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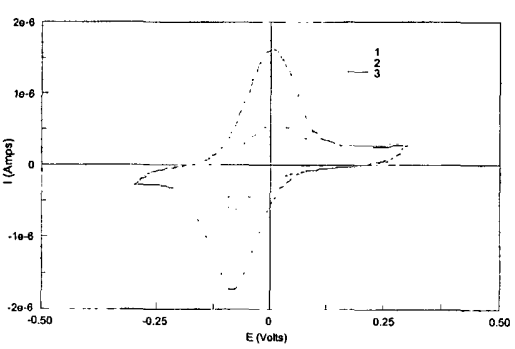
(54) Title: METHODS FOR ENHANCED DETECTION USING SURFACE SENSITIVE TECHNIQUES.




(57) Abstract: Methods for the detection and analysis of specific analytes, using techniques sensitive to surface excess are described. Detection of analytes such as nucleic acids and other biopolymers is achieved through template independent polymerization, or selective degradation. Using analyte specific initiation sites for template independent polymerisation and the measurement thereof by means of a surface sensitive technique, a functional signal amplification method is realised. When combined with immobilised specific affinity sites for the analyte, detailed information with respect to the interaction between analyte and the specific affinity sites may be obtained. Applications chiefly focus on the detection of nucleic acid based reactions and the use of the methods described to characterise nucleic acid analytes. In addition the present invention contains methods for the real time monitoring of nucleic acid substrate based enzymatic activity at the surface. Examples using voltanimetric and impedimetric techniques are described.

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# Methods for enhanced detection using surface sensitive techniques.

## Background to the invention

Methods for examining the behaviour and composition of biopolymers and it's application to molecular diagnostics have traditionally involved the use of different experimental techniques, each having it's specific advantages. This is well exemplified by the study of nucleic acids were sequence composition and interactions are central. Spectroscopy related methods for example have been used to extract thermodynamic information relating to the sequence dependent interactions between nucleic acids (Geiduschek, (1962), J. Mol. Biol.4, 467-487; Spatz, et al., (1969), J. Mol. Biol. 42, 191-219; Pörschke, et al. (1971), J. Mol. Biol., 62, 361-381). Electrophoresis when performed in conjunction with a suitable label (e.g. radiolabeled nucleotides), may unravel the composition of complex macromolecules such as nucleic acids or proteins to a large extend owing to the enzymatic or chemical sequencing techniques respectively developed by Sanger and Maxam-Gilbert (Sanger, et al. (1977), Proc. Natl. Acad. Sci. USA., 74, 5463-5467; Maxam, et al., (1980), Meths. Enzymol., 65, 499-560) yielding quite definite information regarding to particular sequence stretches). Relative infomation may be obtained using particular enzymes or mixtures thereof which cut DNA at predetermined sequences (restriction fragment). Specificity in the generation of nucleic acid fragments may also be obtained by use of a binary system such as oligo/RNaseH directed degradation of RNA (Donnis-Keller, (1979), Nucleic Acids Res., 7, 179-192). Furthermore specific enzymatic and chemical cleavage reactions have been used to detect single nucleotide polymorphisms (e.g. Myers,et al., (1985), Science, 230, 1242-1246; Cotton, et al., (1989), Nucleic Acids Res., 17, 4223-4233). Confidence in the methods applied to nucleic acids spawned the introduction of filter hybridisation methods based on sequence selective affinity, which made it possible to retain parts of the complex information confined in a specie's genetic make-up on a piece of paper. This has made genetic information portable and exploitable and easy to compare for instance to another species. Methods for the analysis of proteins has moved along not to dissimilar lines, with both physical and enzymatic methods playing a crucial role in generating our current understanding, and the development of immuno - affinity assays being a major commercial application. The portable format of molecular diagnostics is now increasingly applied to a wider range of biologically important molecular structures, in order to obtain genetic or extra-genetic molecular correlates with disease. For example particular glycoforms of otherwise similar proteins may for example be responsible for different clinical conditions (e.g. prion glycoprotein, Rudd, et al. (2001), Biochemistry, 40, 3759-3766), and hence the development of diagnostic methods coping with molecular diversity is highly desirable.

Although very useful and reliable, many of the available molecular diagnostic methods see their applications limited because of the format and/or the labelling and detection technique. For example, the reverse hybridisation format frequently involves the incorporation of a label at a particular stage during the experimental protocol in order to visualize a specific hybridisation event. In general the signal amplification or differentiation generated by the labelling system will determine the nature of the measurement technique to evaluate the test, and very often is limited to a discontinuous measurement reflecting the state of hybridised probes at some stage in the hybridisation process. So whenever discontinuous, only one significant measurement per collection of specific nucleic acid probes can be made, representing only one physical or chemical condition under which probe and target interact. This approach has become problematic and quite inflexible, when high resolution tests addressing small variations in a target sequence are being developed. The need for specificity implies that hybridisation of the sample proceeds at a stringent temperature which is calculated from an objective matching sequence. This requires a detailed knowledge and delicate balancing of the melting behaviour of each of the probes to be used in the test. Hence the outcome of experiment is to this extent predetermined, and no positive discrimination of partially matching sequences is possible. Therefore unexpected mutations are easily missed, and mixed populations or heterozygous individuals can be a source of insuperable complexity when designing or interpreting a particular test. Similarly the production of labelled antibodies for a specific sample protein, is often a limiting factor in the development of a protein assay and the nature of this label usually only allows for discontinuous measurement, hence no further discrimination of the signal is possible.

Currently there is an increasing effort to miniaturize the "portable" molecular diagnostic format as well as other techniques, although in many cases the signal generated for detection remains discontinuous. Nonetheless promising new technologies or approaches have for example been applied to the reverse hybridisation format that focus on the measurement of physical or chemical phenomena occurring at transducer surfaces. Methods for immobilizing DNA probes on a transducer surface were described by Flowers and co-workers (Flowers, et al., (1986), Fed. Proc., 45(6), 1516), who used the surface of piezoelectric oscillators to serve as a carrier in the reverse hybridisation assay, offering the possibility for direct detection of DNA hybridisation. Similarly, use of a surface plasmon resonance device wherein the transducer surface is modified with sequence selective probes has been disclosed in U.S. patent 4,889,427 (Van Veen et al.,). Of particular interest is the use of electrochemical techniques to characterize the behaviour of polyelectrolyte modified surfaces. As the detection involves the measurement of electrical signals it may be integrated in relatively inexpensive ways requiring little auxiliary

equipment. Since the pioneering work of Oyama and others (Oyama, et al., (1980), J. Electroanal. Chem., 112, 271-280; (1980) Anal. Chem., 52, 1192-1198) who studied the behaviour of polyelectrolyte modified surfaces in conjunction with transition metal redox complexes carrying an opposite or similar charge (e.g. $Co(phen)_3^{3+/2+}$ or $Fe(CN)_6^{3-/4-}$) the electrochemical method has been increasingly applied for studying biopolymers immobilized to transducer surfaces. Selective binding of transition metal ion complexes to nucleic acids is a subject which has been thoroughly studied both by spectroscopic and electrochemical techniques (Barton, et al., (1984), J. Am. Chem. Soc., 106, 2172-2176; Carter, et al., (1987), J. Am. Chem. Soc. 109, 7528-7530). Mikkelson et al. subsequently disclosed modified electrode surfaces in conjunction with a specific redox probe to distinguish between double and single stranded nucleic acid structures (U.S. Pat. No. 5,312,527, Mikkelson et al.,). Other examples involving the use of faradayic methods (i.e. those involving electron transfer) in conjunction with nucleic acid hybridisation assays have used the electrochemical properties of the DNA bases (Napier, et al., (1997), Bioconjug. Chem., 8(6), 906-913), or draw upon exogenous labels such as redox enzymes (U.S. Pat. No. 4,840,893 , Hill, et al) In close analogy with the faradayic methods applied to transducer surfaces, impedimetric methods draw upon the same instrumentation advantages, and are very convenient. Still, many of the methods described above either require complex instrumentation and are very expensive to employ, encounter sensitivity problems, or see their applications limited because of the labelling or detection technique.

It is the aim of the current invention to provide methods for the detection of nucleic acids as well as other compounds at transducer surfaces by means of surface excess producing reactions, which unify some of the advantages encountered in traditionally different techniques. It is therefore also an aim to increase the sensitivity of detection methods that draw upon surface excess.

Another aim of the invention is to provide a method for continuous measurement of binding, hybridisation, dissociation and similar processes by means of a surface sensitive technique.

## SUMMARY OF THE INVENTION

The present invention provides methods for the detection of the presence of an analyte using template independent polymerisation and or template selective degradation, the products of which are measured as a discrete change in surface excess using a surface sensitive technique.

Detection of the analyte is achieved by means of addressing suitable initiation sites for template independent polymerisation present within the analyte or associated with the analyte. Henceforth analyte specific template independent polymerisation by means of suitable enzymes such as glycosyltransferases and

nucleotidylexotransferases can be used to change the surface excess as measured using a surface sensitive technique.

Using a surface with discrete specific affinity sites which interact with the analyte, a significant change in surface excess is observed upon presence of the analyte by means template independent polymerisation using analyte specific initiation sites.

The present invention further provides a functional signal amplification by means of analyte specific template independent polymerisation, wherein the surface excess may be continuously measured, for example as a function of a physical or chemical gradient. It is a specific advantage that such functional amplification allows for characterisation of the interaction between analyte and discrete specific affinity sites. In practice this makes it possible to positively discriminate mismatches in nucleic acid sequences, or detect multiple interactions between analyte and affinity sites, without the requirement for any further steps.

Additionally the present invention provides methods for the detection of an analyte using template selective degradation. To this extent discrete specific affinity sites containing degradation precursors are degraded in presence of the analyte and a suitable agent catalysing template selective degradation. The presence of the analyte is then detected by measuring a change in surface excess at a transducer surface. Alternatively, the degradation precursors contain in addition also initiation precursors for template independent polymerisation which are activated by said template selective degradation.

Furthermore the processes of template independent polymerisation and template dependent degradation may be combined to detect the presence of an analyte by measurement of the surface excess. For example template selective degradation may be used to create an initiation site for template independent polymerisation. Other reaction schemes combining template independent polymerisation and template selective degradation are described which provide analyte specific signal amplification in an autocatalytic manner.

The present invention further includes methods for the analysis of nucleotide sequences using the advantages of functional signal amplification, and it's measurement by means of a surface sensitive technique.

## BRIEF DESCRIPTION OF THE FIGURES

FIG.1A Cyclic voltammogram of a hybridization using a complementary oligonucleotide 1) before hybridisation 2) after hybridisation and template independent polymerisation

FIG.1B Cyclic voltammogram of a hybridisation using a non complementary oligonucleotide 1) before hybridisation 2) after hybridisation and template independent polymerisation.

FIG.1C Cyclic voltammogram of a hybridisation using a complementary PCR product (sample 1) 1) before hybridisation, 2) after hybridisation, 3) after hybridisation and template independent polymerisation.

FIG. 1D Cyclic voltammogram of a hybridisation using a non-complementary PCR product (sample 2) 1) before hybridisation, 2) after hybridisation, 3) after hybridisation and template independent polymerisation

FIG.1E Bode plot showing the conductivity of a interdigitated impedimetric sensing device 1) before hybridization, 2) after hybridization with complementary nucleotide and template independent polymerization, 3) after hybridization with non complementary oligonucleotide and template independent polymerization.

FIG.2A Shows a van t'Hoff plot obtained from a equilibrium dissociation experiment involving a fully complementary oligonucleotide sequence (Series 1) and an oligonucleotide sequence with one mismatch(Series 2) and a mixture of both complementary oligonucleotides and oligonucleotides with one mismatch (Series 3). The parameter θ represents the relative surface coverage.

FIG.2B Temperature jump experiment showing the course of denaturation of electrodes hybridized with respectively complementary oligonucleotide and a oligonucleotide sequence with a transition mismatch and amplified using DNA nucleotidylexotransferase. θ represents the relative surface coverage.

FIG.3A Differential dissociation plot of homogeneous and heterogeneous surfaces, obtained from equilibrium dissociation using cyclic voltammetry: Homogeneous surface 25-mer (Series 1), Heterogeneous surface containing a 25-mer and 18-mer complementary oligonucleotide (Series 2), Homogeneous surface containing a 18-mer complementary oligonucleotide(Series 3).

FIG.3B Difference differential dissociation plot of a heterogeneous surface containing 18 and 25-mer complement. Series 1: 25-mer homogeneous surface subtracted, Series 2: 18-mer homogeneous surface subtracted.

FIG.4 Temperature jump experiment analyzing the outcome of a restricted length polymerization experiment. The parameter θ represents the relative surface coverage. Series 1 and 2 ACG extension, series 3 and 4 ACT extension (for details see example IV)

FIG.5 Detection of specific endonucleolytic activity of RNase H (duplicates). (1) Chimeric probe and complementary template, (2) Chimeric probe and partially complementary template, (3) Chimeric probe and non complementary template, (4) chimeric probe, no template, (5) chimeric probe and complementary template no RNase H, (6) buffer only (for details see example V). Qi and Q respectively represent the integrated surface charge before and after the experiment.

FIG.6 Real time monitoring of DNase I induced degradation of a transducer surface containing specific affinity sites previously hybridised with complementary DNA oligonucleotides and subjected to template independent polymerization. Series 1: No DNase added, Series 2: DNase added.

## DETAILED DESCRIPTION OF THE INVENTION

### Definitions

The term analyte or specific analyte is used to describe a substance which is the object of detection and further contains, or is made to contain an initiation site or initiation precursor for template independent polymerisation. Alternatively the term is used to describe an analyte which contains a precursor for template dependent degradation. The term analyte is also used to specify those substances which are derived from the analyte by means of a specific process, including particular reaction products further defined below.

The term "nucleic acid sample" refers to a sample containing nucleic acids, which may have been subjected to a suitable purification or extraction method known in the general art of biochemistry. The term is also more widely used to designate the analyte or sample to be investigated further described by the terms "specific reaction product" and "derivative thereof".

The term "specific reaction product" and "derivative thereof" refers to the products obtained in a specific reaction with respect to nucleic acids. It comprises but is not limited to the products obtained from a specific amplification reaction (e.g. polymerase chain reaction, ligase chain reaction, transcription mediated amplification, cycling probe amplification etc...), a nucleic acid hybridization reaction to probes of known origin, or a reaction involving the enzymatic redistribution of nucleic acid sequence information due to for example the activity of specific nucleases, specific chemical cleavages, or polymerase based sequencing reactions. The term specific reaction product also encompasses reactions relating to the combination of specific reactions such as a polymerase based amplification used in conjunction with a specific nuclease reaction or sequencing reaction. In addition the specific reaction product further may include reactions involving the deprotection of soluble nucleic acid probes by means of the specific activity of a DNA or RNA endo- or exonuclease, as such making the said probes suitable for template independent polymerization using for example a RNA or DNA dependent nucleotidylexotransferase. Similarly, the term "specific reaction product" or "derivative thereof" also refers to the products obtained using specific endo- or exoglycosidases, glycanases and other biological or chemical agents causing selective degradation or modification of carbohydrates and related

substances. In analogy to nucleic acids the specific reaction products with respect to carbohydrates may also yield initiation sites for template independent polymerisation using specific glycosyltransferases.

The term "template selective degradation" refers both to the stepwise or processive removal of monomer units of a polymer template and to the single step cleavage of a polymer template, each of them directed by the properties of said template.

The term "initiation site" is used to describe a specific site corresponding to the substrate to which monomers may be added or removed in a template independent or template dependent manner.

The term "template dependent polymerisation" refers to the stepwise addition of monomer units to an initiation site (also referred to as a primer), each of them directed by the properties of a template.

The term "initiation precursor" is used to describe the initiation site which needs activation in order to become a substrate for template dependent or independent polymerization or degradation, and preferably due to a specific event relating to the nature and composition of the said initiation precursor.

The term "degradation precursor" is used to describe the particular entity that is prone to a degradation process. This entity is contained within the analyte, results from binding of analyte to affinity sites, or is contained within the affinity sites but only activated upon binding of the analyte.

The term "template independent polymerization" refers to the stepwise or processive addition of monomer units to the initiation site without the requirement for a template.

The term "functional amplification" refers to the creation of a signal amplification which retains some of the properties of the amplified target.

The term "structural amplification" refers to an amplification of a specific reaction product or sample which retains some of the properties of the amplified target which by amplification induces a significant change in molecular composition, thereby forming the basis for detection of a specific sample or reaction product. In the present context it is used to describe a change in the gross macromolecular composition of a transducer surface.

The term "surface excess" is used to describe a particular surface composition, such as the primary excess of a polyanion, polycation or other relevant species and components associated to them and implies that a difference exists at the interface between transducer surface and bulk medium.

The term "surface sensitive techniques" applies to physical methods that allow one to characterize or measure surface compositions and changes thereof, including but not limited to faradayic and impedimetric techniques, which are sensitive to charge transfer, conductivity and dielectric phenomena. These methods may typically combine chemical specificity and features relating to the overall composition of a surface. Mass sensitive

methods which include but are not limited to resonance methods such as the quartz crystal balance, optical wave guide methods for example resonant mirror and surface plasmon resonance in which the surface itself is transducer and work function based techniques such as the Kelvin microprobe are also included. It should be clear that the list of surface sensitive techniques is bound to be incomplete, and several new techniques are being developed.

The term "transducer surface" relates to the integrated assembly of a surface which may be used in conjunction with one of the above mentioned surface sensitive techniques. The transducer surface in general may be part of a passive transducer (e.g. piezoelectric transducers, optical transducers, mechanical transducers etc...) or an active transducer (e.g. resistance or reactance transducers) or hybrid devices that are classified according to their stimulus requirements.

The terms "modified electrode" and more general "modified transducer surface" is used to specify chemically modified electrode surfaces having specificity for a specific nucleic acid sequence or proteins, or in general have a selective affinity for a particular analyte or improve the resolution of its detection. Typically the modification may consist of nucleic acid sequences, or nucleic acid analogs which are not found in nature. Presently the synthesis of such probes is fully automated and many modifications incorporating chemical functions for the attachment to solid supports are available. Electrode surfaces may thus be modified according to the nature of the electrode material. Several examples relating to the controlled modification, functionalisation of electrode surfaces as well as the general background relating to the art are described in "Integrated chemical systems" by Allen J. Bard (A.J. Bard, Wiley-Interscience 1994).

The term "specific affinity site" is used to describe a molecule or molecular property which has been conferred to an otherwise non-specific carrier, thereby inducing the propensity to specifically associate with a closely defined range of molecules under particular conditions.

The term "peri-electrodic space" is used to specify the space in the immediate vicinity of the electrode, the latter being defined as a conducting substance having a connection to the external measuring circuitry. Apart from the solvent or electrolyte in the immediate vicinity of the electrode surface, a regular reproducible structure having different properties with respect to conductivity and or capacitance may be included into the space defining the boundaries of a particular measurement area. The term peri-electrodic space may thus be applied to both intra and inter electrode configurations, depending on whether this space involves single or multiple distinct connections to the measuring circuitry. For example fibrous carbon electrodes consisting of both conductive and non conducting compounds allowing surface modification as described in "Chemically modified carbon

fibers"(I.N. Ermolenko et al. Eds., VCH 1990), provide a good example of intra peri-electrodic space. Other examples belonging to this class include the patterned or random deposition of for example organo-sulfur compounds on metal electrodes resulting in the deliberate passivation of a portion of the electrode area. The term peri-electrodic space also applies to regular structures allowing deliberate manipulation between at least two independently addressable electrodes. One of the earliest examples of a modular peri-electrodic space is the well known Volta-pile. More recent examples include interdigitated electrodes which may be microscaled using conventional photolithographic procedures, or specific micro-moulding techniques combined with structure-induced metal deposition patterning (Van Gerwen, et al. European patent 0876601)

The term "exogenous label" is used to specify a chemical entity which has a chemical or physical property distinct from the sample molecule to be detected and allows to detect the sample molecule. Typical exogenous labels include radioactive, fluorescent, or enzyme binding sites which may become associated with the sample by means of a probe or are integrated in the probe.

## Embodiments

The general concept behind the present invention, relates directly to the use of surface sensitive techniques. These techniques allow for the measurement of any substance, without the need for labels, to give rise in a measurable signal by assessing parameters that are directly altered by the presence of said substance. It has been found to be very advantageous to exploit this even further by using the presence of said substance as a prerequisite step to magnify the signal, which results from said substance, even further. It has been found that such can be achieved in an analyte specific way by using processes that draw upon the presence of said analyte to either magnify the signal by building more substance or by doing the reverse, and destroying the signal that was already generated by the presence of said analyte. The first can be achieved by template independent polymerisation provided that the analyte present contains an initiation site for said template independent polymerisation. The second can be achieved by template selective degradation provided that the analyte present contains an initiation site for said template selective degradation.

In a first embodiment the present invention provides a method for detecting the presence of an analyte in a sample by means of modifying and measuring the surface excess wherein the said method comprises

a) increasing surface excess by means of template independent polymerization

or

b) decreasing the surface excess by means of template selective degradation

In a embodiment the present invention provides a method for detecting the presence of an analyte in a sample comprising the following steps:

a) Reacting the sample with a reaction mixture that causes template independent polymerisation, by means of addressing suitable initiation sites present within, or specifically associated with, the analyte

b) Assessing the template independent polymerisation, by means of measuring the surface excess using a surface sensitive technique.

In a embodiment the present invention also provides a method for detecting the presence of an analyte in a sample comprising the following steps:

a) Binding the analyte to a surface containing discrete specific affinity sites that are selective for the said analyte.

b) Reacting the said bound analyte with a reaction mixture that causes template independent polymerisation, by means of addressing suitable initiation sites present within, or specifically associated with, the analyte

c) Assessing the template independent polymerisation, by means of measuring the surface excess at the surface containing said specific affinity sites using a surface sensitive technique.

In an embodiment the current invention thus provides a method for detecting specific analytes (containing initiation sites for template independent polymerisation) by means of a transducer surface and template independent polymerization or template selective degradation. The transducer surface may for this purpose either contain, be brought in the vicinity of, or in contact with the analyte in order to measure the surface excess at the said surface. As such a tangible interaction between transducer surface and analyte as defined by the surface excess is understood to be present. In order to detect an appreciable change in surface excess at the transducer surface due to the presence of the analyte, the signal is amplified in a analyte specific way by means of template independent polymerisation of suitable initiation sites contained within the said analyte. Such initiation sites may be present within the specific analyte (being thus a natural property or intrinsic part of the analyte), or associated with the analyte by means of an analyte specific process which couples a natural or synthetic initiation site to the said analyte. The template independent polymerisation process may for example involve the addition of nucleoside or sugar (and their derivatives) residues to the said initiation site by means of an enzyme capable of template independent polymerization belonging for example to the following classes: the nucleotidylexotransferases, hexosyltransferases and pentosyltransferases. The process of template independent polymerisation then readily results in the formation of a homopolymer, copolymer, or block copolymer which

may be a polynucleotide or a polysaccharide and hence gives rise to an appreciable change in surface excess which is analyte specific and is measured at the transducer surface.

When the analyte interacting with the transducer surface is a nucleic acid sample, a specific reaction product or derivative thereof, the said analyte may be detected by template independent polymerization of the initiation sites present within or associated with the said nucleic acid sample. When the nucleic acid sample involves a deoxyribonucleic (DNA) or ribonucleic acid (RNA), initiation sites for template independent polymerisation may for example be the 3'OH termini which are contained within the sample, and are readily amenable to the addition of nucleoside residues by a template independent polymerisation process involving a nucleotidylexotransferase. Consequently a sample containing a ribonucleic or deoxyribonucleic homo or copolymer is synthesized, this structural amplification or increase may then be detected at the transducer surface. Suitable enzymes capable of performing template independent polymerisation may be employed to detect samples containing DNA, RNA or both at a transducer surface by means of template independent polymerisation. These include but are not limited to: DNA deoxyribonucleotidylexotransferase, polyribonucleotide nucleotidyltransferase, polynucleotide adenylyltransferase and specific tRNA nucleotidyltransferases.

Alternatively initiation sites for template independent polymerisation may be specifically associated with the said nucleic acid sample, specific reaction product or derivative thereof. This may for example comprise the hybridisation of suitable initiation sites to the said sample, by means of exploiting the propensity of nucleic acids to form stable and specific hydrogen bonds with oligonucleotides of complementary or partially complementary sequences. Such oligonucleotides may contain initiation sites for template independent polymerisation, or have to them attached initiation sites for template independent polymerisation for example by means of covalent modification, further hybridisation, or enzymatic modification in a analyte specific way. These initiation sites may as before consist of nucleoside residues, and subjected to template independent polymerisation by means of using a suitable nucleotidylexotransferase, in order to detect the said sample at the transducer surface. It will be obvious, that multiple initiation sites may be attached to the analyte by exploiting sequence specific association schemes and hence increase the degree of structural amplification upon template independent polymerisation. The associated initiation sites may however also consist of oligosaccharides which may for example be attached to a oligonucleotide having a degree of specificity for the said nucleic acid sample. Such oligosaccharides may then serve as initiation sites for template independent polymerisation by means of particular hexosyltransferases or pentosyltransferases. For example the initiation site may consist of a number of

(N,N-alpha/beta-glucosyl) residues, which equal or are above the minimum length (e.g. typically 7) to serve as a non reducing end initiation site for template independent polymerisation, by for example: UDP-glucose-glycogen-glucosyltransferase (preferentially devoid of glycogenin glucosyl transferase activity) which may act in concert with a branching enzyme (e.g. the alpha(1,6 branching enzyme) E.C.2.4.1.18). Alternatively the associated initiation site may be a specific oligosaccharide (e.g. galactosyl-galactosyl-xylosyl) which may lead to the formation of more complex polysaccharides such as heparin or heparin sulphate (incorporating D-glucuronic and N-acetylglucosamine residues(see for example: lind, et al., (1993) J. Biol. Chem., 268, 20705-20708)) by means of template independent polymerisation using suitable glycosyltransferases. It may be readily apparent that unlike for nucleic acids, there are numerous types of initiation sites, substrates and enzymes which may be used for the template independent synthesis of polysaccharides, and the concomitant detection of a specific analyte at a transducer surface, all of these are thus considered to be in the scope of the present invention.

In another embodiment the specific analyte may be a protein, carbohydrate, a collection thereof, or a specific reaction product or derivative of said analyte, which contains initiation sites for template independent polymerisation. Such initiation sites for example may be naturally present on specific types of proteins such as glycoproteins as a result of post translational modification, or on proteoglycans, the latter being more complex structures. Oligo or polysaccharides present on such specific proteins may hence be used as initiation sites for template independent polymerisation by means of a suitable hexosyltransferases (or combination thereof), or pentosyltransferases in order to detect a change in surface excess at a transducer surface in an analyte specific way. For example high mannose glycoproteins may be detected using specific glycosyltransferases capable of utilising mannose residues as an initiation site for template independent polymerisation. Similarly proteins having a natural tendency to associate with nucleic acid sequences (the latter being initiation sites for template independent polymerisation) may be detected at a transducer surface by means of template independent polymerisation using a suitable nucleotidylexotransferase.

In still another embodiment the specific analyte is a protein, carbohydrate,a collection thereof, or a specific reaction product or derivative of said analyte, which may be treated to contain associated initiation sites for template independent polymerisation in order to measure a change in surface excess at the transducer surface in a analyte specific way. For this purpose oligosaccharide, or oligonucleotide initiation sites may be associated with the analyte of interest or to another protein or peptide having a specific affinity for the said protein sample (e.g. reporter). Henceforth suitable oligosaccharide initiation sites may be directly associated with the analyte. For example this may be achieved by means of forming an O-linkage to accessible serine or threonine residues, a

reaction which may be catalysed by O-N-acetyl-D-glucosamine transferase (EC.2.5.1.7), or using specific lectins and their conjugates (having initiation sites for template independent polymerisation), and other methods known in the art. Alternatively similar methodologies may be used to couple oligosaccharide or oligonucleotide initiation sites to the said analyte or reporter by means of functional linkers.

In another embodiment the analyte (e.g. a nucleic acid or protein) may be specifically immobilised to a surface containing discrete specific affinity sites for the said analyte. Said surface may be a transducer surface or a surface which may be interrogated by means of measuring the surface excess at the transducer surface. Discrete specific affinity sites which immobilize the analyte in a specific manner include: sequence specific oligo or polynucleotides and derivatives thereof, natural nucleotide sequences, antibodies, peptides, lectins and so on, all of which may be conferred to the said surface in order to specifically immobilize the said analyte. Said immobilized analyte may then be detected by measuring the surface excess at a transducer surface according to the methods described in the current invention. Similarly the said surface may contain discrete specific affinity sites which specifically interact with a product of template independent polymerisation, in order to detect the presence of an analyte by measuring the surface excess at a transducer surface.

In a preferred embodiment, the current invention provides a method for detecting the interaction between a analyte preferably a nucleic acid sample, a specific reaction product or a derivative thereof, and a surface by means of template independent polymerization of the said nucleic acid sample, and the concomitant detection of the surface excess at a transducer surface. The reaction relating to the nucleic acids, involves the interaction (e.g. hybridisation) of a sample nucleic acid sequence or a specific reaction product with complementary or partially complementary oligonucleotide probes immobilized to a surface which may for example be the transducer surface. The oligonucleotide may be DNA, RNA, mixtures of both, or chemical analogs of nucleic acids and their mixtures. The said oligonucleotide probes or derivatives thereof are preferably immobilized to or brought to the vicinity of the surface according to methods known in the art. When the surface sensitive technique used involves a electrochemical detection method, the probe is preferably immobilized to the electrode surface or peri-electrodic space. The said immobilized nucleic acid probes are selected such that they carry no initiation sites for template independent polymerization, typically the probes are immobilized using a 3' modification for chemisorption or covalent attachment to the surface. Following hybridization with the said sample nucleic acid or specific reaction product, the surface is incubated with an enzyme capable of performing template independent polymerization. Depending on the nature of the said nucleic acid sample, initiation sites for template independent polymerisation may be contained within the nucleic acid sample by means of for example

3'OH ends acting as initiation sites for DNA or RNA nucleotidylexotransferases (e.g. DNA nucleotidylexotransferase (E.C.2.7.7.31), polyribonucleotide nucleotidyltransferase (E.C.2.7.7.8), polyribonucleotide adenylyltransferase, and specific tRNA nucleotidyltransferases (E.C.2.7.7.56)). Alternatively initiation sites for template independent polymerisation may be associated with the nucleic acid sample by means of specific affinity sites other than these contained on the said surface, providing thereby analyte specific initiation sites for the template independent synthesis of for example a polysaccharide or polynucleotide catalysed by suitable hexosyltransferases, pentosyltransferases or said DNA or RNA nucleotidylexotransferases. Henceforth the interaction between nucleic acid sample and immobilised discrete specific affinity sites may be detected by means of template independent polymerisation and the concomitant change in surface excess measured at the transducer surface.

Alternatively the nucleic acid sample may be immobilized to the said surface by means of incorporating specific chemical functionalities into the sample allowing a stable interaction with this surface. For instance such functionalisation may involve the incorporation of biotin or modified nucleotides such as phosphorothioates during nucleic acid amplification. This allows the sample to be probed with specific probes having initiation sites for template independent polymerization. Furthermore said nucleic acid sample or specific reaction product may be immobilised by means of oligonucleotide sequences which are complementary to specific oligonucleotide probes, or suitable affinity sites immobilised at the said surface at one end, and have a sample specific sequence at the other end. It will be apparent that for anyone skilled in the art, several methods exist or may be devised to immobilize a nucleic acid sample to a surface in order to be analysed and detected according to the methods described in the current invention.

In one embodiment, it is the particular advantage of the current invention that the analyte specific detection by means of template independent polymerisation provides a functional signal amplification and allows the continuous measurement thereof at a transducer surface. Hence products formed according to the methods described in the present invention may be further analysed, by subjecting the product of template independent polymerisation to conditions involving temperature, hydrophobicity, ionic strength and so on, while measuring the surface excess as a function of the applied conditions. In particular, the interaction(s) between the analyte and discrete specific affinity sites used to immobilise the said analyte, as well as interaction(s) between analyte and associated initiation precursors may be characterised (subsequent to template independent polymerisation). When for example a thermal gradient is applied, both kinetic and equilibrium information relating to the said interactions may be collected by respectively performing temperature jump or equilibrium dissociation

experiments. Using these appropriate methods, thermodynamic parameters relating to the analyte and specific affinity sites and/or associated initiation precursors may hence be extracted. The use of such methodology is most typically desirable when the analyte is a nucleic acid, specific reaction product or a derivative thereof, in which case it is often the objective to detect small variations in nucleotide sequence. For example single nucleotide polymorphisms are easily detected and positively discriminated using detection based on template independent polymerisation in conjunction with the equilibrium dissociation or temperature jump method.

In another embodiment further information regarding the nature of the analyte may be obtained by further subjecting the products obtained using template independent polymerisation to template selective degradation. For this purpose specific DNA or RNA exo or endo-nucleases, specific endo or exoglycosidases, or other specific biological or chemical agents may be used. This for example allows one to investigate the interaction of a specific affinity site such as a oligonucleotide probe and a nucleic acid sample by means of a selective degradation agent such as a restriction endonuclease. Or in the case the analyte is a carbohydrate the initiation sites used for template independent polymerisation may be cleaved off using a specific endo-glycosidase or glycanase.

In a embodiment the present invention provides a method for detecting the presence of an analyte in a sample comprising:

a) Reacting the sample with a reaction mixture that causes template selective degradation, by means of addressing suitable degradation precursors present within the analyte, or specifically associated with, the analyte

b) Assessing the template selective degradation, by means of measuring the surface excess using a surface sensitive technique

In a embodiment the present invention also provides a method for detecting the presence of an analyte in a sample comprising:

a) Binding the analyte to a surface containing discrete specific affinity sites that are selective for the said analyte.

b) Reacting the said bound analyte with a reaction mixture that causes template selective degradation, by means of addressing suitable degradation precursors present within, or specifically associated with, the analyte

In one embodiment the present invention further involves a method for the detection of an analyte by means of template selective degradation and measuring a concomitant change in surface excess at a transducer surface. In contrast to the detection using template independent polymerisation of analyte specific initiation sites resulting in an increase in localised mass, template dependent degradation of specific affinity sites results in a decrease of localised mass and hence also may be used for analyte specific detection. For this purpose specific affinity sites, having the further property of containing degradation precursors may be immobilised to a surface which may be addressed by means of a surface sensitive technique. Upon reaction with a specific analyte said degradation precursors may be cleaved, degraded, or become activated for degradation. Usually such degradation occurs in the presence of a specific agent catalysing the template selective degradation process.

In a preferred embodiment the analyte is a nucleic acid, a specific reaction product or a derivative thereof and the specific agent catalysing template selective degradation is a nucleic acid endo or exonuclease. Degradation precursors having a specific affinity for the said analyte may be immobilised to a surface which may be addressed to measure the surface excess by means of surface sensitive technique. Said specific affinity sites may further also contain components which influence the measured surface excess (e.g. polymeric tails) but have no specific affinity relationship with the said analyte, however they may be degraded or cleaved off, upon reaction with said analyte in the presence of suitable DNA or RNA nuclease or a combination of several nucleases. Schemes for template selective degradation according to this method may for example comprise the analyte (in this case a deoxyribonucleic acid) selective degradation of specific affinity sites (i.e. degradation precursors) immobilised at a surface detectable by means of a transducer surface which are degraded upon hybridisation with the analyte in the presence of a suitable nuclease, for example specific affinity sites consisting of deoxyribonucleic acid and having free 5'-termini may be degraded upon the formation of hybrid with the analyte nucleic acid in the presence of phage lambda induced nuclease, or phage T7 gene 6 exonuclease, or exonuclease III (in the case of 3'-termini). Similarly specific affinity sites immobilised at said surface containinig ribonucleic acid moieties, may be degraded upon the hybridisation with said analyte, in the presence of RNaseH or exonuclease III. In both cases the analyte nucleic acid is released upon degradation of the specific affinity site, and as such may react with other affinity sites causing recurrent hybridisation and hence additional signal increase. Optionally residues contained within the said specific affinity site (e.g. poly ribo-A tail) which do not specifically interact with the analyte, may also be degraded upon the degradation of the specifically interacting residues, due to the activity of a terminally directed exonuclease such as poly A-specific ribonuclease. Other methods for the analyte selective degradation, and the detection thereof by measuring a

change in surface excess, may include the use of one or more specific affinity sites to achieve a particular conformation in the presence of the said analyte and wherein one of the components participating in said conformation is subsequently cleaved by a structure specific nuclease. For example several nucleases (e.g. lambda nuclease, the 5'nuclease of certain DNA polymerases, as well as some nucleases involved in Okazaki-fragment processing) recognise the presence of redundant single strands forming a branch in a otherwise double stranded nucleic acid and may cleave the single strand exonucleolytically or endonucleolytically at a junction containing the 5' or 3'end of strands engaged in a double stranded structure (Kadaba, et al., (1975), J. Biol. Chem., 250, 5438-5445; Setlow, et al., (1972), J. Biol. Chem., 247, 232-240). Therefore at least one of the said components required to form said particular conformation may be a discrete specific affinity site immobilised at the said surface, and may be cleaved upon the activity of a structure specific nuclease when the analyte nucleic acid participates in said conformation. Other components necessary to complete a specific structure may be futher specific affinity sites present in solution or immobilised on the same said surface. In the case of lambda nuclease based degradation, the immobilised specific affinity site may be a oligonucleotide containing a 3' portion not complementary to the sequence of the said analyte which forms a redundant single strand at a junction defined by the analyte hybridised to the said specific affinity site and a auxiliary analyte specific oligonucleotide. When 5' nucleases for example derived from DNA polymerases are used, the method is similar except that the immobilised specific affinity sites contain a 5' portion (i.e. the redundant single strand) which is not complementary to the said analyte nucleic acid. In both cases the conformation may be arranged as such that either the 3' or 5' end of the specific affinity sites is used to immobilise said affinity sites. The combination of nucleases, degradation precursors and analytes described in this particular embodiment is non-exhaustive, and further combinations are possible. However the above examples, show how progressive and structural template selective degradation in an analyte specific manner may be applied for surface excess based detection within the context of the present invention.

In a further embodiment, initiation sites for template independent polymerisation may be produced in a analyte specific manner by means of template selective degradation of soluble or immobilised specific affinity sites interacting with the said analyte. When the analyte is a nucleic acid a specific reaction product or a derivative thereof, template dependent degradation or cleavage of the specific affinity sites interacting with said analyte may be achieved according to the methods previously described in the current invention (previous embodiment). As such immobilised specific affinity sites which are cleaved or degraded upon specific interaction with the analyte (and if necessary auxiliary oligonucleotides), may subsequently present suitable

termini which may serve as initiation sites for template independent polymerisation. For example specific affinity sites having 3' termini which are not capable to serve as initiation sites for template independent polymerisation (either due to their nature, or presence of blocking groups such as dideoxy residues), may be modified by means of analyte specific cleavage or degradation to yield 3'ends which are amenable to template independent polymerisation by providing suitable initiation sites. This may be the case when said specific affinity sites are DNA/RNA chimera where the 3' end may be in the deoxyribonucleoside form, and the ribonucleoside form is exposed upon selective degradation such that the latter may be used as a initiation site for template independent polymerisation using polynucleotide adenylyltransferase. In a similar fashion a 3'blocked end may be removed upon selective degradation yielding an initiation site for various nucleotidylexotransferases. Similar schemes may be devised for analyte specific detection using structure specific nucleases which cleave redundant single strands, which also cause the creation of an initiation site for template independent polymerisation. It may be anticipated that whenever analyte specific detection by means of template selective degradation followed by template independent polymerisation is performed, the degradation may either proceed in solution or at the surface where the surface excess is measured by means of a transducer surface.

In another embodiment the present invention provides additional methods for the analyte specific detection of surface excess at a transducer surface by means of template independent polymerisation in conjunction with template selective degradation. Using the ability to control the composition of the product of a reaction involving template independent polymerisation (e.g. a homopolymer), this product my be used to induce the degradation of specific affinity sites (i.e. degradation precursors) being present at a surface, in solution or both, causing the creation of an initiation site for template independent polymerisation. Alternatively initiation sites may be created from the product of template independent polymerisation due to a specific cleavage thereof. By definition this constitutes an autocatalytic process which may be used to increase the sensitivity of the analyte detection. Depending on the method discrete specific affinity sites having affinity for the analyte or for the product of template independent polymerisation, or a mixture thereof may be immobilised to a surface in order to measure the products of said autocatalytic process by means of determining the surface excess using a surface sensitive technique.

In a preferred embodiment the analyte is a nucleic acid sample, specific reaction product or a derivative thereof which may be detected by measuring the surface excess at a transducer surface, wherein said surface excess is produced by a process involving template independent polymerisation in conjunction with template selective degradation. When for example the analyte is a ribonucleic acid, said analyte may be hybridised to a

sequence specific affinity site at a surface and washed. Following this step the hybridised ribonucleic acid is subjected to a reaction mixture containing polynucleotide adenylyltransferase and ribonuclease IV, the latter of which cleaves the product of template independent polymerisation to yield new initiation sites for template independent polymerisation. The product of the reaction may be detected by means of a surface sensitive technique at a surface containing specific affinity sites for the product of template independent polymerisation. When the said analyte is a deoxyribonucleic acid a similar method may be used involving DNA nucleotidylexotransferase and deoxyribonuclease IV. Other methods wherein a nucleic acid may be detected using a autocatalytic reaction involving the processes of template independent polymerisation and template selective degradation, but wherein reaction products remain confined to the surface where detection proceeds, may also be devised. This may include the detection of a deoxyribonucleic acid sample having an initiation site for template independent polymerisation immobilised to a surface (where detection proceeds) containing discrete specific affinity sites. Following specific immobilisation of the said sample, the surface may be incubated with a reaction mixture containing degradation precursors with a specific affinity for the product of template independent polymerisation (said degradation precursors may thus be present in solution, alternatively they are present on the surface containing the specific affinity sites), a specific nuclease cleaving or degrading said degradation precursors upon interacting with the product of template independent polymerisation and an enzyme capable of performing template independent polymerisation. For example, said degradation precursors may be DNA/RNA chimera which are blocked with respect to template independent polymerisation, and have both DNA and RNA residues complementary to the product of template independent polymerisation, said RNA residues may be degraded when interacting with said product in the presence of RNaseH, and thereby create a initiation site for template independent polymerisation by means of for example DNA nucleotidylexotransferase. Henceforth an appreciable analyte specific change in surface excess may be detected by means of a transducer surface using template independent polymerisation in conjunction with template selective degradation when minimal quantities of the analyte are present.

In another embodiment analyte specific template independent polymerization and the ability to perform continuous detection by means of transducer surface, may be used to obtain information concerning the relative sequence composition of a nucleic acid sample, specific reaction product or a derivative thereof. In particular the specific affinity sites used to immobilise the said nucleic acid sample may serve as primers for a DNA or RNA polymerase reaction in order to perform template dependent polymerisation using said nucleic acid sample as a template. Template dependent polymerisation may then be carried out using different sets nucleoside

triphosphates each consisting of less than four different nucleotide bases. In this manner the length of the polymerized fragment becomes restricted, and the extension pattern using for example 4 different sets each containing 3 different nucleoside triphosphates becomes typical for the sequence immediately flanking the primer binding region. Detection by means of template independent polymerisation is then performed in such a way that the extension of the nucleic acid sample is more favourable than the extension of the specific affinity sites used to prime and immobilise the nucleic acid sample, which is possible by choosing a suitable composition (e.g. RNA or DNA) of said specific affinity sites, template and nucleosides which may be differentiated using the relevant nucleotidylexotransferase. Consequently the fragments generated by the said restricted polymerization may be analyzed in terms of their affinity with the surface by measuring the continuous change in surface excess, using for example equilibrium dissociation or temperature jump methods. Similar methods involving restricted length polymerization reactions may also be carried out in solution using soluble specific probes as primers, the product of the reaction may then be immobilized to a transducer surface and subjected to template independent polymerization. For this particular application, the interaction of said elongated soluble nucleic acid primers with partially or fully complementary probes may be inferred from affinity or structural relationships, in the latter case inhibition with respect to template independent polymerization by for example DNA nucleotidylexotransferase due to the presence of recessive ends in the unreacted primer-probe complex is relieved when a particular length in the restricted length polymerization assay is exceeded. It may be anticipated that the method of restricted length polymerization using soluble primers and predetermined nucleic acid probes will detect minor fluctuations in a target nucleic acid sequence while at the same time taking advantage of the rapid hybridization kinetics of the relatively small nucleic acid primers. When the restricted length polymerization proceeds from a solution based primer and sample a degree of amplification of the solution based reaction may be achieved by allowing several restricted length polymerization cycles. In one particular case the primer used in the reaction may have degradable 5' phosphate end with a flanking sequence of deoxyribonucleoside residues approximately spanning half the sequence of the said primer, this region is bound by a series of non-degradable phosphodiester bond analogues, or ribonucleoside residues succeeded with a region of ribonucleoside or deoxyribonucleoside residues constituting the 3'end of the said primer. When the primer is incubated with the sample, a 5' deoxyexonuclease (e.g. phage lambda induced exonuclease), a polymerase, and a restrictive length nucleoside triphosphate mixture the elongated primers are dissociated from the nucleic acid sample due to the partial digestion of the annealed primer and the competitive annealing of an incoming new primer. Consequently multiple restricted length polymerizations are obtained from one sample

template and as such a substantial degree of amplification may be achieved. Similarly the primer used for the restricted length polymerization may contain a sequence of ribonucleoside residues which are degraded upon sequence specific association with the nucleic acid sample, when the said restricted length polymerisation for example is carried out in presence of ribonuclease H.

Using a rather similar approach variations on the dideoxy sequencing method may be devised in order to be used with the methods described in the current invention. In this case the analyte nucleic acid may be immobilised to specific affinity sites at the surface where detection of the surface excess is possible. Said specific affinity sites may serve as a primer for the dideoxy sequencing reaction or alternatively the immobilised analyte nucleic acid is hybridised with a primer oligonucleotide which further contains initiation sites for template independent polymerisation (and do not interfere with the analyte nucleic acid). The choice for using either immobilised or associated sequencing primers depends mainly on the nature of the template independent polymerisation reaction used to detect the surface excess. Preferably the products obtained after template independent polymerisation are analysed using the equilibrium dissociation method as monitored by the continuous measurement of surface excess under the application of a temperature gradient.

The invention is further described with the following examples, which are given as illustration rather than limitation of the scope of the invention.

## EXAMPLES

### Materials and methods

Gold electrode material either evaporated on silicon wafers or polymer carriers were obtained from both commercial and non-commercial sources. The electrodes were mounted on glass slides or plastic sheet material using a low temperature curable epoxy (Epotek H-54), electrical connections between the electrode material and the electrical wiring were established using silver loaded epoxy and graphite ink, the structure was then thoroughly sealed with epoxy leaving only the desired electrode material exposed. Structures for impedimetric sensing involving a interdigitated electrode architecture were obtained from Imec (Belgium), or commercial suppliers, with interelectrode spacings varying between 1 and 40 μm. The gold electrode surfaces were subsequently inspected and activated by potentiodynamic cycling in a three electrode cell between -0.3 Volt and 1.2 Volt (versus $Hg/HgSO_4$ reference) until steady state voltammograms were obtained. Cyclic voltammograms and other electrochemical measurements were collected using a EGG/PAR 283 potentiostat/galvanostat, equipped with a model 1025 FRA unit for faradayic impedance analysis. Experiments involving impedimetric sensing were analyzed using a HP 4284 A LCR meter.

Oligonucleotide probes and synthetic complements were purchased from Eurogentec (Belgium) and used without further purification. All chemicals used were analytical grade or better, $Co(Phen)_3^{2+/3+}$ was prepared according to the method as described by Dollimore and Gillard (Dollimore, J. Chem. Soc. Dalton, 1973, 933-940.) and modifications thereof. Modification of the gold electrodes was carried out taking advantage of the well known gold sulfur interaction, which will be readily apparent to the person skilled in the art. Briefly 3' or 5' thiol labelled oligonucleotides are dissolved in a high salt acidic buffer (e.g. 1M $KH_2PO_4$ pH 4.5) to a final concentration of 3μM, further containing 2μM 3-mercaptopropylmethyldimethoxysilane (ABCR, Germany). Freshly cleaned electrode surfaces are then fully covered with the modification solution, and left to react for 90 minutes at room temperature. Subsequently the electrode surfaces are rinsed with distilled water and reacted with a solution containing 1M $KH_2PO_4$ and 20 μM 3-mercaptopropylmethyldimethoxysilane for a further 30 minutes at room temperature. The electrodes are then finalized by immersing the surfaces in a nucleic acid hybridisation buffer (e.g. 200mM $Na_2SO_4$, 10mM Tris-$SO_4$) at 73°C for 10 minutes, and stored in the refrigirator in the measurement buffer until use. A similar approach was used for the modification of the impedimetric sensing structures. Modifications were thoroughly checked by cyclic voltammetry or impedance spectroscopy prior to use, confirming successful functionalization of the surfaces.

Electrochemical measurements typically were performed in a low ionic strength buffer containing a affinity redox complex (e.g. 5mM $NaClO_4$, 5mM Tris $HClO_4$ pH 8 and 40 to 80 μM $Co(phen)_3^{2+/3+}$). Typically cyclic voltammetry was used to asses the surface excess and cycling generally is performed between 0.3V and -0.3V versus $Co(phen)_3^{2+/3+}$. Alternatively faradayic impedance is recorded in the same buffer as above and the signal is analyzed in terms of a pseudo-capacity reflecting the surface excess of the electroactive species, the charge transfer resistance or the total impedance. Analysis of the conductivity relating to experiments carried out with impedimetric sensing structures was inferred from the collected bode plots. Briefly the conductivity was taken at the frequencies between respectively the double layer relaxation and dielectric relaxation frequencies, which are bound respectively by the low and high frequency capacities. Also impedimetric measurements were carried out in buffers of low ionic strength or in distilled water.

**Example I**

Amplification of nucleic acid hybridization signal by DNA nucleotidylexotransferase, using electrochemical or impedimetric detection

Sequence selective electrode surfaces, or impedimetric sensing structures using nucleic acid probes with a 3' modification group such as a thiol, are prepared according to the procedure as described in materials and methods section. The response of the modified electrode is checked and hybridised with a sample nucleic acid for example, a oligonucleotide complement (25-mer complementary sequence (5 nM) and 18-mer non complementary sequence (10 nM) Fig 1 A, or 18-mer non complementary sequence (10 nM) alone Fig 1 B), or a polymerase chain reaction product (sample 1 Fig 1 C, sample 2 Fig 1 D), in a suitable hybridisation solution (e.g. 200mM $Na_2SO_4$ and 10 mM Tris-HCl pH 8.5) for about 60 minutes at a temperature roughly corresponding to 0.8 $T_m$ ($T_m$ is the anticipated melting temperature in that solution of the oligonucleotide/target duplex, corresponding to a 50% transition of the double to single stranded conformation). Following hybridisation the electrode may be measured, or is directly incubated with the DNA nucleotidylexotransferase reaction mixture (e.g. 30 Units $\mu l^{-1}$ DNA nucleotidylexotransferase, 5 mM $CoCl_2$, 2 mM dTTP, 200mM potassium cacodylate, 25 mM Tris-HCl pH 6.6 and 1.25 mg $ml^{-1}$ BSA ) preferably for about 10 to 60 minutes. Homopolymeric or copolymer tails of varying composition may be obtained by using a suitable metal activation factor (Kato et al., (1967), J. Biol. Chem. 242, 2780-2789) in the presence of the relevant nucleoside triphosphates. In this way the 3' end of the hybridized nucleic acid sample is extended, creating a surface excess which may be addressed

using for example cyclic voltammetry in the presence of affinity redox probes, or measurement of the surface or localised conductivity.

Details relating to polymerase chain reaction product:

Target amplified : Human Immunodeficiency Virus (HIV) reverse transcriptase fragment, 571 base-pairs.

The target was amplified at Innogenetics Research Labs according to methods known to the art, for convenience one phosphorylated primer was used allowing the generation of single stranded amplicon by means of phage lambda exonuclease. Targets further are used as received and diluted in the hybridisation buffer (1/10). Different samples (sample 1 and sample 2) are used corresponding to variations in the target sequence, and measured with respect to the nucleic acid probe at the electrode surface.

Sequence probe : 5' ATA GAG GAA CTG AGA C

(Partial) sequence sample 1: 3' TAT CTC CTT GAC TCT G

(Partial) sequence sample 2: 3' TAT CTC CTC AAC TCT T

**Example II**

Detection of mismatches using continuous electrochemical monitoring of surface excess, induced by analyte specific template independent polymerisation

Sequence selective electrodes (using a 25-mer oligonucleotide having a 3'- thiol moiety) are prepared as described in the material and methods section. Following modification the surfaces are hybridised under low stringency conditions with sample oligonucleotides which are either fully or partially complementary to the sequence immobilized at the electrode surface (see below). The electrodes are subsequently subjected to template independent polymerisation by DNA nucleotidylexotransferase using thymidine residues. After a brief rinsing step in hybridization solution the electrodes are analyzed using either the equilibrium dissociation or temperature jump method. The response of the electrodes to thermal equilibrium dissociation (figure 2A) or a temperature jump (at 52.8°C) (figure 2B) are reconstructed from the integrated currents as obtained by continuous cyclic voltammetry.

Sample oligonucleotides used:

Fully complementary : 5' TAG ATG CTC GCA ACC ACT ATC CAG T

Single mismatch : 5' TAG ATG CTC GCA CCC ACT ATC CAG T

**Example III**

Heterogeneous surfaces

Sequence selective electrode surfaces are prepared as described in the materials and methods section. The response of the electrode is checked for modification, and challenged with sample or a specific nucleic acid reaction product. In the present example electrode surfaces are incubated with respectively 18-mer and 25-mer complementary oligonucleotides and mixtures thereof. Following hybridisation, the electrode surface is subjected to template independent polymerisation using DNA nucleotidylexotransferase. Subsequently a temperature gradient (for equilibrium dissociation, see Fig. 3A and 3B) or jump is applied to the transducer surface, while the surface excess is continuously monitored using for example cyclic voltammetry.

Sample oligonucleotides used:

25-mer : 5' TAG ATG CTC GCA ACC ACT ATC CAG T

18-mer : 5' TC GCA ACC ACT ATC CAG T

**Example IV**

Determination of sequence dependent restricted polymerization length using dynamic electrochemical monitoring of surface excess

Nucleic acid probe modified electrode surfaces are challenged with a nucleic acid primer which satisfies particular priming criteria known by those skilled in the art. Complementary nucleotides are incorporated using a polymerase such as DNA polymerase I klenow fragment in presence of a restricting nucleotide mixture (0.1mM each dNTP, 0.04 U $\mu l^{-1}$ Klenow fragment, 50 mM Tris-HCl pH 7.5, 10mM $MgCl_2$). In the present example only 3 of the four nucleotides are used, and as such polymerization is terminated when no matching nucleotide is present. When the four possible combinations of the 3 nucleotides are used in conjunction with a primer of a primary specificity a particular extension pattern is obtained. Alternatively the extension reaction is carried out in solution in which a nucleic acid sample is present, and the extended primers are hybridized to the electrode surface. Following extension and hybridization the nucleic acid product is amplified by template independent polymerization, when DNA is used this is readily accomplished with DNA nucleotidylexotransferase. The extension length is subsequently analyzed by voltammetric analysis of the thermally induced dissociation behaviour. The sequence outcomes are outlined below, and the temperature jump responses for the positive reactions are given in Fig. 4. Note that due to the limited length of the primer the extensions in presence of ATG and CTG react weakly due to the lower melting temperature and the properties of DNA

nucleotidylexotransferase, which has decreased affininity for regressive ends as well as steric factors. The latter properties may be used to direct the outcome of the reaction and hence simplify the analysis.

Electrode(Au)-SH-Spacer-ATC TAC GAG CGT TGG TGA TAG CTC A5'

| | |
|---|---|
| primer: | 5'TAG ATG CTC GCA3' |
| ATG extension | A3' |
| CTG extension | |
| ACG extension | ACC AC3' |
| ACT extension | ACC ACT ATC A3' |

**Example V**

Detection of template dependent RNase H activity, using template independent polymerization

In order to demonstrate the activity of a specific endonuclease, sample sequences are incubated in conjunction with chimeric probes and RNase H. Briefly 0,1 μM sample sequence and 0,5 μM chimeric probe are incubated in plastic tubes in the presence of RNase H in a buffer (e.g. Tris HCl pH 7,5) containing 8 to 10 mM $MgCl_2$ at 37°C. Following incubation the reactions are stopped by adding EDTA up to a concentration which equals or is greater than the $MgCl_2$ concentration and adding $Na_2SO_4$ to a final concentration of 200 mM (these steps roughly double the original volume). The reactions are subsequently heated to 75°C for 5 minutes, and put on ice until further analysis. Following the incubation the reaction products are hybridized under low stringency conditions to a transducer surface (e.g. a gold electrode) which is modified with probes which are partially or fully complementary to the chimeric probe, and are not viable to template independent polymerization (e.g. carrying a 3' thiol moiety). The transducer surfaces are now subjected to template independent polymerization using DNA nucleotidylexotransferase. The sequences corresponding to the legend in Fig. 5 are:

| | |
|---|---|
| Chimeric probe: | 5' TAG ATG CTC GCA ACC acu aug gAG T |
| Complementary template | : 3' ATC TAC GAG CGT TGG TGA TAC CTC A |
| Partially complementary template | : 3' ATC TAC GAG CGT TGG TGA TAG GTC A |
| Non complementary template | : 3' AGC GGC GAC GTG ACA CTT CGA G |

Upper case: deoxyribonucleoside residue, Lower case: ribonucleoside residue

**Example VI**

Real time monitoring of nuclease activity

Sequence selective electrode surfaces having 3'thiol moiety are prepared as described in the materials and methods section. Subsequently complementary deoxyoligonucleotide is immobilized to the electrode surfaces and the obtained surface is subjected to template independent polymerization using DNA nucleotidylexotransferase. The obtained electrode surface may now be examined for the activity of a particular nuclease. In this example the electrode is incubated with DNase I (2 Units / 100 µl), in a buffer containing: 10 mM $MgCl_2$, 50 mM Tris-HCl pH 7.6 and 5 mM $Fe(CN)_6^{3-/4-}$. The electrode surface is subsequently continuously cycled in a conventional 3 electrode cell between 0.5 V and -0.7 V versus Ag/AgCl not compensated for IR drop thermostatted at 37°C (see Fig. 6).

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CLAIMS

1. A method for detecting the presence of an analyte in a sample by means of modifying and measuring the surface excess wherein the said method comprises

a) increasing surface excess by means of template independent polymerization

or

b) decreasing the surface excess by means of template selective degradation

2. A method according to claim 1 comprising:

a) Reacting the sample with a reaction mixture that causes template independent polymerisation, by means of addressing suitable initiation sites present within, or specifically associated with, the analyte

b) Assessing the template independent polymerisation, by means of measuring the surface excess using a surface sensitive technique.

3. A method according to claims 1 and 2 comprising:

a) Binding the analyte to a surface containing discrete specific affinity sites that are selective for the said analyte.

b) Reacting the said bound analyte with a reaction mixture that causes template independent polymerisation, by means of addressing suitable initiation sites present within, or specifically associated with, the analyte

c) Assessing the template independent polymerisation, by means of measuring the surface excess at the surface containing said specific affinity sites using a surface sensitive technique.

4. A method according to claims 1 or 2 wherein the template independent polymerisation products comprise a functional amplification, and wherein the surface excess is determined continuously as a function of a physical or chemical gradient.

5. A method according to claims 2 or 3 wherein the analyte is a nucleic acid, or a specific reaction product or a derivative thereof, and wherein said affinity sites are oligonucleotide probes or chemical analogues thereof.

6. A method according to any of claims 1 to 5 wherein said template independent polymerisation comprises reactions catalysed by glycosyltransferases or nucleotidylexotransferases.

7. A method according to any of claims 1 to 5 wherein said suitable initiation site constitutes a free 3' OH group or a nonreducing *N*-OH end.

8. A method according to any of claims 1 to 7 wherein said initiation sites for template independent polymerisation are created by a template selective degradation.

9. A method according to any of claims 2 to 8 wherein the said template independent polymerisation proceeds from initiation sites present on said analyte.

10. A method according to any of claims 2 to 8 wherein the said template independent polymerisation proceeds from initiation sites present on said specific binding sites.

11. A method according to any of claims 9 to 10 wherein said template selective degradation results in a free 3' OH group.

12. A method according to any of claims 1 to 11 wherein said template independent polymerisation yields products that result in secondary initiation sites for template independent polymerisation.

13. A method according to claim 12 wherein said secondary initiation sites are created through a branching enzyme.

14. A method according to claim 12 wherein said secondary initiation sites are created after binding of said products of template independent polymerisation to discrete affinity sites which are selective for said products of template independent polymerisation.

15. A method according to claim 14 wherein said secondary initiation sites for template independent polymerisation are created by a template selective degradation.

16. A method according to claim 1 comprising:

a) Reacting the sample with a reaction mixture that causes template selective degradation, by means of addressing suitable degradation precursors present within the analyte, or specifically associated with, the analyte

b) Assessing the template selective degradation, by means of measuring the surface excess using a surface sensitive technique

17. A method according to claim 16 comprising:

a) Binding the analyte to a surface containing discrete specific affinity sites that are selective for the said analyte.

b) Reacting the said bound analyte with a reaction mixture that causes template selective degradation, by means of addressing suitable degradation precursors present within, or specifically associated with, the analyte

c) Assessing the template selective degradation, by means of measuring the surface excess at the surface containing said specific affinity sites using a surface sensitive technique.

18. A method according to claims 16 or 17 wherein the outcome of template selective degradation is measured as a function of a physical or chemical gradient.

19. A method according to any of claims 16 to 18 wherein the analyte is a nucleic acid, or a specific reaction product or a derivative thereof, and wherein said associated degradation precursor is a oligonucleotide probe or a chemical analogue thereof.

20. A method according to any of claims 16 to 19 wherein said template selective degradation results in a secondary initiation site for a secondary template selective degradation.

21. A method according to any of claims 16 to 20 wherein the said template selective degradation proceeds from initiation sites present on said analyte.

22. A method according to any of claims 16 to 20 wherein the said template selective degradation proceeds from initiation sites present on said specific binding sites or on said associated degradation precursor.

23. A method according to any of claims 16 to 22 wherein said template selective degradation comprises a endonucleolytic cleavage, and said secondary template selective degradation comprises a exonucleolytic degradation.

24. A method according to any of claims 16 to 23 wherein said template selective degradation comprises a endonucleolytic cleavage that creates a free 5' phosphate group that is an initiation site for the said secondary template selective degradation by lambda exonuclease.

25. A method according to any of claims 8 to 24 wherein said selective degradation is catalysed by lambda exonuclease, T7 gene 6 product, DNA polymerase, Rnase H, or exonuclease 3 or a combination thereof.

26. A method according to any of claims 1 to 25 wherein said surface sensitive technique comprises the use of faradayic techniques or impedimetric techniques.

27. A method according to any of claims 2 to 15 and 17 to 26 wherein said discrete specific affinity sites are immobilised directly unto the surface of the electrodes used for said faradayic or impedimetric techniques or in the peri-electrodic spaces.

Figure 1A

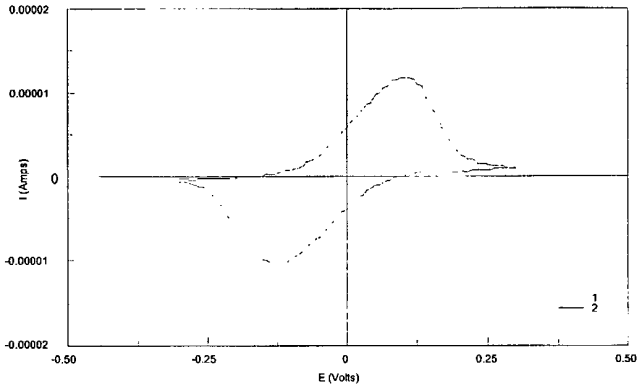



Figure 1B

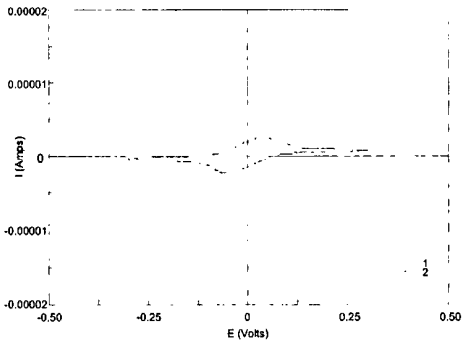


Figure 1C

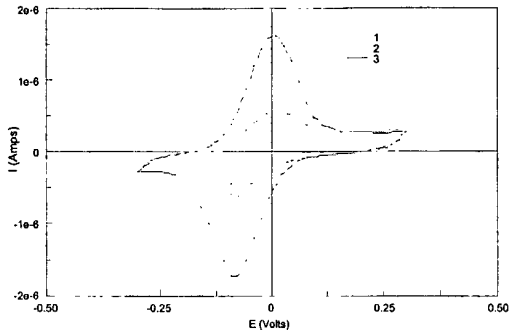



Figure 1D

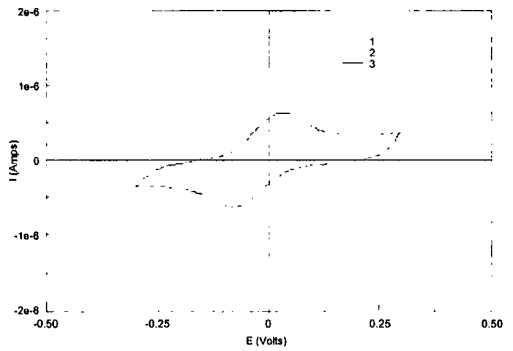


Figure 1E

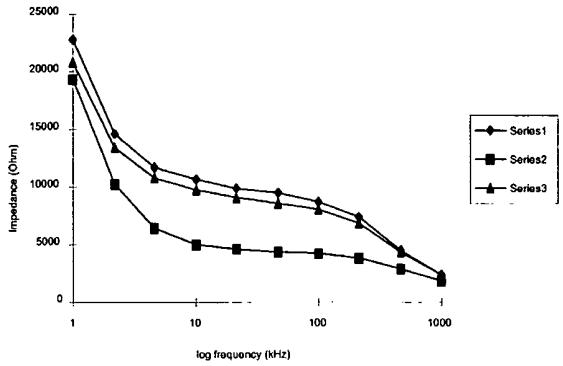



Figure 2A

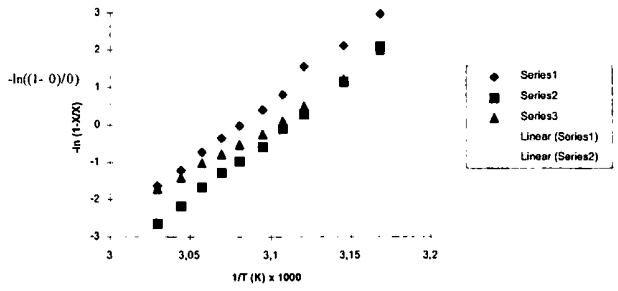


Figure 2B

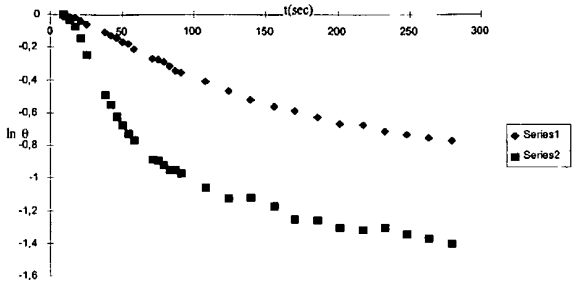



Figure 3A

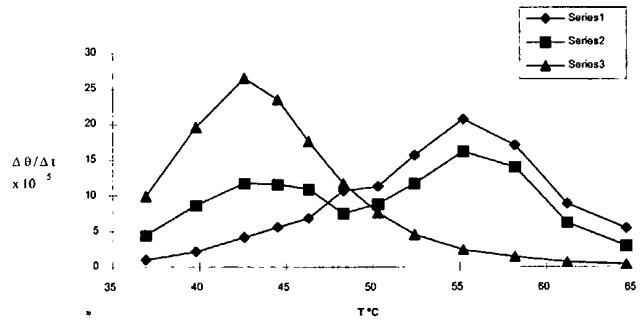


Figure 3B

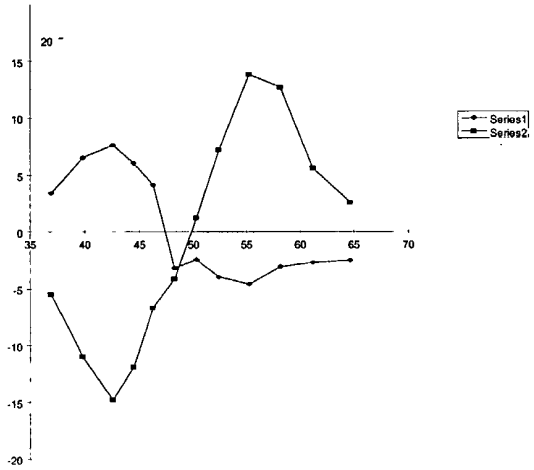



Figure 4

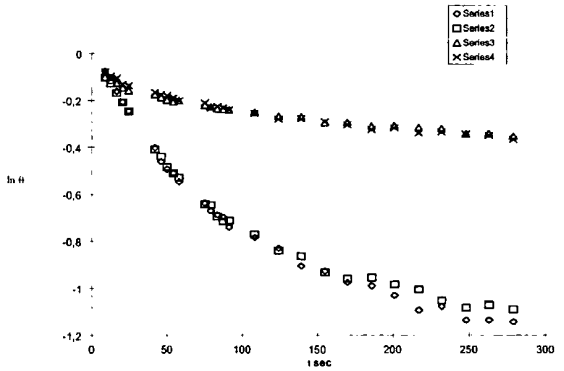


Figure 5

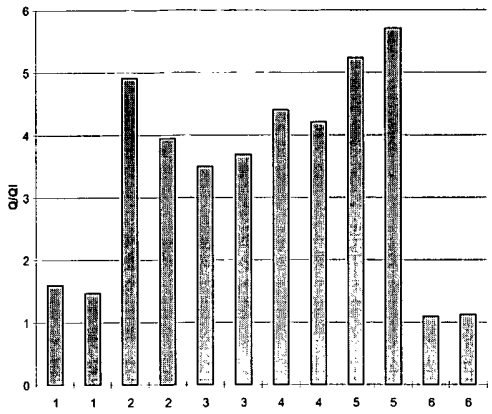
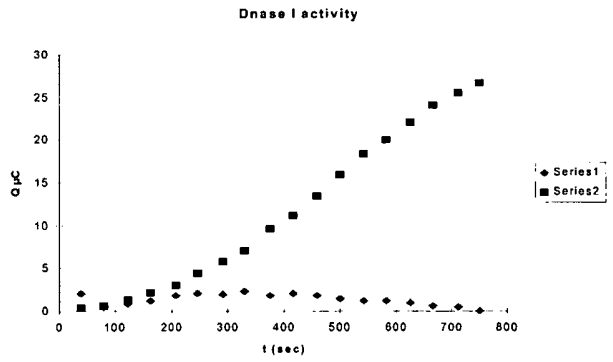



Figure 6



# INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/050842

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 G01N33/53 G01N33/543

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, MEDLINE, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | MAESAWA CHIHAYA ET AL: "A rapid biosensor chip assay for measuring of telomerase activity using surface plasmon resonance." NUCLEIC ACIDS RESEARCH. ENGLAND 15 JAN 2003, vol. 31, no. 2, 15 January 2003 (2003-01-15), pages E4-E4, XP002258866 ISSN: 1362-4962 * the whole document, in particular Material and Methods, Fig. 1 * | 1-3,6,7, 9,12 |

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 1 October 2004 | 12/10/2004 |

| Name and mailing address of the ISA | Authorized officer |
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| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Pinta, V |

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# INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/050842

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 01/31057 A (MUTH JOCHEN; WINDHAB NORBERT (DE); AVENTIS RES & TECH GMBH & CO (DE)) 3 May 2001 (2001-05-03)<br><br>* the whole document, in particular p. 10 l. 10-12, p. 15 l. 25 to p. 16 l. 13, p. 25 l. 25 to p. 26, l. 8, p. 32 l. 1-13, Fig. 1, Fig. 6 * | 1,2,7,8, 11,16, 17,20, 21,25-27 |
| A | WO 97/04129 A (PHARMACIA BIOSENSOR AB; MALMQVIST MAGNUS (SE); PERSSON BJOERN (SE)) 6 February 1997 (1997-02-06)<br>the whole document | |
| A | WO 90/13666 A (AMERSHAM INT PLC)<br>15 November 1990 (1990-11-15)<br>the whole document | |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2004/050842

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [X] in written format

   [X] in computer readable form

   c. time of filing/furnishing

   [ ] contained in the international application as filed

   [ ] filed together with the international application in computer readable form

   [X] furnished subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP2004/050842

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| | | JP 4505251 T | 17-09-1992 |