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(54) Titre : ARSENITE OXYDASE MODIFIE ET BIOCAPTEUR POUR DETECTER L'ARSENITE
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(54) Title: MODIFIED ARSENITE OXIDASE AND A BIOSENSOR FOR DETECTING ARSENITE

(57) Abstract: The present invention provides an arsenite oxidase enzyme modified to prevent translocation by modification of a translocation signal sequence. A microorganism modified to express the heterologous arsenite oxidase enzyme is also provided by the invention, together with a device for detecting the presence of arsenite in a sample.



WO 2013/057515 A1

Modified arsenite oxidase and a biosensor for detecting arsenite

Field of the Invention

5 The present invention relates to modified enzymes for detecting the presence of arsenic derivatives, in particular arsenite, and methods for expressing such enzymes in heterologous organisms. The invention is also directed to devices for detecting arsenic, and derivatives thereof, in a sample.

10 Background to the Invention

 It is said that the third major challenge for a sustainable future (together with food security and energy) will be making the best use of limited supplies of pure water for both agricultural use and human consumption, and the remediation of marginal and contaminated water will be essential in achieving
15 this. Already groundwater contamination, resulting from either natural geochemical processes or industrial activities such as mining, is a major problem in many countries.

 Arsenic (As) is a groundwater contaminant that is ubiquitous in the environment and the two soluble forms, arsenite (As^{III}) and arsenate (As^{V}), are
20 toxic. Anthropogenic activity has resulted in widespread contamination of both soluble forms but As^{III} is prevalent in anoxic environments, including most sources of drinking water. Countries affected include India, Bangladesh, Vietnam, USA, Germany, France, Hungary, Australia, Argentina, Mexico, Canada.

25 An important aspect of remediation is assessment and monitoring, and whilst laboratory methods exist that demonstrate high specificity and sensitivity (e.g. ICP-MS or HPLC) it is also possible, and indeed desirable, to measure analytes such as arsenite in the field using sensors. Ideally, the sensors should be low-cost, disposable and able to be readily adapted to multiple analytes that
30 are commonly found together in contaminated water.

 Many As field test kits are commercially available (e.g. from Industrial Systems, Inc, Hydrodyne) but these only detect total As, rather than the most

toxic form, As^{III}, which is dominant in anoxic drinking waters. Moreover, because As^{III} remediation preferentially removes As^V (e.g. by binding to iron hydroxide) and requires the pre-oxidation of As^{III}, it is crucial to determine whether any As^{III} remains in the water. The chemically based arsenic field kits rely on a colorimetric method which reduces the As^{III} and As^V to the gas arsine which reacts with the mercuric bromide test strips. These kits require the training of personnel, are expensive (e.g. Arsenic, Quick II™ Hydrodyne kit US\$4.24 per test and Ultra Low Quick II™, Industrial Test Systems, Inc. US\$6 per test) and have low sensitivity and reproducibility.

Whole cell biosensors have been developed for the detection of As^{III} by a number of groups (e.g. Stacker et al. (2003) *Environ. Sci. Technol.* 37, 4743-4750). These methods are all based on colorimetric assays that sometimes require the use of a luminometer. They all use the regulatory mechanism of the *Escherichia coli* arsenic resistance system which detects both As^{III} and antimonite (Sb^{III}). The regulatory gene in this system, *arsR*, is fused to a reporter gene (e.g. luciferase gene, *luxB*) that when expressed after induction with As^{III} produces a visible signal (e.g. fluorescence).

There are many problems with whole cell based As^{III} biosensors, including: 1) the system is too complex and because of this has a slow response time (i.e. As^{III} must enter cells followed by induction of regulator-reporter gene protein - this can take up to 24 hours for a response); 2) lack of specificity as the system does not discriminate between As^{III} and Sb^{III}; 3) incubation temperatures of 30°C are often required; 4) colorimetric assay often requires use of a luminometer, which is not feasible at most field sites; and 5) use of genetically modified organisms always presents an additional problem. No whole cell biosensors for the detection of As^{III} are commercially available.

A biosensor for As^{III} has been developed using molybdenum-containing arsenite oxidase (known as "Aio" and also previously known as Aro and Aso; see Lett et al., Unified Nomenclature for Genes Involved in Prokaryotic Aerobic Arsenite Oxidation; *J. Bacteriology*, 4 Nov 2011 ; p.207-208) which is a member of the DMSO reductase family, prepared from chemolithoautotrophic

Alphaproteobacterium *Rhizobium* sp. strain NT-26 (Santini *et al.* (2000) Appl. Environ. Microbiol. 66, 92-97).

As^{III} oxidase catalyses the oxidation of As^{III} to As^V and the suitability of this native enzyme for use as a biosensor has been tested and shown to detect
5 down to 1 ppb As^{III}, which is 10 times lower than the recommended WHO MCL (maximum contaminant level) of As in drinking water. Furthermore the native enzyme shows specificity for As^{III} (Male *et al.* (2007) Anal. Chem. 79, 7831-7837). The biosensor comprises the enzyme directly linked to the surface of a
10 multiwalled carbon nanotube-modified electrode, in which electron transfer proceeds directly from enzyme to electrode. The authors noted, however, that certain commonly-used electrode materials, in particular glassy carbon (GC), were not suitable for direct electron transfer in this configuration.

Heterologous expression of molybdenum-containing enzymes, especially members of the DMSO reductase family, is notoriously difficult. Recently, the
15 dissimilatory arsenate reductase from *Shewanella* sp. str. ANA-3 was expressed in *Escherichia coli* but comparisons with the native enzyme were not made (Malasarn *et al.* (2008) J. Bacteriol. 190, 135-142). Expression was optimal when *E. coli* was grown anaerobically with DMSO although other electron acceptors for anaerobic growth were not tested and neither were different
20 strains.

Since the entire native Aio is poorly expressed in a heterologous expression system, such as *E. coli*, use of this enzyme in routine detection of As^{III} is not commercially viable.

As such, there is a need for improved methods and sensors for cheap
25 and effective detection of As^{III} in liquids such as drinking-water, waste-water and biological samples.

Summary of the Invention

According to a first embodiment, the present invention provides an
30 arsenite oxidase enzyme modified to prevent translocation to the periplasm, wherein the enzyme comprises the native AioA subunit from NT-26 and the native AioB subunit from NT-26, wherein a portion of the native AioB subunit

corresponding to the translocation signal sequence, or a functional fragment thereof, is modified.

According to a second embodiment, the present invention is directed to the use of a modified As^{III} oxidase according to the first aspect of the invention as a biosensor to detect
5 the presence of As^{III} in a sample.

According to a third embodiment, the present invention provides a microorganism, modified to express a heterologous As^{III} oxidase according to the first aspect of the invention.

According to a fourth embodiment, the present invention provides method of
10 producing recombinant As^{III} oxidase, comprising expressing the modified enzyme heterologously according to the first aspect of the invention in a microorganism.

According to a fifth embodiment, the present invention provides a device for detecting the presence of As^{III} in a sample, comprising at least one electrode, an arsenite oxidase enzyme and a redox mediator.

15 According to an aspect of the invention is microorganism expressing a heterologous arsenite oxidase enzyme which catalyses the oxidation of As^{III} to As^{IV}, wherein the enzyme comprises:

a native AioA subunit from *Rhizobium sp.* NT-26, or a homologue thereof that has at least 90% sequence similarity to the polypeptide of SEQ ID NO:4; and

20 a native AioB subunit from *Rhizobium sp.* NT-26 or a homologue thereof that has at least 90% sequence similarity to the polypeptide of SEQ ID NO:3;

wherein a portion of a nucleotide sequence encoding the native AioB subunit corresponding to a translocation signal sequence, or a homologue thereof, comprises a functional fragment of the translocation signal sequence having a mutation or deletion

25 which upon expression prevents translocation of the enzyme to the periplasm; or

wherein a portion of a nucleotide sequence encoding the native AioB subunit corresponding to a translocation signal sequence, or a homologue thereof, does not comprise a functional fragment of the translocation signal sequence.

According to a further aspect is a method of producing recombinant arsenite oxidase
30 which catalyses the oxidation of As^{III} to As^{IV} comprising expressing a gene sequence encoding an arsenite oxidase enzyme in a heterologous microorganism, wherein the arsenite oxidase enzyme comprises:

a native AioA subunit from *Rhizobium sp.* NT-26, or a homologue thereof that has at least 90% sequence similarity to SEQ ID NO:4; and

a native AioB subunit from *Rhizobium sp.* NT-26 or a homologue thereof that has at least 90% sequence similarity to SEQ ID NO:3;

wherein a portion of a nucleotide gene sequence encoding the native AioB subunit
5 corresponding to a translocation signal sequence, or a homologue thereof, comprises a functional fragment of the translocation signal sequence having a mutation or deletion which upon expression prevents translocation of the recombinant arsenite oxidase enzyme to the periplasm; or

wherein a portion of a nucleotide sequence encoding the native AioB subunit
10 corresponding to a translocation signal sequence, or a homologue thereof, does not comprise a functional fragment of the translocation signal sequence.

According to a further aspect is a system for detecting the presence of arsenite in a sample, the system comprising at least one electrode, an arsenite oxidase enzyme which catalyses the oxidation of As^{III} to As^{IV} and a redox mediator, wherein the redox mediator
15 transfers electrons from the arsenite oxidase enzyme to the electrode surface, wherein the enzyme comprises:

a native AioA subunit from *Rhizobium sp.* NT-26, or a homologue thereof which has at least 90% sequence similarity to SEQ ID NO:4; and

a native AioB subunit from *Rhizobium sp.* NT-26 or a homologue thereof which has
20 at least 90% sequence similarity to SEQ ID NO:3;

wherein a portion of a nucleotide sequence encoding the native AioB subunit which corresponds to a translocation signal sequence, or a homologue thereof, comprises a functional fragment of the translocation signal sequence having a mutation or deletion which upon expression prevents translocation of the arsenite oxidase enzyme to the
25 periplasm; or

wherein a portion of a nucleotide sequence encoding the native AioB subunit corresponding to a translocation signal sequence, or a homologue thereof, does not comprise a functional fragment of the translocation signal sequence.

30 Description of the Figures

Figure 1 shows the micro-structured surface of a device according to the invention;

Figure 2 is an AsIII dose-response curve showing the comparable results of the sensor of the invention over two consecutive days;

Figure 3 is a comparison of AsIII oxidase activities in total cell extracts of *E. coli* strains DH5 α and JM109 λ pir grown with different electron acceptors;
35

Figure 4 shows a SDS-Polyacrylamide gel (12%) of purified recombinant NT-26 AsIII
oxidase (M: Molecular weight marker: phosphorylase b (94 kDa), albumin (67 kDa), oval
albumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), β -lactalbumin (14
5 kDa) (GE Healthcare) 1: Purified recombinant AioBA, two subunits AioA (~94 kDa) AioB with
N-terminal His-tag (~19 kDa));

Figures 5 (a-j) show cyclic voltammogram measurements and chrono amperometry
for a device according to the invention including ferrocene methanol as the mediator;

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Figures 6 (a-d) shows show cyclic voltammogram measurements for a device according to the invention including Tris(2,2'-bipyridine) dichlororuthenium(II) as the redox mediator;

Figures 7a and 7b show cyclic voltammogram and chrono amperometry measurements for a device according to the invention including feccocene carboxylic acid as the mediator;

Figures 8 (a-f) show cyclic voltammograms measurements for a device according to the invention including 2,6-dichlorophenolindophenol as the redox mediator; and

Figure 9 shows cyclic voltammograms of TTF as the redox mediator.

Detailed Description of the Invention

The present inventors have developed a modified version of the native As^{III} oxidase from *Rhizobium* sp. NT-26 (GenBank Accession Number AY345225) which can be successfully expressed in heterologous expression systems such as *E. coli*. This has been achieved by modifying a translocation signal sequence present in the native enzyme, or a functional fragment thereof, to prevent translocation.

The native As^{III} oxidase consists of two heterologous subunits: AioB is the small subunit largely composed of beta sheets and AioA is the large subunit largely composed of alpha-helices (Santini & vanden Hoven (2004) J. Bacteriology. 186(6):1614-1619). The polypeptide sequence of wild-type AioB from *Rhizobium* sp. NT-26 is shown in SEQ ID No. 1 and SEQ ID No. 5 shows the corresponding nucleotide sequence. The AioB subunit comprises a Tat leader sequence (also referred to herein interchangeably as a Tat translocation signal sequence) which corresponds to the first 25 amino acids of SEQ ID No 1. This translocation signal sequence is shown as SEQ ID No. 2 and SEQ ID No. 6 shows the nucleotide sequence of the portion of the *aioB* gene encoding the translocation signal sequence. The signal sequence directs the transport of the protein to the periplasm using the Twin Arginine Translocation (Tat) pathway.

The present inventors have modified the native As^{III} oxidase by modifying the translocation signal sequence in the AioB subunit. As a result of the

modification, the modified enzyme is not exported from the host cytoplasm, and as a result can be expressed in large, commercially-viable quantities.

The translocation signal sequence can be modified by various methods which will be apparent to a person skilled in the art, including frame-shift
5 mutations, substitution mutations or deletion. Any modification that results in loss of function of the native translocation signal sequence is within the scope of the invention, however deletion of the translocation signal sequence or a functional fragment thereof, is preferred.

Accordingly, a first aspect of the invention provides an As^{III} oxidase
10 modified to prevent translocation by modification of a translocation signal sequence, or a functional fragment thereof.

The modified As^{III} oxidase comprises two subunits. The first subunit corresponds to the native AioA subunit from NT-26, or a variant, derivative or homologue thereof. The second subunit corresponds to the native AioB subunit
15 from NT-26, or a variant, derivative or homologue thereof; however a portion of the native AioB subunit which corresponds to the translocation signal sequence, or a functional fragment of the translocation signal sequence, is modified in the enzyme of the invention. Preferably, the portion of the native AioB subunit which corresponds to the translocation signal sequence, or a functional fragment
20 thereof, is modified by deletion.

In one embodiment, at least a portion of the *aioB* gene which encodes the translocation signal sequence, or a portion thereof encoding a functional fragment of the translocation signal sequence, is modified, preferably by deletion.

The portion of the *aioB* gene sequence encoding the translocation signal
25 sequence is identified herein as SEQ ID NO. 6. Either the complete sequence identified as SEQ ID NO. 6 or a homologue of this sequence encoding a functional fragment of the translocation signal sequence may be modified according to the invention. As a result of the modification to the nucleotide sequence, the modified enzyme of the invention does not comprise the amino acid sequence identified
30 herein as SEQ ID No. 2, or does not comprise a portion thereof that is required for a functional translocation signal sequence.

As used herein, the term 'functional fragment' means that the portion of the nucleotide sequence that is modified (e.g. by deletion) encodes a polypeptide

having Tat translocation signal sequence activity, preferably having at least the same activity of the polypeptide shown as SEQ ID NO: 2. As a result of the modification, the recombinant enzyme of the invention lacks such Tat translocation signal sequence activity.

5 As used herein, the term "homologue" refers to a nucleotide sequence that encodes a polypeptide which has Tat translocation signal sequence activity. With respect to sequence similarity, preferably there is at least 60%, more preferably at least 70%, more preferably at least 75%, more preferably at least 85%, more preferably at least 90% sequence similarity between SEQ ID NO. 6 and the
10 sequence of the native *aioB* gene that is modified according to the invention. More preferably there is at least 95%, more preferably at least 98%, sequence similarity. These terms also encompass allelic variations of the sequences.

 In another embodiment, the portion of the *aioB* gene that is modified according to the invention comprises or consists of the sequence identified
15 herein as SEQ ID NO. 6.

 SEQ ID No. 3 corresponds to the amino acid sequence of the AioB subunit excluding the entire native leader region. SEQ ID No. 7 shows the corresponding nucleotide sequence. If the entire AioB leader region is deleted, then the AioB subunit of the modified enzyme of the invention will correspond to
20 SEQ ID No. 3. However, it is within the scope of the invention for a portion of the leader region within the AioB subunit to remain unmodified, provided that the portion of the leader region that is modified renders the remaining portion non-functional.

 SEQ ID No. 4 shows the amino acid sequence of the AioA subunit and
25 SEQ ID No. 8 shows the corresponding nucleotide sequence.

 It is within the scope of the invention for the modified As^{III} oxidase to comprise homologues, variants or derivatives of SEQ ID Nos. 3 and 4.

 The terms "variant", "homologue", "derivative" or "fragment" as used herein include any substitution, variation, modification, replacement, deletion or addition of
30 one (or more) amino acid from or to a sequence. The variant may have a deletion, insertion or substitution variation. The variation may produce a silent change and a functionally equivalent polypeptide, or may result in improved catalytic function or other characteristics of the resulting enzyme. Deliberate amino acid substitutions

may be made on the basis of similar physio-chemical properties such as size, charge and hydrophobicity in order to alter the catalytic function or other properties or characteristics of the enzyme.

Unless the context admits otherwise, references to "AioA" and "AioB" include
5 references to such variants, homologues and derivatives of the native subunits. The term "homologue" covers identity with respect to structure and/or function and is used to refer to peptides that share levels of sequence identity or similarity to SEQ ID Nos. 3 and 4 and retain at least the functionality of the native amino acid sequences. The variants may result in improvements in the catalytic activity or
10 other properties of the resulting enzyme. These terms also encompass polypeptides derived from amino acids which are allelic variations of the aioA and/or aioB nucleic acid sequences (SEQ ID Nos. 7 and 8).

Levels of identity or similarity between amino acid sequences can be calculated using known methods. Publicly available computer based methods
15 include BLASTP, BLASTN and FASTA (Atschul *et al.*, Nucleic Acids Res., 25: 3389-3402 (1997)), the BLASTX program available from NCBI, and the GAP program from Genetics Computer Group, Madison WI.

It is preferable if there is at least 60% sequence identity or similarity to the specified peptides of SEQ ID Nos. 3 and 4, preferably 70%, more preferably
20 80% and most preferably greater than 90%, e.g. at least 95% to the sequences of SEQ ID Nos. 3 and 4. The removal of the Tat leader sequence or a functional fragment thereof to prevent export to the periplasm is a known technique, and has been used previously in the production of heterodimeric molybdenum-containing enzymes (Malasarn *et al.* (2008), J Bacteriol, 190, 135-142).
25 However, As^{III} oxidase differs from other molybdenum-containing enzymes in that it is a much larger $\alpha_2\beta_2$ heterotetramer containing additional co-factors (i.e. a 3Fe-4S cluster and a Rieske 2Fe-2S cluster) not present in other molybdenum-containing enzymes). It has been demonstrated that the native enzyme is localised to the periplasm (Santini *et al.* (2000), J Bacteriol, 66, 92-97), given
30 size of the assembled heterotetrameric complex it is therefore reasonable to speculate that two $\alpha\beta$ heterodimers formed in the cytoplasm are exported (*via* the Tat system) to the periplasm prior to heterotetrameric complex formation. Therefore, the present inventors were surprised to find that that following

removal of the Tat leader sequence, the modified As^{III} oxidase could achieve complete assembly (both subunit assembly and cofactor addition) to form a heterotetramer in the cytoplasm of the various *E. coli* strains.

For the avoidance of doubt, the abbreviations "aio", "Aio", "aro", "Aro", "aso" and "Aso" can be used interchangeably and all refer to the arsenite oxidase enzyme (gene or protein). The different abbreviations are the result of different nomenclature that has been used in the art (Lett *et al.*, J. Bacteriology, 4 Nov 2011; p.207-208).

The advantage of modifying the native As^{III} oxidase according to the invention is that it can be expressed successfully in heterologous expression systems such as *E. coli* at high, commercially-viable yields. Once expressed, the enzyme can be used to detect the presence of As^{III}. Therefore, a second aspect of the invention is directed to the use of the modified arsenite oxidase enzyme according to the first aspect of the invention as a biosensor to detect the presence of As^{III} in a sample. The modified enzyme of the invention has been found to be equally effective as the native enzyme in catalysing the oxidation of As^{III} to arsenate As^V (Warelow & Santini, unpublished data). The absence of the translocation sequence does not impact upon the catalytic activity of the enzyme. The modified enzyme is therefore suitable for use in biosensors to detect the presence of As^{III}.

Preferably the sample is a liquid sample. The liquid sample may be any type of liquid that is susceptible to As^{III} contamination, including, but not limited to, ground-water, drinking-water, environmental liquids such as mining effluent and sewage, waste-water, biological samples.

Preferably the modified enzyme of the invention may be used in laboratory-based biosensors or, preferably, in low-cost disposable biosensor suitable for field use (i.e. detecting As^{III} in a sample at the source of the sample rather than in a laboratory).

The advantage of utilising the modified enzyme according to the first aspect of the invention in a biosensor for detecting As^{III} in a sample is that it can be expressed in commercially-viable quantities in a variety of heterologous expression systems, including, unexpectedly, host strains that are normally used for cloning rather than protein expression.

Therefore, according to a third aspect the present invention provides a microorganism modified to express the heterologous As¹" oxidase of the first aspect of the invention. The wild-type microorganism may be selected from a range of species including, but not limited to, *E. coli*. Preferably, the wild-type microorganism is *E. coli*, in particular *E. coli* K12 strains DH5aTM and JM109Ap/r.

It is surprising that these strains have been found to be the most suitable for expressing the recombinant enzyme of the invention, since they are usually used in the art for cloning, rather than protein expression. As detailed in Example 1 below, the inventors found that unexpectedly high yields were achieved using *E. coli* strains DH5a and JM109Ap/r rather than strains BL21 (protease-deficient) and Origami, which are commonly used for heterologous expression of recombinant enzymes.

E. coli strain DH5a is a Hoffman-Berling 1 100 strain derivative and can be purchased from InvitrogenTM. The properties of this strain are described by the following standard nomenclature:

F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG 4>80dlacZAM15 A(lacZYA-argF)U169, hsdR17(rK- mK+), λ-.

The DH5aTM strain is described in the following publications: *FOCUS* (1986) 8:2, 9.; Hanahan, D. (1985) in *DNA Cloning: A Practical Approach* (Glover, D.M., ed.), Vol. 1, p. 109, IRL Press; Grant, S.G.N, et al. (1990) *Proc. Natl. Acad. Sci. USA* 87: 4645-4649 PMID 2162051 ; and Meselson M. and Yuan R. (1968) *Nature* 217:11 10 PMID. 4868368.

E. coli strain JM109Ap/r can be purchased from Promega and has the following standard nomenclature properties:

endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB+ A(lac-proAB) e14- [F' traD36 proAB+ lacIq lacZAM15] hsdR17(rK-mK+).

This strain is described in Yanisch-Perron et al (1985) *Gene*, 33(1):103— 19; and Penfold et al (1992) *Gene* 118:145-146.

According to a fourth embodiment, the present invention provides a method of producing recombinant As¹" oxidase, comprising expressing the modified enzyme according to the first aspect of the invention

novel primers identified herein as SEQ ID NO. 9 and SEQ ID NO. 10 can be used to clone the NT-26 *aioB* and *aioA* genes (termed *aioBA*) into the commercially-available pPROEX Htb vector (Invitrogen). As described in relation to the first aspect of the invention, the *aioB* gene is modified to delete at least the
5 portion of the nucleotide sequence (SEQ ID NO. 6) encoding at least the functional portion of the TAT leader sequence shown herein as SEQ ID NO. 2.

Preferably the heterologous microorganism is *E. coli*, and preferably *E. coli* strains DH5 α or JM109 λ *pir*. The present inventors have found that strain DH5 α provides optimal expression of the heterologous arsenite oxidase enzyme
10 when grown aerobically and JM109 λ *pir* provides optimal expression of the heterologous enzyme when grown anaerobically with nitrate as the electron acceptor. Furthermore the inventors have found that use of an affinity tag at the N-terminus of AioB allows for simple purification. This embodiment is described in more detail in Example 1 below.

15 According to a fifth aspect, the present invention provides a device for detecting the presence of As^{III} in a sample. The device comprises an electrode (termed the "test electrode") incorporating an As^{III} oxidase enzyme and a redox mediator.

The device may comprise wild-type As^{III} oxidase or the modified As^{III}
20 oxidase according to the first aspect of the present invention. Preferably, the enzyme is the modified As^{III} oxidase. As discussed above, high yields of the modified enzyme can be obtained through heterologous expression in *E. coli*. Therefore, it is preferable to use the modified enzyme to keep the manufacturing cost of the device low and to enable the device to be manufactured in
25 commercially-viable quantities.

In one embodiment, the device comprises a test strip made of polymer or ceramic materials. Preferably, the device comprises two or more planar electrodes. Preferably the device comprises a "reference electrode" in addition to the test electrode. At least the test electrode incorporates the As^{III} oxidase.

30 The device includes a redox mediator. The term "redox mediator" is defined as any moiety capable of transferring electrons from the enzyme to the electrode surface. Artificial redox mediators such as 2,6-

dichlorophenolindophenol (DCPIP) are often used in solution in laboratory-based spectrophotometric measurements; however spectrophotometric measurements are not routinely used in field test equipment. The inclusion of a redox mediator is advantageous in a number of different ways, compared with existing devices
5 in which the enzyme is directly linked to an electrode. The presence of a redox mediator improves the efficiency of electron transfer and reduces the effects of electrode surface chemistry on the currents measured. This enables a much wider range of electrode materials to be used. As the background currents due to sample components are dependent on the electrode material this gives
10 greater versatility in optimising signal to background.

As shown in Example 2, a wide range of mediators can be used in the in the electrochemical As^{III}-sensing device of the invention. Examples of suitable redox mediators include, but are not limited to, metal complexes where the metal exists in two or more different redox states, for example iron, ruthenium or
15 osmium complexes, organic molecules that can exist in two or more accessible redox states, for example cytochromes, conducting organic polymers, conducting organic salts, 2,6-dichlorophenolindophenol, ferrocene and ferrocene derivatives including ferrocene carboxylic acid and hydroxymethyl ferrocene (ferrocene methanol), Tris(2,2'-bipyridine)dichlororuthenium(II), tetrathiafulvalene
20 (TTF) and quinones including, but not limited to, benzoquinone and hydroquinone. However not all redox mediators are effective with Aio (arsenite oxidase), for example tetraammine ruthenium (III) chloride, methylene blue, ferricyanide and oxygen have been found not to be effective.

Advantageously, the device of the invention is versatile and has been
25 shown to work with a variety of test electrode materials (see Example 2 below). Preferably, the test electrode is made of one or more conducting materials including, but not limited to, carbon, carbon nanotubes, graphene, graphite, gold, platinum, palladium, glassy carbon, nanostructured metal oxides or nanostructured metal.

30 Preferably, the reference electrode comprises a reference redox couple, such as Ag/AgCl.

The electrode materials can be deposited on the test strip by a variety of methods including, but not limited to, screen-printing or evaporation. The electrodes may be open or covered by a lid so forming a defined volume cell. There may, or may not, be a membrane covering the electrodes.

5 The test strip is suitable for laboratory use and, preferably, field-based use (i.e. As^{III} can be detected in a sample at the source using the test strip or device). The device may be suitable for multiple uses or a single use, and may be disposable.

10 Preferably, the device comprises a micro-structured surface, with the enzyme entrapped thereon with a mediator (see Figure 1). A micro-structured surface, for example pillars rising from the base of the electrode, improves the performance of the electrode.

15 Preferably the sample in which As^{III} is detected using the device of the invention is a liquid sample. The liquid sample may be any type of liquid that is susceptible to As^{III} contamination, including, but not limited to, ground-water, drinking-water, environmental liquids such as mining effluent and sewage, waste-water, biological samples.

20 In use, the test sample is brought into direct contact with the test strip. Operation of the sensor device involves applying an electrical potential between the test and reference electrodes and measuring the current. A number of methods would be apparent to those skilled in the art, and include, but are not limited to, chronoamperometry, square wave voltammetry and coulometry. The response to As^{III} can be measured from 5 μM to 40 μM with the same sensor showing comparable results the next day (see Figure 2).

25 The advantages of using the device of the invention as described above to test for the presence of As^{III} in a sample are that the sensitivity for the analyte is high and the test is specific for As^{III} (i.e. the most toxic soluble form of arsenic) due to the presence of the enzyme; the test is quick and results are obtained almost instantaneously; the device is simple to use; the results are reproducible; 30 and the device can be produced and purchased cheaply (particularly important for use in developing countries).

The present invention is further described with reference to the following examples.

Example 1

5

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmid and growth conditions

Escherichia coli strains DH5 α , JM109 λ pir, RK4353, C43, BL21 and Origami were used to test expression of the NT-26 arsenite oxidase. The expression vector pPROEX-HTb (Invitrogen) was used for expression purposes. All expression conditions involved growing *E. coli* in LB containing 100 μ g/ml ampicillin either aerobically (with aeration at 170 rpm (1:5 ratio liquid to head space) or anaerobically with nitrate (14 mM) or DMSO (14 mM) as electron acceptors and sodium lactate (20 mM) as the electron donor.

15

Cloning and Expression

The NT-26 *aioB* and *aioA* (*aioBA*) genes were amplified without the native Tat leader sequence using the following primers:

Forward:

20 PROEXAroBFHis 5'-GCGAATTCAAGCTACCGCGGCGGCAGGGGTC-3' (SEQ ID No. 9)

Reverse:

25 PROEXAroAR 5'-GCCTGCAGTCAAGCCGACTGGTATTCTTTTCGA-3' (SEQ ID No. 10)

The restriction enzymes *EcoRI* and *PstI* (underlined above) were used to facilitate cloning into the expression vector, pPROEX-HTb. The pPROEX-HTb carrying the *aioBA* genes was transformed into a variety of *E. coli* strains to determine which one gave optimal expression. A variety of IPTG (Isopropyl β -D-1-thiogalactopyranoside) and sodium molybdate (Mo) concentrations as well as time of induction were tested. The final optimum expression conditions used for

purification of the Aio involved growing DH5a aerobically at 21°C for 24 hrs in LB containing 40 µM IPTG and 1 mM Mo.

Purification of Recombinant Arsenite Oxidase

Recombinant Aio was purified from DH5a using a combination of affinity and size exclusion chromatography. Cells were harvested by centrifugation at 9,700 x g for 10 minutes. The cell pellets were pooled and washed by suspending in binding buffer (10 ml/gm wet weight cells) (20 mM potassium phosphate, 500 mM sodium chloride, 20 mM imidazole, pH 7.2) and centrifuged at 12,000 x g for 15 minutes. The cell pellet was then re-suspended in binding buffer (10 ml/gm wet weight cells). The *E. coli* cells were disrupted by a single pass through a French pressure cell (12,000 psi) and the cell debris removed by centrifugation at 30,000 x g for 30 minutes. The supernatant was loaded onto a GraviTrap™ prepacked Ni charged affinity chromatography column (GE Healthcare) as per the manufacturer's instructions except with one minor modification; the wash volume used was 120 ml. The eluent was desalted in 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 5.5) buffer resulting in the precipitation of non-target co-eluted protein(s) which were removed by centrifugation at 10,000 x g for 5 minutes. The supernatant was filtered through a 0.22 µm filter (Millipore), concentrated using a Vivaspin™ 20 (MWCO 100,000) (GE Healthcare) centrifugal concentrator and loaded onto a Superdex™ 200 gel filtration column (GE Healthcare) pre-equilibrated with 50 mM MES, 100 mM NaCl, pH 5.5 buffer. Chromatography was carried out at a flow rate of 0.3 ml/min. The 0.25 ml fractions containing Aio activity were pooled and concentrated using a Vivaspin™ 20 centrifugal concentrator (MWCO 100,000).

Confirmation of the native molecular mass of the recombinant Aio was done using a Superdex™ 200 gel filtration (GE Healthcare) chromatography column. A calibration curve was created using a gel filtration calibration kit (GE Healthcare) of known molecular mass proteins (Thyroglobulin 669 kDa, Ferritin 440 kDa, Aldolase 158 kDa, Conalbumin 75 kDa, Ovalbumin 43 kDa) and the void volume of the column was determined using Blue dextran 2000 (2,000 kDa).

Chromatography conditions used were as per the manufacturers' instructions with a flow rate of 0.3 ml/min.

RESULTS AND DISCUSSION

5 Heterologous expression and purification of the recombinant arsenite oxidase

In this study the *aioB* and *aioA* (designated *aioBA*) genes were cloned without the *aioB* Tat leader sequence allowing for expression in the *E. coli* cytoplasm. A combination of different strains and growth conditions were tested to optimise
10 Aio expression. Surprisingly, the highest Aio specific activity was obtained with *E. coli* K12 strains DH5 α and JM109 λ *pir*.

Interestingly, there was also a variation of Aio activity when different electron acceptors were used (Figure 3), with nitrate the optimal electron acceptor. Given
15 the greater cell yield obtained from aerobic growth, DH5 α and oxic conditions were chosen for further studies. Recombinant As^{III} oxidase was purified from *E. coli* using a combination of Ni-NTA and size exclusion chromatography. Based on SDS polyacrylamide gel electrophoresis, the recombinant enzyme was >99% pure and contained the two known Aio subunits, AioA (93 kDa) and AioB (21
20 kDa) (see Figure 4). Based on size exclusion chromatography the native molecular mass of the enzyme was 219 kDa which is consistent with the $\alpha_2\beta_2$ oligomeric state of the native enzyme purified from NT-26 (Santini & vanden Hoven, 2004, J. Bacteriology. 186(6):1614-1619).

25 Example 2

A number of experiments were performed to demonstrate that the enzyme described within the patent application can be used in an enzyme mediated electrochemical device to detect arsenite in water. The results demonstrate that a range of redox mediator molecules can be used in the device and that the
30 electrode material can also be varied.

MATERIALS & METHODS

Screen printed electrodes were purchased from Dropsens (Oviedo, Spain).

Carbon screen printed electrodes have a carbon working electrode (4mm diameter) and counter electrode and silver/silver chloride reference electrode (product ref. DRP-C110).

5 Carbon nanotube screen printed electrodes have a multi-walled carbon nanotube working electrode (4 mm diameter), carbon counter electrode and silver/silver chloride reference electrode (product reference DRP-110CNT). Gold screen printed electrode have a gold working electrode (4 mm diameter) and counter electrode and a silver/silver chloride reference electrode (product reference DRP-220AT). Electrochemical

10 measurements (cyclic voltammetry and chrono amperometry) were recorded using a CompactStat.e™ potentiostat instrument (Ivium Technologies) and IviumSoft software (Ivium Technologies). 2,6-dichlorophenolindophenol, Ferrocene Carboxylic acid, hydroxymethyl ferrocene (ferrocene methanol), tetrathiafulvalene (TTF) and Tris(2,2'-bipyridine)dichlororuthenium(II) mediators were all purchased from Sigma Aldrich.

15 mM solution of sodium arsenite (Fluka) was also purchased from Sigma Aldrich.

In all experiments the working, counter and reference electrodes were completely covered with a 200µl buffered solution containing the required amount of mediator, arsenite oxidase enzyme and arsenite for each experiment.

20 Cyclic voltammograms were recorded using carbon, carbon nanotube and gold electrodes with a 0.5 mM hydroxymethyl ferrocene solution (10 mM PBS, pH 7.1 , 100 mM KCl) containing 0.05 U arsenite oxidase (0.17 nanomoles) with and without 500 micromolar sodium arsenite. A scan rate of 5 mV/s was used. Chrono amperometry was performed using a 0.25 mM hydroxymethyl ferrocene solution (10 mM PBS, pH 7.1 , 100
25 mM KCl) containing 0.05 U arsenite oxidase (0.17 nanomoles) with 0 and 50 micromolar sodium arsenite when using a carbon electrode and 0, 12.5, 25, 50 and 100 micromolar sodium arsenite when using a gold electrode. The solution was mixed between each chrono amperometry run. Triplicate runs were recorded for each concentration of

arsenite and the average values plotted. A one step potential of 0.35 V (vs Ag/AgCl) was applied for each run.

5 Cyclic voltammograms were recorded using carbon and carbon nanotube electrodes with a 1 mM Tris(2,2'-bipyridine)dichlororuthenium(II) solution (10 mM PBS, pH 7.1, 100 mM KCl) containing 0.05 U arsenite oxidase (0.17 nanomoles) with and without 500 micromolar sodium arsenite. A scan rate of 5mV/s was used.

10 Cyclic voltammograms were recorded using carbon and gold electrodes with a 1 mM ferrocene carboxylic acid solution (10 mM PBS, pH 7.1, 100 mM KCl) containing 0.1 U arsenite oxidase (0.34 nanomoles) with and without 1 mM sodium arsenite. A scan rate of 5 mV/s was used. Chrono amperometry was performed using a 1 mM ferrocene carboxylic acid solution (10 mM PBS, pH 7.1,
15 100 mM KCl) containing 0.1 U arsenite oxidase (0.34 nanomoles) with 0, 50 and 100 micromolar sodium arsenite with a carbon electrode. A single step run was performed applying a potential of 0.38 V (vs Ag/AgCl reference electrode). The solution was mixed between each chrono amperometry run. Duplicate runs were recorded for each concentration of arsenite.

20 Cyclic voltammograms were recorded using carbon, carbon nanotube and gold electrodes with a 1 mM 2,6-dichlorophenolindophenol (DCPIP) solution (50 mM MES, pH 5.5, 100 mM KCl) containing 0.05 U arsenite oxidase (0.17 nanomoles) with and without 500 micromolar sodium arsenite. A scan rate of 5 mV/s was
25 used.

Cyclic voltammograms were recorded using carbon nanotube electrode by first depositing 6 μ l of 2 mM tetrathiafulvalene (TTF) in acetone onto the working electrode and allowing to dry for 10 minutes. The electrode screen printed
30 electrode was then immersed in 200 μ l solution (10 mM PBS, pH 7.1, 100 mM KCl) containing 0.05 U arsenite oxidase (0.17 nanomoles) with and without 500 micromolar sodium arsenite. A scan rate of 50mV/s was used.

RESULTS

2,6-dichlorophenolindophenol, Ferrocene Carboxylic acid, Ferrocene Methanol and Tris(2,2'-bipyridine)dichlororuthenium(II) mediators all showed reversible redox behaviour in the presence of the enzyme (very similar cathodic and anodic peak currents) and an increased cathodic peak current relative to the anodic peak current in the presence of the enzyme and arsenite. This is a typical response of an enzyme mediated electrochemical device.

Figures 5 (a-f) show cyclic voltammograms of ferrocene methanol as the mediator with carbon (a and b), carbon nanotube (c and d) and gold (e and f) screen printed electrodes without (a, c, e) and with 500 micromolar arsenite (b,d,f). Figure 5g shows chronoamperometry plots of ferrocene methanol plus enzyme, and ferrocene methanol plus enzyme plus 50 μ M arsenite recorded using a carbon electrode. Figures 5h and 5i show chronoamperometry plots of ferrocene methanol plus enzyme, and ferrocene methanol plus enzyme plus 0, 12.5, 25, 50 and 100 micromolar arsenite recorded using a gold electrode. Figure 5i is a zoomed portion of Figure 5h. Figure 5j is a plot showing a linear dose response of current to arsenite concentration and refers to the Chrono amperometry plots in Figures 5h and 5i, plotting current values taken at four seconds from the chrono amperometry plots.

Figures 6 (a-d) show cyclic voltammograms of Tris(2,2'-bipyridine) dichlororuthenium(II) as the mediator with carbon (a and b) and carbon nanotube (c and d) screen printed electrodes, without (a and c) and with (b and d) 500 micromolar arsenite.

Figures 7a and 7b show cyclic voltammogram's of ferrocene carboxylic acid as the mediator with carbon (2a) and gold (2b) screen printed electrodes. Figure 2c shows a chrono amperometry plots of ferrocene carboxylic acid plus enzyme with 0, 50 micromolar and 100 micromolar arsenite.

Figures 8 (a-f) show cyclic voltammograms of 2,6-dichlorophenolindophenol as the mediator with carbon (a and b), gold (c and d) and carbon nanotube (e and f) screen printed electrodes, without (a,c,e) and with (b,d,f) 500 micromolar arsenite.

5

Figure 9 shows cyclic voltammograms of TTF as the mediator with carbon nanotube screen printed electrodes, without and with 500 micromolar arsenite.

Chronoamperometry plots show a shift to high current values in the presence of arsenite and there was a linear dose response in the current shift to the arsenite concentration.

10

CLAIMS

1. A microorganism expressing a heterologous arsenite oxidase enzyme which catalyses the oxidation of As^{III} to As^{IV}, wherein the enzyme comprises:
 - a native AioA subunit from *Rhizobium sp.* NT-26, or a homologue thereof that has at least 90% sequence similarity to the polypeptide of SEQ ID NO:4; and
 - a native AioB subunit from *Rhizobium sp.* NT-26 or a homologue thereof that has at least 90% sequence similarity to the polypeptide of SEQ ID NO:3;wherein a portion of a nucleotide sequence encoding the native AioB subunit corresponding to a translocation signal sequence, or a homologue thereof, comprises a functional fragment of the translocation signal sequence having a mutation or deletion which upon expression prevents translocation of the enzyme to the periplasm; or
 - wherein a portion of a nucleotide sequence encoding the native AioB subunit corresponding to a translocation signal sequence, or a homologue thereof, does not comprise a functional fragment of the translocation signal sequence.
2. The microorganism according to claim 1, wherein the microorganism is *E. coli*.
3. The microorganism according to claim 2, wherein the microorganism is *E. coli* strain DH5 α TM or JM109 λ *pir*.
4. The microorganism according to any one of claims 1 to 3, wherein a portion of the native *aioB* gene encoding the translocation signal sequence is identified as SEQ ID NO:6, or a homologue thereof that has at least 90% sequence similarity to SEQ ID NO:6, and the translocation signal sequence is identified as SEQ ID NO:2.
5. The microorganism according to any one of claims 1 to 4, wherein the AioB subunit comprises a peptide with the amino acid sequence of SEQ ID NO:3 or a homologue that has at least 95% sequence similarity to SEQ ID NO:3.

6. The microorganism according to any one of claims 1 to 5, wherein the AioB subunit is encoded by the polynucleotide sequence of SEQ ID NO:7 or a homologue thereof that has at least 90% sequence similarity to SEQ ID NO:7.
7. The microorganism according to any one of claims 1 to 6, wherein the AioA subunit comprises a peptide with the amino acid sequence of SEQ ID NO:4 or a homologue thereof that has at least 95% sequence similarity to SEQ ID NO:4.
8. The microorganism according to any one of claims 1 to 7, wherein the AioA subunit is encoded by the polynucleotide sequence of SEQ ID NO:8 or a homologue thereof that has at least 90% sequence similarity to SEQ ID NO:8.
9. A method of producing recombinant arsenite oxidase which catalyses the oxidation of As^{III} to As^{IV} comprising expressing a gene sequence encoding an arsenite oxidase enzyme in a heterologous microorganism, wherein the arsenite oxidase enzyme comprises:
- a native AioA subunit from *Rhizobium sp.* NT-26, or a homologue thereof that has at least 90% sequence similarity to SEQ ID NO:4; and
 - a native AioB subunit from *Rhizobium sp.* NT-26 or a homologue thereof that has at least 90% sequence similarity to SEQ ID NO:3;
- wherein a portion of a nucleotide gene sequence encoding the native AioB subunit corresponding to a translocation signal sequence, or a homologue thereof, comprises a functional fragment of the translocation signal sequence having a mutation or deletion which upon expression prevents translocation of the recombinant arsenite oxidase enzyme to the periplasm; or
- wherein a portion of a nucleotide sequence encoding the native AioB subunit corresponding to a translocation signal sequence, or a homologue thereof, does not comprise a functional fragment of the translocation signal sequence.
10. The method according to claim 9, wherein the native AioB subunit corresponding to the translocation signal sequence, or the portion of the nucleotide

sequence encoding the native AioB subunit corresponding to the translocation signal sequence, or the homologue thereof comprising the functional fragment of the translocation signal sequence, has a deletion which upon expression prevents translocation of the recombinant arsenite oxidase enzyme to the periplasm.

11. The method according to claim 9 or 10, wherein the heterologous microorganism is *E. coli*.

12. The method according to claim 11, wherein the *E. coli* is strain DH5 α TM or JM109 λ *pir*.

13. The method according to any one of claims 9 to 12, wherein said gene sequence is present in a vector.

14. The method according to claim 13, wherein the vector is a pPROEX HTb vector.

15. The method according to claim 13 or 14, wherein nucleotide primer sequences of SEQ ID NO:9 and/or SEQ ID NO:10 are used to clone *aioA* and *aioB* gene sequences into the vector.

16. The method according to any one of claims 9 to 15, wherein the portion of the native *aioB* gene which encodes the translocation signal sequence is identified as SEQ ID NO:6, or a homologue thereof has at least 90% sequence similarity to SEQ ID NO:6, and wherein the translocation signal sequence has the polypeptide sequence of SEQ ID NO:2.

17. The method according to any one of claims 9 to 16, wherein the AioB subunit comprises a peptide with the amino acid sequence identified as SEQ ID NO:3, or a homologue thereof that has at least 95% sequence similarity to SEQ ID NO:3.

18. The method according to any one of claims 9 to 17, wherein the AioB subunit is encoded by the polynucleotide sequence of SEQ ID NO:7, or a homologue thereof that has at least 90% sequence similarity to SEQ ID NO:7.

19. The method according to any one of claims 9 to 18, wherein the AioA subunit comprises a peptide with the amino acid sequence identified as SEQ ID NO:4, or a homologue thereof that has at least 95% sequence similarity to SEQ ID NO:4.

20. The method according to any one of claims 9 to 19, wherein the AioA subunit is encoded by the polynucleotide sequence of SEQ ID NO:8, or a homologue thereof that has at least 90% sequence similarity to SEQ ID NO:8.

21. A system for detecting the presence of arsenite in a sample, the system comprising at least one electrode, an arsenite oxidase enzyme which catalyses the oxidation of As^{III} to As^{IV} and a redox mediator, wherein the redox mediator transfers electrons from the arsenite oxidase enzyme to the electrode surface, wherein the enzyme comprises:

a native AioA subunit from *Rhizobium sp.* NT-26, or a homologue thereof which has at least 90% sequence similarity to SEQ ID NO:4; and

a native AioB subunit from *Rhizobium sp.* NT-26 or a homologue thereof which has at least 90% sequence similarity to SEQ ID NO:3;

wherein a portion of a nucleotide sequence encoding the native AioB subunit which corresponds to a translocation signal sequence, or a homologue thereof, comprises a functional fragment of the translocation signal sequence having a mutation or deletion which upon expression prevents translocation of the arsenite oxidase enzyme to the periplasm; or

wherein a portion of a nucleotide sequence encoding the native AioB subunit corresponding to a translocation signal sequence, or a homologue thereof, does not comprise a functional fragment of the translocation signal sequence.

22. The system according to claim 21, wherein the portion of the native *aioB* gene which encodes the translocation signal sequence is identified as SEQ ID NO:6, or a homologue thereof that has at least 90% sequence similarity to SEQ ID NO:6, and wherein the translocation signal sequence has the polypeptide sequence of SEQ ID NO:2.

23. The system according to claim 21 or 22, wherein the AioB subunit comprises a peptide with the amino acid sequence identified as SEQ ID NO:3, or a homologue thereof that has at least 95% sequence similarity to SEQ ID NO:3.

24. The system according to any one of claims 21 to 23, wherein the AioB subunit is encoded by the polynucleotide sequence of SEQ ID NO:7, or a homologue thereof that has at least 90% sequence similarity to SEQ ID NO:7.

25. The system according to any one of claims 21 to 24, wherein the AioA subunit comprises a peptide with the amino acid sequence identified as SEQ ID NO:4, or a homologue thereof that has at least 95% sequence similarity to SEQ ID NO:4.

26. The system according to any one of claims 21 to 25, wherein the AioA subunit is encoded by the polynucleotide sequence of SEQ ID NO:8, or a homologue thereof that has at least 90% sequence similarity to SEQ ID NO:8.

27. The system according to any one of claims 21 to 26, wherein the at least one electrode is made of one or more conducting materials selected from the group consisting of carbon, gold, palladium, nanostructured metal oxides and nanostructured metal.

28. The system according to claim 27, wherein the carbon is selected from the group consisting of carbon nanotubes, graphene, graphite and glassy carbon.

29. The system according to any one of claims 21 to 28, wherein the redox mediator is selected from the group consisting of metal complexes where the metal

exists in two or more accessible redox states, and organic molecules that can exist in two or more accessible redox states.

30. The system according to claim 29, wherein the redox mediator comprises iron complexes, ruthenium complexes or osmium complexes.

31. The system according to claim 29, wherein the organic molecules that can exist in two or more accessible redox states are cytochromes, conducting organic polymers, conducting organic salts, 2,6-dichlorophenolindophenol, ferrocene or ferrocene derivatives, Tris(2,2'-bipyridine)dichlororuthenium(II), tetrathiafulvalene (TTF) or quinones.

32. The system according to claim 31, wherein the ferrocene or ferrocene derivatives are ferrocene carboxylic acid or hydroxymethyl ferrocene (ferrocene methanol).

33. The system according to any one of claims 21 to 32 further comprising a reference electrode.

34. The system according to claim 33, wherein the reference electrode comprises an Ag/AgCl reference redox couple.

35. The system according to any one of claims 21 to 34, wherein the system is a test strip with a micro-structured surface.

36. The system according to any one of claims 21 to 35, wherein the system is for laboratory use or field based use for detecting the presence of arsenite in a sample.

37. The system according to claim 36, wherein the sample is a liquid susceptible to As^{III} contamination.

1/14

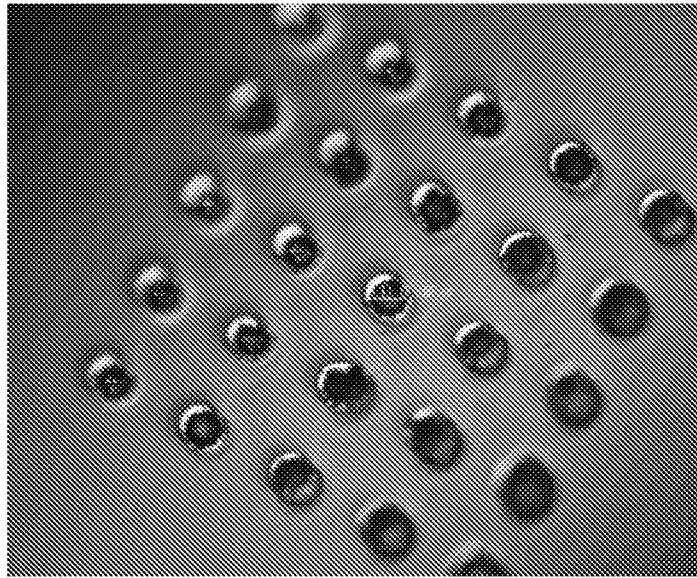


Figure 1

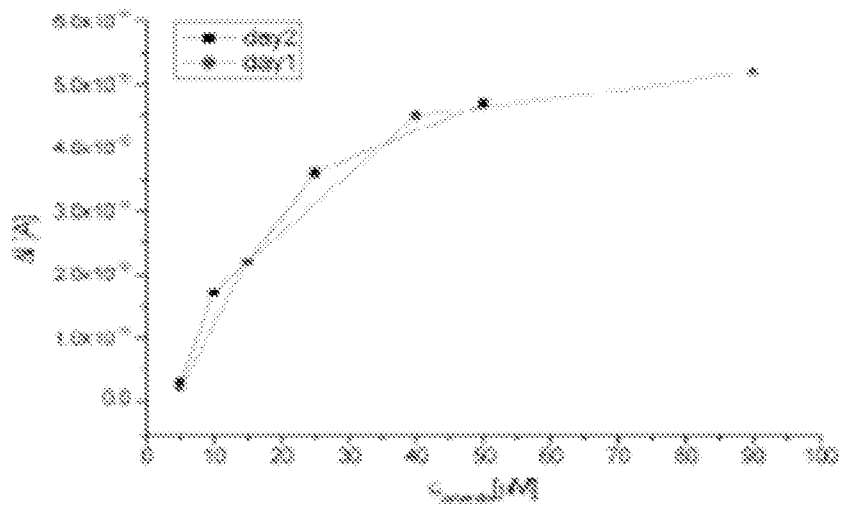


Figure 2

2/14

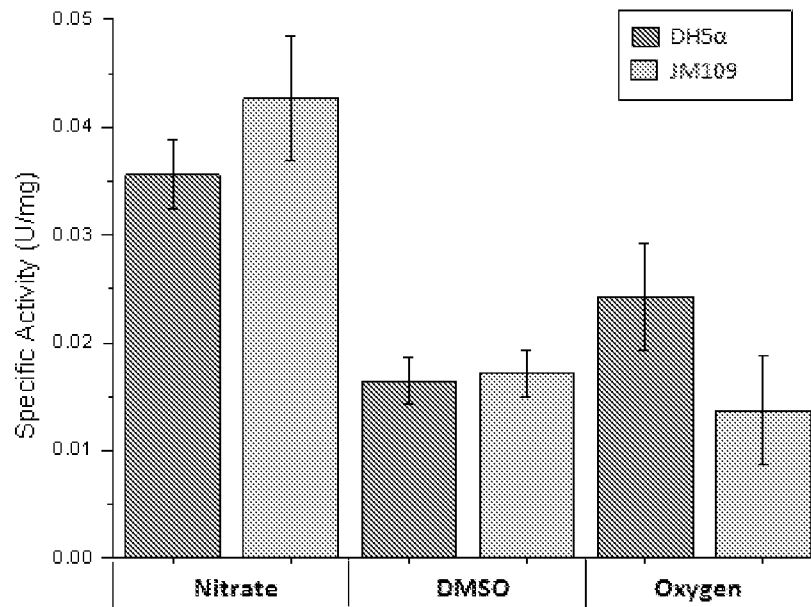


Figure 3

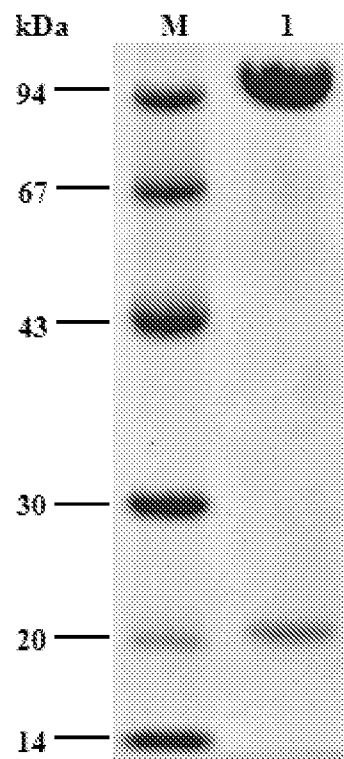


Figure 4

3/14

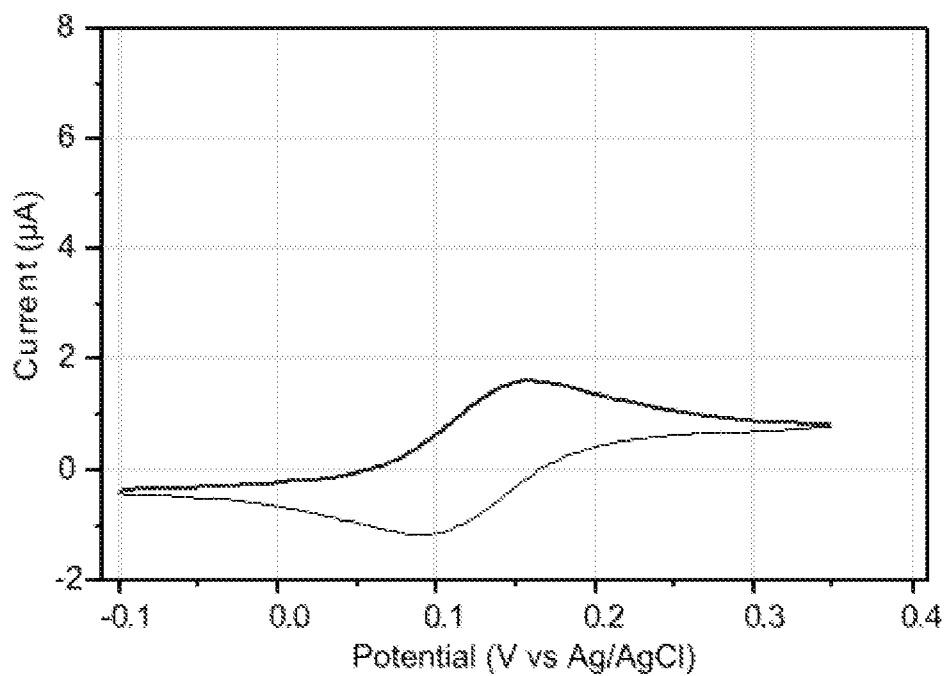


Figure 5a

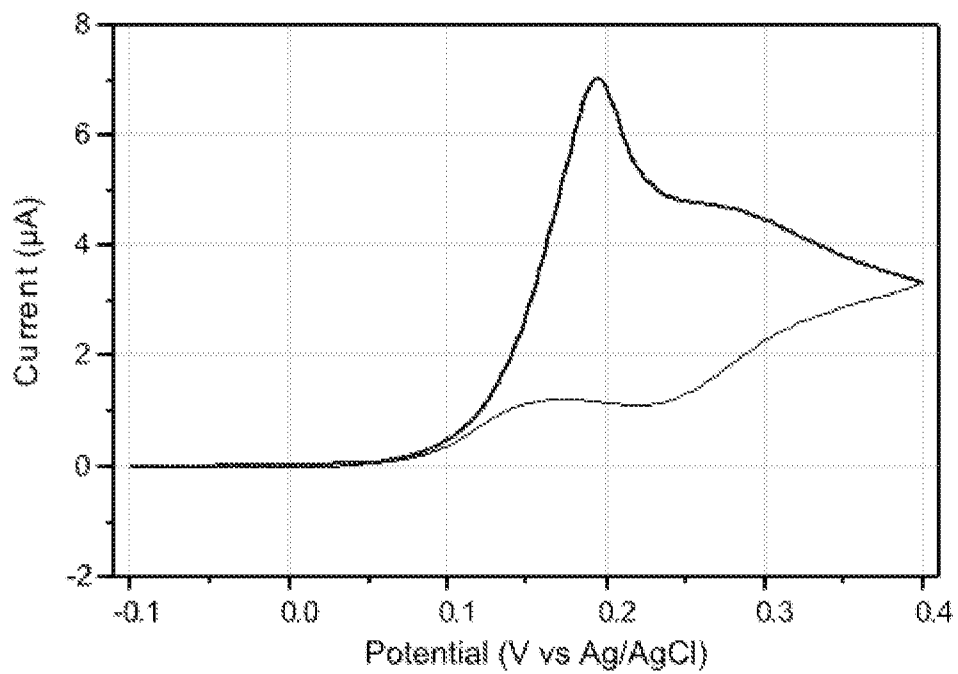


Figure 5b

4/14

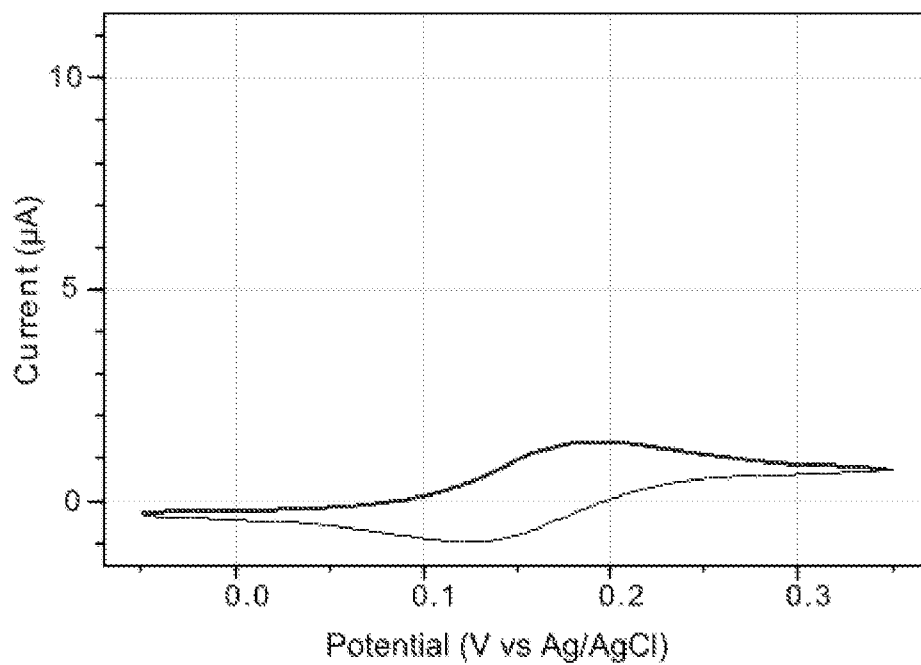


Figure 5c

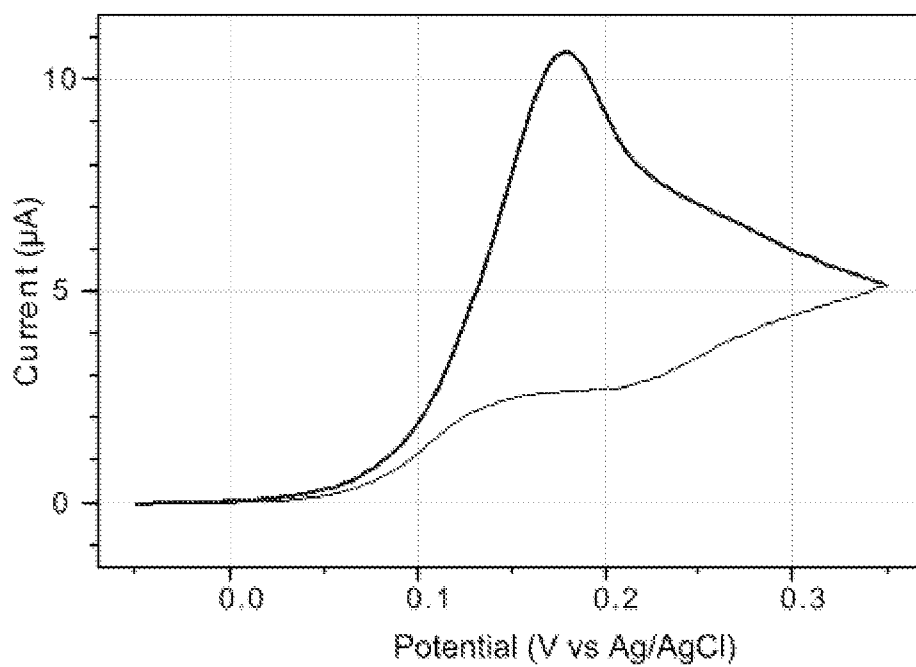


Figure 5d

5/14

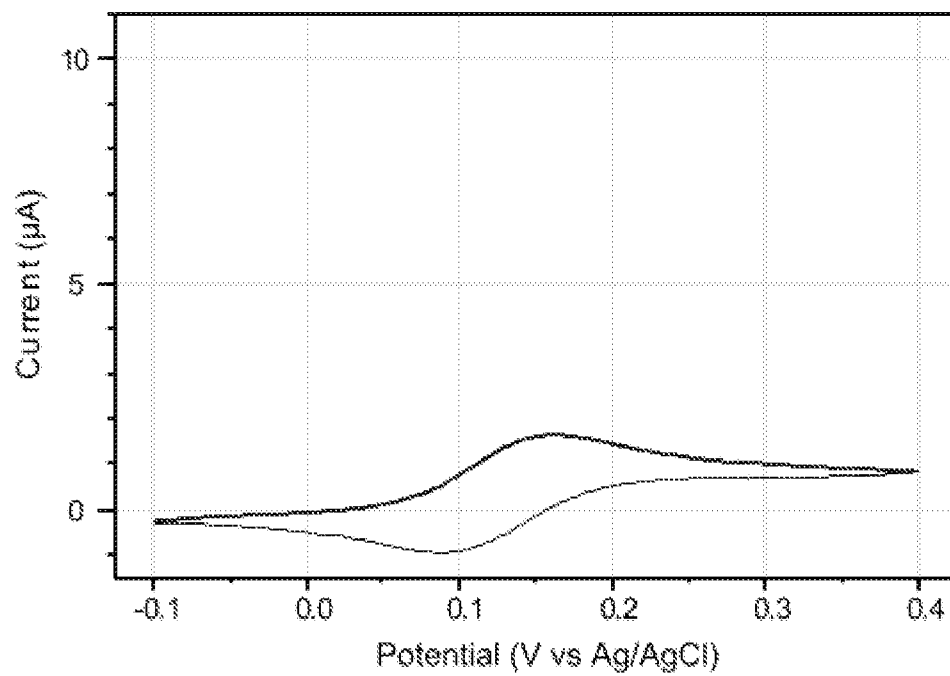


Figure 5e

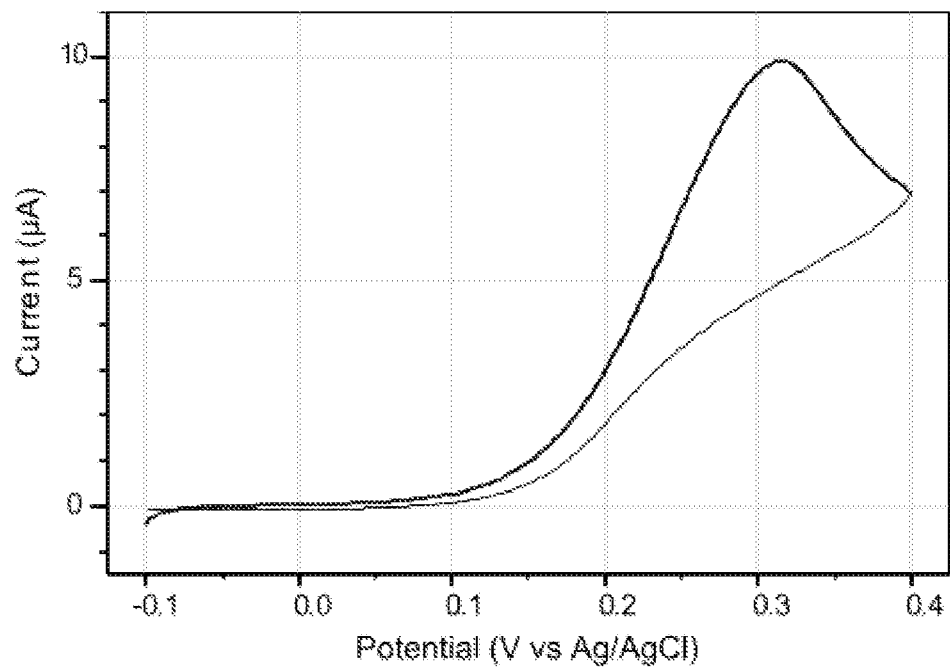


Figure 5f

6/14

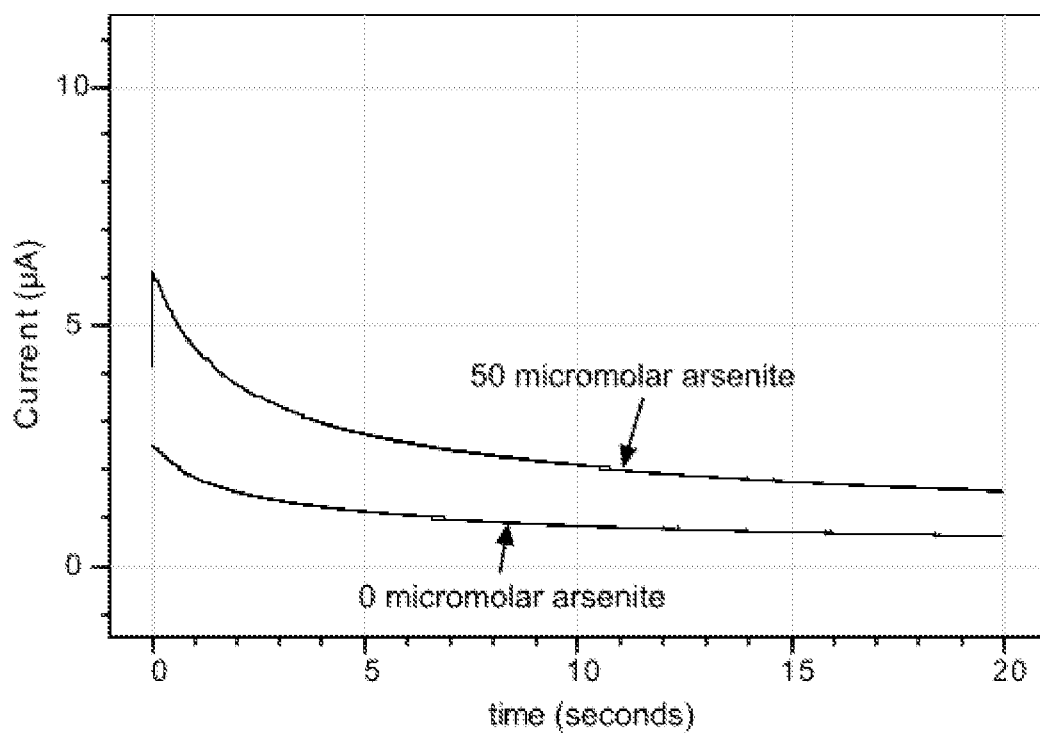


Figure 5g

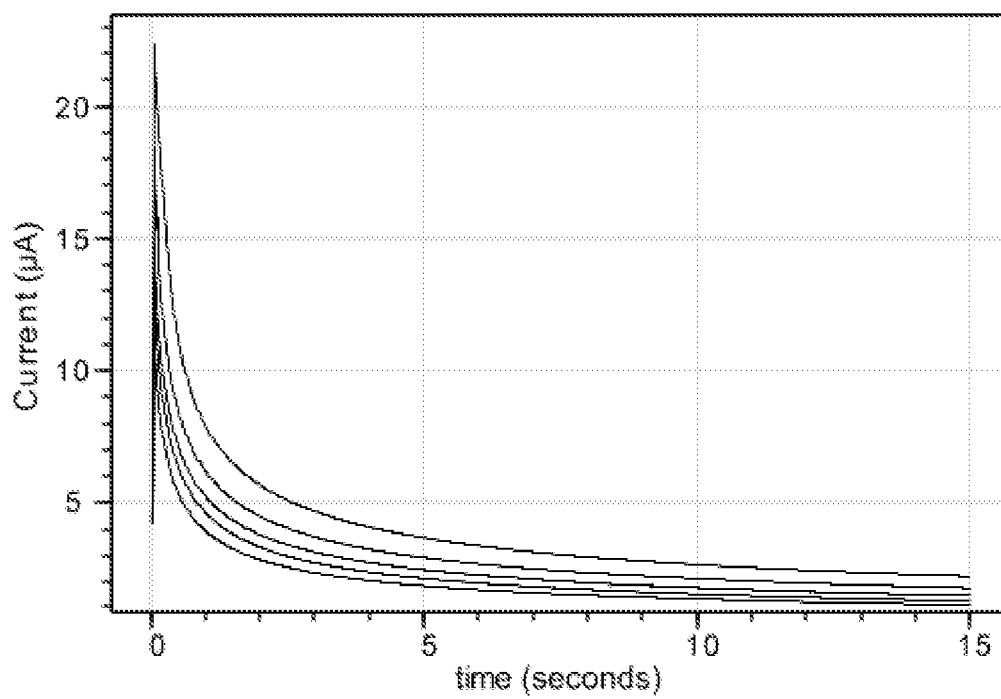


Figure 5h

7/14

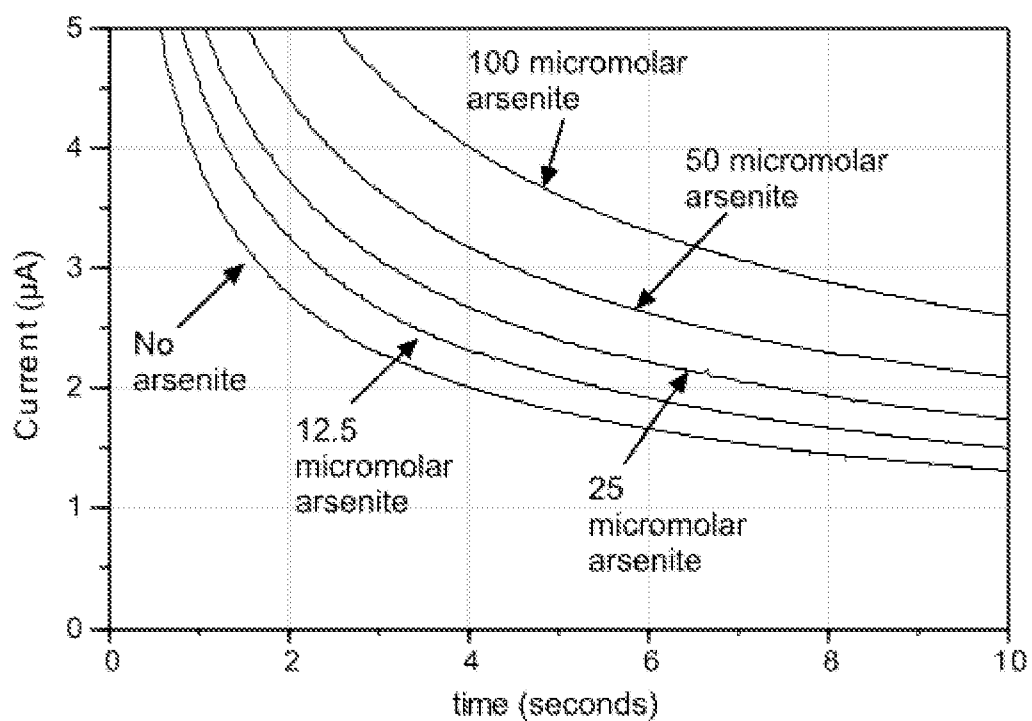


Figure 5i

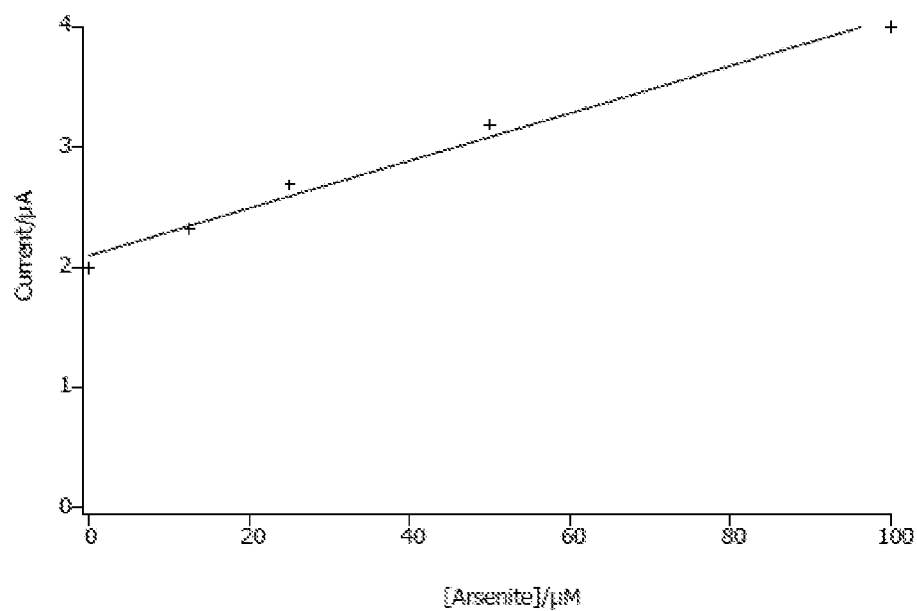


Figure 5j

8/14

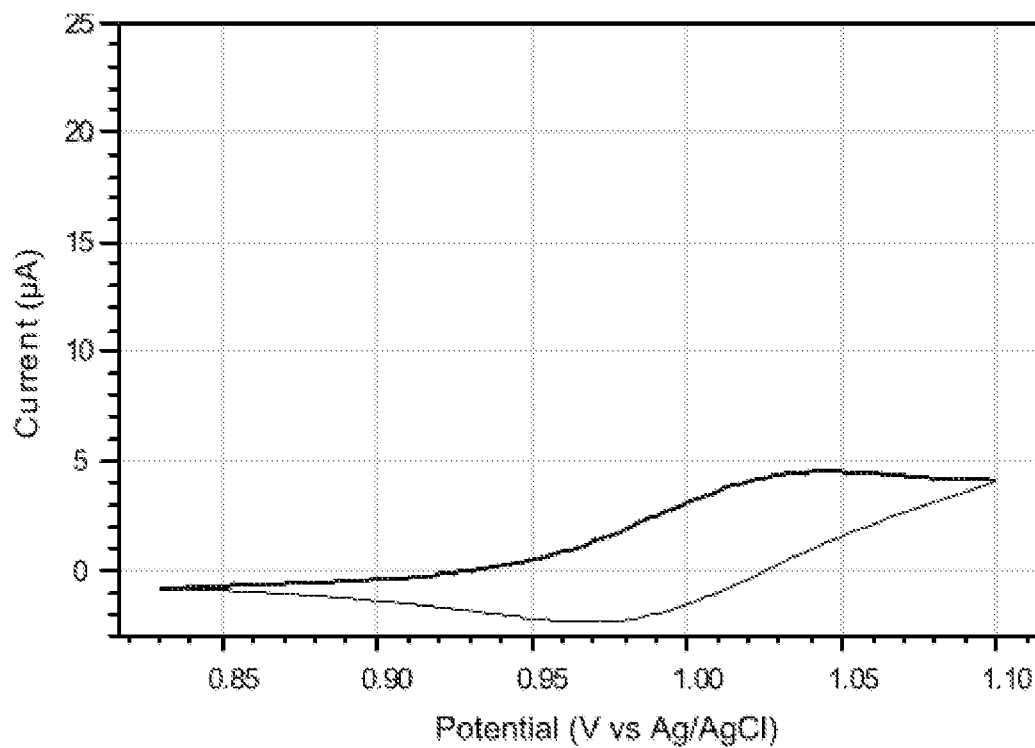


Figure 6a

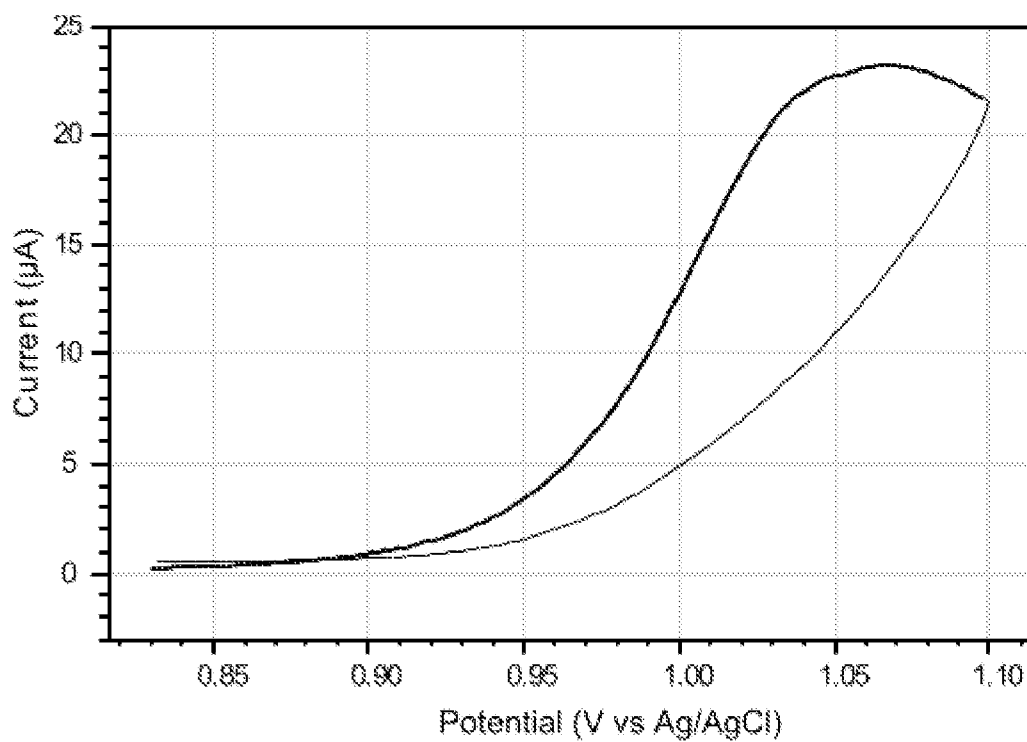


Figure 6b

9/14

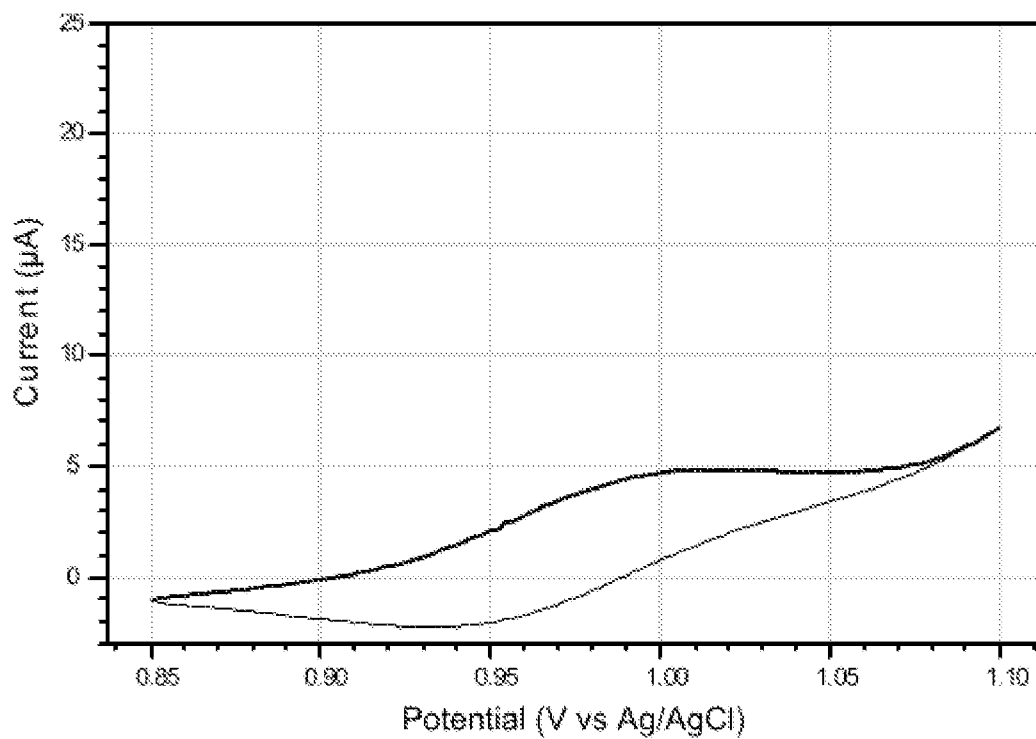


Figure 6c

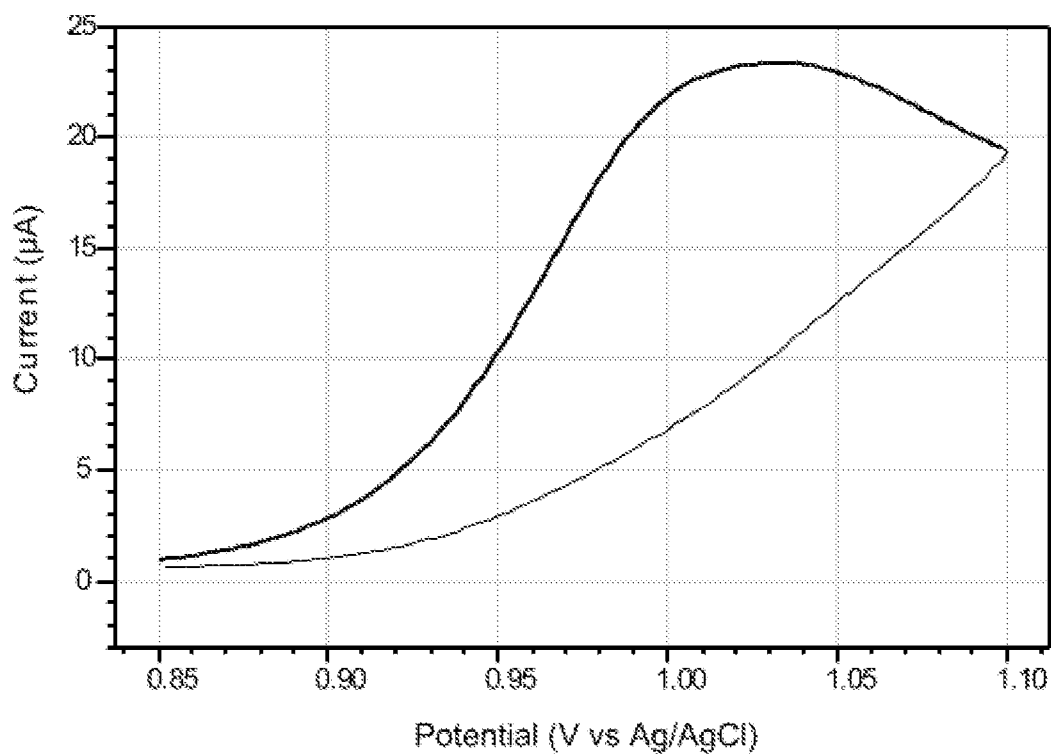


Figure 6d

10/14

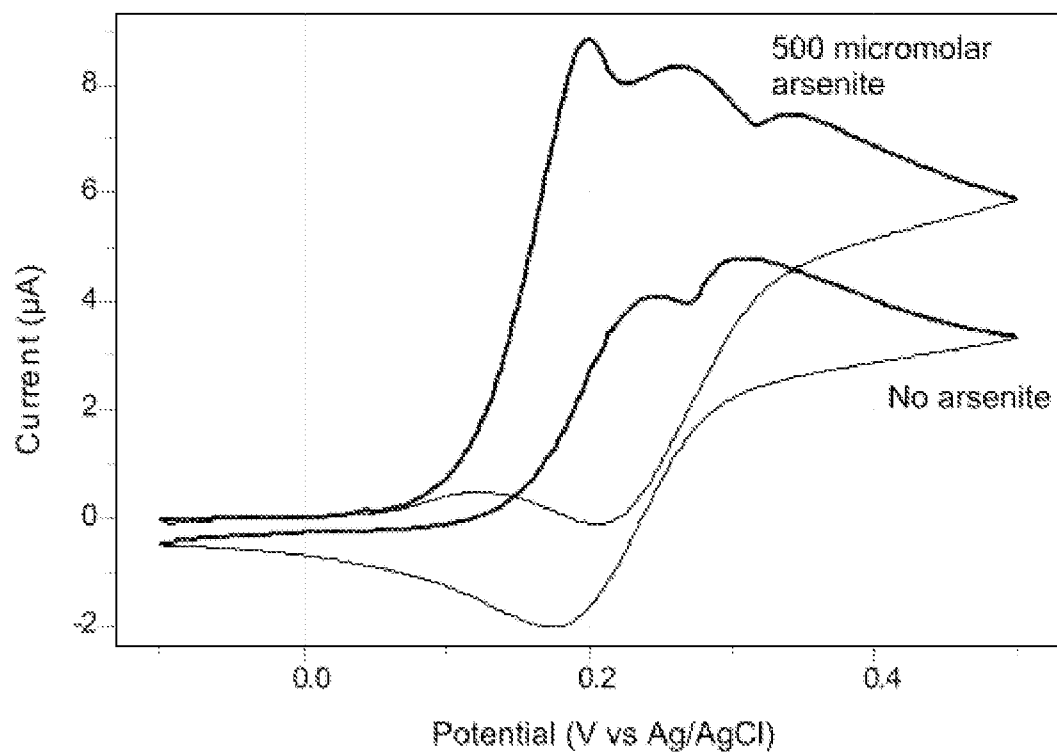


Figure 7a

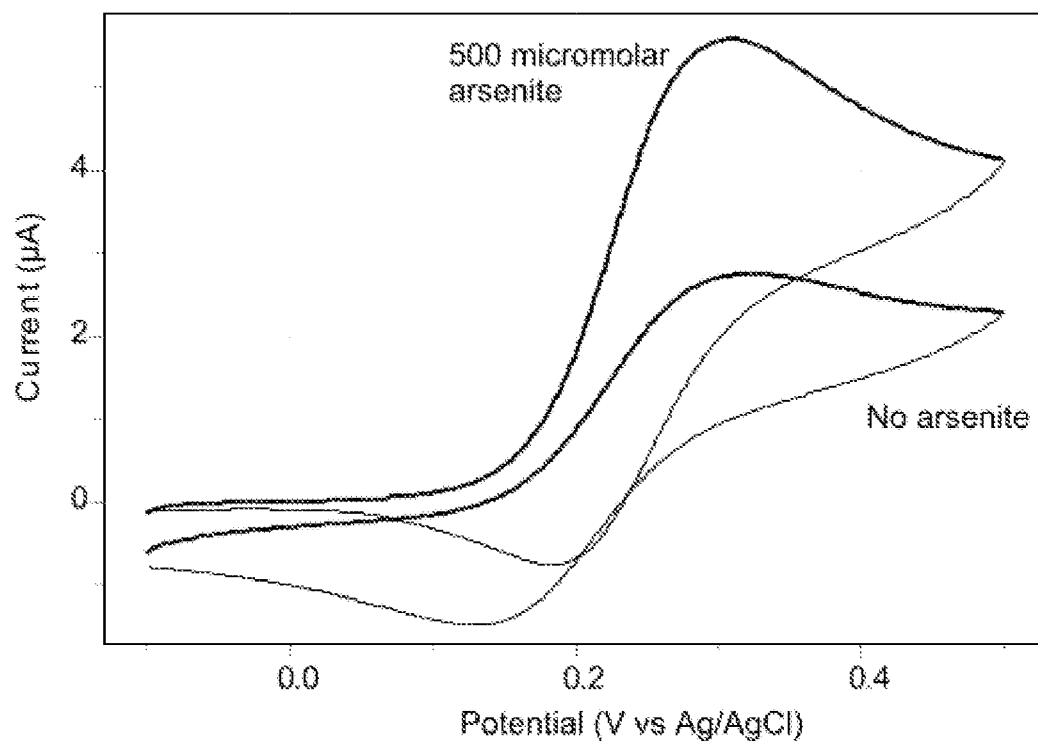


Figure 7b

11/14

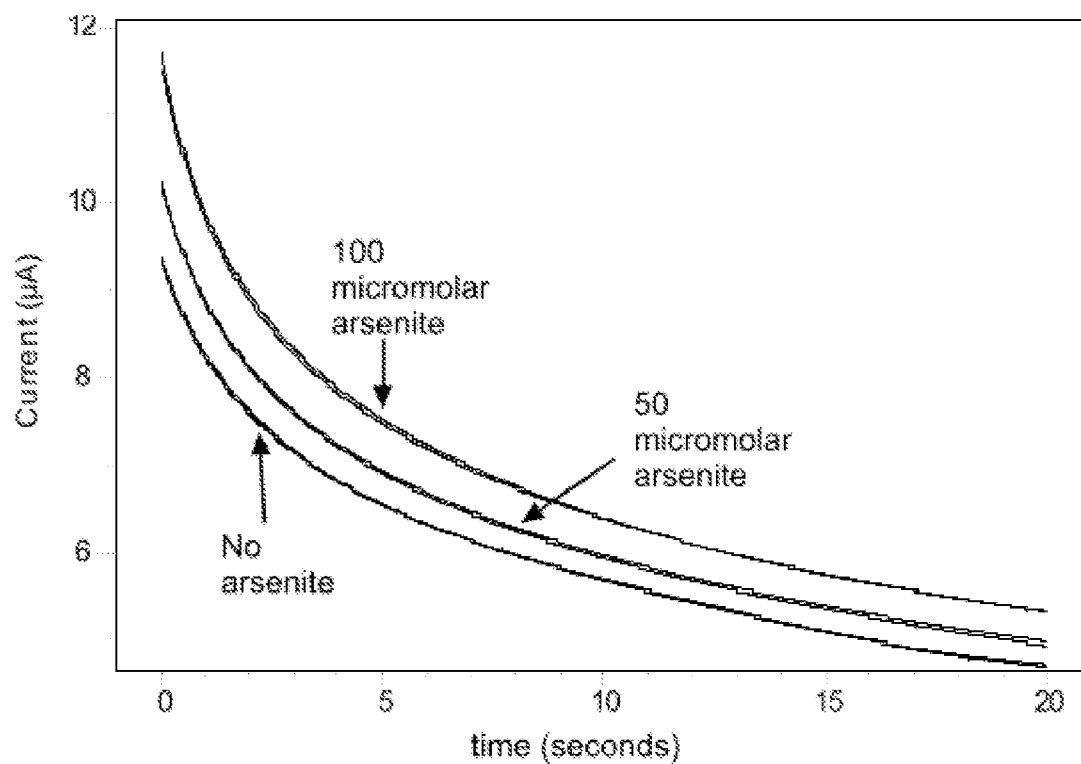


Figure 7c

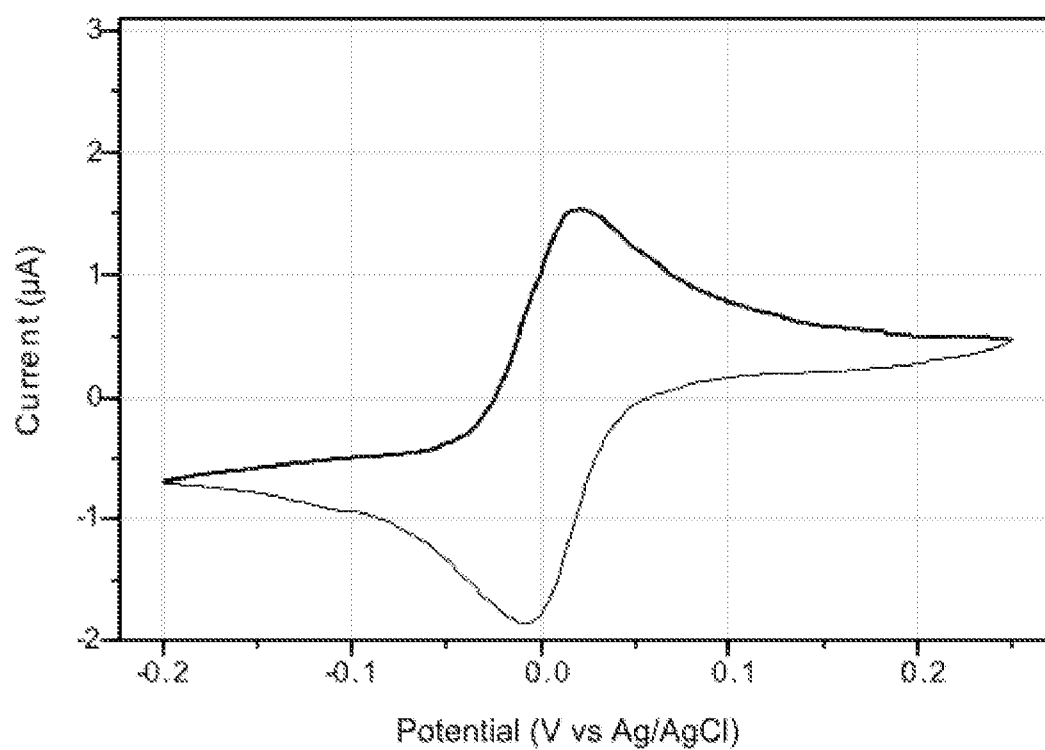


Figure 8a

12/14

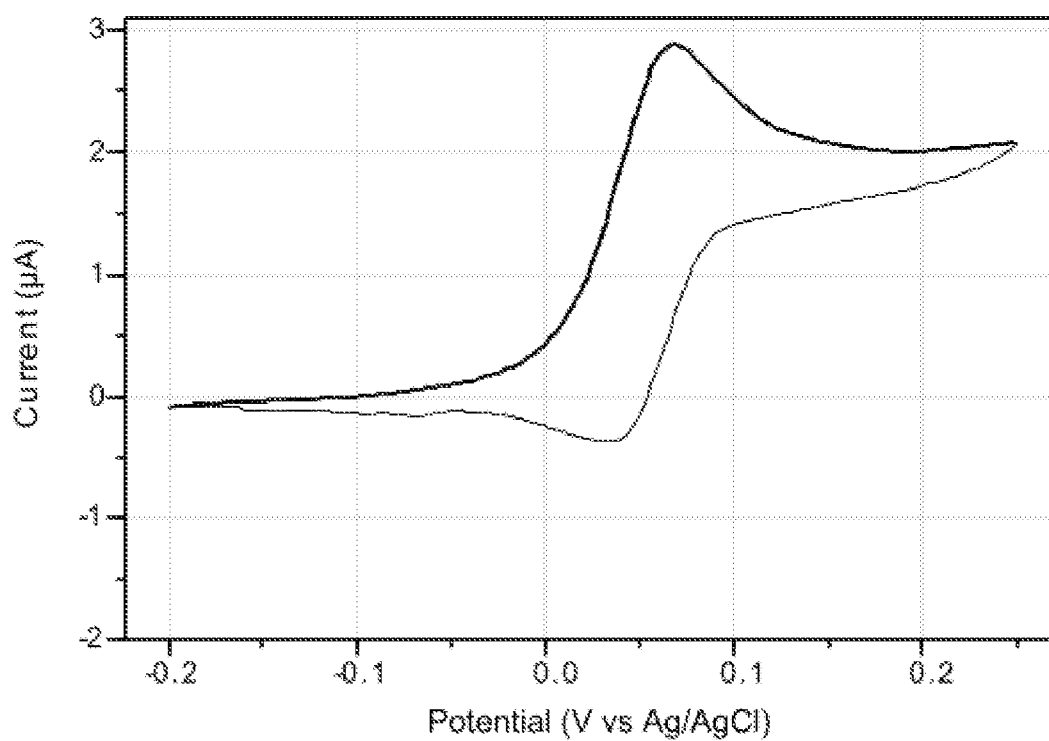


Figure 8b

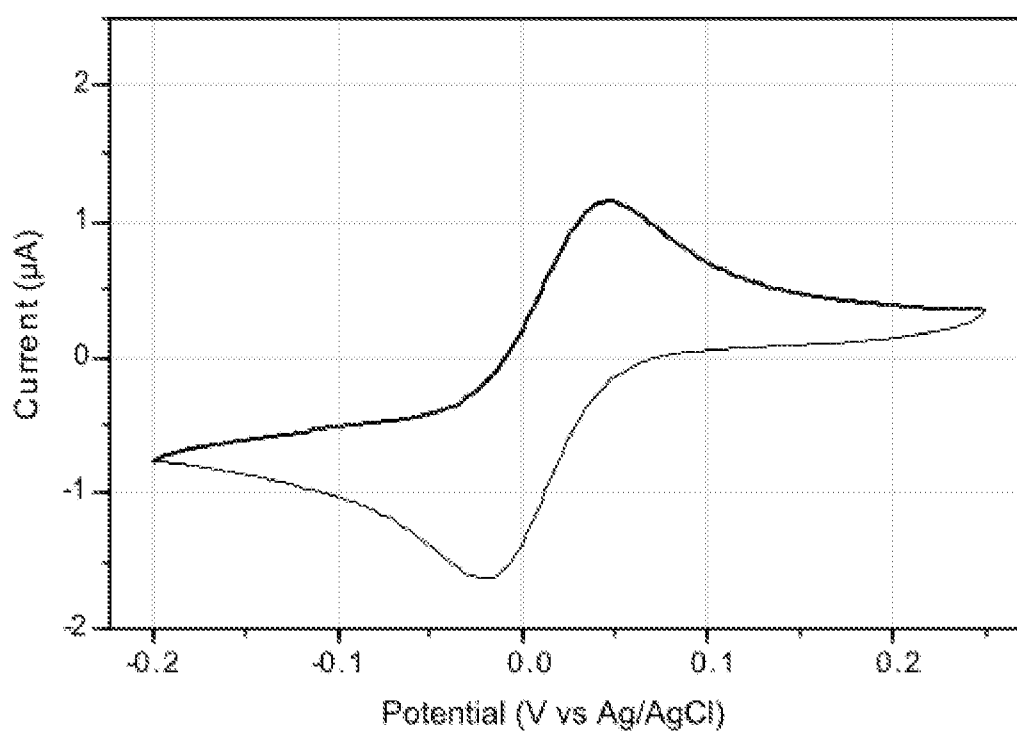


Figure 8c

13/14

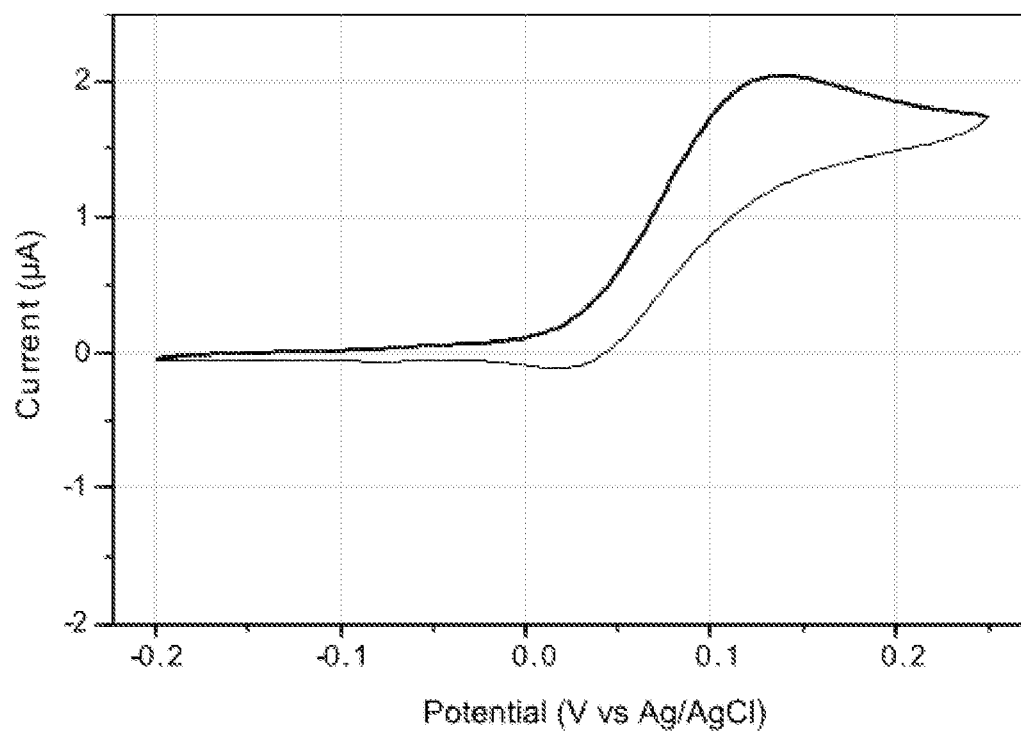


Figure 8d

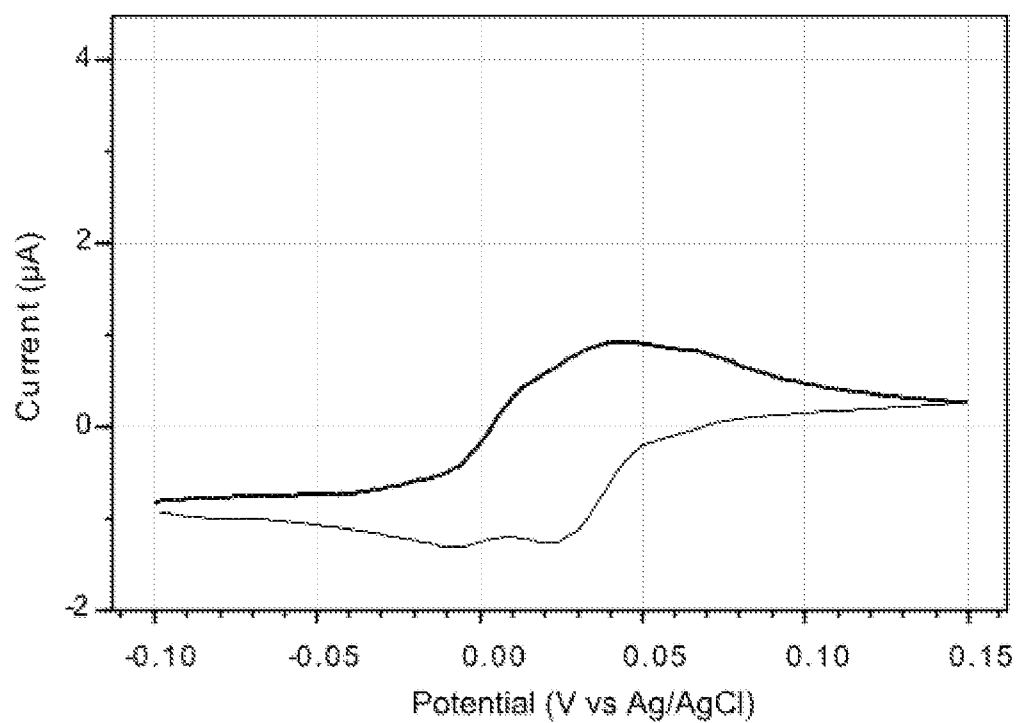


Figure 8e

14/14

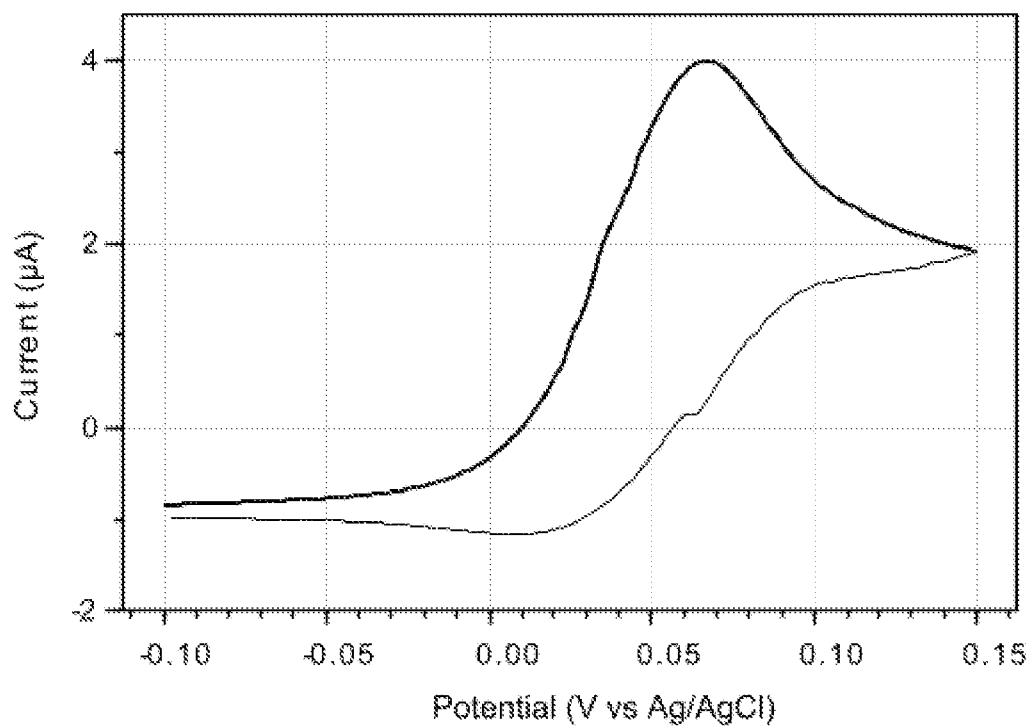


Figure 8f

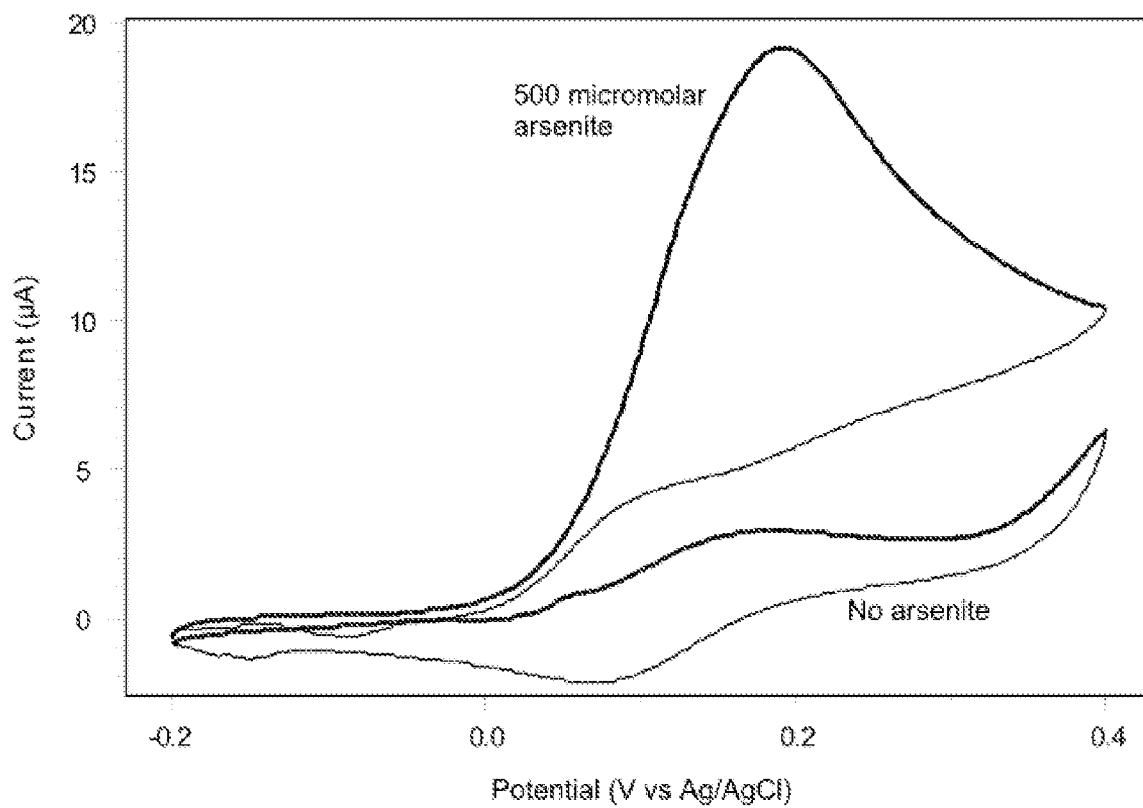


Figure 9