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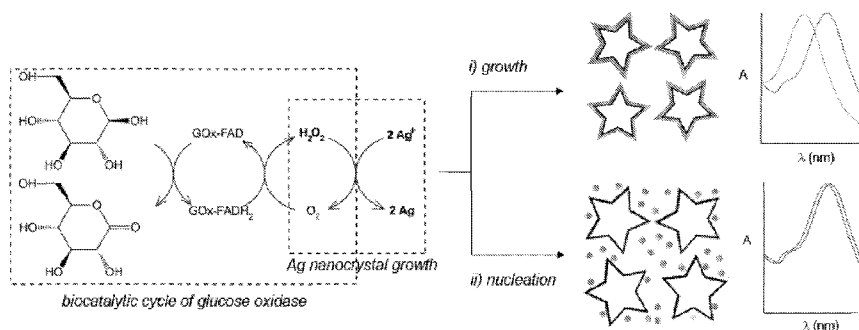
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(54) Title: ASSAY

Figure 1



(57) Abstract: The present invention provides a method of determining the presence of an analyte in a liquid sample. The method comprises contacting a first binding moiety with the liquid sample, the first binding moiety being linked to signal moiety which yields a signal and specifically binding the analyte to form a reaction conjugate. A detection conjugate is formed by binding the reaction conjugate to a reducing agent-generating moiety. The detection conjugate is contacted with a substrate for the reducing agent-generating moiety and metal ions; and the signal yielded by the signal moiety is measured. A change in the signal indicates the presence of the analyte.

Assay

The present invention relates to an assay and a kit for determining the presence of an analyte in a liquid sample.

5 Lowering the limit of detection is a fundamental aspect in the design of sensors important to food safety regulations,¹⁻² environmental policies³⁻⁵ and the diagnosis of severe diseases.⁶⁻¹⁰ However, conventional transducers generate a signal that is directly proportional to the concentration of the target molecule. As such, the presence of chemical species at ultralow concentrations results in
10 tiny variations in the physical properties of the sensor and these are difficult to detect with confidence.

Metal nanoparticles can be used as building blocks for the fabrication of biosensors when their localized surface plasmon resonance (LSPR) shifts in response to a biorecognition event.¹¹ In these detection platforms, the largest variations in the LSPR are observed when another metallic nanostructure interacts in close proximity with the nanoparticle.

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Methods of detecting analytes that are present at very low concentrations are highly sought after in a wide range of clinical and commercial fields.

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According to a first aspect, the present invention provides a method of determining the presence of an analyte in a liquid sample, comprising:

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- (i) contacting a first binding moiety with the liquid sample, the first binding moiety being linked to a signal moiety which yields a signal and specifically binding the analyte to form a reaction conjugate;
- (ii) forming a detection conjugate by binding a reducing agent-generating moiety to the
25 reaction conjugate;
- (iii) contacting the detection conjugate with a substrate for the reducing agent-generating moiety and metal ions; and
- (iv) measuring the signal yielded by the signal moiety, wherein a change in the signal indicates the presence of the analyte.

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In the present invention, the concentration of the reducing agent-generating moiety around the signal moiety is directly related to the concentration of the analyte. This is achieved by binding the reducing agent-generating moiety to the reaction conjugate. The reducing agent-generating moiety may be linked to a second binding moiety which binds the reaction conjugate directly, and preferably binds the analyte in the reaction conjugate. Alternatively, the second binding moiety may bind the reaction conjugate indirectly via one or more intermediate binding moieties, at least

one of which binds the reaction conjugate, preferably the analyte. When the second binding moiety or the one or more intermediate binding moieties binds the analyte in the reaction conjugate, the method preferably includes the step of removing analyte which is not bound to the first binding moiety prior to binding any further binding moieties to the analyte in the reaction conjugate. When the detection conjugate is contacted with a substrate for the reducing agent-generating moiety and metal ions, reducing agent is generated which reduces the metal ions. Depending on the supply of the reducing agent, the reduction of metal ions to metal atoms causes either the formation of a homogeneous metal coating on the signal moiety or the formation of free-standing metal nanocrystals. When the reducing agent-generating moiety is present at low concentrations (due to a low concentration of analyte), the short supply of reducing agent dictates slow crystal growth conditions. This favours the growth of a homogeneous metal coating on the signal moiety acting as a seeding point,¹⁶ which yields a change in the signal yielded by the signal moiety.^{17,18} However, when the concentration of the reducing agent-generating moiety is high (due to a high concentration of analyte), the abundant supply of reducing agent favours nucleation instead of epitaxial growth and free-standing metal nanocrystals are obtained.¹⁶ Consequently, less metal is deposited on the signal moiety and the signal change is less than in the presence of reducing agent-generating moiety at low concentrations. If no analyte is present, the reducing agent-generating moiety will not be localized to the signal moiety. Therefore the metal ions will not be reduced and there will be no change in the signal generated by the signal moiety because no coating will form on the signal moiety.

Thus, the presence of the target analyte at low concentrations yields a larger change in the signal yielded by the signal moiety via the formation of a metal coating. This inverse relationship between concentration and signal, herein referred to as "inverse sensitivity", makes this approach highly suitable for the fabrication of ultrasensitive sensors. This is because the change in signal yielded by the signal moiety when the analyte is present at very low concentrations is the highest, making it easy to differentiate it from when there is no change in signal due to an absence of the analyte.

Advantageously, the assay can be used to determine the presence or concentration of an analyte even when the analyte is present in a very low concentration and, for example, in a complex matrix such as bodily fluids. The assay of the present invention can be used to detect analyte at a concentration of as low as about 1×10^{-12} g ml⁻¹ or less, about 1×10^{-13} g ml⁻¹ or less, about 1×10^{-14} g ml⁻¹ or less, about 1×10^{-15} g ml⁻¹ or less, about 1×10^{-16} g ml⁻¹ or less, about 1×10^{-17} g ml⁻¹ or less and most preferably about 1×10^{-18} g ml⁻¹ or less (which is close to the single-molecule level).

The first binding moiety is contacted with a liquid sample. If present, analyte binds to the first binding moiety to form a reaction conjugate. The liquid sample may be incubated with the first binding moiety for a period of time, for example about 0.5 to about 5 hours inclusive, about 1 hour to about 4 hours inclusive, about 1.5 hours to about 3 hours inclusive and preferably about 2 hours. The reaction conjugate may then be washed, for example by centrifugation, and then may be re-dispersed in buffer. Following this, a detection conjugate is formed by binding the reaction conjugate to a reducing agent-generating moiety. In one embodiment, the reducing agent-generating moiety is linked to a second binding moiety which binds the reaction conjugate. Once the detection conjugate has been formed, unbound second binding moiety linked to the reducing agent-generating moiety may be washed away. Alternatively, the second binding moiety may bind the reaction conjugate indirectly via one or more intermediate binding moieties, at least one of which binds the reaction conjugate. In such embodiments, any unbound intermediate binding moieties may be washed away prior to the second binding moiety binding the one or more intermediate binding moieties.

The detection conjugate is then contacted with a substrate for the reducing agent-generating moiety. This causes a reducing agent to be generated. The reaction mixture is contacted with metal ions which are reduced by the reducing agent to metal atoms. As discussed above, when the analyte concentration is low, the enzyme concentration around the signal moiety is low, and the short supply of reducing agent dictates slow crystal growth conditions. This leads to the deposition of a metal coating on the signal moiety (one embodiment is shown schematically in Figure 1 of the accompanying drawings). The coating causes a change in the signal yielded by the signal moiety.

The presence of an analyte can therefore be detected by determining whether there has been a change in the signal yielded by the signal moiety, thus providing a qualitative test. It will be appreciated that the method can readily be transformed into a quantitative test whereby the concentration of the analyte in the sample can be determined. This may be achieved using a calibration curve such as the one shown in Figure 2 of the accompanying drawings. Importantly, since the change in signal is larger when the analyte is less concentrated (i.e. the assay is inversely sensitive) and there is no change in signal when analyte is not present, the invention allows one to differentiate samples containing no analyte from samples containing very low levels of analyte with a high degree of confidence.

The sample may be tested and the signal interpolated in calibration curves similar to those provided in Figure 2 of the accompanying drawings. Alternatively, the sample may be serially diluted 1:10 several times. These dilutions should show an inversely proportional relationship with the concentration, as shown in Figure 2 of the accompanying drawings. Although the

second methodology is more elaborate, it has several benefits. First, given that inverse sensitivity is such a unique phenomenon, the observation of a negative slope would increase the confidence in the measurement. Second, it increases the dynamic range of the approach, that is, it allows the quantification of those samples whose concentration is higher than the upper limit of the dynamic range. Finally, it minimizes background effects due to complex matrices since these potential interferences are also diluted in the process.

The signal moiety, which is linked (covalently or non-covalently) to the first binding moiety can be, for example, a plasmonic sensor, a fluorescent moiety or an electrochemical sensor. A plasmonic sensor is preferably a coinage metal nanostructure that shows the localized surface plasmon resonance effect. Suitable plasmonic sensors include nanoparticles, for example spherical silver nanoparticles, gold nanoparticles, silver prisms, gold nanorods, gold nanoflowers and gold nanostars as well as other nanostructures such as core-shell silica-gold colloids. In a preferred embodiment, the plasmonic sensor is a gold nanostar. When the signal moiety is a plasmonic sensor, the signal to be measured is preferably the Localized Surface Plasmon Resonance (LSPR) of the plasmonic sensor. When metal coating is deposited on the plasmonic sensor, this yields a shift in the LSPR of the plasmonic sensor.^{17,18} This shift in LSPR indicates the presence of the analyte. The shift in LSPR is caused by the hybridization of the dielectric constants of the plasmonic sensor and the metal coating. For example, when a silver coating is deposited on a gold nanostar, the LSPR of the gold nanostar can be shifted by as much as 150 nm.

In another embodiment, the signal moiety is a fluorescent moiety. The fluorescent moiety may be a fluorescent particle such as a fluorescent nanoparticle. The fluorescent moiety is preferably made from a fluorescent material. In some embodiments, the fluorescent material is a semiconductor material, such as, for example, CdS, CdSe, CdTe, ZnS, AgS, PbS, ZnO, TiO₂ as well as core-shell structures of two different materials (e.g. a CdSe core surrounded by a ZnS shell). The fluorescent moiety can also be an upconverting nanoparticle such as a lanthanide-doped nanoparticle. When the signal moiety is a fluorescent moiety, the signal to be measured is preferably the fluorescence of the fluorescent moiety. When metal coating is deposited on the fluorescent moiety this quenches the fluorescence of the fluorescent moiety and a change in fluorescence indicates the presence of the analyte.

In yet another embodiment, the signal moiety is an electrochemical sensor. The electrochemical sensor may be an electrode such as a noble metal (e.g. gold, platinum) electrode, a mercury drop electrode or a carbon-based electrode. In some embodiments, a potentiometric sensor, for example a field-effect transistor or a glass electrode may be used. When the signal moiety is an electrochemical sensor, the signal to be measured is preferably the electrochemical potential of

the electrochemical sensor, and a change in electrochemical potential indicates the presence of the analyte. The signal may be the reduction potential.

5 The first, second and, where present, the one or more intermediate binding moieties can each be any moiety which binds a target. Such binding may be specific. In the case of the first binding moiety, the target is the analyte. In the case of the second binding moiety, the target is the reaction conjugate, preferably the analyte in the reaction conjugate or the one or more intermediate binding moieties where these are used. In the case of the one or more intermediate binding moieties, at least one of these binds the reaction conjugate. Further intermediate binding
10 moieties may be added in series, each binding to the intermediate binding moiety preceding it with the second binding moiety binding the final intermediate binding moiety added.

The binding moiety may be naturally derived or wholly or partially synthetically produced. The binding moiety and target together form a pair of binding partners. One member of the pair has
15 an area on its surface, which may be a protrusion, cavity or a particular chemical function which specifically binds to and is therefore complementary to a particular spatial and polar organisation of the other member of the pair. Thus, the members of the pair have the property of binding specifically to each other. Examples of types of specific binding pairs are antigen-antibody, biotin-avidin, hormone-hormone receptor, DNA-DNA, receptor-ligand and enzyme-substrate. The
20 present invention is generally concerned with antigen-antibody type reactions. Thus, preferred binding moieties of the invention are antibodies.

The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site
25 that specifically binds an antigen, whether natural or partly or wholly synthetically produced. The term also covers any polypeptide or protein having a binding domain which is, or is homologous to, an antibody binding domain. These can be derived from natural sources, or they may be partly or wholly synthetically produced. Examples of antibodies are the immunoglobulin isotypes (e.g., IgG, IgE, IgM, IgD and IgA) and their isotypic subclasses; fragments which comprise an
30 antigen binding domain such as Fab, scFv, Fv, dAb, Fd; and diabodies. Antibodies may be polyclonal or monoclonal. A monoclonal antibody may be referred to herein as "mab".

It is possible to take monoclonal and other antibodies and use techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the
35 original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementary determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP-A-184187, GB 2188638A or EP-A-239400. A hybridoma or other cell producing an

antibody may be subject to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any specific binding member or substance having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, humanised antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023. A humanised antibody may be a modified antibody having the variable regions of a non-human, e.g. murine, antibody and the constant region of a human antibody. Methods for making humanised antibodies are described in, for example, US Patent No. 5225539.

It has been shown that fragments of a whole antibody can perform the function of binding antigens. Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. *et al.*, *Nature* **341**:544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')₂ fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird *et al.*, *Science* **242**:423-426 (1988); Huston *et al.*, *PNAS USA* **85**:5879-5883 (1988)); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA* **90**: 6444-6448 (1993)).

Diabodies are multimers of polypeptides, each polypeptide comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being linked (e.g. by a peptide linker) but unable to associate with each other to form an antigen binding site: antigen binding sites are formed by the association of the first domain of one polypeptide within the multimer with the second domain of another polypeptide within the multimer (WO94/13804).

Where bispecific antibodies are to be used, these may be conventional bispecific antibodies, which can be manufactured in a variety of ways (Hollinger & Winter, *Current Opinion Biotechnol.* **4**:446-449 (1993)), e.g. prepared chemically or from hybrid hybridomas, or may be any of the

bispecific antibody fragments mentioned above. It may be preferable to use scFv dimers or diabodies rather than whole antibodies. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction. Other forms of bispecific antibodies include the single chain "Janusins" described in Traunecker
5 *et al.*, *EMBO Journal* **10**:3655-3659 (1991).

Bispecific diabodies, as opposed to bispecific whole antibodies, may also be useful because they can be readily constructed and expressed in *E. coli*. Diabodies (and many other polypeptides such as antibody fragments) of appropriate binding specificities can be readily selected using
10 phage display (WO94/13804) from libraries. If one arm of the diabody is to be kept constant, for instance, with a specificity directed against antigen X, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected.

An "antigen binding domain" is the part of an antibody which comprises the area which
15 specifically binds to and is complementary to part or all of an antigen. Where an antigen is large, an antibody may only bind to a particular part of the antigen, which part is termed an epitope. An antigen binding domain may be provided by one or more antibody variable domains. An antigen binding domain may comprise an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

20 "Specific" is generally used to refer to the situation in which the binding moiety will not show any significant binding to molecules other than its target(s), and, e.g., has less than about 30%, preferably 20%, 10%, or 1% cross-reactivity with any other molecule. The term is also applicable where e.g. an antigen binding domain is specific for a particular epitope which is carried by a
25 number of antigens, in which case, the binding moiety carrying the antigen binding domain will be able to bind to the various antigens carrying the epitope.

The first, second and one or more intermediate binding moieties do not necessarily have to be the same type of binding moiety, although they may be in some embodiments. In a preferred
30 embodiment, the first binding moiety is a polyclonal antibody and the second binding moiety is a monoclonal antibody. If one or more intermediate binding moieties are used, it is preferred if the first binding moiety is a polyclonal antibody and at least one intermediate binding moiety which binds the analyte is a monoclonal antibody.

35 The reducing agent-generating moiety can be any moiety that, when supplied with its substrate, is capable of generating a reducing agent that reduces metal ions. Examples of suitable reducing agents include hydrogen peroxide (H₂O₂), para-nitrophenol and the reduced form of nicotinamide adenine dinucleotide (NADH₂). Preferably the reducing agent-generating moiety is an enzyme.

Examples of suitable enzymes include glucose oxidase (GOx) which generates H_2O_2 in the presence of glucose, alkaline phosphatase which generates para-nitrophenol in the presence of para-nitrophenylphosphate and alcohol dehydrogenase which generates $NADH_2$ in the presence of ethanol, although the skilled person will be aware of other enzymes which can perform the function of generating a reducing agent that reduces metal ions. In a preferred embodiment, the enzyme is GOx.

In the presence of the reducing agent, the metal ions are reduced to metal atoms which may form a metal coating on the signal moiety or free-standing metal nanocrystals. Any metal ions can be used in the invention provided that the deposition of the metal atom formed by reduction of the metal ion results in a change in size, morphology, state of aggregation, fluorescence emission or electrochemical potential of the signal moiety, causing a change in the signal yielded by the signal moiety. For example, gold, silver, copper, cobalt, nickel, lead or mercury ions could be used. The skilled person will understand that metal ions may be generated using an ionic compound by adding this to the reaction mixture. In some cases, a reagent which reacts with the ionic compound to form metal ions may be used. In a preferred embodiment, silver nitrate and ammonia are added to generate silver ions.

The skilled person will understand that the method described herein can be adapted to detect any analyte of interest. The analyte of interest may be chosen from any species capable of undergoing a binding event with the first binding moiety. The analyte may be a biomarker for a disease, a nucleic acid, a pollutant, an allergen, a contaminant or an antigen derived from a pathogen. In some embodiments, the assay is used to detect a cancer biomarker such as prostate-specific antigen (PSA).

In the present invention, the term "liquid sample" includes any liquid which may contain an analyte of interest. Any raw sample may be pre-treated if necessary to obtain and/or release the analyte of interest and the treatment process may involve treating a solid sample to yield a liquid sample containing analyte of interest. The term "liquid sample" includes bodily liquids that can be obtained from a mammalian body, including, for example, blood, plasma, urine, lymph, gastric juices, bile, serum, saliva, sweat, interstitial fluid and spinal and brain fluids. Furthermore, the bodily liquids may be either processed (e.g., serum) or unprocessed. Depending upon the analyte of interest, other fluid and liquid samples may be contemplated such as ones from industrial, environmental or agricultural sources.

According to a second aspect, the present invention provides a kit for determining the presence of an analyte in a liquid sample, comprising:

- (i) a signal moiety which yields a signal linked to a first binding moiety which specifically binds the analyte to form a reaction conjugate;
- (ii) a reducing agent-generating moiety; and
- (iii) one or more binding moieties for binding the reducing agent-generating moiety to the reaction conjugate.

In certain embodiments, the reducing agent-generating moiety is linked to a second binding moiety that is adapted to bind the reaction conjugate directly, preferably by binding the analyte. In other embodiments, the reducing agent-generating moiety is linked to a second binding moiety that binds the reaction conjugate indirectly, preferably by binding one or more intermediate binding moieties, at least one of which binds the reaction conjugate, preferably the analyte. In such cases, the kit may comprise one or more intermediate binding moieties. The kit may also comprise one or more of the following: calibration data e.g. a calibration curve showing analyte concentration in relation to change in signal (for example the shift in LSPR when a plasmonic sensor is used), one or more washing buffers, one or more working buffers, one or more substrates for the reducing agent-generating moiety, one or more ionic compounds used to generate metal ions, such as, for example, silver nitrate and one or more reactants that react with ionic compounds to generate metal ions, such as for example, ammonia.

Preferred features of each aspect of the invention are as for each other aspect *mutatis mutandis*. The prior art documents mentioned herein are incorporated to the fullest extent permitted by Law.

Examples

The invention will now be described further in the following non-limiting examples. The research described in these examples was supported by a Marie Curie Intra European Fellowship within the 7th European Community Framework Programme and a FPU scholarship from Ministerio de Educación, Spain. Reference is made to the accompanying drawings in which:

Figure 1 shows one embodiment of a signal generation mechanism via enzyme-guided crystal growth. Glucose oxidase (GOx) generates hydrogen peroxide, which reduces silver ions to grow a silver coating around plasmonic nanosensors (gold nanostars); i) at low concentrations of GOx, the nucleation rate is slow, which favours the growth of a conformal silver coating that induces a large blue shift in the LSPR of the nanosensors; ii) when GOx is present at high concentrations, the fast crystal growth conditions stimulate the nucleation of Ag nanocrystals and less silver is deposited on the nanosensors, therefore generating a smaller variation of the LSPR. When the concentration of GOx is related to the concentration of a target molecule via immunoassay, this signal generation step induces inverse sensitivity because condition (i) is fulfilled at low

concentrations of analyte. FAD and FADH₂ are the oxidized and reduced forms of flavin adenine dinucleotide.

Figure 2 relates to an immunoassay for the ultrasensitive detection of PSA with GOx-labeled antibodies. The graphs show blue shift of the LSPR absorbance band ($\Delta\lambda_{\text{max}}$) as a function of the concentration of: a) PSA in PBS and BSA in PBS, b) PSA spiked into whole serum and BSA spiked into whole serum. Error bars are the standard deviation ($n = 3$). The spectral shift was calculated with respect to the control experiment in the absence of the analyte (see Figures 10 and 11).

Figure 3 shows SERS spectra of: (a) PVP-stabilized gold nanostars, (b) after modification with glutaraldehyde to yield **2** (see reaction scheme in methods section), (c) after covalent attachment of proteins (in this example GOx) to obtain **3** (see reaction scheme in methods section). Inset: amplification of the spectral region between 600 and 200 cm⁻¹.

Figure 4 shows intensity of the band at 288 cm⁻¹ (a) and 383 cm⁻¹ (b) with respect to the concentration of GOx when the protein immobilization step is performed in the presence or in the absence of glutaraldehyde.

Figure 5 shows Vis-NIR spectra of protein-modified gold nanostars in water (●) and in 0.3 M NaCl (○).

Figure 6 shows XEDS analysis of free-standing silver nanoparticles found in the solution containing 10⁻¹⁴ g·mL⁻¹ GOx after the signal amplification step

Figure 7 shows XEDS analysis of gold nanostars that were modified with no GOx after the signal amplification step.

Figure 8 shows XEDS analysis of gold nanostars modified with 10⁻²⁰ g·mL⁻¹ GOx after the signal amplification step.

Figure 9 shows XEDS analysis of gold nanostars modified with 10⁻¹⁴ g·mL⁻¹ GOx after the signal amplification step.

Figure 10 shows Vis-NIR spectra of gold nanostars after immunodetection of PSA diluted in PBS to the final concentration of 0 g·mL⁻¹, 10⁻¹³ g·mL⁻¹, 10⁻¹⁴ g·mL⁻¹, 10⁻¹⁵ g·mL⁻¹, 10⁻¹⁶ g·mL⁻¹, 10⁻¹⁷ g·mL⁻¹ and 10⁻¹⁸ g·mL⁻¹.

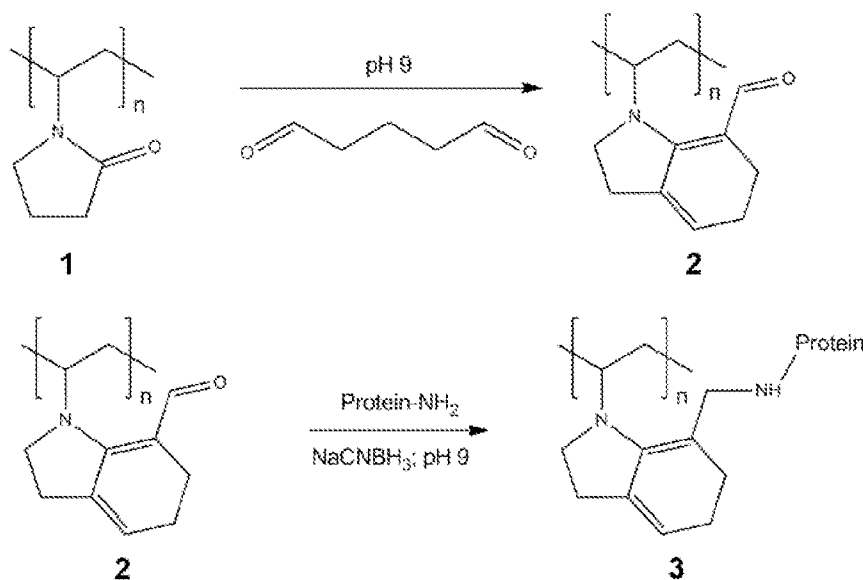
Figure 11 shows Vis-NIR spectra of gold nanostars after immunodetection of PSA diluted in whole serum to the final concentration of $0 \text{ g}\cdot\text{mL}^{-1}$, $10^{-14} \text{ g}\cdot\text{mL}^{-1}$, $10^{-15} \text{ g}\cdot\text{mL}^{-1}$, $10^{-16} \text{ g}\cdot\text{mL}^{-1}$, $10^{-17} \text{ g}\cdot\text{mL}^{-1}$ and $10^{-18} \text{ g}\cdot\text{mL}^{-1}$.

- 5 **Figure 12** shows inverse sensitivity in plasmonic nanosensors. In particular, this figure shows a) TEM image of gold nanostars (scale bar: 50 nm); b) Visible/near infrared spectra of the nanosensors, modified with $10^{-14} \text{ g}\cdot\text{mL}^{-1}$ GOx, $10^{-20} \text{ g}\cdot\text{mL}^{-1}$ GOx and without GOx after the signal generation step; c) blue shift of the LSPR absorbance band ($\Delta\lambda_{\text{max}}$) as a function of the concentration of GOx in the immobilization solution when the signal generation step is performed
- 10 in the absence or in the presence of the enzyme substrate glucose (semilogarithmic scale). The spectral shift was calculated with respect to the control experiment in the absence of GOx (Figure 12b). The high aspect ratio spikes localize the low energy plasmon mode at their tips, which results in a dominant LSPR band in the near infrared region (Figure 12b).^{19,20}
- 15 **Figure 13** shows TEM and XEDS analysis of silver coated gold nanostars. In particular, this figure shows TEM pictures after the signal generation step when gold nanostars were modified with a) $10^{-20} \text{ g}\cdot\text{mL}^{-1}$ GOx, and b) $10^{-14} \text{ g}\cdot\text{mL}^{-1}$ GOx (scale bar: 50 nm); STEM image (c) and XEDS map (d) showing the distribution of gold and silver around nanostars modified with $10^{-14} \text{ g}\cdot\text{mL}^{-1}$ GOx (scale bar: 20 nm). XEDS spectra are shown in Figures 6-9.

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Methods

Covalent attachment of proteins to gold nanostars



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The chemical procedure for the modification of PVP-stabilized gold nanostars with proteins is summarized in the above reaction scheme. After removing excess layers of PVP (**1**) by centrifugation and resuspension in isopropanol (3000 rpm, 3 times), gold nanostars were dispersed in 10 mL of sodium bicarbonate buffer solution (100 mM, pH 9). Then, 1 mL of 50% glutaraldehyde was added and the suspension was stirred for 3 h to yield **2**.²³ The nanoparticles were then separated by centrifugation, washed and re-suspended in 10 mL of bicarbonate buffer solution. Appropriate concentrations of the protein diluted in bicarbonate buffer were reacted with the nanoparticles dispersion (0.5 mM) containing NaCNBH₃ (2 mM) for 3 h at room temperature to obtain **3**.²⁴ Subsequently, non-reacted aldehyde sites were blocked with bovine serum albumin (BSA, 0.1 mg/mL) and ethanolamine (10 mM) in bicarbonate buffer for 1 h. The protein-modified nanostars were then washed by centrifugation and re-dispersed in phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.4, tablets, Sigma).

The formation of **2** and **3** can be confirmed by measuring the surface-enhanced Raman spectroscopy (SERS) spectra of the nanostars after each modification step, as shown in Figure 3.²⁵ The inelastic scattered radiation was collected with a Renishaw Invia Reflex system, equipped with a two-dimensional Peltier charge-coupled device (CCD) detector and a confocal Leica microscope. The spectrograph has 1200 g/mm grating with additional band-pass filter optics. Samples were excited with a 785 nm (diode) laser line. Samples for SERS were prepared by drop-casting 10 μ L of the resulting dispersions on glass slides. Spectra were collected by focusing the laser line onto the sample by using a 50 $\times$ objective (N.A. 0.75), providing a spatial resolution of about 1 μ m², with accumulation times of 10 s. The formation of **2** is proven by the observation of the characteristic peaks for out of plane ring deformation at 343 and 726 cm⁻¹, H-C=O wagging; ring rocking at 383 cm⁻¹, CH₂ ring twisting at 1196 and 1223 cm⁻¹, CH₂ (cyclohexadiene group) wagging at 1343 cm⁻¹, C=C stretching at 1594 cm⁻¹ and C=C-C=O stretching 1642 cm⁻¹.²⁶

The covalent coupling of the protein to the nanostar can be inferred through the spectral changes observed in their corresponding SERS spectra (see Figure 3). First after glutaraldehyde addition the SERS spectrum completely changes its vibrational profile. This is typical of the generation of high SERS cross-section moieties in the low cross-section aliphatic polymer. Thus, the spectrum clearly shows characteristic bands due to the ring including ring stretching and CCH in plane bendings (region from 1400 to 1600 cm⁻¹) and ring breathings (996 and 1070 cm⁻¹). Notably, after the protein coupling, the SERS spectrum changes slightly. The vibrational variations are accumulated in those regions described and are mainly due to the change in the orientation of the aromatic ring with respect to the plasmonic surface because of the steric hindrance induced by the protein. Thus, the vibrational change can be completely explained in full agreement with the

surface selection rules.²⁷ Further, additional evidence of the coupling is also shown in the change of the relative intensity of the bands between 1500 and 1600 cm^{-1} . This spectral window contains the contribution of the aldehyde C=O stretching. Notably, after the coupling of the protein, the relative intensity remarkably decreases while the vibrational profile varies as a consequence of the disappearance of the C=O group due to the reductive amination. Further, the new profile shows ring stretching contributions together with the characteristic amide bands of proteins, especially those of the amide I at around 1650 cm^{-1} .²⁸ These spectral features are fully reproducible within the same sample and in different samples which further supports the covalent coupling of the protein with no evidence of physisorption.

To confirm that the attachment mainly arises through the formation of covalent bonds and not only by non-specific adsorption of proteins on the nanostars, the intensity of the bands at 288 and 383 cm^{-1} was measured in the presence or in the absence of the glutaraldehyde linker. The band at 288 cm^{-1} corresponds to the CH_2 rocking characteristic of amines; the band at 383 cm^{-1} was selected for its higher intensity. In Figure 4, the intensity of the bands increases as the concentration of GOx in the immobilization solution increases when the process is performed in the presence of glutaraldehyde as described above. However, in the absence of glutaraldehyde, only a small increase is registered, which is attributed to minor physisorption of the protein on the nanosensors.

Signal generation via enzyme-guided crystal growth

The synthesis of Au nanostars has been reported elsewhere.¹⁹ Briefly, nanostars were prepared by adding PVP-coated gold seeds ($[\text{Au}] = 9.29 \times 10^{-4} \text{ M}$) in ethanol to a mixture of HAuCl_4 ($2.73 \times 10^{-4} \text{ M}$) and poly(vinylpyrrolidone) (PVP, 10 mM) in DMF under rapid stirring at room temperature. The prepared nanostars have an average total size of $60 \pm 8 \text{ nm}$. Protocols for covalent attachment of biomolecules to PVP-stabilized gold nanostars can be found above. The protein-modified nanosensors are stable in solutions containing highly concentrated electrolytes as discussed below. The production of H_2O_2 was initiated by adding glucose (100 mM) to GOx-modified nanostars in MES buffer (10 mM, pH 5.9) for 1 h. Subsequently, AgNO_3 (0.1 mM) and NH_3 (40 mM) were added to trigger the reduction of silver ions on the gold nanosensors. Spectral changes were measured with a Jasco V-670 UV-vis-NIR spectrophotometer after 2 h. High resolution (HRTEM) and scanning transmission electron microscopy (STEM) images were obtained with a JEOL JEM 2010 FEG-TEM microscope operating at an acceleration voltage of 200 kV. The samples were prepared by depositing a droplet on carbon-coated grids and evaporating the solvent in air at room temperature. X-ray energy dispersive spectra (XEDS) were acquired using an Inca Energy 200 TEM system from Oxford Instruments, and elemental mapping was acquired by coupling the X-ray spectrometer to a STEM unit, equipped with a high

angle annular dark field (HAADF) detector. Mapping was performed with a 0.7 nm probe size, and the acquisition time was limited to 60 s to avoid sample drift. The corresponding quantitative EDS measurements were performed using an INCA EDS microanalysis system from Oxford Instruments. Determination of the relative amount of silver in the coatings was carried by standardless analysis using the Cliff-Lorimer correction with absorbance. Calculations were performed on three different sites on the grid.

Stability of protein-modified gold nanostars

It is well known that gold nanoparticles can aggregate in solutions containing high salt concentrations when their surface is not adequately engineered. These aggregation phenomena can shift the LSPR of the nanosensors to longer wavelengths, therefore interfering in the detection step. This is particularly worrying when working under physiological conditions, which usually imply high ionic strength solutions. To prove the stability of protein-modified gold nanostars in solutions containing high salt concentrations, the nanoparticles were centrifuged and resuspended in 0.3 M NaCl. In Figure 5, no variation of the LSPR of the nanosensors is observed, which demonstrates the stability of protein-modified gold nanostars in solutions containing ions at high concentrations.

PSA detection with antibody-modified gold nanostars

The conjugation of GOx to anti-mouse IgG was performed by converting amino groups in the antibody to thiolate groups with 2-iminothiolane followed by conjugation with GOx via the heterofunctional linker sulfosuccinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC).²⁹ To 5 mg of glucose oxidase in 1 mL of PBS buffer pH 7.6, 200 μ L of sulfo-SMCC in water (5 mg/mL) was added two times at 30 min intervals. The reaction mixture was incubated for 1 hour at room temperature with periodic mixing. Immediately after, the maleimide-activated GOx was purified with a P10 desalting column using PBS as eluate. Protein-rich fractions were identified by their characteristic absorbance peak at 280 nm. Parallel to the enzyme activation, polyclonal anti-mouse IgG developed in goat (Sigma) was dissolved to a concentration of 1 mg/mL in PBS. Then, 100 μ L of 1.5 mg/mL 2-iminothiolane solution was reacted with the antibody for 1 hour at room temperature. The resulting thiolated antibody was purified with the desalting column using PBS as eluate; 1 mL fractions were collected and monitored for protein at 280 nm. Finally, the fractions containing antibody were pooled and immediately mixed with the maleimide-modified glucose oxidase. After overnight incubation at 4°C, the GOx-modified antibodies were stored as single-use aliquots at 4°C until needed.

To detect PSA, gold nanostars were modified with polyclonal anti-PSA raised in rabbit (Abcam) as described above. Then, the antibody-modified gold nanostars ([Au] = 0.25 mM) were

incubated with PSA (Sigma) in the concentration range between 10^{-19} and 10^{-12} g·mL⁻¹ in a final volume of 1 mL for 2 h. PSA samples were obtained by diluting a concentrated solution either in PBS or in whole serum from a female donor (Sera Lab). This serum was found to contain endogenous PSA with the concentration of ca. 10^{-18} g·mL⁻¹ (see also the section on endogenous levels of PSA in female serum below). The particles were then washed by centrifugation (2000 rpm, 10 min) and re-dispersed with PBS. Next, 10 µL of monoclonal anti-PSA developed in mouse (0.13 mg·mL⁻¹) was added to detect the PSA with a sandwich immunoassay format. After 2 h, the particles were washed and re-dispersed in PBS and 20 µL of GOx-labeled anti-mouse IgG added for 2 h at room temperature. After washing once with MES buffer containing Tween-20 (0.05%) and once with MES buffer, the signal generation step was carried out as described above. To demonstrate that the signal was specific and generated by the antibody-antigen recognition, control experiments were performed by following exactly the same procedure but substituting PSA for bovine serum albumin (BSA) during the analyte incubation step (Figs. 4a and 4b, respectively).

X-ray energy dispersive spectroscopy (XEDS) spectra

Figure 6 shows the XEDS spectrum of free-standing silver nanoparticles obtained with 10^{-14} g·mL⁻¹ GOx. Figures 7, 8 and 9 show XEDS spectra obtained from gold nanostars modified with 0, 10^{-20} and 10^{-14} g·mL⁻¹ GOx, respectively after the signal amplification step. Each experiment was repeated 10 times at random sites of the grid with identical results. Figure 6 shows that the free-standing round nanoparticles are made of silver but not gold, which demonstrates that they are generated via nucleation in solution triggered by the biocatalytic activity of GOx. In Figure 7, the XEDS spectrum obtained with gold nanostars that were not modified with GOx after addition of glucose and silver does not show any signal for silver. This experiment, along with the absence of a sensor response in Figure 12b demonstrates that neither glucose nor the proteins that decorate the nanostars are responsible for the reduction of silver ions, therefore proving that the enzyme-generated hydrogen peroxide is the reducing agent, as initially hypothesized. Conversely, silver was detected when the experiment was performed with 10^{-20} and 10^{-14} g·mL⁻¹ GOx, which confirms that the presence of the enzyme, even at ultralow concentrations, is crucial for the deposition of silver.

Endogenous levels of PSA in female serum

It has been suggested that serum from female donors may contain PSA.³⁰ The concentration of endogenous PSA in the serum used in Figure 2b can be estimated from the experiments in the absence of PSA performed in PBS and serum, as shown in Figure 10 and 11. It should be noted that this is an approximation because we are not taking into account the contribution of non-specific interactions in this calculation. After calculating the difference in the LSPR absorbance position for both cases and interpolating this value in the calibration curve shown in Figure 2a, it

was estimated that the serum from the female donor used here contains approximately 10^{-18} g·mL⁻¹ PSA. The same serum from the same batch extracted from the same donor was used for all experiments.

5 Example 1

To test the signal generation mechanism depicted in Figure 1, gold nanostars were covalently modified with GOx (as described above). After adding glucose to trigger the enzymatic production of hydrogen peroxide, silver nitrate was added to initiate crystal growth. In Figure 12b, the LSPR of gold nanostars modified with 10^{-14} g·mL⁻¹ GOx undergoes a blue shift after silver reduction, as
10 expected from the formation of a silver coating around gold nanosensors.^{13, 17-18} The same experiment performed with gold nanostars modified with a non-catalytic globular protein (BSA) does not alter the optical properties of the nanosensors, which indicates that the presence of GOx is essential for the formation of a silver coating. The modification of the nanosensors with as low as 10^{-20} g·mL⁻¹ GOx yields a drastic change in the optical properties of the nanoparticle solution
15 that is much larger than the one obtained with 10^{-14} g·mL⁻¹ GOx. Figure 12c represents the shift of the LSPR band position as a function of GOx concentration, which clearly shows that the shift increases when the concentration of GOx decreases in the range between 10^{-13} and 10^{-20} g·mL⁻¹. Higher concentrations of GOx do not result in a further shift of the LSPR absorbance band. Conversely, control experiments performed with the same GOx-modified nanostars but without
20 adding the enzyme substrate glucose have a negligible effect on the optical properties of the nanosensors. This result, along with the absence of signal in the experiment in the presence of glucose but in the absence of GOx (Fig. 2b), demonstrates that the production of hydrogen peroxide by the biocatalytic activity of the enzyme is the key factor for the reduction of silver ions.

25 Example 2

In the signal generation mechanism depicted in Figure 1, the magnitude of the signal registered by plasmonic nanosensors depends on the rate of crystallization, which favours either the growth of a silver coating on the existing nanocrystals or the nucleation of free-standing small particles. To demonstrate this theory, the presence of silver nanostructures in the solutions containing
30 GOx-modified nanostars was determined after the crystal growth step. Figures 3a and 3b show representative TEM images obtained after silver reduction in the presence of 10^{-20} g·mL⁻¹ and 10^{-14} g·mL⁻¹ GOx, respectively. Free-standing quasi-spherical nanoparticles were observed in abundance when the nanostars were modified with 10^{-14} g·mL⁻¹ GOx, and these nanoparticles were found to be made of silver (see the section on XEDS spectra above). In Figures 3c and 3d,
35 X-ray energy dispersive spectroscopy (XEDS) maps demonstrate the presence of a uniform silver coating around Au nanostars modified with 10^{-14} g·mL⁻¹ GOx. Coatings containing on average 4 % more silver were observed around nanostars modified with 10^{-20} g·mL⁻¹ GOx, which is in agreement with the larger LSPR shift observed in Fig. 2b. These observations agree with the

proposed mechanism that low concentrations of reducing agent result in a slow rate of crystallization, which in turn favours the epitaxial growth of a silver coating on gold seeds, as schematically shown in Figure 1. These silver coatings shift the LSPR of the gold nanostars by as much as 150 nm, as shown in Figure 12b. This effect is due to the hybridization of the dielectric constants of gold and silver in these segregated nanoalloys. The dielectric function of silver has a larger imaginary part than that of gold, which means that it is more absorptive.²¹ By contrast, high concentrations of reducing agent favour the nucleation of free-standing nanoparticles (Fig. 3b). Under these conditions, the lower amount of silver around the nanostars shifts the LSPR by only 80 nm, as shown in Figure 12b. These experiments confirm that the growth of silver on the nanosensors is responsible for the blue shift observed in the LSPR, and that the inverse sensitivity recorded at different enzyme concentrations originates from harnessing crystal growth to favour either the nucleation or the growth of silver nanostructures, as depicted in Figure 1.

Example 3

After demonstrating the mechanism of signal generation via enzyme-guided crystal growth, an experiment was designed to prove the extreme sensitivity and robustness of plasmonic nanosensors when working in the inverse sensitivity regime. Gold nanostars modified with polyclonal antibodies against prostate specific antigen (PSA) captured this cancer biomarker, which was then detected with monoclonal antibodies and labeled with secondary antibodies bound to GOx. PSA was chosen as the model analyte because the detection of this biomarker at ultralow concentrations is crucial for the early diagnosis of cancer recurrence in patients that have undergone total prostatectomy, which makes it an interesting clinical target for testing ultrasensitive sensors.²² Moreover, the detection of PSA in human serum provides a complex matrix to prove the robustness of these plasmonic nanosensors against interferences. In Figure 2a the calibration curve for the detection of PSA in buffered solution shows the negative slope that is characteristic of inverse sensitivity in the concentration range between 10^{-18} and 10^{-13} g·mL⁻¹ (see also Figures 10 and 11). The limit of detection, defined here as the lowest concentration of analyte in the inverse sensitivity regime, was 10^{-18} g·mL⁻¹. Although the signal generation time is longer (ca. 3 h), this result is one order of magnitude lower compared to recently proposed ultrasensitive digital ELISA assays.⁷ The same experiments performed with a control protein (BSA) did not yield any significant signal, which proved that the effect of non-specific interactions between the GOx-labeled antibodies and the nanosensors were minimal. When PSA was spiked into non-diluted human serum, the absolute value of the slope in the calibration curve decreased and the dynamic range was reduced by one order of magnitude (Figure 2b). This is attributed to endogenous levels of PSA and non-specific interactions between the antibodies and proteins present in the serum, which increase the concentration of GOx and therefore decrease the signal of the bioassay (see also the section on endogenous levels of PSA in female serum above). However, the limit of detection did not change and the solution

containing PSA at the ultralow concentration of 10^{-18} g·mL⁻¹ still yielded the highest signal, therefore demonstrating the suitability of this approach for the detection of minute concentrations of target proteins in complex matrices such as body fluids.

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Claims:

1. A method of determining the presence of an analyte in a liquid sample, comprising:
 - (i) contacting a first binding moiety with the liquid sample, the first binding moiety being linked to a signal moiety which yields a signal and specifically binding the analyte to form a reaction conjugate;
 - (ii) forming a detection conjugate by binding a reducing agent-generating moiety to the reaction conjugate;
 - (iii) contacting the detection conjugate with a substrate for the reducing agent-generating moiety and metal ions; and
 - (iv) measuring the signal yielded by the signal moiety, wherein a change in the signal indicates the presence of the analyte.
2. The method of claim 1, wherein the signal moiety is a plasmonic sensor, a fluorescent moiety or an electrochemical sensor.
3. The method of claim 2, wherein the plasmonic sensor is a gold nanostar.
4. The method of any one of claims 1-3, wherein the reducing agent-generating moiety is an enzyme.
5. The method of claim 4, wherein the enzyme is glucose oxidase.
6. The method of any preceding claim, wherein the reducing agent-generating moiety is linked to a second binding moiety which binds the reaction conjugate either directly or indirectly via one or more intermediate binding moieties, at least one of which binds the reaction conjugate.
7. The method of claim 6, wherein one or more of the binding moieties is an antibody.
8. The method of any preceding claim, further comprising determining the concentration of the analyte by measuring a change in the signal yielded by the signal moiety, wherein the greater the change in the signal, the lower the concentration of the analyte.
9. The method of any preceding claim, wherein the metal ions are silver ions
10. The method of any preceding claim, wherein the analyte is a biomarker for a disease.

11. The method of claim 10, wherein the disease is cancer.
12. The method of claim 11, wherein the analyte is prostate-specific antigen (PSA).
- 5 13. A kit for determining the presence of an analyte in a liquid sample, comprising:
(i) a signal moiety which yields a signal linked to a first binding moiety which
specifically binds the analyte to form a reaction conjugate;
(ii) a reducing agent-generating moiety; and
10 (iii) one or more binding moieties for binding the reducing agent-generating moiety
to the reaction conjugate.
14. The kit of claim 13, wherein the signal moiety is a plasmonic sensor, a fluorescent moiety
or an electrochemical sensor.
- 15 15. The kit of claim 14, wherein the plasmonic sensor is a gold nanostar
16. The kit of any one of claims 13-15, wherein the reducing agent-generating moiety is an
enzyme.
- 20 17. The kit of claim 16, wherein the enzyme is glucose oxidase.
18. The kit of any one of claims 13-17, wherein one or more of the binding moieties is an
antibody.

Figure 1

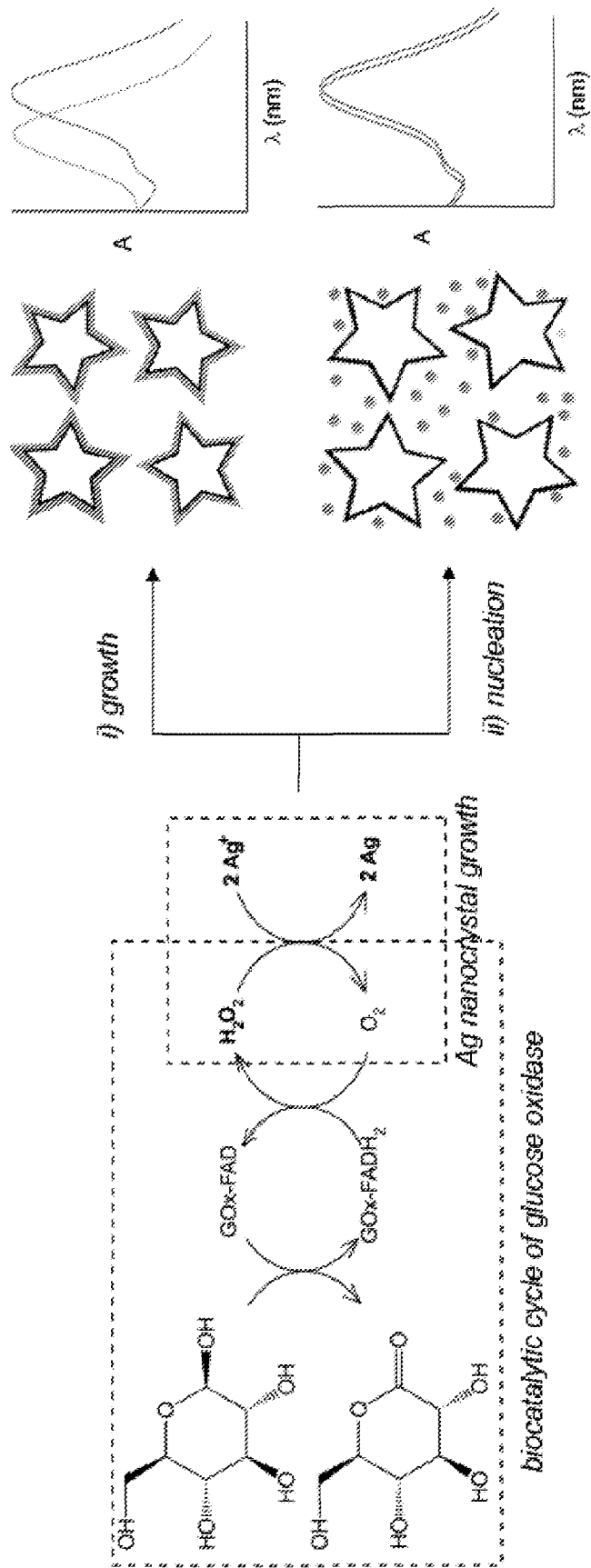
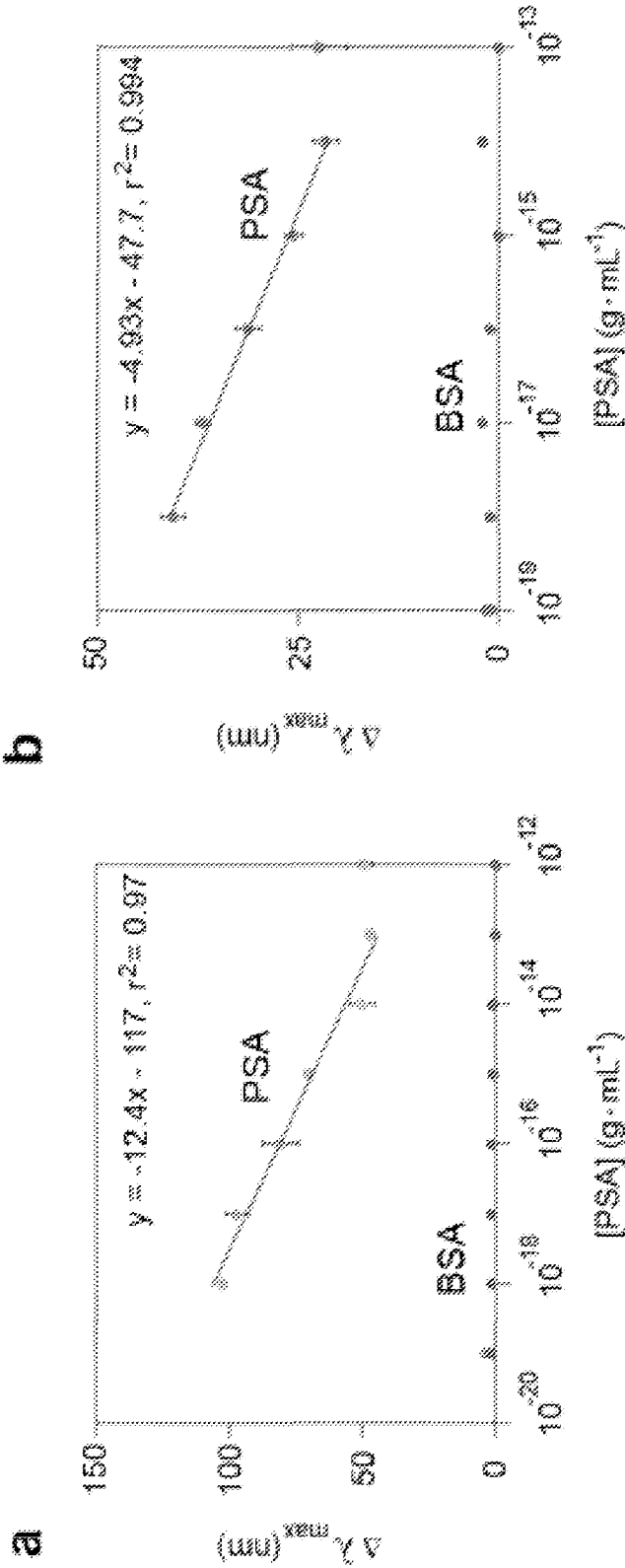


Figure 2



3/9

Figure 3

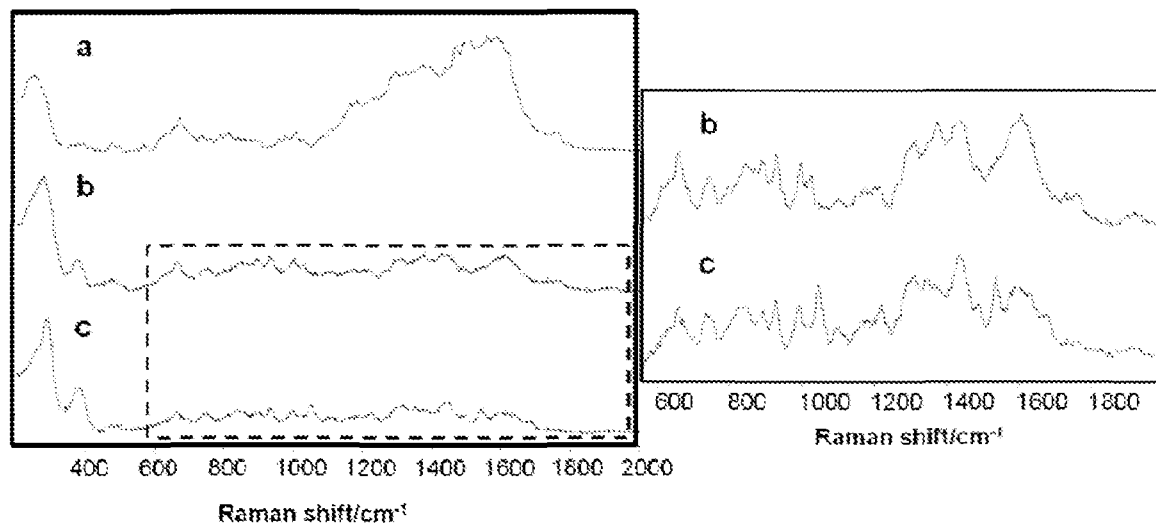
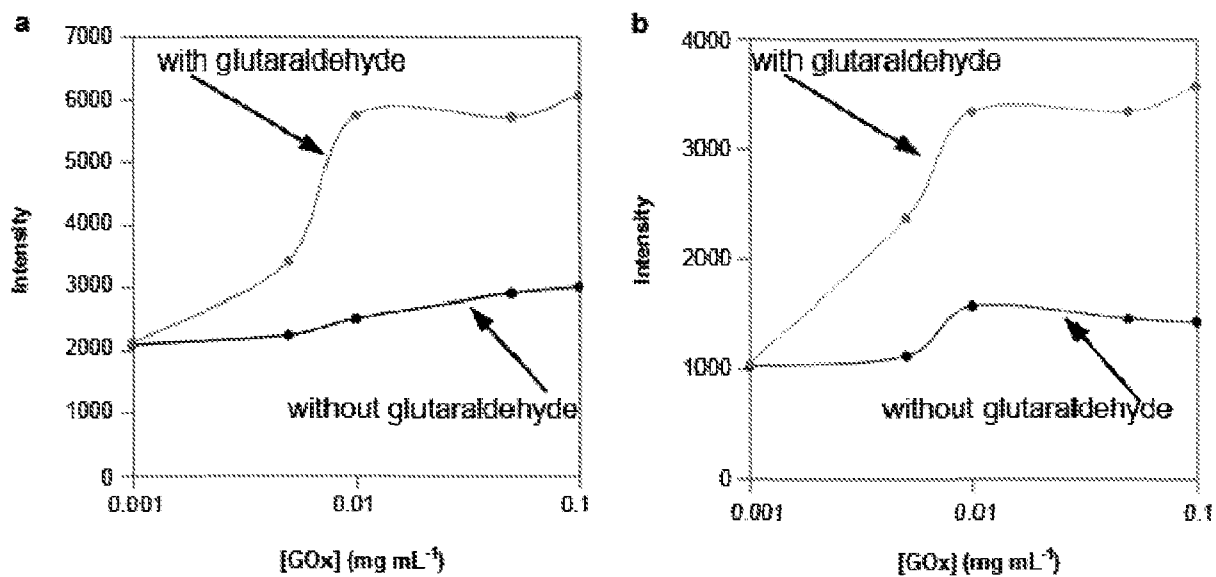


Figure 4



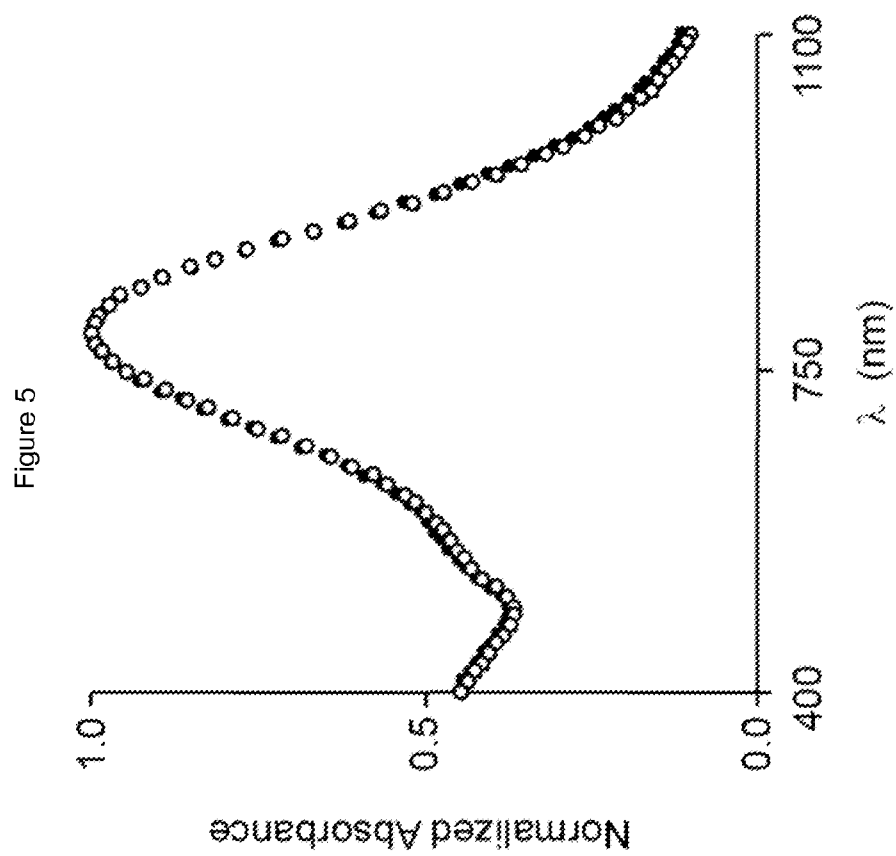


Figure 6

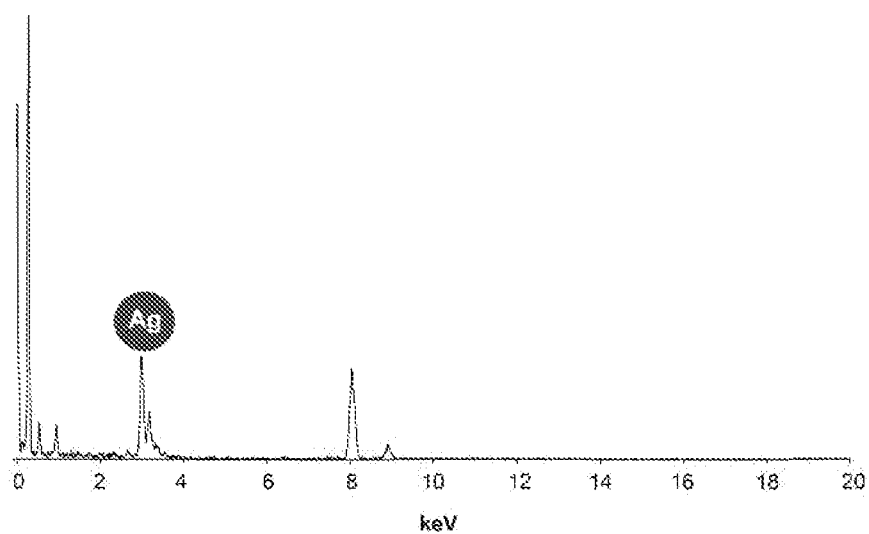
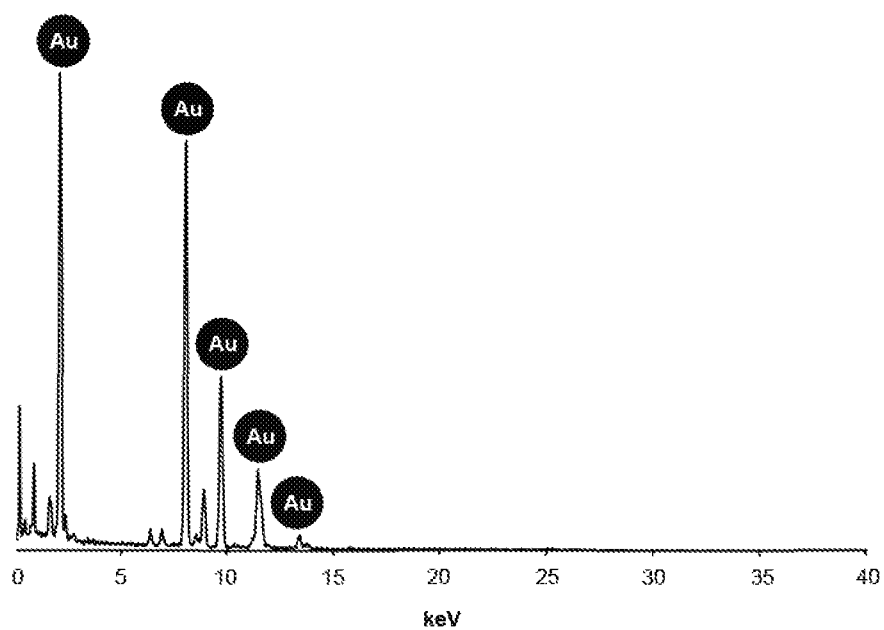


Figure 7



6/9

Figure 8

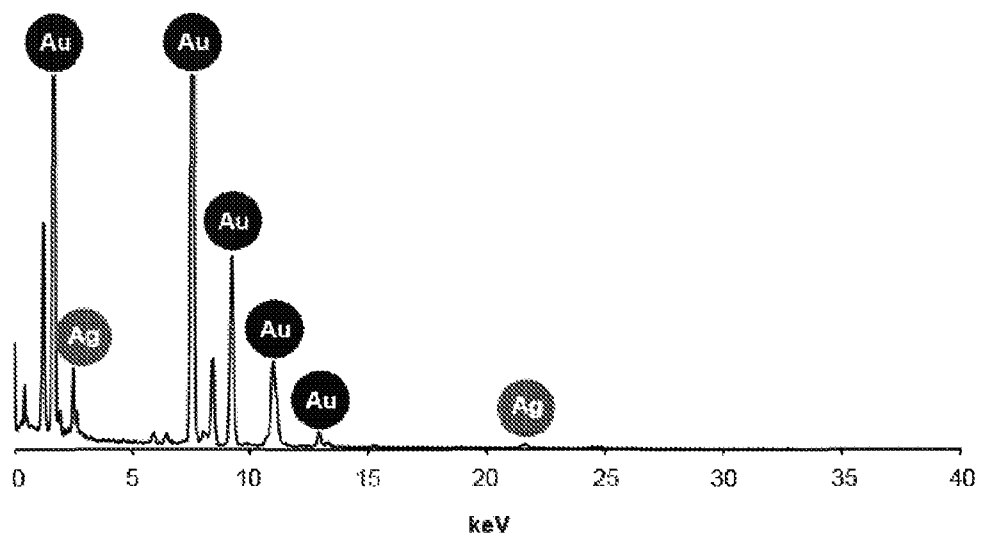
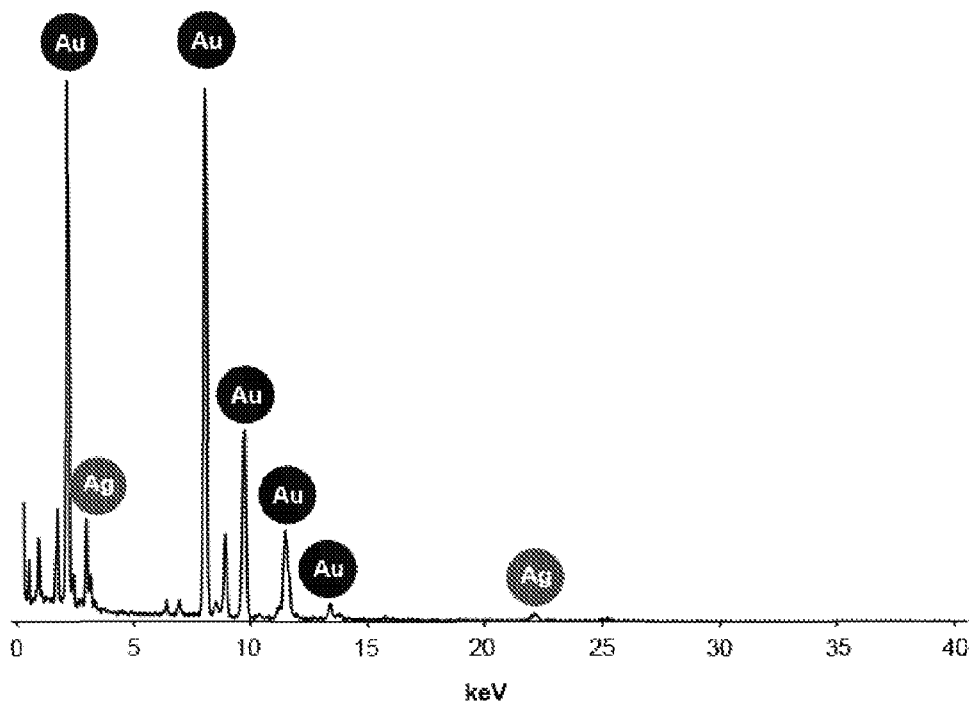


Figure 9



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Figure 10

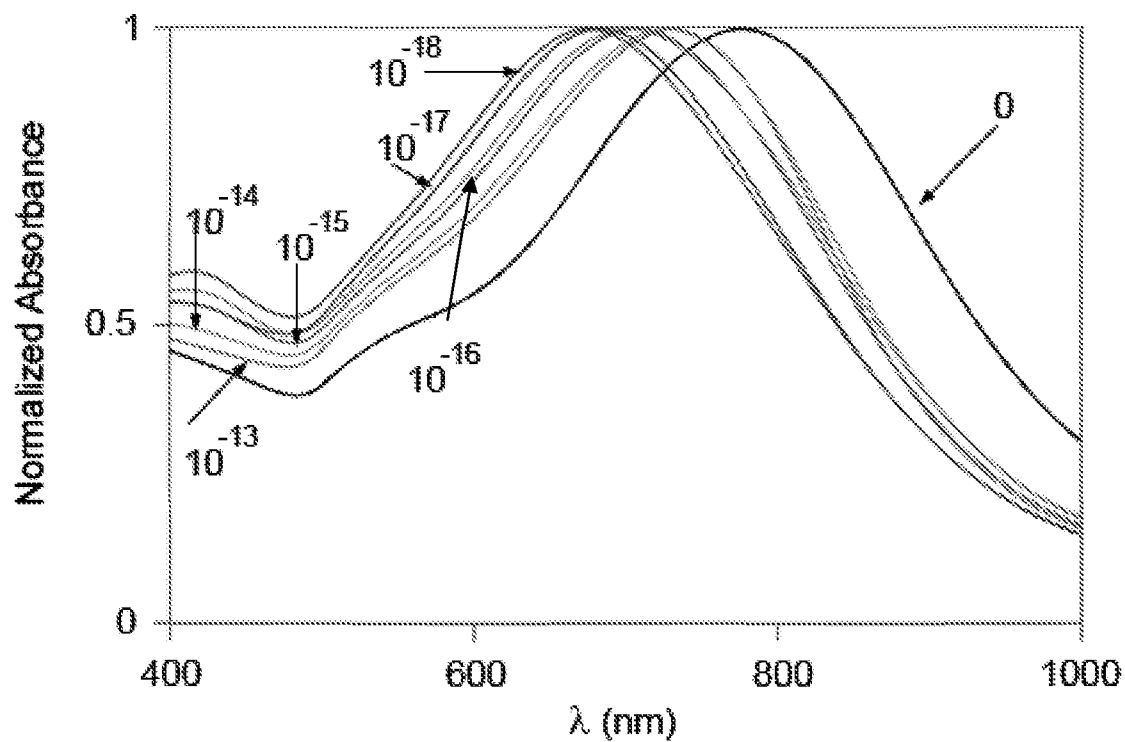


Figure 11

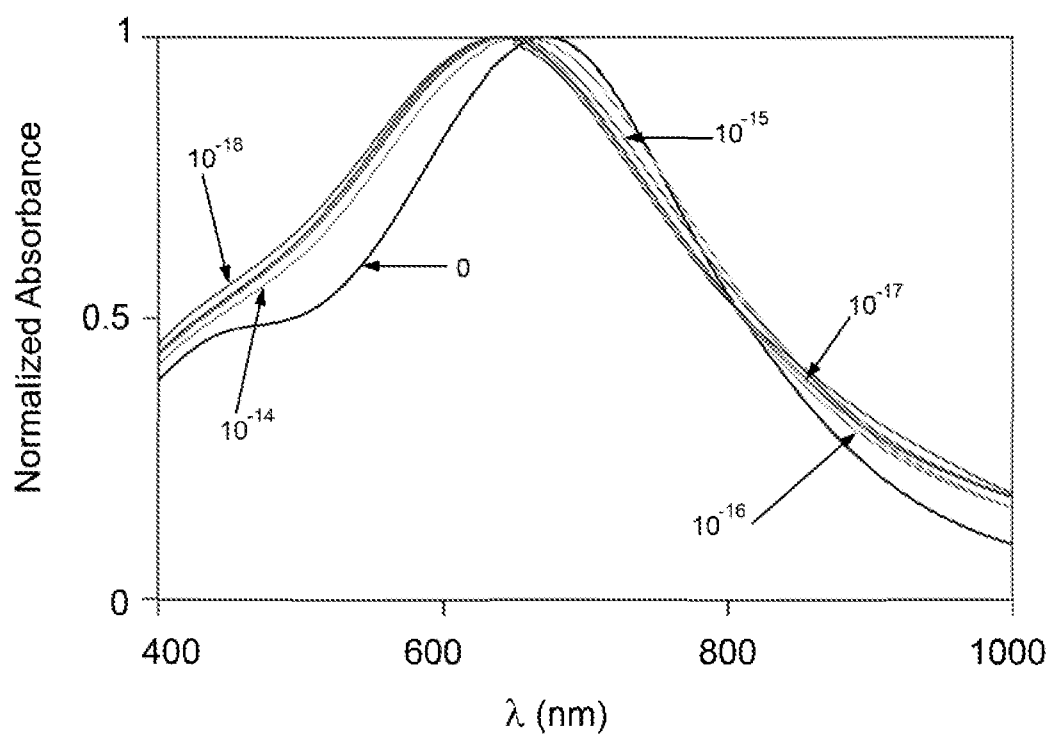


Figure 12

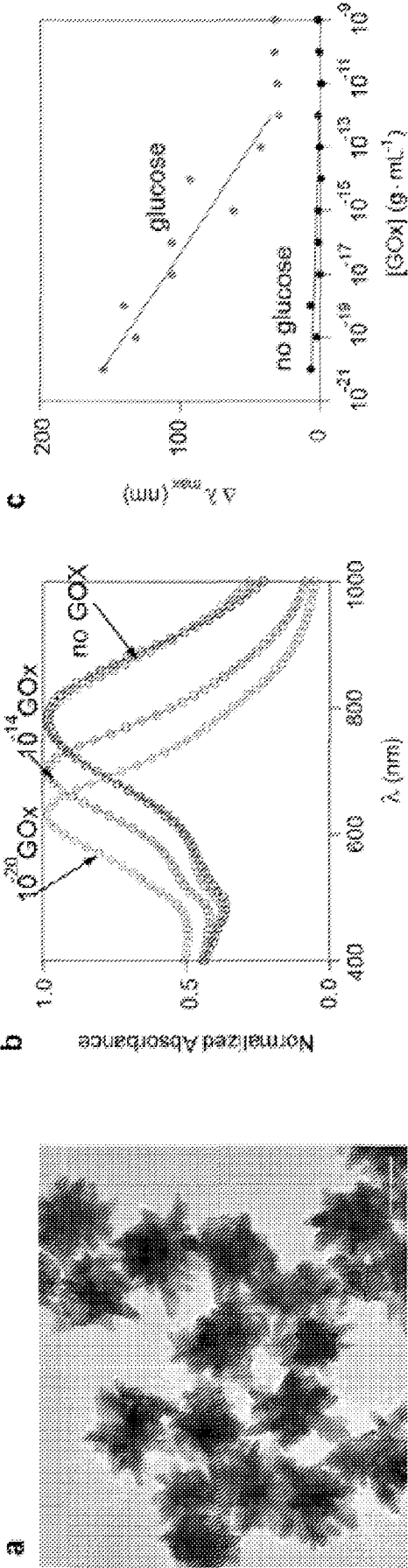
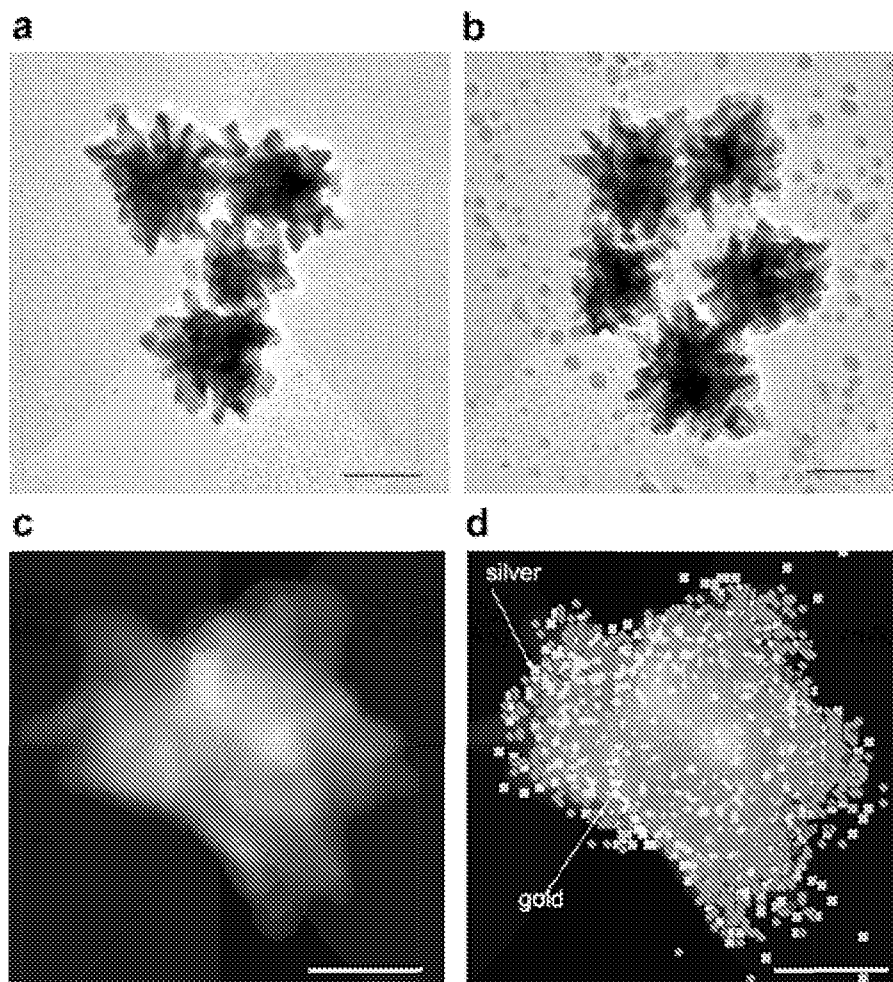


Figure 13



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2012/051852

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/58
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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 practicable, search terms used)

EPO-Internal, BIOSIS, 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	CHEN ET AL: "Successively amplified electrochemical immunoassay based on biocatalytic deposition of silver nanoparticles and silver enhancement", BIOSENSORS AND BIOELECTRONICS, ELSEVIER BV, NL, vol. 23, no. 4, 17 October 2007 (2007-10-17), pages 485-491, XP022302118, ISSN: 0956-5663, DOI: 10.1016/J.BIOS.2007.06.005	1,4,6,7, 9,13,16, 18
Y	abstract page 487, left-hand column ----- -/--	2,3,5,8, 10-12, 14,15,17

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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