Average (n=3) onset time (mins)	>60	45.9	24.9	12.4	7.4
Std. Dev. Log ₁₀ onset time (Log ₁₀ seconds)	-	0.009	0.049	0.019	0.018
% RSD Log ₁₀ onset time	-	0.26	1.55	0.68	0.69

Table 11 Reproducibility obtained when using further optimised settings

10 Effect of testing voltage range on reproducibility

Endotoxin Concentration (EU/mL)		0	0.005	0.05	0.5	5
Voltage range = -0.3 V to 0.4 V	% RSD Log ₁₀ onset time (n=3, each taken on a different day)	22.6	21.8	9.9	8.4	9.6
Voltage range = -0.1 V to 0.2 V	% RSD Log ₁₀ onset time (n=3, each taken on a different day)	10.4	3.9	16.3*	7.2	5.2

* % RSD higher owing to an outlier in one of four repeats. If the outlier is excluded then the % RSD is 8.8%.

Table 12 Effect of testing voltage range on reproducibility of results

The voltage range of -0.1 V to 0.2 V provided more reproducible results, particularly at lower endotoxin concentrations of 0 and 0.005 EU/mL.

5 Although the present invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention. The disclosures of all patent and scientific literature cited herein are expressly incorporated herein in their entirety by reference.

CLAIMS:

1. A method of detecting an optionally substituted nitrobenzene, the method comprising:
- 5 (i) providing a first compound comprising an optionally substituted nitrobenzene conjugated to a cleavable group and contacting the first compound with a cleaving agent such that the cleaving agent cleaves the cleavable group from the first compound to provide the optionally substituted nitrobenzene; and then:
- (ii) applying a pre-conditioning voltage to a working electrode such that an
10 optionally substituted nitrobenzene on or in proximity to the working electrode is converted to a first state;
- (iii) applying a testing voltage to the working electrode such that the first state is converted to a second state, wherein the testing voltage is different to the pre-conditioning voltage; and
- 15 (iv) sensing an electrical response.
2. A method of detecting a target in a sample, wherein the target is a cleaving agent, or the presence of the target generates a cleaving agent, the method comprising:
- (i) contacting a first compound, comprising an optionally substituted nitrobenzene
20 conjugated to a cleavable group, with a sample such that when the target is present, the cleaving agent cleaves the cleavable group from the first compound to provide the optionally substituted nitrobenzene;
- (ii) applying a pre-conditioning voltage to the working electrode such that cleaved optionally substituted nitrobenzene, when present, is converted to a first state;
- 25 (iii) applying a testing voltage to the working electrode such that the first state, when present, is converted to a second state; and
- (iv) sensing an electrical response.
3. The method of claim 1 or claim 2, wherein the pre-conditioning voltage is different
30 to the testing voltage, optionally wherein the pre-conditioning voltage reduces the optionally substituted nitrobenzene to the first state.
4. The method of any one preceding claim, wherein the pre-conditioning voltage is:
- (i) about -0.3 to about -2 V, about -0.65 to about -1.1 V, or about -0.8 to about -1.0 V,
35 optionally about -1.0 V; and/or

(ii) applied for a duration of about 0.01 to about 40 seconds, about 0.1 to about 30 seconds, or about 1 second to about 20 seconds, optionally about 20 seconds.

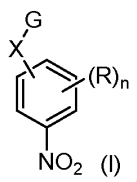
5. The method of any one preceding claim, wherein the pre-conditioning voltage is between about -0.8 and about -1 V and is applied for a duration of about 5 to about 30 seconds, such as about -1 V for about 20 seconds.

6. The method of any one preceding claim, wherein the testing voltage oxidises the first state to the second state, optionally wherein the testing voltage is any one voltage or a potential window of about -1 to about 1 V, about -0.5 V to about 0.5 V, about -0.4 V to about 0.3 V, about -0.1 to about 0.1 V, or about -0.1 to about 0.2 V.

7. The method of any one preceding claim, wherein the testing voltage is applied immediately after, about 0 to about 10 s after, or about 0.01 to 10 s after the pre-conditioning voltage.

8. The method of any one of claims 2 to 7, which is capable of detecting the presence of a target in a sample at concentrations less than or equal to about:
 (i) 100 pM, about 50 pM, about 10 pM, about 1 pM, about 100 fM, about 50 fM, or about 1 fM; or
 (ii) about 5 ng/mL, about 500 pg/mL, about 50 pg/mL, about 5 pg/mL, about 500 fg/mL or about 100 fg/mL.

9. The method of any one preceding claim, wherein the first compound is of formula (I):



wherein:

X is NH, O, S -C(CR¹₂)-, -NR¹-, -SO₂-, -C(O)-, -C(O)O-, -OC(O)-, -C(O)NR¹-, or NR¹C(O)-, wherein R¹ is H or an organyl group (such as C₁₋₆ alkyl);

each R is independently selected from the group consisting of C₁₋₆alkyl, halo, C₁₋₆haloalkyl, hydroxy, C₁₋₆alkoxy, C₁₋₆alkylol and phenyl;

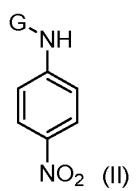
n is 0 to 4; and

G is the cleavable group.

10. The method of claim 9, wherein X is selected from NH, O and S, optionally wherein X is NH and/or n is 0.

5

11. The method of any one preceding claim, wherein the first compound is of formula (II):



wherein G is the cleavable group.

10

12. The method of any one preceding claim, wherein the cleavable group is a biological molecule, or a leaving group such as phosphate, sulfonate, halide, alkoxide, aryloxy, amine-based leaving group, or carbonyl-based leaving group (e.g. carboxyl) and/or a photocleavable group.

15

13. The method of claim 12, wherein the biological molecule comprises any one or more selected from the group consisting of an amino acid, a nucleotide, a saccharide, a phospholipid and a triglyceride, optionally wherein the biological molecule comprises any one or more selected from the group consisting of a peptide, an oligonucleotide, DNA, RNA, a peptide nucleic acid (PNA), a locked nucleic acid (LNA), a glycol nucleic acid (GNA), a xeno nucleic acids (XNA), a phosphorodiamidate morpholino oligomer (PMO), and a molecularly imprinted polymer (MIP).

20

14. The method of claim 12, wherein the biological molecule is a peptide, optionally wherein the cleavable group is a peptide.

25

15. The method of any one preceding claim, wherein the cleaving agent is irradiation, or a catalyst, such as an enzyme, an organic catalyst, or an inorganic catalyst.

16. The method of any one of claims 2 to 15, wherein the cleaving agent is the product, of a reaction or a series of reactions, initiated by the presence of the target,

30

optionally wherein the cleaving agent is clotting enzyme and is the product of a cascade reaction initiated by an endotoxin.

17. The method of claim 16, wherein the strength of the electrical response is proportional to the amount of cleaving agent produced in the reaction, or series of reactions, and/or is proportional to the amount of target present in the sample.

18. The method of any one preceding claim, wherein a background electrical response is measured by:

(i) applying a pre-conditioning voltage to the working electrode such that the optionally substituted nitrobenzene is converted to a first state;

(ii) applying a testing voltage such that the first state is converted to a second state; and

(iii) sensing an electrical response,

before, or at the same time, the sample and/or cleaving agent is contacted with the first compound.

19. The method of any one preceding claim, wherein the first compound is deposited on or in proximity to a working electrode on or within a test strip prior to addition of the cleaving agent and/or sample.

20. The method of any one preceding claim, wherein the contacting is carried out in a liquid.

21. The method of any one preceding claim, wherein the working electrode is part of an electrode system comprising at least one further electrode and/or the electrode system is electronically connected to a sensor such as a potentiostat.

22. The method of any one preceding claim, wherein the testing voltage is applied and the electrical response is sensed by any one selected from square wave voltammetry, differential pulse voltammetry, cyclic voltammetry, electrochemical impedance spectroscopy, chronoamperometry, coulometry, and potentiometry.

23. The method of any one preceding claim, wherein the testing voltage is applied and the electrical response is sensed by square wave voltammetry, optionally wherein:

- 5 (i) the frequency of the square wave pulse is less than or equal to about 200 Hz, about 150 Hz, or about 125 Hz, or about 10 to about 125 Hz, or about 25 to about 100 Hz, such as about 25 Hz, about 70 Hz, about 80 Hz, or about 90 Hz;
- (ii) the amplitude of the square wave pulse is about 0.001 V to about 5 V, about 0.01 V to about 2.5 V, or about 0.05 V to about 0.15 V, such as about 0.05 V or about 0.1 V; and/or
- 10 (iii) the step potential increments of the square wave pulse are about 0.001 V to about 0.1 V, or about 0.01 V to about 0.025 V, such as about 0.01 V.

24. The method of any one preceding claim, wherein the electrical response is a current, voltage, resistance and/or impedance.

- 15 25. A system comprising:
- (a) a test-strip comprising:
- (i) a working electrode; and
- (ii) a first compound comprising an optionally substituted nitrobenzene conjugated to a cleavable group; and
- 20 (b) a potentiostat,
- wherein the first compound is on or in proximity to the working electrode;
- the potentiostat is configured to apply a pre-conditioning voltage to the working electrode such that the optionally substituted nitrobenzene is converted to a first state; and
- 25 the potentiostat is configured to apply a testing voltage to the working electrode such that the first state is converted to a second state; and
- wherein the pre-conditioning voltage is different to the testing voltage, and optionally wherein the system further comprises one or more reagents capable of reacting with a target to generate a cleaving agent capable of cleaving the cleavable
- 30 group from the first compound.

26. A kit of parts comprising, as separate components:

- (a) a test-strip comprising:
- (i) a working electrode; and

(ii) a first compound comprising an optionally substituted nitrobenzene conjugated to a cleavable group;

(b) a potentiostat; and

5 (c) one or more reagents capable of reacting with a target to generate a cleaving agent capable of cleaving the cleavable group from the first compound;

wherein the first compound is on or in proximity to the working electrode;

the potentiostat is configured to apply a pre-conditioning voltage to the working electrode such that the optionally substituted nitrobenzene is converted to a first state; and

10 the potentiostat is configured to apply a testing voltage to the working electrode such that the first state is converted to a second state; and

wherein the pre-conditioning voltage is different to the testing voltage.

27. The system of claim 25 or the kit of claim 26, wherein the one or more reagents
15 are positioned at the same position as, or in proximity to the first compound.

28. The system or kit of any one of claims 25 to 27, wherein the one or more reagents comprise limulus clotting factor C, limulus clotting factor B, and proclotting enzyme.