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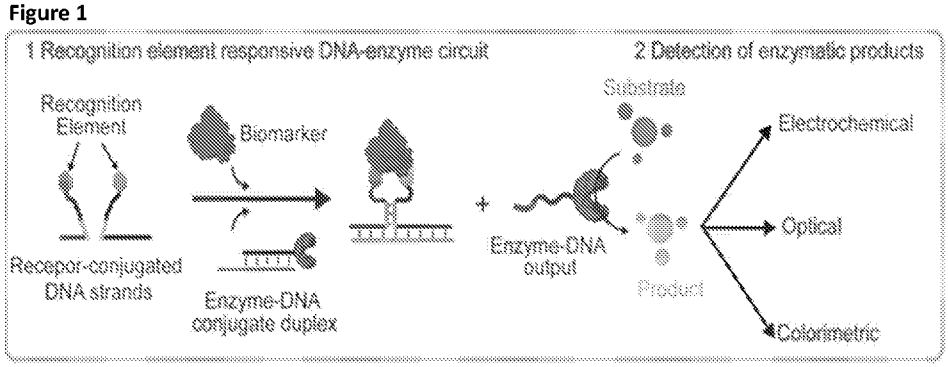
(54) Title: DNA-ENZYME CONJUGATES FOR SPECIFIC AND ULTRA-SENSITIVE DETECTION OF A TARGET ANALYTE, AND METHOD THEREOF

Figure 1



(57) Abstract: The present invention relates to a method for the detection and quantification of target analytes such as, for example, antibodies, proteins, enzymes, small molecules, or ions directly in complex matrices of samples, with very high specificity and sensitivity. In particular, said method is preferably based on the use of a programmable DNA-based circuit which, in the presence of a specific target analyte, triggers the release of an enzyme-conjugated DNA filament through a DNA filament displacement reaction. Said enzymeconjugated DNA filament is then detected using suitable optical, electrochemical, or enzymatic methods.

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DESCRIPTION

Annexed to Patent Application for INDUSTRIAL INVENTION entitled:

"DNA-ENZYME CONJUGATES FOR SPECIFIC AND ULTRA-SENSITIVE DETECTION OF A TARGET ANALYTE, AND METHOD THEREOF"

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DESCRIPTION

Technical Field of the Invention

The present invention relates to a method for the detection and quantification of target analytes such as, for example, antibodies and/or proteins and/or enzymes and/or small molecules and/or ions, or other

ones, with very high specificity and sensitivity, directly in complex matrices of samples. In particular, said method is preferably based on the use of a programmable DNA-based circuit which, in the presence of a specific target analyte, triggers the release of an enzyme-conjugated DNA filament through a DNA filament displacement reaction. Said enzyme-conjugated DNA filament is then detected using suitable optical, electrochemical, or enzymatic methods.

Description of the Prior Art

The detection of target analytes such as, for example, antibodies and/or proteins and/or enzymes and/or small molecules and/or ions, or so on, plays a fundamental role in the diagnosis of a large number of diseases, such as, for example, infections, autoimmune diseases, and oncologic diseases.

In addition to being important as disease markers, the antibodies (like, for example, monoclonal and/or bi-specific antibodies) and proteins are being increasingly used also as drugs within therapeutic contexts[1].

As is well known in the art, standard analytical methods employed for detecting such types of markers are, for example, based on complex processes requiring multiple

wash steps, or on the use of multiple reagents (like, e.g., the enzyme-linked immunosorbent assay, commonly referred to as ELISA), or on qualitative or semi-quantitative methods like, for example, lateral flow tests (usually, LFT). ELISA assays are both sensitive and quantitative, but require time-consuming measurements to be carried out in a laboratory through the use of special instruments, so that the applicability of this technique for "point-of-care" diagnostics is rather limited. In turn, lateral flow tests are fast and easy to use, but their essentially qualitative (or, in the best of cases, semi-quantitative) nature limits the possibility to benefit from quantitative information, which may sometimes be very important.

In recent times, sensors based on synthetic DNA have acquired increasing importance as promising analytical tools for fast, economical and quantitative measurement of a wide range of analytes, such as, for example, nucleic acids, proteins, antibodies, and small molecules. The unique characteristics of synthetic nucleic acids, which include low cost, easy synthesis, and high capability of predicting base-pair interaction, have been exploited for programming switches, circuits and devices that can be employed in

applications for detecting molecular targets and/or for releasing drugs.

In particular, the chemical versatility of synthetic nucleic acids has been exploited to conjugate different molecules, such as, for example, antigens, small molecules, peptides, proteins, enzymes, antibodies, etc., as well as "tag" (or "label") molecules capable of providing a signal that can be measured (e.g., using optical or electrochemical methods), in order to develop devices which can detect and monitor a wide range of molecular targets, including, for example, antibodies[2].

As a demonstration of the importance of such systems for clinical applications, several classes of DNA-based sensors have been developed so far in the industry, wherein the detection of specific antibodies is based on optical and electrochemical methods[3,4].

In a recent embodiment, the present inventors have proposed a novel programmable approach for the detection of antibodies and/or other analytes, which employs a DNA-based circuit. Said circuit is based on the use of a so called reaction of displacement of a DNA filament, and has been engineered in such a way as to induce the release of a DNA filament modified with an optical or electrochemical reporter only in the

presence of a specific target antibody. More particularly, said circuit uses three synthetic elements: a DNA duplex and two single-stranded DNAs conjugated with an antigen and designed in such a way that, upon formation of a divalent bond with a target antibody, they come in close vicinity to each other and, as a consequence, can hybridize to form a functional bi-molecular complex. Said resulting functional bi-molecular complex, as it binds with the single-stranded free portion of the pre-hybridized DNA duplex, invades it and triggers the release of a DNA filament modified with a reporter. Said released filament can produce an optical or electrochemical signal which is directly proportional to the concentration of the target antibody[5].

Drawbacks of the Prior Art

- *Drawbacks of the biomarker detection methods currently in use*

Notwithstanding their high sensitivity, the current gold standards (e.g., ELISA assays) for detecting antibodies, proteins, small molecules, etc. are based on multi-step processes that are rather complex, since they require many wash steps and large amounts of reagents. Therefore, the analysis is time-consuming

and, in some cases, undesired delays in patient treatments may result, thus having limited usefulness for fast point-of-care applications. On the contrary, lateral flow tests (LFTs) require no wash steps and are easy to carry out, but they can normally provide qualitative results only, thus not permitting a precise quantification of the target analyte.

- *Drawbacks of prior-art DNA-based detection platforms*

Detection platforms based on synthetic DNA have been developed and employed to meet the increasing demand for new analytical diagnostic tools with high sensitivity and specificity to be used for detecting biological targets. Most synthetic-DNA-based sensors known to date for detection of clinically relevant biomolecules, including, for example, antibodies, make use of optical or electrochemical methods. DNA-based optical sensors offer several advantages in terms of versatility and sensitivity, but they are affected by several limitations such as, for example, poor performance when used for direct analysis of complex matrices of samples (e.g., blood serum, whole blood, saliva, urine, etc.) and lack of low-cost portable instrumentation.

DNA-based electrochemical sensors overcome the above-described limitations, with the additional advantage

that the surface of the electrode can be miniaturized. However, the DNA-based sensors for biomarker detection known in the art can only reach limits of detection (LOD) in the low nano-molar range (nM) (1 nM-50 nM) or, at most, in the high pico-molar range (pM) (100-500 pM) for their specific targets. Unfortunately, this level of experimental sensitivity is of orders of magnitude higher than the concentration levels of many biomarkers that need, or are expected, to be detected in clinical samples; as a consequence, DNA-based sensors do not provide the hoped usefulness in the detection of such biomarkers.

The development of DNA-based sensors/platforms capable of detecting different target antibodies of clinical interest in a programmable manner and having much higher sensitivity (e.g., in the low pico-molar range pM) than prior-art ones would therefore be crucial to permit the practical use of such platforms for facing many health challenges.

Technical Problem

The need is therefore still felt in the field for a fast, versatile, specific, ultra-sensitive and low-cost method which can, advantageously, effectively detect a wide range of biomarkers including, for example,

antibodies and/or proteins and/or enzymes and/or small molecules and/or ions (said target analytes) directly in complex matrices of samples (e.g. blood, serum, or plasma samples) at the clinically relevant levels of said biomarkers.

The aim of the present invention is to provide an adequate solution to the above-described technical problem.

Summary of the Invention

The subject of the present invention is a method for carrying out the detection (e.g., optical and/or colorimetric and/or electrochemical) of target analytes such as, for example, antibodies and/or proteins and/or enzymes and/or small molecules and/or ions, with very high specificity and sensitivity, directly in complex matrices of samples. Said method is based on the use of a programmable synthetic-DNA-based circuit/platform that, in the presence of a specific target analyte (e.g. an antibody, a protein, an enzyme, a small molecule, an ion, etc.), triggers the release of an enzyme-conjugated DNA filament (hereafter referred to as "output filament") through a reaction of displacement of the DNA filament. Said DNA filament, conjugated with an enzyme, is then detected and quantified by means of

suitable optical, electrochemical, or enzymatic methods.

Detection principle

The detection platform is based on a DNA circuit that uses two DNA filaments conjugated with a recognition element (e.g. an antigen, a protein, a protein epitope, an aptamer, a small molecule, etc.), and designed in such a way that, upon formation of a link with a suitable target analyte, e.g. selected from those previously described herein, colocalize and form a functional bi-molecular complex capable of triggering a reaction of displacement of an enzyme-conjugated DNA filament, as detailed in the annexed Figure 1. In order to reach this goal, the two DNA filaments, conjugated with the above-mentioned recognition element, are designed in such a way that, in the absence of the target analyte (and at the concentration values commonly used or usable in the medical practice), they will remain separate, and only upon formation of a link with the target analyte they will form a stable bi-molecular complex. Subsequently, this bi-molecular complex will be able to bind with the single-stranded portion of a DNA duplex connected to an enzyme, thereby triggering a filament displacement reaction resulting in controllable release of the DNA-enzyme conjugated

filament (the output filament). The released DNA-enzyme conjugated filament will be proportional to the concentration of the target analyte inducing the displacement reaction. The released output filament can then be detected by means of known electrochemical, optical, or colorimetric methods in the presence of the related enzymatic substrate (as shown in the annexed Figure 1).

Brief Description of the Drawings

Figure 1 shows a general diagram of a DNA-based circuit using DNA-enzyme conjugates to provide ultrasensitive and specific detection of a target analyte. The DNA-based circuit is designed to be activated exclusively in the presence of the target analyte, thereby triggering the release of a DNA filament conjugated with an enzyme. In its turn, the released DNA-enzyme conjugated filament reacts with a specific enzymatic substrate, which catalyzes its conversion into a product that can be easily measured by means of known electrochemical and/or optical and/or colorimetric methods.

Figure 2 shows an illustrative general diagram of a circuit based on a DNA-enzyme conjugate for electrochemical detection of a target anti-digoxigenin

(anti-DIG) antibody; a) Two DNA filaments conjugated with an antibody recognition element (an antigen), modified with the digoxigenin small molecule, are designed in such a way that, upon formation of a link between the target anti-DIG antibody and two recognition elements, they will hybridize to form a complex capable of triggering a filament displacement reaction, thereby inducing the release of a DNA filament conjugated with the glucose-oxidase enzyme (DNA-GOx). Following hybridization with a complementary-DNA probe immobilized on the surface of a screen-printed gold-based electrode, the released enzyme-conjugated filament will output a current signal in the presence of a fixed concentration of glucose (200 mM); b) Chrono-amperograms obtained in the absence and in the presence of the anti-DIG antibody (100 nM); c) Values of the current signal recorded at increasing concentrations of the anti-DIG antibody; d) Percent signal variation values obtained at saturation concentrations (100 nM) of anti-DIG antibody, non-specific antibodies, and other control tests.

Figure 3 shows: a) Diagram of a circuit based on a DNA-GOx conjugate for detection of the target anti-dinitrophenol (anti-DNP) antibody; b) Chrono-amperograms obtained in the absence and in the presence

of the anti-DNP antibody (100 nM); c) Current signals recorded at increasing concentrations of the anti-DNP antibody; d) Percent signal variation values obtained at saturation concentration (100 nM) of anti-DNP antibody, non-specific antibodies, and other control tests.

Figure 4 shows circuits based on DNA-GOx conjugates which utilize, as conjugated recognition elements, peptide-PNA (Peptide Nucleic Acid) and protein-DNA for the recognition and measurement of three different target antibodies, namely a) Anti-MUC (anti-mucin-1) antibody; d) Cetuximab (chimeric monoclonal antibody, which recognizes the receptor of the epidermal growth factor (EGFR)); g) Bi-specific antibody recognizing a peptide of 15 amino acids of the mucin-1 protein and the EGFR protein; b), e), h) Dose-response curves at increasing concentrations of the specific target antibody; c), f), i) Percent signal variation values obtained at saturation concentration (100 nM) of target antibody, non-specific antibodies, and other control tests.

Figure 5 shows: a) A comparison between dose-response curves obtained with the circuit based on DNA-GOx conjugates and a commercial ELISA kit for detection of Cetuximab; b) Quantification of Cetuximab assessed by

analyzing serum samples supplemented with different known concentrations of Cetuximab (1 pM to 100 nM), with the percent recovery values obtained.

Figure 6 shows: a) General diagram for multiple detection of anti-DIG antibody and Cetuximab using two circuits based on DNA-enzyme conjugates in the same solution. Both targets are detected simultaneously and independently by means of two screen-printed gold-based electrodes, each one modified with a probe complementary to one of the two filaments released by the two circuits; b) Current signal values obtained with different combinations of the two targets.

Figure 7 shows: a) General diagram for optical detection of anti-DIG antibody using a circuit based on DNA-HRP (horseradish peroxidase) conjugates. The DNA-HRP filament released in the presence of the anti-DIG antibody reacts with its specific substrate (tetramethyl-benzidine (TMB)) and H_2O_2 to give a colored product, which can be measured with optical approaches; b) Absorbance signal increase calculated from the dose-response curve of the anti-DIG antibody.

Figure 8 shows: a) General diagram for colorimetric detection of anti-DIG antibody using a circuit based on DNA-GOx conjugates. The DNA-GOx filament released in the presence of the anti-DIG antibody is captured using

magnetic beads modified with streptavidin and a biotinylated DNA probe complementary to the released filament. In the presence of glucose, phenol, 4-aminotriptyn and HRP, the GOx enzyme catalyzes the formation of a colored product visible to the naked eye, which can be measured by means of known colorimetric methods; b) Absorbance signal increase dependent on anti-DIG antibody concentration.

Detailed Description of the Invention

As briefly described above, the method of the present invention is based on the use of a circuit based on DNA-enzyme conjugates, which provides a quantitative determination of antibodies and/or proteins and/or enzymes and/or small molecules and/or ions and/or other specific molecular targets. The method exploits the signal amplification caused by the presence of the enzyme, which guarantees high sensitivity (pM or pg/mL) of the platform for detecting specific antibodies and/or proteins and/or other target molecules. The DNA-based circuit comprises a set of three synthetic elements: a DNA duplex and two DNA filaments conjugated with a specific recognition element for the target analyte as previously described herein. The DNA duplex is designed to contain a single-stranded portion that

will act as "anchor" region for the DNA filament displacement reaction. In addition to this, said filament released upon activation of the filament displacement reaction is conjugated with an enzyme. In order to attain the activation of the DNA-based circuit, induced by the target analyte, two DNA filaments, conjugated with recognition elements for the target analyte, are rationally designed to form a bi-molecular complex only upon formation of a divalent bond between the target analyte and the two recognition elements. This functional bi-molecular complex can efficiently hybridize to the single-stranded portion of the DNA duplex, thereby triggering the release of the enzyme-conjugated output filament (the DNA-enzyme conjugate). The enzyme-conjugated filament thus released can be detected by means of electrochemical, optical or colorimetric methods (as shown in the annexed Figure 1 et seq.) in the presence of a specific enzymatic substrate that catalyzes its conversion into an easily measurable product.

Experimental Section

Example 1: detection of a target antibody by means of electrochemical methods

- *Reaction of displacement of the enzyme-conjugated DNA filament induced by the target antibody*

All reactions of displacement of the enzyme-conjugated DNA filament took place in a solution of 10 µL of PBS (50 mM Na_2HPO_4, 150 mM NaCl, pH 7.0) containing 100 nM of DNA duplex, 160 nM of both filaments conjugated with the recognition element, and different concentrations of the target antibody. The solution was incubated at 25 °C for 30 minutes to permit the reaction of displacement of the enzyme-conjugated DNA filament. For the electrochemical tests, a part of the displacement reaction solution (10 µL) was placed onto the working electrode (WE) of a screen-printed gold electrode for 30 min, then the same was washed with PBS and supplemented with 50 µL of 200mM glucose in PBS. The electrochemical signal was measured 10 min after the addition of said enzymatic substrate (200mM glucose) and monitored for 1 minute.

- *Modification of the screen-printed electrodes with the probe complementary to the DNA-enzyme conjugate*

The probe complementary to the DNA-enzyme conjugate (100 µM) was reduced for 1 hour in a solution of 0.4 mM of TCEP (tris(2-carboxyethyl)phosphine hydrochloride) prepared in a buffer solution containing 150 mM NaCl and 50 mM NaH_2PO_4, pH 7.0 to allow the reduction of the disulfide bonds. This solution was then diluted to the final concentration of 250 nM in

the same buffer. This latter solution (10 µL) was then placed onto the working electrode (WE) made of gold of the screen-printed electrode. After 1 hour of incubation, the screen-printed electrode was washed with distilled water to remove the excess unbound DNA; subsequently, 10 µL of 2 mM mercaptohexanol (prepared in 150 mM NaCl, 50 mM NaH_2PO_4, pH 7.0) were placed onto the working electrode to passivate the electrode's surface. After 1 hour and 30 minutes of incubation, the screen-printed electrode was washed with distilled water.

- *Electrochemical experiments*

All electrochemical measurements were taken at room temperature using an MUX8-R2 multiplexer potentiostat (Palmsens Instruments, the Netherlands). The DNA-based circuit was left to react in PBS and/or blood serum in a test tube for 30 minutes at 25 °C, and then a part thereof was transferred onto the surface of the screen-printed electrode as previously described. Following the addition of the enzymatic substrate (200mM glucose), experimental data were obtained by chrono-amperometry[6] at a potential of 0.6 V using the PSTrace 5.7v software (Palmsens Instrument).

The circuit based on DNA-enzyme conjugates is activated exclusively in the presence of the specific antibody,

or target analyte, which, by linking the conjugated recognition elements (e.g., digoxigenin (DIG) and dinitrophenol (DNP)) to the end of the synthetic DNA filaments, triggers the release of the enzyme-conjugated DNA filament (as shown in the annexed Figures 2 and 3).

The platforms were characterized as a function of the concentration of the target antibodies by drawing the titration curves of the anti-DIG and anti-DNP antibodies at concentrations ranging from 1 fM to 1 µM (as shown in the annexed Figures 2 and 3). An increased current signal was observed with increasing concentrations of the target antibody, up to a maximum percent signal variation of approximately 200% at saturation concentrations of the target antibody (100 nM). The detection achieved a sensitivity within the low femto-molar (fg/mL) concentration range for the target antibody, and turned out to be highly specific, in that no current signal was observed in the presence of non-specific antibodies or in the absence of one or two antigen molecules (as shown in the annexed Figures 2 and 3).

The platform can be adapted into a modular version also allowing the introduction of more complex recognition elements (peptide epitopes, proteins, etc.), e.g. by

using synthetic oligonucleotides of peptide nucleic acid (PNA) to achieve conjugation of peptide epitopes, since conjugation with synthetic DNA filaments would otherwise require complex and costly procedures. In the modular approach, said filaments conjugated with the recognition elements hybridize to the complementary synthetic oligonucleotides for detecting clinically relevant antibodies (as described by way of example in the annexed Figure 4).

This modular platform permits the detection of three distinct target antibodies directly in blood serum, namely: Cetuximab, the anti-MUC1 antibody, and a bi-specific antibody that simultaneously recognizes the EGFR protein (receptor of the epidermal growth factor) and a peptide residue of 15 amino acids of the MUC1 protein (mucin associated with the cell surface). The modular version of the platform shows levels of sensitivity and specificity comparable with those of its non-modular counterpart (as shown in the annexed Figure 4). The recovery percentages of serum samples supplemented with different concentrations of the target analyte (1 pM to 100 nM) for the platform programmed for detecting Cetuximab were also evaluated, obtaining recovery percentages ranging from 81% to 125%. Recovery is assessed by measuring different

analyte concentrations added to the actual samples (referred to as spiked samples).

$$\text{Recovery } (\%) = [(C1 - C2)/C3] \cdot 100$$

where:

C1: analyte concentration measured after addition

C2: analyte concentration measured before addition

C3: added concentration

The closer the recovery is to 100%, the better the result, although a 100% recovery does not necessarily indicate the absence of errors. The final result of the analysis can be expressed considering the correction made as a function of recovery. Values in excess of 100% may also be obtained.

Usually, any analytical method requires staying within a well-defined interval of recovery values.

The same serum samples, like those previously described herein, were also analyzed with a commercial ELISA kit used for Cetuximab quantification. The platform of the invention showed better sensitivity, detecting concentrations of target analyte below 10 pM; such low concentration values provide no signal that can be measured by the ELISA kit (as shown in the annexed Figure 5). Within the concentration range measurable also by the ELISA kit, comparable recovery percentage values were obtained.

The platform also allows for multiple detection, which occurs simultaneously and independently of the presence of different target antibodies in the same sample solution (as shown in Figure 6). In order to demonstrate this, two orthogonal (i.e., with no cross-reactivity) DNA circuits and two screen-printed electrodes were used, each one modified with a probe complementary to the filament released by each circuit in response to the presence of the specific antibody.

Example 2: detection of a target antibody by means of optical methods

- Reaction of displacement of the enzyme-conjugated DNA filament

All reactions of displacement of the enzyme-conjugated DNA filament took place in a solution of 100 µL of PBS (50 mM Na_2HPO_4, 150 mM NaCl, pH 7.0) using 100 nM of DNA duplex, 160 nM of both filaments conjugated with the recognition element, and different concentrations of the target antibody. The solution was incubated at 25 °C for 30 minutes to permit the reaction of displacement of the enzyme-conjugated DNA filament. For the optical tests, a part of the displacement reaction solution (100 µL) was placed for 30 minutes into a well of an immunoassay plate, where there was a capture probe complementary to the DNA-enzyme filament released in

the presence of the target antibody, followed by washing with PBS and addition of 100 µL of pre-dosed commercial 3,3',5,5'-tetramethylbenzidine (TMB) and H_2O_2. The optical signal was recorded 10 minutes after the addition of the enzymatic substrate, measuring the absorbance at 655 nm.

- *Optical tests*

All optical measurements were taken at room temperature by means of a spectrophotometer. The DNA-based circuit was programmed in such a way that, upon formation of a divalent bond between a target antibody and the two antigen-conjugated DNA filaments, an output DNA filament conjugated with horseradish peroxidase (HRP) enzyme would be released. The DNA-based circuit was left to react in a test tube for 30 minutes at 25 °C, and then a part thereof was transferred into a cuvette (or an immunoassay plate), where there was a capture probe complementary to the conjugated DNA-enzyme filament released in the presence of the antibody. After the addition of a standard concentration of pre-dosed commercial 3,3',5,5'-tetramethylbenzidine (TMB) and H_2O_2, HRP catalyzes the oxidation of TMB, generating a product having a blue color. The latter can then be measured using known optical approaches (e.g., by measuring the absorbance at 655 nm) (as shown in the

annexed Figure 7). The system was characterized by drawing a calibration curve with increasing concentrations of the target antibody ranging from 3 pM to 1 μM, and a concentration-dependent increase in the absorbance signal was observed.

All tests were carried out in a buffer solution containing 50 mM Na_2HPO_4, 150 mM NaCl, pH 7.0 at 25°C, as described in Example 1.

Example 3: detection of a target antibody by means of colorimetric methods

- Reaction of displacement of the enzyme-conjugated DNA filament

All reactions of displacement of the enzyme-conjugated DNA filament took place in a solution of 100 μL of PBS (50 mM Na_2HPO_4, 150 mM NaCl, pH 7.0) using 100 nM of DNA duplex, 160 nM of both filaments conjugated with the recognition element, and different concentrations of the target antibody. The solution was incubated at 25 °C for 30 minutes to permit the reaction of displacement of the enzyme-conjugated DNA filament. For the colorimetric tests, a part of the displacement reaction solution (100 μL) was placed into a test tube containing 10 μL of magnetic beads modified with streptavidin and a biotinylated DNA probe complementary to the released filament (1 mg/ml) for 30 min under agitation.

Afterwards, the magnetic beads were separated from the sample by means of a magnet, washed with PBS, and supplemented with 10 µL of a fixed concentration of a solution of glucose (200 mM), phenol (5 mM), 4-aminotriptyn (0.4 mM), and HRP (1 U mL^{-1}). The colorimetric signal was recorded after 10 minutes, measuring the absorbance at 505 nm.

- *Colorimetric tests*

All optical measurements were taken at room temperature using a spectrophotometer as in Example 2. The DNA-based circuit was left to react in a test tube for 30 minutes at 25 °C. In this example, after having been released from the DNA-based circuit, the DNA filament conjugated with the glucose oxidase (GOx) enzyme was isolated from the solution using magnetic beads modified with streptavidin and a biotinylated DNA probe complementary to the released filament. After the reaction, the magnetic beads were separated from the sample by means of a magnet. In the presence of a fixed concentration of a solution of glucose (ranging between 10 and 500 mM), phenol (5 mM), 4-aminotriptyn (0.4 mM), and HRP (1 U mL^{-1}), the GOx enzyme catalyzes the reduction of O_2 to H_2O_2, which in turn starts the reduction of phenol and 4-aminotriptyn, catalyzed by HRP, to form a quinonic product. The latter takes a

pink color, visible to the naked eye, which can be measured using colorimetric methods, e.g. by measuring the absorbance at 505 nM. The system was tested for detection of anti-DIG antibodies by drawing a calibration curve with increasing concentrations of the anti-DIG antibody (from 3 nM to 300 nM) and observing the increase in the colorimetric signal as a function of the concentration of the antibody (as shown in the annexed Figure 8).

Advantages of the Present Invention

The method of the present invention, as described above in detail, allows for the fast, specific and highly sensitive detection of antibodies and/or proteins and/or enzymes and/or small molecules and/or ions and/or other molecular targets in complex matrices of samples by exploiting the activation, induced by the target, of the DNA-based circuit based on DNA-enzyme conjugates. The advantages of the present method include rapidity (less than 70 minutes), high sensitivity (possibility of detecting low values, down to the low pico-molar (pM) or lower range, the possibility of using different detection techniques (electrochemical, optical, or colorimetric techniques), low cost, and possibility of

implementation at the point of care. The method of the present invention may also be useful to develop a multiplex platform for simultaneous detection of multiple different targets.

In comparison with the DNA-based sensors for target detection previously known in the art, the method of the present invention provides, by exploiting the enzymatic amplification of the signal, ultrasensitive detection of different targets. This makes the platform of the present invention extremely advantageous when used for detecting different classes of targets that are present at very low concentrations (pg/mL) in blood or in other complex clinical samples.

Exemplary Variants of the Present Invention

The method of the invention described herein can be implemented for detecting a wide range of targets in complex matrices of samples. The DNA-based circuit can be activated through the formation of a link with any target, e.g. an antibody, a protein, an enzyme, a small molecule, an ion, or any biomolecule, whose specific recognition element (the receptor) can be conjugated with synthetic oligonucleotides.

The method can be implemented for detecting small molecules by designing assays/tests that are

competitive with, and much more sensitive than, those currently known in the art (similar to those used in, for example, ELISA).

The method can also be implemented for detecting clinically relevant proteins recognizing specific DNA or RNA sequences as transcription factors (MITF, TFE3, TFEB, and TFEC) or as DNA repair enzymes (i.e. UDG, FPG).

The method can also be implemented for detecting targets recognized by aptamers.

The method can also be implemented for detecting important targets recognized by lectins, such as, for example, pathogenic bacteria, glycoproteins, or cells.

Applications of the method of the present invention other than the detection of clinically relevant targets are possible as well.

As a non-limiting example of the broad range of application of said method, the enzyme-DNA circuit reactive to an analyte (target) may also be used for controlling the translation and transcription of cell-free systems.

Industrial Applicability

The present invention has made it possible to provide a method for the specific and quantitative detection of

target antibodies and/or proteins and/or small molecules and/or ions in complex matrices of samples, which is based on the activation, induced by the target itself, of a circuit based on synthetic DNA and DNA-enzyme hybrids.

List of Cited Literature

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[5] S. Bracaglia, S. Ranallo, K.W. Plaxco, F. Ricci. Programmable, Multiplexed DNA Circuits Supporting Clinically Relevant, Electrochemical Antibody Detection. *ACS Sensors* **2021** 6 (6), 2442-2448

[6] K. Phasuksom, A. Sirivat. Chronoamperometric detection of enzymatic glucose sensor based on doped polyindole/MWCNT composites modified onto screen-printed carbon electrode as portable sensing device for diabetes. RSC Adv. **2022** 12 (44), 28505-28518.

CLAIMS

1. A method for the detection and quantification of target molecular analytes, with high sensitivity and specificity, directly in complex matrices of samples, said method comprising the following phases:

- activating a DNA-based circuit through induction by said target analytes, giving a functional bi-molecular complex capable of forming a link with a DNA-enzyme conjugated duplex;
- forming said link between said functional bi-molecular complex and said DNA-enzyme conjugated duplex;
- triggering a filament displacement reaction giving the release of a DNA-enzyme conjugated filament, wherein the amount of DNA-enzyme conjugated filament released is proportional to the concentration of the target analyte which induces the displacement reaction;
- detecting and quantifying the released filament, through known electrochemical, or optical, or colorimetric methods, in the presence of the related enzymatic substrate.

2. The method according to claim 1, wherein said target molecular analytes are selected from the group

comprising, or consisting of, antibodies and/or proteins and/or enzymes and/or small molecules and/or ions or others.

3. The method according to claim 2, wherein said molecular analytes are selected from antibodies.

4. The method according to any one of claims from 1 to 3, wherein said complex matrices of samples are selected from the group comprising, or consisting of, plasma, or serum, or blood, or saliva, or sweat, or analogues.

5. The method according to any one of the preceding claims, wherein said DNA-based circuit is formed of two DNA filaments conjugated with recognition elements of the analyte.

6. The method according to any one of the preceding claims, for simultaneous and independent detection of multiple different target analytes.

7. The method according to any one of the preceding claims, wherein the sensitivity of the method is within the low pico-molar or lower (1-10 fM) range.

8. The method according to any one of the preceding claims, the duration of which is less than 70 minutes.

9. Use of the method according to any one of the preceding claims for detecting and quantifying target molecular analytes, with high sensitivity and

specificity, directly in complex matrices of samples at the point of care.

Figure 1

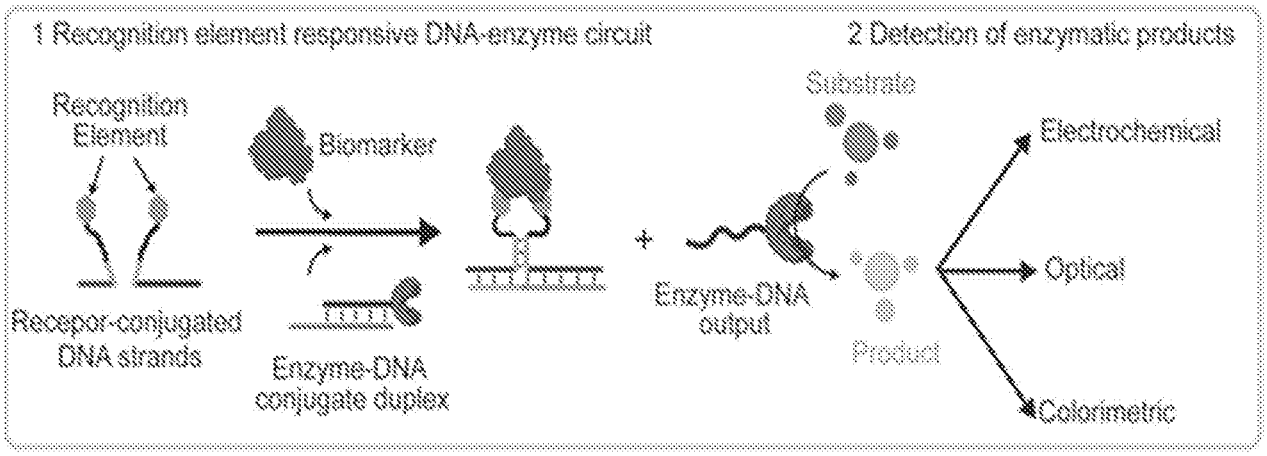


Figure 2

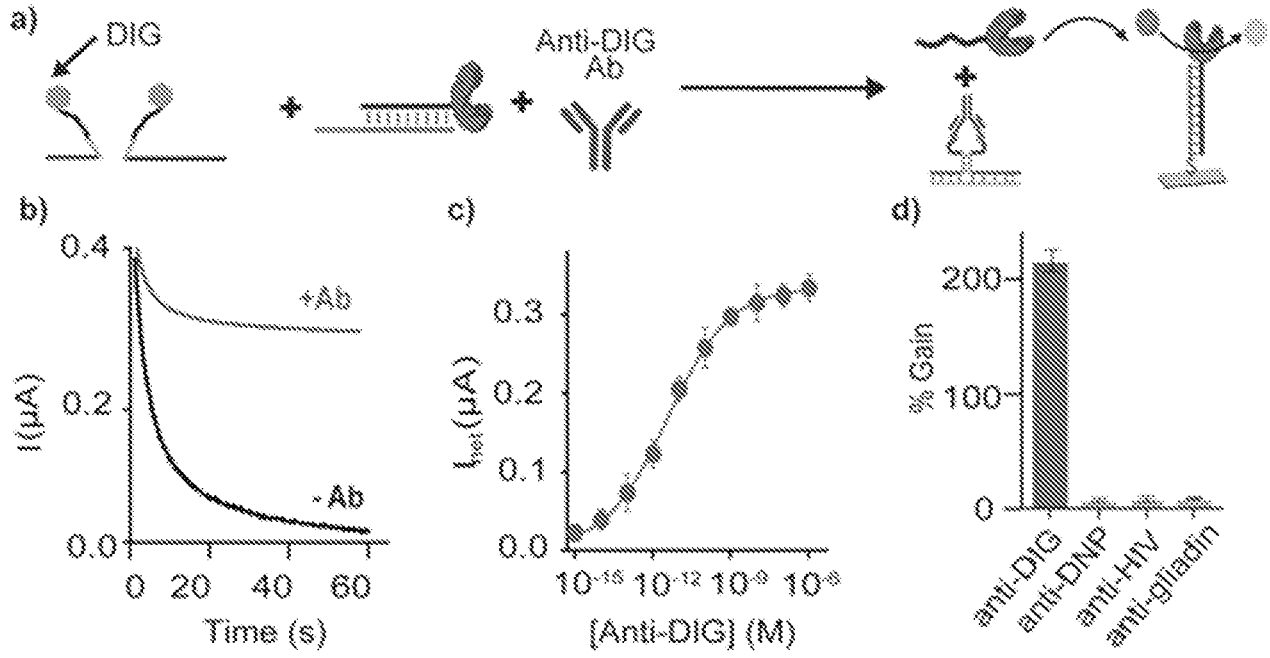
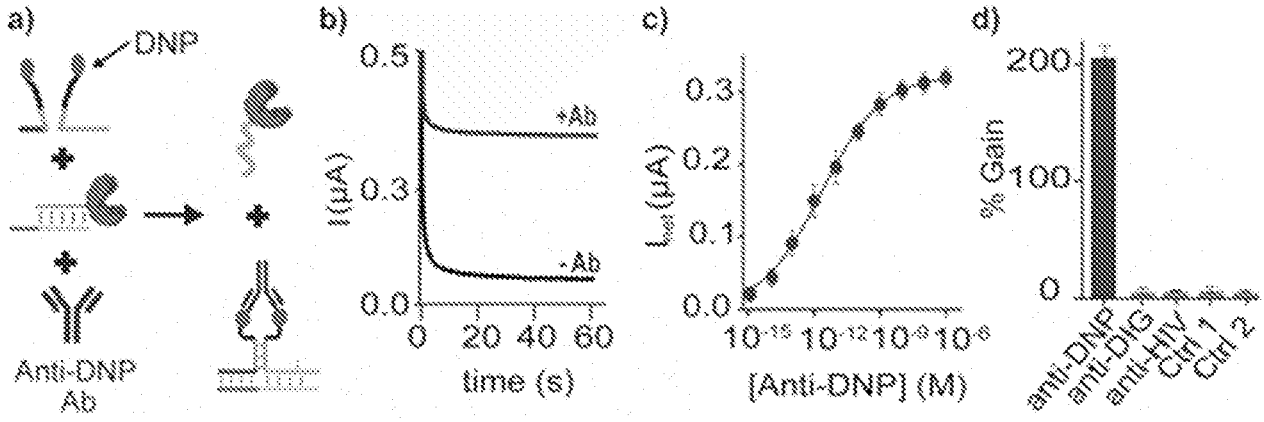


Figure 3



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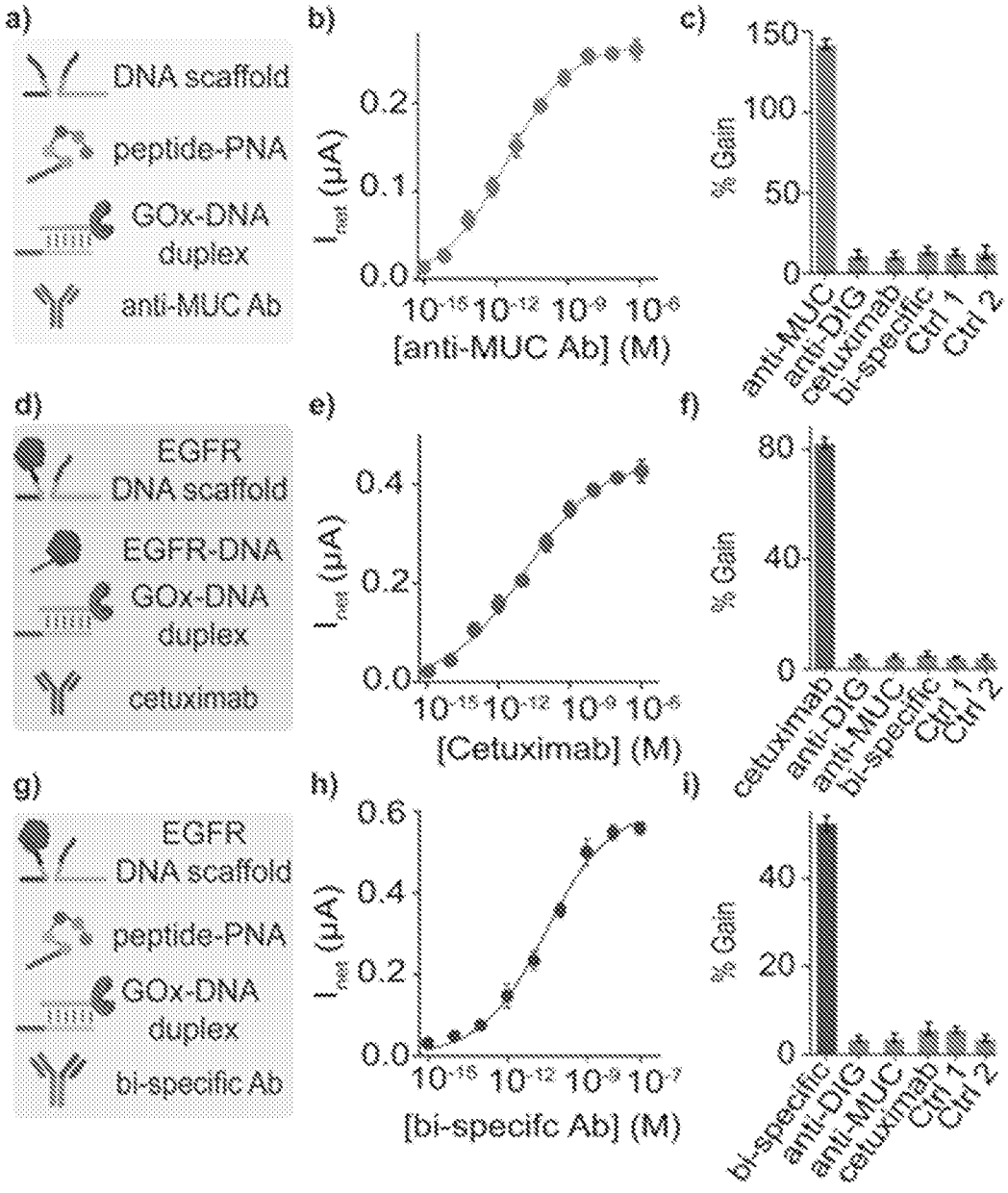
a)
DNA scaffold
peptide-PNA
GOx-DNA duplex
anti-MUC Ab
b)
I_{read} (µA)
0.0
0.1
0.2
10^{-15} 10^{-12} 10^{-9} 10^{-6}
[anti-MUC Ab] (M)
c)
% Gain
150
100
50
0
anti-MUC
anti-DIG
cetuximab
bi-specific
Ctrl 1
Ctrl 2
d)
EGFR DNA scaffold
EGFR-DNA
GOx-DNA duplex
cetuximab
e)
I_{read} (µA)
0.0
0.2
0.4
10^{-15} 10^{-12} 10^{-9} 10^{-6}
[Cetuximab] (M)
f)
% Gain
80
40
0
cetuximab
anti-DIG
anti-MUC
bi-specific
Ctrl 1
Ctrl 2
g)
EGFR DNA scaffold
peptide-PNA
GOx-DNA duplex
bi-specific Ab
h)
I_{read} (µA)
0.0
0.2
0.4
0.6
10^{-15} 10^{-12} 10^{-9} 10^{-7}
[bi-specifc Ab] (M)
i)
% Gain
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20
0
bi-specific
anti-DIG
anti-MUC
cetuximab
Ctrl 1
Ctrl 2

Figure 5

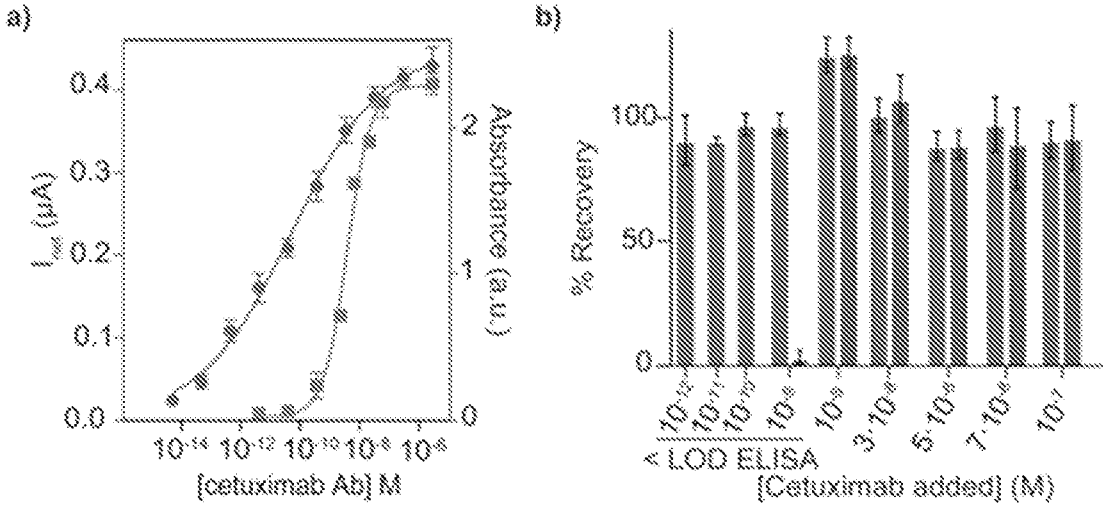


Figure 6

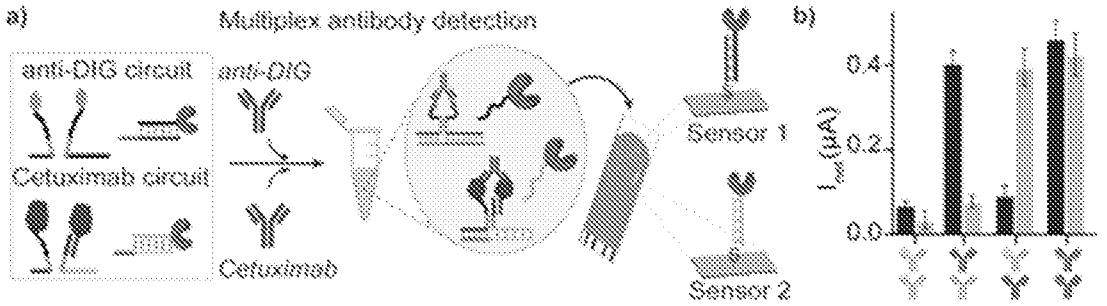


Figure 7

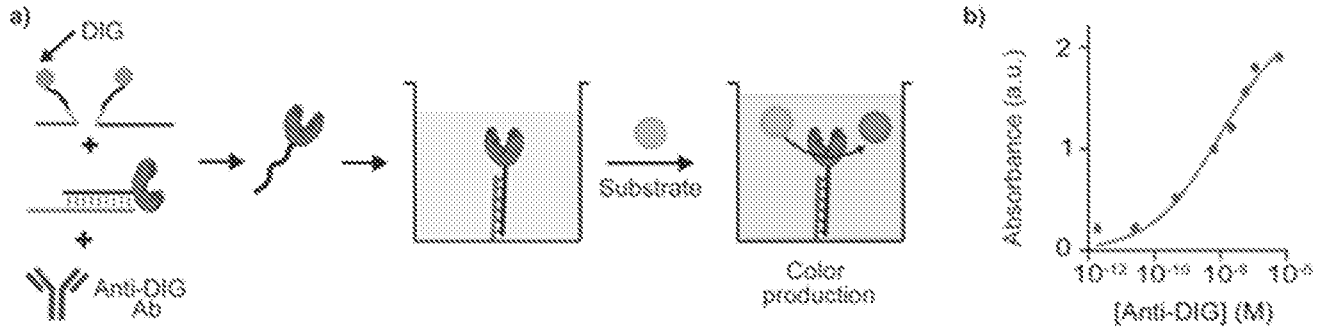
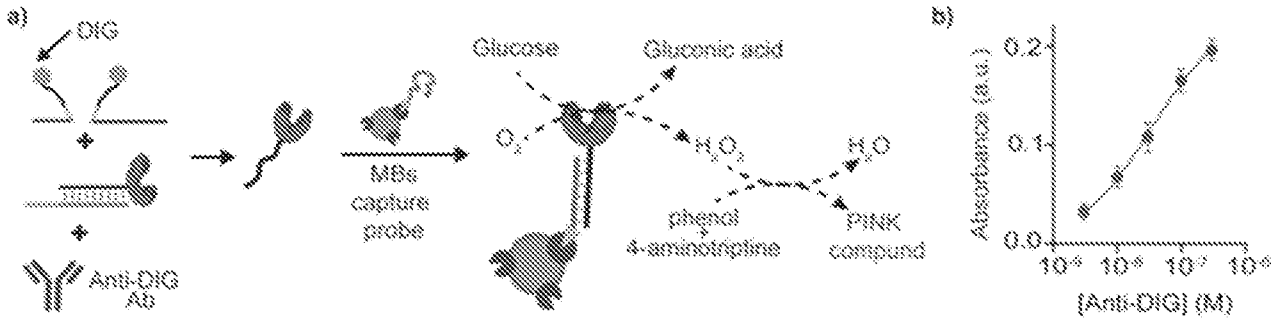


Figure 8


a)
DIG
Anti-DIG Ab
MBs capture probe
Glucose
Gluconic acid
O_2
H_2O_2
H_2O
phenol 4-aminotripline
PINK compound
b)
Absorbance (a.u.)
0.2
0.1
0.0
10^{-9} 10^{-8} 10^{-7} 10^{-6}
[Anti-DIG] (M)

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2024/056486

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/682 C12Q1/6825
ADD.

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)
C12Q

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)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FORTUNATI SIMONE ET AL: "Design of Specific Nucleic Acid-Based Biosensors for Protein Binding Activity", ANALYSIS & SENSING, vol. 2, no. 6, 9 August 2022 (2022-08-09), XP093104819, ISSN: 2629-2742, DOI: 10.1002/anse.202200037 abstract, Item 1.,p. 6/11 col. 1 and Fig. 2c, p. 7/11	1-9

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[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"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

"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

"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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 October 2024	31/10/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lapopin, Laurence

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2024/056486

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DESMET CLOÉ ET AL: "Paper electrodes for bioelectrochemistry: Biosensors and biofuel cells", BIOSENSORS AND BIOELECTRONICS, ELSEVIER SCIENCE LTD, UK, AMSTERDAM , NL, vol. 76, 28 June 2015 (2015-06-28), pages 145-163, XP029300770, ISSN: 0956-5663, DOI: 10.1016/J.BIOS.2015.06.052 abstract and p. 160 col. 1 4.	1-9
Y	US 2015/031014 A1 (LU YI [US] ET AL) 29 January 2015 (2015-01-29) [0003] and [0090]	1-9

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2024/056486

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2015031014 A1	29-01-2015	NONE	