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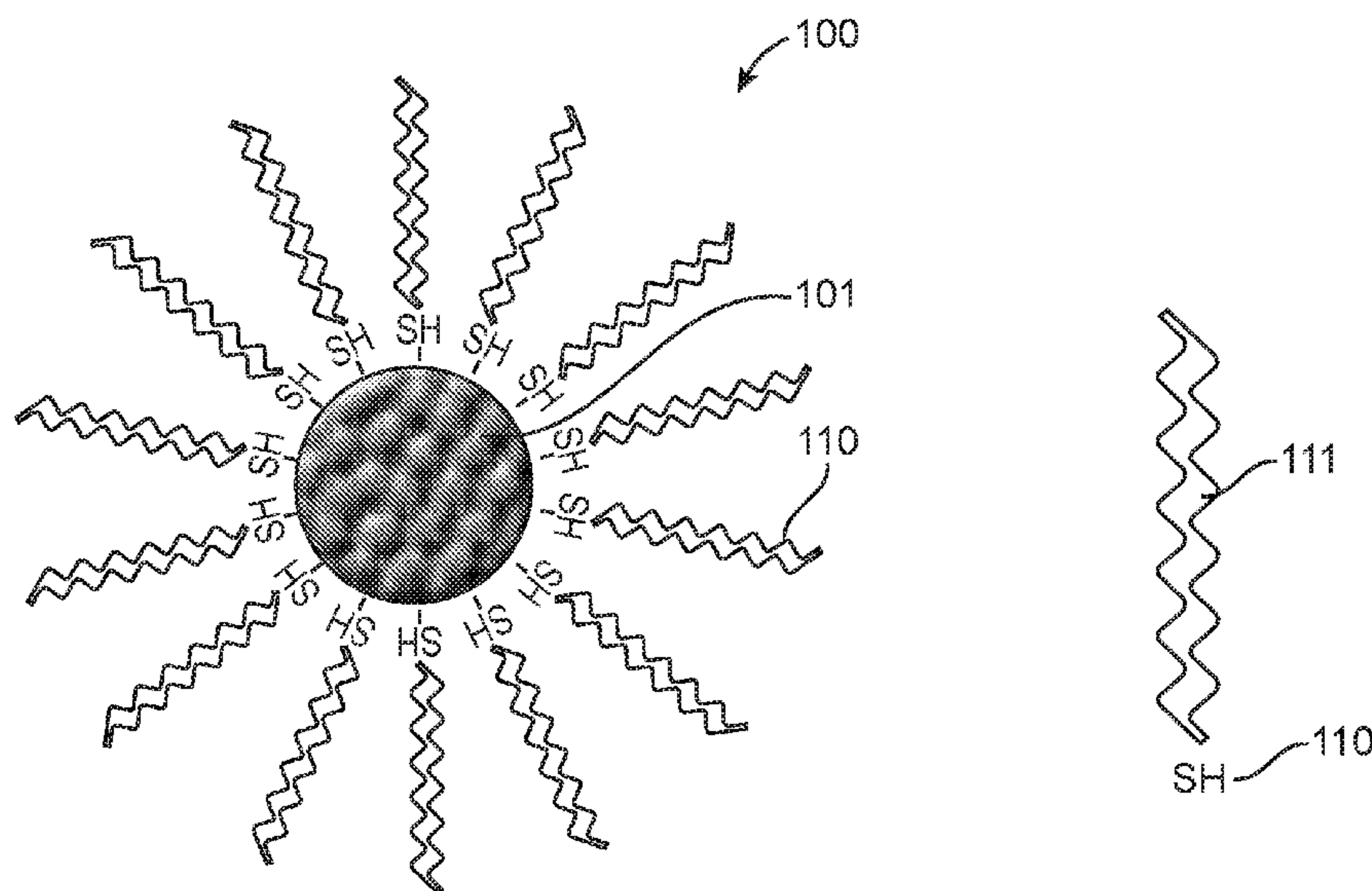


FIG. 1

(57) Abstract: The invention provides a nanozyme for detecting one or more nucleic acid biomarkers and method for preparation thereof. The nanozyme includes a metallic nanoparticle, and a plurality of single-stranded oligonucleotide probes with thiol modification at a 5' end thereof, wherein each of the single-stranded oligonucleotide probes are conjugated via thiol chemisorption on the nanoparticle surface. The present invention also discloses nanozyme linked oligo probe assay (NLOPSA) for detection of one or more nucleic acid biomarkers.

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**NANOZYME LINKED OLIGO PROBE SORBENT ASSAY (NLOPSA) FOR THE
DETECTION OF NUCLEIC ACID BIOMARKERS**

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority to Indian patent application no. 201941049535 filed on 02 December 2019.

FIELD OF THE INVENTION

[0002] The present invention relates to a nanozyme for detecting one or more nucleic acid biomarkers, and a method to detect the specific nucleic acid biomarker sequences by nanozyme linked oligo probe-sorbent assay (NLOPSA).

DESCRIPTION OF RELATED ART

[0003] Horseradish peroxidase (HRPase) is the most extensively used reporter enzyme in enzyme linked immunosorbent assay (ELISA) technique in the area of clinical diagnosis. It is present in the form of a chimeric protein with antibodies or protein receptors for the detection of target proteins/antigens/ligands. However, the natural enzyme has its own drawbacks like stability at external environment or outdoor condition, and substrate level inhibition by excessive H₂O₂. Furthermore, the conjugation of HRPase with oligonucleotide probes for the detection of specific nucleic acid biomarker is not yet established so far. Also, the conjugation of HRPase with oligo probes is a great challenge at present due to molecular weight and complexity of structure.

[0004] CN103808926B, discusses magnetic nanoparticle nanozyme, and a method of immuno chromatographic detection of biological molecules using the same.

[0005] US20180216158A1, discusses a colorimetric method for the detection of nucleic acid sequences, by aggregation of gold nanoparticles. Further, it discusses about the compositions and methods employing DNA hybridization chain reaction for catalytic aggregation of gold nanoparticles.

[0006] The patent CN101706504A, discusses use of gold nanoparticles to label antibody to construct ‘gold nanoparticle - enzyme labeled antibody’ for use in biological testing. The gold nanoparticle mimetic enzyme imitates HRP to catalyze peroxide and hydrogen donating substrate to perform a color development reaction.

[0007] H Zhao et al., Analytical Methods, Vol.8, 2016, page no.2005-2012 discusses a visible and label-free colorimetric sensor for microRNA-21 (miRNA-21) detection based on the peroxidase-like activity of graphene/gold-nanoparticle (Au-NP) hybrids which could be flexibly controlled by using single-stranded PNA-21 (ssPNA-21)..

[0008] The invention provides a novel synthetic enzyme and synthetic enzyme-based assay for detecting specific oligonucleotides that addresses some of the drawbacks of existing methods.

SUMMARY OF THE INVENTION

[0009] The invention in various embodiments discloses a nanozyme for detecting one or more nucleic acid biomarkers that includes a metallic nanoparticle, and a plurality of single-stranded oligonucleotide probes with thiol modification at a 5' end thereof, wherein each of the single-stranded oligonucleotide probes are conjugated via thiol chemisorption on the nanoparticle surface. The nanozyme mimics HRPase like activity in harsh environment and is free of substrate level inhibition, unlike natural HRPase.

[0010] In various embodiments a method for synthesizing nanozyme for detecting one or more nucleic acid biomarkers is described. The method includes capping a metallic nanoparticle by treating a metal precursor with an agent, which acts as a capping and /or reducing agent to provide metallic nanoparticle.

[0011] In various embodiments a nanozyme linked oligonucleotide probe sorbent assay (NLOPSA) for detecting a nucleic acid biomarker is described. The method

includes preparing polystyrene (PS) multiwell plate functionalized with amine groups using silane monolayer for the terminal specific immobilization of capture probe with 5' phosphate modification complementary to 3' end of the nucleic acid and adding nanozyme to the amine functionalized PS multiwell plate for nucleic acid hybridization and adding hydrogen peroxide (H_2O_2) and hydrogen donor substrate to the PS multiwell plate; and detecting the nucleic acid biomarker by identifying reaction of the nanozyme with the substrate.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] The invention has other advantages and features which will be more readily apparent from the following detailed description of the invention and the appended claims, when taken in conjunction with the accompanying drawings, in which:

[0013] FIG. 1 shows the structure of the gold nanoparticles (AuNPs) conjugated oligonucleotide probes.

[0014] FIG. 2 illustrates a method of synthesizing nanozyme.

[0015] FIG. 3 illustrates nanozyme linked oligo probe sorbent assay (NLOPSA) method

[0016] FIG. 4A depicts the UV/Visible spectra of citrate-capped AuNPs

[0017] FIG. 4B depicts the XRD of citrate-capped AuNPs

[0018] FIG. 4C shows the particle size distribution of citrate-capped AuNPs

[0019] FIG. 4D shows HR-TEM image of citrate-capped AuNPs

[0020] FIG. 4E shows EDX spectra image of citrate-capped AuNPs

[0021] FIG. 5A depicts the optical absorbance spectra of citrate-capped AuNPs

[0022] FIG. 5B shows correlation and regression analysis of peroxidase like activity of AuNP nanozyme.

[0023] FIG. 6A shows the stability of the specific oligo-probes conjugated AuNPs before the salt aging process.

[0024] FIG. 6B shows the stability of the specific oligo-probes conjugated AuNPs after the salt aging process.

[0025] FIG. 7A depicts HR-TEM analysis of the oligo probe conjugate of AuNP-miR-21.

[0026] FIG. 7B depicts HR-TEM analysis image of oligo probe conjugate of AuNP-miR-155.

[0027] FIG. 7C shows HR-TEM analysis image of oligo probe conjugate of of AuNP-miR-144.

[0028] FIG. 8A shows UV-visible absorbance spectra of TMB oxidation by oligo-probes conjugated AuNPs

[0029] FIG. 8B shows optical absorbance spectra of oligo-probes conjugated AuNPs

[0030] FIG. 9 shows the graphical representation on the comparison of TMB oxidization between citrate capped AuNPs and ss-oligo-probes conjugated AuNPs.

[0031] FIG. 10A shows UV-visible absorbance spectra image of TMB oxidation of different type's ss-oligo probes conjugated AuNPs nanozymes specific for miRNA-21, miRNA-155 and miRNA-144.

[0032] FIG. 10B shows optical absorbance spectra of TMB oxidized by nanozymes -oligo conjugates.

[0033] FIG. 11A shows the absorbance of different concentrations of custom-made miRNA-21 with miRNA derived S1 and S2 cDNA samples at 650 nm in the NLOPSA

[0034] FIG. 11B shows the absorbance of different concentrations of custom-made miRNA-155 with miRNA derived S1 and S2 cDNA samples at 650 nm in the NLOPSA.

[0035] FIG. 11C shows the absorbance of different concentrations of custom-made miRNA-144 with miRNA derived S1 and S2 cDNA samples at 650 nm.

DETAILED DESCRIPTION

[0036] While the invention has been disclosed with reference to certain embodiments, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the scope of the invention. In addition, many modifications may be made to adapt to a particular situation or material to the teachings of the invention without departing from its scope.

[0037] Throughout the specification and claims, the following terms take the meanings explicitly associated herein unless the context clearly dictates otherwise. The meaning of "a", "an", and "the" include plural references. The meaning of "in" includes "in" and "on." Referring to the drawings, like numbers indicate like parts throughout the views. Additionally, a reference to the singular includes a reference to the plural unless otherwise stated or inconsistent with the disclosure herein.

[0038] The invention in its various embodiments discloses a nanozyme which mimics HRPase like activity, a method of synthesizing nanozyme used for detecting one or more nucleic acid, and a nanozyme linked oligonucleotide probe sorbent assay (NLOPSA) for detecting a nucleic acid biomarker.

[0039] In various embodiment the nanozyme **100** for detecting one or more nucleic acid biomarkers comprises a metallic nanoparticle **101** and a plurality of single-stranded oligonucleotide probes with thiol modification at a 5' end (**110**), wherein each of the single-stranded oligonucleotide probes with thiol modification at a 5' end is conjugated via thiol chemisorption on the surface of the nanoparticle.

[0040] In various embodiments the metallic nanoparticle is selected from gold (Au), ruthenium (Ru) or transition metal or metal oxide nanoparticle.

[0041] In various embodiments, the metallic nanoparticle of the present invention mimic HRPase like activity and oxidizes the substrate in presence of an oxidizing agent, and yields a characteristic color change that is detectable by spectrophotometric

methods or ELISA readers. In various embodiments the characteristic color change is configured to be detected by absorption in the optical wavelength range 400-800 nm. In some embodiments the characteristic color change is configured to be detected by absorption in the optical wavelength range 500-700 nm. The blue colored oxidized substrate in some embodiments is configured to show maximum optical absorbance in the range 550 nm to 700 nm when the metallic nanoparticle is a gold nanoparticle.

[0042] The gold nanoparticles are widely used plasmonic nanoparticles in biosensor development due to their attractive and tunable physicochemical properties like biocompatibility, strong surface plasmon resonance and optical absorption properties.

[0043] In various embodiments, the nanozyme (metallic nanoparticle conjugated with single stranded oligonucleotide) of the present invention are configured to show stronger HRPase like activity in comparison to unconjugated metallic nanoparticles, and oxidize the substrate in presence of an oxidizing agent, to yield a characteristic color change that is detectable by spectrophotometric methods or ELISA readers. The blue colored oxidized substrate in some embodiments is configured to show maximum optical absorbance in the range 550 nm to 700 nm when the metallic nanoparticle is a gold nanoparticle.

[0044] The nanozyme is intended to be used as a sensing probe in the development of colorimetric and scanometric detection methods for the detection of DNA, RNA, miRNA, Single Nucleotide Polymorph (SNP), and other nucleic acids.

[0045] In various embodiments the nucleic acid biomarkers are designed for miRNA 21, miRNA 155 and miRNA 144. In various embodiments the nanozyme consists of the single stranded oligonucleotide probe with thiol modification nucleic acid strand 111 designed for miRNA 21, miRNA 155 and miRNA 144.

[0046] In various embodiments the miRNA-21, 155, and 144 specific oligo-probe sequences were designed with poly (A) tail at 5' end in order to enhance the hybridization efficiency and accessibility using BioEdit 7.0 software. The designed

oligonucleotide probes were custom synthesized with thiol modification at 5' end. Positive control for miRNA-21, 144 and 155 were prepared with 5' phosphate modification. For sandwich hybridization model, capture probes were designed with 5' phosphate modifications which were partially complementary to 3' end of miRNAs. Signal probes were designed with thiol modification at 3' end which were partially complementary to 5' end of miRNAs. The nucleotide of customized miRNA is provided in table 1 below:

Table 1. Nucleotide Sequence of Customized miRNAs and ss Oligo Probes

Seq. ID	Sequence (5'— 3')	Accession No.
miR-21	5'-PO ₄ - AAAAATAGCTTATCAGACTGATGTTG A-3'	hsa-miR-21-5p MIMAT0000076*
miR-21 SP1	5'SH- AAAAATCAACATCAGTCTGATAAGCT A-3'	Customized oligo probe**
miR-155	5'-PO ₄ - AAAAATTAATGCTAATCGTGATAGG GGT-3'	hsa-miR-155-5p MIMAT0000646*
miR-155 SP1	5'-SH- AAAAAACCCTATCACGATTAGCATT AA-3'	Customized oligo probe**
miR-144	5'-PO ₄ - AAAAAGTCCAGTTTTCCAGGAATCC CT-3'	hsa-miR-144-5p MIMAT0004600*
miR-144 SP1	5'-SH- AAAAAAGGGATTCTGGGAAACTG GAC-3'	Customized oligo probe**
miR-21 CP	5'-PO ₄ -AAAATCAACATCAGT-3'	Customized oligo probe**
miR-21 SP2	5'-CTGATAAGCTAAAAA-SH-3'	Customized oligo probe**
miR-155 CP	5'- PO ₄ -AAAACCCCUAUCACG-3'	Customized oligo probe**
miR-155 SP2	5'-AUUAGCAUUAUAAAAA-SH-3'	Customized oligo probe**

miR-144 CP	5-PO ₄ -AAAAAGGGATTCCTG-3'	Customized oligo probe**
miR-144 SP2	5'-GGAAAACCTGGACAAA-SH-3'	Customized oligo probe**

* retrieved from <http://www.mirbase.org/>.

** customized oligo probes were synthesized by VBC-Biotech Service GmbH, (Vienna, Austria)

[0047] The invention in its various embodiments describes a method for synthesizing nanozyme **200** for detecting one or more nucleic acid biomarkers as depicted in Fig. 2. Nanozyme is synthesized by capping a metallic nanoparticle by treating a metal precursor with an agent, which acts as a capping and /or reducing agent to provide metallic nanoparticle **201**; synthesizing a single-stranded oligonucleotide probe with thiol modification at a 5' end thereof **202**; and conjugating the metallic nanoparticle with a plurality of the single-stranded oligonucleotide probe with thiol modification at the 5' end via thiol chemisorption to provide the nanozyme **203**.

[0048] In various embodiments the agent which acts as a capping and/or reducing agent is selected from alkali metal citrate, ascorbic acid, tannic acid, or alkali metal borohydrides. In various embodiments the unconjugated nanoparticles has a size of 10-25nm in diameter and are spherical in shape. Fig.4D and Fig. 4E depicts the particle size distribution and shape of the gold nanoparticle as analysed by High-resolution transmission electron microscopy (HR-TEM) respectively.

[0049] The invention in its various embodiments describes a nanozyme linked oligonucleotide probe sorbent assay (NLOPSA) for detecting a nucleic acid biomarker as depicted in Fig. 3. The assay method comprises preparing polystyrene (PS) multiwell plate functionalized with amine groups using silane monolayer for terminal specific immobilization **301**; immobilizing a miRNA/ capture probe with 5' phosphate

modification complementary to 3' end of the nucleic acid **302**; adding nanozyme (100) to the amine functionalized PS multiwell plate for nucleic acid hybridization **303**; adding hydrogen peroxide (H_2O_2) and hydrogen donor substrate to the PS multiwell plate **304**; and detecting the nucleic acid biomarker by identifying reaction of the nanozyme with the substrate **305**.

[0050] The NLOPSA assay works similarly to enzyme linked immunosorbent assay (ELISA) and is used detect nucleic acid targets and biomarker for biomedical application and diagnosis. Multiple samples can be analysed simultaneously in a shorter period of time (~ 1-2 hrs.) using NLOPSA.

[0051] In various embodiments the polystyrene (PS) multi-well plate is functionalized with amino groups by silane monolayer selected from 3-aminopropyl triethoxy silane (APTES), 3-aminopropyl trimethoxy silane (APTMS), or N-[3-(trimethoxysilyl)propyl]ethylenediamine (TMSPED).

[0052] After the functionalization of metallic nanoparticles, the surface atoms are engaged with formation of self-assembled thiol-monolayer. The catalytic property of metallic nanoparticles is enhanced or suppressed after the functionalization with oligonucleotide probes through thiol chemisorption.

[0053] In various embodiments the nucleic acid hybridization can be a direct DNA hybridization or sandwich DNA hybridization. For sandwich hybridization model, capture probes were designed with 5' phosphate modifications which were partially complementary to 3' end of miRNAs. Signal probes were designed with thiol modification at 3' end which were partially complementary to 5' end of miRNAs.

[0054] In various embodiments the hydrogen donor substrate used in NLOPSA is selected from 3,3',5,5' Tetramethylbenzidine (TMB), tetramethylbenzidine sulfate (TMBS), o-phenylenediamine (OPD), diaminobenzidine (DAB), diaminobenzidine tetrahydrochloride (DAB-4HCl), 5-aminosalicylic acid (5- AS), o-tolidine (OT) or diazonium diamine salt (ABTS).

[0055] In various embodiments the oxidation reaction of the nanozyme with the hydrogen donor substrate in presence of hydrogen peroxide is configured to be detected by color change and with optical absorbance as analyzed by UV-visible spectroscopy.

[0056] In various embodiments the NLOPSA based detection of miRNA biomarkers is configured to operate with high detection sensitivity down to 100zM target concentration with high specificity in less time and at low cost. Another advantage of the assay of the present invention is the terminal specific immobilization of unmodified nucleic acid fragments.

[0057] Though, the current state of the art discusses the use of magnetic nanoparticles and gold nanoparticles for biomedical detection, the conjugation of nanoparticles with oligonucleotide probe to detect nucleic acid biomarkers is not disclosed. The invention addresses the conjugation of natural HRPase with oligonucleotide probe that is a challenge due to the molecular weight and complex structure. The present invention provides a stable nanozyme comprising metallic nanoparticle conjugated with single-stranded oligonucleotide which mimics HRPase like activity in harsh environment and doesn't possess substrate level inhibition like natural HRPase. The nanozyme is used for detecting one or more nucleic acid biomarkers in high concentration of hydrogen peroxide. The present invention provides an easy, cost effective and sensitive method to detect the specific nucleic acid biomarker sequences by nanozyme linked oligo probe-sorbent assay (NLOPSA) through direct or sandwich nucleic acid hybridizations. The NLOPSA assay of the present invention is suitable to analyze multiple samples simultaneously in a shorter period of time and has high sensitivity and sequence specificity.

[0058] The nanozyme, novel assay and methods disclosed present several advantages in early detection of cancer. Generally, miRNA-21 act as an anti-apoptotic factor which suppresses the programmed cell death of cancer cells by down regulating 1-p53

gene through interacting with the HMG-box transcription factor 1-p53-sterol regulatory element-binding transcription factor 1 pathway which leads to increase the malignant nature through cell proliferation and migrations. Therefore, miRNA-21 is considered as one of the oncogenic miRNA biomarkers which are circulating in body fluids and it helps to detect cancers at its early stage of development. Another circulating oncogenic miRNA biomarker is miRNA-155, which is also over expressed in colon cancer, breast cancer, and leukemia. It is associated with FOXO3a and PIK3R1 regulation pathway and directly suppresses the expression of FOXO3a genes. In result, the malignancy develops and leads to the recurrence of cancers. miRNA-144 is one of the tumor suppressor biomarkers found in circulating body fluid which is down regulated in most of the cancer cells including colon and breast cancers.

[0059] Although the detailed description contains many specifics, these should not be construed as limiting the scope of the invention but merely as illustrating different examples and aspects of the invention. It should be appreciated that the scope of the invention includes other embodiments not discussed herein. Various other modifications, changes and variations which will be apparent to those skilled in the art may be made in the arrangement, operation and details of the system and method of the present invention disclosed herein without departing from the spirit and scope of the invention as described here. While the invention has been disclosed with reference to certain embodiments, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the scope of the invention. In addition, many modifications may be made to adapt to a particular situation or material the teachings of the invention without departing from its scope.

EXAMPLES

[0060] **Example 1: Synthesis of oligonucleotide probes conjugated gold nanoparticles (AuNP) nanozyme**

[0061] 100 mL of 1 mM chloroauric acid (HAuCl_4) was heated and added 10 mL of 38.8 mM tri-sodium citrate at the boiling point and this solution was stirred continuously for 15 min. The solution was cooled at room temperature to obtain citrate capped gold nanoparticles and characterized by UV– visible spectrophotometer (Shimadzu UV-1600, Japan) and HR-TEM [Model Fei Technai G2 F30 S-TWIN with 250 kV high-resolution (UHR) pole piece].

[0062] Citrate capped gold nanoparticles was used to prepare oligo-probe conjugated AuNPs using thiol modified ss-oligo-probe (1 nM/mL) through Au-SH based chemistry. The synthesized oligo-probe conjugated AuNPs were characterized by UV/visible spectrophotometer and high-resolution transmission electron microscope (HR-TEM) as shown in FIG. 4.

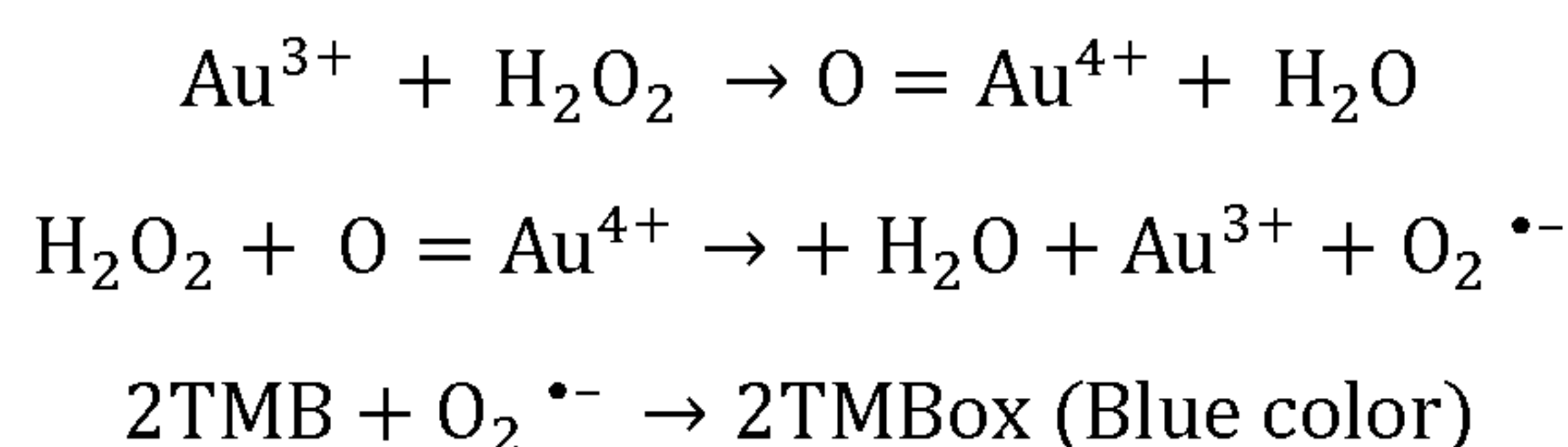
[0063] Result: Yellow color of gold salt solution gradually changed to cherry red color on the addition of tri-sodium citrate at the boiling point, indicating the formation of AuNPs. In details, Au^{3+} ions were reduced to Au^0 metal by citrate ions which leads to the formation of nuclei cluster continue nucleation growth. Simultaneously, citrate molecules stabilize growing Au particles at nanoscale and prevent the further growth. Formed citrate-capped AuNPs showed maximum optical absorbance at a wavelength of 520 nm which corresponds to the cherry red color of its solution (Fig. 4A). XRD spectra indicated that the synthesized AuNPs are under crystalline state (Fig. 4B). The size and shape of these NPs were analyzed by HR-TEM which was ~ 17 nm in diameter and spherical in shape respectively (Fig. 4C and Fig. 4E). Energy-dispersive X-ray spectroscopy (EDX) analysis indicated the presence of Au element prominently (Fig. 4E).

[0064] Example 2: Peroxidase like activity of citrate-capped AuNPs nanozyme

[0065] Peroxidase like activity of citrate-capped AuNPs was studied by adding different concentrations of AuNPs (0, 10, 20, 30, 40, and 50 μL) with 50 μL of H_2O_2 (1 mM), 50 μL TMB (1mM) and 200 μL of sodium acetate buffer (NaAc buffer, pH =

4.3), then the total reaction volume was made up to 300 μL using deionized water. The obtained solutions were kept in dark condition for 20 mins. The color of the solution was changed to blue which indicated that oxidation of TMB happened in the presence of H_2O_2 by AuNPs and it was confirmed that AuNPs possess peroxidase like catalytic activity of nanozyme activity of AuNPs at different concentrations (0-50 μL). The color intensity of each concentration was measured using UV/visible spectrophotometer as shown in FIG. 5A in that the optical absorbance of spectra were observed to the maximum absorbance at 650 nm for blue color. Based on the absorbance band and concentration of AuNPs the Correlation and regression analysis of peroxidase like activity of AuNP nanozyme are calculated as shown in FIG. 5B.

[0066] The rate of TMB oxidation increases with an increase in the concentration of AuNPs from 0 to 50 μL . In detail, Au^{3+} atoms on the surface of AuNPs involved in this oxidation reaction. First, Au^{3+} atom reduces the hydrogen peroxide into water (H_2O) and reactive oxygen species (ROS) which is turn to oxidize TMB substrate into blue color product. The following reaction is depicted below-



Oxidized TMB (TMB_{ox}) shows maximum absorbance at 650 nm wavelength which corresponds to the blue color. FIG. 5A shows the UV/Visible optical absorbance spectra of different concentrations of AuNPs and peaks intensity at 650 nm wavelength was increased significantly from 0 to 50 μL .

[0067] Example 3: Preparation of oligonucleotide probes (specific for miRNA-21, 155, and 144) conjugated AuNPs

[0068] The synthesized AuNP solution was used for the preparation of oligonucleotide probes conjugated AuNPs. In detail, miRNA-21, 155, and 144 specific oligo-probe sequences were designed with poly (A) tail at 5' end in order to enhance the

hybridization efficiency and accessibility by BioEdit 7.0 software. The designed oligonucleotide probes were custom synthesized with thiol modification at 5' end. Similarly, positive control for miRNA-21, 144 and 155 were also prepared with 5' phosphate modification. For sandwich hybridization model, capture probes were designed with 5' phosphate modifications which were partially complementary to 3' end of miRNAs. Signal probes were designed with thiol modification at 3' end which were partially complementary to 5' end of miRNAs. All the designed oligonucleotide probes and positive control sequences were summarized in Table 1.

[0069] AuNP-oligo probe conjugates were prepared by Au-S chemistry. In detail, 10 μ L of 100 pM of thiol modified oligonucleotide probes mixed with 10 μ L of 0.1 M PBS was added to 1 mL of aqueous AuNP solution ($\sim$ 10 nM) to reach a final concentration of oligonucleotide probes around 1 pM. The result solution was incubated at RT for 24 h. After incubation, the solution was subjected to "aging" process with 100 mM PBS buffer at pH = 7 (contains NaCl at a final concentration of 0.1 M) for an additional 24 h. The excess and unbound probes were removed by centrifugation at 14,000 rpm for 30 min. While, the supernatant was discarded, the cherry red oily precipitate was washed twice with 10 mM PBS (pH = 7) containing 0.3 M NaCl by successive centrifugation and finally dissolved in 500 μ L of deionized water. The prepared AuNP- oligo probe conjugates were characterized by UV/visible spectrophotometer, HR-TEM, and Dynamic Light Scattering (DLS).

[0070] The oligonucleotide probes (specific for miRNA 21 and 155) conjugated AuNPs were prepared by our earlier report based on the Au-S based chemistry. The thiol modified oligonucleotide probes at 5' ends were chemically adsorbed on AuNPs surface and formed a self-assembled oligo thiol monolayer. AuNPs and thiol modified oligonucleotide probes were mixed and then incubated in PBS buffer containing 0.3 M NaCl. Here, NaCl was added to reduce the repulsion among the oligonucleotide probes as well as AuNPs which might enhance the chemo-adsorption. After incubation, the

NaCl salt concentration was increased up to 0.1 M as final concentration for the aggregation of unbounded AuNPs in the solution. The optical absorbance properties of AuNP-oligo probe conjugates with control and AuNPs reference before and after the salt aging process were analyzed by UV/visible spectrophotometer (FIG. 6A and B). AuNPs showed different light absorbance properties with and without salt treatment. In absence of salt, AuNPs show sharp bands at 520 nm with high intensity but in the presence of salt, AuNPs display the broadened peak towards longer wavelength region (above 600 nm) with small peak at 520 nm. The absorbance spectra of AuNP-oligo probe conjugates exhibit surface-plasmon resonance (SPR) band at 524 nm even after the salt treatment. The red shift of AuNPs was found from 520 to 524 nm after their conjugations with thiol modified oligonucleotide probes and this can be further implied to the successful adsorption of ss-oligonucleotide probes onto the surface of AuNPs.

[0071] The size of oligo probes (for miRNA-21, miRNA-155 and miRNA-144) conjugated AuNPs were investigated by HR-TEM analysis (FIG. 7A, B, and C). The unconjugated AuNPs were found to be monodispersed with a size of ~ 17 nm in diameter before the salt treatment and it was aggregated with increased size (more than ~ 400 nm) after the salt treatment. The oligo probes functionalized AuNPs were not aggregated in the high salt environment and they were found to be in 2D array arrangements. In addition, the faint bands were clearly observed surrounding the AuNP-oligo probe conjugates. This is due to the conjugation of AuNPs with thiol modified oligonucleotide probes. Such functionalization prevents the particle overlapping and aggregation by maintaining the inter-particle distance and it proves that thiol modified oligonucleotide functionalized AuNPs were not aggregated and resist the salt induced aggregation. 2D array arrangements of AuNP-oligo probe conjugates were mainly due to the weaker interactions between the oligo probes.

[0072] The number of oligonucleotide probes chemically adsorbed on single AuNP (~ 17 nm) was calculating using the UV-visible spectroscopy. The oligonucleotide probes

conjugated AuNPs were treated with β -mercaptoethanol (a strong reducing agent) to break Au-S bond, and salt for the release of surface bound probes and aggregation of AuNPs. The numbers of oligonucleotide probes immobilized on single AuNP was calculated using molar concentration of AuNPs and ss-oligos probes and it was found to ~ 105 , 109 and 103 for miRNA-21, miRNA-155, and miRNA-144 signal probes, respectively. The absorbance efficiency of ss-oligonucleotide probes depends on the size and surface volume ratio of AuNPs, length of probes and ionic strength of the medium. The higher surface area of AuNPs facilitates the absorbance of a greater number of ss-oligonucleotide probes.

[0073] Example 4: Peroxidase like activity of oligonucleotide probes conjugated AuNPs

[0074] Different concentrations of oligo-probe conjugated AuNPs specific for miRNA-21 from 0, 10, 20, 30, 40, and 50 μL added to 50 μL of H_2O_2 (1 mM), 50 μL TMB (1 mM) and 200 μL of sodium acetate buffer (NaAc buffer, pH = 4.3), then the total reaction volume was made up to 300 μL using deionized water. Then, these solutions were kept in dark condition for 20 mins. The color of the solution was changed to blue indicating that the oxidation of TMB occurred in the presence of H_2O_2 by oligo-probe conjugated AuNPs. This confirms that oligo-probe conjugated AuNPs possess peroxidase like catalytic activity. The color intensity of each concentration was measured by UV-visible spectrophotometer.

[0075] Experimental evidence shows that manipulated AuNPs surface exhibit HRP like activity and oxidize TMB effectively (FIG. 8). The development of blue color indicates the oxidation of TMB by ROS produced by ss-oligo-probes conjugated AuNPs through catalytic splitting of H_2O_2 . The blue color intensity of TMB was increased by increasing the concentration of ss-oligo probes conjugated AuNPs from 0-50 μL (FIG. 8A) and their corresponding optical absorbance was found at 650 nm wavelength with a gradual increase in peak intensities. Their correlation coefficient

graph was showed in FIG. 8B. An effective oxidation of TMB by citrate capped AuNPs and ss-oligonucleotide probes conjugated AuNPs was analyzed.

[0076] The result showed that ss-oligo-probe conjugated AuNPs showed more potential in oxidation of TMB than the citrate capped AuNPs (FIG. 9). The covalently adsorbed ssDNA-oligonucleotide probes on AuNPs surface show more HRP like activity than the citrate capped AuNPs. This could be due to the interaction of TMB substrate with ssDNA-oligonucleotide probes through electrostatic interactions and intermolecular forces. These forces bring the substrates very close to AuNP surface, increase the localized TMB concentration and accelerate the rate of oxidation reactions.

[0077] Example 5: HRP like activity of different oligonucleotide probes conjugated AuNPs

[0078] 50 μ L of oligonucleotide probes conjugated AuNPs specific for miRNA-21, miRNA-155 and miRNA-144 were added to 50 μ L of H_2O_2 (1 mM), 50 μ L of TMB (1mM) and 200 μ L of sodium acetate buffer (NaAc buffer, pH = 4.3), then the total reaction volume was made up to 300 μ L using deionized water. These solutions were kept in dark condition for 20 mins. The color of the solution was changed to blue indicating that oxidation of TMB occurred in the presence of H_2O_2 by oligonucleotide probes conjugated AuNPs and it is confirming that oligonucleotide probes conjugated AuNPs possess peroxidase like catalytic activity. The color intensity of each concentration was measured using UV-visible spectrophotometer.

[0079] ss-oligonucleotide probes conjugated AuNPs show 1.75 times higher HRP like catalytic activity than unmodified AuNPs. Specifically, ss-oligo-probe conjugated AuNP for miRNA-155 shows more potential than miRNA-21 followed by miRNA-144 specific ss-oligonucleotide probes conjugated AuNPs. This could be due to the presence of a greater number of ss-oligonucleotide probes on AuNP's surface specific for miRNA-155 than oligonucleotide probes specific for miRNA-21 followed by

miRNA-144. Further, ssDNA-oligo-probe enhance the catalytic activity of AuNPs to oxidize TMB in high level than unmodified AuNPs by increasing localized TMB substrate concentration close to the AuNP surface. Overall results showed that ss-oligoprobe functionalized AuNPs (spherical nucleic acid) possess HRP like catalytic activity which can effectively catalyze H_2O_2 splitting and oxidation of TMB simultaneously when compared to unmodified AuNPs.

[0080] Example 6: Preparation of ss-oligo arrays for NLOPSA.

[0081] 6a. Surface modification of polystyrene multiwall plate for the terminal specific immobilization of nucleic acids/ ss-oligo probes/ fragmented miRNA

[0082] Unmodified polystyrene 96 well plate surface was modified with amine functional groups using 3-aminopropyltriethoxysilane (APTES) (NH_2 -PS plate). In details, the plates were treated with freshly prepared HNO_3 and H_2SO_4 mixtures (47:53 ratio, 250 μL /well) at RT for 30 min in a fume hood with constant shaking. The plates were washed three times with dd H_2O followed by the addition of 5 % of APTES (250 μL /well, pH = 6.9) and incubated at room temperature for 2 h. The excess APTES was washed three times with dd H_2O and incubated at 60 °C for 2 h to enhance the APTES binding over the PS surface. Then the modified plates were kept at 4 °C for overnight before to use.

[0083] 6b. Preparation of ss-DNA array

[0084] Different concentrations of 5'-phosphate-modified custom synthesized miRNA-21, miRNA-155 and miRNA-144 were immobilized on the amine modified PS multiwell (NH_2 -PS) plate surface. In detail, the ice-cold 10 mM 1- methyl imidazole (pH = 7.0) was prepared as the final concentration from ice-cold 0.1 M 1- methyl imidazole stock. The miRNA oligo solutions were loaded on amine-functionalized PS plate (50 μL per well) on ice. EDC (0.2 M) was dissolved in freshly prepared ice-cold 10 mM 1- methyl imidazole, and then 50 μL of EDC was added to each well and incubated at 50 °C for 5 h. After incubation, the wells were washed three times with washing solution

1 (0.4 N NaOH + 0.25 % sodium dodecyl sulfate) for 5 min at 50 °C followed by three times washing with sterile water. Finally, the unbounded surface was blocked by adding 150 µL of 1% PEG and allowed to rest for 30 min at RT, and then the plate was washed with sterile water.

[0085] Hybridization of oligonucleotide probes conjugated AuNPs was optimized through direct DNA hybridization (DDH) assay. Hybridization was performed with optimized parameters like temperature, pH, and time, as reported earlier with slight modifications. The oligonucleotide probes conjugated AuNPs (50 µL) specific for miRNA-21, 155 and 144 were mixed with 50 µL of hybridization buffer (4 x saline sodium citrate (SSC) buffer, 0.05% Tween- 20, and 35% formamide: 0.6 M NaCl and 0.06 M trisodium citrate were dissolved, pH = 7.0 was adjusted with HCl, and the final volume was made up to 100 mL with ddH₂O) and added to each well of different concentrations of custom made miRNA-21, 155 and 144 (100 pM, 100 fM, 100 aM, and 100 zM) immobilized on PS plate. It was incubated for hybridization at 40 °C for 1 h. After incubation, the plate was subjected to high-stringency wash with 200 µL of washing solution 2 (0.5 M NaNO₃ containing 0.05% of Tween-20) twice at RT (2 min for each washing step) and onetime low-stringency wash with 0.4 x SSC buffer for 30 s. Then, 50 µL of H₂O₂ (1 mM), 50 µL TMB (1 mM) and 200 µL of sodium acetate buffer (NaAc buffer, pH = 4.3) were added to each well and the total reaction volume was made up to 300 µL using deionized water. Further, the plate was kept in dark condition for 20 mins. Finally, the development of blue color was observed and their intensities were measured using UV-visible spectrophotometer.

[0086] Similarly, 50µL of heat fragmented cDNA was obtained from total RNA isolated from two different cancer cell lines which were immobilized on amine modified PS plate. Then, miRNAs specific oilgo-probes conjugated AuNPs were hybridized with cDNA array. NLOPSA was performed following aforementioned protocol.

[0087] Result: Different concentrations (100 pM, 100 fM, 100 aM, 100 zM and 0 zM) of 5'-PO₄ terminal specific custom-made miRNAs were immobilized on amine modified PS multi-well plate using EDC/ 1- methyl imidazole based conjugation chemistry along with sample cDNA arrays. miRNA-21, miRNA- 155 and miRNA-144 specific oligonucleotide probes conjugated AuNPs were hybridized with miRNA/cDNA array. Where, oligonucleotide probes conjugated AuNPs hybridize with its complementary sequence and forms miRNA-oligonucleotide probes conjugated AuNPs complex. In the absence of target sequence, the oligonucleotide probes conjugated AuNPs were washed out. After the addition of H₂O₂ and the chromogenic substrate TMB, the blue color was developed due to breaking down of hydrogen peroxide into water and ROS. ROS turn to oxidize the chromogenic substance TMB which leads to the development of blue color. The intensity of blue color depends upon the number of miRNA-oligonucleotide probes conjugated AuNPs complex which directly correlates with the amount of target miRNA in each well.

[0088] NLOPSA based detection of miRNAs biomarkers in PS multi-well plate:

The intensity of blue color was increased from 100 zM to 100 pM and no color was found in 0 zM (control). Similarly, the pattern of color intensity was observed in all three rows of PS plate represent miRNA-21 (FIG. 11A), miRNA-155 (FIG. 11B) and miRNA-144 (FIG. 11C). Sample cDNA obtained from colon cancer cell lines S1 and S2 shows the significant blue color development with high intensity. However, the blue color intensity of S1 and S2 are not same for all three rows and significant variations were observed. Specifically, the high intensity of blue color was found in S2 than S1 in row 1 and row 2 after the hybridization with miRNA-21 and miRNA-155 specific oligonucleotide probes conjugated AuNPs (FIG. 11A, 11B) but in the row, S1 shows more intensity than S2 after the hybridization with miRNA-144 specific oligonucleotide probes conjugated AuNPs (FIG. 11C). Experimental results evidence that miRNA-21 and miRNA-155 were over expressed in sample S2 than S1. However, miRNA-144 was highly expressed in S1 than S2 cell line. This result confirms that S1

cell line is noncancerous and S2 is cancerous cell line. Because, miRNA-21 and miRNA-155 are well known tumorigenic miRNA biomarkers were highly expressed in cancer cells including breast, colon cancers. Also, miRNA-144 is tumor suppressor biomarker which generally express in normal cells.

[0089] Optical absorbance at 650 nm for the detection of miRNA- 21, miRNA-155 and miRNA-144, respectively: High detection signal was found in miRNA-155 followed by miRNA-21 than miRNA-144. Further, miRNA-21 and miRNA-155 were found at lower concentration than miRNA-144 in S1 sample. But in S2, the concentration of miRNA-144 was found very low than miRNA-21 and miRNA-155. Because, miRNA-21 and miRNA-155 are oncogenic miRNA biomarker, they are over expressed in colon cancer, and breast cancer cells.

WE CLAIM:

1. A nanozyme (100) for detecting one or more nucleic acid biomarkers, comprising:
a metallic nanoparticle (101), and a plurality of single-stranded oligonucleotide probes with thiol modification at a 5' end (110) thereof, wherein each of the single-stranded oligonucleotide probes are conjugated via thiol chemisorption on the nanoparticle surface.
2. The nanozyme as claimed in claim 1, wherein said metallic nanoparticle is selected from gold (Au), ruthenium (Ru), transition metal or metal oxide nanoparticle.
3. The nanozyme as claimed in claim 1, wherein the said nucleic acid biomarker is designed for miRNA 21, miRNA 155 or miRNA 144.
4. The nanozyme as claimed in claim 1, wherein the single stranded oligonucleotide probe with thiol modification at 5' end is a nucleic acid strand (111) designed for miRNA 21, miRNA 155 and miRNA 144.
5. The nanozyme as claimed in claim 1 having size of 10 - 25 nm.
6. A method of synthesizing nanozyme (100) for detecting one or more nucleic acid biomarkers, comprising:
capping a metallic nanoparticle by treating a metal precursor with an agent, which acts as a capping and /or reducing agent to provide metallic nanoparticle (101);
synthesizing a single-stranded oligonucleotide probe with thiol modification at a 5' end (110) thereof, a capture probe with 5' phosphate modification

complementary to 3' end of nucleic acid, signal probe with 3' thiol modification complementary to 5' end of nucleic acid; and
conjugating the metallic nanoparticle (101) with the single-stranded oligonucleotide probe with thiol modification at 5' end (110) via thiol chemisorption to provide the nanozyme (100).

7. The method as claimed in claim 6, wherein the metallic nanoparticle is selected from gold (Au), ruthenium (Ru) or transition metal or metal oxide nanoparticle.

8. The method as claimed in claim 6, wherein the agent which acts as a capping and / or reducing agent is selected from alkali metal citrate, ascorbic acid, tannic acid, or alkali metal borohydrides.

9. The method as claimed in claim 6, wherein the nanozyme (100) has size of 10 - 25 nm.

10. A nanozyme linked oligonucleotide probe sorbent assay (NLOPSA) for detecting a nucleic acid biomarker, comprising:

preparing polystyrene (PS) multiwell plate functionalized with amine groups using silane monolayer for terminal specific immobilization;

immobilizing a capture probe with 5' phosphate modification complementary to 3' end of the nucleic acid;

adding nanozyme (100) to the amine functionalized PS multiwell plate for nucleic acid hybridization;

adding hydrogen peroxide (H₂O₂) and hydrogen donor substrate to the PS multiwell plate; and

detecting the nucleic acid biomarker by identifying reaction of the nanozyme with the substrate.

11. The nanozyme linked oligonucleotide probe sorbent assay as claimed in claim 10, wherein the silane monolayer is selected from 3-aminopropyltriethoxysilane (APTES), 3-aminopropyltrimethoxysilane (APTMS), or N-[3-(trimethoxysilyl)propyl]ethylenediamine (TMSPED).

12. The nanozyme linked oligonucleotide probe sorbent assay as claimed in claim 10, wherein the nucleic acid hybridization is by direct DNA hybridization or sandwich DNA hybridization.

13. The nanozyme linked oligonucleotide probe sorbent assay as claimed in claim 10, wherein the hydrogen donor substrate is selected from 3,3',5,5'-Tetramethylbenzidine (TMB), tetramethylbenzidine sulfate (TMBS), o-phenylenediamine (OPD), diaminobenzidine (DAB), diaminobenzidine tetrahydrochloride (DAB-4HCl), 5-aminosalicylic acid (5-AS), o-tolidine (OT) or diazonium diamine salt (ABTS).

14. The nanozyme linked oligonucleotide probe sorbent assay as claimed in claim 10, wherein the assay is configured to detect miRNA 21, miRNA 155, miRNA 144 or other nucleic acid biomarkers.

15. The nanozyme linked oligonucleotide probe sorbent assay as claimed in claim 10, wherein the oxidation reaction and its optical absorbance are analyzed in the range of 550-700nm.

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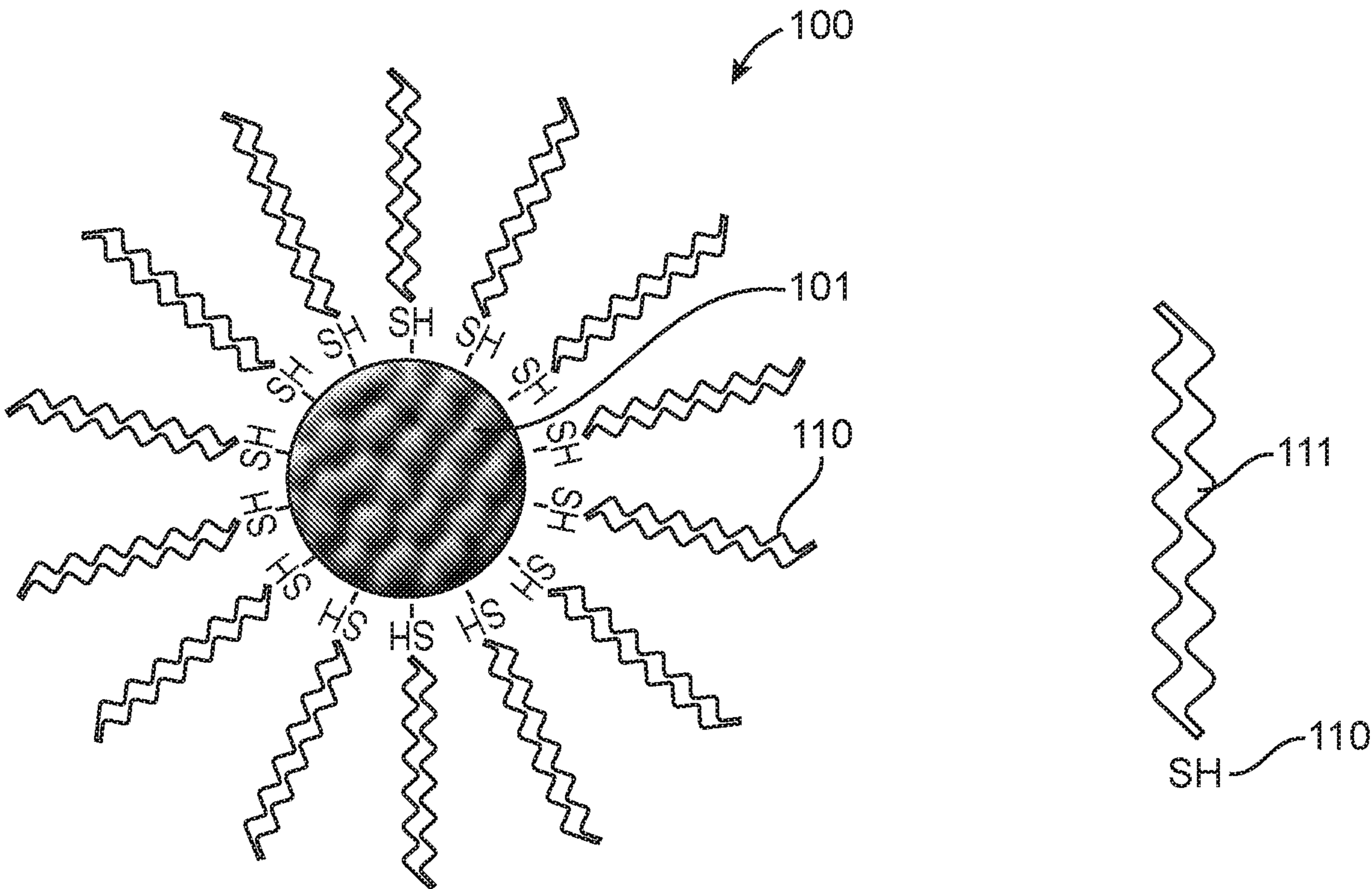


FIG. 1

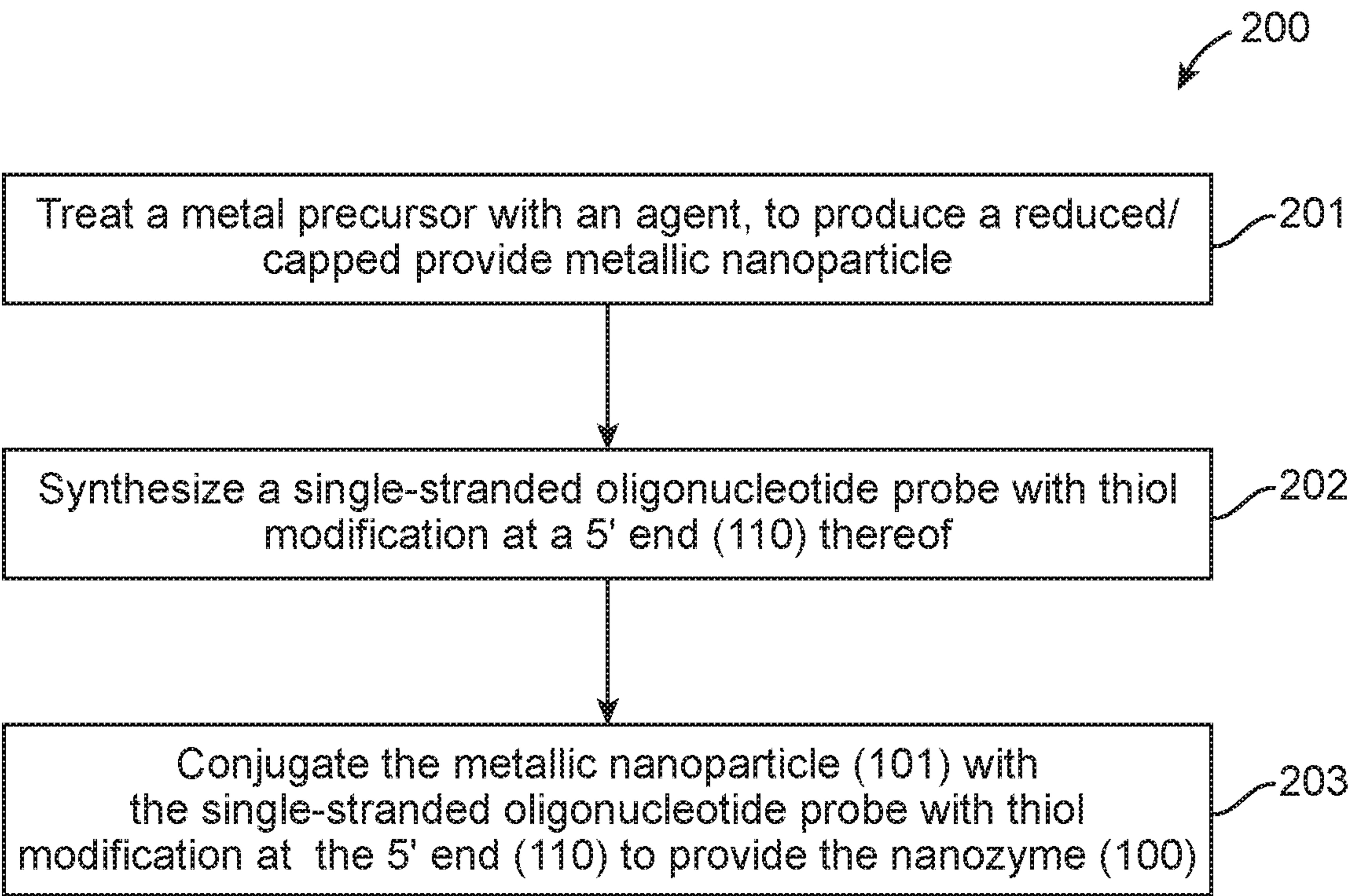


FIG. 2

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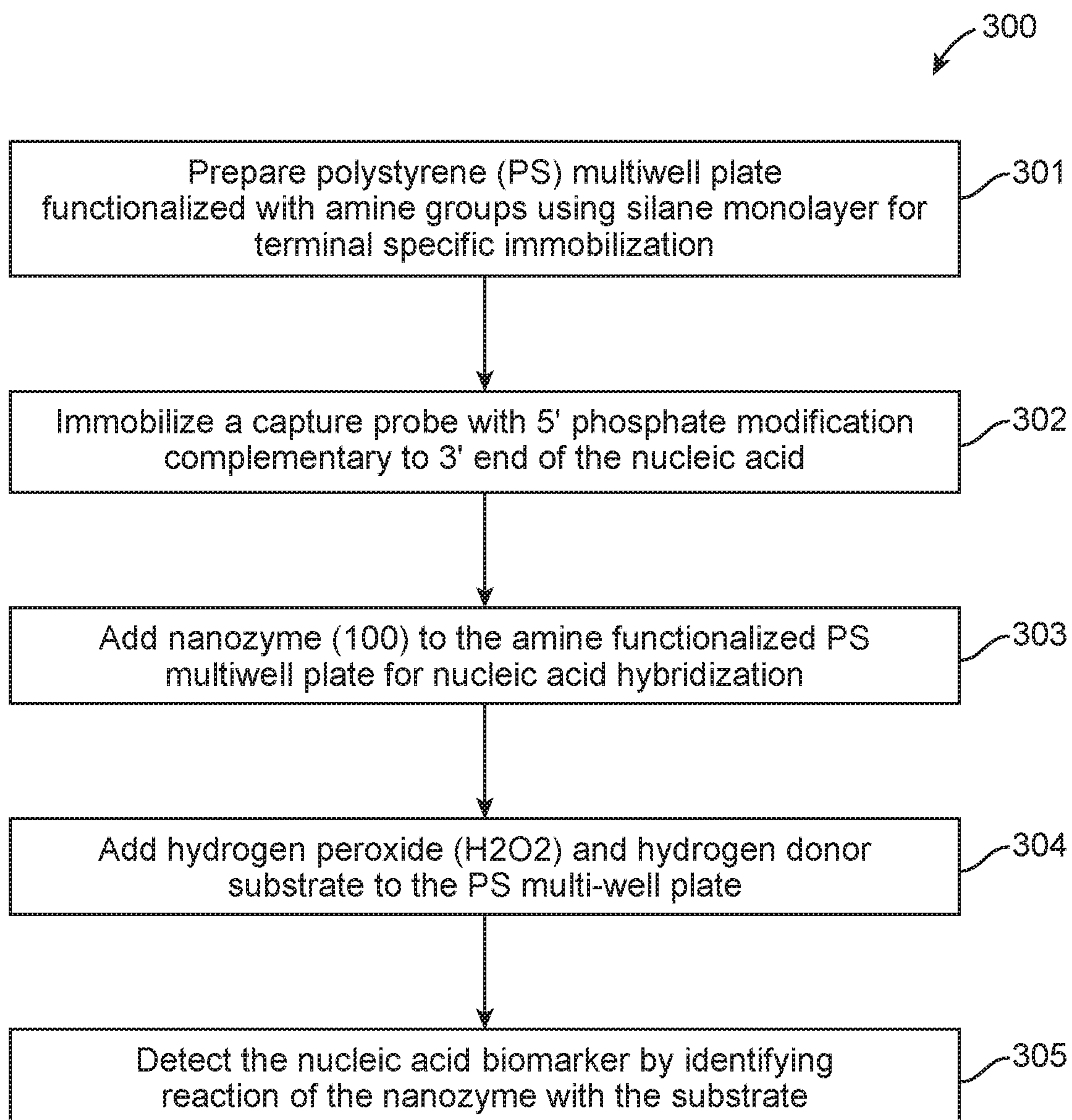


FIG. 3

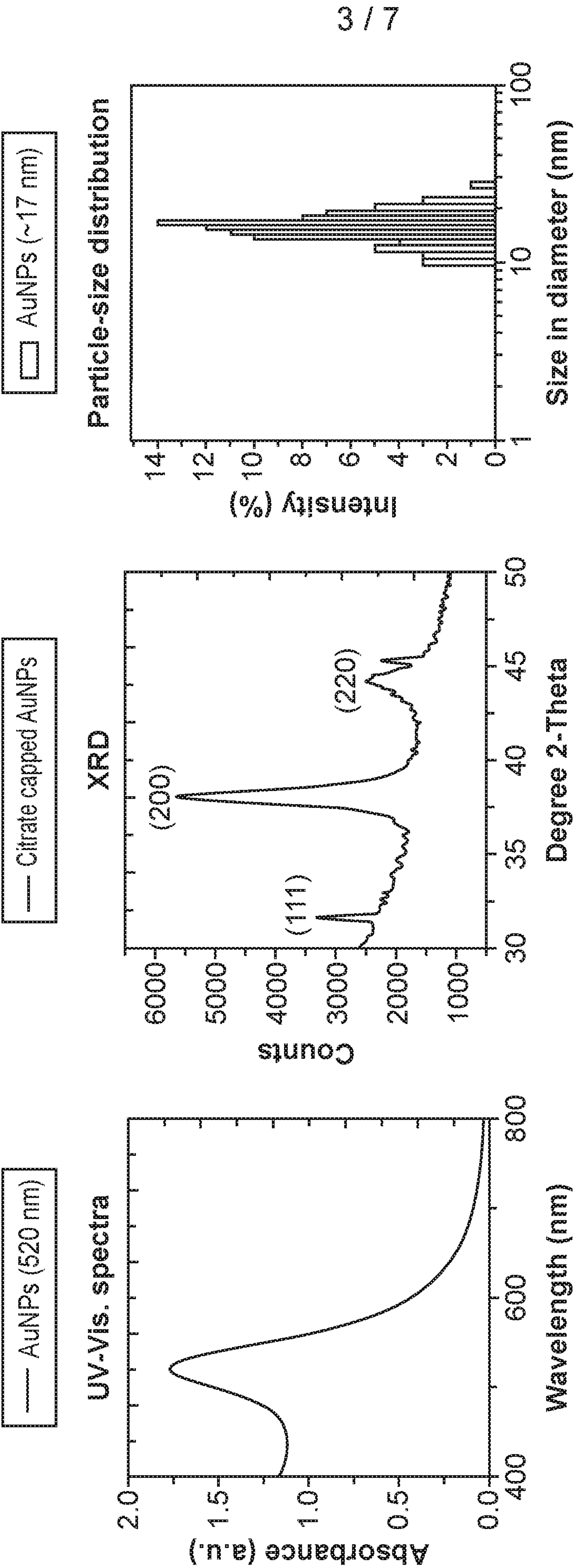


FIG. 4A

FIG. 4B

FIG. 4C

EDX

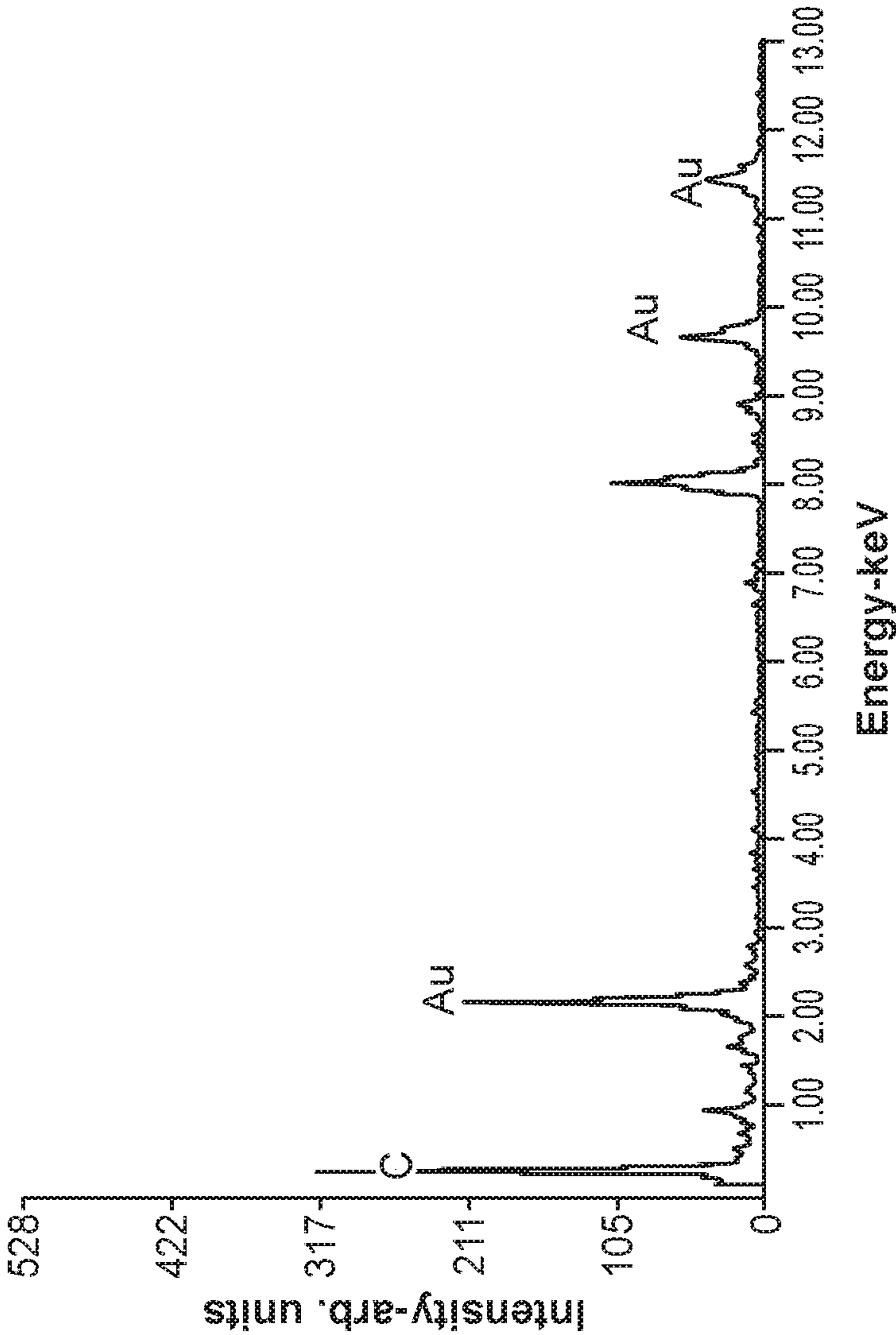


FIG. 4E

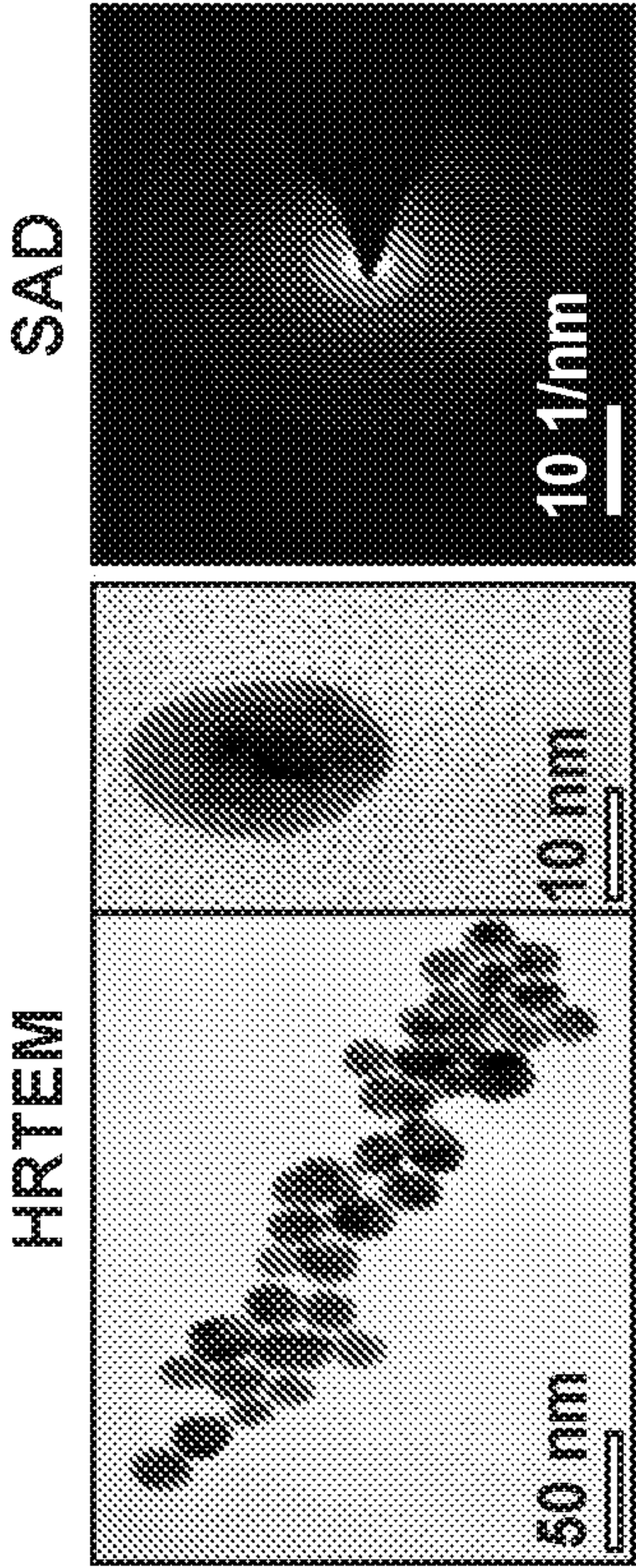


FIG. 4D

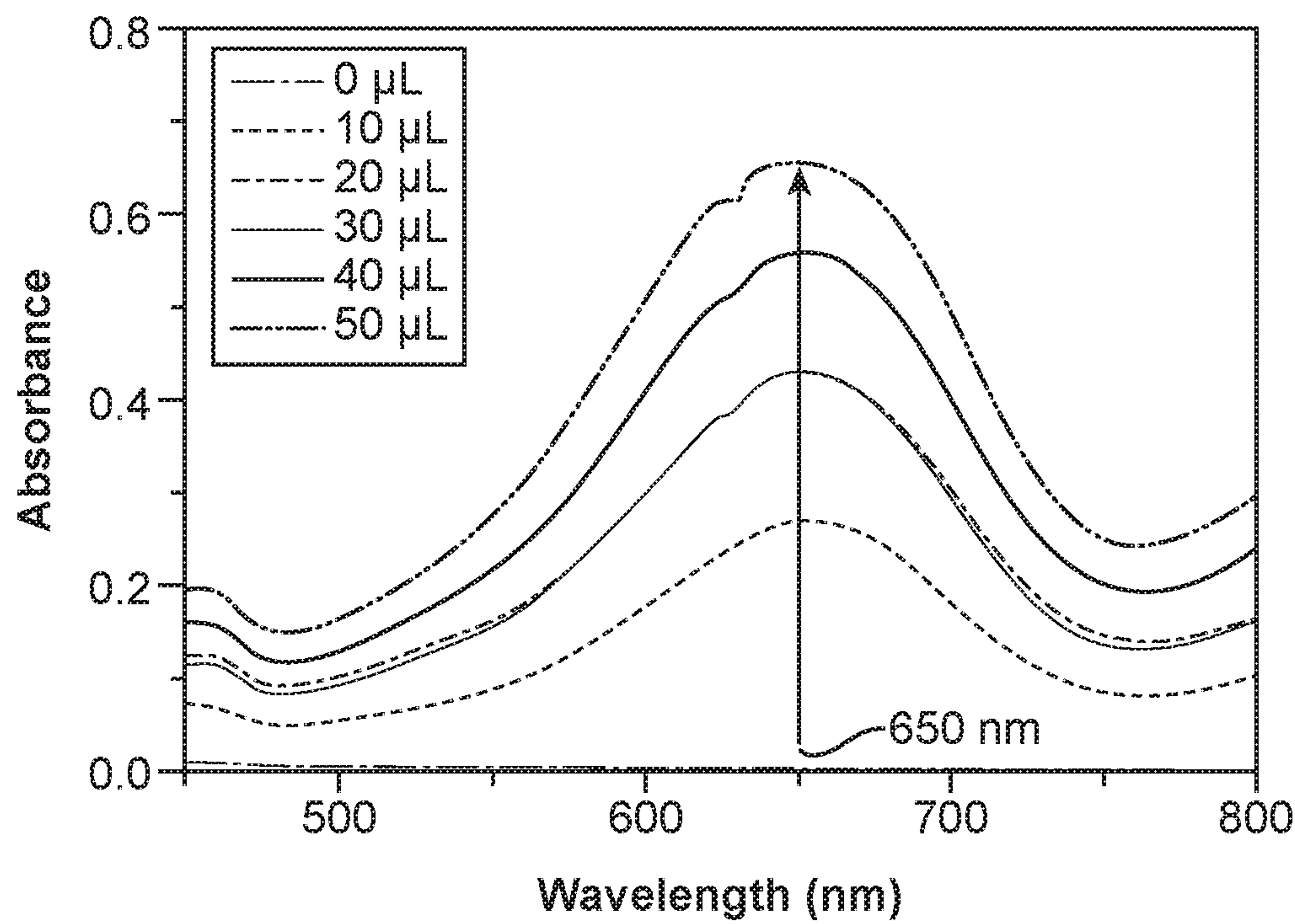


FIG. 5A

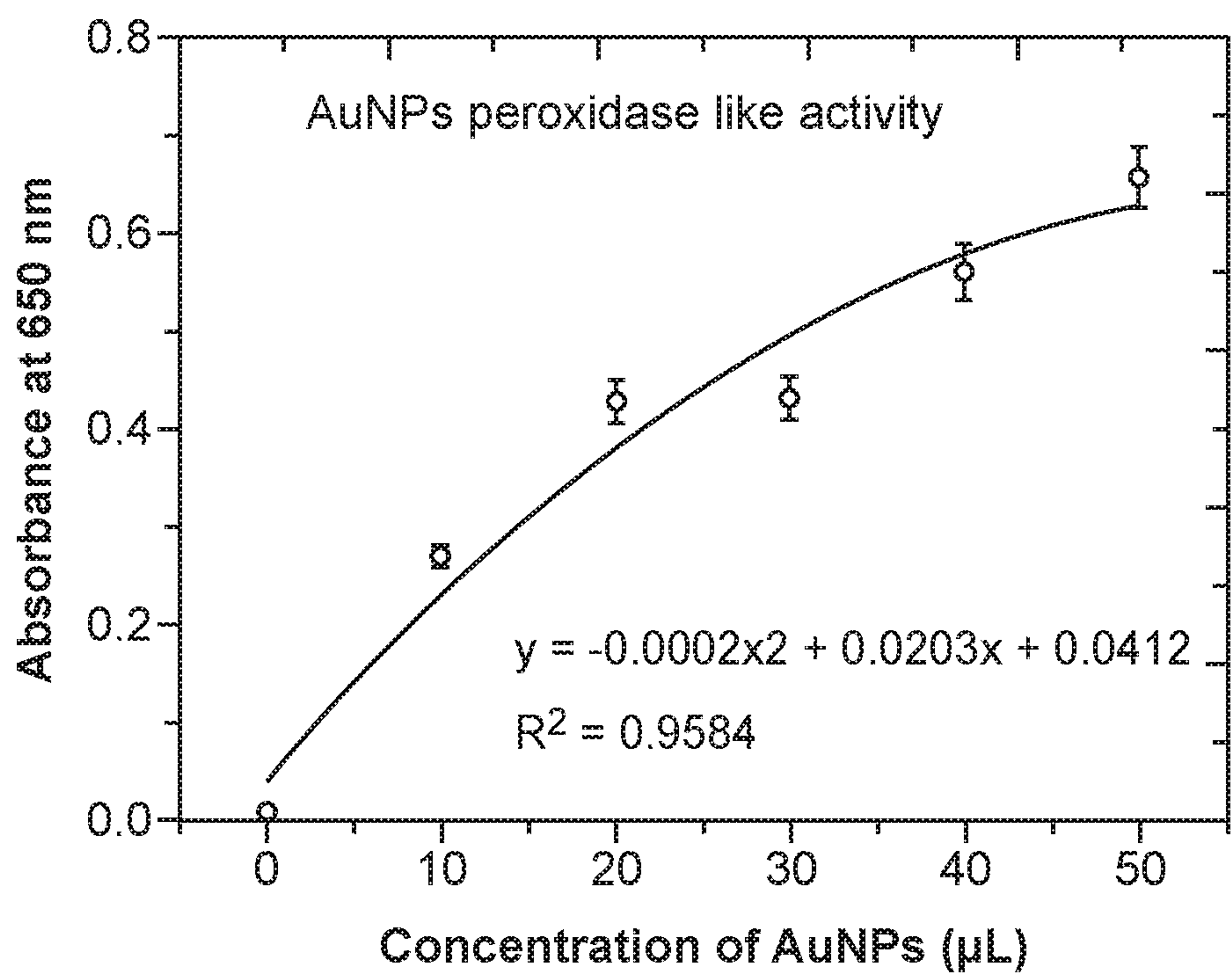


FIG. 5B

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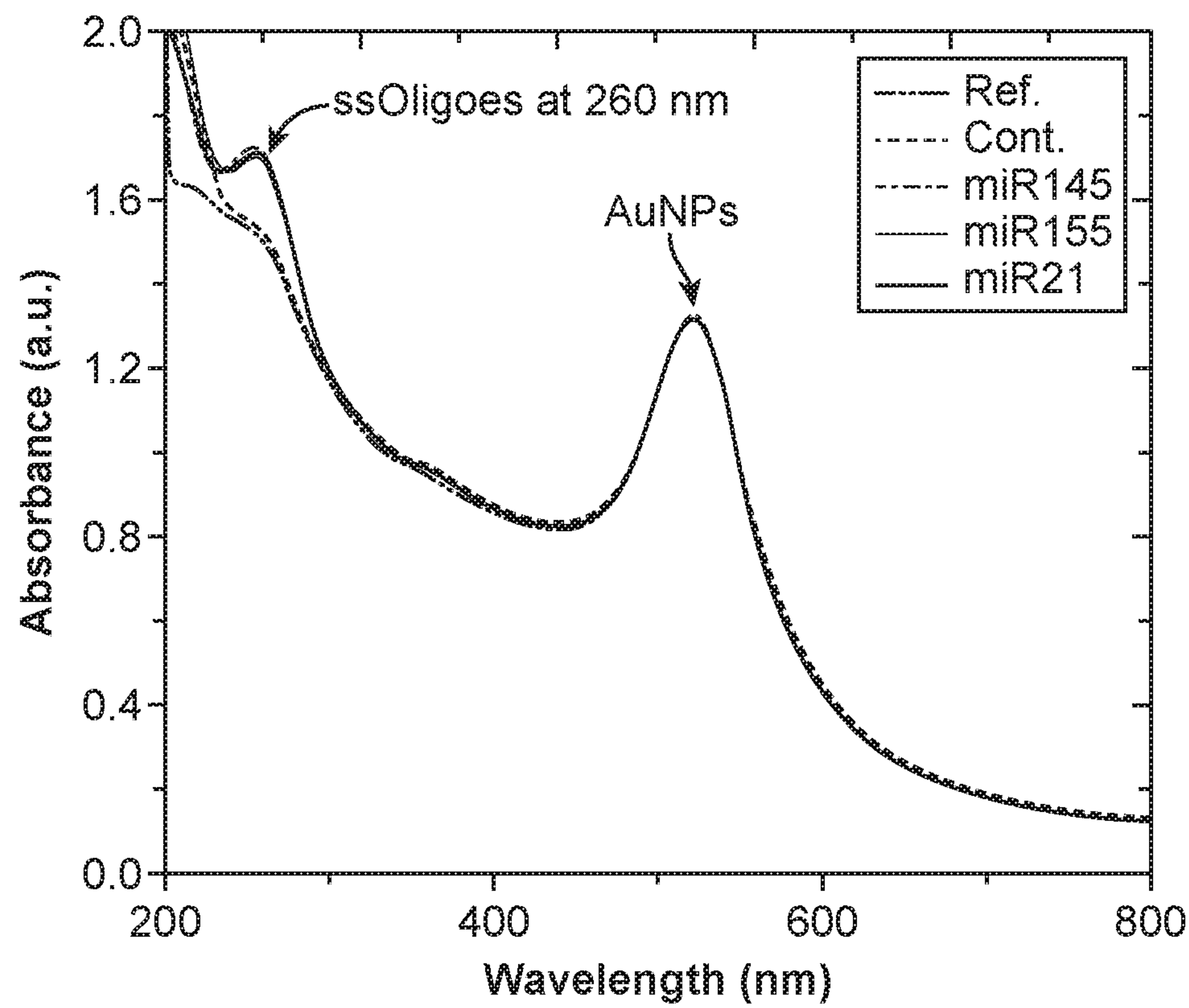


FIG. 6A

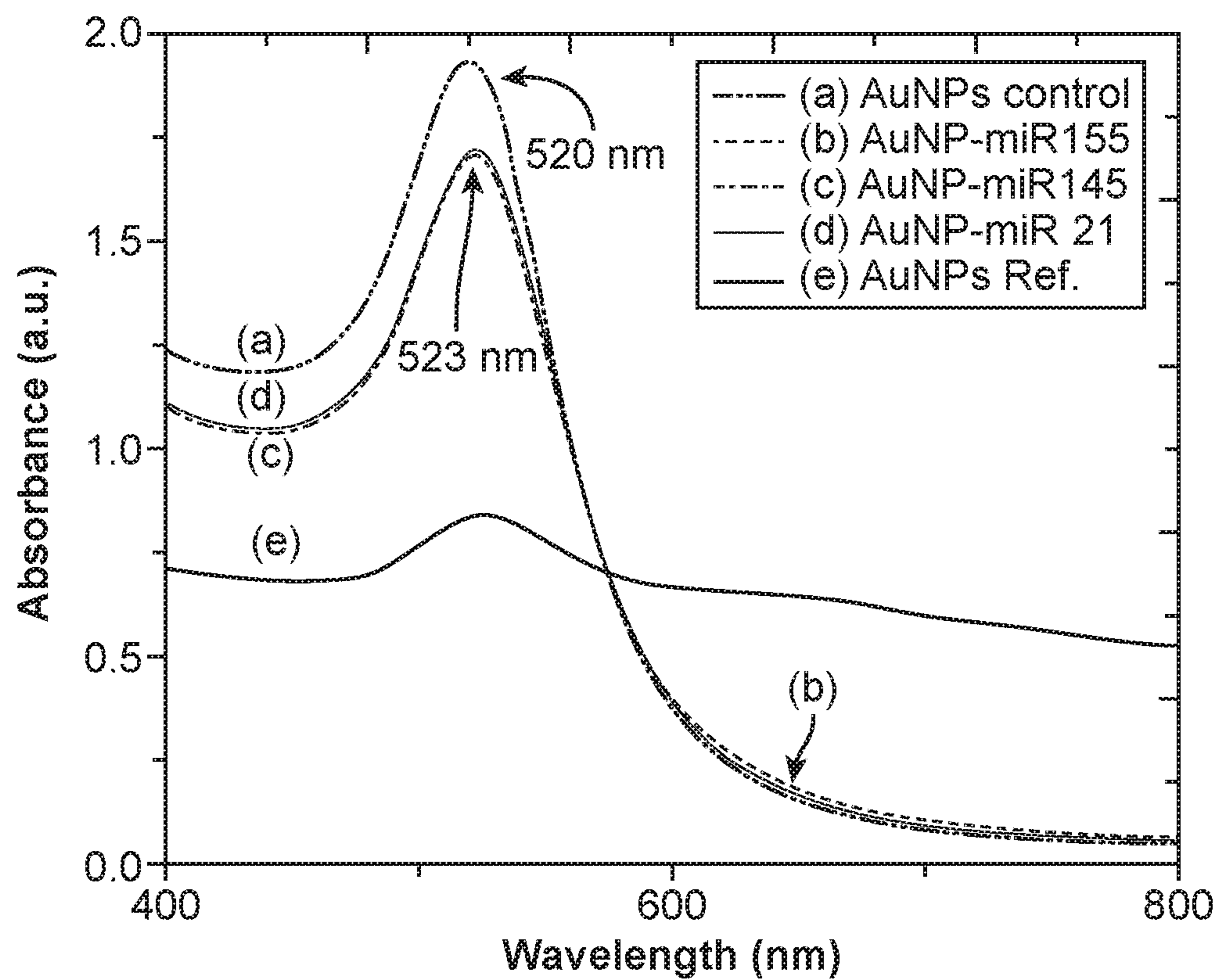


FIG. 6B

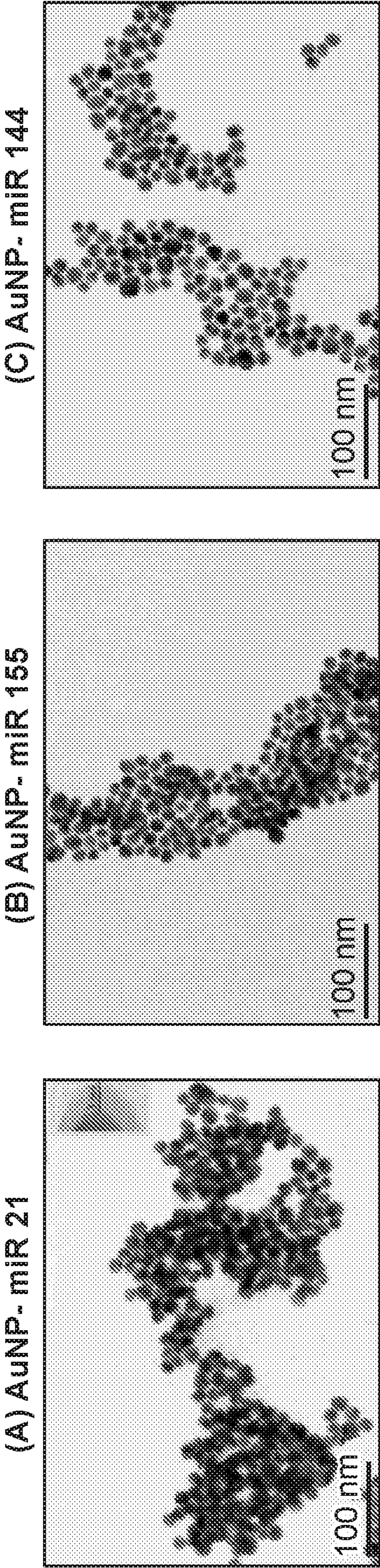


FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2020/050970

A. CLASSIFICATION OF SUBJECT MATTER

C12Q1/6816 Version=2021.01

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q

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

G01N

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

TotalPatent One, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Hizir et al. 2016. Multiplexed Activity of perAuxidase: DNA-Capped AuNPs Act as Adjustable Peroxidase, Analytical Chemistry, 88(1):600-605 11 December 2015 Abstract, Introduction, Results and Discussion, Supporting Information	1, 2 & 5-9
Y	Abstract, Introduction, Results and Discussion -----	3, 4 & 10-15
X	CN 101706504 A (SOUTHEAST UNIVERSITY), 12 May 2010 (12-05-2010) Abstract, Section 'Methods', Figures 4 & 6, Claims 1-4 -----	1-15
Y	US 20150024961 A1 (CARIS LIFESCIENCES SWITZERLAND HOLDINGS GMBH), 22 January 2015 (22-01-2015) Paragraphs [0129]-[0131], [0144], [0145] & [0946] and Claims 1, 2, 10, 12, 18 & 21 -----	1-15
Y	WO 2017214145 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA), 14 December 2017 (14-12-2017) Abstract, Page 5, Figure-1, Example-1 and Claims 1, 4, 19, 35, 36, 46, 52 & 53 -----	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

16-03-2021

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/IN2020/050970

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Dharanivasan et al. 2015. Gold nanoparticles assisted characterization of amine functionalized polystyrene multiwell plate and glass slide surfaces, Applied Nanoscience, 5: 39-50 9 January 2014 Abstract, Methods and Page 40	10 & 11
Y	Hakimian et al. 2018. Ultrasensitive optical biosensor for detection of miRNA-155 using positively charged Au nanoparticles, Scientific Reports, 8:2943 13 February 2018 The whole document	1-15
Y	Zhao et al. 2016. A visible and label-free colorimetric sensor for miRNA-21 detection based on peroxidase-like activity of graphene/gold-nanoparticle hybrids, Analytical Methods, 8(9):2005-2012 01 February 2016 The whole document	1-15
A	Huang et al. 2019. Colorimetric and fluorescent dual-mode detection of microRNA based on duplex-specific nuclease assisted gold nanoparticle amplification, The Analyst, 144(16):4917-4924 05 July 2019 The whole document	1-15
A	Chang et al. 2015. Gold Nanoparticle-Based Colorimetric Strategies for Chemical and Biological Sensing Applications, Nanomaterials (Basel), 9(6):861 6 June 2019 The whole document	1-15
A	Ying et al. 2017. Colorimetric detection of microRNA based hybridization chain reaction for signal amplification and enzyme for visualization, Analytical Biochemistry, 528:7-12 20 April 2017 The whole document	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/IN2020/050970

Citation	Pub.Date	Family	Pub.Date
WO 2017214145 A1	14-12-2017	US 20190136302 A1	09-05-2019