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(71) Applicant: **THE UNIVERSITY COURT OF THE UNIVERSITY OF GLASGOW** [GB/GB]; University Avenue, Glasgow, Strathclyde, G12 8QQ (GB).

(72) Inventors: **COOPER, Jonathan**; University of Glasgow, School of Engineering, James Watt South Building, Glasgow, Strathclyde G12 8QQ (GB). **REBOUD, Julien**; University of Glasgow, School of Engineering, James Watt South Building, Glasgow, Strathclyde G12 8QQ (GB).

(74) Agents: **WILLS, Jonathan** et al.; Mewburn Ellis LLP, 33 Gutter Lane, London, Greater London, EC2V 8AS (GB).

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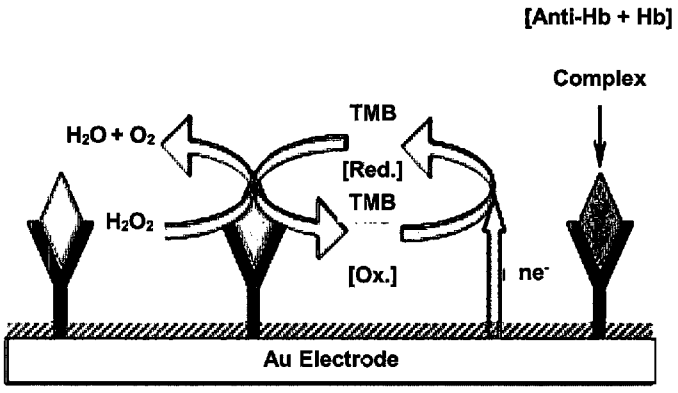
(54) Title: METHODS FOR DETECTING PROTEINS

Figure 1



(57) Abstract: Provided is a method for the detection of a protein, such as Hb, in a test sample, the method comprising the steps of: (i) exposing the sample to a surface having a binding moiety and permitting the binding moiety to specifically immobilise the protein in the sample; (ii) permitting the immobilised protein to catalyse a reaction of a first reagent with a second reagent; (iii) detecting, such as electrochemically detecting, the reaction of the first reagent with the second reagent. The binding moiety may be provided on the surface of an electrode.

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METHODS FOR DETECTING PROTEINS

Related Application

The present case claims the benefit and priority of GB 1315532.0 filed on 30 August 2013 (30/08/2013), the contents of which are hereby incorporated by reference in their entirety.

Field of the Invention

The present invention relates to a method for detecting a protein, in a sample. In particular, the invention relates to a label-free electrochemical assay for the detection of haemoglobin.

Background

The presence of blood in clinical samples is a proxy for many clinically important conditions. Haemoglobin (Hb) is a significant biomarker and a proxy for the presence of blood in such samples.

For example, in urine, haematuria can be sign of an infection, or, together with other markers, inform on the diagnosis of cancer. In faeces, the presence of blood has been linked to bowel cancer.

Assays for detecting Hb are routinely carried out in clinical laboratories, usually using spectroscopy. However, spectroscopy does not have the required sensitivity or specificity for more complex samples. In faeces, the sample's turbidity prevents any optical detection, while the complex mixture of components in urine means that very high sensitivity is needed. Consequently, more complex biochemical assays have been developed based on non-optical techniques, such as electrochemistry, to allow the analysis of such opaque or turbid samples.

Current electrochemical detection methods include direct assays where Hb is captured non-specifically within a matrix. A redox mediator is provided on a working electrode surface to enable electron transfer. A commercial system based on this principal is available: BeneCheck (see Hsieh *et al.*, *Clinica Chimica Acta* **2011**, *412*, 2150). This strategy performs well for high concentrations in blood for example, but is limited at low sensitivity owing to the lack of specificity of the haemoglobin capture.

A modified electrode for use as a haemoglobin detector has been described by Sun *et al.* (*Colloids and Surfaces B: Biointerfaces* **2013**, *101*, 177). Here, haemoglobin is non-specifically immobilised onto mesoporous spheres, and the functionalised spheres are in turn combined with a carbon ionic liquid electrode. The electrode is further modified with a

chitosan layer. The immobilised haemoglobin is capable of reacting with the added reagent TCA to give rise to a detectable signal.

A second type of assay is the sandwich assay or ELISA-type assay where Hb is first captured by a first binding moiety, such as an antibody. A second Hb-specific antibody is then added to create a sandwich, this second antibody having attached an enzyme label, such as HRP, which is used to generate a detectable signal. In sandwich assays, as is common to all heterogeneous immunoassay formats, there is a requirement for fluid manipulation (e.g. a washing step to separate bound and unbound labelled antibodies) which limits the speed with which samples, particularly complex ones, can be processed.

An example of such an assay is described by Stöllner *et al.* (*Analytica Chimica Acta* **2002**, *470*, 111), where a haptoglobin protein is used to selectively enrich Hb at a sensor surface in front of a Pt working electrode. A particular Hb form, HbA1c, is detected using a glucose oxidase (GOx) labelled anti-HbA1c antibody. Hydrogen peroxide generated by the glucose oxidase label is detected electrochemically.

There are currently half a million deaths per year from bowel cancer, with a lifetime mortality risk of 5-6 % (1 in 18), in the Developed World. This risk can be mitigated by early diagnosis, with subsequent treatment resulting in remission. As a consequence, there is a need to provide a more streamlined and specific electrochemical method for the analysis of Hb in faecal samples, as well as a more general streamlined and specific method for the detection of other analytes in complex biological samples.

It is noted the Hb has previously been used in a variety of biosensors for the electrochemical detection of hydrogen peroxide. See for example Zhang *et al.* (*Analytical Letters*, 2007, **40**, 661-676), Chen *et al.* (*Biosensors & Bioelectronics*, 2007, **22**, 1268-1274) and Lai *et al.* (*Sensors and Actuators B-Chemical*, 2008, **129**, 497-503).

Summary of the Invention

At its most general, the present invention provides a method for detecting a protein in a sample, wherein the method comprises the step of specifically immobilising the protein and subsequently permitting the immobilised protein to catalyse the reaction of a reagent, and detecting the reaction of the reagent.

The present invention uses the inherent catalytic activity of the protein as a reporter for the presence of that protein. Thus, the presence of the protein, including the quantitative amount of that protein, may be gauged from the reaction of a standard reagent. In this regard the present invention provides a significant advantage over sandwich-based assays, which require an immobilised protein of interest to be subsequently labelled for detection. Typically, the label includes an antibody to bind specifically to the immobilised protein. This

additional labelling step adds a layer of complication to the detection method. It is clear that the labelling step must be quantitative, otherwise detection of the label will not be representative of the actual quantity of the immobilised protein. The labelling step requires additional preparative steps, which include the labelling itself and the subsequent washing steps.

It is a requirement of a sandwich-based assay that the protein for detection is capable of associating with a binding moiety, such as an antibody, that is present on the surface, and additionally the protein must be capable of associating with the label, typically including a further antibody. Thus if a protein is to be detected in a sandwich-based assay that protein must be capable of orthogonally binding two different binding moieties. Moreover, for a particular protein of interest that protein may be used in the sandwich assay only if there are two known binding moieties that are capable of orthogonally binding to that protein. For many proteins of interest this may not be the case, and the sandwich assay is not suitable for use in detecting that protein.

In a first aspect of the invention there is provided a method for determining the presence of a protein, the method comprising the steps of:

(i) providing a surface having a binding moiety that is associated with a protein, wherein the binding moiety is specific for the protein;

(ii) permitting the protein to catalyse a reaction of a first reagent with a second reagent;

(iii) detecting the reaction of the first reagent with the second reagent, such as electrochemically detecting the reaction of the first reagent with the second reagent.

The present invention may include the step of immobilising the protein from a sample. Accordingly, in a further aspect of the invention there is provided a method for determining the presence of a protein in a sample, the method comprising the steps of:

(i) exposing the sample to a surface having a binding moiety and permitting the binding moiety to specifically immobilise the protein in the sample;

(ii) permitting the immobilised protein to catalyse a reaction of a first reagent with a second reagent;

(iii) detecting the reaction of the first reagent with the second reagent.

The binding moiety is specific for the protein and the immobilisation step allows the protein to be separated from other components of the sample, such as other proteins, which may be subsequently washed away. This immobilised protein is then exposed to a reagent. The protein catalyses the reaction of the reagents. The reaction of the reagents is detectable and is indicative of the presence of the immobilised protein. Additionally, the amount of protein present may be determined from the change in reaction rate.

The method of the present case requires the protein in the sample to associate with a binding moiety. The requirements for this association are that it should be specific for the protein and that the association should permit the protein, whilst associated, to catalyse the reaction of the reagents.

The protein is specifically immobilised onto a surface, thereby allowing the protein to be purified from the test sample in which it was present. This advantage is also known from the sandwich-based assays.

Advantageously, the surface to which the protein is attached may form part of a sensor for the detection of the reaction of the reagent that is catalysed by the protein.

In one embodiment, the sample is a biological sample. The sample may be a stool or urine sample. The sample may comprise blood.
In one embodiment, the method comprises the preliminary step of processing the sample with surface acoustic wave actuation.

In one embodiment, the protein is a metalloprotein.
In one embodiment, the protein is haemoglobin (Hb).

In one embodiment, the binding moiety is a polypeptide, such as an antibody or a protein. In one embodiment, the protein is Hb and the specific binding moiety is anti-Hb antibody.
In one embodiment, the binding moiety is attached directly or via a linker to an electrode.

In step (ii), the reagent is made available for reaction catalysed by the immobilised protein.
In one embodiment, the protein catalyses the reaction of a first reagent with a second reagent, and the reaction of the first reagent with the second reagent is detectable.
In one embodiment, the first reagent is, or comprises, a compound selected from tetramethylbenzidine (TMB), alpha guaiaconic acid, 2,2' azino bis(3-ethylbenzothiazolidine-6-sulphonic acid), hydroquinone, phenylenediamine, o-dianisidine, o-tolidine (dimethylbenzidine), 6-methoxyquinoline, and 3,3'-diaminobenzidine, 3-amino-9-ethylcarbazole.
In one embodiment, the second reagent comprises hydrogen peroxide or a precursor thereof. For example, the second reagent is or comprises hydrogen peroxide, urea peroxide or sodium perborate.

In one embodiment, the surface is an electrically conductive surface. The electrically conductive surface may be or contain Ag, Au, Pt, Pd, Fe or carbon and mixtures thereof.
In one embodiment, the surface is part of an electrochemical sensor. In this embodiment, the reaction of the first reagent with the second reagent can be detected electrochemically, for example by amperometric measurement. In one embodiment, the surface is a working electrode.

In one embodiment, step (i) includes a preliminary blocking step wherein the surface is exposed to one or more blocking agents thereby to minimise non-specific adsorption of the protein.

In one embodiment, step (i) further comprises separating the immobilised protein from the protein-depleted sample. This may be a washing step, where the surface is washed thereby to remove non-bound material from the surface. The reagents are made available after this time such that immobilised protein (only) is permitted to catalyse the reaction of the reagent.

In one embodiment, the surface is a bead or film. In one embodiment, step (ii) includes the step of providing the surface at or close to a working electrode prior to the step of permitting the immobilised protein to catalyse the reaction of a first reagent with a second reagent. For example, a bead or film having immobilised protein may be placed on or in close proximity to a working electrode prior to the catalysis reaction.

In one embodiment, the electrochemical sensor comprises a working electrode and optionally further comprises one or more of a reference electrode, a counter electrode, a power supply and a meter for measuring potential and/or current. The electrochemical sensor may include a controller, for example to control potential over time.

The electrochemical sensor is capable of measuring the change in current between the working electrode and the counter electrode over time.

In a further aspect of the invention, an immobilised protein having catalytic activity may be used as a reporter for an analyte that is a binding partner, such as a specific binding partner, to that protein. In the absence of the binding partner the protein is capable of catalysing the reaction of a reagent. The reaction of the reagent is detectable. When a binding partner is present, the protein binds to the binding partner, thereby rendering the protein incapable of catalysing the reaction of the reagent. A drop or loss in reagent reactivity may be associated with the binding of the binding partner to the protein.

Accordingly, in a further aspect of the invention there is provided a method for detecting an analyte in a sample, which analyte is a binding partner for a protein, the method comprising the steps of:

(i) providing a protein that is immobilised, such as specifically immobilised, to a surface;

(ii) exposing the immobilised protein to the analyte in the sample, thereby to bind the analyte to the immobilised protein; and

(iii) exposing the immobilised protein to a reagent, such as first and second reagents;

(iv) detecting the reaction of the reagent, such as first and second reagents,

wherein the immobilised protein is a catalyst for the reaction of the reagent in the absence of the analyte. The binding of the analyte to the immobilised protein alters the catalytic activity of the protein for the reaction of the first reagent with the second reagent.

In one embodiment, the protein is a metalloprotein.
In one embodiment, the protein is haemoglobin (Hb).

In one embodiment, the protein is specifically immobilised to a surface by a binding moiety. The surface may be an electrically conductive surface.

In one embodiment, the binding partner is a receptor for the protein. The receptor may be present on a pathogen, including a bacterium such as *S. aureus*.
In one embodiment, the binding partner is or includes an antibody for the protein, such as a bispecific antibody.

Other aspects and embodiments of the invention are described in further detail below.

Description of the Figures

Figure 1 shows a schematic for 'label-free' electrochemical immunoassay for Hb detection according to one embodiment of the invention. The assay uses the intrinsic peroxidase activity of immobilised haemoglobin to catalyse the reaction of TMB.

Figure 2 are cyclic voltammograms recorded on a bare Au-disk electrode in phosphate-citrate, pH 4.5, containing KCl (0.1 M) and: a) containing 2 mM TMB; b) 2 mM TMB and 2 mM H_2O_2; and c) 2 mM TMB, 2 mM H_2O_2 and 2.5 mg/mL Hb. Scan rate: 50 mV/s.

Figure 3 shows typical responses of an electrochemical immunoassay of the invention at different concentrations of Hb (equivalent to a range between 0 and 8.0 mg/mL Hb), measured *in vitro* using model analytical solutions.

Detailed Description of the Invention

The present invention provides a method for detecting a protein in a sample, particularly a biological sample. The method provided is a specific label-free assay. Because of the specific capture of the protein, the method provides sensitivity at low concentrations of the protein.

Furthermore, as the method uses the intrinsic enzyme activity of the analyte to generate a signal, secondary antibodies and enzyme labels are not needed. As a result, the method has fewer processing steps than traditional assays, making the method amenable to

miniaturisation onto microfluidic Lab-on-a-Chip devices for faster processing of large numbers of samples.

The present invention relates in particular to a method for the detection of a protein such as Hb, where the activity of the protein may be detected electrochemically.

Further streamlining of the assay can be achieved by integrating the method within a surface acoustic wave (SAW) platform for the disruption of solid samples, such as faeces.

Faster sample processing has the potential to have a significant impact on, for example, the detection of Hb in bowel cancer screening.

In another aspect a change in the reaction rate catalysed by the immobilised protein is used as a reporter for the presence of a binding partner to the immobilised protein. The formation of a complex of the binding partner with the immobilised protein alters the catalytic activity of the immobilised protein, and the change in reaction rate may be detected.

US 5,506,114 describes the immobilisation of Hb to a surface using an Hb-specific antibody. Once bound, the Hb is used to catalyse the reaction of hydrogen peroxide with another reagent. That reaction is followed spectroscopically, detecting the amount of product formed and comparing it to a standard. US 5,506,114 does not describe the use of an electrode as an immobilisation surface, and there is no suggestion that the reaction could be measured electrochemically.

US 4,806,468 broadly describes the immobilisation of Hb to a surface using an Hb-specific antibody. The glycosylated Hb that is bound to the surface is permitted to catalyse the reaction of hydrogen peroxide with another reagent. The reaction is also followed spectroscopically. As with US 5,506,114, US 4,806,468 does not describe the use of an electrode as an immobilisation surface, and there is no suggestion that the reaction could be measured electrochemically.

WO 2013/005003. This also refers to the use of an immobilised enzyme to catalyse a reaction. In this work the reaction yields an insoluble product, and the insoluble product is detected by spectroscopy. Again, there is no reference to electrochemical detection. Furthermore this document does not refer to Hb detection.

Assay kits are available for the immobilisation and detection of certain proteins. For example, the InnoZyme™ Myeloperoxidase Activity Kit from Calbiochem makes use of an (undisclosed) antibody to immobilise myeloperoxidase. The immobilised myeloperoxidase is then permitted to catalyse the reaction a detection reagent comprising TMB and hydrogen peroxide. The reaction is followed spectroscopically based on a colour change in the reaction mixture (associated with the formation of oxidised TMB, detectable at 450 nm).

However, this commercial assay does not describe or suggest the use of electrochemical techniques for the detection of an immobilised protein activity. Moreover, the assay is not apparently suitable for the detection of Hb.

Protein and Analyte

The method of the invention may be used to detect a protein. The protein may itself be an analyte of interest or in other embodiments the protein may be associated with an analyte of interest as described below. In this embodiment, the protein acts as a reporter (or label) for the analyte of interest.

The protein is immobilised to a surface using a binding moiety that is specific for that protein. In one embodiment, the method of the invention includes the step of immobilising the protein to a surface. As described below, the immobilisation step permits the protein to be purified from a sample. The immobilisation of the protein may be a preliminary step in the method for the detection of the protein. The protein is preferably held non-covalently to the binding moiety. The inherent reactivity of the protein may therefore be used as a reporter to identify and quantity that protein. Whilst the protein is immobilised to the surface it retains its catalytic activity

In one embodiment, the protein is capable of coupling oxidation of a first reagent to reduction of the second reagent. The first reagent and the oxidising agent are selected with a target protein (or analyte) in mind. The reaction of the first reagent and the second reagent in the presence of the protein is detectable, for example by electrochemical means.

Preferably, the protein is a catalyst for the reaction of hydrogen peroxide, or a derivative of hydrogen peroxide, with the first reagent.

Preferably, the reagents have low reactivity in the absence of the protein. For example, in the presence of the protein, the reaction rate is 100 times or more, 500 times or more, or 1,000 times or more than the reaction rate in the absence of the protein.

The rate of reaction in the presence and absence of the protein may be determined electrochemically, for example by amperometric measurement.

The protein may comprise a polypeptide, and the protein is capable of catalysing the reaction of the first reagent and the second reagent.
The protein may be a peroxidase.
The protein may be a metalloprotein. The protein may be an oxygen-binding protein.
In one embodiment the protein is a haem protein i.e. a protein having a haem group.
Preferably, the protein is haemoglobin (Hb) or horseradish peroxidase.

More preferably, the protein is haemoglobin. In this most preferred embodiment, the method of the invention is suitable for use in the detection of blood in sample.

In one embodiment, the haemoglobin is human haemoglobin or a variant thereof.
In one embodiment, the human haemoglobin is selected from the group consisting of haemoglobin A, haemoglobin A_2, haemoglobin F.
In one embodiment, the human haemoglobin is a haemoglobin associated with the human embryo or fetus, for examples a haemoglobin selected from the group consisting of Gower 1, Gower 2 Hemoglobin Portland and haemoglobin F.
In one embodiment, the human haemoglobin is a haemoglobin variant associated with a disease state, for example with sickle cell disease. In one embodiment, the human haemoglobin is a haemoglobin variant selected from the group consisting of haemoglobin H (β_4), haemoglobin Barts (γ_4), haemoglobin S ($\alpha_2\beta^S_2$), haemoglobin C ($\alpha_2\beta^C_2$), and haemoglobin E ($\alpha_2\beta^E_2$).
References to a protein may also include references to the glycosylated versions of that protein. The methods described herein may be used to directly detect such proteins if the glycosylation does not interfere with the ability of the protein to catalyse the reaction of the reagents.

In one embodiment, the haemoglobin is a glycosylated haemoglobin.
For example, in one embodiment, the haemoglobin is HbAc1. The detection and quantification of HbAc1 levels in a biological sample is used as a means for determining plasma glucose concentrations. Thus, the methods of the invention may be used to determine plasma glucose concentrations in a subject.

A reference to haemoglobin may be taken as a reference to a single haemoglobin type as described above, or a plurality of haemoglobin types. Binding moieties are available that are capable of selectively binding one haemoglobin type over other of haemoglobin types. Binding moieties are available that are capable of selectively binding haemoglobins generally over other polypeptides.
For example, Stöllner *et al.* describe the use of haptoglobin as a binding moiety to immobilise haemoglobins. Stöllner *et al.* also describe the use of anti-HbAc1 as specifically targeting HbAc1 haemoglobin over other haemoglobin types.

The protein may itself be associated with an analyte of interest. The analyte may be a biomolecule, such as those biomolecules that are, or comprise, a polynucleic acid, a polypeptide, or a polysaccharide. The protein may be considered as a label for the analyte that allows the detection, and optionally quantification, of the analyte in a sample. Such detection and analysis is based on the catalytic activity of the protein label. It follows that the protein, whilst it is associated with the analyte, retains its ability to catalyse the reaction of the reagents. The analyte itself does not catalyse the reaction of the reagents.

In the method for detecting a protein according to an aspect of the invention, the protein may itself be associated with an analyte of interest as noted above. This association does not result in the loss of the catalytic activity of the protein for the reaction of the reagents. The increase in the reaction rate of the reagents is an indicator for the presence of the protein, bound to the binding moiety.

In an alternative aspect of the invention, for detecting an analyte which is a binding partner for the protein, the association of the binding partner with the protein results in the loss of the catalytic activity of the protein for the reaction of the reagents. The decrease in the reaction rate of the reagents is an indicator for the presence of the binding partner. This is described in further detail in the *Detection of a Binding Partner* section below.

In one embodiment, the protein is covalently connected to the analyte of interest.

As described herein, a protein may be bound to a surface, such as an electrically-conductive surface, *via* a binding moiety such as an antibody.
In one embodiment the protein is associated with a binding moiety, and that binding moiety is connected to the surface, such as an electrically-conductive surface. Here, the protein is not connected to any other binding moieties.
In another embodiment, the protein is associated with a binding moiety that is connected to the surface and may be associated with an additional binding moiety that is not connected to the surface. The protein, whilst it is associated with the additional binding moiety, retains its ability to catalyse the reaction of the first reagent and the second reagent.

The additional binding moiety may be an antibody for the protein.
Where an additional binding moiety is present, it is preferred that the additional binding moiety does not catalyse the reaction of the first and second reagent. In one embodiment, the reaction rate of the first and second reagents in the presence of the additional binding moiety is substantially unchanged compared with the reaction rate of the first and second reagents in the absence of the additional binding moiety. Thus, where there is an increase in the rate of reaction between the first and second reagents, the increase in the reaction rate is predominantly or entirely associated with the protein itself, and not with the additional binding moiety.
The additional binding moiety may itself be labelled to allow detection or isolation of the binding moiety and the protein by other means. As noted above, however, the binding moiety does not catalyse the reaction of the first and second reagent, therefore if follows that the label is not capable of catalysing this reaction.
In contrast to the sandwich assays previously reported, the present case uses the intrinsic activity of the immobilised protein to catalyse the reaction of the reagents. In contrast, the sandwich assays rely on the activity of the additional binding moiety to provide a reporter activity for the immobilised protein.

In a preferred embodiment, the protein is not associated with an additional binding moiety.

As noted herein, the catalytic improvement in the reaction rate of the reagents is associated with the protein itself, and not any binding moiety, label or analyte that is connected to it. Thus, the methods for the detection of the protein involve the step of measuring the reaction catalysed by the protein. Thus, the method of the invention may include the step of determining the contribution of the protein to the change in the reaction rate.

The method of the invention is based on the measurement of the reaction of the reagents catalysed by the protein. Typically, the binding moiety, a protein label or an analyte associated with the protein (where present) are selected based on their relative inability to catalyse this reaction. However, under some circumstances another agent, such as the binding moiety, a protein label or an analyte associated with the protein, may also catalyse the reaction. Here, the detectable signal includes the contribution of the another agent to the catalysis of the reaction. Where the method of the invention looks to quantify the amount of protein present, the contribution of the another agent is deducted from the measured signal. Thus the method of the invention looks solely at the contribution of the protein to increase the rate of reaction between the first and second reagents.

The contribution of the another agent may be determined using standard reference experiments, for example with and without the presence of the protein and the binding moiety, label or analyte.

Preferably, only the protein is capable of acting as a catalyst for the reaction of the first reagents and the second reagent. Thus, the binding moiety, the surface and so on have a negligible catalytic activity.

As explained previously, the present invention uses the catalytic activity of the protein as an identifiable characteristic to allow the presence of that protein to be detected and quantified.

Sample

The method of the invention may be used to detect the presence or absence of a protein in a sample. In one embodiment, the protein is the analyte of interest. In another embodiment, the protein is associated with the analyte of interest, and therefore acts as a label.

The sample may be a biological sample. The sample may be obtained from a subject such as a mammal, including a human, rodent, canine, feline, bovine, avian or equine subject, such as described further below.

For example, the sample may be a faecal sample, a saliva sample, a hair sample, a blood sample, a urine sample, or a skin sample, amongst others. In one embodiment, the sample is a faecal sample.

The sample may be a liquid sample or a solid sample, or it may be a sample having solids and liquids, for example a fluid, such as a liquid, having solids suspended within it. Intermediate samples of this type may be referred to as semi-solids. A faecal sample is an example of a sample having solid and liquid parts.

In one embodiment the method is for detecting and optionally quantifying the amount of a biological molecule in a sample. That biological molecule may be a polypeptide (protein), polynucleotide, or polysaccharide. Preferably, the biological molecule is a protein, such as a metalloprotein. Where the biological molecule is a protein, the method may use the inherent catalytic ability of that protein as the means for detecting and quantifying the protein. In other embodiments, the biological molecule is labelled with a protein that is capable of catalysing the reaction the first and second reagents. The protein therefore acts as a reporter for the biological molecule. This arrangement is preferred when the biological molecule is incapable of catalysing the reaction of the reagents.

In one embodiment the method is for detecting and optionally quantifying the amount of blood in a sample. Here, the blood component haemoglobin may be used as the protein.

The present invention is particularly suitable for analysing stool samples, and may be used to detect blood within that sample, for example by detecting the presence of haemoglobin.

The sample for analysis in the methods described herein is preferably a fresh sample. Thus, the sample is one obtained from a subject (such as a human subject) and is subjected to analysis very shortly afterwards. In one embodiment, the sample is analysed at most 60 minutes, at most 3 minutes, at most 15 minutes, at most 10 minutes, at most 5 minutes, or at most 1 minute after the sample is obtained or deposited from the subject.

The sample may be obtained from a subject who is known or is suspected to have a clinically relevant disease or injury. In one embodiment, the subject is one suspected or known to have a disease associated with intestinal bleeding. In one embodiment, the subject is a human having Crohn's disease, ulcerative colitis, ulcers and/or cancer, such as bowel cancer. Here, a stool sample may be tested. The presence of blood, as indicated by the presence of haemoglobin in the sample, may be an indication of intestinal bleeding. Preferably the stool sample is taken from the inner portion of the stool, and is not limited to samples taken from the outer surface of the stool. The presence of blood on the surface of the stool sample is not necessarily indicative of a disease as described above. Typically the presence of blood within a core of the stool has a greater association with the diseases described above.

In one embodiment, the subject is one suspected or known to have a disease associated with a blood disorder, for example diabetes or haemophilia.
The subject may also be a subject who is undergoing a routine health check. Thus, the methods described herein may be used to identify subjects having a disease or at risk of having a disease. The methods may also be used to verify the well-being of a healthy subject.

It is not necessary for the sample to be modified in any particular way prior to its use in the methods of the invention. However, in preferred embodiments of the invention the sample, such as a faecal (stool) sample, is processed in order to ensure that material within the core and the surface of the sample is accessible. As described herein, surface acoustic wave actuation (SAW) methods may be used to prepare a stool sample for analysis.

In one embodiment, the sample may be diluted for analysis, for example with water. The purpose of the dilution step is to reduce the concentration of the protein in the sample thereby to minimise non-specific binding of the protein to the surface.

Where the sample for analysis comprises cells, such as blood cells, those cells may be lysed prior to the immobilisation step. The lysis step allows protein within the cell to become available for interaction with the binding moiety, and therefore immobilisation. Methods for lysing cells are well known and include centrifugation, the use of chemical reagents such as Baso-Haemolysis-WBC reagent (as described by Hseih *et al.*), amongst other methods.

In one embodiment, the method of the invention includes the step of determining the protein concentration in a plurality of samples, where a second sample is a diluted version of a first sample.

In some embodiments, it is not necessary to establish the optimal concentration of the sample for analysis. The source and expected range of concentrations of protein in a sample will allow a skilled person to prepare a suitable mixture for analysis.

In one embodiment, the concentration of the protein in the sample is at most 100 nM, at most 150 nM, at most 200 nM, at most 500 nM, at most 1 μM, at most 5 μM, at most 10 μM or at most 50 μM.
In one embodiment, where the sample is a blood sample, the sample is diluted at least 10-fold, at least 100-fold, at least 200-fold, at least 500-fold, at least 1,000-fold or at least 10,000-fold prior to analysis.
In one embodiment, the concentration of the protein, such as haemoglobin, in the sample is at most 6 mg/mL. In the exemplified methods of the invention, concentrations above this are associated with there is a saturation in the detectable signal, which may be associated with transport of the catalysis reaction product, which is likely to be diffusions-limited at higher

concentrations. Additionally or alternatively, the saturation may be linked to the depletion of a reagent, such as the second reagent.

The subject from which a biological sample is taken may be an animal, a mammal, a placental mammal, a rodent (e.g., a guinea pig, a hamster, a rat, a mouse), murine (e.g., a mouse), a lagomorph (e.g., a rabbit), avian (e.g., a bird), canine (e.g., a dog), feline (e.g., a cat), equine (e.g., a horse), porcine (e.g., a pig), ovine (e.g., a sheep), bovine (e.g., a cow), a primate, simian (e.g., a monkey or ape), a monkey (e.g., marmoset, baboon), an ape (e.g., gorilla, chimpanzee, orang-utan, gibbon), or a human.

Furthermore, the subject may be in any of its forms of development, for example, a foetus.

In one preferred embodiment, the subject is a human.

Surface

In the methods of the invention, the protein is specifically attached to a surface, i.e. the surface has a high specificity for the particular protein for detection. The surface is provided with a binding moiety that is suitable for binding the protein with high specificity. The binding moiety is immobilised to the surface. The binding of the protein to the binding moiety therefore also immobilises the protein to the surface.

In one embodiment the specificity can be achieved by an antibody immobilised on the surface, the antibody having specificity for the analyte as described above.

In one embodiment, the binding moiety is attached directly to the surface. However, in alternative embodiments the binding moiety is attached to the surface via a linker. This may be appropriate where the binding moiety is not suitably functionalised to attach to the surface, for example where the surface is an electrically conductive surface, such as a metal surface.

Methods for the immobilisation binding moieties to surfaces are well known in the art, and are discussed in relation to the known sandwich (ELISA) assays, where a binding moiety is provided on a surface, such as an electrically conductive surface. For example, standard techniques for antibody immobilisation and analysis are discussed in *Current Advances in Antibody Immobilization on Different Surfaces and Beads* (Hahn *et al. Current Proteomics* **2008**, *5*, 115-128).

For example, where the surface is a gold surface, a thiol-containing linker may provide a connection between the binding moiety and the gold surface. The modification or patterning of surfaces in this way for connection to binding moieties is well known in the art. Described herein, by way of example, is a gold surface having a monolayer of mercaptoundecanoic

acid linkers (see also Bioanalytical Applications of Self Assembled Monolayers, J.M. Cooper and B. Leidberg. *BioAnalytical Techniques,* 1999 Ed. A.E.G. Cass, Oxford University Press). The amino groups in the linker may be used to form a covalent bond to a binding moiety such as an antibody, thereby immobilising the binding moiety to the surface. The surface may be part of an analytical device that is suitable for measuring the reaction of the first reagent and the second reagent. The analytical device may be one that is based on the detection of reactivity at, or close to, a surface.

In one embodiment, the surface is an electrically conductive surface, such as the surface of a working electrode. Thus, the surface may form part of an electrochemical sensor. In one embodiment the protein is immobilised on a surface at, or in close proximity to, a working electrode. Here, *close proximity* is intended to refer to the location of the surface and therefore the immobilised protein, sufficiently close to the working electrode such that the catalysed reaction of the reagents is detectable at the working electrode. Therefore it is not essential for electrochemical analysis that a binding moiety be attached to a conducting surface. For example, Stöllner *et al.* describe the immobilisation of a binding moiety, haptoglobin, on a cellulose membrane. The binding moiety is used to immobilise Hb. Such a construct may be used in the present case. Note that Stöllner *et al.* describe a sandwich-based assay and the construct is subsequently exposed to a particular anti-Hb form. The cellulose surface is later placed on top of a working electrode for amperometirc measurements.

In one embodiment, the surface is that of the electrochemical sensor. The analyte can be specifically attached to the sensor by a specific binding moiety immobilised at the sensor surface, such as the surface of a working electrode. Such constructs are generally preferred as the binding moiety and the protein it immobilises are located immediately adjacent to the active electrode surface. Using the electrode as the surface also simplifies the design of the system.

For example, an anti-analyte antibody can be immobilised on the surface of the sensor using standard immobilisation techniques. Such techniques include carboxylic acid NHS/EDC coupling or any other coupling chemistry known in the art.

Following antibody immobilisation on the sensor, or any of the other surfaces, a blocking step may be used to mitigate against non-specific adsorption of the analyte onto the surface. In one embodiment, the blocking agent comprises milk protein and/or BSA, which are standard blocking agents known in the art.

For the avoidance of doubt, it is noted that the blocking agents, where they include proteins, are not specifically bound to the surface. Moreover, these proteins do not substantially catalyse the reaction of a reagent, such as a first reagent with a second reagent.

Microbead

The analyte can also be specifically attached to the surface via a microbead which has a specific binding moiety, for example an anti-analyte antibody, immobilised on its surface. The microbeads can then be dispersed in the sample enabling a high volume coverage thus increasing capture efficiency. Such dispersion can be achieved by using microfluidic mixers, magnetic handling of magnetic beads or using surface acoustic waves.

To achieve sensitive detection, the beads may then be gathered next to the electrochemical sensor. This can be achieved in the same way as for dispersion, i.e. using hydrodynamics (for example, weir type structures or valves), magnetic fields or acoustics (for example, particle concentration in rotating flow).

Bead-like structures which comprise rough surfaces or porous structures (for example, zeolites) can be used as these advantageously increase the available surface for specific binding moieties.

As noted previously, Sun *et al.* describe the use mesoporous spheres in an electrochemical Hb-containing sensor. Such spheres may be adapted to include a binding moiety specific for a protein such as Hb.

Porous Structure

The specific binding moiety may also be incorporated into a porous structure such as a membrane or matrix, which may be part of an electrode itself or the structure may be located at or in close proximity to an electrode.

The use of porous structures for immobilising proteins is well known, and is described, for example, by Stöllner *et al.* A binding moiety may be provided on a membrane, such as a cellulose membrane, for use in the immobilisation of a protein. That membrane, once suitably exposed to protein, may be placed on or close to an electrode surface. The porous nature of the structure permits a reaction product, such as the product of the reaction between the first reagent and the second reagent catalysed by the immobilised protein, to pass through the structure and to become exposed to the electrode surface.

Binding moiety

The method of the present invention makes use of a binding moiety that is immobilised on a surface. The surface may be an electrically conductive surface. That binding moiety is specific for a protein, and may be referred to as a specific binding moiety. The binding moiety is therefore capable of holding the protein close to the surface.

Typically, the binding moiety immobilises the protein by non-covalent interactions. The binding moiety is bound to a surface, either covalently or non-covalently, preferably covalently.

The binding moiety may be connected to the surface using techniques described in the art. In one embodiment the binding moiety is covalently bound to the surface.
In one embodiment the binding moiety may be directly bonded to the surface or the binding moiety may be indirectly connected to the surface *via* a linker. The linker is not a catalyst for the reaction of the first reagent and the second reagent. In one embodiment the linker is not or does not contain a polypeptide.

The analyte is specifically attached to a surface by a binding moiety, the binding moiety being specific for the analyte.

The binding moiety may be an antibody specific for the protein. For example, and as described in further detail below, where the protein is Hb, the binding moiety may be an anti-Hb antibody, such as an anti-human Hb.
In one embodiment, the binding moiety is an antibody for any one of the proteins mentioned in the Protein and Analyte section above.
Alternatively, the binding moiety may itself be a protein having specificity for the protein. For example, where the protein is Hb, the binding moiety may be haptoglobin, a strong Hb-binding protein. The use of haptoglobin to bind Hb proteins is described, for example, by Stöllner *et al.*

Where the binding moiety is a polypeptide, such as an antibody or a protein, the binding moiety may be connected to the surface via free amino, thiol hydroxy or carboxy groups present within the binding moiety, such as on the surface of the binding moiety.

Preferably, the binding moiety has a specificity, or K_D, for the protein of at least 10^{-15} M, at least 10^{-12} M, at least 10^{-10} M, at least 10^{-9} or at least 10^{-6} M.
Where the methods of the invention involve exposing the binding moiety to a mixture (such a biological sample), the binding moiety preferentially binds to protein in the mixture, and has essentially no affinity for any other components in the mixture. Thus, the specificity or K_D for each of the other components is at most 10^{-5} M, at most 10^{-3} M, or at most 10^{-1} M.
Where the binding moiety is an antibody, the preferred binding affinity for the protein is simply the inverse of the K_D values given above.

Antibodies for binding metalloproteins are well known in the art. For example, antibodies for haemoglobin are readily available from commercial sources, such as Santa Cruz Biotechnology, Inc. and Abcam. Also known in the art are antibodies that are specific for particular forms of haemoglobin, such as the anti-HbA1c antibody described by Stöllner *et al.* for use in a sandwich assay.

Electrochemical Sensor

The electrochemical sensor is suitable for use in the electrochemical detection of the reaction catalysed by the protein, such as the reaction of the first reagent with the second reagent.

Working Electrode

The electrochemical sensor includes a working electrode. The working electrode is not particularly limited and may be or include Pd, Au, Ag, Pt, Fe and mixtures thereof. The working electrode may be or comprise steel. Other materials that are suitable for use are carbon or graphite, and include these materials as matrix forms.

Where the binding moiety is to be connected to a working electrode, it is preferred that the electrode is of a material suitable for modification. Thus, Au and Ag electrodes are suitable for modification. Pd and Pt electrodes are less suitable, and electrodes having these metals may be used together with another surface for immobilising the binding moiety. As described herein, a surface, such as cellulose, may be used to immobilise the binding moiety, and the surface may be placed in close proximity to, including on, the electrode.

The electrode may be in the form of a plate, disc, mesh or wire, amongst others.

Exemplified in the present case is an Au electrode. The electrode is provided with a self-assembled monolayer of thiol-containing linker for connection of the electrode to the binding moiety.

The working electrode is stable. It does not degrade substantially over time or degrade substantially on prolonged exposure to the sample or the test solution.

The working electrode, optionally including the binding moiety, may be stored for at least 14 days, at least 28 days, or at least 6 months without significant loss of electrode activity. Preferably the electrode is stored for this time in a dry state to minimise degradation. It is preferred that the working electrode, and optionally the other components of the sensor, is stored in an atmosphere of an inert gas, such as nitrogen or argon.

The activity of the working electrode may be gauged by electrochemical analysis of a standard sample solution comprising a protein. A loss of electrode activity corresponds to a fall in the average recorded current recorded at the working electrode in comparison to a recorded current at a control working electrode. Preferably, the fall in average recorded current is about 50 % or less, about 30 % or less, about 10 % or less, or about 5 % or less.

The environment against which the stability of the working electrode is tested may include a biological sample as described herein. The working electrode may also be tested against a sample approximating the conditions to which the working electrode is intended to be exposed. The sample may be a simulated intestinal fluid, for example, or a simulated stool sample.

Electrochemical Circuit

In one embodiment, the working electrode is part of an electrochemical circuit which includes a counter electrode. The counter electrode is connectable to a power source. The counter electrode is in electrical communication with the working electrode.

There are no specific limitations on the type of counter electrode that may be used in the electrochemical sensor of the invention. Preferred electrode materials include steel and platinum. Steel is the most preferred electrode material for use in disposable and one shot sensors and apparatus owing to its relatively low cost.

Other means of supplying current to the working electrode are also envisaged.

Reference Electrode

A reference electrode may be included in the electrochemical sensor of the invention.

The reference electrode may be a standard silver / silver chloride (Ag/AgCl) electrode. The reference electrode may be a pseudo reference electrode, which is operable as a reference electrode in the presence of a suitable buffer comprising appropriate ions. In one embodiment, the pseudo reference electrode may be a silver-based electrode that is obtained, or is obtainable from, a silver electrode that is treated with about 1% aqueous $FeCl_3$ solution.

The reference electrode is in electrical communication with the working electrode and the counter electrode.

Electrochemistry

The electrochemical sensor may further comprise a voltage supply (or power supply). The voltage supply is preferably adapted to supply a constant bias between electrodes, such as the working electrode and the reference electrode, where present.

In one embodiment, the electrochemical sensor is for amperometric measurement.

Preferably the voltage supply is adapted to supply a constant bias in the range -100 to -1,000 mV, for example against a Ag/AgCl reference electrode. In one embodiment the bias is in the range -200 to -900 mV.
Thus, in one embodiment, the voltage is at least -50, at least -100, at least -200 or at least -400 mV, for example against a Ag/AgCl reference electrode.
In one embodiment, the voltage is at most -600, at most -800, at most -1,000 or at most 1,500 mV, for example against a Ag/AgCl reference electrode.

Preferably the voltage supply is adapted to supply a constant bias of about -500 mV as against an Ag/AgCl reference electrode.

The electrochemical sensor may further comprise a detector for monitoring current.
The electrochemical sensor may further comprise a controller for controlling the voltage supply and timing of that supply.

The amount of protein in a sample may be determined from the change in the measured current over time, for the reaction of the reagents as catalysed by immobilised protein. The present inventors have found that for a protein such as haemoglobin, the rate of current increase increases linearly with increasing protein concentration.

The methods of the invention may include the step of conducting a reference analysis in the absence of immobilised protein. Thus, an immobilised binding moiety that is not associated with protein may be exposed to reagent, and the reaction of the reagent, if any such occurs, may be detected. The reference analysis provides a background signal which may be deducted from the signal recorded in the presence of the specifically immobilised protein. Such techniques are familiar to those of skill in the art.

The background signal may be associated with one or more of the reaction of the reagent in the absence of catalyst, the catalytic activity of one or more of the surface, binding moiety, linker, and the blocking agent, analyte, label, where such are present. Typically these factors are minor, and the skilled person selects these features such that they have a minimal influence on the reaction of the reagent. Thus, the reaction of the reagent may be primarily associated with the presence of the immobilised protein.

Reagents

The method of the invention uses the intrinsic catalytic activity of a protein to generate an analytical response, which negates the need for secondary antibodies and enzyme labels.

In one embodiment, the protein catalyses the reaction of a reagent, and the product of that reaction is detected, for example by electrochemical methods.

In one embodiment, the reaction of the reagent is associated with the reaction of hydrogen peroxide, including the production and consumption of hydrogen peroxide.

The reagent, such as first and second reagents, is made available to the immobilised protein. The protein catalyses the reaction of the reagent and gives rise to a detectable signal.

The amount of reagent made available to the immobilised protein is not particularly limited. Typically, the reagent is provided as a sufficient level to allow for detection, for example by electrochemical analysis.
In one embodiment, the protein catalyses the reaction of a first reagent with a second reagent thereby to generate a reaction product, and various embodiments are described below. It follows that the method of the invention includes the step of detecting the reaction of the first reagent with the second reagent. This may include the detection of the reaction product as it is formed, and/or may include the detection of the first or second reagent as it is consumed. The protein couples oxidation of the first reagent to reduction of the second reagent, this coupling reaction providing a detectable signal at the electrochemical sensor. The first reagent is therefore a redox mediator.

In one embodiment, the protein catalyses the reaction of a reagent thereby to generate a reaction product. Here, the method of the invention of detecting the reaction of the reagent. This may include the detection of the reaction product as it is formed, and/or may include the detection of the reagent as it is consumed.

For example, Hsieh *et al.* (*Clinica Chimica Acta* **2011**, *412*, 2150) describe a haemoglobin sensor having a GLBM reagent for reaction with haemoglobin. The GLBM, provided at a known concentration, is permitted to react with haemoglobin. After reaction, the test solution is analysed electrochemically. This electrochemical step oxidises remaining (unreacted) reagent. The intensity of the recorded current is proportional to the remaining mediator concentration, and therefore inversely proportional to the haemoglobin concentration in the blood sample.

First Reagent

The first reagent is reactable with the second reagent in the presence of the analyte, such as a protein. Preferably the second agent is hydrogen peroxide, or a precursor thereof. Therefore, the first reagent is preferably a compound that reacts with hydrogen peroxide in the presence of the protein.

Preferably the first reagent is, or comprises, a compound selected from tetramethylbenzidine, alpha-guaiaconic acid, 2,2'azino-bis(3-ethylbenzothiazolidine-6-sulphonic acid), hydroquinone, phenylenediamine, o-dianisidine, o-tolidine

(dimethylbenzidine), 6-methoxyquinoline, and 3,3' diaminobenzidine, 3-amino-9-ethylcarbazole.

Preferably the first reagent is, or comprises, tetramethylbenzidine.
Preferably the first reagent is, or comprises, 3,3',5,5' tetramethylbenzidine.

In the most preferred embodiment, the first reagent is, or comprises, tetramethylbenzidine. This reagent is particularly suitable for use in the invention as it is not believed to be harmful to the user. TMB is also used within other assay systems, and will therefore be familiar to those of skill in the art. For example, TMB is described by Liem *et al.* (*Analytical Biochemistry*, 1979, **98**, 388-393) for use as a cytochemical indicator for the optical detection of Hb in cells.

TMB is also a common reagent for use in HRP-based assays, such as described by Josephy *et al.* (*J. Biol. Chem.*, 1982, **257**, 3669-3675).

In one embodiment, the methods of the invention measure the oxidation product of TMB. In one embodiment, the one electron oxidation product of TMB is measured.
It is possible to measure the two electron oxidation product of TMB (the diimine product). However, this requires the termination of the enzyme-mediated reaction with an acid, which involves an additional sample manipulation step (see, for example, Fanjul-Bolado *et al.* 13th European Conference on Analytical Chemistry, Salamanca, SPAIN, 2004).

Second Reagent

The second reagent is an oxidising agent or a precursor thereof. It is reactable with the first reagent in the presence of the protein.

Preferably the second reagent is or comprises hydrogen peroxide or a precursor thereof.

The hydrogen peroxide is reactable with the first reagent in the presence of the protein.

In one embodiment, the second reagent is hydrogen peroxide.
In one embodiment, the second reagent is, or comprises, a compound selected from urea peroxide, a perborate compound and a periodate compound. In one embodiment, the second reagent is, or comprises, urea peroxide. In another embodiment, the second reagent is, or comprises, a perborate compound, preferably sodium perborate.

Test Solution

The method of the present invention includes the step of permitting the immobilised protein to catalyse a reaction of a reagent, such as a first reagent with a second reagent. Thus, it is necessary to make the reagents available to the immobilised protein.

Once the protein is immobilised, material remaining in the sample may be separated from the immobilised protein. Typically, this is achieved in a simple washing step. Subsequently the reagents are then made available to the immobilised protein. The reagents may be provided in a test solution. In one embodiment, the test solution is also an electrolyte suitable for electrochemistry.

In one embodiment, the reagent, such as a first or second reagent, is present in the test solution at a concentration of at least 0.1 mM, at least 0.5 mM, or at least 0.1 mM. In one embodiment, the first reagent is present in the test solution at a concentration of at most 5 mM, at most 10 mM, at most 50 mM, or at most 100 mM. In one embodiment the concentration is in a range selected from the upper and lower values mentioned.
In one embodiment, the reagent is present in the test solution at a concentration of about 2 mM.

In one embodiment, the test solution contains a buffer to substantially maintain the pH of the test solution during the analysis steps.
In one embodiment, the test solution is acidic. In one embodiment, the pH of the test solution is at most 5 or at most 6. In one embodiment, the pH of the test solution is at least 2, is at least 3, or is at least 4. In one embodiment the pH of the test solution is in a range selected from the upper and lower pH values mentioned.

The test solution may include other components, such as one or more salts.
In one embodiment, the salt is present in the test solution at a concentration of at least 1 mM, at least 5 mM, at least 10 mM or at least 50 mM. In one embodiment, the salt is present in the test solution at a concentration of at most 0.2 M, at most 0.5 M or at most 1 M. In one embodiment the concentration is in a range selected from the upper and lower values mentioned.

Optionally other component may be included in the test solution, for example to improve electrochemical analysis.

Microfluidic Platform

In one embodiment, the method of the invention is performed within a microfluidic device. Such devices carry out laboratory assays in a miniaturised format. The microfluidic device may be generally small enough to fit on a laboratory bench, and in exemplary embodiments

is small enough to be carried by a user. For example, the device may be a "lab-on-a-chip" device.

A microfluidic device can comprise a support in which one or more channels are formed to provide a channel network capable of directing flow, and optionally controlling flow, of liquid through part, or all, of the network. Typically the channel network will have multiple channel portions. In exemplary embodiments the microfluidic device is configured to interact with a meter in order to provide the results of the method.

In exemplary embodiments, channels and channel portions are generally enclosed spaces defined by surrounding walls. The channel can have any cross-sectional shape (e.g. rectangular, trapezoidal, or circular). Channels can be in fluid communication with the atmosphere external to the microfluidic device by means of apertures (e.g., inlets, outlets or vents) formed in the channel network. Channels or channel portions can be open to the atmosphere for part or all of their length, e.g. by not having an enclosing lid. Channels or channel portions can comprise a capillary, i.e. a channel of small internal diameter capable of holding or transporting liquid by capillary action, wherein capillary action is (at least in part) the effect of surface tension that draws a liquid into or along the channel.

Use and Methods

In a general aspect the present inventions provides a method of detecting an immobilised protein by using the intrinsic activity of that protein to catalyse the reaction of the first reagent with a second reagent.

In one embodiment, the method includes the step of immobilising the protein. Thus, the method comprises the steps of:

(i) exposing a sample to a surface having a binding moiety and permitting the binding moiety to specifically immobilise a protein in the sample;

(ii) permitting the immobilised protein to catalyse a reaction of a first reagent with a second reagent;

(iii) detecting the reaction of the first reagent with the second reagent.

The reaction of the first reagent and the second reagent may be detected electrochemically.

In one embodiment, the method includes the step of immobilising the protein. Thus, the method comprises the steps of:

(i) exposing a sample to an immobilised binding moiety, thereby to immobilise protein from the sample;

(ii) providing first and second reagents and permitting the immobilised protein to catalyse the reaction of the first reagent with the second reagent;

(iii) detecting the reaction of the first reagent and the second reagent.

In one embodiment, the method of the invention comprises the preliminary step of treating the sample with surface acoustic waves, such as described herein.

After a protein is immobilised to a surface, the surface may be washed thereby to remove components of the sample that are not immobilised.

In preferred embodiments of the invention, electrochemical methods are used to detect the presence of an immobilised protein.

To detect a protein in a sample, the sample is exposed to an immobilised binding moiety which has specificity for the protein. The binding moiety is permitted to immobilise the protein. The protein-depleted sample is subsequently separated from the immobilised protein, for example by washing.

The binding moiety is attached to a surface, as described herein. The surface may be a surface of a working electrode. Where the surface is not that of a working electrode, the methods of the invention includes the step of providing the surface, after immobilisation of the protein, at or in close proximity to a working electrode.

The immobilised protein is then permitted to catalyse the reaction of a reagent, such as the reaction of a first regent with a second reagent. The reaction provides a detectable signal at the working electrode.

The working electrode is held at a bias as described herein. The change in current within the electrochemical cell is monitored over time, and is attributable to the electrochemical reaction of a reaction product or a reagent in the catalysis reaction. The change in current over time may be directly linked to the concentration of the reaction product or reagent, which may then be directly linked to the concentration of protein in the original sample.

In the present invention, the immobilised protein is haemoglobin, and the immobilised haemoglobin catalyses the reaction of TMB with hydrogen peroxide. The oxidised TMB product is reduced at the working electrode to give rise to a detectable signal.

Processing of Sample with Surface Acoustic Wave Actuation (SAW)

A sample, such as a biological sample and particularly a blood-containing sample, may be process with SAW methods prior to the exposure of that sample to the binding moiety.

One of the major advantages of this technique lies in its capability to work with turbid samples, with limited sample handling. In particular, the method can be integrated with

Surface acoustic wave actuation (SAW) to perform the sample processing. A SAW system may be used to lyse blood cells without the need for chemical reagents.

The present inventors have previously described the use of SAW techniques for sample processing - see WO 2011/023949, the contents of which are hereby incorporated by reference in their entirety.

Surface acoustic waves (SAWs, the most common being Rayleigh waves) are acoustic waves that can be caused to travel along the surface of a material. SAWs can be used to rapidly homogenise a sample, for example, a stool sample, to increase the confidence in the results of the diagnostic test, and can be readily integrated with other microfluidic functions.

For example, a stool sample (1-10 mg) is deposited on the surface of piezoelectric wafer, onto which an IDT has been patterned to drive a SAW at a specific frequency (>1 MHz). A drop (20 µL) of buffer (MOPS or HEPES for example) is then added to the stool sample. This can be driven by the SAW. The buffer may include a lysis agent or solubilising agent (such as saponin for example), but using SAW may allow the use of such agents to be avoided.

Once the sample is merged with the buffer, the SAW is actuated to break down the stool sample and release the analyte to be detected, haemoglobin. The mechanical energy propagated by the SAW is used to disrupt the solid matter, while the efficient mixing enabled by streaming flows leads to homogenisation.

Such homogenisation can be carried out either as a pre-step before detection, the resulting homogenised sample then exposed to the specific binding moiety on the surface.

Alternatively, the sample can be homogenised *in situ*, i.e. when the sample is already in contact with the specific binding moiety. The working electrode can then detect the Hb as it is released from the sample.

Prior to the processing of the stool, a sample may be taken from the core of the stool, and a sample may also be taken from an outer portion. Testing of both samples can show what proportion of blood in the total sample is derived from non-serious bleeding (blood from the outer part of the stool, which may be associated with e.g. haemorrhoids) and serious bleeding (blood from the core, which may be associated with e.g. bowel cancer). A sample volume of 20 µL is sufficient for the methods of the present invention.

Detection of a Binding Partner

In an alternative aspect of the invention there is provided the use of an immobilised protein for the detection of an analyte that is a binding partner for that protein. In the absence of the

binding partner, the protein is capable of catalysing the reaction of a reagent, such as a first and second reagent, to give rise to a detectable signal. When a binding partner is present, the protein binds to the binding partner, thereby rendering the protein incapable of catalysing the reaction of the reagent. A change in the reaction rate of the reagents may be associated with the presence of the binding partner, and the amount of change may be correlated to the quantity of binding partner that is present.

The inherent catalytic activity of the protein, and the absence of this catalytic activity, may be used as an indicator for the presence of components that bind to the protein. The protein may be immobilised, for example it may be specifically immobilised by a binding moiety, to a surface.

Accordingly, the present invention provides a method for detecting an analyte in a sample, which analyte is a binding partner for a protein, the method comprising the steps of:

(i) providing a protein that is immobilised, such as specifically immobilised, to a surface;

(ii) exposing the immobilised protein to the analyte in the sample, thereby to bind the analyte to the immobilised protein; and

(iii) exposing the immobilised protein to a reagent, such as first and second reagents;

(iv) detecting the reaction of the reagent, such as first and second reagents,

wherein the immobilised protein is a catalyst for the reaction of the reagent in the absence of the analyte. The binding of the analyte to the immobilised protein alters the catalytic activity of the protein for the reaction of the first reagent with the second reagent.

In one embodiment, the method may comprise the preliminary step of exposing a protein to a surface having a binding moiety and permitting the binding moiety to specifically immobilise the protein.

The reaction of the first reagent and the second reagent may be detected electrochemically.

The detected reaction rate may be compared against a standard reaction rate recorded for the reaction of the reagent in the presence of the immobilised protein and in the absence of the analyte. In one embodiment, the method compromises the step of detecting the reaction of the reagent, such as first and second reagents, in the presence of the immobilised protein, where the immobilised protein is not bound to analyte.

An immobilised protein may be immobilised as described above for the methods of detecting a protein. The proteins that are suitable for detection in those methods are suitable for use as protein in this aspect of the invention. Thus, the proteins, binding moieties, surfaces, reagents and electrochemical sensors may all be used in the method for detecting an analyte that is binding partner for the protein.

Thus, the protein may be a peroxidase. The protein may be a metalloprotein. The protein may be an oxygen-binding protein.
In one embodiment the protein is a haem protein i.e. a protein having a haem group.
Preferably, the protein is haemoglobin (Hb) or horseradish peroxidase.
More preferably, the protein is haemoglobin.

The binding moiety is specific for the protein. The binding moiety may be an antibody specific for the protein. For example, and as described in detail above, where the protein is Hb, the binding moiety may be an anti-Hb antibody, such as an anti-human Hb.
The binding moiety is an antibody for any one of the proteins mentioned above.
Alternatively, the binding moiety may itself be a protein having specificity for the protein. For example, where the protein is Hb, the binding moiety may be haptoglobin, a strong Hb-binding protein.

In one embodiment, the surface is an electrically conductive surface, such as the surface of a working electrode. Thus, the surface may be part of an electrochemical sensor. Such surfaces and sensors may be as described above.

The analyte can also be specifically attached to the surface via a microbead which has a specific binding moiety, for example an anti-analyte antibody, immobilised on its surface. The specific binding moiety may also be incorporated into a porous structure such as a membrane or matrix, which may be part of an electrode itself or the structure may be located at or in close proximity to an electrode. Such microbeads and porous structures may be as described above.

The reagents for use in the methods of detection are such as described previously. For example, the reagent may be a first reagent, such as TMB, and a second reagent, such as hydrogen peroxide.

Binding Partner

The analyte is a binding partner for the protein. Thus, the protein is capable of binding to analyte in a sample. The binding of the analyte to the protein reduces the catalytic activity of the protein. A reduction in catalytic activity may be used as an indicator for the presence of the analyte bound to the protein.

In the absence of the binding partner, the protein is a catalyst for the reaction of a reagent, such as first and second reagents.

The protein may be specific for the analyte. Thus, the protein has a specificity, or K_D, for the analyte of at least 10^{-15} M, at least 10^{-12} M, at least 10^{-10} M, at least 10^{-9} or at least 10^{-6} M.

Where the methods of the invention involve exposing the protein to a mixture (such a biological sample), the analyte preferentially binds to analyte in the mixture, and has essentially no affinity for any other components in the mixture. Thus, the specificity or K_D for each of the other components is at most 10^{-5} M, at most 10^{-3} M, or at most 10^{-1} M.

The analyte may be or comprise a polypeptide for binding to the immobilised protein. The analyte may itself be or comprise a protein. In one embodiment, the analyte is a receptor for the protein. The receptor may be located on a cell surface. The cell may be part of a pathogen, including a microorganism, such as a bacterium such as *S. aureus*.

It is known that certain parasites and bacteria are capable of acting upon proteins such as haemoglobin, for example as a source of iron or its ions (such as Fe^{2+} or Fe^{3+}), or amino acids. Thus, the present methods may be used to detect those pathogens that target proteins such as haemoglobin.

For example, Gaudin *et al.* (*Biochemistry* **2011**, *50*, 5443–5452) describe the hemoglobin receptor IsdB from *S. aureus* that binds Hb via the N-terminal NEAT domain (IsdB-N1). IsdB is a cell wall-anchored protein, and therefore an immobilised protein that is capable of binding to this receptor may act as a reporter for the presence of *S. aureus*.

In one embodiment, the analyte comprises a NEAT (near iron transporter) domain.

In one embodiment, the analyte is or comprises an antibody ("antibody analyte"). The antibody may be specific for the protein. In one embodiment, the antibody analyte is a bispecific antibody. Thus, the antibody analyte has specificity for the immobilised protein and specificity for another component, such as another protein, which may be a receptor, such as a receptor on a cell surface. The another protein may be present on a pathogen, such as a virus. The bispecific antibody analyte may be associated with that other component prior to its exposure to the immobilised protein. In this way, the bispecific antibody may be used to allow the indirect detection of the other component.

In on embodiment, the method comprises the step of modifying an analyte thereby to provide a group suitable for binding to the immobilised protein, and subsequently exposing the immobilised protein to the analyte, thereby to bind the analyte to the immobilised protein. The modifying step may take the form of attaching a polypeptide with affinity for the protein to the analyte.

Alternatively, the method of the invention may comprise the step of exposing the analyte, which analyte has specificity for the protein and another component, to the other component thereby to bind the analyte to the other component, and subsequently exposing the immobilised protein to the analyte, thereby to bind the analyte to the immobilised protein.

Thus, the methods of the invention may be used to detect components to which the protein cannot bind, or binds to with low affinity. Here, the analyte is bispecific for the protein and the other component. The analyte therefore acts as a bridge to allow the detection of the component using the methods of the invention. The bispecific analyte may be a bispecific antibody. Examples of bispecific antibodies are described by May *et al.* (*Biochemical Pharmacology* **2012**, *84*, 1105-112).

For the avoidance of doubt it is noted that the analyte, which may be or include a protein, receptor or antibody, is not a catalyst for the reaction of the reagent. Rather, it is the immobilised protein that catalyses this reaction, and the binding of the analyte to the immobilised protein reduces the catalytic activity of the immobilised protein.

Other Preferences

Each and every compatible combination of the embodiments described above is explicitly disclosed herein, as if each and every combination was individually and explicitly recited.

Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure.

"and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. For example "A and/or B" is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein.

Unless context dictates otherwise, the descriptions and definitions of the features set out above are not limited to any particular aspect or embodiment of the invention and apply equally to all aspects and embodiments which are described.

Certain aspects and embodiments of the invention will now be illustrated by way of example and with reference to the figures described above.

Examples

The following examples explain the use of the method of the invention for the detection of an analyte that catalyses the oxidation of a first reagent. As an exemplary system, Hb is used as the analyte of interest.

3,3',5,5'-Tetramethylbenzidine (TMB) is one of the most commonly used chromogen for HRP-based assays. The mechanism of TMB catalysed oxidation by HRP/H_2O_2 is well established and generates a blue-coloured intermediate complex (following a one electron oxidation) and subsequently, when fully oxidised, a yellow di-imine product (as a two

electron oxidation). The catalysed oxidation of TMB by Hb occurs in a similar manner, and, in fact, TMB has previously been used as a cytochemical indicator for the optical detection of Hb in cells.

In this study, TMB was the mediator of choice due to its low reactivity with H_2O_2. Initially, cyclic voltammetry was used to explore the coupling between TMB, H_2O_2 and Hb at a gold electrode and to optimise the optimal parameters for electrochemical measurements, to be associated with the Hb immunoassay.

Voltammograms were recorded between -200 and -900 mV vs. a Ag|AgCl reference electrode in solutions containing: (a) 2 mM TMB; (b) 2 mM TMB and 2 mM H_2O_2; and (c) 2 mM TMB, 2 mM H_2O_2 and 2.5 mg/mL Hb in phosphate-citrate buffer, pH 4.5, containing 0.1 M KCl, Fig. 1. The small reduction peak of TMB in buffer (Fig. 1(a)) and in the same solution containing H_2O_2, (Fig. 1 (b)), both at ca. -500 mV demonstrated a small reactivity between TMB and H_2O_2. Indeed, the choice of the mediator, in this case TMB, was made in order to minimise this background signal, as it ultimately determines sensitivity of the assay, in the presence of Hb. Addition of Hb to the assay produced a large increase in the magnitude of the reduction current, Fig 1(c).

In order to maximise the signal and minimise potential electrochemical interference, an immunoassay at -500 mV using chronoamperometric detection of Hb bound to an immobilised immunoglobulin was developed as shown in the general scheme in Fig 2. The one-electron oxidation product of TMB in the immunoassay was measured instead of the two elextron diimine product as measurement of the latter product would require the termination of the enzyme mediated reaction with 0.5M H_2SO_4 (involving an additional sample manipulation step).

The immunoassay involved the immobilisation of anti-human Hb (Abcam, UK) on the surface of an Au-disc electrode using a standard immobilisation based upon N-hydroxysuccinimide activation of a self-assembled monolayer of 11-mercaptoundecanoic acid (leading to reaction with free amine groups on the surface of the antibody). Following a blocking step with a mixture of milk protein and BSA, to mitigate against non-specific adsorption of Hb, the immunosensor electrode was then immersed in a solution of Hb. After washing away unbound Hb, the bound-Hb antibody construct was then immersed in a solution containing TMB (2 mM), H_2O_2 (2 mM) and KCl (0.1 M) in phosphate-citrate buffer (pH 4.5). Current time transients were measured for various Hb concentrations between 0.0 and 8.0 mg/mL (w/v), covering a clinically significant range. The immunoassay may also be performed using sheep anti-human Hb antibody, such as the affinity purified (A80-135A) product available from Bethyl Laboratories Inc.

Fig. 3 shows typical chronoamperometric plots for the reduction of TMB(Ox.), produced during the signal generation step of these immunoassays. An increase in magnitude of the

reduction current was observed for increasing Hb concentration between 0.0 and 8.0 mg/mL, with a limit of detection (mean, n=3, > 3 sd of mean background) of 0.25 mg/mL (3.7 μM). The rate of current increase (μA/s) increased linearly with increasing Hb concentration (although this saturated at concentrations of Hb greater than ca. 6.0 mg/mL, due either to the depletion of H_2O_2 in solution or as the rate of transport of TMB(Ox.) became diffusion-limited). The relationship with Hb concentration (between 0.0 and 6.0 mg/mL) was described by the general linear equation (μA/s) = $-1.5\times10^{-3} \pm 6\times10^{-5}$ [Hb] (mg/mL), r^2=0.98. The explanation for the observed increase in current with time lies in the relative rates of turnover of TMB(red)-H_2O_2 and the effective rate of electron transfer of TMB(ox) to the underlying electrode (including mass transfer effects). The effective electron transfer rate is slow due to the large amount of immobilised (antibody) and adsorbed (milk protein and BSA) on the electrode. As a consequence, TMB(ox) will accumulate in a concentration layer at the surface of the electrode, increasing the local flux and hence the current. This is consistent both with the increase in current observed with time and increased amounts of bound Hb, and indeed saturates as expected.

These examples demonstrate the development of a new, sensitive, label-free electrochemical immunoassay for Hb that utilises the intrinsic peroxidase activity of this clinically important analyte. The concentration range for Hb measurement of this immunoassay accommodates the clinically relevant levels of Hb in fluids from the lower GI tract (ca. > 0.5 mg/mL). Importantly, the results also show that Hb retains its intrinsic peroxidase activity once bound by anti-Hb on the electrode surface. The assay not only provides a better biosensing route for determining Hb in turbid or opaque samples, when compared with its optical counter-part, but as a label-free assay it requires fewer liquid handling steps (making it a promising candidate for successful miniaturisation onto a microfluidic platform).

References

All documents mentioned in this text are incorporated herein by reference.

Bioanalytical Applications of Self Assembled Monolayers, J.M. Cooper and B. Leidberg. *BioAnalytical Techniques,* 1999 Ed. A.E.G. Cass, Oxford University Press

Chen *et al. Biosensors & Bioelectronics*, 2007, **22**, 1268-1274

Fanjul-Bolado *et al.* 13th European Conference on Analytical Chemistry, Salamanca, SPAIN, 2004

Gaudin *et al. Biochemistry* **2011**, *50*, 5443–5452

Hsieh *et al. Clinica Chimica Acta* **2011**, *412*, 2150

Josephy *et al. J. Biol. Chem.*, 1982, **257**, 3669-3675

Lai *et al. Sensors and Actuators B-Chemical*, 2008, **129**, 497-503

Liem *et al. Analytical Biochemistry*, 1979, **98**, 388-393

May *et al. Biochemical Pharmacology* **2012**, *84*, 1105-112

Stöllner *et al. Analytica Chimica Acta* **2002**, *470*, 111
Sun *et al. Colloids and Surfaces B: Biointerfaces* **2013**, *101*, 177
WO 2011/023949
Zhang *et al. Analytical Letters*, 2007, **40**, 661-676

Claims

1. A method for determining the presence of a protein in a sample, the method comprising the steps of:

(i) exposing the sample to a surface having a binding moiety and permitting the binding moiety to specifically immobilise the protein in the sample;

(ii) permitting the immobilised protein to catalyse a reaction of a first reagent with a second reagent;

(iii) detecting the reaction of the first reagent with the second reagent.

2. The method of claim 1, wherein the reaction of the first reagent with the second reagent is detected electrochemically

3. The method of claim 1 or claim 2, wherein the protein is a metalloprotein.

4. The method of claim 1 or claim 2, wherein the protein is haemoglobin.

5. The method of any one of the preceding claims, wherein the sample is a biological sample.

6. The method of claim 5, wherein the biological sample is a faecal sample.

7. The method of claim 5 or claim 6, wherein the biological sample contains blood.

8. The method of any one of the preceding claims, wherein the sample is subjected to surface acoustic wave actuation prior to its exposure to the surface having a binding moiety.

9. The method of any one of the preceding claims wherein the binding moiety is an antibody, and the antibody having specificity for the protein.

10. The method of any one the preceding claims wherein the surface is an electrically conductive surface.

11. The method of claim 10, wherein the electrically conductive surface is a surface of a working electrode.

12. The method of claim 10, wherein the working electrode is or comprises Au.

13. The method of claim 9, wherein step (i) additionally includes providing the immobilised protein at or in close proximity to a working electrode.

14. The method of claim 13, wherein the surface is a microbead, membrane or matrix.

15. The method of any one of the preceding claims, wherein the second reagent is hydrogen peroxide, or a precursor thereof.

16. The method of any one of the preceding claims, wherein the first reagent is or comprises TMB (tetramethylbenzidine).

17. The method of any one of the preceding claims, wherein step (i) further comprises separating the immobilised protein from the protein-depleted sample.

18. The method of any one of the preceding claims, wherein the method is performed within a microfluidic platform.

19. A method for detecting an analyte in a sample, which analyte is a binding partner for a protein, the method comprising the steps of:

(i) providing a protein that is immobilised, such as specifically immobilised, to a surface;

(ii) exposing the immobilised protein to the analyte in the sample, thereby to bind the analyte to the immobilised protein; and

(iii) exposing the immobilised protein to a first reagent and a second reagent;

(iv) detecting the reaction of first reagent with the second reagent,

wherein the immobilised protein is a catalyst for the reaction of the first reagent with the second reagent in the absence of the analyte, and

the binding of the analyte to the immobilised protein alters the catalytic activity of the protein for the reaction of the first reagent with the second reagent.

20. The method of any one claim 19, wherein the reaction of the first reagent with the second reagent is detected electrochemically.

21. The method of claim 19, wherein the protein is a metalloprotein.

22. The method of claim 19, wherein the protein is haemoglobin.

23. The method of any one of claims 19-21, wherein the protein is specifically immobilised to an antibody on the surface.

24. The method of any one of claims 19-23 wherein the surface is an electrically conductive surface.

25. The method of claim 24, wherein the electrically conductive surface is a surface of a working electrode.

26. The method of claim 24, wherein the working electrode is or comprises Au.

27. The method of claim 23, wherein step (i) additionally includes providing the immobilised protein at or in close proximity to a working electrode.

28. The method of claim 27, wherein the surface is a microbead, membrane or matrix.

29. The method of any one claims 19-28, wherein the second reagent is hydrogen peroxide, or a precursor thereof.

30. The method of any one of claims 19-29, wherein the first reagent is or comprises TMB (tetramethylbenzidine).

Figure 1

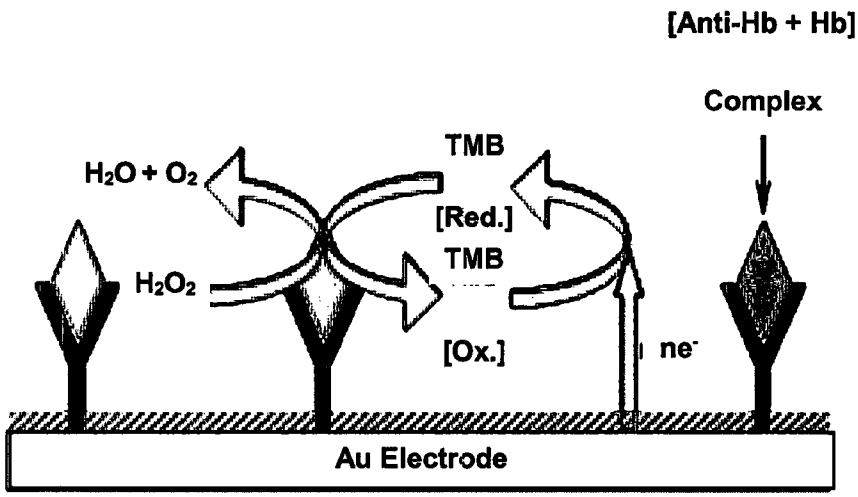


Figure 2

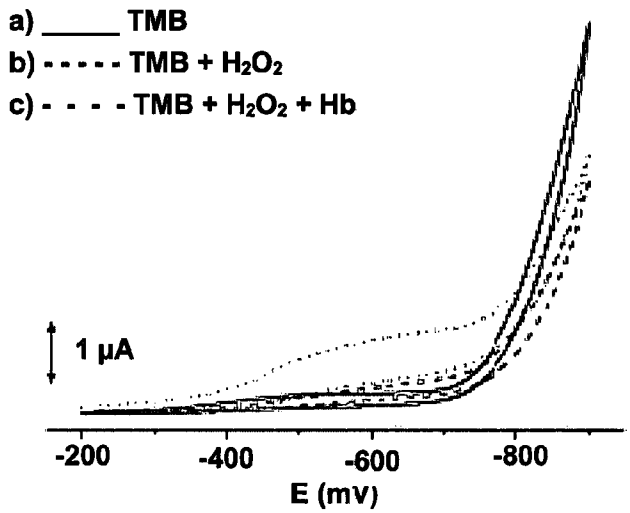
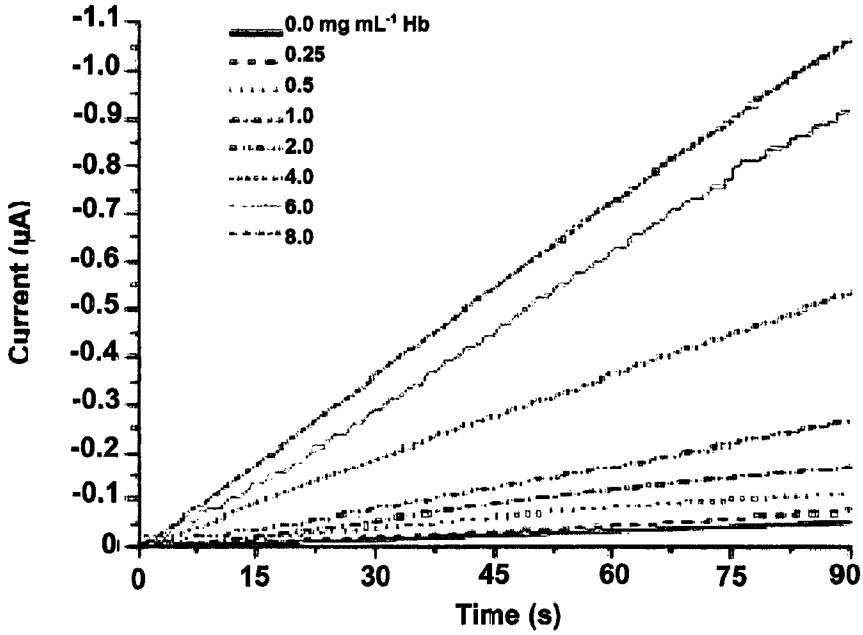


Figure 3


0.0 mg mL-1 Hb
0.25
0.5
1.0
2.0
4.0
6.0
8.0
Current (μA)
-1.1
-1.0
-0.9
-0.8
-0.7
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
0
0
15
30
45
60
75
90
Time (s)