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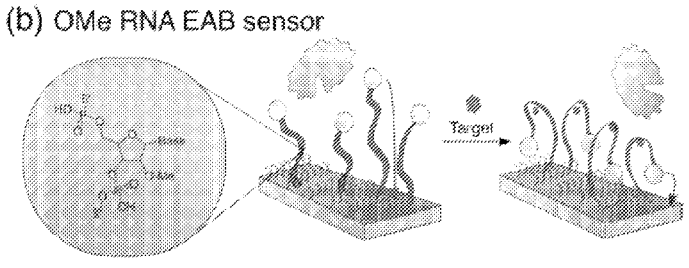


Figure 2B

(57) Abstract: Electrochemical aptamer-based (EAB) sensors support the high-frequency, real-time monitoring of analyte in vivo. Methods for reducing the sensor drift during in vivo placements are described. EAB sensors with non-natural nucleic acid can be used to reduce sensor signal drift. The EAB sensors have improved stability and reduced signal loss.

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# SYSTEMS AND METHODS FOR ELECTROCHEMICAL SENSORS WITH REDUCED SIGNAL LOSS

## CROSS-REFERENCE TO RELATED APPLICATIONS

**[001]** The current application claims the priority to U.S. Provisional Patent Application No. 63/506,560 entitled "Electrochemical Sensor Having Reduced Signal Loss" filed June 6, 2023. The disclosure of U.S. Provisional Patent Application No. 63/506,560 is hereby incorporated by reference in its entirety for all purposes.

## STATEMENT OF FEDERALLY SPONSORED RESEARCH

**[002]** This invention was made with government support under AI164483 and R01AI145206 awarded by the National Institutes of Health. The government has certain rights in the invention.

## SEQUENCE LISTING

**[003]** The instant application contains a Sequence Listing which has been submitted electronically in XML format and is hereby incorporated by reference in its entirety. Said XML copy, created on May 20, 2024, is named 08507PCT.xml and is 7 kilobytes in size.

## FIELD OF THE INVENTION

**[004]** The present invention relates to electrochemical sensors having nucleic acids configured to specifically detect an analyte in a biological fluid. The signal output by the present sensors is configured to resist undesirable loss of signal, and are therefore useful for monitoring of analytes over an extended period of time.

## BACKGROUND

**[005]** Electrochemical aptamer-based (EAB) sensors are known for the detection of a target analyte in biological fluids. The aptamer portion of the sensor is an oligonucleotide of defined base sequence that can selectively interact with a target analyte. In one version, the aptamer is coupled to the surface of a working electrode and a redox reporter is coupled to the free end of the aptamer. Binding of analyte to the aptamer causes a

conformational change in the aptamer thereby causing the redox reporter to move more proximal to the electrode. That movement in turn causes an increase in the rate of electron transfer ($k_{et}$) between the redox reporter and the electrode. This change in $k_{et}$ informs on the target analyte concentration in real-time and without the addition of exogenous reagents.

**[006]** EAB sensors can perform seconds and/or sub-seconds resolved measurements of multiple drugs and metabolites *in situ* in the living body, and to support closed-loop, feed-back controlled drug delivery. For *in vivo* applications, the sensor may include an aptamer-coated microneedle or wire as a working electrode. The microneedle or wire may be inserted through the surface of the skin such that the aptamer-coated portion contacts a biological fluid of the subcutaneous tissues. Alternatively, the sensor may be placed in another bodily compartment, such as a vein. The sensor can also include a counter electrode and a reference electrode. These electrodes can also be in the form of a microneedle, or a wire similarly inserted under the skin.

**[007]** The sensor electrodes may remain *in situ* for minutes, hours or even days and over that period provide clinically valuable information on the amount of analyte in the bodily fluid. Reasonable extrapolation to estimate amounts of analyte in the general circulation may be made. In this way, an EAB sensor can provide clinically relevant information on the amount of an exogenous analyte (such as a drug) or an endogenous analyte (such as a hormone) in the subject. The information may be used in the diagnosis, treatment, and/or monitoring of a disease. For example, where the analyte is a drug, information provided by the sensor may be used to optimize pharmacotherapy. For example, the amount of drug may be monitored to ensure the levels remain above a minimum effect concentration, but below a concentration where a toxic effect may arise. Drug dosage may be adjusted to ensure appropriate levels are maintained.

**[008]** Interrogation of an EAB sensor to detect target analyte requires the application of electrical potential waveform, with resulting current output by the working electrode being used to determine the amount of target analyte in solution. Square wave voltammetry (SWV) can be used for the *in vivo* detection of analyte given the ability to correct for loss (also termed "drift") in current output. To explain, the amount of an analyte reported by an EAB sensor tends to drift downwardly over time. Measuring sequential square wave

voltammograms at two different frequencies enables drift correction in an approach called kinetic differential measurements (KDM). KDM utilises the difference between relative SWV measurements taken at two frequencies to subtractively correct for drift.

**[009]** While drift correction methods are effective, after some time the current output by the sensor will inevitably decrease to a level at or below the noise floor. At that point, there is no signal detectable above the level of noise inherent in the system, and the sensor is no longer able to function in target analyte detection. It is generally accepted in the art that the electrical potential delivered to the sensor during interrogation has a role in causing the loss of aptamer from the surface of the working electrode. Fouling of the working electrode with biological material in the biological fluid under analysis (such as proteins, lipids, and whole cells) is also thought to be causative.

**[0010]** The problem of signal drift with the eventually loss of signal negatively impacts the usefulness of EAB sensors, especially for *in vivo* clinical applications where the accurate determination of analyte amount over an extended time is of great importance. As an example of one such application, the sensor may be configured to detect an antibiotic *in situ* in a subject by the electrodes contacting the blood plasma. Monitoring of the antibiotic over an extended time may be required to ensure that the dosage regime maintains concentrations of the drug *in vivo* over the minimum inhibitory concentration, while ensuring toxic concentrations are not reached. Typically, the antibiotic is monitored over a period of at least 24 hours, with prior art sensors being unable to sustain signal over such an extended period without significant loss of signal and a concomitant reduction in signal-to-noise ratio. Loss of signal may be addressed by continual replacement of the sensor, however there is a cost penalty in that approach. Repeated replacement of the sensor would require ongoing disturbance of the subject. Furthermore, replacement of a new sensor (and additionally the calibration of the sensor) takes time and can result in loss of data over the relevant changeover period.

**[0011]** The discussion of documents, acts, materials, apparatus, articles, and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the

present invention as it existed before the priority date of each provisional claim of this application.

## BRIEF SUMMARY

**[0012]** Summarized here and described in detail below are EAB sensors with improved performance such as (but not limited to) reduced signal loss.

**[0013]** Some embodiments include a working electrode for an electrochemical sensor, the working electrode comprising an electrically conductive element and an analyte sensing element associated therewith configured to specifically interact with a target analyte, wherein the sensing element comprises a non-natural nucleic acid coupled with a redox reporter.

**[0014]** In some embodiments, the non-natural nucleic acid is incapable of being read and/or duplicated by any natural mammalian cell.

**[0015]** In some embodiments, the non-natural nucleic acid has a greater resistance to degradation by a component of a biological fluid as compared with a similar natural nucleic acid.

**[0016]** In some embodiments, the similarity is in relation to any one of more of: length, base sequence, secondary structure, tertiary structure, and ability to interact with the target analyte.

**[0017]** In some embodiments, the biological fluid comprises a nuclease.

**[0018]** In some embodiments, the non-natural nucleic acid is formed from a single strand.

**[0019]** In some embodiments, the non-natural nucleic acid is a polymer.

**[0020]** In some embodiments, the non-natural nucleic acid comprises between 10 and 100 subunits.

**[0021]** In some embodiments, the non-natural nucleic acid is a chemical variant of a natural deoxyribose nucleic acid or a natural ribose nucleic acid.

**[0022]** In some embodiments, the chemical variant is to a sugar backbone and/or one or more bases.

**[0023]** In some embodiments, the non-natural nucleic acid is made by, or with assistance, of a human.

**[0024]** In some embodiments, the non-natural nucleic acid is a xeno nucleic acid (XNA) or a peptide nucleic acid (PNA).

**[0025]** In some embodiments, the non-natural nucleic acid is a DNA or RNA aptamer having an altered chemical structure.

**[0026]** In some embodiments, the non-natural nucleic acid is an altered form of a natural nucleic acid capable of specifically interacting with a target analyte, and the non-natural nucleic acid has an interacting capability of at least 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140%, or 150% that of the natural nucleic acid.

**[0027]** In some embodiments, the non-natural nucleic acid is an altered form of a natural nucleic acid capable of specifically interacting with a target analyte, and the non-natural nucleic acid has a sensitivity, precision, or specificity for recognition of the target analyte of at least 50%, 60%, 70%, 80%, 90%, or 100% of that of the natural nucleic acid.

**[0028]** In some embodiments, the non-natural nucleic acid is an altered form of a natural nucleic acid capable of specifically interacting with a target analyte, and the non-natural nucleic acid has an uncorrected signal loss of less than 90%, 80%, 70%, 60%, or 50% of that of the natural nucleic acid.

**[0029]** In some embodiments, the working electrode is a portion of an electrochemical sensor with an uncorrected signal drift rate, and the uncorrected signal drift rate is determined or averaged over a period of at least 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, or 72 hours.

**[0030]** In some embodiments, the non-natural nucleic is bound at a first end to the electrically conductive element.

**[0031]** In some embodiments, the redox reporter is bound to a second end of the non-natural nucleic acid.

**[0032]** In some embodiments, the electrically conductive element comprises a skin penetrating portion having the non-natural nucleic acid bound thereto.

**[0033]** In some embodiments, the electrically conductive element is a needle, a microneedle, or a wire.

**[0034]** Some embodiments include an electrochemical sensor apparatus comprising the working electrode of any one of claims 1 to 21, and a counter electrode.

**[0035]** Some embodiments comprise a reference electrode.

**[0036]** Some embodiments having associated therewith a retainer configured to retain the working electrode in contact with a bodily fluid of a subject.

**[0037]** In some embodiments the retainer is configured to retain the working electrode in contact with a bodily fluid of a subject for a period of at least 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours , 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, or 72 hours.

**[0038]** Some embodiments having associated therewith a housing configured to enclose a power source and/or electronics for functioning of the sensor.

**[0039]** Some embodiments include a method for monitoring a target analyte in a biological fluid of a subject, comprising contacting the working electrode of any one of claims 1 to 21 to the biological fluid for a period of time.

**[0040]** In some embodiments, the working electrode is contacted with a biological fluid that remains *in situ* within the subject for a duration of the method.

**[0041]** In some embodiments, the biological fluid is blood or interstitial fluid.

**[0042]** In some embodiments, the biological fluid has not been removed from the subject.

**[0043]** In some embodiments, the period of time is at least 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, or 72 hours.

**[0044]** Additional embodiments and features are set forth in part in the description that follows, and in part will become apparent to those skilled in the art upon examination of the specification or may be learned by the practice of the disclosure. A further understanding of the nature and advantages of the present disclosure may be realized by reference to the remaining portions of the specification and the drawings, which forms a part of this disclosure.

## BRIEF DESCRIPTION OF THE FIGURES

**[0045]** The description will be more fully understood with reference to the following figures, which are presented as embodiments of the invention and should not be construed as a complete recitation of the scope of the invention, wherein:

**[0046]** FIG. 1A is a diagrammatic representation of the response of EAB sensors to the presence of a target analyte in accordance with prior art.

**[0047]** FIG. 1B illustrates immersion of an EAB sensor employing a DNA sequence in whole blood (*in vitro*) at 37 ˚C in accordance with prior art.

**[0048]** FIG. 1C illustrates disposition of an EAB sensor employing a DNA sequence in the jugular vein (*in vivo*) of a rat in accordance with prior art.

**[0049]** FIG. 1D and FIG. 1E show graphs of normalised current *in vitro* and *in vivo* as a function of time obtained by SWV collected at two frequencies in accordance with prior art.

**[0050]** FIG. 1F and FIG. 1G show graphs of KDM signal *in vitro* and *in vivo* as a function of time in accordance with prior art.

**[0051]** FIG. 2A shows diagrammatically and chemically an EAB sensor employing a natural DNA molecule, and the destruction thereof by an agent in a biological fluid in accordance with an embodiment.

**[0052]** FIG. 2B shows diagrammatically and chemically an EAB sensor having a non-natural nucleic acid functioning similarly to the aptamer of FIG. 2A in interacting with a target analyte, although being resistant to degradation by the agent of FIG. 2A in accordance with an embodiment.

**[0053]** FIG. 2C shows a graph of relative sensor output signal as a function of time, comparing the output of sensors have natural versus non-natural nucleic acids as sensing elements in the presence of a nuclease in accordance with an embodiment.

**[0054]** FIG. 3A shows KDM signal as a function of tobramycin with natural and non-natural nucleic acids as sensing elements in accordance with an embodiment.

**[0055]** FIG. 3B shows a graph of normalized current as a function of time for sensors having separately natural and non-natural nucleic acids as sensing elements, when

disposed on the left and right jugular veins of a single rat challenged with an aminoglycoside antibiotic in accordance with an embodiment.

**[0056]** FIG. 3C shows KDM signal as a function of time for the sensors as used in FIG. 3B in accordance with an embodiment.

**[0057]** FIG. 3D shows concentration of the aminoglycoside as a function of time for the sensors as used in FIG. 3B in accordance with an embodiment.

**[0058]** FIG. 3E illustrates signals of DNA and Ome RNA aptamer sensors in accordance with an embodiment.

**[0059]** FIG. 3F and FIG. 3G illustrate charge transfer kinetics of the DNA aptamer sensor and the OMe RNA aptamer sensor respectively in accordance with an embodiment.

**[0060]** FIG. 3H illustrates normalized signal of the DNA aptamer sensor and the OMe RNA aptamer sensor in accordance with an embodiment.

**[0061]** FIG. 4A shows diagrammatically *in vitro* whole blood having immersed therein EAB sensors having either natural or non-natural nucleic acids as sensing elements in accordance with an embodiment. The graphs to the right show the relative sensor signal strength as a function of time for each sensor type.

**[0062]** FIG. 4B shows diagrammatically EAB sensors having either natural or non-natural nucleic acids as sensing elements disposed in the jugular vein of a rat in accordance with an embodiment. The non-natural nucleic acid sensor was inserted before the natural nucleic acid sensor. The graphs to the right show relative sensor signal strength as a function of time for each sensor type, and for each of the two rats treated.

**[0063]** FIG. 4C shows diagrammatically sensors having either natural or non-natural nucleic acids as sensing elements disposed in the jugular vein of a rat in accordance with an embodiment. The natural nucleic acid sensor was inserted before the non-natural nucleic acid sensor. The graphs to the right show relative sensor signal strength as a function of time for each sensor type, and for each of the two rats treated.

**[0064]** FIGs. 5A through 5F show a series of plots of charge transfer versus frequency (Lovric plots) extending over a 5-hour period demonstrating the time evolution of the electron transfer kinetics of EAB sensors placed under various conditions in accordance with an embodiment. FIG. 5A shows a Lovric plot for sensors fabricated from a natural nucleic acid being challenged with DNase *in vitro*. FIG. 5B shows a Lovric plot for sensors

fabricated from a non-natural nucleic acid being challenged with DNase *in vitro* in PBS buffer. FIG. 5C shows a Lovric plot for sensors fabricated from a natural nucleic acid being challenged with DNase *in vitro* in whole blood. FIG. 5D shows a Lovric plot for sensors fabricated from a non-natural nucleic acid being challenged with DNase *in vitro* in whole blood. FIG. 5E shows a Lovric plot for sensors fabricated from a natural nucleic acid *in vivo* in whole blood. FIG. 5F shows a Lovric plot for sensors fabricated from a non-natural nucleic acid *in vivo* in whole blood.

**[0065]** FIG. 6 illustrates an upper perspective view a microneedle embedding apparatus in accordance with an embodiment. The embodiment relies on the user to provide the motive force for insertion of the microneedles into the skin. The arm is shown in the first position as it is presented to the user, and before embedment of the microneedles in the skin.

**[0066]** FIG. 7A illustrates a lower perspective view of FIG. 6 in accordance with an embodiment.

**[0067]** FIG. 7B illustrates an upper perspective view of FIG. 6 in accordance with an embodiment.

**[0068]** FIG. 8 illustrates a lower perspective view of FIG. 6 in accordance with an embodiment, more completely showing the removable flexible layer that is removed to expose the dermatologically acceptable adhesive.

**[0069]** FIG. 9 illustrates in lower perspective view the microneedle embedding apparatus of FIG. 6 having the removable flexible layer removed to expose the dermatologically acceptable adhesive in accordance with an embodiment.

**[0070]** FIG. 10 illustrates in lower perspective view the microneedle embedding apparatus of FIG. 9 with the microneedles in an extended position for embedment in the skin of a subject in accordance with an embodiment.

**[0071]** Unless otherwise indicated herein, features of the drawings labelled with the same numeral are taken to be the same features, or at least functionally similar features, when used across different drawings.

**[0072]** The drawings are not prepared to any particular scale or dimension and are not presented as being a completely accurate presentation of the various embodiments.

## DETAILED DESCRIPTIONS

**[0073]** After considering this description it will be apparent to one skilled in the art how the invention is implemented in various alternative embodiments and alternative applications. However, although various embodiments of the present invention will be described herein, it is understood that these embodiments are presented by way of example only, and not limitation. As such, this description of various alternative embodiments should not be construed to limit the scope or breadth of the present invention. Furthermore, statements of advantages or other aspects apply to specific exemplary embodiments, and not necessarily to all embodiments, or indeed any embodiment covered by the claims.

**[0074]** Throughout the description and the claims of this specification the word "comprise" and variations of the word, such as "comprising" and "comprises" is not intended to exclude other additives, components, integers, or steps.

**[0075]** Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment, but may.

**[0076]** The term "subject" is used to refer to an animal (including a human and a non-human animal) to which the present invention may be applied.

**[0077]** As used herein, a "bodily fluid" may be any biological fluid of a subject, including but not limited to, interstitial fluid (ISF), blood, saliva, a lacrimal secretion, a lactational secretion, a nasal secretion, a tracheal secretion, a bronchial secretion, an alveolar secretion, a gastric secretion, a gastric content, a glandular secretion, a vaginal secretion, a uterine secretion, a prostate secretion, semen, urine, sweat, cerebrospinal fluid, a glomerular filtrate, an hepatic secretion, bile, or an exudate, any of which are contacted in use with an electrode of the invention.

**[0078]** The present invention is predicated at least in part on the inventors' discovery that non-natural nucleic acids can be used as detecting elements, having similar functionality to natural nucleic acid aptamers (such as DNA and RNA), but exhibiting significantly lower

rates of signal loss when used in an *in vivo* EAB sensor. The result is a sensor that can provide signal output above the noise floor for extended periods of time.

**[0079]** Many embodiments provide that degradation of aptamers in an EAB sensor by agents present in biological fluids is a significant cause, and possibly the chief cause of the signal loss seen *in vivo*. In several embodiments, the agent is a nuclease. These findings represent a revelation of the previously unknown underlying cause of the problem of signal drift. In light of that revelation, the inventors discovered that non-natural nucleic acids resisted signal drift in the context of an EAB sensor, whilst functioning similarly to (and in some circumstances better than) a natural nucleic acid aptamer in detecting an analyte.

**[0080]** These findings are at odds with the accepted dogma in the art that signal loss is due to loss of the aptamer occasioned by interrogating currents and fouling with biological materials.

**[0081]** Significant advantage is provided in applications where degradation of sensing aptamers in EAB sensors is problematic. Apart from advantages in *in vivo* applications as discussed above, certain *ex vivo* applications may benefit. In some embodiments, electrochemical sensors used to monitor the presence of an analyte in an industrial process involving biological materials may resist degradation over the course of a process run. Some embodiments provide *in vitro* testing of clinical samples in a pathology facility. While a sensor may only momentarily contact the clinical sample, significant degradation may nevertheless occur, leading to an unstable output signal. Moreover, a sensor may be washed and used for a number of samples and in such circumstances the service life of the sensor will be extended given its resistance to degradation.

**[0082]** As used herein, the term “non-natural nucleic acid” is intended to include a polymer that is biosimilar to a natural nucleic acid polymer such as DNA or RNA, but having a chemical structure that is altered and not found in nature. As a result of the altered structure, the non-natural nucleic acid may be more resistant than a natural nucleic acid against degradation (such as cleavage of a chemical bond) occasioned by agents found in biological fluids such as blood and the ISF. The agent may be a nuclease, such as a DNase or an RNase.

**[0083]** A non-natural nucleic acid may derive from a naturally occurring nucleic acid, but having had an alteration to its chemical structure such that the chemical structure is considered non-natural. More typically, the non-natural nucleic acid will be synthesised *de novo* in an altered form.

**[0084]** The term "non-natural nucleic acid" is not intended to include a nucleic acid that has been synthesised by, or with the assistance of man, but nevertheless having a natural chemical structure. While such molecules are not the product of nature, they nevertheless have the same chemical structure as a nucleic acid found in nature.

**[0085]** A non-natural nucleic acid molecule useful in the context of the present invention (i.e., as a sensing element associated with a working electrode of an EAB sensor) may be an altered form of an aptamer. For example, a DNA aptamer known to detect an analyte (such as an antibiotic drug) may be chemically modified to retain the ability to detect the analyte and yet resist degradation by an agent in a biological fluid. The non-natural nucleic acid may be an oligomer having a non-natural backbone, being a molecular analogue to DNA or RNA. Examples of non-natural backbone oligomers include, but are not limited to, 2'-fluoroarabinoside nucleic acid (FANA), 2'-O-methyl RNA, locked nucleic acid (LNA), and threose nucleic acid (TNA). Collectively, these non-natural backbone oligomers are referred to as xeno nucleic acids (XNAs). Because they are not produced in nature, XNAs are generally highly resistant to enzymatic degradation. In some embodiments, the non-natural nucleic acid may be a peptide nucleic acid (PNA).

**[0086]** Apart from the altered chemical structure which confirms stability in biological fluids, the present non-natural nucleic acids may share one or more features of prior art aptamer. Such features include, but are not limited to, length, base sequence (primary structure), secondary structure and tertiary structure. Aptamers are small (usually from 20 to 60 nucleotides) RNA or DNA oligonucleotides formed from a single strand and able to bind a target analyte with high affinity and specificity. Aptamers may be considered as nucleotide analogues of antibodies, but aptamer production is an *in vitro* cell-free process that is easier and cheaper than the production of antibodies by cell culture or *in vivo* methods.

**[0087]** One method of identifying aptamers useful in the context of the present invention is to use a method (such as SELEX) to identify a natural DNA or RNA aptamer, and to

then modify the identified aptamer to have a non-natural chemical structure. Alternatively, methods such as SELEX may be adapted by the use enzymes configured to synthesize and amplify non-natural nucleic acids in the first instance.

**[0088]** Aptamers can be selected from combinatorial library having a vast number (up to 1018) of different oligonucleotides. While RNA aptamers provide a greater structural diversity compared to DNA aptamers, their application is complicated by stability issues in the presence of RNases, high temperature and unfavorable pH.

**[0089]** Selection of an aptamer that is selective for a given analyte may be facilitated by a process known as SELEX (systematic evolution of ligands by exponential enrichment). The process may be considered as two alternating stages. In the first stage, the library oligonucleotides are amplified by a polymerase chain reaction (PCR) to the desired concentration. For the selection of RNA aptamers, the single-chained oligoribonucleotides are generated by *in vitro* transcription of double-stranded DNA with T7 RNA-polymerase. For DNA aptamers, a pool of oligodeoxyribonucleotides is generated by strand separation of double-stranded PCR products. In the second stage, the products of amplification are incubated with target analyte and oligonucleotides which bind the analyte used in the next SELEX round.

**[0090]** Separation of oligonucleotides with higher affinity for target drug and removal of unbound oligonucleotides are achieved through intense competition for binding sites. The selection pressure rises with every SELEX round. Sufficient enrichment of the oligonucleotide pool with aptamers with the strongest affinity for the target analyte can be achieved after about 5 to 15 rounds.

**[0091]** Once identified as useful, a non-natural nucleic acid may be associated with a working electrode of an EAB sensor. The working electrode may have at least one associated counter electrode and at least one associated reference electrode. Each working electrode may have a dedicated counter electrode, however in some embodiments the counter electrode is shared amongst some or all the assembled working electrodes. Each working electrode may have a dedicated reference electrode, however in some embodiments the reference electrode is shared amongst some or all the assembled working electrodes.

**[0092]** In many embodiments, an EAB sensor useful in the context of the disclosure may be voltametric, chronoamperometric, or impedimetric. In a voltametric sensor, a potential waveform is applied to the sensor interface, and the resulting current response is recorded. In chronometric approaches, a step potential is applied, and the resulting time-evolving current response is recorded. In impedimetric sensing, a sinusoidal potential waveform is applied, and the resulting sinusoidal current response is recorded.

**[0093]** EAB sensors are typically of the voltametric type, with the aptamer (or non-natural nucleic acid in accordance with many embodiments) being bound to the working electrode. Gold can be used as the probe surface for the working electrode. The aptamer has an associated redox-active species which acts as a reporter. The redox reporter can be (but not limited to) methylene blue. Upon target (e.g., drug) binding, the aptamer undergoes a conformational change, bringing the redox reporter more proximal to the working electrode surface. This increase in proximity increases electron transfer from the redox reporter to the electrode. The increase in speed of electron transfer contributes to a change in Faradaic current that is detected by a potentiostat. EAB sensors can be incorporated into a circuit having a reference electrode. The reference electrode is the site of a known chemical reaction that has a known redox potential. For example, a reference electrode based on the silver-silver chloride (Ag/AgCl) redox pair has a fixed and known potential forming the point against which the redox potential of the working electrode is measured. Also typically included in the circuit is a counter electrode which functions as a cathode or an anode to the working electrode. Because current does not pass through the reference electrode (due to an impedance of the potentiostat), any current generated is attributed to the working and counter electrodes. Current is measured as a function of potential of the interrogating electrode versus the reference electrode. The difference in potential produces the current in the circuit thereby generating an output signal. The signal quantifies target binding depending on electron transfer that is ideally stoichiometrically proportional to target binding.

**[0094]** The present apparatus, when assembled, is particularly suitable for use as a wearable apparatus, allowing measurements to be performed whilst the subject is undergoing normal activities and/or over a prolonged period. In several embodiments, the wearable apparatus may be a collar, a bracelet or other suitable jewellery piece, a watch,

a garment, a strap, an adhesive, or a patch. A person skilled in the art would appreciate that means may be provided to assist adhering and/or securing the wearable apparatus, when in use, to a subject, e.g., micro-anchors, or the like.

**[0095]** In some embodiments, the wearable apparatus may comprise a housing structure comprising one or more other components, such as (but not limited to) electronics processing unit. The electronics processing unit is configured to be in direct or indirect electrical communication with at least one conductive element (such as an electrode), and generally will include any one or more of a power source, a data processing unit, an analogue front-end, and a wireless transmitter.

**[0096]** In several embodiments, the housing structure may be configured to encase, at least partially, the apparatus, where the electrodes (such as microneedles) are exposed from a plane of the housing structure. The electrodes may be protected by a protective cover, which may be removed to expose the protruding electrodes before use.

**[0097]** In many embodiments, the apparatus may further comprise means for monitoring temperature or pH of the bodily fluid where validity of an output is dependent thereon, or where adjustment to operation or output is possible.

**[0098]** In several embodiments, the housing structure may be configured to encase and be coupled to the apparatus by any appropriate mechanism. Examples of the mechanisms include (but are not limited to) electromagnetic coupling, mechanical coupling, adhesive coupling, magnetic coupling, or the like. In some embodiments, the coupling mechanism enables the apparatus and the housing structure to be attached and detached, which would enable the housing structure and its other components to be re-usable, while the apparatus can be discarded and replaced with another apparatus as necessary.

**[0099]** In many embodiments, the wearable apparatus may further comprise a computer program product executable as a software application, resident on a mobile communication apparatus in communication with the electronics processing unit, wherein the computer program product is able to control one or more of (i) detection of electrochemical measurements conducted at the electrode-based platform, (ii) data analysis, (iii) data transmission, (iv) apparatus configuration, and (v) apparatus power management. Examples of suitable mobile communication apparatus include, but are not

limited to, smartphones, smartwatches, tablets, smart glasses, laptops or other personal computers.

**[00100]** In some embodiments, the apparatus itself comprises a processor with program instructions configured to drive onboard functions such as voltammetry and transmitting output to a remote apparatus via a wireless module, such as a Bluetooth™ module.

**[00101]** In some embodiments, the working electrode or any other electrode may be a wire, a needle, a microneedle, an electrode array, a microneedle array, which contact the ISF, blood or any other relevant bodily material of a subject. Microneedles and/or microneedle arrays are preferred for transdermal applications where piercing of the skin is necessary to contact the ISF.

**[00102]** Electrodes in accordance with many embodiments can be fabricated in a range of various shapes and geometries, although their specific geometry for transdermal applications be optimised to breach the stratum corneum for reliable skin penetration. In some embodiments, the apparatus may be configured to be urged into the skin of a subject to facilitate the electrodes breaching the stratum corneum and to penetrate through the skin layers. For non-human applications, the stratum corneum may be replaced by an analogous, or even a non-analogous layer on the surface of the subject.

**[00103]** Generally, each electrode will have the shape of a protruding pointed structure extending from the mount. Typically, the electrodes will extend generally perpendicular from the mount.

**[00104]** The protruding structure of each electrode can be of any needle-type shape. In some embodiments, protruding structure may taper smoothly from a base to form a pointed tip (e.g., cone shape), may have multiple lateral sides extending from a base that converge to form a pointed tip (e.g., pyramid shape or triangular prism), be tapered in just one dimension, or have a base with curved sides of relatively constant diameter, which is segmented to form a pointed tip (e.g., a segment of a cylindrical shape). Typically, the pointed tip will be sharp. The electrode may or may not include shape changes along its length. Further, any edge or side of the shape may be bevelled, curved, or rounded.

**[00105]** In some embodiments, the shape of the electrode is a cone, or a pyramid such as a triangular pyramid, square pyramid, or hexagonal pyramid. In other

embodiments, the shape of the electrode is a tetrahedron or a triangular prism. In further embodiments, it may take the shape of a rocket, turret, arrowhead, spike, or spear.

**[00106]** It will be appreciated that a range of other shapes could be used. In certain embodiments, the shape may a circular or an elliptical cylinder, which is truncated. Any of the other shapes described herein may or may not be truncated. The term "truncated", as used in this context, may refer to a shape cut on a plane parallel to the base, which may be referred to as a parallel-truncated shape or more specifically, a frustum, or a shape cut at an angle relative to an axis of the electrode, which may be referred to as an angular-truncated shape. For angular-truncated shapes, the angle of truncation relative to an axis of the shape will be at least about 50° and no more than about 75°. It will be appreciated that the same or different shapes could be provided on a single EAB sensor.

**[00107]** The exterior wall of the microneedle may have a smooth or rough surface, and can include surface features, such as (but not limited to) raised portions, etchings, serrations, anchors, barbs, or the like, which may assist engaging a biological tissue once the electrodes have breached the stratum corneum to secure them within the subject. It will be appreciated that the ability of an EAB sensor to remain *in situ* is particularly beneficial, as this ensures that continuous measurements over a prolonged period are made at the same site within the subject. Furthermore, constraining the location in which measurements are performed ensures more accurate longitudinal monitoring. In some embodiments, the EAB sensor is configured to remain *in situ* for at least one minute, or at least one hour, or at least about 8 hours, or at least about 18 hours, or at least one day (about 24 hours), or at least about 3 days, or at least about 4 days, or at least one week. In some applications it may be necessary or desirable to remain *in situ* for one month or more.

**[00108]** It will be appreciated that the size of the electrodes, and their arrangement on the mount, may vary depending upon the intended application.

**[00109]** In many embodiments, the electrodes may be of a length at least greater than the thickness of the stratum corneum and to penetrate the skin layers to a depth of at least 100 μm, to be positioned in a biological tissue to contact a bodily fluid of a subject. In some embodiments, the length will be at least about 10% greater than the thickness of the stratum corneum, or at least about 20% greater than the thickness of the stratum

corneum, or at least about 50% greater than the thickness of the stratum corneum, or at least about 75% greater than the thickness of the stratum corneum, or at least about 100% greater than the thickness of the stratum corneum. In some embodiments, the length is less than about 1500 μm, or less than about 1000 μm, or less than about 500 μm, or greater than about 100 μm, or greater than about 50 μm, or greater than about 20 μm, or greater than about 10 μm. In some embodiments, the length is between about 100 μm and about 1000 μm.

**[00110]** In some embodiments, the electrodes have a tiered arrangement and thus would not all be of the same length.

**[00111]** The base width of the electrodes may be at least less than about 50% of the length, or less than about 25% of the length, or less than about 20% of the length, or less than about 15% of the length, or less than about 10% of the length, or less than about 5% of the length. In some embodiments, the base width is at least about 100 μm but no more than about 400 μm. In some embodiments, the diameter is about 200 μm, or about 300 μm.

**[00112]** The diameter of the electrodes may be at least less than about 50% of the length, or less than about 25% of the length, or less than about 20% of the length, or less than about 15% of the length, or less than about 10% of the length, or less than about 5% of the length. In some embodiments, the diameter is between at least about 0.1 mm and no more than about 5 mm. In some embodiments, the diameter is between about 0.5 mm and about 1 mm.

**[00113]** It may be desirable for one microneedle to penetrate more deeply into the skin as compared to another microneedle. The two microneedles may therefore terminate at different distances from the skin surface, or at different distances from an electrode mounting portion. In some embodiments, the two microneedles are different lengths. In some embodiments, the microneedles are the same length, and a mounting portion is configured to axially displace one microneedle relative to the other. In certain embodiments, the mounting portion may be multi-levelled with a first electrode extending from a first level and a second electrode extending from a second level.

**[00114]** In many embodiments, the electrodes may be arranged in pairs, in groups, or as a matrix. A pair arrangement would comprise an even number of electrodes. A group

arrangement may comprise between 1 and about 5 groups, with each group comprising between about 4 to about 8 electrodes. A matrix arrangement may comprise either an even or odd number of electrodes as such an arrangement may or may not have the same number of rows and/or columns. In some embodiments, the electrodes are arranged in matrix selected from the group consisting of 2x2, 2x3, 2x4, 2x5, 2x6, 3x2 3x3, 3x4, 3x5, 3x6, 4x2, 4x3, 4x4, 4x5, 4x6, 5x2, 5x3, 5x4, 5x5, 5x6, 6x2, 6x3, 6x4, 6x5, and 6x6. In any arrangement, the electrodes may be spaced less than about 5 mm, or less than about 4 mm, or less than about 3 mm, or less than about 2 mm, or less than about 1 mm, or less than about 0.5 mm, and more than about 0.1 mm, from each other. The space may be measured from the centre-to-centre point of each respective electrodes.

**[00115]** The present invention will now be more fully described by reference to the following non-limiting examples.

EXEMPLARY EMBODIMENTS

**[00116]** The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for.

EXAMPLE 1: Materials and sources

**[00117]** The following materials were used in Examples 2 to 5.

**[00118]** *In vitro* sensors were made from 0.2 mm diameter gold wire (99.99) insulated with polyolefin heat-shrink tubing (0.05",0.017", 0.007"). For *in vitro* tests, a commercial Ag|AgCl(s) reference electrode and a commercial platinum reference electrode were used. Sensors used for *in vivo* measurements consisted of 0.2 mm diameter gold wire, 0.005 in. diameter platinum wire (99.99% purity) and 0.005 in. diameter silver wire (99.99% purity). The insulation used for these sensors was polytetrafluoroethylene heat-shrink (PTFE, 0.02", 0.005", 0.003", black).

**[00119]** Sodium hydroxide, 6-mercapto-1-hexanol, Tris (2-carboxyethyl) phosphine, sulfuric acid, and DNase were used. Phosphate buffered saline (PBS) was diluted from a 20x stock. Heparinised whole bovine blood was used. Tobramycin Sulphate was used. The T37 methylene blue- and HO-C6S-S-C6-modified DNA sequence and other methylene blue- and HO-C6S-S-C6-modified sequences including the 2'-O-methyl sequences were used.

EXAMPLE 2: Fabrication of EAB sensors

**[00120]** *In vitro* sensors were made with 3 mm length exposed and insulated with polyolefin. (See, e.g., Leung et al; ACS Sensors 2021, 6 (9), 3340–3347; the disclosure is incorporated by reference.) Following manufacturing of these sensors, they were then electrochemically cleaned. *In vivo* sensors were made by bundling gold (working electrode), platinum (counter electrode) and silver (reference electrode) wires in parallel to one another. These wires were insulated from one another using the polytetrafluoroethylene heat-shrink and bundled together in a staggered manner. The gold wire was located at the bottom with exposed length of 3 mm, followed by the 6 mm exposed platinum and then finally, the 1 cm exposed length of silver. Once bundled together, this three-electrode sensor was immersed overnight in household bleach to chlorinate the silver electrode. The three electrodes were subsequently rinsed with Millipore water prior to electrochemical cleaning.

**[00121]** The gold portion of both the *in vitro* and *in vivo* sensors were electrochemically cleaned in NaOH followed by roughening in $H_2SO_4$ using a CH1040C potentiostat. The cleaning involved cycling the potential between -1.0 V and -2 V at 2 V/s for 1000 times in 0.5 M NaOH. This was followed by roughening of the gold wire in 0.5 M $H_2SO_4$ with the application of 20 ms pulses at 0 V and 2.2 V for 32000 times to increase the electrode's microscopic surface area. The gold electrodes were subsequently analysed by cyclic voltammetry in 0.5 M $H_2SO_4$ (between 1.5 and -0.35 V at 1 V/s) to determine their electroactive surface area. The electrodes were then thoroughly rinsed with Millipore water prior to DNA deposition. At this point, *in vivo* sensors were then inserted into a 20G catheter.

**[00122]** Prior to depositing DNA onto the gold, the HO-C6S-S-C6 modified aptamers were firstly deprotected by combining 14 µL of 10 mM tris (2-carboxyethyl) phosphine with 2 µL of 100 µM DNA for 1 h in the dark. Following this deprotection, the DNA was diluted to 500 nM in PBS. The electrochemically cleaned sensors were then immersed and rinsed into the DNA solution in the dark. Following a 1-hour incubation period, the sensors were transferred to a 10 mM solution of 6-mercapto-1-hexanol made in PBS and stored overnight before use.

| | |
|---|---|
| Unstructured DNA | HO-$C_6$S-S-$C_6$-5' ATTAT TTTTT ATTTA TTTTT ATTTT ATTTT ATTTT TT 3' MB |
| Unstructured 2'-O-methyl RNA | HO-$C_6$S-S-$C_6$-5' OMeA OMeU OMeA OMeU OMeU OMeU OMeU OMeU OMeU OMeA OMeU OMeU OMeU OMeA OMeU OMeU OMeU OMeU OMeU OMeA OMeU OMeU OMeU OMeU OMeA OMeU OMeU OMeU OMeU OMeA OMeU OMeU OMeU OMeU OMeU 3' MB |
| Aminoglycoside DNA Aptamer | HO-$C_6$S-S-$C_6$-5'-GGGACTTGGTTTAGGTAATGAGTCCC-Me 3' MB |
| Aminoglycoside 2'-O-methyl RNA Aptamer | HO-$C_6$S-S-$C_6$-5'-MeOG-MeOG-MeOG-MeOA-MeOC-MeOU-MeOU-MeOG-MeOG-MeOU-MeOU-MeOU-MeOA-MeOG-MeOG-MeOU-MeOA-MeOA-MeOU-MeOG-MeOA-MeOG-MeOU-MeOC-MeOC-MeOC-3' MB |

EXAMPLE 3: Electrochemical measurements

**[00123]** *In vitro* electrochemical measurements were done generally using SWV. (See, e.g., Leung et al; ACS Sensors 2021, 6 (9), 3340–3347; the disclosure is incorporated by reference.) All sensors were first tested at room temperature in a shot glass with 1x PBS prior to use as a quality check. They were then transferred to a shot glass of whole blood at 37˚C or PBS containing 2.5 mM $CaCl_2$ and 1.5 mM $MgCl_2$ with immediate commencement of electrochemical measurements. For the DNase experiment, after obtaining a stable baseline, the measurement was paused, the sensors quickly removed, 5 µg/mL DNase added, mixed the solution, and then resumed the measurement. The sensors were repeatedly interrogated at multiple square wave frequencies (5, 7, 10, 15, 30, 50, 70, 100, 200, 250, 300, 600 and 1000 Hz) and the square wave frequency close to the methylene blue electron transfer rate used to monitor the sensor degradation.

**[00124]** *In vivo* electrochemical sensors were deployed in the jugular veins of live rats. For experiments involving monitoring the degradation of DNA and 2'-O-methyl RNA sensor analogues (using the T37 sequence and OMe37 sequence), the sensors were repeatedly interrogated at multiple square wave frequencies, using the square wave frequency close to the methylene blue transfer rate for analysis. For experiments involving the tobramycin detecting sensors, SWV was performed at frequencies 100 Hz and 200 Hz in an alternating fashion. Signal drift was corrected by using these two frequencies to calculate a KDM signal as follows:

$$KDM = \frac{\left(\frac{i_{200Hz}}{i_{200Hz,t=0}} - \frac{i_{100Hz}}{i_{100Hz,t=0}}\right)}{0.5 \times \left(\frac{i_{200Hz}}{i_{200Hz,t=0}} + \frac{i_{100Hz}}{i_{100Hz,t=0}}\right)}$$

where $i_{200Hz}$ and $i_{100Hz}$ are the peak heights at their respective frequencies. Peak heights $i_{200Hz,t=0}$ and $i_{100Hz,t=0}$ denote peak heights measured prior to injection of target. Concentrations of tobramycin found in the vein were back-calculated using a Langmuir fit of the calibration curve.

EXAMPLE 4: *In vivo* surgeries and dosage

**[00125]** All *in vivo* experiments were performed in male Sprague-Dawley rats (4-5 months old). The rats weighed between 350-500 g and were pair-housed in a standard light cycle room (12:12 regular light cycle with lights on at 8AM). They were allowed ad libitum access to food and water.

**[00126]** Prior to the measurement, the rats were anesthetized using 4% isofluorane in a Plexiglas anesthesia chamber and then maintained anesthesia via a nose cone during the entire length of the experiment using a 2-2.5% isofluorane/oxygen mixture. The neck was shaved and dissected to surgically isolate the left and right jugular veins. A small incision was made in each vein using spring-loaded microscissors that allowed for insertion of the in-vivo sensor and catheter with an infusion line (for heparin delivery). Both the sensors and infusion lines were anchored using two sterile 6-0 silk sutures. Prior to any recording, 30 units of heparin were infused through the infusion line (jugular delivery for tobramycin experiments and femoral vein for the aminoglycoside). To

intravenously dose the rats (30 mg/kg) with tobramycin, a precalculated volume of 0.1 M tobramycin sulphate diluted in PBS was injected using a syringe pump via the femoral vein.

EXAMPLE 5: Experiments and discussion of results

**[00127]** The EAB sensors support the high-frequency, real-time measurement of specific molecules *in situ* in the living body. Signaling in this class of sensors occurs when a binding-induced conformational change in the target analyte-recognizing aptamer alters the rate of electron transfer from an attached redox reporter. This signal transduction mechanism renders EAB sensors independent of the specific chemical structure or reactivity of their target, as aptamers can be generated to bind to a wide range of small-molecule analytes. Thus, EAB sensors are the first *in vivo* molecular monitoring approach that is generalizable across multiple types of analytes. Consistent with this, EAB sensors can perform seconds and/or sub-seconds resolved measurements of multiple drugs and metabolites *in situ* in the veins, brains, and subcutaneous ISF of live animal subjects and support closed-loop, feed-back controlled drug delivery.

**[00128]** FIGs. 1A through 1G illustrate electrochemical Aptamer-based (EAB) sensors support continuous, real-time molecular monitoring in unprocessed biological fluids both *in vivo* and *in situ* in the living body in accordance with prior art. FIG. 1A shows EAB sensors each having a gold electrode to which a redox-reporter-modified aptamer is attached via the formation of an alkane-thiol-on-gold self-assembled monolayer. Upon exposure to target, the aptamer undergoes a conformational change that alters the electrochemical behavior of the redox reporter (here methylene blue), producing an easily measured electrochemical signal. When an EAB sensor is challenged in 37˚C whole blood (FIG. 1B) *in vitro* (commercially sourced bovine blood) or (FIG. 1C) *in vivo* (*in situ* in the rat jugular). FIG. 1D and FIG 1E show the electrochemical peak currents it produces drifts downward over time (shown are data collected at the indicated pair of square wave frequencies). FIG. 1F and FIG. 1G show that this drift can be corrected by taking the difference between the relative currents observed at pairs of square-wave frequencies at which the signal drifts in concert, a drift correction approach termed Kinetic Differential Measurements (KDM). The good return to baseline *in vitro* as the sensor is placed back

in drug-free blood or *in vivo* as the drug is eliminated from the body indicates the accuracy with which this approach corrects drift. However, although KDM drift correction ensures that EAB sensors remain accurate, the loss in peak current associated with the drift reduces their signal-to-noise ratio, ultimately reducing precision (e.g., the standard deviation of the signal in FIG. 1G increases from 0.008 prior to the drug injection to 0.020 after 4 h in vivo). The data shown here are for vancomycin-detecting EAB sensors, which were challenged with vancomycin at the indicated times. The return to baseline as the sensor is (*in vitro*) placed back in drug-free blood or as (*in vivo*) the drug is eliminated from the body indicates the accuracy with which KDM corrects drift.

**[00129]** A limitation of EAB sensors is that they suffer from time-dependent loss in signal when deployed in bodily fluids, an effect that occurs both *in vitro* (FIG. 1D) and *in vivo* (FIG. 1E). As this "drift" can be accurately corrected using dual-frequency square wave approaches (FIG. 1F, FIG. 1G), such as KDM, or circumvented by using interrogation method like chronoamperometry or electrochemical impedance spectroscopy, it does not affect measurement accuracy. Nevertheless, the loss in signaling current reduces signal-to-noise ratios (irrespective of the approaches used to correct or circumvent it), an effect that ultimately harms measurement precision (see, e.g., the increased noise at the right-hand side of FIG. 1G).

**[00130]** Prior artisans have characterized the mechanisms underlying EAB sensor drift *in vitro* in undiluted whole blood held at 37°C. Under these conditions, the drift is dominated by electrochemically induced loss of their target-recognizing aptamer (presumably due to loss of the attaching, thiol-on-gold monolayer) and fouling of the electrode surface by proteins and cells, with former easily avoided by judicious selection of the potential window employed in their interrogation. In the following experiments, however, it is demonstrated that the *in vivo* drift behavior of EAB sensors differs from that seen in whole blood *in vitro*. And by uncovering the origins of this difference, many embodiments provide hardware and methods by which the *in vivo* drift of EAB sensors can be significantly reduced.

**[00131]** In bodily fluids *in vitro*, enzymatic degradation of the aptamers that EAB sensors employ for target recognition is only a minor contributor to the drift. It has been previously shown, for example, that the significant signal loss seen for EAB sensors

challenged in undiluted whole blood at 37˚C is about 80% recoverable upon washing the sensors with concentrated urea, indicating that the physical removal of DNA from the surface, such as would occur upon enzymatic degradation, contributes relatively little to the drift seen under these conditions. Some prior artisans have likewise shown that, despite the exceptional DNase resistance of non-natural L-enantiomer DNA oligonucleotides ("spiegelmers"), the in vitro blood and serum drift properties of sensors fabricated using this polymer are similar to those seen for natural D-DNA-based sensors deployed under the same conditions. It is worth noting, however, that the days-old, commercially sourced bovine blood and plasma used in these studies could differ significantly from blood *in situ* in the veins of a live animal. For example, DNases are known to be active in circulating blood. Given the potential for important differences between these two experimental conditions, many embodiments provide the mechanisms underlying the drift seen for EAB sensors emplaced in the jugulars of live rats.

**[00132]** The blood-driven drift of EAB sensors *in vivo* differs significantly from that seen *in vitro*. In several embodiments, sensor-like constructs were first employed comprised of an unstructured oligonucleotide sequence (i.e., lacking significant internal complementarity) fabricated using either DNA or 2' methoxy-ribonucleic acid (OMe RNA), a non-natural xeno nucleic acid (XNA) that is relatively resistant to enzymatic degradation.

**[00133]** A device employing an unstructured oligomer of the XNA 2' methoxy RNA (OMe RNA) is less prone to degradation by DNase than a device employing the equivalent, unstructured DNA oligonucleotide. FIGs. 2A through 2C illustrate the enzymatic degradation of oligonucleotides significantly reduced by the introduction of non-natural oligonucleotide XNAs in accordance with an embodiment. To confirm that this holds for the 2' methoxy-ribose backbone (OMe RNA) XNA employed here, some embodiments fabricate EAB sensor analogs employing either DNA (FIG. 2A) or OMe RNA (FIG. 2B) oligonucleotides modified on their 3' ends with methylene blue and on their 5' ends with a thiol for surface attachment. The sequences used lack any significant self-complementarity to avoid any effects that might arise from the differential population of secondary structure. In FIG. 2C, when challenged with DNase in PBS (containing the necessary divalent cations and held at 37°C), the signal from the DNA-employing device falls monotonically. In contrast, after small initial drop the signal from the OMe RNA-

employing device stabilizes. Shaded areas indicate the standard deviation of (n=4) independently fabricated devices; no significant difference can be observed in the reproducibility of devices fabricated using either DNA or XNA.

**[00134]** Given this observation and the likely greater activity of DNases in fresh blood *in vivo* than aged blood *in vitro*, several embodiments explore whether the use of OMe RNA might reduce the *in vivo* drift seen for EAB sensors when placed *in vivo*. To test this, however, may need the availability of an XNA aptamer that binds a molecular target suitable for use in *in vivo*. That is, a target that is non-toxic at concentrations well above the sensor's limit of detection and is cleared from the blood at a pace rapid relative to the few-hour time frames afforded by experiments on anesthetized animals. While the development of aptamer selection schemes is amenable to the selection of XNA aptamers, no XNA aptamer can simultaneously fulfill both needs. For example, most of the XNA aptamers reported to date bind proteins, a class of targets that generally clears rather slowly from plasma. There is one small-molecule-binding XNA aptamer that can bind the mycotoxin orchratoxin, but the toxicity precludes its use in *in vivo* studies.

**[00135]** Many embodiments generate new, small-molecule-binding XNA aptamers for the desired *in vivo* applications. Several embodiments use nucleic acid receptor binding the aminoglycoside antibiotics. The aminoglycosides, including tobramycin, gentamicin and kanamycin, bind the prokaryotic ribosomal RNA, disrupting translation and inhibiting bacterial growth. Previous report included that a 14-base RNA hairpin excised from the prokaryotic ribosomal RNA folds in vitro into a conformation that binds to this class of drugs with micromolar affinity. Motivated by the clinical importance of measuring the aminoglycosides (the therapeutic windows of these nephrotoxic and ototoxic drugs are quite narrow), the utility of using adapting this RNA into an EAB sensor was investigated. (See, e.g., A. A. Rowe, et al., Anal. Chem. 2010, 82, 7090–7095; the disclosure of which is incorporated by reference.) Unfortunately, although the resulting sensor achieved clinically relevant limits of detection, precision, and specificity, the RNA "aptamer" rapidly degraded when challenged in unprocessed biological fluids. An unexpected solution to this problem became apparent upon inspection of the atomically detailed structure of the RNA/drug complex. Specifically, (1) the hairpin forms a structure between the B form of a DNA helix and the A form of an RNA helix and (2) the

aminoglycoside binds in the major groove of the hairpin, on the opposite side of the double helix from the 2' hydroxyls that distinguish RNA from DNA. Given these observations, it may be worth testing whether, in this unusual and specific case, the otherwise naïve approach of simply replacing the RNA sequence with DNA might not significantly alter the structure or the drug-binding properties of the nucleic acid. The test showed that while its affinity is reduced significantly (by a factor of 4 relative to that of the equivalent RNA construct), the DNA construct also binds aminoglycosides.

**[00136]** Several embodiments provide that the same sequence synthesized from OMe RNA might also bind to the aminoglycosides, given the likelihood that methoxy-RNA resembles RNA more closely than DNA does. In several embodiments sensors employing the equivalent OMe RNA sequence not only bind the antibiotic tobramycin but with higher affinity than that of sensors employing the equivalent DNA aptamer. This increase in affinity significantly improves the *in vivo* limit of detection for the OMe RNA aptamer. Specifically, when placed in vivo the root-mean-squared noise observed prior to drug challenge corresponds to limits of detection (at a coefficient of variation of 3) of 1.2 μM and 9.9 μM for sensors employing the OMe RNA or DNA aptamers, respectively.

**[00137]** FIGs. 3A through 3D illustrate the use of an aminoglycoside binding OMe RNA sequence significantly reduces the drift seen for an EAB sensor deployed *in situ* in the jugular vein of a live rat in accordance with an embodiment. In FIG. 3A, the aminoglycoside detecting EAB sensors using either OMe RNA or DNA sequences that bind to the aminoglycoside antibiotics are shown and their binding to the drug tobramycin *in vitro* in whole bovine blood at 37°C is characterized. Error bars indicate the standard deviation of (n=4) independently fabricated sensors. In FIG. 3B, a DNA-employing and an OMe RNA-employing sensor are placed into the left and right jugular veins, respectively, of a rat. After 3 h, the animal was challenged with a 30 mg/kg dose of tobramycin via a femoral catheter. The signal arising from the OMe RNA-employing sensor drifts relatively little under these conditions (shown are the peak currents at 100 and 200 Hz, normalized to the first voltammogram). Specifically, at just 7%, the drift seen for the OMe RNA employing sensor is nearly 7-fold less than the 48% signal loss seen for the DNA-employing sensor over the course of this 5 h in vivo experiment. FIG. 3C shows that while the drift seen for both sensors can be corrected using KDM, the loss in

signal associated with the DNA construct ultimately reduces precision by reducing signal-to-noise ratios. Note that the peak KDM signal seen (upon drug challenge) for the sensor employing OMe RNA is due to the higher tobramycin-binding affinity of this sequence (FIG. 3A). FIG. 3D shows that conversion of these KDM signals to concentrations enables the reproducible estimation of plasma drug levels. The slightly lower $C_{max}$ seen for the OMe RNA sensor is associated with a slower rise to $C_{max}$, suggesting that it may be due to a restriction (such as the formation of a blood clot or the sensor intruding into the vein wall) slowing transport of drug to that sensor. The duration of these experiments was limited by animal welfare concerns, which restrict the time we can maintain animals under anesthesia. FIG. 3E illustrates signals of DNA and Ome RNA aptamer sensors in accordance with an embodiment. FIG. 3E shows a zoomed in view of the signal change immediately before and after 30 mg/kg intravenous tobramycin injection into the femoral vein of the rat. The injection is delivered to the DNA and OMe RNA aptamer sensors into the left and right jugulars, respectively, of a single rat. The slight delay (about 2.5 min) before $C_{max}$ is reached for the OMe RNA sensor suggests that the transport of drug to this sensor may be restricted, due to a blood clot or the working electrode having intruded into the vein wall. Either effect would slow transport of drug to that sensor, leading to the observed, somewhat lower $C_{max}$.

**[00138]** To ascertain whether the *in vivo* drift performance of an OMe RNA-employing sensor is improved relative to that of a DNA-employing sensor, one of each is placed in the left and right jugular veins, respectively, of an anesthetized rat. While the signal of the DNA-employing device fell by 48% after 5 h under these conditions, the signal of the equivalent OMe RNA device fell by just 7% (FIG. 3B), a difference that holds across a range of square-wave frequencies. Although KDM drift correction accurately corrects the drift seen for both (FIG. 3C), after just a few hours the greater signal loss from the DNA-employing sensor noticeably degrades its signal-to-noise ratio. Critically, both sensors remain functional in the animal, as demonstrated by an intravenous tobramycin (30 mg/kg) challenge. Due to the higher affinity of the OMe RNA aptamer (FIG. 3A), the KDM signal change this challenged produced is notably larger for the OMe RNA sensor (FIG. 3C). When these signals are converted to concentration, however, the two data sets produce closely similar concentration-time profiles (FIG. 3D). FIGs. 3F and

3G illustrate charge transfer kinetics of the DNA aptamer sensor and the OMe RNA aptamer sensor respectively in accordance with an embodiment. When the sensors employing aminoglycoside-binding DNA (FIG. 3F) or OMe RNA (FIG. 3G) constructs *in vivo* are challenged, the drift seen for both is largely independent of the square-wave frequency employed. Charge transfer kinetics shown here are recorded prior to dosing of tobramycin. FIG. 3H illustrates normalized signal of the DNA aptamer sensor and the OMe RNA aptamer sensor in accordance with an embodiment. Discordant *in vivo* drift is seen between the aminoglycoside-binding constructs at the frequency that best matches the electron transfer rate, 70 Hz.

**[00139]** The use of OMe RNA significantly improves the *in vivo* drift properties of the aminoglycoside detecting EAB sensor contrasts with the prior observation that the use of this backbone reduces drift only slightly when EAB sensors are employed *in vitro* in undiluted whole blood held at 37°C. (See, e.g., K. K. Leung, et al., *ACS Sensors* 2021, *6*, 3340–3347; the disclosure of which is incorporated by reference.) To elucidate the origins of this discrepancy, several embodiments characterize the *in vivo* performance of devices employing unstructured constructs of DNA and OMe RNA, to eliminate any complexities that might arise due to differences in secondary or tertiary structure. Previously, devices employing these simple constructs exhibit significant initial drift when placed in vitro in 37°C whole blood, albeit with the OMe RNA-employing device drifting somewhat less. Following this rapid decline, the signals produced by both devices then "level off," suggesting that it arises due to some saturable effect, such as fouling. Building on this foundation, some embodiments repeated these experiments *in vivo* in the left and right jugulars of four live rats. Under these conditions, both DNA- and OMe RNA-employing devices once again exhibit a rapid initial loss in signal. As was true *in vitro*, the signals arising from OMe RNA-employing devices then level off and remain relatively steady. The signal from the DNA-employing device, however, continued to fall for the duration of our experiments, behavior that contrasts with the same device's behavior in blood *in vitro*. These observations suggest that, while both the *in vivo* and *in vivo* drift seen for the OMe RNA devices is dominated by a mechanism that eventually saturates, such as fouling, the *in vivo* drift seen for the DNA-employing device arises predominantly due to some non-saturable process, such as enzymatic degradation.

**[00140]** FIGs. 4A through 4C illustrate EAB sensor behaviors in *in vitro* and *in vivo* tests in accordance with an embodiment. In FIG. 4A, when challenged *in vitro* in whole blood at 37°C, sensors fabricated using DNA and OMe RNA both exhibit a rapid loss in signal, after which their signals remain stable. Shaded areas indicate standard deviations for (n=4) independently fabricated sensors. In FIG. 4B and FIG. 4C, the OMe RNA analog behaves similarly when placed *in vivo* in the rat jugular. In contrast, the DNA analog continues to lose signal for the duration of these *in vivo* experiments. The surgical insertion of each probe inside the lumen of the jugular vein takes approximately 3-4 min, causing a delay between when the sensors are exposed to the *in vivo* environment and when measurements start. Hence, shown here are examples in which FIG. 4B the OMe RNA sensor was inserted before the DNA sensor, and FIG. 4C when this order was reversed.

**[00141]** Several embodiments provide characterization of the electron transfer kinetics of the EAB sensors under various *in vitro* and *in vivo* conditions. These characterizations provide support that DNase-driven degradation dominates the *in vivo* drift seen for DNA-employing devices. To see this, charge transfer from each device can be measured as a function of square-wave frequency, exploring first a DNA-employing device challenged with DNase in buffer.

**[00142]** FIGs. 5A through 5F illustrate the time evolution of the electron transfer kinetics of EAB sensors placed under various conditions in accordance with an embodiment. These measurements provide insights into the origins of the signal loss seen when they are deployed in blood *in vitro* and *in vivo*. Lovric plots of the amount of charge transfer as a function of frequency over 5 h measurement durations are shown in FIGs 5A through 5F. The arrow indicates the magnitude of the signal loss overtime and whether it corresponds to a decrease in the electron transfer rate as indicated by a shift in the peak of the charge transfer distribution. When challenged *in vitro* with DNAs in phosphate buffered saline at 37°C the signals from sensors employing an unstructured (FIG. 5A) DNA or (FIG. 5B) OMe RNA construct decrease at all frequencies, indicating that, while the number of methylene blue molecules on the surface is falling, the transfer kinetics of the reporters that remain on the surface is otherwise unchanged. Notably, the magnitude of this drop is smaller for the OMe RNA construct, reflecting its relative

nuclease resistance. FIGs. 5C and 5D, in contrast, when immersed in whole bovine blood in vitro at 37˚C both devices exhibit a rapid, initial reduction in charge transfer and a concomitant shift to lower frequencies. After this, the electron transfer properties of both devices stabilize. FIGs. 5E and 5F show that when challenged *in situ* in rat jugular veins the behavior of the two devices diverges radically. Specifically, the DNA-employing device exhibits a dramatic drop in charge transfer at all frequencies, an effect that closely resembles the behavior seen upon enzymatic degradation *in vitro* (FIG. 5A). This contrasts with the charge transfer behavior of the OMe RNA sensor, which instead mimics that seen for the OMe RNA sensor when challenged in whole blood *in vitro* (FIG. 5D), suggesting that fouling dominates this sensor's in vivo drift.

**[00143]** Under these conditions, the magnitude of the charge transfer decreases dramatically and continuously throughout the experiment with the rate of transfer remaining constant (FIG. 5A). This observation is consistent with the loss of methylene blue due to DNA cleavage where the number of methylene blue redox reporters are reduced without alteration of the transfer kinetics of those that remain. Under these same conditions, the decrease in charge transfer seen for the OMe RNA-employing device is much smaller. Like the case for DNA, the decrease also occurs equally at all frequencies (FIG. 5B), suggesting that it too, arises due to the (more limited nuclease-driven degradation of this oligonucleotide. When the two devices are challenged *in vitro* in 37˚C whole blood, conditions under which have previously shown that fouling dominates drift, their charge transfer initially falls and shifts to lower frequencies before ultimately stabilizing (FIGs. 5C and 5D). This presumably occurs as proteins adsorb to the surface, inhibiting access of the redox reporter and slowing electron transfer. In contrast, the behavior of the two devices diverges more significantly when they are challenged in blood *in vivo* (FIG.s 5E and 5F). Specifically, the behavior of the DNA-employing device *in vivo* is similar to its behavior when it is challenged *in vitro* in DNase-containing buffer: the signal drifts downward throughout the duration of our experiments without any significant change in transfer rate (compare FIGs. 5A and 5E). In contrast, the behavior of the OMe RNA-employing device *in vivo* is similar to its behavior when challenged *in vitro* in whole blood: the magnitude of the charge transfer falls rapidly at first, with a concomitant reduction in transfer rate, before both effects then level off (compare FIGs. 5D and 5F).

These observations argue that the *in vivo* behavior of devices employing DNA and OMe RNA differ at an important, mechanistic level, and suggest that, while the *in vivo* drift seen for the OMe RNA devices is dominated by fouling, which eventually saturates, the drift seen for the DNA-employing device arises due DNase-driven loss of the oligonucleotide.

**[00144]** In many embodiments, the use of a DNase-resistant OMe RNA aptamer significantly reduces the drift observed when an aminoglycoside-detecting EAB sensor is employed intravenously. In several embodiments, after 5 h in the jugular vein of a live rat the in vivo drift of an aminoglycoside sensor employing an OMe RNA aptamer is 7-fold less than that of a sensor employing the equivalent DNA aptamer. Follow-up mechanistic studies in accordance with several embodiments employing simple, model oligonucleotides (i.e., lacking secondary or tertiary structure) suggest that this occurs because enzymatic degradation is a major contributor to the drift seen for EAB sensors deployed *in vivo*, an observation that contrasts with prior studies suggesting that fouling dominates the drift seen *in vitro* in 37˚C blood. Several embodiments provide the applications of XNA aptamers in *in vivo* duration of EAB sensors and other aptamer-based technologies.

EXAMPLE 6: Wearable microneedle-based sensor apparatus

**[00145]** The working electrode (and any other electrodes) described herein may be configured as microneedles and incorporated into a wearable sensor apparatus, and exemplary type being shown in FIG. 6, FIG. 7A, FIG. 7B, FIG. 8, FIG. 9, and FIG. 10.

**[00146]** The apparatus comprises an upper housing portion (25) and a skin contacting portion (30). Also provided is a removable flexible layer (90) being graspable by way of the tab (95), the removal of which exposes a dermatologically acceptable adhesive on the skin contacting surface (35). The adhesive is for the purpose of retaining the apparatus on the subject's skin for an extended period. The flexible layer (90) functions to prevent curing or drying of the adhesive, prevent contamination of the adhesive layer before use and/or premature attachment of the adhesive to packaging, or to other surfaces. In a particularly preferred embodiment, in addition to covering the adhesive layer, the flexible layer (90) extends over the spaces (45) to prevent

contamination of the microneedles (15) and also help prevent unintended needle-stick injuries to a user.

**[00147]** The apparatus may have a retaining portion functioning to retain the apparatus on the skin such that the projecting portions remain in contact with a biological fluid of the subject. The retaining portion may be dedicated to that function or may perform another function.

**[00148]** In many embodiments, a retaining portion being or comprising a dermatologically acceptable adhesive will be useful. Adhesives allow for simplicity in application of the apparatus by a user, often requiring only the removal of a protective backing sheet to expose the adhesive and then contacting the exposed adhesive to the skin. This method of application is similar to the application of a sticking plaster and is therefore already a familiar process to users.

**[00149]** As an alternative to the use of adhesives, the retaining portion may be some mechanical means for maintaining the apparatus in the required position on the skin. For example, the apparatus may comprise a dedicated strap that engages about limb that is adjustable so as to keep the apparatus firmly applied to the subject. As an alternative, the apparatus may be incorporated into a wearable item such as a glove or a shirt, or an item of jewellery such as a ring which functions to retain the apparatus in position. The apparatus may be configured to engage with a discrete wearable item (such as by complimentary hook-and-loop means) or may have the wearable item integral therewith.

**[00150]** In some embodiments, the apparatus is retained simply by the wearable item bearing against the housing. For example, the retaining portion may be a snug-fitting elasticised glove which is worn over the apparatus.

**[00151]** In some embodiments, the retaining portion is any surface or part of the apparatus which contacts the skin of the subject, with a feature of the subject being at least partially responsible for maintaining the apparatus in place on the subject. For example, the apparatus may be configured to be retained between two parts of the body normally in close apposition, or within an existing anatomical structure. The apparatus may be shaped and/or dimensioned to be retained between the toes, the buttocks, in the groin, in the buccal cavity, in a nostril, in the ear canal, or in the umbilicus.

**[00152]** In some embodiments the apparatus housing is shaped and/or dimensioned to snugly fit over a digit, a toe, or an ear, for example. The apparatus housing may be elastically deformable, composed of a rubberised material for example, and configured to be stretched over any anatomical part (such as a finger).

**[00153]** Each of the embodiments can be a retaining portion in the context of the present invention.

**[00154]** The apparatus further comprises a releasing member (100) having a grasping portion (105) and a wedging portion (110), the function of which will be more fully described *infra*.

**[00155]** Turning now to the exploded views of FIG. 7A and FIG. 7B components that are analogous to those in earlier figures will be immediately apparent.

**[00156]** In certain embodiments, the motive force responsible for moving the arm (205) thereby urging the microneedles (15) into the underlying skin is provided by the user. In use, the user places a finger on the upper housing (25) and pushes downwardly. Furthermore, the arm (205) is movable by way of a hinging arrangement.

**[00157]** The hinging arrangement is provided by way of opposing lugs (115) extending from skin contacting portion (30), each lug comprising an aperture. The arm (205) comprises opposing laterally extending discs (120), each of which seats into an aperture of the lugs (115). It will be apparent that the arm (205) is able to hinge relative to skin contacting portion (30) to allow movement from the first position to the second position.

**[00158]** The arm (205) is presented to the user having the arm in the first position. The arm (205) is maintained in the first position by the wedging portion (110) of the releasing member (100). Before removal of the releasing member (100) the wedging portion inserts between the skin contacting portion (30) and the arm (205), thereby keeping the microneedles within the apparatus.

**[00159]** When intending to apply the apparatus to the subject's skin, the user removes the flexible layer (90) by pulling on the tab (95) to expose the adhesive layer on the skin contacting surface (35). The apparatus is then applied to the skin, with the adhesive maintaining it *in situ* for an extended period.

**[00160]** Once the apparatus has been applied to the skin, the user grasps the grasping portion (105) and pulls laterally to the left (as drawn), to completely remove the releasing member (100). The releasing member (100) has no further function and is discarded at this juncture. By removal of the releasing member (100) the arm (205) is released from the first position and permitted to move (under a downward force exerted by the user) into the second position whereby the lower face of arm (205) contacts the upper face of the skin contacting portion (30). In the second position, the microneedles (15) extend through the spaces (45) and into the underlying skin.

**[00161]** As will be appreciated, the releasing member (100) may be configured to prevent the upper housing (25) of the apparatus from closing to the skin contacting portion (30) when not intended by the user. The releasing member (100) is inserted or otherwise juxtaposed between the upper housing (25) and the skin contacting portion (30) to prevent closure of the upper housing (25) towards the skin contacting portion (30) sufficient to allow the tips of the microneedles (i.e., projecting portions) to protrude from the base of the holes in the skin contacting portion (30). Preventing closure also prevents movement of the arm (205) from the first position to the second position. Thus, when the releasing member (100) is in place, the tips of the microneedles cannot be inadvertently accessed to cause microneedle contamination or injury. In using the apparatus, the user removes the releasing member (100) as a step in the use process. In a preferred embodiment of apparatus use, the user first adheres the apparatus to the subject's skin and then removes the releasing member (100), prior to pressing the upper housing (25) to insert the microneedles into the skin.

**[00162]** Prior to removal by the user, the releasing member (100) can be kept in place by any one of a variety of features. In one example the releasing member (100) comprises protrusions that fit into recesses in either the upper housing (25), the skin contacting portion (30) or both the upper housing (25) and the skin contacting portion (30) to assist in retaining it in place until intentionally removed. In another example the releasing member (100) is designed to be slidably assembled to the skin contacting portion (30) or upper housing (25), such that friction between the releasing member (100) and either the upper housing (25) or the skin contacting portion (30) assists in keeping it in place until intentionally removed. In yet another example magnetic force may be used

to assist in keeping the releasing member (100) in place. In a one embodiment of the invention, a magnet mounted within the releasing member (100) is positioned so as to be proximal to a Hall effect sensor positioned in either the upper housing (25) or the skin contacting portion (30), when the releasing member (100) is in place. According to this embodiment, when the releasing member (100) is removed by the user, the Hall effect sensor detects the removal of the magnet and causes the apparatus to take some action, such as powering up the electronic circuitry ready for use, converting it from sleep mode to active mode. It is to be understood that the above are examples of possible methods for assisting in retaining the releasing member (100) in place prior to intentional removal that may be used alone or in combination and that other methods as known in the art can also be used alone or in combination with the examples given.

**[00163]** In some embodiments of the invention, the releasing member (100) can also function as a covering element that is used to cover the microneedles after the apparatus has been removed from the subject. In a preferred example of this embodiment the locking element is located on the upper housing (25), extending down towards the skin contacting portion (30). The releasing member (100) comprises a groove that allows the releasing member (100) to slide past the locking element when the releasing member (100) is being withdrawn from the apparatus, while keeping the face of the releasing member (100) facing the upper surface of the skin contacting portion (30) continuous. In use, a releasing member (100) according to this preferred embodiment is removed by the user prior to pressing the upper housing (25) to insert the microneedles into the subject's skin and retained by the user. After the apparatus is removed from the subject post use, the user is instructed to adhere the releasing member (100) to the adhesive layer on the lower surface of the skin contacting portion (30) to cover the protruding microneedles. In another example of this embodiment, the releasing member (100) is flexibly attached to the apparatus such that the releasing member (100) can remain attached to the apparatus after it has been withdrawn by the user and then repositioned to cover the protruding microneedles after the apparatus has been removed from the subject post use. In yet another example of this embodiment, the releasing member (100) and the upper housing (25) are designed such that the releasing member (100) can be slidably or otherwise engaged with the upper housing (25) once it has been removed, where it is intended that

the releasing member (100) be stored while the apparatus is in use and removed to be used as a covering element after the apparatus has been removed from the subject.

**[00164]** In some embodiments, of the apparatus is configured to facilitate the user in removing the apparatus from the subject. As will be appreciated, the use of an adhesive layer may result in difficulty in removal of the apparatus from the skin. Examples of such configuration include leaving a portion of the skin contacting surface (35) uncoated with adhesive, such that a gap is present between the subject's skin and the surface (35), wherein the user uses the gap as a leverage point to assist in pulling the apparatus away from the skin by breaking the adhesive bond. In another example, a leverage mechanism not located on the skin contacting surface is incorporated to allow a taller gap than that created by the absence of adhesive on a portion of the skin contacting surface. In yet another example, a tab extending beyond at least one edge of the skin contacting portion (30) and attached to the adhesive layer can be incorporated, where the user pulls on the tab with sufficient force to cause the adhesive layer to stretch and yield, further causing the adhesive to delaminate from the skin contacting surface (35) and the skin.

**[00165]** In some embodiments, the apparatus is designed such that the releasing member (100) is locked into place in its position prior to apparatus use unless pressure is applied to the upper housing (25). This embodiment is intended to further ameliorate the risk of the releasing member (100) being prematurely withdrawn. In an example of this embodiment, there are features on the releasing member (100) and on at least one of the upper housings (25) and skin contacting portion (30) that are lockably engaged when the upper housing (25) is not being pressed. When the upper housing (25) is depressed, the feature on at least one of the upper housings (25) and skin contacting portion (30) is distorted to disengage the releasing member (100) and allow it to be withdrawn.

**[00166]** In yet other embodiments, the releasing member (100) need not be removed from the apparatus by the user. According to these embodiments, the releasing member (100) comprises a flexible element of sufficiently high stiffness that it does not substantially deflect when subjected to closing forces likely to be present on the apparatus during manufacture, storage and in the user's hands prior to application to the subject, but flexible enough that it deflects when the user intentionally applies a closing force to

the apparatus when it is applied to the subject's skin. In so flexing, the releasing member (100) is deflected, allowing the upper housing (25) to close towards the skin contacting portion (30). In these embodiments, the releasing member (100) could also function as the locking element, or the releasing member (100) could be separate from a locking portion. In some of these embodiments, a feature such as that labelled as (220) in FIG. 7A, FIG. 7B, forms the releasing member (100).

**[00167]** Each space (45) of the apparatus is dimensioned such that a microneedle can extend through it clearly, with at least a tapered part of the microneedle not impacting the sides of the hole during insertion. In some embodiments the holes may be of sufficient cross-section such that no part of the microneedle will contact the sides of the space during insertion. In several embodiments, at least a part of the hole along its length will have a cross-section such that a portion of the length of the microneedle contacts the sides of the hole during insertion. According to this embodiment the hole functions to help support a portion of the length of the microneedle to assist in preventing bending of the microneedle as it is inserted.

**[00168]** In some embodiments, the skin contacting portion (30) comprises further spaces or depressions configured to accept protrusions on the releasing member, to assist in retaining the releasing member until it is removed by the user. In addition, or alternatively, the skin contacting portion (30) comprises protrusions designed to be accepted into recesses in the releasing member to assist in retaining the releasing member in place until deliberate removal by the user.

**[00169]** The apparatus comprises a locking portion in the form of a latch (220) which permanently locks the arm (205) in the second position preventing the arm (205) from any hinging movement. In the drawn embodiment, the latch (220) is a simple unitary member capable of deflecting in response to movement of the arm (205) toward the closed position, but then returning to its original position when the arm (205) is in the second position (205b), thereby locking the arm (205) in place.

**[00170]** Rather than act on the arm (205), the locking portion may act on another component of the apparatus, that component in turn locking the arm in place. For example, the locking portion may act on the upper housing (25), with the upper housing (25) in turn retaining the arm (205) in the second position. In a further alternative the

locking portion may act on the PCB (65), with the PCB (65) in turn retaining the arm (205) in the second position.

**[00171]** In some embodiments, the locking portion comprises a recess into which a protrusion on the upper housing (25) is inserted to lock the upper housing (25) in a closed position (i.e., with the arm (205) in the second position). In one embodiment, the locking portion comprises a flexible element that is designed to allow the locking portion to move when impinged upon by the upper housing (25), so at to allow the housing (25) to close relative to the skin contacting portion (30) and whereby once the upper housing (25) has closed, allows the locking portion to move to lock in place the upper housing (25) in the closed position. In one embodiment, the apparatus comprises a protrusion on the upper housing (25), designed to be inserted into a recess in the locking portion, the protrusion comprising a flexible element to allow the protrusion to move, allowing the upper housing (25) to close relative to the skin contacting portion (30) and whereafter the housing (25) has closed relative to the skin contacting portion (30) the protrusion moves to be inserted in the recess in the locking portion, so as to lock the upper housing (25) in the closed position. The flexible element may comprise a shaft that is sufficiently deformable to allow the upper housing (25) to close without yielding of the shaft, so that the flexible element will try to return to its original position post the upper housing (25) closing. In a less preferred, but nonetheless functional embodiment, the flexible element comprises a coil spring.

**[00172]** A flexible element of the locking portion may be fabricated from any suitable material having the necessary stiffness and yield point. Examples of suitable material include non-crystalline plastics, crystalline plastics, sprung steel, unsprung steel, stainless steel, or other materials as are known if the art with suitable mechanical properties.

**[00173]** In several embodiments. the locking portion is fabricated from the same material as the skin contacting portion (30), to facilitate the fabrication of a skin contacting portion with an integral locking portion.

**[00174]** In certain embodiments, the force required to deflect or otherwise move the flexible element is designed to be large enough that the pressure the user needs to supply to deform the flexible element and thus cause the upper housing (25) to close towards

the skin contacting portion, is sufficient to insert the microneedles into the skin. According to this embodiment, the flexible element of the locking portion is used to set the force necessary to close the apparatus (thereby causing the arm to assume the second position) and ensure that the force is sufficient to insert the microneedles in their intended position embedded in the skin.

**[00175]** In some embodiments, the locking portion comprises at least one adhesive region located on at least one of the lower surfaces of the upper housing (25) and the upper surface of the skin contacting surface (35). When the apparatus is closed, the one or more adhesive regions adhere the upper housing (25) to the skin contacting portion (30), locking the apparatus in the closed position.

**[00176]** In several embodiments, the locking portion can assume three different stable states. In a first state, the locking portion is in a disengaged configuration, before the upper housing (25) is pushed downwardly towards the skin contacting portion (30) to close the apparatus. In a second state, the locking portion is in a first engaged position. When the locking portion is in the first engaged position it serves to lock the microneedles (15) in the embedded position in the skin (i.e., the arm (205) being in the second position). In a third state, the locking portion is in a second engaged position. In this state, the locking portion locks the apparatus in the open position (i.e., with the arm (205) in the first position) with the microneedles withdrawn into the apparatus to ameliorate the possibility of needle-stick injury resulting from microneedles protruding after apparatus use. In several embodiments, the locking portion comprises a user engagement portion, that can be gripped or otherwise engaged by the user, for example by engaging a fingernail under an overhanging ledge, so that the user can deflect the flexible portion of the locking portion. According to this embodiment, to close the apparatus the user presses on the upper housing (25) and locks it in place, as in other embodiments disclosed herein. When it is desired to remove the apparatus from the subject, the user engages with the locking portion and deflects it in a first direction, so as to unlock the upper housing (25) from the skin contacting portion (25), and then deflect the locking portion in a second direction, to lock the apparatus in the open position (i.e., with the arm in the first position) with the microneedles in the withdrawn position. In a preferred embodiment of this example, in the first direction, the locking portion is moved is away from the body of the apparatus, and

in the second direction, is towards the body of the apparatus. When deflected sufficiently in the second direction, the locking portion is designed, for example, to be stably engaged in a recess to prevent closure of the apparatus without intentionally doing so.

**[00177]** In some embodiments, a downward force on the microneedles when inserted into the skin is provided via the flexible element of the locking portion applying a downward force when the apparatus is locked in the closed position (i.e., with the movable arm in the second position). In some embodiments, effective locking of the movable arm in the second position is provided by a dedicated spring or other suitable biasing means. In some embodiments, the spiring or other biasing means is not dedicated to a locking function and may, for example, act also as a motive force in the movement of the arm from the first position to the second position. For example, a torsion spring may apply a closing torque at a pivot point (where present). In yet another example a flat, disk or coil spring is mounted to the rear of microneedles, such that when the apparatus is closed the spring is distorted or compressed to apply a downward force on the microneedles when the apparatus is in the closed position.

**[00178]** Although not an essential feature of the invention, the PCB (65) will be required for many applications where the microneedles are for the purpose of conducting electrical current to, from or through the skin. In that regard, the PCB may carry a microprocessor, and/or volatile electronic memory (such as RAM) and/or non-volatile electronic memory (such as ROM) and/or a wireless networking module (such as a Bluetooth™ module). The apparatus will of course comprise a power source, typically by way of a button battery.

**[00179]** Those skilled in the art will appreciate that the invention described herein is susceptible to further variations and modifications other than those specifically described. It is understood that the invention comprises all such variations and modifications which fall within the spirit and scope of the present invention.

**[00180]** Accordingly, the spirit and scope of the present invention is not to be limited by the foregoing examples, but is to be understood in the broadest sense allowable by law.

EXAMPLES

**[00181]** Example 1: A working electrode for an electrochemical sensor, the working electrode comprising an electrically conductive element and an analyte sensing element associated therewith configured to specifically interact with a target analyte, wherein the sensing element comprises a non-natural nucleic acid coupled with a redox reporter.

**[00182]** Example 2: The working electrode of example 1, wherein the non-natural nucleic acid is incapable of being read and/or duplicated by any natural mammalian cell.

**[00183]** Example 3: The working electrode of example 1 or 2, wherein the non-natural nucleic acid has a greater resistance to degradation by a component of a biological fluid as compared with a similar natural nucleic acid.

**[00184]** Example 4: The working electrode of example 1, or 2, or 3, wherein the similarity is in relation to any one of more of: length, base sequence, secondary structure, tertiary structure, and ability to interact with the target analyte.

**[00185]** Example 5: The working electrode of any one of examples 1 to 4, wherein the biological fluid comprises a nuclease.

**[00186]** Example 6: The working electrode of any one of examples 1 to 5, wherein the non-natural nucleic acid is formed from a single strand.

**[00187]** Example 7: The working electrode of any one of examples 1 to 6, wherein the non-natural nucleic acid is a polymer.

**[00188]** Example 8: The working electrode of any one of examples 1 to 7, wherein the non-natural nucleic acid comprises between 10 and 100 subunits.

**[00189]** Example 9: The working electrode of any one of examples 1 to 8, wherein the non-natural nucleic acid is a chemical variant of a natural deoxyribose nucleic acid or a natural ribose nucleic acid.

**[00190]** Example 10: The working electrode of any one of examples 1 to 9, wherein the chemical variant is to a sugar backbone and/or one or more bases.

**[00191]** Example 11: The working electrode of any one of examples 1 to 10, wherein the non-natural nucleic acid is made by, or with assistance, of a human.

**[00192]** Example 12: The working electrode of any one of examples 1 to 11, wherein the non-natural nucleic acid is a xeno nucleic acid (XNA) or a peptide nucleic acid (PNA).

**[00193]** Example 13: The working electrode of any one of examples 1 to 12, wherein the non-natural nucleic acid is a DNA or RNA aptamer having an altered chemical structure.

**[00194]** Example 14: The working electrode of any one of examples 1 to 13, wherein the non-natural nucleic acid is an altered form of a natural nucleic acid capable of specifically interacting with a target analyte, and the non-natural nucleic acid has an interacting capability of at least 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140%, or 150% that of the natural nucleic acid.

**[00195]** Example 15: The working electrode of any one of examples 1 to 14, wherein the non-natural nucleic acid is an altered form of a natural nucleic acid capable of specifically interacting with a target analyte, and the non-natural nucleic acid has a sensitivity, precision, or specificity for recognition of the target analyte of at least 50%, 60%, 70%, 80%, 90%, or 100% of that of the natural nucleic acid.

**[00196]** Example 16: The working electrode of any one of examples 1 to 15, wherein the non-natural nucleic acid is an altered form of a natural nucleic acid capable of specifically interacting with a target analyte, and the non-natural nucleic acid has an uncorrected signal loss of less than 90%, 80%, 70%, 60%, or 50% of that of the natural nucleic acid.

**[00197]** Example 17: The working electrode of any one of examples 1 to 16, wherein the working electrode is a portion of an electrochemical sensor with an uncorrected signal drift rate, and the uncorrected signal drift rate is determined or averaged over a period of at least 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, or 72 hours.

**[00198]** Example 18: The working electrode of any one of examples 1 to 17, wherein the non-natural nucleic is bound at a first end to the electrically conductive element.

**[00199]** Example 19: The working electrode of any one of examples 1 to 18, wherein the redox reporter is bound to a second end of the non-natural nucleic acid.

**[00200]** Example 20: The working electrode of any one of examples 1 to 19, wherein the electrically conductive element comprises a skin penetrating portion having the non-natural nucleic acid bound thereto.

**[00201]** Example 21: The working electrode of any one of examples 1 to 20, wherein the electrically conductive element is a needle, a microneedle, or a wire.

**[00202]** Example 22: An electrochemical sensor apparatus comprising the working electrode of any one of examples 1 to 21, and a counter electrode.

**[00203]** Example 23: The apparatus of example 22, comprising a reference electrode.

**[00204]** Example 24: The apparatus of example 22 or 23 having associated therewith a retainer configured to retain the working electrode in contact with a bodily fluid of a subject.

**[00205]** Example 25: The apparatus of example 22, or 23, or 24, wherein the retainer is configured to retain the working electrode in contact with a bodily fluid of a subject for a period of at least 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours , 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, or 72 hours.

**[00206]** Example 26: The apparatus of any one of examples 22 to 25 having associated therewith a housing configured to enclose a power source and/or electronics for functioning of the sensor.

**[00207]** Example 27: A method for monitoring a target analyte in a biological fluid of a subject, comprising: contacting the working electrode of any one of examples 1 to 21 to the biological fluid for a period of time.

**[00208]** Example 28: The method of example 27, wherein the working electrode is contacted with a biological fluid that remains *in situ* within the subject for a duration of the method.

**[00209]** Example 29: The method of example 27 or 28, wherein the biological fluid is blood or interstitial fluid.

**[00210]** Example 30: The method of example 27, or 28, or 29, wherein the biological fluid has not been removed from the subject.

**[00211]** Example 31: The method of any one of examples 27 to 30, wherein the period of time is at least 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, or 72 hours.

WHAT IS CLAIMED IS:

1. A working electrode for an electrochemical sensor, the working electrode comprising an electrically conductive element and an analyte sensing element associated therewith configured to specifically interact with a target analyte, wherein the sensing element comprises a non-natural nucleic acid coupled with a redox reporter.

2. The working electrode of claim 1, wherein the non-natural nucleic acid is incapable of being read and/or duplicated by any natural mammalian cell.

3. The working electrode of claim 1 or claim 2, wherein the non-natural nucleic acid has a greater resistance to degradation by a component of a biological fluid as compared with a similar natural nucleic acid.

4. The working electrode of claim 3, wherein the similarity is in relation to any one of more of: length, base sequence, secondary structure, tertiary structure, and ability to interact with the target analyte.

5. The working electrode of claim 3 or claim 4, wherein the biological fluid comprises a nuclease.

6. The working electrode of any one of claims 1 to 5, wherein the non-natural nucleic acid is formed from a single strand.

7. The working electrode of any one of claims 1 to 6, wherein the non-natural nucleic acid is a polymer.

8. The working electrode of claim 7, wherein the non-natural nucleic acid comprises between 10 and 100 subunits.

9. The working electrode of any one of claims 1 to 8, wherein the non-natural nucleic acid is a chemical variant of a natural deoxyribose nucleic acid or a natural ribose nucleic acid.

10. The working electrode of claim 9, wherein the chemical variant is to a sugar backbone and/or one or more bases.

11. The working electrode of any one of claims 1 to 10, wherein the non-natural nucleic acid is made by, or with assistance, of a human.

12. The working electrode of any one of claims 1 to 11, wherein the non-natural nucleic acid is a xeno nucleic acid (XNA) or a peptide nucleic acid (PNA).

13. The working electrode of any one of claims 1 to 12, wherein the non-natural nucleic acid is a DNA or RNA aptamer having an altered chemical structure.

14. The working electrode of any one of claims 1 to 13, wherein the non-natural nucleic acid is an altered form of a natural nucleic acid capable of specifically interacting with a target analyte, and the non-natural nucleic acid has an interacting capability of at least 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140%, or 150% that of the natural nucleic acid.

15. The working electrode of any one of claims 1 to 14, wherein the non-natural nucleic acid is an altered form of a natural nucleic acid capable of specifically interacting with a target analyte, and the non-natural nucleic acid has a sensitivity, precision, or specificity for recognition of the target analyte of at least 50%, 60%, 70%, 80%, 90%, or 100% of that of the natural nucleic acid.

16. The working electrode of any one of claims 1 to 15, wherein the non-natural nucleic acid is an altered form of a natural nucleic acid capable of specifically interacting with a target analyte, and the non-natural nucleic acid has an uncorrected signal loss of less than 90%, 80%, 70%, 60%, or 50% of that of the natural nucleic acid.

17. The working electrode of claim 1, wherein the working electrode is a portion of an electrochemical sensor with an uncorrected signal drift rate, and the uncorrected signal drift rate is determined or averaged over a period of at least 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, or 72 hours.

18. The working electrode of any one of claims 1 to 17, wherein the non-natural nucleic is bound at a first end to the electrically conductive element.

19. The working electrode of any one of claims 1 to 18, wherein the redox reporter is bound to a second end of the non-natural nucleic acid.

20. The working electrode of any one of claims 1 to 19, wherein the electrically conductive element comprises a skin penetrating portion having the non-natural nucleic acid bound thereto.

21. The working electrode of claim 20, wherein the electrically conductive element is a needle, a microneedle, or a wire.

22. An electrochemical sensor apparatus comprising the working electrode of any one of claims 1 to 21, and a counter electrode.

23. The apparatus of claim 22, comprising a reference electrode.

24. The apparatus of claim 22 or claim 23 having associated therewith a retainer configured to retain the working electrode in contact with a bodily fluid of a subject.

25. The apparatus of claim 24, wherein the retainer is configured to retain the working electrode in contact with a bodily fluid of a subject for a period of at

least 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours , 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, or 72 hours.

26. The apparatus of any one of claims 22 to 25 having associated therewith a housing configured to enclose a power source and/or electronics for functioning of the sensor.

27. A method for monitoring a target analyte in a biological fluid of a subject, comprising: contacting the working electrode of any one of claims 1 to 21 to the biological fluid for a period of time.

28. The method of claim 27, wherein the working electrode is contacted with a biological fluid that remains *in situ* within the subject for a duration of the method.

29. The method of claim 27 or claim 28, wherein the biological fluid is blood or interstitial fluid.

30. The method of any one of claims 27 to 29, wherein the biological fluid has not been removed from the subject.

31. The method of any one of claims 27 to 30, wherein the period of time is at least 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 36 hours, 48 hours, or 72 hours.

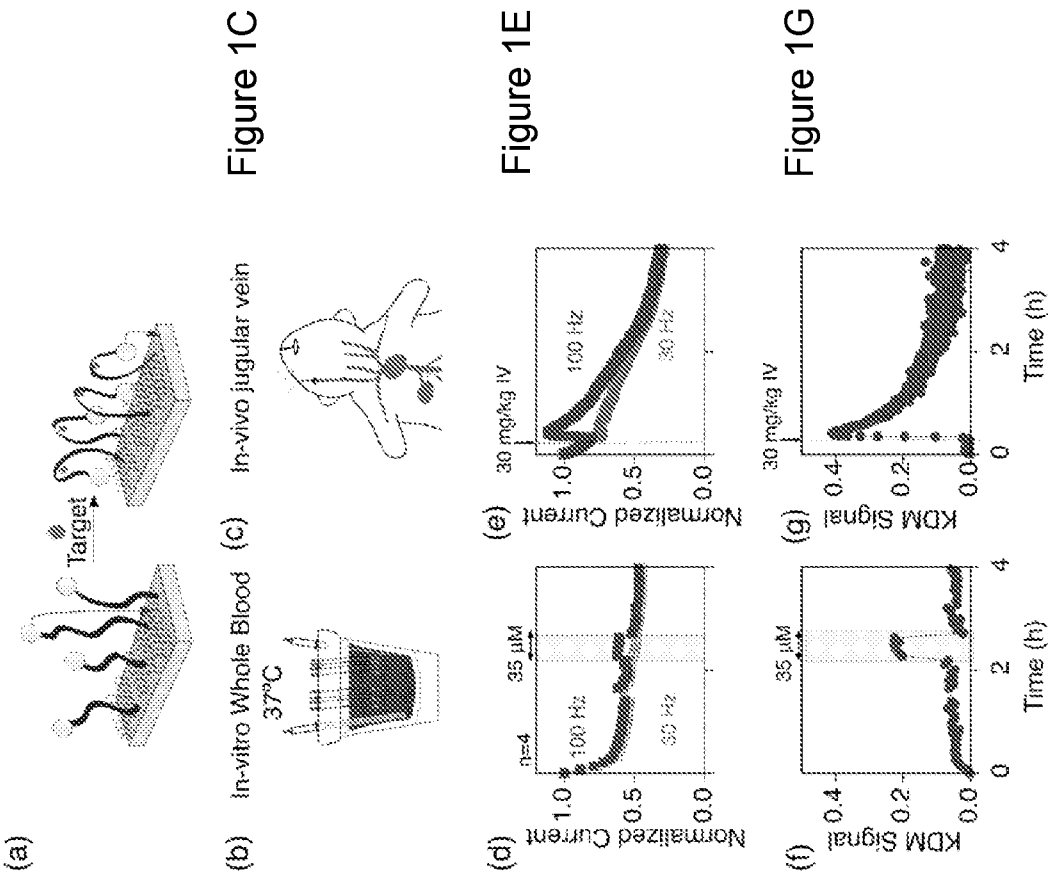



Figure 1A

Figure 1B

Figure 1C

Figure 1D

Figure 1E

Figure 1F

Figure 1G

Prior Art

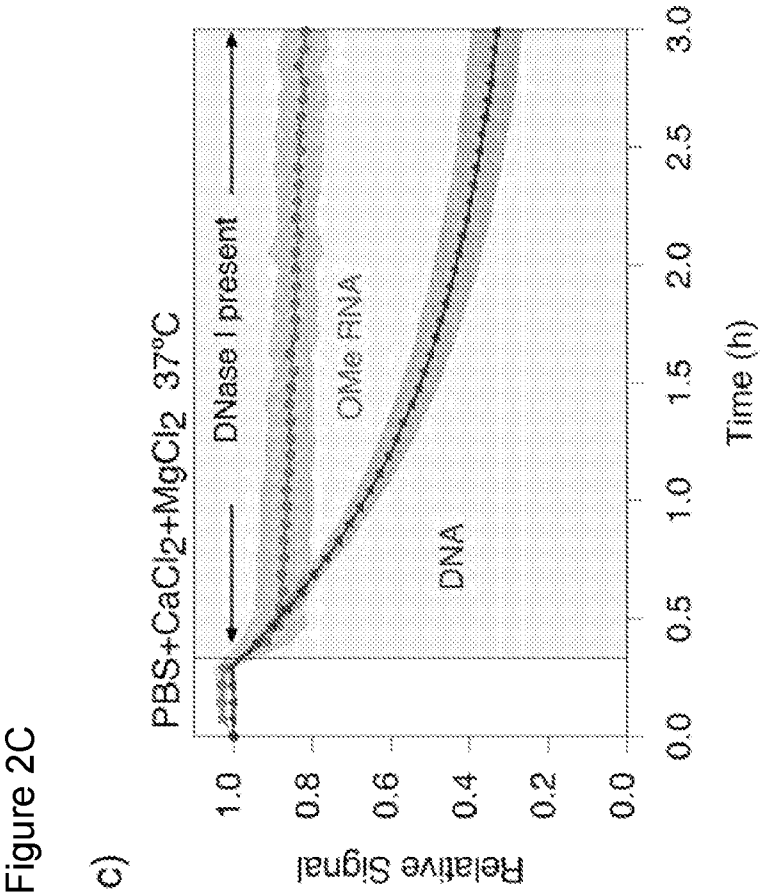



Figure 2C

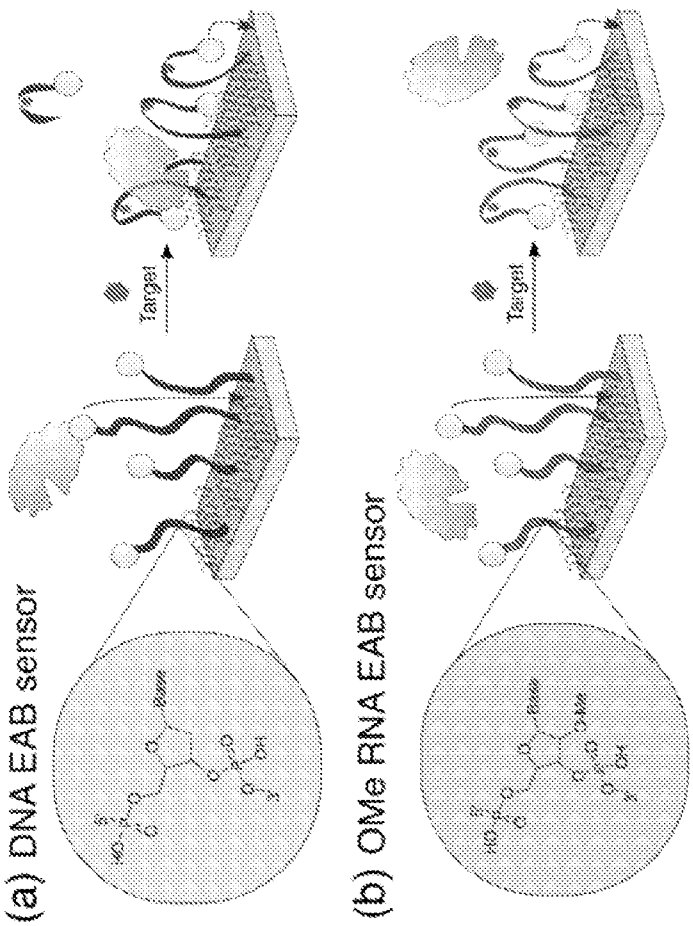



Figure 2A

Figure 2B

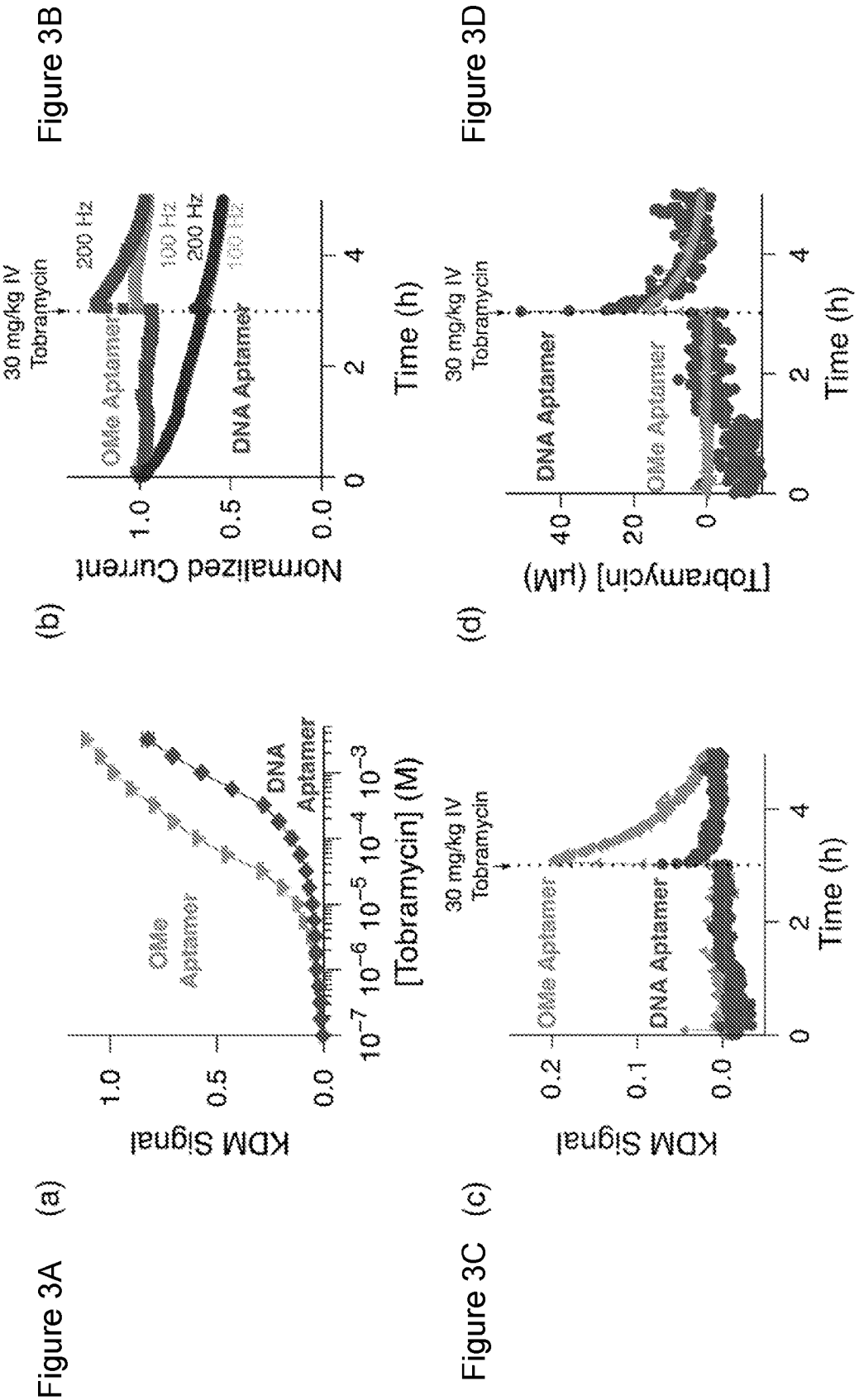



Figure 3A

Figure 3B

Figure 3C

Figure 3D

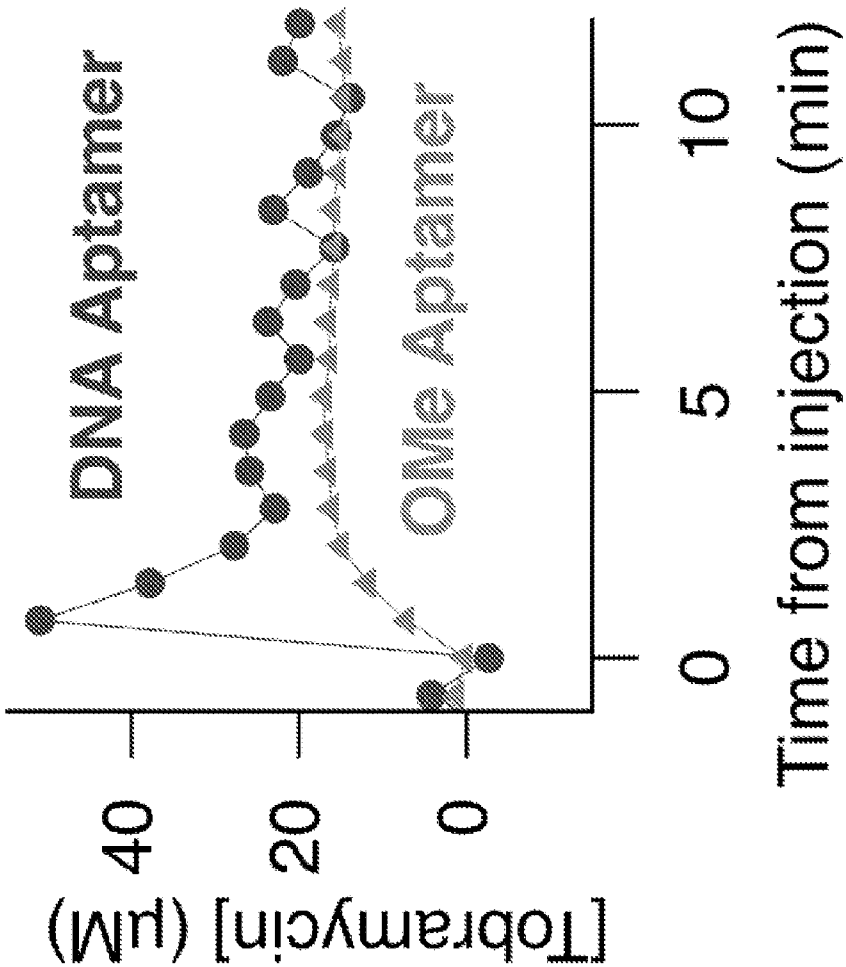

DNA Aptamer
OMe Aptamer
[Tobramycin] (μM)
40
20
0
0
5
10
Time from injection (min)

Figure 3E

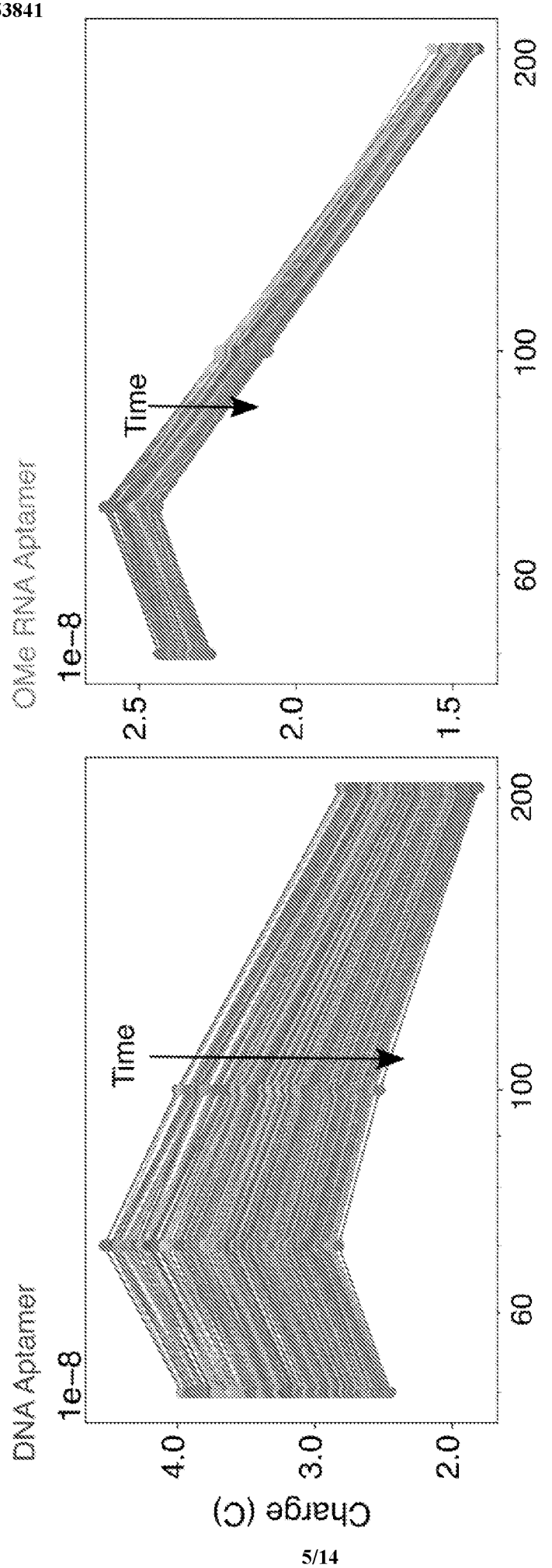



Figure 3F

Figure 3G

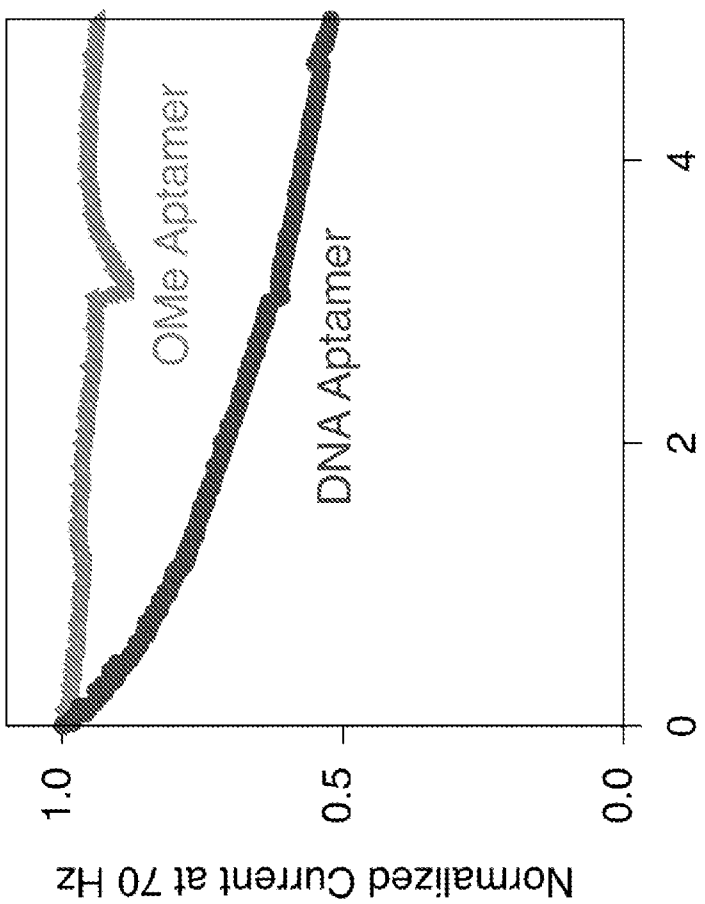
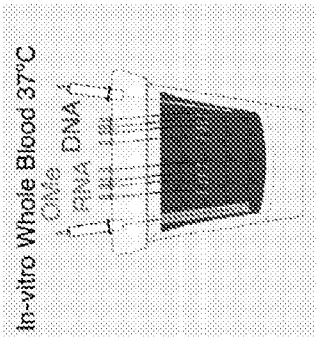

Normalized Current at 70 Hz
1.0
0.5
0.0
0
2
4
Time (h)
OMe Aptamer
DNA Aptamer

Figure 3H

Figure 4A



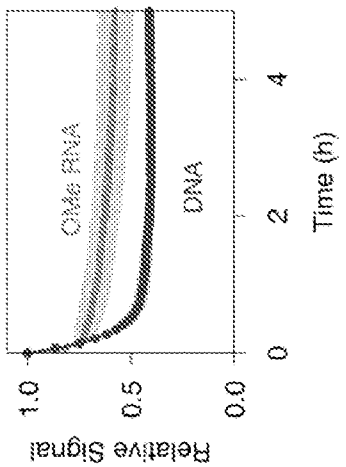
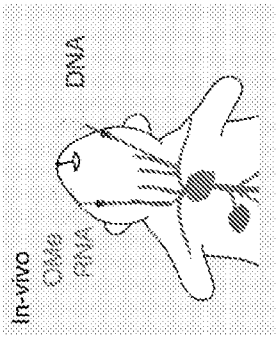




Figure 4B



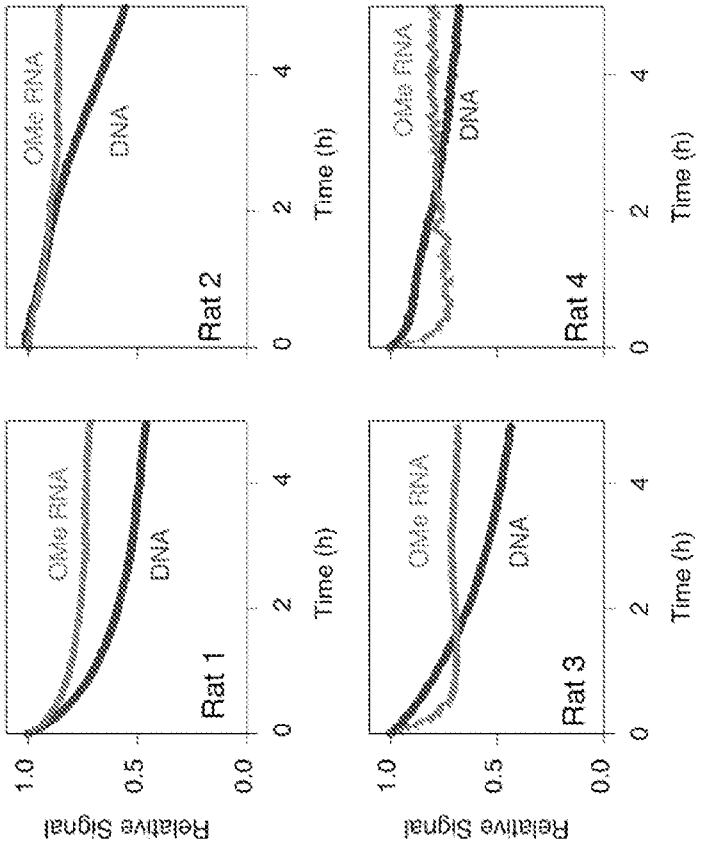
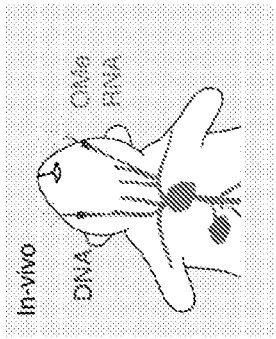




Figure 4C



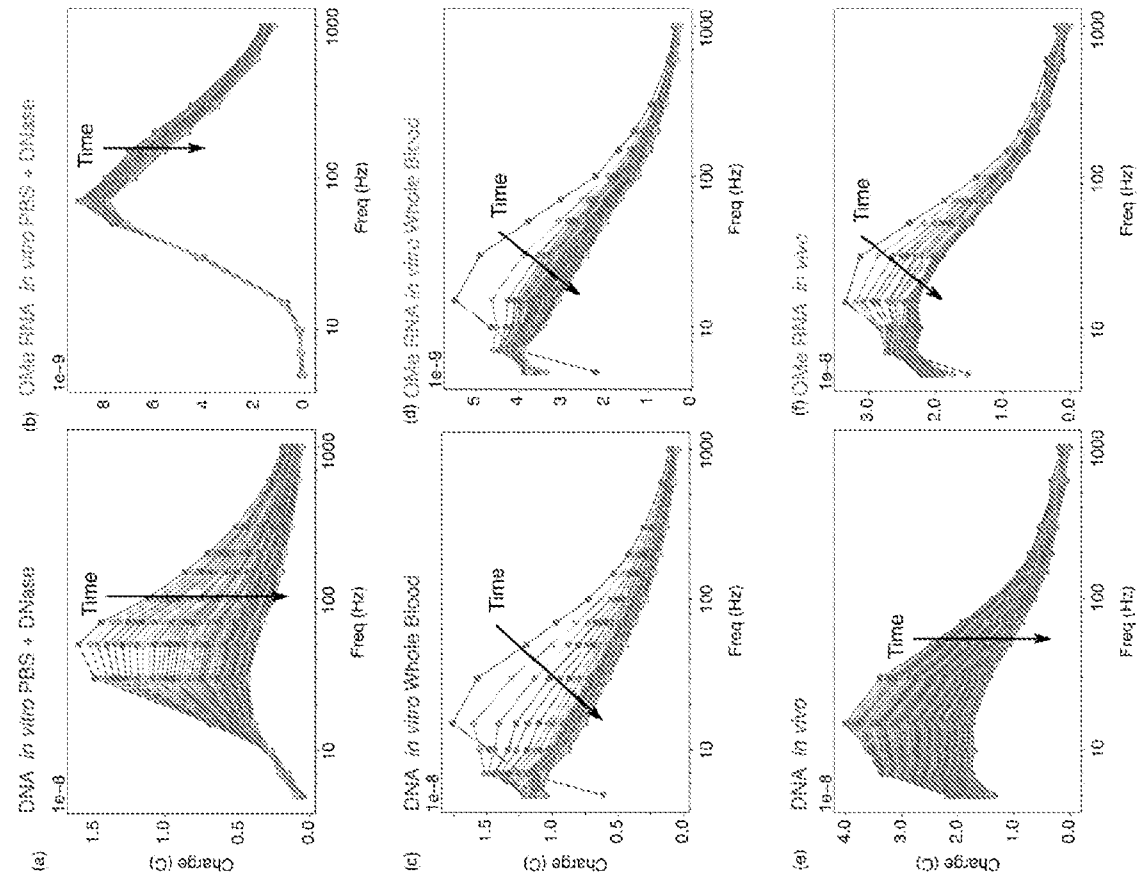



Figure 5A

Figure 5B

Figure 5C

Figure 5D

Figure 5E

Figure 5F

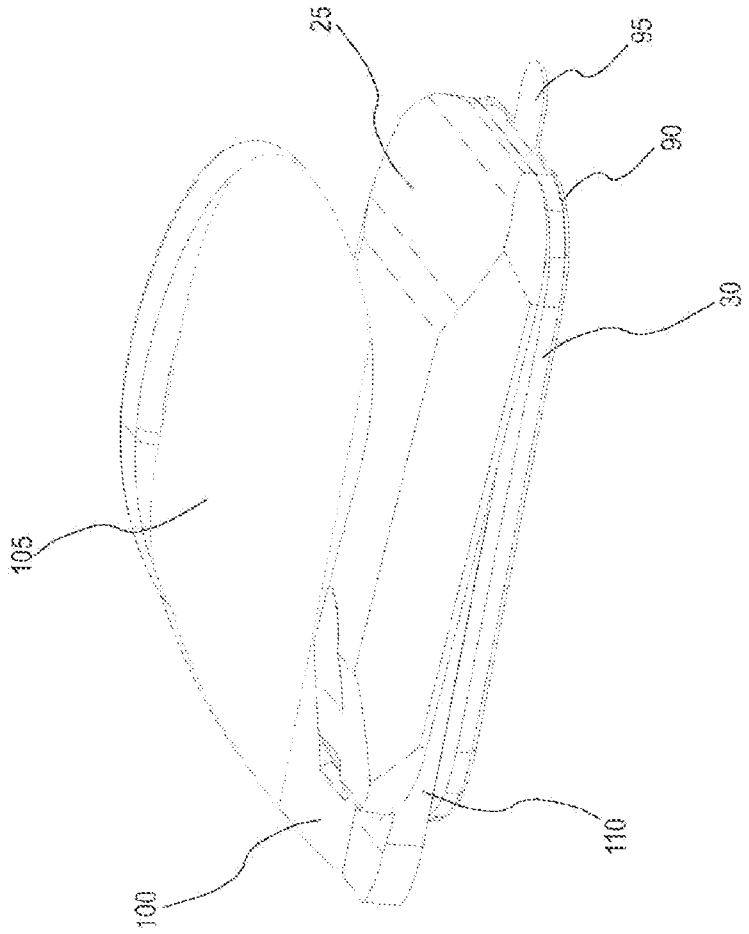



Figure 6

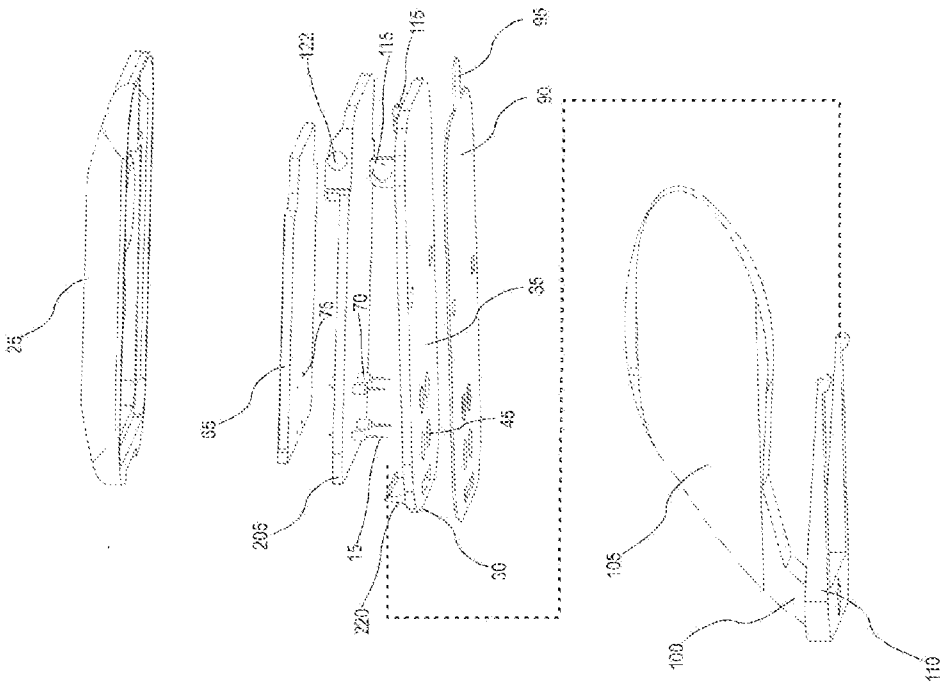



Figure 7A

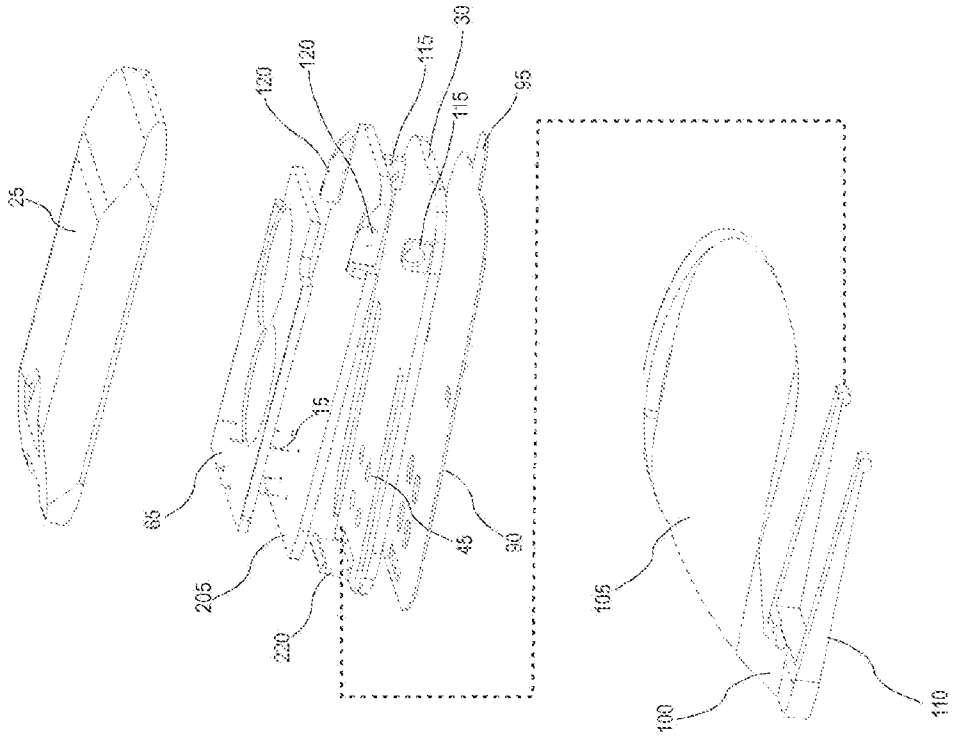



Figure 7B

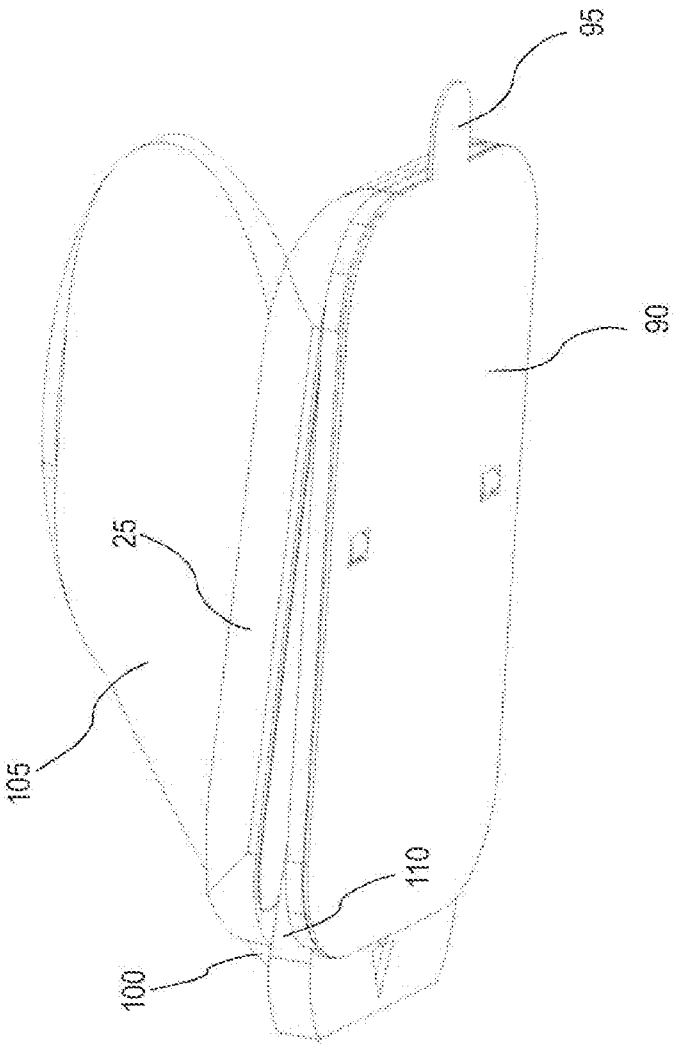



Figure 8

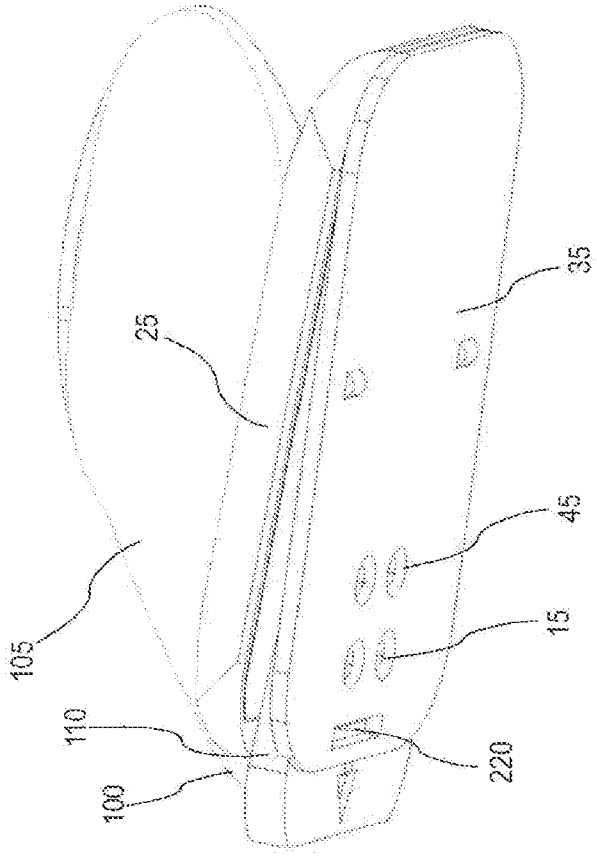



Figure 9

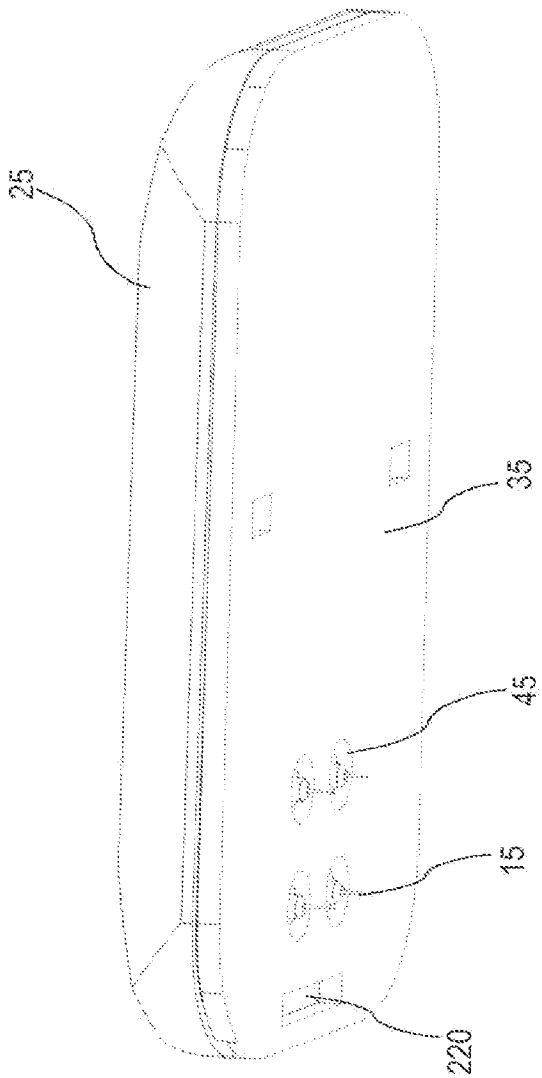



Figure 10