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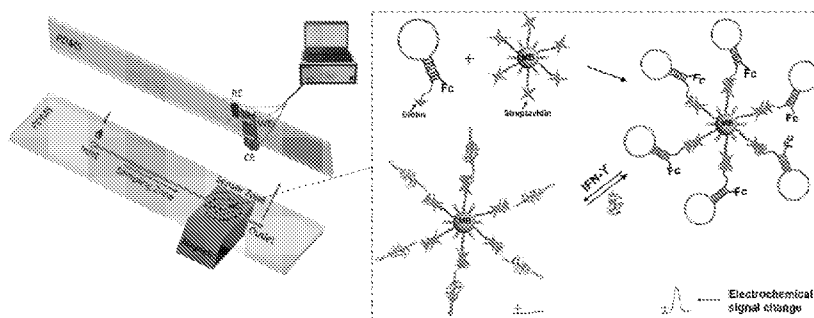
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(54) Title: A COMPOSITE AND SENSING DEVICE FOR POINT-OF-CARE BIOANALYSIS

Figure 1



(57) **Abstract:** The present invention relates to a composite for detecting the presence and concentration of an analyte in a sample. The composite comprises an aptamer comprising an analyte recognition region and at least one self-assembly region; a magnetic particle linked to a first position of the aptamer; and a redox-active tag linked to a second position of the aptamer. The aptamer interchanges between a disrupted conformation and a self-assembled conformation in response to interaction of an analyte with the analyte recognition region of the aptamer. The present invention also relates to a sensing device for use with the composite, manufacturing methods of the composite, uses of the composite in diagnosis or treatment and kits comprising the composite.



A composite and sensing device for point-of-care bioanalysis

Field of invention

The invention relates to a composite for detecting the presence and concentration of an analyte in a sample. The invention also relates to a sensing device for use with the composite. The invention further relates to diagnostic methods, manufacturing methods and kits.

Background

Real-time monitoring of key analytes (e.g. biomarkers of medical conditions) has medical importance. For example, continuous monitoring of troponin I, can predict a heart attack, and real-time tracking of cytokines can assist some cancer patients achieve the best treatment effect and reduce the severity of associated side effects. To our knowledge, continuous, real-time monitoring is currently possible only for a handful of molecular targets, such as glucose, lactose, and oxygen. The few existing platforms for continuous molecular specific measurement rely on very specific chemistries and are not generalizable for the monitoring of other analytes, such as cytokines.

Cytokines are crucial cellular signaling molecules which are secreted by cells, and are present in body fluids and tissues at the picomolar (pM) concentration range. The elevated concentrations of cytokines normally accompany inflammation or disease. Interferon gamma (IFN- γ) is a strong proinflammatory cytokine with direct antiviral activity, antiproliferative effects, as well as differentiation inducing and immunoregulatory properties. Subsequently elevated levels of IFN- γ can be an early indicator of multiple diseases (such as tuberculosis, HIV, Crohn's disease, and paratuberculosis) and cancers. The quantification of IFN- γ concentrations in serum and blood samples through various analytical techniques has been explored to further understand the immunological response during disease progression.

Current laboratory methods for detecting IFN- γ are based on a sandwich enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot assay (ELISPOT), or reverse transcriptase polymerase chain reaction (RT-PCR). Though these techniques can detect IFN- γ at biologically relevant concentrations (10 pg mL⁻¹ for tuberculosis, 15 pg mL⁻¹ for HIV, and 10 pg mL⁻¹ for Crohn's disease), they are relatively expensive and time consuming. Furthermore, these techniques, in most cases, require transportation of samples and trained technicians to conduct the diagnostic tests in the laboratory setting.

Thus, ELISA, ELISPOT, and PCR are generally not amenable to rapid, in-field biodetection. These techniques also usually only perform single point measurements, and are incapable of monitoring molecular analytes continuously.

One approach achieving real-time molecular sensing in complex environments involves biomolecular switches. A biomolecular switch is a biomolecule that undergoes binding-induced changes in conformation or oligomerization state to transduce chemical information into specific biochemical outputs.

A microfluidic device for the detection of local IFN- γ release from primary human leukocytes in real time with the sensitivity of less than 60 pM (1 ng mL^{-1}) has been described. This device involved the self-assembly of a DNA-hairpin comprising an IFN- γ aptamer on a gold electrode. However, the sensitivity of this device is compromised due to the limited capture of the aptamers on the electrode.

There is a continuing need to develop systems capable of real-time monitoring of analytes, including in complex systems such as biological samples. There also exists a continuing need to develop devices capable of providing point-of-care real-time monitoring of analyte concentrations.

Summary

In one aspect, there is provided a composite comprising:

- an aptamer comprising an analyte recognition region and at least one self-assembly region;
- a magnetic particle linked to a first position of the aptamer; and
- a redox-active tag linked to a second position of the aptamer;

wherein the aptamer interchanges between a disrupted conformation and a self-assembled conformation in response to interaction of an analyte with the analyte recognition region of the aptamer.

In another aspect, there is provided a sensing device comprising:

- an inlet for receiving a sample and the composite of the invention;
- a sensor zone comprising an electrochemical system and a magnetic retainer.

In a further aspect, there is provided a method for detecting an analyte, comprising

- providing a composite of the invention and a sample to the inlet of the sensing device of the invention,
- 5 - retaining the composite after it has interacted with the sample in the sensor zone, and
- detecting the response of the retained composite to an electrochemical signal delivered from the electrochemical system.

In yet another aspect, there is provided a method for detecting an analyte, comprising:

- 10 - exposing a composite of the invention to a sample potentially comprising the analyte;
- detecting a response of the exposed composite to an electrochemical signal.

In a further aspect, there is provided a method of manufacturing the composite of the invention, comprising:

- 15 conjugating an aptamer linked to a redox-active tag with a magnetic particle; or
- conjugating a redox-active tag with an aptamer conjugated with a magnetic particle.

In another aspect, there is provided a method for the diagnosis and/or prognosis of a medical condition, comprising:

- 20 - exposing a composite of the invention to a biological sample potentially comprising a biomarker for the medical condition;
- detecting a response of the exposed composite to an electrochemical signal.

In a further aspect, there is provided a method of treatment of a medical condition, comprising:

- 25 - exposing a composite of the invention to a biological sample obtained from a subject;
- detecting a response of the exposed composite to an electrochemical signal; and

- administering one or more drugs to the subject based on the detected response.

In another aspect, there is provided a kit comprising in separate parts:

- (a) the composite of the invention; and
- (b) a buffer solution.

5 In a further aspect, there is a kit comprising in separate parts:

- a. a first composite of the invention; and
- b. a second composite of the invention.

Before describing the present invention in detail, it is to be understood that this invention is not limited to particularly exemplified embodiments, such as the composites, sensing
10 devices, diagnostic methods and methods of manufacture, which may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety. However, publications mentioned herein
15 are cited for the purpose of describing and disclosing the protocols and reagents which are reported in the publications and which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

In this specification where reference has been made to patent specifications, other external
20 documents, or other sources of information, this is generally for the purpose of providing a context for discussing the features of the invention. Unless specifically stated otherwise, reference to such external documents is not to be construed as an admission that such documents, or such sources of information, in any jurisdiction, are prior art, or form part of the common general knowledge in the art.

25 The term "aptamer" as used herein describes a single-stranded nucleic acid molecule or peptide molecule that is capable of adopting a conformation that binds a ligand. Typically, the aptamer-ligand binding is highly selective and preferably specific.

It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

Thus, for example, a reference to “a particle” and/or “at least one particle” may include one or more particles, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any materials and methods similar or equivalent to those described herein can be used to practice or test the present invention, the preferred materials and methods are now described.

The term “(s)” following a noun contemplates the singular or plural form, or both.

The term “and/or” can mean “and” or “or”.

Unless the context requires otherwise, all percentages referred to herein are percentages by weight of the composite.

Unless the context requires otherwise, all amounts referred to herein are intended to be amounts by weight.

Various features of the invention are described with reference to a certain value, or range of values. These values are intended to relate to the results of the various appropriate measurement techniques, and therefore should be interpreted as including a margin of error inherent in any particular measurement technique. Some of the values referred to herein are denoted by the term “about” to at least in part account for this variability. The term “about”, when used to describe a value, may mean an amount within $\pm 25\%$, $\pm 10\%$, $\pm 5\%$, $\pm 1\%$ or $\pm 0.1\%$ of that value.

The term “comprising” is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention. When interpreting statements in this specification that include that term, the features, prefaced by that term in each statement, all need to be present but other features can also be present. Related terms such as “comprise” and “comprised” are to be interpreted in the same manner.

Brief Description of Drawings

The present invention will be further described, by way of example only, with reference to the accompanying drawings, in which:

Figure 1 shows a schematic of the customized microfluidic chip integrated with the

functionalized particles of Example 1 for continuous monitoring of IFN- γ .

Figures 2A-D show a) cyclic voltammograms of the functionalized particles of Example 1 in buffer C before and after reaction with 5 μ M aptamer solution; b) UV-visible (UV-Vis) spectra of magnetic nanobeads (MBs) and the functionalized particles of Example 1; c) Dynamic light scattering (DLS) distribution of MBs; and d) DLS distribution of the functionalized particles of Example 1.

Figure 3A shows cyclic voltammograms of on-chip sensing device of Example 1 before and after the presence of IFN- γ .

Figures 3B-D show bar charts showing the effect of varying b) pH, c) salt concentration, and d) temperature on the signal strength obtained by electrochemistry of the on-chip sensing device without the presence of IFN- γ .

Figures 4A-E show a) square wave voltammograms of the functionalized particles of Example 1 in the microfluidic device after exposure to different concentration of IFN- γ (0, 5, 10, 20, 40, 60, 80, 100, 200, 400, 500, 800, 1000 pg mL^{-1}); b) a plot demonstrating the relationship of the absolute peak current with the log concentration of IFN- γ ; c) a chart of current as a function of time for the functionalized particles of Example 1 in the microfluidic device in buffer C solution at a constant potential of 0.2 V after adding IFN- γ with different concentrations; d) a bar chart of the percentage interference on the functionalized particles of Example 1 in the microfluidic device sensing interface responding to 500 pg mL^{-1} IFN- γ with the presence of the following nonspecific proteins BSA, PSA, CA-125, IL-6, IgG, and TNF- α ; and e) a bar chart of the percentage interference on the functionalized particles of Example 1 in the microfluidic device sensing interface responding to the presence of the following proteins BSA, PSA, CA-125, IL-6, IgG, and TNF- α in 100 mM Tris(hydroxymethyl)aminomethane (Tris) buffer.

Figures 5A-D show a) a bar chart of signal intensity over time (I_0 is the signal obtained from the fresh sensing interface at day 0); b) chronoamperometry recording of the functionalized particles of Example 1 sensing interface on magnetic GC electrodes at a constant potential of 0.2 V after spiking in 100 pg mL^{-1} IFN- γ and buffer B at the indicated time points; c) chronoamperometry recording of the functionalized particles of Example 1 in the microfluidic device in cell culture medium of peripheral blood mononuclear cells (PBMCs) at a constant potential of 0.2 V after spiking in IFN- γ with different concentration and after spiking in PBS as control; and d) chart of determined IFN- γ concentration in supernatant of PBMCs after lipopolysaccharide (LPS) stimulation for different period of time

using the functionalized particles of Example 1 in the microfluidic device and by ELISA.

Figure 6 shows a photograph of a microfluidic device annotated with: (a) pattern was drawn as dash line; (b) the gold surfaces serve as the working electrode (WE) and counter electrode (CE), respectively with the dimension of 2.5 mm x 2.5 mm; (c) the silver wire works as the reference electrode (RE), with dimension of 1.2 mm x 10 mm; (d) the front view schematic of the fabricated microfluidic system.

Figure 7 shows a bar chart of current versus MB volume showing the relationship between the square wave voltammetry (SWV) current and the volume of 100 ng mL⁻¹ magnetic beads solution reacted with 30 µL 5 µM solution of the functionalized particles of Example 1.

Figure 8 shows square wave voltammogram of the functionalised particle of Example 1 before exposure to IFN-γ, after exposure to IFN-γ and after stirring IFN-γ bound surface in buffer B for 1 h.

Figure 9 shows a schematic of the composite of Example 2.

Figure 10 shows square wave voltammograms of the composite of Example 2 before addition of IFN-γ, vascular endothelial growth factor (VEGF) and tumour necrosis factor-α (TNF-α) obtained with the sensing device of Example 1.

Description of embodiment(s)

The inventors have developed a customized sensing device for use with functionalized magnetic particles. This device is capable of continuously tracking the concentration of a target analyte (e.g. IFN-γ) in complex media, such as cell culture medium or blood serum. This device is simple and easy to operate and may provide a universal point-of-care sensing platform for continuous screening of a spectrum of analytes *ex vivo*.

Central to operation of the device is a composite that comprises a structure-switching signaling aptamer linking a redox-active tag with a magnetic particle. The aptamer changes conformation on binding its ligand – selected to be the analyte of interest – and the conformational change affects the electrochemical response of the redox-active tag/magnetic nanoparticle system.

Composite

In one aspect, the invention provides a composite comprising an aptamer, a magnetic particle linked to a first position of the aptamer and a redox-active tag linked to a second position of the aptamer. The aptamer comprises an analyte recognition region and at least one self-assembly region. The aptamer interchanges between a disrupted conformation and a self-assembled conformation in response to interaction of an analyte with the analyte recognition region of the aptamer.

The composite may serve as a sensor able to detect the presence and concentration of an analyte that is recognized by the analyte recognition region of the aptamer.

The composite may also be referred to as a functionalized particle as the magnetic particle is functionalized with the aptamer linked to the redox-active tag.

Aptamer

The aptamer serves as a signaling switch and allows the composite to detect the concentration of analyte in a solution.

The aptamer comprises an analyte recognition region. The analyte recognition region is capable of adopting a conformation that selectively recognizes the analyte, typically by forming non-covalent bonds between the analyte and the analyte recognition region, similar to a ligand-protein interaction. The recognition is typically governed by spatial recognition between the surface of the analyte recognition region and the analyte.

The structure/sequence of analyte recognition regions selective for various analytes of interest have been described. For example, analyte recognition regions that recognize various cytokines, including interferons (e.g. IFN- γ), interleukins (e.g. IL-1 β and IL-6), tumour necrosis factors (e.g. TNF- α), and a vascular endothelial growth factor (VEGF) (e.g. VEGF-1), cancer biomarkers, including human epidermal growth factor receptor-2 (HER2) and epithelial cell adhesion molecule (EpCAM), and antibodies (e.g. immunoglobulin G (IgG)) have been previously described. Any of these previously described analyte recognition regions may be incorporated into the aptamer. The analyte recognition region may alternatively be designed by any technique known in the art. For example, one suitable technique is Systematic Evolution of Ligand by EXponential enrichment (SELEX), where a target analyte is systematically screened against a library of potential analyte recognition

regions to identify an analyte recognition region that interacts sufficiently selectively with the target analyte.

The signaling switch function of the aptamer is achieved through the interchange between a self-assembled conformation and a disrupted conformation in response to interaction of an analyte with the analyte recognition region.

In some embodiments, the self-assembled conformation is a closed conformation and the disrupted conformation is an open conformation. Typically, the self-assembled conformation describes the conformation adopted by the aptamer when directed by the at least one self-assembly region, and the disrupted conformation relates to a conformation where the self-assembled conformation is disrupted, typically by interaction of an analyte with the analyte recognition region. Typically, the first position and second position on the aptamer are spatially closer together in the self-assembled conformation and further apart in the disrupted conformation.

The transition from self-assembled confirmation to disrupted confirmation affects the interaction between the redox-active tag and the magnetic particle sufficiently to result in a detectable change in the composite's response to an electrochemical signal. In some embodiments, the self-assembled conformation may be a signaling conformation where the response of the redox-active tag to an electrochemical signal is strongest, and the disrupted conformation results in a reduction of the response, preferably complete loss of response to the electrochemical signal.

Typically, the aptamer is a single-stranded nucleic acid molecule. The aptamer may therefore be a ribonucleic acid molecule (RNA), a deoxyribonucleic acid molecule (DNA), or a combination thereof (XNA).

Alternatively, in some embodiments, the aptamer may be a peptide. The peptide aptamer may comprise any combination of amino acids, including a proteinogenic amino acid, a non-proteinogenic amino acid (e.g. a non-natural amino acid), and combinations thereof. Non-proteinogenic amino acids include D-amino acids and beta-amino acids.

In some embodiments, the aptamer comprises a minimum number of bases or amino acid residues of at least about 5, 10, 15, 20, 25 or 30 units. The maximum length of the aptamer is not particularly limited provided the aptamer is stable within the composite. In some embodiments, the maximum number of bases or amino acid residues may be not more than about 100, 90, 80, 70, 60, 50, 45, 40, 35, 30, 25, or 20 units. The number of bases or

amino acid residues within the aptamer may be from any of these minimum amounts to any of these maximum amounts provided that the minimum number is lower than the maximum number selected. For example, the aptamer may comprise a number of bases or amino acid residues from about 5 units to about 100 units, about 15 units to about 50 units, about 5 units to the about 20 units or about 20 units to about 45 units.

The aptamer comprises an analyte recognition region. The analyte recognition region contains a number and sequence of bases or amino acid residues sufficient to selectively interact with the analyte typically through non-covalent binding. In some embodiments, the sequence length of the analyte recognition region will be the same as that described for the aptamer above. In these embodiments, the aptamer may consist of the analyte recognition region. However, typically, the aptamer comprises bases in addition to the analyte recognition region. In these embodiments, the analyte recognition region may comprise a minimum of at least about 5, 10, 15 or 20 bases or amino acid residues. The analyte recognition region may comprise a maximum of not more than about 100, 75, 50, 40, 35 or 30 bases or amino acid residues. The analyte recognition region may comprise a number of bases or amino acid residues between any of these minimum amounts to any of these maximum amounts, such as from about 5 units to about 100 units, from about 10 units to about 50 units or from about 20 units to about 30 units.

The aptamer comprises at least one self-assembly region. The self-assembly region is a sequence of bases or amino acid residues that interacts with another part of the aptamer to guide its self-assembly into a conformation capable of interchanging between the self-assembled conformation and disrupted conformation. The aptamer may comprise 2, 3, 4, 5, 6 or more self-assembly regions depending on what self-assembled conformation is desired. Each self-assembly region may comprise a minimum of at least about 2, 3, 4, 5, 6, 7, 8, 9, or 10 bases or amino acid residues. Each self-assembly region may comprise a maximum of not more than 20, 19, 18, 17, 16, 15, 14, 13, 12, 11 or 10 bases or amino acid residues. Each self-assembly region may comprise a number of bases or amino acid residues between any of these minimum amounts to any of these maximum amounts, such as from about 2 units to about 20 units, about 2 units to about 15 units, or about 4 units to about 10 units. In some embodiments, the self-assembly region comprises nucleic acid bases that form complimentary base pairing interactions within the aptamer. In some embodiments, the self-assembly region comprises amino acid residues capable of forming a hydrogen bond, a disulfide bond, a salt bridge, or a combination thereof within the aptamer.

In some embodiments, the sequence of a self-assembly region and the analyte recognition region overlap. Overlapping these sequences may be advantageous in certain embodiments as binding of the analyte with the overlapping sequence of the analyte recognition region and a self-assembly region may assist in the transition from the self-assembled conformation to the disrupted conformation of the aptamer by disrupting the intramolecular interaction of the self-assembly region.

The self-assembled conformation of an aptamer comprising nucleic acid bases may be selected from a hairpin loop, a G-quadruplex or a combination thereof. Suitable self-assembled conformations of various nucleic acid aptamers are described in Cao, C. *et al.*, *Trends in Analytical Chemistry* 2018;102;379-396, which is entirely incorporated by reference.

The self-assembled conformation of an aptamer comprising amino acid residues may be a scaffold comprising a loop region (sometimes referred to as "loop on a frame") and a scaffold containing a rigid combinatorial motif. Suitable self-assembled conformations of various peptide aptamers are described in Reverdatto, S., Burz, D. S., and Shekhtman, A., *Curr Top Med Chem* 2015;15(12);1082-1101, which is entirely incorporated herein by reference.

In some embodiments, the self-assembled conformation of the aptamer comprises a loop, such as a hairpin loop or a loop on a frame. In such embodiments, the aptamer typically comprises two self-assembly regions that interact with each other in the self-assembled conformation and wherein the intervening bases or amino acid residues define the loop. In some embodiments, the loop comprises at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 or more bases or amino acid residues. In some embodiments, the loop comprises from about 3 bases or amino acid residues to about 50 bases or amino acid residues, about 5 bases or amino acid residues to about 40 bases or amino acid residues, or about 15 bases or amino acid residues to about 35 bases or amino acid residues. In some embodiments, the analyte recognition sequence will be entirely contained within the loop, while in others the analyte recognition sequence will overlap with at least one of the self-assembly regions. Typically, the loop will comprise at least a part of the analyte recognition sequence. In some embodiments, the two self-assembly regions are positioned apart within the aptamer, such as at opposite ends of the aptamer. In some embodiments, the two self-assembly regions comprise complementary nucleic acid bases. In these embodiments, in the self-assembled conformation, the complementary bases of the two self-assembly regions may form

complimentary base-pair interactions, and the sequence between the two self-assembly regions forms a single-stranded loop.

Typically, the intramolecular binding of at least one self-assembly region is disrupted in the disrupted conformation. In some embodiments, the self-assembly regions do not form any intramolecular interaction in the disrupted conformation. However, in some embodiments, some intramolecular interactions are maintained in the disrupted conformation, provided that the distance between the redox-active tag and the magnetic particle is sufficiently different when in the disrupted conformation than when in the self-assembled conformation. Typically, the distance between the redox-active tag and the magnetic particle is greater in the disrupted conformation than in the self-assembled conformation. In some embodiments, the aptamer adopts a linear conformation in the disrupted conformation.

In addition to the analyte recognition region and at least one self-assembly region, the aptamer may comprise a spacer region. The spacer region may comprise a minimum of at least about 5, 6, 7, 8, 9 or 10 bases or amino acid residues. The spacer region may comprise a maximum of not more than about 40, 35, 30, 25, 20 or 10 bases or amino acid residues. The spacer region may comprise between any of these minimum number of bases or amino acid residues to any of these maximum number of bases or amino acid residues, such as from about 5 units to about 40 units or about 5 units to about 10 units. Inclusion of a spacer region may be advantageous to provide sufficient degrees of freedom to the analyte recognition region to adopt its required conformation when the aptamer is in the self-assembled conformation.

Magnetic particle

The composite comprises a magnetic particle.

Any magnetic particle capable of being linked to the aptamer and interacting with the redox-active tag in response to an electrochemical signal may be employed. In addition, the magnetic particles enable the use of magnetic fields, such as a high gradient magnetic field, to retain and concentrate the composites within a sensing zone of a device.

In some embodiments, the magnetic particles comprise a magnetic material selected from a magnetic oxide (such as a magnetic iron oxide, for example maghemite or magnetite), a magnetic metal or a combination thereof.

The particles may have any suitable morphology and/or shape. The magnetic material selected for the magnetic particle will influence the morphology and shape. In some

embodiments, the magnetic particle is substantially spherical. The magnetic particles may be core-shell particles, wherein at least one of the core or a shell layer comprises a magnetic material.

In some embodiments, the minimum average particle size of a population of the magnetic particles may be about 1nm, 5nm, 10nm, 20nm, 50nm, 100nm, 150nm, 200nm, 220nm, 250nm, 300nm, 350nm, 400nm, 410nm, 420nm, 430nm, 440nm or 450nm. The maximum average particle size of a population of the magnetic particles may be not more than about 100 μ m, 50 μ m, 1 μ m, 950nm, 900nm, 850nm, 800nm, 750nm, 700nm, 650nm, 600nm, 550nm, 500nm, 490nm, 460nm, or 450nm. The average size of a population of the magnetic particles may be between any of these minimum and maximum sizes without limitation save for the minimum size being smaller than the maximum size. For example, in some embodiments, the average particle size of a population of magnetic nanoparticles may be from about 1nm to about 100 μ m, about 100nm to about 600nm or about 350nm to about 550nm.

In some embodiments, the minimum average particle size of a population of the composites (i.e. functionalized magnetic particles) may be about 1nm, 5nm, 10nm, 20nm, 50nm, 100nm, 150nm, 200nm, 220nm, 250nm, 300nm, 350nm, 400nm, 410nm, 420nm, 430nm, 440nm, 450nm or 460nm. The maximum average particle size of a population of the functionalized magnetic particles may be not more than about 100 μ m, 50 μ m, 1 μ m, 950nm, 900nm, 850nm, 800nm, 750nm, 700nm, 650nm, 600nm, 550nm, 500nm, 490nm, 480nm, 470nm or 460nm. The average size of a population of the functionalized magnetic particles may be between any of these minimum and maximum sizes without limitation save for the minimum size being smaller than the maximum size. Further, the average size of the functionalized magnetic particles is larger than for the unfunctionalized magnetic particles. For example, in some embodiments, the average particle size of a population of magnetic nanoparticles may be from about 1nm to about 100 μ m, about 220nm to about 1 μ m or about 400nm to about 600nm.

In some embodiments, the magnetic particles are magnetic nanobeads (MBs). MBs have an average size of less than 1000nm and have a substantially spherical shape. MBs have been steadily gaining interest in analytical science for recognition of numerous analyte molecules with high sensitivity. Besides providing a large surface area to capture recognition molecules, the merits of MBs include their flexibility due to functionalization by means of surface modification and specific binding, simplicity of washing and separation

steps to exclude unspecific adsorptions, and the possibility of manipulating them inside microfluidic channels by utilizing high gradient magnetic fields.

The magnetic particle typically comprises surface functionalization. Any functionalization suitable to link the particle to the first position of the aptamer and allow the particle to
5 interact with the redox-active tag when the aptamer is in the self-assembled conformation, and the magnetic field in the device may be employed.

In some embodiments, the magnetic nanoparticle is linked directly to the first position of the aptamer. In other embodiments, the magnetic nanoparticle is linked to the first position of the aptamer through a linker group. The linker group may comprise a polymer chain
10 optionally interrupted by a conjugation site. Suitable polymers for the linker group include polyesters, peptides, polysaccharides, and combinations thereof. The conjugation site may comprise a covalently conjugated group selected from an amide, an ester, a triazole, or an olefin. The conjugation may comprise the conjugation product of conjugation partners selected from biotin and a biotin recognition protein, and an antigen and antibody. The
15 biotin recognition protein may be selected from avidin, streptavidin and deglycosylated avidin.

Redox-active tag

The composite comprises a redox-active tag. Any redox-active tag capable of interacting with the magnetic particle when the aptamer is in the closed position may be employed. In
20 some embodiments, the redox active tag is a redox active moiety covalently bound to the second position of the aptamer optionally through a second linker group. The second linker group may be selected from any of the linker groups described above.

Suitable redox-active tags include ferrocene, methylene blue, an anthraquinone, Nile blue and redox-active derivatives thereof. Suitable redox-active derivatives of the redox-active
25 tags may comprise 1-3 covalent substituents in addition to the bond linking the tag to the aptamer or the second linker group (if present), provided that the substituents do not cause loss of redox-activity.

The composite may comprise more than one kind of redox-active tag. Typically, each redox-active tag will provide a different response to the electrochemical signal, e.g.
30 exhibiting a peak at different voltage. This difference in response allows selective detection of each tag. Therefore, linking different redox-active tags to the same aptamer may enable more accurate detection of the concentration of the analyte as the various electrochemical

signals for each redox-active tag can be analyzed separately. However, typically, each unique aptamer within the composite will be linked to a single kind of redox-active tag. Such combinations of aptamer and redox-active tag may be referred to herein as an “aptamer-redox-active tag pair”. For example, a first aptamer (e.g. comprising a recognition region selective for INF- γ) may be linked with a first redox-active tag (e.g. ferrocene), and a second aptamer (e.g. comprising a recognition region selective for TNF- α) with a second redox-active tag (e.g. methylene blue). In some embodiments, the composite comprises more than one redox-active tag pair, which may enable the composite to detect the presence and concentration of different analytes within the same solution (see Example 2 and Figures 9 and 10). Alternatively, multi-analyte detection may be achieved by providing a first population of composites comprising a first aptamer-redox-active tag pair together with a second population of composites comprising a second aptamer-redox-active tag pair.

The magnetic particle is linked to the first position of the aptamer and the redox-active tag is linked to the second position of the aptamer. In the self-assembled conformation, the first and second positions of the aptamer allow the redox-active tag and magnetic particle to interact in response to an electrochemical signal. In the disrupted conformation (e.g. when an analyte is bound to the analyte recognition region of the aptamer) the first and second positions are typically spatially further apart, and accordingly the interaction between the redox-active tag and the magnetic particle changes in response to the electrochemical signal. Preferably, the change in response to the electrochemical signal is complete loss of signal.

In some embodiments, the first and second positions are at opposite ends of the aptamer sequence. In some embodiments, the first position is at the 3' end of the aptamer. In some embodiments, the second position is at the 5' end of the aptamer. In some embodiments, the first position is at the 5' end of the aptamer. In some embodiments, the second position is at the 3' end of the aptamer. In other embodiments, the first position may be the N-terminal end of the aptamer and the second position may be at the C-terminal end. Conversely, in some embodiments, the first position may be at the C-terminal end of the aptamer and the second position may be at the N-terminal end.

Process of manufacturing composites

In another aspect, there is provided a method of manufacturing the composite of the invention.

In some embodiments, the method comprises conjugating an aptamer linked to a redox-active tag with a magnetic particle. The conjugation may be any suitable conjugation to form a stable link between the aptamer and the magnetic particle.

The conjugation may involve forming a covalent link between the magnetic particle and aptamer. The covalent link may be selected from an amide, an ester, a triazole, or an olefin. In some embodiments, the covalent link is an amide formed by reaction of a carboxylic acid functionalized magnetic particle and an aptamer-redox-active tag comprising an amine functional group, typically a primary amine functional group ($-NH_2$). For example, a free amine group may be substituted for the biotin moiety of the aptamer-redox-active tag pairs described in Examples 1 and/or 2 and conjugated with a carboxylic acid functionalized magnetic particle.

Alternatively, the conjugation may involve formation of one or more non-covalent bonds, such as ionic bonds, hydrogen bonds or London bonds. Thus, in some embodiments, the conjugation may comprise the formation of a conjugation product by interaction of conjugation partners selected from biotin and a biotin recognition protein, and an antigen and antibody. The biotin recognition protein may be selected from avidin, streptavidin and deglycosylated avidin.

The conjugation of the magnetic particle and the aptamer linked to the redox-active tag may be between:

- a. the surface of the magnetic particle and the aptamer directly,
- b. the surface of the magnetic particle and a linker group bonded to the aptamer at the first position;
- c. a linker group bonded to the surface of the magnetic particle directly with the aptamer; or
- d. part of the linker group bonded to the surface of the magnetic particle and part of the linker group bonded to the first position of the aptamer.

In some embodiments, the method comprises conjugating a redox-active tag with an aptamer conjugated with a magnetic particle. Any means known in the art for conjugating the redox-active tag with the second position of the aptamer may be employed, including those described above. This conjugation may also optionally be through a linker group.

Accordingly, the conjugation of the redox-active tag to the aptamer linked to the magnetic particle may be between:

- a. the redox-active tag and the aptamer directly,
- b. the redox-active tag and a linker group bonded to the aptamer at the second position;
- c. a linker group bonded to redox-active tag and the aptamer; or
- d. part of the linker group bonded to the redox-active tag and part of the linker group bonded to the second position of the aptamer.

Sensing device

10 In a further aspect, there is provided a sensing device. The sensing device comprises an inlet and a sensor zone. The inlet is in fluid communication with the sensor zone. In some embodiments, the sensing device is a microfluidic device.

The inlet is adapted to receive a sample and the composite of the invention. The sample may be a biological sample, such as a blood sample, plasma sample, saliva sample, swap sample, culture sample or a combination thereof. The sample and the composite may be introduced into the inlet by any suitable means, for example by syringe, dropper or cannula. The sample and the composite may be introduced separately or may be mixed into a solution prior to introduction into the device. The sample and the composite may be suspended in a carrier prior to introduction to the device. Suitable carriers include water, dimethylformamide, acetonitrile, methanol, ethanol, dimethylsulphoxide, a buffer solution or a combination thereof. Suitable buffers include phosphate buffered saline (PBS), Dulbecco's PBS (DPBS) and Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH=7.4). The Tris-HCl buffer may comprise about 100 mM Tris-HCl, about 100 mM NaCl, about 1 mM MgCl₂, and about 5 mM KCl in deionized (Milli-Q) water.

25 The sensing device also comprises a sensor zone comprising an electrochemical system and a magnetic retainer. The electrochemical circuit may be a two-electrode or a three-electrode system. Typically, the electrochemical circuit is a three-electrode system comprising a working electrode (WE), a counter electrode (CE) and a reference electrode (RE). A suitable two-electrode system may comprise an anode and a cathode. Any suitable electrodes may be used. In some embodiments, the electrodes a glassy-carbon electrodes

30

or gold electrodes. Gold electrodes are preferred in embodiments where the device is a microfluidic device as they may conveniently be printed onto the etched device.

The magnetic retainer is adapted to retain the composite in the sensor zone. Conveniently, the sample and the composites are made to flow from the inlet through the sensor zone before exiting the device. Prior to entering the sensor zone, the composites and sample should be given a suitable time to mix and for any analyte present in the sample to be recognized by the aptamer. This mixing may occur either prior to introducing into the device, or the device may further comprise a sampling zone. The sampling zone may be located between the inlet and the sensor zone. Then, as the reacted composites flow through the sensor zone, they are retained by the magnetic retainer for analysis by the electrochemical system. The flow and retention of composites in the sampling zone also purifies the composites prior to analysis, as any contaminant should not be retained. The magnetic retainer is able to retain the composites in the sensor zone by creating a magnetic field that acts on the magnetic particle of the composites. For multi-use devices, the magnetic retainer also releases the composites from the device. This may be achieved by use of an electromagnet or by allowing for the removal of the magnetic retainer from the device after completion of the sensing operation.

In some embodiments, the device further comprises an outlet. The outlet assists remove components of the sample that are not being analyzed from the device, and allows for recovery of the composites after sensing are re-use of the device. The outlet may be omitted in single-use devices, where it is desirable to retain the biological sample within the device, for example, within a reservoir located downstream of the sensor zone.

One embodiment of a sensing device is shown in Figure 1 in schematic and Figure 6 as an image. The embodiment shown in Figure 6 comprises an inlet (d), a sampling zone in the form of a channel extending from the inlet to the sensor zone. The magnetic retainer is provided in the form of a magnet, and the electrochemical system is provided in the form of a printed three electrode system (b). An outlet (a) is also shown. The details of fabrication of this embodiment are described in Example 1.

The sensing device may be used with any of the composites described herein. As noted above, multianalyte sensing may be achieved using this device with either (a) a composite comprising two or more aptamer-redox-active tag pairs; or (b) a population of composites comprising an aptamer-redox-active tag pair and one or more further populations of composites each comprising a different aptamer-redox active tag pair.

Also provided herein is a method for detecting an analyte, comprising:

- exposing a composite of the invention with the analyte; and
- detecting a response of the exposed composite to an electrochemical signal.

The composite and the analyte are combined for a time sufficient to allow the analyte
5 recognition region to bind the analyte.

The electrochemical signal may be provided through square wave voltammetry (SQW). SQW is considered a relatively gentle electrochemical technique. The voltage of the electrochemical signal should be selected based on the redox-active tag of the composite. For example, in single scanning mode, in embodiments comprising ferrocene as the redox-
10 active tag, the electrochemical signal may be by SQW across a voltage range from about -0.2V to about 0.6V. Whereas for the same embodiments, in continuous analysis mode, measurement of the composites at about -0.2V indicates changes in concentration of the analyte over time.

The response to the electrochemical signal is typically concentration dependent. Therefore,
15 by pre-testing known concentrations of analyte bound to the composites of the invention, a concentration/signal curve may be created allowing for concentration determination. Accordingly, the method may further comprise comparing the response to a concentration/signal curve prepared for the composite and analyte.

The method may be conveniently carried out on the sensing device. Thus, the method may
20 comprise providing the composite and a sample to the inlet of the sensing device, retaining the composite after it has interacted with the sample in the sensor zone, and detecting a response of the retained composite to an electrochemical signal delivered by the electrochemical system. After detection, the composites may be released from the device and the device washed to reset it for further use.

Also provided herein is a method for the diagnosis and/or prognosis of a medical condition,
25 comprising:

- exposing a composite of the invention to a biological sample potentially comprising a biomarker for the medical condition;
- detecting a response of the exposed composite to an electrochemical signal.

These diagnostic and/or prognostic methods rely on matching the analyte recognition region of an aptamer with a biomarker associated with the medical condition.

The method may further comprise comparing a response to the electrochemical signal against a concentration/signal curve prepared for the composite and the biomarker prior to analysis. However, it will be appreciated, that in some embodiments, the presence of the biomarker in the sample may be sufficient to diagnose the condition.

Also provided is a method of treatment of a medical condition, comprising:

- combining a composite of the invention with a biological sample potentially comprising a biomarker for the medical condition;
- scanning the composite with an electrochemical signal to detect the concentration of the biomarker in the biological sample; and
- administering one or more drugs to a subject that provided the biological sample based on the diagnosis/prognosis of the medical condition.

The term "medical condition" is intended to relate to a disease, disorder and/or condition, including symptoms thereof.

As used herein, the terms "treating", "treatment", "treat" and the like mean affecting a subject (e.g. a patient), tissue or cell to obtain a desired pharmacological and/or physiological effect. The effect may be prophylactic in terms of completely or partially preventing, or reducing the severity of, a disease or associated symptom, and/or may be therapeutic in terms of a partial or complete cure of a disease. For example, a reference to "treating" a medical condition therefore encompasses: (a) arresting the progress of the disease, e.g. preventing worsening of a symptom or complication over time; (b) relieving or ameliorating the effects of the medical condition, e.g. causing an improvement of at least one symptom or complication of the medical condition; (c) preventing additional symptoms or complications of the medical condition from developing; (d) preventing the medical condition or a symptom or complication associated with the medical condition from occurring in a subject at risk of the medical condition; and/or (e) preventing an increased risk of developing the medical condition.

Kits

Also provided is a kit comprising in separate parts:

- the composite of the invention; and
- a buffer solution

The buffer solution may be used to suspend the composite and/or to mix the composite with a sample for analysis. Alternatively, the buffer may be used to wash the device of the invention before and/or after use with the composite.

Also provided is a kit comprising in separate parts:

- a first composite of the invention; and
- a second composite of the invention.

In some embodiments, the first composite comprises an aptamer comprising an analyte recognition region that selectively recognizes a first analyte, and the second composite comprising an aptamer comprising an analyte recognition region that selectively recognizes a second analyte. Typically, the first composite comprises a first redox-active tag and the second composite comprises a second redox-active tag that is different to the first redox-active tag. In some embodiments, the first composite and/or the second composite are suspended in a buffer solution.

Examples

The invention will be further described by way of non-limiting example(s). It will be understood to persons skilled in the art of the invention that many modifications may be made without departing from the spirit and scope of the invention.

Example 1

This example describes a composite of the invention designed to detect interferon- γ (INF- γ) within a biological sample. This example also describes the fabrication of an embodiment of a device of the invention and its use in detecting the concentration of INF- γ in various samples.

Experimental

Chemicals and materials. Magnetic nanobeads (300 nm) was purchased from Ocean NanoTech, American. Tris(hydroxymethyl)aminomethane(Tris), hydrochloric acid, potassium chloride, magnesium chloride, sodium nitrite, potassium ferricyanide, lyophilized human serum, and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich. Aqueous

solutions were prepared using Mill-Q water. Recombinant Human IFN- γ was purchased from R&D company. All DNA with the following sequences were synthesized and purified by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). The hairpin aptamer probe sequence is 5'-Fc- GGG GTT GGT TGT GTT GGG TGT TGT GTC CAA CCCC-biotin-3' where underline part indicates the sequence with affinity to IFN- γ . The compositions of the buffers used for the experiments are as follows: buffer for bead washing, 100 mM Tris-HCl, 1 mM EDTA, and 2 M NaCl pH 7.5 (buffer A); buffer for the immobilization of the primary aptamer onto the beads, 5 mM Tris-HCl, 0.5 mM EDTA, and 100 mM NaCl pH 7.5 (buffer B); detection buffer, Tris-HCl buffer (pH=7.4) contains 100 mM Tris-HCl, 100 mM NaCl, 1 mM MgCl₂, and 5 mM KCl (buffer C).

Instrumentation. All electrochemical experiments were conducted using a CHI660E system (CH Instruments, Inc., Shanghai). Magnetic glassy carbon (GC) electrodes were 3 mm disks embedded in epoxy resin (Gaoss Union, China). All experiments utilized a Pt secondary electrode and a Ag/AgCl reference electrode. UV-Vis absorption data were collected on a Shimadzu UV-Vis spectrophotometer model 2450. Dynamic light scattering (DLS) data were acquired using a Nano-ZS90 (Malvern) apparatus (Malvern Instruments, Malvern, UK).

Preparation of magnetic bead (MB)-aptamer nanocomposites. MBs coated with streptavidin were washed with 500 μ L of buffer A before use, as advised by the manufacturer. A suspension of 50 μ L of the nanobeads was introduced in a tube containing 50 μ L of buffer A. After 15 min of incubation, the tube was positioned on a magnetic block to allow the precipitation of the nanobeads on the bottom of the test tube; the supernatant was then removed and the nanobeads were washed twice with 500 μ L of buffer B. The nanobeads were then washed with buffer B and resuspended in 500 μ L of buffer B. These could also be prepared in advance and kept at 4 °C for several weeks before usages. To prepare for MB-aptamer nanocomposites, the aptamer solution (5 μ M) was added to MB solution (100 ng mL⁻¹) with the volume ratio of 1:2 to react for 2 h with shaking at room temperature. Then the reaction tube was positioned on a magnetic block to allow nanobeads to settle down before removing the supernatant, and subsequent washing with buffer B twice. The MB-aptamer nanocomposites were achieved after repeating washing steps twice, and finally dispersed in buffer C for further usage. For quantitation of aptamers loaded on MBs, supernatant of 10 μ L of MBs was dropped to clean surface of glassy carbon electrode, and then cyclic voltammetry (CV) was scanned in the buffer C solution

from -0.2 V to 0.6 V. The size distribution of MB-aptamer nanocomposites was characterized by DLS in buffer C.

Fabrication of microfluidic based sensing devices. The customized microfluidic chip (Figure 1) includes two layers: bottom PDMS layer and top PDMS layer. For the bottom PDMS layer with the microfluidic pattern which was achieved by photolithography on the 100 μm thickness Su-8 on silicon wafers. The pattern includes the inlet sampling zone (1.2 mm in diameter), sampling channel zone with the length of 25 mm x 0.3 mm, and the sensor loading zone (1.2 cm in diameter) connecting to the sampling outlet zone with the diameter of 0.8 mm. Afterwards, PDMS were poured directly on the pattern which was left in an oven at 65° for 2 h for curing. Finally, the PDMS microfluidics chip were peeled off from the Su-8 mould for further use. For the top PDMS layer, three electrode sensors were fabricated on the top of a single 525 μm thick crystal silicon wafer; over native silicon dioxide (SiO_2) insulated layer: 500 nm of gold were sputtered on top of 20 nm Chromium seed layer to provide defect free adhesion of thin film gold electrodes as working electrode (WE) and counter electrode (CE), respectively; silver ink was deposited onto 20 nm Chromium seed layer to form the silver electrode as reference electrode (RE). The WE and CE are opposite to each other and RE is beside the WE. Liquid PDMS will be poured around each electrode sensor to form a uniform layer with exposure sensor surfaces. There electrode sensors were connected to the 3 separated electrostatic wires by the conductive glue, which connected to the external potentiostat. The final step is to bond the bottom PDMS pattern layer to the processed up sensor/PDMS layer to form enclosed microchannels to make sure the two PDMS layers matching to each other. A square shaped magnet with a side length of 1.5 mm was mounted below the keyhole-shaped compartmentalized microchamber as shown in Figure 1. In Figure 1, the bottom part of the PDMS-based chip is the microfluidic pattern including sampling zone and sensor zone, and the top part included WE, RE and CE on a silicon wafer which was diced and put on the glass slide. Both parts were bonded and combined by liquid PDMS. The image of the fabricated microfluidic device is illustrated in Figure 6. All flow to the device was controlled via a syringe pump (PhD 2000, Harvard Apparatus). Sample was continuously drawn into the device by engaging the waste pump at 1 mL/hour.

Electrochemistry measurement of IFN- γ . The MB-aptamer nanocomposite solution flew through the microfluidic device, and kept for absorption step for 10 min to make sure MB-aptamer was completely adsorbed to the sensor zone. The oxidation signal of ferrocene was measured by using SWV in buffer C scanning from -0.2 V to 0.6 V. The real-time

measurement was carried out by chronoamperometry in a series of different concentrations of IFN- γ by fixing the potential at 0.2 V for 5000 s.

Procedure to measure reversible capacity of sensing interface. To study the regeneration of MB-aptamer nanocomposites, 1000 pg mL⁻¹ IFN- γ was injected into the inlet port and flowed down the channel to the sensor zone to react with MB-aptamer on the sensor zone to achieve the aptamer-IFN- γ conjugates. Then the microfluidic chamber was washed with buffer B followed by the flowing with buffer C for 2 h. Due to the reversible binding between aptamer and IFN- γ , flowing through with buffer C will induce dissociation of IFN- γ from the aptamer-IFN- γ conjugates to regenerate MB-aptamer nanocomposites. The regenerated MB-aptamer nanocomposites were available to bind IFN- γ again.

Cell culture and measurement. Informed consent in this study was obtained under approved Human Research Ethics Committee protocols. Peripheral blood mononuclear cells (PBMCs) were purchased from Procell (Wuhan, China). They were cultured in a T25 cm² flask containing the completed Roswell Park Memorial Institute 1640 (RPMI-1640) medium supplemented with 10% Human Serum AB Heat Inactivated, 10 U mL⁻¹ of penicillin, 1000 U mL⁻¹ IL-2 and 1100 μ g mL⁻¹ of streptomycin, 10 μ g mL⁻¹ Gentamycin, 2 mM Gentamine and 25 mM HEPES. The 6 well plate was used to tune the density cells of 1×10^6 /mL, and incubated in 37 °C 5% CO₂ incubator to culture a period of time. The cells were cultured to about 80–90% confluence before harvesting. During harvesting, the cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS) followed by trypsinization using 2 mL of trypsin to detach the cells from the flask. The trypsin was neutralized by adding 4 mL of fresh supplemented medium, and the harvested cells in the RPMI-1640 medium suspension were transferred into a centrifuge tube and centrifuged at 1500 relative centrifugal force (rcf) for 10 min. The supernatant was discarded, and the cells were resuspended in fresh RPMI-1640 medium. For preparation of IFN- γ samples, the cells with the density of 1×10^6 /mL were suspended in 1 mL of warm medium containing 0.1 μ g mL⁻¹ LPS to secret IFN- γ for 0, 2, 4, 6, 8, and 20 h, respectively. Supernatants from cells were collected in triplicate. The Nunc MaxiSorp 96 well plate and Galaxy plate reader were used for ELISA.

Cytokine detection in human blood serum. Since under physiological conditions cytokine concentrations normally are low or undetectable, we spiked IFN- γ in the serum samples. Lyophilized serum was firstly reconstituted by dissolving 100 mg in 5 mL of deionized water mixing with gentle stirring. Then, the serum was spiked with 50 pg mL⁻¹, 100 pg mL⁻¹,

150 pg mL⁻¹ and 200 pg mL⁻¹ IFN- γ , and recovery of a spiked sample was measured using the prepared sensing device.

RESULTS AND DISCUSSION

Characterization of MB-aptamer nanocomposite. The streptavidin of MBs tended to selectively bind to biotin labeled on aptamers, mainly through strong non-covalent interactions. In order to confirm the successful loading of aptamer on MBs, the prepared MB-aptamer nanocomposites were pumped down to the homemade microfluidic chip followed by electrochemical measurement in buffer C (Figure 2a). As expected, MBs did not show any Faradaic peaks between -0.2 and 0.6 V versus Ag/AgCl. For the MB-aptamer nanocomposites, a redox peaks centered at 0.2 V were observed, which corresponded to the oxidation and reduction peaks of ferrocene, respectively, hence showing that aptamer were successfully attached to the surface. These results were further supported by UV-Vis spectra (Figure 2b) and DLS (Figure 2c and Figure 2d). An adsorption peak at 260 nm, characteristic of DNA, was observed in the UV-Vis spectrum of MB-aptamer but not in that of the unconjugated magnetic beads. In addition, the adsorption peak of magnetic beads was shifted from 300 nm to 360 nm upon aptamer conjugation. Further, the average diameter of MBs increased from 300 nm to 458 nm after aptamer conjugation according to the DLS data in Figures 2c and 2d. According to redox peak of Fc in Figure 2a, the surface coverage of Fc labeled aptamer can be calculated to be 4.01×10^{-11} cm² mol⁻¹). Thus, the total number of aptamers (about 93500) on MBs was calculated on the sensor zone.

Optimization of parameters for preparation of MB-aptamer

Figure 7 shows the SWV curves that 30 μ L 5 μ M aptamer solution reacted with magnetic beads solution in different volume for 2 h, respectively. After reaction, the tube was positioned on a magnetic block to allow the precipitation of the beads on the bottom of the test tube. After removing the supernatant, the beads were washed twice with buffer B, to obtain MB-aptamer complex. It was observed that the oxidation peak current of ferrocene increased with the volume of magnetic beads from 30 to 120 μ L, and it reached a plateau when more than 60 μ L of aptamer solution was added. Thus, the optimized volume of magnetic beads solution was 60 μ L which reacted with 30 μ L 5 μ M aptamer solution.

Response of the microfluidic on-chip device to IFN- γ .

Figure 3 illustrates that the electrochemical response of MB-aptamer modified microfluidic device after reaction with IFN- γ . Without the presence of IFN- γ , obvious Faradic current

was observed at 0.3V which is characteristic of ferrocene (Figure 3a). However, redox peaks disappeared after exposure to the analyte IFN- γ followed by washing with a copious amount of buffer B. The loss of electrochemical signal at 0.3V was due to the binding of IFN- γ to the loop part of the aptamers leading to the opening of the aptamer and subsequent shift of ferrocene away from the MB-based electrode surface. It suggests the prepared on-chip sensor was feasible for detection of IFN- γ .

The sensor in this work is based on the structure-switching aptamers, and the configuration change in structure is induced by the analyte resulting in electrochemical signal switching. Thus, any factors which affect the folding of aptamer modified on MBs will have an effect on the sensor performance. The effect of pH, salt concentration and temperature on the stability of MB-aptamer sensing interface was investigated without the presence of IFN- γ . It was observed that the current of the sensing interface increased with pH from 5.0 to 7.5 after incubation with Tris-buffer solution for 5 min under different pH conditions (Figure 3b). Under acidic conditions, the DNA was deprived of the purines leading to the stem unfold. Thus, a smaller number of the ferrocene molecules were away from the interfaces. However, when the pH reached 8, aptamers were denatured in these alkaline conditions, which neutralized the charge of acids but also caused hydrolysis of bases upon prolonged treatment. Thus pH 7.5 was found to provide the optimized conditions to the combination between MB and aptamer. In addition, Figure 3c shows that the maximum current was achieved when salt concentration was about 100 mM. Temperature also has effect on the signal detected (Figure 3d). The unfolding of aptamer, and therefore release of ferrocene molecules, was negligible before the reaction with IFN- γ at temperatures below 60°C. Heating up the MB-aptamer solution to 90°C led to significant release of ferrocene molecules without the presence of IFN- γ . The MB-aptamer sensing interface performed well across a range of temperatures, including at physiological temperature – about 37°C.

Electrochemistry measurement of IFN- γ .

The performance of the on-chip device for detection of IFN- γ was studied under the optimized condition (room temperature, pH 7.5, and salt concentration of 100 mM). The square wave voltammetry (SWV), a more sensitive electrochemical technique can be used to quantitatively monitor IFN- γ concentrations. Figure 4a shows the SWV of microfluidic chip device after incubation with IFN- γ at different concentrations. The Fc peak current decreased with the concentration of IFN- γ with a linear range of 10-500 pg mL⁻¹, and the lowest detectable concentration of 10 pg mL⁻¹ was obtained in buffer C. The detection limit was calculated to be 6 pg mL⁻¹ based on three times of signal to noise with confidence

factor of 3. As determined from the midpoint (100 pg mL^{-1}) of the calibration curve in Figure 4b, the affinity constant between IFN- γ (molecular weight 16.9 kDa) and MB-aptamer is calculated to be around $2.1 \times 10^{10} \text{ M}^{-1}$. The typical values of the affinity constants K_a for antigen-antibody reactions is in the range of 10^8 to 10^{12} M^{-1} , so this value indicates that the MB-aptamer probe has very high affinity to IFN- γ . Additionally, a chronoamperometry experiment at a constant potential of 0.2 V which is close to the oxidation potential of ferrocene, was carried out by adding different concentrations of IFN- γ (Figure 4c). Upon the addition of IFN- γ , the monitored current of MB-aptamer microfluidic device decreased accordingly, further suggesting that IFN- γ induced the configuration change of the aptamer leading to Fc far away from the interface. As a control experiment, PBS was added to the sensor zone and no current switching was observed. To study the specificity of aptamer sensors for IFN- γ , the electrochemical signal was monitored by incubation of the MB-aptamer sensor with the nonspecific proteins BSA (2 mg mL^{-1}), PSA (1 mg mL^{-1}), CA-125 (1 mg mL^{-1}), IgG (1 mg mL^{-1}), IL-6 (1 ng mL^{-1}), and TNF- α (1 ng mL^{-1}), without the presence of IFN- γ . Compared to the original signal which was the response of MB-aptamer sensor to 500 pg mL^{-1} IFN- γ in the absence of interfering proteins (Figures 4d and 4e), no significant signal (less than 15%) was observed for the interfering proteins. It suggests this sensing interface has the satisfied specificity to IFN- γ . The selectivity of the prepared microfluidic device sensing interface was also studied by monitoring the electrochemical response of MB-aptamer sensor to 500 pg mL^{-1} IFN- γ with the presence of the nonspecific proteins BSA (2 mg mL^{-1}), PSA (1 mg mL^{-1}), CA-125 (1 mg mL^{-1}), IgG (1 mg mL^{-1}), IL-6 (1 ng mL^{-1}), and TNF- α (1 ng mL^{-1}) (Figure 4d). The response of sensing device to 500 pg mL^{-1} IFN- γ retained 95% of the original signal of 500 pg mL^{-1} IFN- γ in the absence of interfering proteins (Figure 4e), suggesting a negligible degree of interference for these species tested in relation to the control test (<5%).

Stability and reversibility of the MB-aptamer sensors for detection of IFN- γ .

The stability of MB-aptamer was investigated by recording time-dependent current changes of the MB-aptamer sensing interface which was stored on PBS solution at room temperature over 30 days. It was observed that I/I_0 decreased slowly (Figure 5a) and the signal (I) obtained at day 30 was $92\% \pm 2\%$ of signal (I_0) from the fresh sensing interface at day 0, indicating low and acceptable amounts ($\leq 10\%$) of aptamer loss from MB-aptamer nanocomposites after storing in PBS for 30 days and the non-covalent interactions between streptavidin and biotin were sufficiently stable. The binding between aptamers and proteins was a reversible process, which was studied on the magnetic glassy carbon electrode. The

electrochemical signal before and after stirring the IFN- γ bound MBs in buffer solution for 1 h (Figure 8) was recorded. This performance of on-chip sensing device was further investigated by recording chronoamperometry with different circles of the regeneration (Figure 5b), and it showed the electrochemistry of on chip device retained 90% of the signal of the fresh prepared MB-aptamer based sensing interface for detection of 100 pg mL⁻¹ IFN- γ , suggesting that on-chip sensing interface could be regenerated. It was observed the electrochemical signal switched off after IFN- γ binding. However, the signal recovered after incubation of MBs in buffer B with stirring. It suggests that upon binding to its target molecule, the probe underwent a reversible conformational rearrangement that modulated the redox current and generated an electrochemical signal.

Measurement of IFN- γ in cell culture media and while human blood serum.

MB-aptamer microfluidic device was used as the device for detection of IFN- γ secreted by live PBMCs (Figure 5c). Firstly, the chronoamperometry experiment at a constant potential of 0.2V was carried out in the cell culture medium of PBMCs, to which IFN- γ with the concentration of 10-1000 pg mL⁻¹ was added. It was observed that the current decreased continuously after adding IFN- γ until it reached a plateau when the concentration of IFN- γ was 500 pg mL⁻¹. The control was carried out by recording the continuing current after spiking in PBS without the presence of IFN- γ . The supernatant of 1×10^6 PBMCs was collected for IFN- γ analysis after LPS stimulation for 2, 4, 6, and 12 h, respectively by both of the commercial ELISA kit and herein prepared microfluidic MB-aptamer device. As shown in Figure 5d, the concentration of IFN- γ increased with the duration of the LPS treatment and the highest content of IFN- γ was obtained after 12h LPS stimulation. The similar trend was observed using the ELISA kit. The concentration values found by both methods were statistically compared by means of the Student's t-test. The experimental t values (t_{exp} , 0.30), were lower than the tabulated t value (2.11) and, therefore, no significant differences were found between both methodologies at a significance level of 0.05. Thus, the sensing device has the capability of real-time cytokine sensing.

Finally, the developed microfluidic sensing device was applied to the determination of IFN- γ in human serum spiked at clinically relevant concentration levels. Regarding serum, the possible existence of matrix effects was initially evaluated by constructing a calibration plot in the sample (lyophilized serum from Sigma) which was spiked with IFN- γ . The equations obtained for the respective calibration graphs were $I(\mu\text{A}) = 0.0023 C (\text{pg mL}^{-1}) + 0.3198$ for detection of IFN- γ in serum. The comparison of the slope values with that (0.0026) calculated for the calibration plot constructed with standard solutions by applying the

Student's t-test showed t_{exp} value 1.465 lower than that tabulated t , 2.426, therefore indicating that no statistically significant differences existed between the slopes values for both types of calibration plots. Therefore, the determination of IFN- γ could be carried out by interpolation of the current values measured for the serum samples into the calibration plots constructed with the standard solutions. Table 1 summarizes the results obtained by triplicate analysis of samples spiked at four different concentration levels: 50, 100, 150 and 200 pg mL^{-1} IFN- γ . Recoveries ranged between 98% and 101% with low relative standard deviations indicating the reliability of the approach to determine low IFN- γ concentrations in serum following a simple working protocol. These results suggest the herein developed microfluidic sensing device is potential to determine IFN- γ at clinically relevant concentrations in biological fluids such as serum for point-of-care analysis.

Table 1 Recovery studies on human serum samples after spiking IFN- γ in different concentration.

Serum samples	Added IFN- γ / pg mL^{-1}	Found IFN- γ / pg mL^{-1}	RSD /%	Recovery /%
1	50	49.9	3.18	99.7
2	100	101	1.15	101
3	150	147	2.00	98
4	200	199.3	2.30	99.6

CONCLUSIONS

This Example describes a customized disposable microfluidic chip modified with MB-aptamer nanocomposites for continuous detection of IFN- γ in a wide range of concentration with excellent precision. The microfluidic electrochemical detector for *in vitro* continuous monitoring requires no exogenous reagents, operates at room temperature, and can be reconfigured to measure different target molecules by exchanging probes in a modular manner. A DNA hairpin containing IFN- γ binding aptamer was biotinylated, conjugated with ferrocene(Fc) redox tag, and immobilized on magnetic beads by non-covalent interactions between streptavidin and biotin. Binding of IFN- γ caused the aptamer hairpin to unfold, pushing Fc redox molecules away from the electrode and decreasing electron-transfer efficiency. The change in redox current was quantified using square wave

voltammetry and was found to be highly sensitive to IFN- γ concentration. The limit of detection for optimized biosensor was 10 pg mL⁻¹ with linear response extending to 500 pg mL⁻¹. This microfluidic sensing device was specific to IFN- γ in the presence of overabundant serum proteins and allowed for the continuous monitoring of IFN- γ without adding exogenous reagents. It provided a universal point-of-care sensing platform for continuous screening of a spectrum of analytes *ex vivo*.

Example 2

This example describes an embodiment of the invention designed to detect IFN- γ , vascular endothelial growth factor (VEGF) and tumour necrosis factor- α (TNF- α) within a biological sample.

Chemicals and materials. Unless otherwise noted, the chemicals and materials, and instrumentation used in Example 2 are as described above in Example 1.

The aptamer-redox-active tag pair selective for IFN- γ (referred to herein as “aptamer1-redox1”) sequence is 5'-biotin-T10-GGG GTT GGT TGT GTT GGG TGT TGT GTC CAA CCCC-methylene blue-3'.

The aptamer-redox-active tag pair selective for VEGF (referred to herein as “aptamer2-redox2”) sequence is 5'-biotin-A10-CCG TCT TCC AGA CAA GAG TGC AGG GAA TCT GGA AGAC-NB-3', wherein NB denotes a Nile blue redox-active tag.

The aptamer-redox-active tag pair selective for TNF- α (referred to herein as “aptamer3-redox3”) sequence is 5'-biotin-TGA GGG TTG TCG CCT GGT GGA TGG CGC AGT CGG CGA CAA -Fc-3', wherein Fc denotes a ferrocene redox-active tag.

Preparation of magnetic bead (MB)-aptamer nanocomposites. MBs coated with streptavidin were washed with 500 μ L of buffer A before use. A suspension of 50 μ L of the nanobeads was introduced into a tube containing 50 μ L of buffer A. After 15 min of incubation, the tube was positioned on a magnetic block to allow the precipitation of the nanobeads on the bottom of the test tube; the supernatant was then removed and the nanobeads were washed twice with 500 μ L of buffer B. The nanobeads were then washed with buffer B and resuspended in 500 μ L of buffer B. These could also be prepared in advance and kept at 4 °C for several weeks before use. A mixture of three different aptamer solutions (5 μ M, aptamer1-redox1, aptamer2-redox2, aptamer3-redox3) were added to the MB-buffer B solution (100 ng mL⁻¹) with the volume ratio of 1:2 (aptamer-redox

solution : MB solution) and allowed to react for 2 h with shaking at room temperature (about 25 °C). Then the reaction tube was positioned on a magnetic block to allow nanobeads to settle before removing the supernatant, and subsequent washing with buffer B twice. Following two further washing steps (buffer B), and finally dispersion in buffer C, MB-aptamer nanocomposites were furnished. A schematic of this MB-aptamer with each aptamer in its self-assembled conformation and in its disrupted conformation is shown in Figure 9.

Electrochemistry measurement of IFN- γ , VEGF, and TNF- α . Electrochemistry

measurements were obtained using the fabricated device as described in Example 1. The MB-aptamer nanocomposite solution flew through the microfluidic device, and kept for absorption step for 10 min to make sure MB-aptamer1, MB-aptamer2, MB-aptamer3 were completely adsorbed to the sensor zone by magnetic field. The oxidation signal of ferrocene, methylene blue and Nile blue (NB) was measured by using SWV in buffer C scanning from 0.5 V to -0.6 V after adding solutions containing known concentrations of the three cytokines, IFN- γ , VEGF, and TNF- α .

The results of these electrochemistry measurements are shown in Figure 10. The SWV clearly shows three distinct signals that can be attributed to the presence of the three analytes interacting with the MB-aptamer composite.

Claims

1. A composite comprising:
 - an aptamer comprising an analyte recognition region and at least one self-assembly region;
 - a magnetic particle linked to a first position of the aptamer; and
 - a redox-active tag linked to a second position of the aptamer;wherein the aptamer interchanges between a disrupted conformation and a self-assembled conformation in response to interaction of an analyte with the analyte recognition region of the aptamer.
2. The composite of claim 1, wherein the first position is at one end of the aptamer and the second position is at the other end of the aptamer.
3. The composite of claim 1 or 2, wherein the aptamer is a nucleic acid molecule.
4. The composite of claim 3, wherein the first position is at the 3' end of the aptamer.
5. The composite of claim 3 or 4, wherein the second position is at the 5' end of the aptamer.
6. The composite of any one of claims 3 to 5, wherein the aptamer comprises about 10 bases to about 100 bases.
7. The composite of any one of claims 3 to 6, wherein the analyte recognition region comprises about 5 to about 100 bases.
8. The composite of any one of claims 3 to 7, wherein the self-assembled confirmation is a hairpin loop confirmation.
9. The composite of claim 8, wherein the aptamer comprises two complimentary sequences that bind to form the hairpin loop, and the analyte recognition sequence comprises at least one of the two complimentary short sequences.
10. The composite of any one of claims 1 to 9 wherein the aptamer is linked to the magnetic particle by a linker group.
11. The composite of claim 10, wherein the linker comprises a polymer chain interrupted by a conjugation site.

12. The composite of claim 11, wherein the conjugation site the conjugation product of conjugation partners selected from biotin and a biotin recognition protein, and an antigen and antibody.
13. The composite of claim 12, wherein the biotin recognition protein is selected from avidin, streptavidin and deglycosylated avidin.
14. A method of manufacturing the composite according to any one of claims 1 to 13, comprising:
 - conjugating an aptamer comprising a redox-active tag with a magnetic particle; or
 - conjugating a redox-active tag with an aptamer conjugated with a magnetic particle.
15. A sensing device comprising:
 - an inlet for receiving a sample and the composite of any one of claims 1 to 13;
 - a sensor zone comprising an electrochemical system and a magnetic retainer.
16. The sensing device of claim 14, wherein the electrochemical system is a three-electrode system comprising a working electrode, a counter electrode and a reference electrode.
17. A method for detecting an analyte, comprising:
 - providing a composite of any one of claims 1 to 13 and a sample to the inlet of the sensing device of claim 15 or 16,
 - retaining the composite after it has interacted with the sample in the sensor zone,and
 - detecting a response of the retained composite to an electrochemical signal delivered by the electrochemical system.
18. A method for detecting an analyte, comprising:
 - exposing a composite of any one of claims 1 to 13 to the analyte;
 - detecting a response of the exposed composite to an electrochemical signal.
19. A method for the diagnosis and/or prognosis of a medical condition, comprising:
 - exposing a composite of any one of claims 1 to 13 to a biological sample potentially comprising a biomarker for the medical condition;
 - detecting a response of the exposed composite to an electrochemical signal.
20. A method of treatment of a medical condition, comprising:

- exposing a composite of any one of claims 1 to 13 to a biological sample obtained from a subject;
 - detecting a response of the exposed composite to an electrochemical signal; and
 - administering one or more drugs to the subject based on the detected response.
21. A kit comprising in separate parts:
- (a) the composite of any one of claims 1 to 13; and
 - (b) a buffer solution.
22. A kit comprising in separate parts:
- c. a first composite of any one of claims 1 to 13; and
 - d. a second composite of any one of claims 1 to 13.

Figure 1

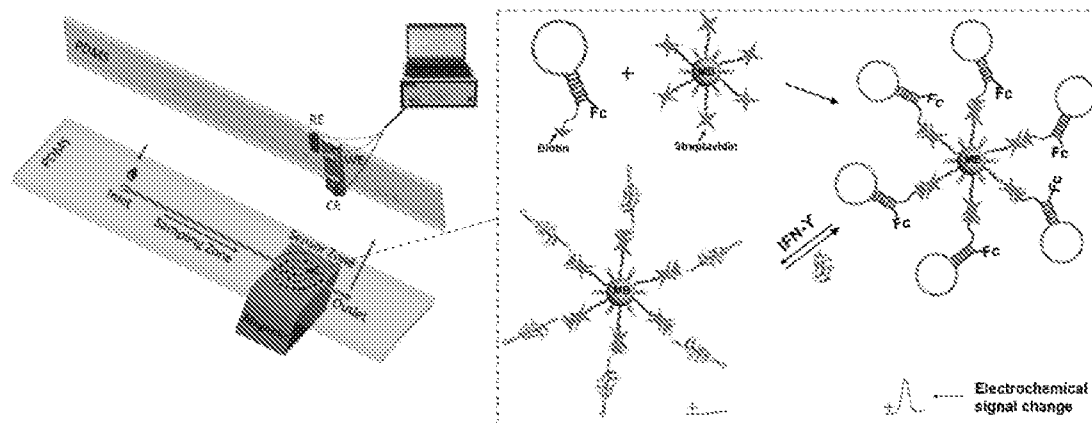


Figure 2

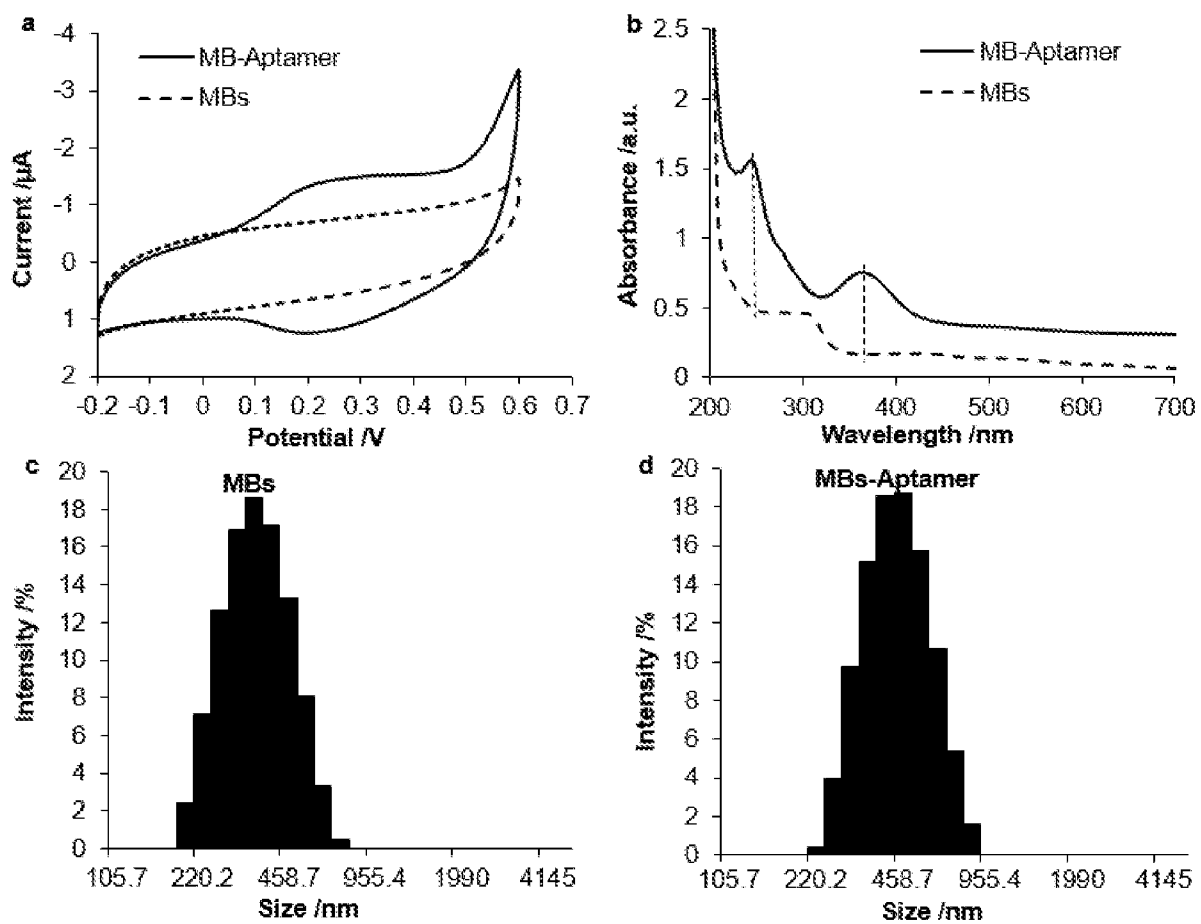


Figure 3

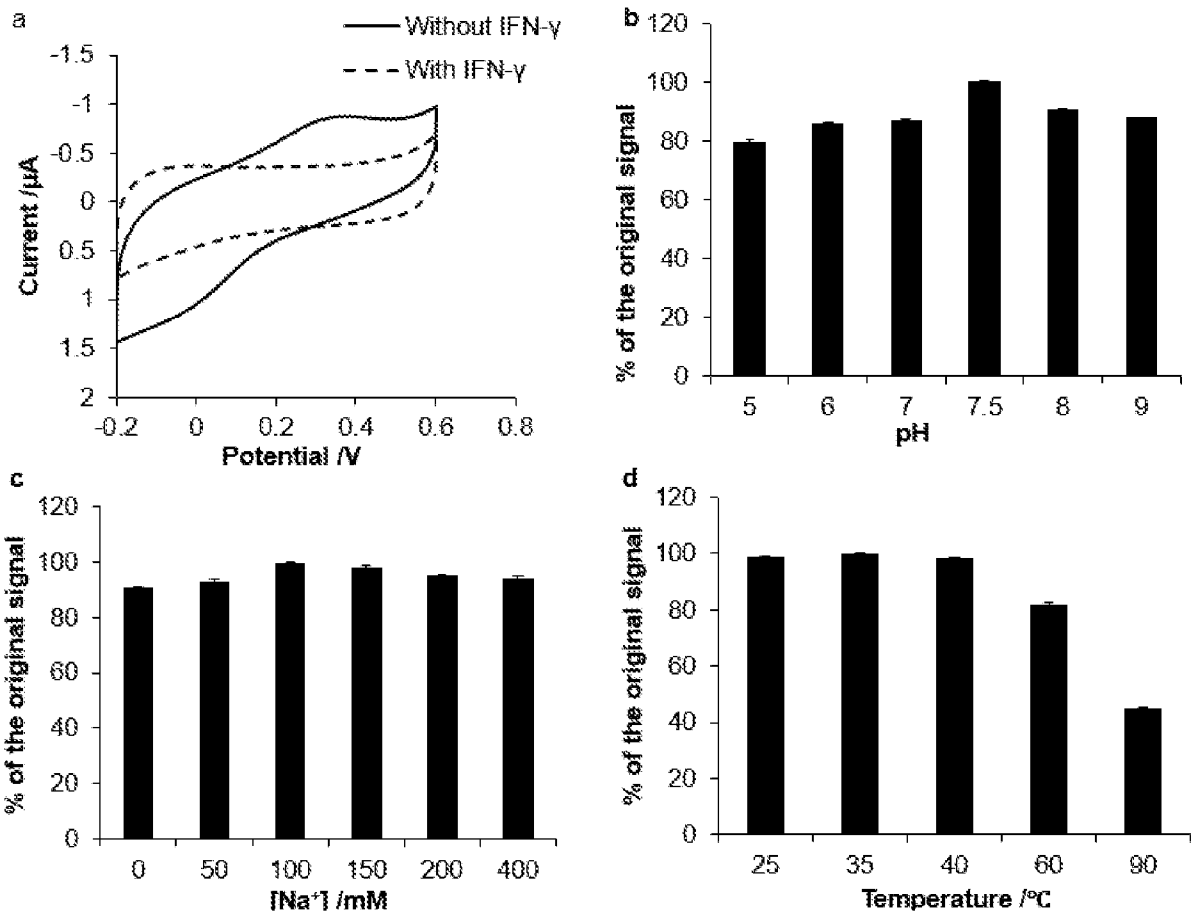


Figure 4

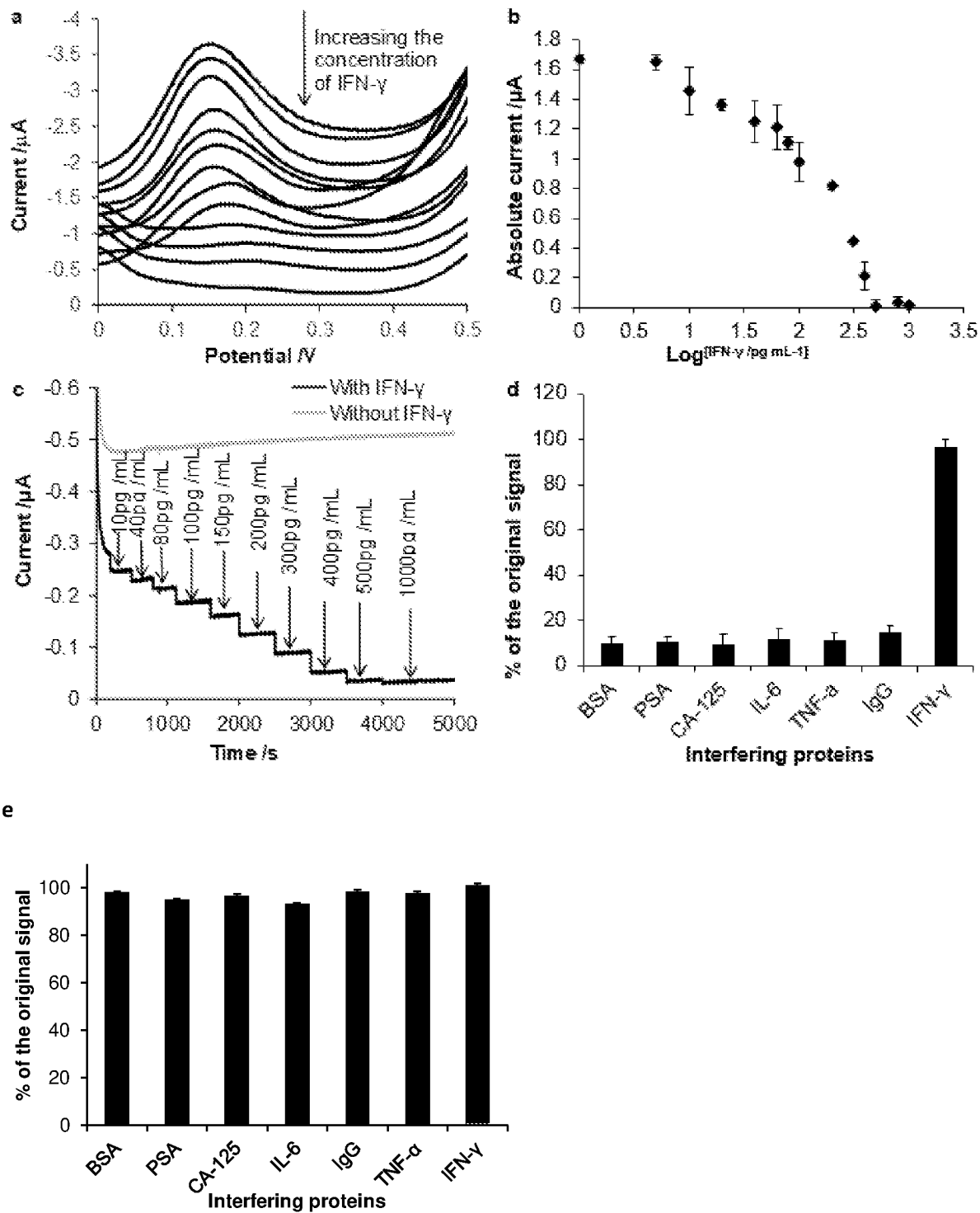


Figure 5

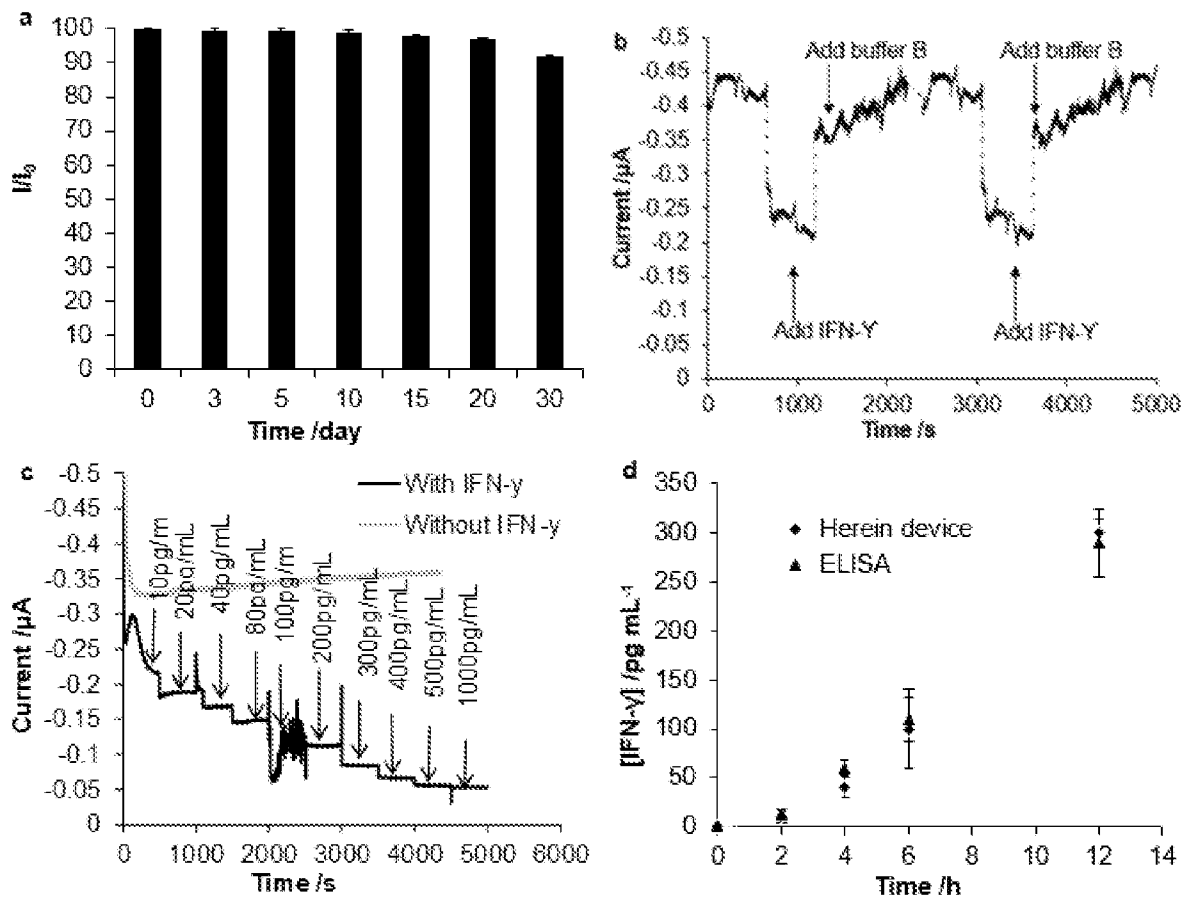


Figure 6

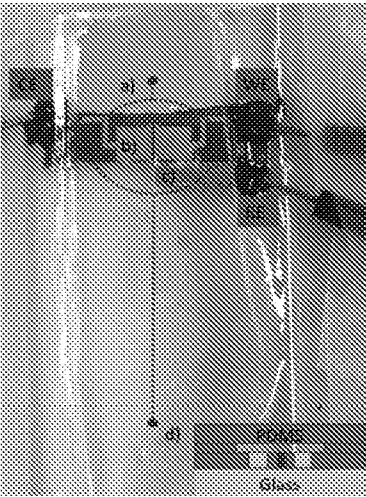


Figure 7

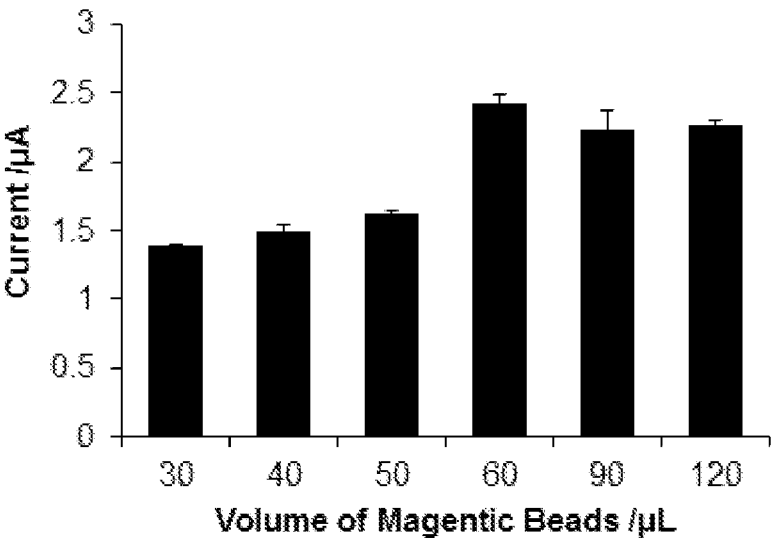


Figure 8

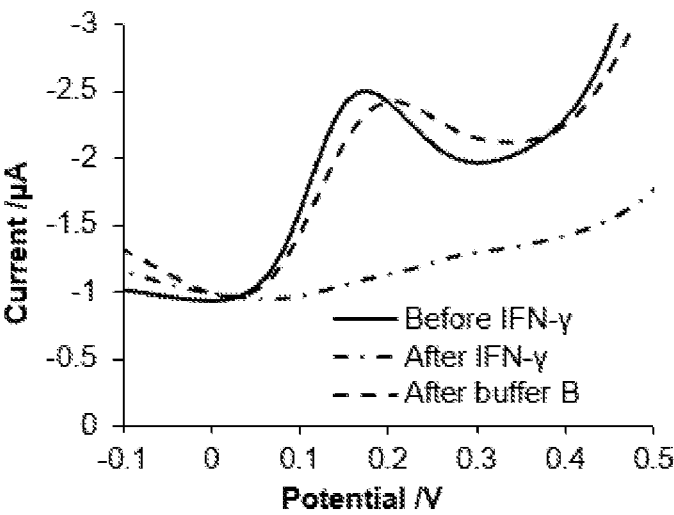


Figure 9

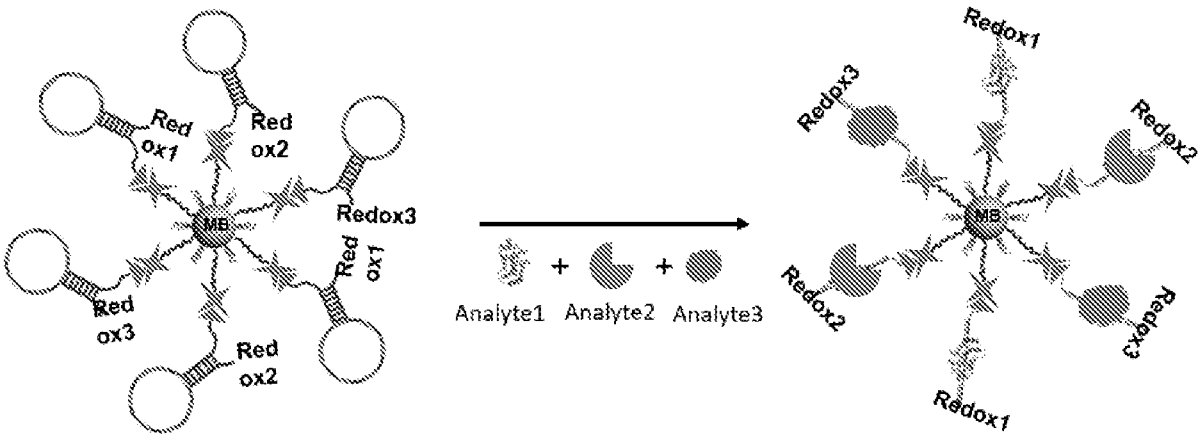
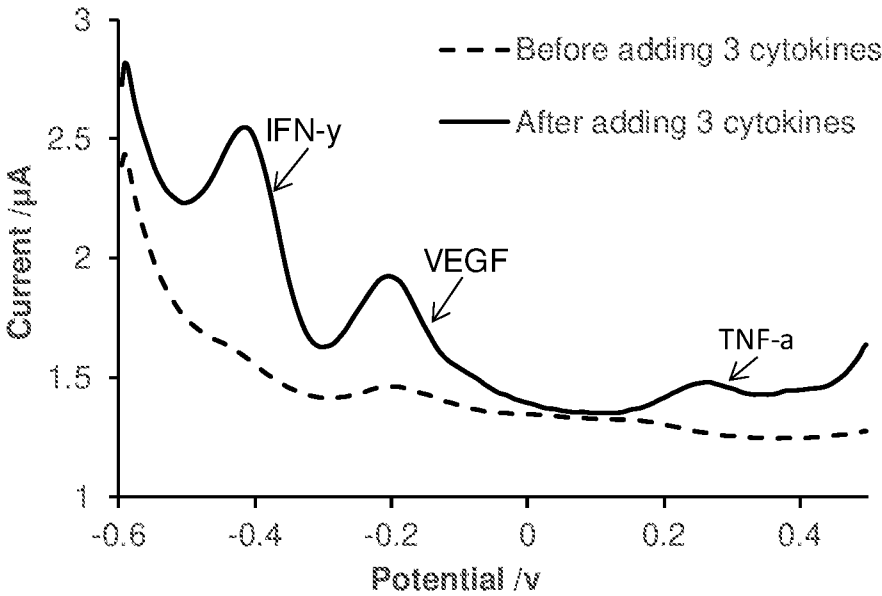


Figure 10



INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2020/050105

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/53 (2006.01) C12Q 1/6825 (2018.01) G01N 27/26 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PATENW, MEDLINE, CAPLUS, BIOSIS, EMBASE & keywords: aptamer, magnetic particle, switch, self-assemble, redox, electrochemical;
CPC/IPC: C12Q2525/205, C12N15/115, C12N2310/16, C12Q2563/143, G01N33/54326, C12Q2563/113

AUSPAT, ESPACENET, PUBMED, Internal databases: Inventor names

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"D" document cited by the applicant in the international application	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 27 March 2020	Date of mailing of the international search report 27 March 2020
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au	Authorised officer Richard Filmer AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832735

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2020/050105
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 108309995 A (CENTRAL CHINA NORMAL UNIVERSITY) 24 July 2018 [0017]-[0021], [0048], Fig. 1a, Fig. 1b	1-14, 18-22
X	CHENG, G. et al., "A new electrochemically active–inactive switching aptamer molecular beacon to detect thrombin directly in solution", Biosensors and Bioelectronics, 2010, vol. 25, pages 2265-2269 Sections 2.4, 3.6; Fig. 1; Table 1	1-3, 5-11, 14-22
P,X	LIU, G. et al., "On-chip structure-switching aptamer-modified magnetic nanobeads for the continuous monitoring of interferon-gamma ex vivo", Microsystems & Nanoengineering, 2019, vol. 5, no. 35 entire document	1-22

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/AU2020/050105	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
CN 108309995 A	24 July 2018	CN 108309995 A	24 Jul 2018
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2019)			