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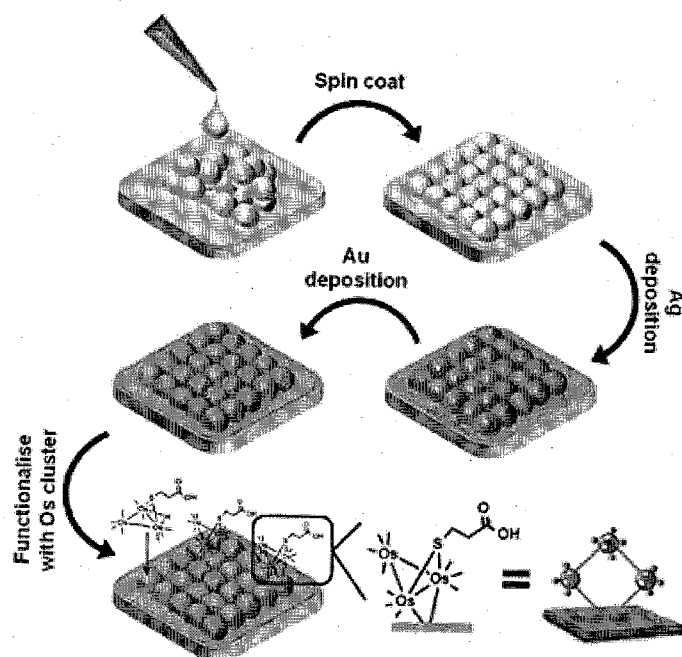
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(54) Title: METHOD FOR DETECTING ANALYTE USING SURFACE ENHANCED RAMAN SPECTROSCOPY, BIOSENSOR, AND METHOD OF MANUFACTURING THEREOF



(57) Abstract: A method for detecting one or more analytes using surface enhanced Raman spectroscopy (SERS) is provided. The method includes a) providing a SERS-active substrate having at least one metal carbonyl cluster compound covalently attached thereon; b) contacting one or more analytes with the at least one metal carbonyl cluster compound; and c) detecting changes in surface enhanced Raman signal from the at least one metal carbonyl cluster compound as an indication of the presence of the one or more analytes. A biosensor for the detection of an analyte using SERS and method of manufacturing the biosensor are also provided.



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**METHOD FOR DETECTING ANALYTE USING SURFACE ENHANCED RAMAN
SPECTROSCOPY, BIOSENSOR, AND METHOD OF MANUFACTURING
THEREOF**

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority of Singapore patent application no. 201301637-3 filed on 5 March 2013, the content of which is incorporated herein by reference in its entirety for all purposes.

TECHNICAL FIELD

[0002] The invention relates to spectroscopy and molecular diagnostics, and relates to a method of detecting analytes by surface enhanced Raman spectroscopy (SERS). In further aspects, the invention relates to a biosensor suitable for the invented SERS-based analyte detection method, and a method of manufacturing the biosensor.

BACKGROUND

[0003] In medical practice, for example, identification of a disease requires recognition of associated symptoms, as well as detection of specific features that indicate its presence unambiguously.

[0004] Identification of a disease may be carried out through biomarker screening, which may only be carried out through an analysis of biological fluids, such as blood, urine and cerebral spinal fluid. An accurate diagnostic may rarely be accomplished through detection of a single biomarker, hence a panel of markers has to be analyzed for reliable results, such as in a multiplexed assay. Monitoring the expression patterns of a variety of biomarkers at various stages of a disease assist prognosis, and allows tracking of disease progression. Early detection in asymptomatic populations is of utmost importance to facilitate early treatment and to reduce health-care costs.

[0005] State of the art protein biomarker assays are largely based on immunoassays. Platforms made of polymer or glass and bearing several immobilized antibodies spotted on different well-defined locations are usually provided. These assays involve exposure of the platform to the sample, followed by incubation with one or two further antibodies, and several washing and blocking steps in between to increase specificity of the assay results.

Detection is usually via fluorescence detection, chromophoric absorption, or a colorimetric readout.

[0006] Although a number of immunosensor arrays have been developed in recent years, a truly rapid, accurate and miniaturizable system is still non-existent. Taking ELISA, for example, it is the current gold standard for bio-assay. However, it requires multiple steps, each with separate reagents. Each ELISA analysis requires a separate distinct reaction and, in addition, requires a label for detection of the analyte.

[0007] Vibrational spectroscopic techniques, such as infrared (IR), normal Raman and Surface Enhanced Raman (SER) spectroscopy, have been considered for analyte detection. Of these, surface enhanced Raman spectroscopy (SERS) has evolved as an important method for analyte detection, where spectral intensity is enhanced tremendously by interaction of SERS-active analyte molecules with a substrate surface. However, detection of the analyte molecules remains very much dependent on properties of the analyte molecule-substrate ensemble, and is currently limited to certain classes of SERS-active molecules.

[0008] In view of the above, there remains a need for an improved method for detecting one or more analytes that alleviates at least one or more of the above-mentioned problems.

SUMMARY

[0009] In a first aspect, the invention refers to a method for detecting one or more analytes using surface enhanced Raman spectroscopy (SERS). The method comprises

- a) providing a SERS-active substrate having at least one metal carbonyl cluster compound covalently attached thereon;
- b) contacting one or more analytes with the at least one metal carbonyl cluster compound; and
- c) detecting changes in surface enhanced Raman signal from the at least one metal carbonyl cluster compound as an indication of the presence of the one or more analytes.

[0010] In a second aspect, the invention refers to a biosensor for the detection of an analyte using surface-enhanced Raman spectroscopy, the biosensor comprising at least one metal carbonyl cluster compound covalently attached to a SERS-active substrate. The at least one metal carbonyl cluster compound has general formula (I)



wherein M at each occurrence denotes a metal selected from Group 6 to Group 11 of the Periodic Table of Elements; x is an integer from 10 to 12; and each L is independently selected from the group consisting of -H, -SH and -A-(CH₂)_n-COOH, wherein A is selected from the group consisting of S, O, C and N; and n is an integer from 1 to 10.

[0011] In a third aspect, the invention refers to a method of manufacturing a biosensor according to the second aspect, the method comprising

- a) attaching a plurality of nanospheres on a substrate;
- b) depositing a first metallic layer on the plurality of nanospheres;
- c) depositing a second metallic layer on the first metallic layer to form a metallic bilayer; and
- d) covalently attaching the at least one metal carbonyl cluster compound to the metallic bilayer to form the biosensor.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0013] **FIG. 1** depicts molecular transducer system on a chip. In **FIG. 1A**, a photograph of a bimetallic film over nanosphere (BMFON) chip disclosed herein is shown. The inset shows an optical microscope image (50x objective). In **FIG. 1B**, a schematic representation of an osmium carbonyl cluster immobilized on BMFON in a planar rhombus manner is shown. **FIG. 1C** shows a finite element method (FEM) simulation of electric field enhancement $|E_{local}/E_0|$ of osmium carbonyl cluster-gold film stress sensor system, to provide an understanding of distribution of electric field on BMFON. $|E_0|$ is normalized to 1. The directions of k and E for incident light are provided. **FIG. 1D** shows a SERS spectra of carbonyl region present on BMFON coated with osmium carbonyl clusters. Y-axis denotes intensity (counts) and x-axis denotes Raman shift (cm⁻¹). Labels on Y-axis read 0, 1000, 2000, 3000, and 4000, while labels on x-axis read 1800, 1900, 2000, 2100, and 2200.

[0014] **FIG. 2** depicts response of a molecular transducer disclosed herein. **FIG. 2A** shows a mechanical experiment carried out using 4 μm plain-surface polystyrene (PS) microspheres. The inset shows an optical image under microscope of the chip. **FIG. 2B**

shows SERS spectra measured from chip with bead ("polystyrene") and without bead ("beside polystyrene") (scale bar = 4 μm). **FIG. 2C** shows principal component analysis of spectral data for bound PS beads (o) ("polystyrene"), region adjacent to PS microsphere (x) ("beside polystyrene") and osmium carbonyl clusters only (*) ("osmium").

5 [0015] **FIG. 3** depicts density-functional theory (DFT) calculation of osmium carbonyl cluster. **FIG. 3A** shows molecular structure of osmium carbonyl cluster for DFT calculation. **FIG. 3B** shows computed Raman activity spectra with Os-S bond lengths ($\text{\AA}$) of I) 2.52260, 2.52238; II) 2.49926, 2.49927; III) 2.49139, 2.49139; IV) 2.48358, 2.48352; V) 2.47628, 2.47603; VI) 2.44715, 2.44716. Y-axis denote in the graphs denote intensity. **FIG. 3C** shows
10 optimized geometries (side-view) of B of Os-S bond lengths ($\text{\AA}$) of I) 2.52260, 2.52238; II) 2.49139, 2.49139 and III) 2.44715, 2.44716. Compression of Os-S bonds lead to changes in the geometries with the S atom more inclined towards the triosmium core. **FIG. 3D** shows calculated HOMO diagrams (A-front view, B-top view) of geometries with Os-S bond lengths ($\text{\AA}$) of 1) 2.52260, 2.52238; 2) 2.49139, 2.49139. This illustrates that shortening of
15 Os-S bonds can result in changes in the electronic distribution on the structures.

[0016] **FIG. 4** depicts response of molecular transducer to varying molecular mass. **FIG. 4A** shows SERS spectra of biomolecules of different molecular weight of 5 kDa, 50 kDa, 120 kDa, and 160 kDa on chip. **FIG. 4B** shows PCA of spectral data for proteins with different molecular weights on osmium clusters ("insulin", "Antigen", ConA" and "Antibody"), and
20 free osmium clusters ("osmium").

[0017] **FIG. 5** depicts molecular transducer for label-free sensing. **FIG. 5A** shows schematic representation of antigen binding onto antibody-conjugated osmium carbonyl clusters on BMFON. **FIG. 5B** shows SERS spectra of osmium carbonyl clusters functionalized with anti-p53 and various concentration of p53 antigen of 0.001 nM, 0.005 nM, 0.01 nM, 0.05 nM, 0.1 nM, 0.5 nM, 1 nM, 10 nM, and 20 nM. **FIG. 5C** shows PCA of bound
25 p53 proteins with different concentrations ranging from 0.001 nM to 20 nM, free osmium clusters ("osmium") and free antibodies ("antibody").

[0018] **FIG. 6** shows (A) plot representing the intensity of CO stretching frequency versus different concentration of A1AT protein, with the interpolated value for the spiked urine sample at concentration of 0.4 mg/mL; and (B) SERS spectrum of osmium coated chip obtained after binding of A1AT protein. In **FIG. 6B**, y-axis denotes intensity (counts) and x-axis denotes Raman shift (cm^{-1}) in the range of 1000 to 2200.
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DETAILED DESCRIPTION

[0019] As disclosed herein, molecular geometry and bonding interactions within metal carbonyl cluster compounds may be characterized by surface enhanced Raman spectroscopy (SERS). Changes in bond length of the metal carbonyl cluster compounds due to interaction with analyte molecules affect electron distribution in the compound. This translates into changes in pattern and/or intensity of the SERS signal, particularly in the $1,800\text{ cm}^{-1}$ to $2,200\text{ cm}^{-1}$ region unique to the metal carbonyl cluster compounds. The changes in pattern and/or intensity of the SERS signal form basis of the method for detecting one or more analytes using SERS disclosed herein.

[0020] Accordingly in a first aspect, the present invention refers to a method for detecting one or more analytes using SERS.

[0021] The method includes providing a SERS-active substrate having at least one metal carbonyl cluster compound covalently attached thereon. As used herein, the term "metal carbonyl cluster compound" refers to metal cluster compounds comprising carbon monoxide in complex combination with metal atoms, wherein the metal atoms in the metal carbonyl cluster are held together entirely or at least substantially by bonds between metal atoms.

[0022] The carbonyl ligands and/or other ligands in the metal carbonyl cluster compound may be bonded to some or all of the metal atoms to form a complex. In some embodiments, a carbonyl ligand is bonded to two metal atoms to form a bridge between the two metal atoms. Other suitable bridging groups may include, for example, phosphine, arsine, and mercapto groups.

[0023] The at least one metal carbonyl cluster compound may have general formula (I)

[0024]



[0025] wherein M at each occurrence denotes a metal selected from Group 6 to Group 11 of the Periodic Table of Elements; x is an integer from 10 to 12; and each L is independently selected from the group consisting of $-\text{H}$, $-\text{SH}$ and $-\text{A}-(\text{CH}_2)_n-\text{COOH}$, wherein A is selected from the group consisting of S, O, C and N; and n is an integer from 1 to 10.

[0026] In various embodiments, M is independently selected from the group consisting of Cr, Mo, W, Mn, Tc, Re, Fe, Ru, Os, Co, Rh, Ir, Ni, Pd, Pt, Cu, Ag, and Au. In some

embodiments, M is independently selected from the group consisting of Fe, Ru, and Os. In specific embodiments, M is Os.

[0027] x is an integer from 10 to 12. For example, x may be 10, 11, or 12. In specific embodiments, x is 10.

5 [0028] L denotes a ligand in the metal carbonyl cluster compound. L at each occurrence may be the same or different. Each L is independently selected from the group consisting of –H, –SH and –A-(CH₂)_n-COOH, wherein A is selected from the group consisting of S, O, C and N. n is an integer from 1 to 10, such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

[0029] In various embodiments, A is S.

10 [0030] In various embodiments, n is 10.

[0031] In various embodiments, L includes –H and –A-(CH₂)₁₀-COOH.

[0032] In various embodiments, the metal carbonyl cluster compound is Fe₃(CO)₁₀(μ-H)[μ-S(CH₂)₁₀COOH], Ru₃(CO)₁₀(μ-H)[μ-S(CH₂)₁₀COOH], or Os₃(CO)₁₀(μ-H)[μ-S(CH₂)₁₀COOH]. In specific embodiments, the metal carbonyl cluster compound is
15 Os₃(CO)₁₀(μ-H)[μ-S(CH₂)₁₀COOH].

[0033] The at least one metal carbonyl cluster compound is covalently attached to a SERS-active substrate. This may take place by interaction between the SERS-active substrate and organic ligand comprised in the metal carbonyl cluster compound. To facilitate covalent coupling of the metal carbonyl cluster compound to the surface of the SERS-active substrate,
20 the metal carbonyl cluster compound may include a functional group.

[0034] In various embodiments, the metal carbonyl cluster compound comprises a functional group selected from the group consisting of mercapto, carboxy, and amino. For example, the organic ligand comprised in the metal carbonyl cluster compound may comprise a functional group selected from the group consisting of mercapto, carboxy, and amino, for
25 attaching the metal carbonyl cluster compound to the surface of the SERS-active substrate.

[0035] A preferred functional group is a mercapto (–SH) group. The terms “thiol group” and “mercapto group” are used interchangeably herein and both relate to the –SH group. The mercapto group may facilitate covalent attachment to the metal surface by forming a covalent bond between the sulfur atom and a metal surface atom.

30 [0036] The SERS-active substrate may comprise a metallic bilayer deposited on a plurality of nanospheres which are attached on a support. In various embodiments, the SERS-active substrate is provided by attaching a plurality of nanospheres on a support; depositing a

first metallic layer on the plurality of nanospheres; depositing a second metallic layer on the first metallic layer to form a metallic bilayer; and covalently attaching at least one metal carbonyl cluster compound to the metallic bilayer.

[0037] Advantageously, it has been found by the inventors that the SERS-active substrate, having a metallic bilayer deposited on a plurality of nanospheres which are attached on a support, provide excellent signal enhancement of Raman signals, to allow detection of biomolecules using only an extremely low sample volume of about 20 μ L. As demonstrated herein, osmium carbonyl cluster compounds are able to bind strongly on the SERS-active substrate. Furthermore, fabrication of the SERS-active substrate does not require clean room environment or sophisticated instruments for fabrication, which facilitates scaling up and adoption of the fabrication process in industry.

[0038] The support that is used to form the SERS-active substrate may generally be formed from any material. Examples of material that may be used to form the support include, but are not limited to, glass, ceramic and organic polymers. In various embodiments, the support is glass.

[0039] A plurality of nanospheres is attached to the support, whereby the term "plurality" as used herein means more than one, such as at least 2, 20, 50, 100, 1000, 10000, 100000, 1000000, 10000000, or even more.

[0040] The plurality of nanospheres may be formed from a SERS-active material or a non-SERS active material. Examples of a SERS-active material include, but are not limited to, noble metals such as silver, palladium, gold, platinum, iridium, osmium, rhodium, ruthenium; copper, aluminium, or alloys thereof. For example, the nanospheres may be formed entirely from a SERS metal, and may for example, consist of a metal selected from the group consisting of a noble metal, copper, aluminium, and alloys thereof. In some embodiments, the nanospheres are formed from a non-SERS active material, such as plastic, ceramics, composites, glass or organic polymers. In specific embodiments, the nanospheres comprise or consist of polystyrene.

[0041] Size of the nanospheres may be characterized by their mean diameter. The term "diameter" as used herein refers to the maximal length of a straight line segment passing through the center of a figure and terminating at the periphery, whereas the term "mean diameter" refers to an average diameter of the nanospheres, and may be calculated by dividing the sum of the diameter of each nanosphere by the total number of nanospheres. In

various embodiments, the nanospheres have a mean diameter of about 30 nm to about 60 nm, for example about 40 nm.

[0042] The plurality of nanospheres attached to the surface of the support may be monodisperse. The term "monodisperse" refers to nanospheres having a substantially uniform size and shape. In some embodiments, the standard deviation of diameter distribution of the nanospheres is equal to or less than 20 % of the mean diameter value, such as equal to or less than 15 %, 10 %, 5 % or 3 % of the mean diameter value. In some embodiments, the diameter of the nanospheres is essentially the same.

[0043] The nanospheres may be attached to a substrate by any suitable means. For example, the nanospheres may be attached to the surface of the support by means of linker molecules, which are molecules having one or more functional groups that can bind or link the nanospheres to the support. Type of linker molecules may depend on the nanospheres and support. Generally, any functional group that can bind the nanospheres to the surface of the support may be used. Examples of functional groups include, but are not limited to, a thiol group, an amine group, and a 2-diphenylphosphino group.

[0044] The functional groups on the linker molecules may allow covalent bonding of the nanospheres to the surface of the support. The strong covalent bonds used to attach the nanospheres to the support may prevent their dislodgement, thereby resulting in mechanical stability of the nanospheres on the support.

[0045] After attaching the plurality of nanospheres on the support, a first metallic layer may be deposited on the nanospheres, followed by deposition of a second metallic layer on the first metallic layer. The first metallic layer and the second metallic layer may comprise or consist of a SERS-active metal, such as silver, palladium, gold, platinum, iridium, osmium, rhodium, ruthenium; copper, aluminium, or alloys thereof. In specific embodiments, the first metallic layer that is deposited on the plurality of nanospheres comprises or consists of silver, and the second metallic layer that is deposited on the first metallic layer comprises or consists of gold.

[0046] The first metallic layer and the second metallic layer are present as a metallic bilayer on the plurality of nanospheres. In embodiments where the nanospheres are formed from a non-SERS active material, presence of the metallic bilayer on the nanospheres renders the nanospheres SERS-active. In such embodiments, the nanospheres may have a core-shell structure, in which the core of the nanospheres is formed from any material such as plastic,

ceramics, composites, glass or organic polymers, and the shell of the nanospheres is formed from a SERS-active metal such as a noble metal.

[0047] The at least one metal carbonyl cluster compound is covalently attached to the metallic bilayer. As mentioned above, this may take place by interaction between the metallic bilayer on the SERS-active substrate and organic ligand comprised in the metal carbonyl cluster compound. Examples of organic ligand that may be present in the metal carbonyl cluster compound have already been described above.

[0048] Method of the first aspect includes contacting one or more analytes with the at least one metal carbonyl cluster compound. The terms "contacting" or "incubating" as used interchangeably herein refer generally to providing access of one component, reagent, analyte or sample to another.

[0049] Contacting may be carried out by dispensing one or more drops of a sample containing the one or more analytes to at least one metal carbonyl cluster compound covalently attached on a SERS-active substrate. The solution comprising the analyte or sample may also comprise another component or reagent, such as dimethyl sulfoxide (DMSO) or a detergent, which facilitates mixing, interaction, uptake, or other physical or chemical phenomenon advantageous to the contact between the analytes and/or samples.

[0050] In various embodiments, contacting the one or more analytes with the at least one metal carbonyl cluster compound is carried out by placing the one or more analytes on the at least one metal carbonyl cluster compound without attaching the one or more analytes to the at least one metal carbonyl cluster compound. Advantageously, this allows recycling or reuse of the SERS-active substrate, since the analytes are not attached to the SERS-active substrate, and may be removed so as to regenerate the SERS-active substrate.

[0051] After contacting the one or more analytes with the at least one metal carbonyl cluster compound, changes in surface enhanced Raman signal from the at least one metal carbonyl cluster compound are detected as an indication of the presence of the one or more analytes.

[0052] In various embodiments, detecting changes in surface enhanced Raman signal from the at least one metal carbonyl cluster compound comprises detecting changes in pattern and/or intensity of SERS signal in the region of 1800 cm^{-1} to 2200 cm^{-1} .

[0053] Advantageously, the at least one metal carbonyl cluster compound is able to provide a unique SERS signal at a region of 1800 cm^{-1} to 2200 cm^{-1} , thereby avoiding

interference with signals emitted by biomolecules which are in the 800 cm^{-1} to 1800 cm^{-1} region. This allows identification of biomolecules without the need to decouple signals emitted from the metal carbonyl cluster compound. This attribute may be used to provide a more complex spectrum for multiplex detection.

5 [0054] In various embodiments, changes in the surface enhanced Raman signal of the at least one metal carbonyl cluster compound are correlated with mass of the one or more analytes.

[0055] Without wishing to be bound by theory, it is postulated that analyte molecules, which are in contact with or placed on the metal carbonyl cluster compounds, compress
10 metal-ligand and/or metal-carbonyl bonds in the metal carbonyl cluster compounds. This compression results in geometric distortion of the metal carbonyl cluster compounds. For example, the metal-ligand and/or metal-carbonyl bonds in the metal carbonyl cluster compounds may be shortened.

[0056] In light of the above, analyte molecules of different masses result in differing
15 extent at which metal-ligand and/or metal-carbonyl bonds in the metal carbonyl cluster compound are shortened. The different extent at which the bonds are shortened result in a shift in the vibrational frequencies, thereby changing the pattern and/or intensity of SERS signal. This effect is particularly pronounced in the region of 1800 cm^{-1} to 2200 cm^{-1} , which offers a unique SERS signal for analyte detection.

20 [0057] In various embodiments, the metal carbonyl cluster compound is conjugated or attached to an analyte-binding molecule. The term "analyte binding molecule" as used herein refers to any molecule capable of binding to an analyte of choice so as to form a complex consisting of the analyte binding molecule and the analyte.

[0058] In one embodiment of such a conjugate, the analyte binding molecule is covalently
25 coupled to the metal carbonyl cluster compound, which is in turn covalently attached to SERS-active substrate. Preferably, the binding between the analyte binding molecule to the analyte molecule is specific so that a specific complex between analyte and analyte binding molecule is formed.

[0059] "Specifically binding" and "specific binding" as used herein mean that the analyte
30 binding molecule binds to the target analyte based on recognition of a binding region or epitope on the target molecule. The analyte binding molecule preferably recognizes and binds to the target molecule with a higher binding affinity than it binds to other compounds in the

sample. In various embodiments, "specifically binding" may mean that an antibody or other biological molecule, binds to a target molecule with at least about a 10^6 -fold greater affinity, preferably at least about a 10^7 -fold greater affinity, more preferably at least about a 10^8 -fold greater affinity, and most preferably at least about a 10^9 -fold greater affinity than it binds molecules unrelated to the target molecule. Typically, specific binding refers to affinities in the range of about 10^6 -fold to about 10^9 -fold greater than non-specific binding. In some embodiments, specific binding may be characterized by affinities greater than 10^9 -fold over non-specific binding. The binding affinity may be determined by any suitable method. Such methods are known in the art and include, without limitation, surface plasmon resonance and isothermal titration calorimetry. In a specific embodiment, the analyte binding molecule uniquely recognizes and binds to the target analyte.

[0060] Examples of analyte binding molecules include, but are not limited to, an antibody, antibody fragment, or antibody like molecules. In various embodiments, the analyte binding molecule is a proteinaceous molecule, such as an antibody, for example a monoclonal or polyclonal antibody, which immunologically binds to the target analyte at a specific determinant or epitope. The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies as well as antibody variants, fragments or antibody like molecules, such as for example, Fab, F(ab')₂, scFv, Fv diabodies and linear antibodies, so long as they exhibit the desired binding activity.

[0061] In some embodiments, the analyte binding molecule is a monoclonal antibody. The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. The monoclonal antibodies can include

"chimeric" antibodies and humanized antibodies. A "chimeric" antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

[0062] Monoclonal antibodies may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to the human B-cell hybridoma technique, and the EBV-hybridoma technique, for example. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb may be cultivated *in vitro* or *in vivo*. Production of high titres of mAbs *in vivo* makes this a very effective method of production.

[0063] In some embodiments, the analyte binding molecule is a polyclonal antibody. "Polyclonal antibodies" refer to heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as rabbits, mice and goats, may be immunized by injection with an antigen or hapten-carrier conjugate optionally supplemented with adjuvants.

[0064] In various embodiments, targeting ability of the at least one metal carbonyl cluster compound is achieved by incorporating a variety of analyte binding molecule. Examples of analyte binding molecules include, but are not limited to, p53 antigen and A1AT antibody.

[0065] The terms "analyte", "target compound", "target molecule" or "target" as interchangeably used herein, refer to any substance that can be detected in an assay by binding to a binding molecule, and which, in one embodiment, may be present in a sample. Therefore, the analyte can be, without limitation, any substance for which there exists a naturally occurring antibody or for which an antibody can be prepared. The analyte may, for example, be an antigen, a protein, a polypeptide, a nucleic acid, a hapten, a carbohydrate, a lipid, a cell or any other of a wide variety of biological or non-biological molecules, complexes or combinations thereof. Generally, the analyte will be a protein, peptide, carbohydrate or lipid derived from a biological source such as bacterial, fungal, viral, plant or animal samples. Additionally, however, the target may also be a small organic compound such as a drug, drug-metabolite, dye or other small molecule present in the sample.

[0066] The term "sample", as used herein, refers to an aliquot of material, frequently biological matrices, an aqueous solution or an aqueous suspension derived from biological

material. Samples to be assayed for the presence of an analyte include, for example, cells, tissues, homogenates, lysates, extracts, and purified or partially purified proteins and other biological molecules and mixtures thereof.

[0067] Non-limiting examples of samples include human and animal body fluids such as whole blood, serum, plasma, cerebrospinal fluid, sputum, bronchial washing, bronchial aspirates, urine, semen, lymph fluids and various external secretions of the respiratory, intestinal and genitourinary tracts, tears, saliva, milk, white blood cells, myelomas and the like; biological fluids such as cell culture supernatants; tissue specimens which may or may not be fixed; and cell specimens which may or may not be fixed. The samples used may vary based on the assay format and the nature of the tissues, cells, extracts or other materials, especially biological materials, to be assayed. Methods for preparing protein extracts from cells or samples are well known in the art and can be readily adapted in order to obtain a sample that may be used in the method disclosed herein. Detection in a body fluid can also be *in vivo*, i.e. without first collecting a sample.

[0068] "Peptide" generally refers to a short chain of amino acids linked by peptide bonds. Typically peptides comprise amino acid chains of about 2-100, more typically about 4-50, and most commonly about 6-20 amino acids. "Polypeptide" generally refers to individual straight or branched chain sequences of amino acids that are typically longer than peptides. "Polypeptides" usually comprise at least about 20 to 1000 amino acids in length, more typically at least about 100 to 600 amino acids, and frequently at least about 200 to about 500 amino acids. Included are homo-polymers of one specific amino acid, such as for example, poly-lysine. "Proteins" include single polypeptides as well as complexes of multiple polypeptide chains, which may be the same or different.

[0069] Multiple chains in a protein may be characterized by secondary, tertiary and quaternary structure as well as the primary amino acid sequence structure, may be held together, for example, by disulfide bonds, and may include post-synthetic modifications such as, without limitation, glycosylation, phosphorylation, truncations or other processing.

[0070] Antibodies such as IgG proteins, for example, are typically comprised of four polypeptide chains (i.e., two heavy and two light chains) that are held together by disulfide bonds. Furthermore, proteins may include additional components such associated metals (e. g., iron, copper and sulfur), or other moieties. The definitions of peptides, polypeptides and

proteins includes, without limitation, biologically active and inactive forms; denatured and native forms; as well as variant, modified, truncated, hybrid, and chimeric forms thereof.

[0071] The term "detecting" as used herein refers to a method of verifying the presence of a given molecule. The technique used to accomplish this is surface enhanced Raman spectroscopy (SERS). The detection may also be quantitative, i.e. include correlating the detected signal with the amount of analyte. The detection includes *in vitro* as well as *in vivo* detection.

[0072] The method for detecting analytes using SERS may also be a multiplex method for detecting more than one analyte, i.e. two or more different analytes. This usually requires the use of more than one analyte binding molecule in the contacting step so that each analyte is bound by a specific analyte binding molecule.

[0073] In a second aspect, the invention relates to a biosensor for the detection of an analyte using surface-enhanced Raman spectroscopy. The biosensor comprises at least one metal carbonyl cluster compound covalently attached to a SERS-active substrate, the at least one metal carbonyl cluster compound having general formula (I)

[0074]



[0075] wherein M at each occurrence denotes a metal selected from Group 6 to Group 11 of the Periodic Table of Elements; x is an integer from 10 to 12; and each L is independently selected from the group consisting of -H, -SH and -A-(CH₂)_n-COOH, wherein A is selected from the group consisting of S, O, C and N; and n is an integer from 1 to 10.

[0076] In various embodiments, A is S.

[0077] In various embodiments, n is 10.

[0078] In various embodiments, L includes -H and -A-(CH₂)₁₀-COOH.

[0079] The metal carbonyl cluster compound may be Fe₃(CO)₁₀(μ-H)[μ-S(CH₂)₁₀COOH], Ru₃(CO)₁₀(μ-H)[μ-S(CH₂)₁₀COOH], or Os₃(CO)₁₀(μ-H)[μ-S(CH₂)₁₀COOH]. In specific embodiments, the metal carbonyl cluster compound is Os₃(CO)₁₀(μ-H)[μ-S(CH₂)₁₀COOH].

[0080] As mentioned above, SERS-active substrate having at least one metal carbonyl cluster compound covalently attached thereon may comprise a metallic bilayer having a first metallic layer and a second metallic layer deposited on a plurality of nanospheres which are attached on a support. Examples of materials that may be used to form the first metallic layer, second metallic layer, nanospheres, and support have already been mentioned above.

[0081] The biosensor described herein may be used in a method for the detection of an analyte, wherein the method comprises contacting the biosensor with the analyte containing medium, for example a sample or body fluid, and detecting changes in SERS signal from the sensor. The changes in SERS signal may be in the form of changes in pattern and/or intensity of SERS signal in the region of 1800 cm^{-1} to 2200 cm^{-1} .

[0082] The metal carbonyl cluster compound may be conjugated or attached to an analyte-binding molecule. Examples of analyte-binding molecules have already been mentioned above. In specific embodiments, the analyte-binding molecule is A1AT antibody.

[0083] In some embodiments, the biosensor is configured for a multiplex method that allows the detection of more than one analyte.

[0084] In a third aspect, the invention refers to a method of manufacturing a biosensor according to the second aspect. The method includes attaching a plurality of nanospheres on a support; depositing a first metallic layer on the plurality of nanospheres; depositing a second metallic layer on the first metallic layer to form a metallic bilayer; and covalently attaching the at least one metal carbonyl cluster compound to the metallic bilayer to form the biosensor.

[0085] Examples of materials that may be used to form the first metallic layer, second metallic layer, nanospheres, support, and metal carbonyl cluster compound have already been mentioned above. In specific embodiments, the metal carbonyl cluster compound is $\text{Os}_3(\text{CO})_{10}(\mu\text{-H})[\mu\text{-S}(\text{CH}_2)_{10}\text{COOH}]$.

[0086] Hereinafter, the present invention will be described more fully with reference to the accompanying drawings, in which exemplary embodiments of the invention are shown. This invention may, however, be embodied in many different forms and should not be construed as limited to the exemplary embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art. In the drawings, lengths and sizes of layers and regions may be exaggerated for clarity.

[0087] As used herein, the term "and/or" includes any and all combinations of one or more of the associated listed items. It will be understood that, although the terms first, second, third, etc., may be used herein to describe various elements, components, regions, layers and/or sections, these elements, components, regions, layers and/or sections should not be limited by these terms. These terms are only used to distinguish one element, component, region, layer or section from another region, layer or section. Thus, a first element,

component, region, layer or section discussed below could be termed a second element, component, region, layer or section without departing from the teachings of the present invention.

5 [0088] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. It will be further understood that the terms "comprises" and/or "comprising," when used in this specification, specify the presence of stated features, integers, steps, operations, elements, and/or components, but do not preclude the presence or
10 addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof.

[0089] Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. It will be further understood that terms, such as those defined in
15 commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the relevant art and will not be interpreted in an idealized or overly formal sense unless expressly so defined herein.

[0090] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed
20 herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible
25 within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

30 [0091] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or

negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0092] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

EXPERIMENTAL SECTION

[0093] Nanomechanical biosensors couple mechanical and chemical phenomena on a molecular scale. Ideally, such biosensors are able to combine sensitivity, specificity, versatility, robustness, and portability with advantages of modern nanotechnology.

[0094] As disclosed herein, a novel mechano-chemistry concept with organometallic chemistry for nanomechanical biosensing is presented. In various embodiments, tunable carbonyl signal is used for surface enhanced Raman scattering based biosensing, and molecular interaction is used as basis for developing a model, which takes into account of structure deformation and electron distribution in metal carbonyl, for development of a mechano-responsive chip.

[0095] Theoretical formulation, its experimental validation, and potential practical applications of such a molecular scale system have been demonstrated. It is postulated by the inventors that carbonyl signals may be manipulated by varying the bond length in osmium carbonyl clusters. This postulation was validated with experimental data obtained. In embodiments, the prepared systems were made protein-sensitive by incorporating antibodies on the molecular "springs". Modulation of geometry and electron distribution may serve as a wavelength-based biodetection tool, demonstrating the useful mechano-chemical concept.

[0096] **Example 1: General procedure**

[0097] All manipulations for chemical synthesis were carried out using standard Schlenk techniques under an argon or nitrogen atmosphere. $\text{Os}_3(\text{CO})_{12}$ was purchased from Oxkem; all other chemicals were purchased from other commercial sources and used as supplied.

[0098] Infrared (IR) spectra were obtained using a Bruker Alpha Fourier transform infrared spectrometer.

[0099] ^1H NMR spectra were recorded in deuterated chloroform (CDCl_3) on a JEOL ECA400 or ECA400SL spectrometer and were referenced to residual solvent resonances.

[00100] The Raman spectral measurements were carried out using a Renishaw InVia Raman (UK) microscope with a Peltier cooled charge coupled device (CCD) detector and an excitation wavelength at 633 nm, where the laser beam is directed to the sample through a 50× objective lens, which was used to excite the sample and also to collect the return Raman signal. All Raman spectra were processed with WiRE3.0 software. The maximum laser power at the sample was measured to be 6.2 mW and the exposure time was set at 10 s throughout the measurements. Prior to each measurement, the instrument was calibrated with a silicon standard whose Raman peak is centered at 520 cm⁻¹.

[00101] **Example 2: Functionalization of the bimetallic film over nanosphere (BMFON) chip with osmium carbonyl clusters**

[00102] The BMFON chip was prepared as follows.

[00103] During preparation, the PS sphere solution was added with 15 wt% surfactant sodium dodecyl sulphate (SDS), to composite solution containing 2.36 wt% PS spheres and 0.85 wt% SDS. Clean microscope glass slides used as supporting substrate were cut into square pieces (10 mm x 10 mm x 1 mm). Using the spin-coating method, PS sphere monolayers were fabricated onto the clean glass slides. This was done by dispersing 10 µL of the prepared PS colloidal solution onto the center of a glass slide. Each glass slide was spin coated in the spin coater preset at 2000 rpm for 20 s. The coated glass slides were then dried in vacuum desiccators overnight at 0.6 Pa pressure. The substrates were first coated with Ag (99.999 % purity, JEOL) at thickness that amount to 120 s of sputtering (JEOL JFC-1600 Auto fine coater) before being sputtered with a layer of Au (99.999 % purity, JEOL) for 80 s. Each metal layer was deposited at a rate of 1.33 nm/s.

[00104] The chip was cleaned thoroughly with ethanol and then immersed for 24 hours in a 1 mM of Os₃(CO)₁₀(µ-H)[µ-S(CH₂)₁₀COOH] solution prepared in dichloromethane. The Os₃(CO)₁₀(µ-H)[µ-S(CH₂)₁₀COOH] solution was prepared as follows.

[00105] Os₃(CO)₁₀(NCCH₃)₂ (0.028 mmol) and 3-mercaptopropanoic acid (0.034 mmol) were dissolved in dichloromethane (6 mL) and left to stir at room temperature overnight. Solvent was removed in vacuo and the residue was purified by TLC using ethyl acetate:hexane (1:1, v/v) as eluant to yield one major yellow band as cluster Os₃(CO)₁₀(µ-H)[µ-S(CH₂)₂COOH].

[00106] The substrates were then removed from the solution and washed thoroughly with dichloromethane to remove unbound osmium carbonyl clusters. Polystyrene bead solution

(200 μ L) was added on the chip and dried under argon flow. For immobilization of different molecular weight of biomolecules, the chip was immersed in a solution of biomolecules mixed with EDC/NHS for 4 hours. The same coupling procedures were applied to prepare immobilization of anti-p53 antigen. Upon completion of the incubation, the chip was thoroughly washed with phosphate buffered saline (PBS) and incubated with p53 antigen.

[00107] **Example 3: Computational Studies**

[00108] The geometries were studied using DFT utilizing the Becke's three parameter hybrid function and Perdew-Wang's gradient-corrected correlation function (B3PW91). The basic set employed was LanL2DZ (Los Alamos Effective Core Potential Double- ζ) for all atoms. Vibrational frequencies calculations of the systems were performed at 298.15 K and 1 atm pressure. All geometries were fully optimized and evaluated for the correct number of imaginary frequencies. All calculations were performed using Gaussian 09 suite of program.

[00109] **Example 4: Simulation**

[00110] A Lenovo desktop was used to perform high mesh density simulation of nanoparticles. The specification of the computer was Intel(R) Core(TM)2 Quad CPU Q9650 at 3.00 GHz with 8GB RAM. The operating platform was 64-bit Windows 7 professional. For the simulation of plasmonic properties of gold nanoparticles, radio frequency (RF) module, an extended version of COMSOL Multiphysics 3.5a version, was used. The desired particle size and shape in 3D were drawn in draw mode using the Cartesian coordinate system. Boundary conditions and perfectly matched layer (PML) were also defined in the draw mode. Simulation duration for a single nanoparticle took about 4 hours.

[00111] **Example 5: Data Analysis**

[00112] Principal component analysis (PCA) is a method of analyzing complex sets of data with multiple variables. The technique facilitates identification of hidden relationships between data sets by reducing their dimensionality and representing the data in the new coordinate system. Raman spectrum can be considered as a data matrix where the first column represents the Raman shift and the second column contains the corresponding signal intensity. For PCA of n spectra with p data points, an n -by- p matrix is constructed, where each row represents a Raman intensity spectrum. The purpose of the PCA is to find a new p -dimensional orthogonal coordinate system where the data projection on each coordinate axis has a sequentially maximal variance. Each projection corresponds to a linear combination of

the original data, with the first projection having the maximum variance and representing the first principal component. Here, PCA was performed using Matlab calculation environment.

[00113] **Example 6: Results and Discussion**

[00114] To create a surface enhanced Raman spectroscopy-based (SERS-based) nanomechanical biosensor chip (**FIG. 1B**), an osmium carbonyl cluster was covalently immobilized on a SERS-active substrate, bimetallic film over nanosphere (BMFON), in a planar rhombus manner, thus acting as a molecular “spring”.

[00115] Theoretical calculations for the plasmonic enhancement of osmium carbonyl cluster on BMFON were performed by adopting finite element methods simulation with COMSOL.

[00116] In the model used, distance between the two osmium atoms and the gold film was set at an estimated value of 275 pm. In the simulation, the refractive index of gold and osmium are $0.99958884+1.1257e^{-4}i$ and $0.999626+3.3600e^{-4}i$, respectively. A plane wave with wavelength at 633 nm is incident perpendicular to the gold film, as shown by the k vector direction in **FIG. 1C**. As a result, the electric field enhancement was calculated by $|E_{local}/E_0|$.

[00117] Both the coupling between Os atoms and the coupling between Os atoms and gold film were enhanced by the highest factor of 26 were noted, making the first step towards a mechanochemical approach to sensing by monitoring the changes in the carbonyl signal due to variation in electron distribution of osmium carbonyl clusters as a transduction mechanism.

[00118] The strong SERS carbonyl signal of molecular spring assemblies made from osmium carbonyl clusters and BMFON has also been determined experimentally (**FIG. 1D**).

[00119] In general, three peaks are observed in the carbonyl region at about $1,950\text{ cm}^{-1}$, about $2,030\text{ cm}^{-1}$ and a sharp $2,109\text{ cm}^{-1}$ peak.

[00120] To validate the mechano-chemical concept, an experiment was performed where a four-micron unfunctionalised polystyrene (PS) microsphere was loaded on the sensing surface (**FIG. 2A**), allowing its weight to exert a downward mechanical pressure on the osmium carbonyl cluster layer. SERS spectra were then obtained by focusing the excitation laser beam through the PS microsphere on the point of contact between the microsphere and the osmium carbonyl cluster, followed by another excitation with the sample slightly translated adjacently to focus on an unbound (PS free) osmium carbonyl cluster.

[00121] The SERS spectra corresponding to the two beam positions are shown in **FIG. 2B** as “polystyrene” and “beside polystyrene” respectively.

[00122] When SERS measurement was made through the PS, a dramatic change in the carbonyl signal pattern and reduced intensities were observed. This may be attributed to the forces exerted by the microsphere at the point of contact. The spectral data in the wavenumber range 1,900 to 2,080 cm^{-1} corresponding to the PS-osmium carbonyl cluster and unbound osmium carbonyl cluster were also analyzed using principal component analysis (PCA).

[00123] PCA is a statistical method which uses an orthogonal transformation to reduce the multi-dimensional dataset to its main principal components, such that the maximum variance within the dataset can be obtained in mutually orthogonal dimensions. **FIG. 2C** shows a plot of principal component 2 (PC2) against principal component 1 (PC1) for this set of data, in which PC1 accounted for about 90 % of the total variance within the dataset. As displayed in the figure, data points corresponding to PS-osmium carbonyl cluster are clearly differentiated from those corresponding to free osmium carbonyl cluster. In addition, data points corresponding to unloaded osmium carbonyl clusters and loaded unbound (PS free) osmium carbonyl clusters are clustered together, implying spectral similarity between the two classes and thus validating the sensor technique as a means of observing significant mass-induced wavelength changes.

[00124] One advantage of using metal carbonyl mechanical transducer is the potential reversibility of the spectral processes in such structures. To demonstrate that, since there is no covalent bond between osmium carbonyl clusters and PS, the molecular mass added to the chip which resulted in carbonyl signal changes was washed with ethanol, and it shows a recovery of the original carbonyl.

[00125] To validate the explanation provided herein, a theoretical approach was used to calculate Raman shifts of the model with different bond lengths.

[00126] A simplified structure (**FIG. 3A**), was selected as the model for density-functional theory (DFT) calculations with various Os-S bond lengths ranging from 2.52 Å to 2.45 Å. **FIG. 3B** shows the progressive decrement in the Os-S bond lengths, with changes in the calculated Raman frequencies of the carbonyl stretches, notably in the 1,950 cm^{-1} to 2,020 cm^{-1} region. The original geometry (**FIG. 3B(I)**), which is derived, shows two strong absorption at 1,978 cm^{-1} and 1,991 cm^{-1} . Shortening of the Os-S leads to changes in the

Raman frequencies, resulting in the merging of peaks and intensity decrease (**FIG. 3B(VI)**). This phenomenon could be due to geometry distortion as the optimized structures revealed the progressive geometries changes with the μ -S atom being more inclined towards the triosmium plane when the Os-S bonds are shortened (**FIG. 3C**). This demonstrates that bond compression can lead to geometric distortion, causing a shift in the vibrational frequencies and, hence, changing the Raman spectra.

[00127] The experiment was subsequently repeated with various biomolecules of different molecular masses ranging between 5,000 Dalton and 160,000 Dalton (5 kDa insulin, 50 kDa p53 antigen, 120 kDa Concavalin A and 160 kDa IgG antibody). Their SERS spectra are depicted in **FIG. 4**.

[00128] Upon adding biomolecules, similar observation experienced with PS microsphere was made, in which there was a change in carbonyl signal pattern and intensity with the molecular mass. When higher mass was added, a dramatic change in carbonyl signal was observed; the relative intensities and the peak widths broaden. This is also in complete agreement with the theoretical description previously, describing the deformation of osmium carbonyl cluster. These changes are all indication of stronger interactions among osmium carbonyl clusters and mass of interest.

[00129] It has been shown herein that compression of metal-metal bonds length of metal clusters causes a corresponding signal alteration of carbonyl signal and thus reflects the electron distribution of osmium carbonyl cluster. Such an observation was interpreted as metal-metal bond distance reduction or deformation of cluster structure affecting electron distribution, resulting in alternation of carbonyl signal, rendering it useful to utilise the correlations between the changes in metal-metal bond or deformation of cluster structure and the frequency shifts.

[00130] The spectral data in the wavenumber range $1,900\text{ cm}^{-1}$ to $2,080\text{ cm}^{-1}$ corresponding to bound proteins with different molecular weights (MW) as well as free osmium carbonyl clusters were once again analyzed using PCA.

[00131] **FIG. 4B** shows a plot of principal component 2 (PC2) against principal component 1 (PC1), in which PC1 accounted for greater than 99 % of the total variance within the dataset. After performing k-means clustering on the data points, it is clearly demonstrated that the data points forming 3 distinct clusters, namely the osmium cluster only (blue cluster), bound proteins with low MWs of less than 100 kDa (green cluster), as well as bound proteins

with relatively higher MWs of more than 100 kDa (red cluster). This confirmed the ability of the biosensor to discriminate proteins with different MWs from one another. In addition, the gap between the green and blue cluster strongly suggests potential of the biosensor to detect proteins with MWs even lower than 5 kDa.

5 [00132] To demonstrate the versatility of the chip, the chip was functionalized for antigen detection in which p53 protein was chosen for this study (**FIG. 5A**).

[00133] On the basis of the current study, the same design principle was adopted to use a osmium carbonyl cluster as the basic building block of the mechanical transducer comprising a carboxylic acid functional group. The biodetection functionalities may be acquired by
10 incorporating an antibody to the carboxylic acid moiety. Such structures may be made by following standard bioconjugation protocols.

[00134] A change in the wavelength of the carbonyl signal was observed after the bioconjugation of antibody onto osmium carbonyl cluster (**FIG. 5B**), closely matching our expectations, and as such may be attributed to the bond shortening and electron distribution
15 changes of osmium carbonyl cluster.

[00135] As the chip was incubated with p53 antigen, carbonyl signal was further changed. It is important to note that the wavelength changes depend on the concentration of the molecule of interest. As such, the change in wavelength when different amounts of p53 antigen were added was monitored.

20 [00136] The spectra were monitored over a broad concentration range (0.001 nM to 20 nM) until spectral saturation where change was no longer observed. The wavelength was changed with an increase in p53 antigen concentration between 0.001 nM and 1 nM. The amplitude of the shifts gradually decreased owing to the progressive overloading of the protein; 1 μ M above showed similar results. The concept of specificity of the chip is demonstrated here.

25 [00137] Lastly, the spectral data in the same wavenumber range $1,900\text{ cm}^{-1}$ to $2,080\text{ cm}^{-1}$ corresponding to bound p53 proteins with different concentrations as well as free osmium clusters were analyzed with PCA.

[00138] **FIG. 5C** shows a plot of principal component 2 (PC2) against principal component 1 (PC1) for this set of data, in which PC1 accounted for about 90 % of the total variance
30 within the dataset.

[00139] As shown in the figure, the data points are shifting in the direction of decreasing PC1 scores, with increasing p53 concentration, reaching a saturation limit beyond 1 nM

concentration, where the data points for 1 nM, 10 nM and 20 nM concentrations are very close together. This implies a trend in the spectral waveform variation as a function of p53 concentration, up to a threshold of 1 nM. The resulting graph shows a decreasing trend in signal intensity, eventually reaching a minimum threshold at very high p53 concentrations.

5 [00140] Thus, with regard to these results, it is hypothesized that at higher p53 concentrations beyond 1 nM, almost all the anti-p53 binding sites on the osmium clusters have been fully occupied with p53 proteins, resulting in little or no variation in the corresponding spectra.

10 [00141] Osmium carbonyl clusters were conjugated with p53 antibody and loaded with different biomolecules. It was observed that carbonyl intensity did not vary much with respect to the concentration of loaded H1 antigen as compared to that of p53 antigen. It reveals that the BMFON chip could be a potential ultra sensitive sensor chip with SERS technology to detect analytes far below physiological concentration ranges.

15 [00142] Carbonyl signal of osmium carbonyl clusters reduces and shifts in the presence of p53 antigen, arising in the variance in the bond length. For bond length variations, which are attributed to deformation of cluster structure resulting in change in carbonyl signal. This usually occurs because of variations in the bond length of metal clusters or dimensional characteristics; this process leads to a physically controllable carbonyl signal shift, serving as a foundation for the future development of nanoscale structures with sensing and other
20 functionalities.

[00143] The degree of compression of bond depends on the nature of the metal. From the DFT calculation for the osmium carbonyl cluster model, it can be seen that the small change in bond length can create a noticeable carbonyl signal shift. It is noteworthy that the actual magnitude of the shift cannot be determined without knowing the degree of compression of
25 the metal-metal bond which has proved difficult to study currently.

[00144] ELISA is the current gold standard for bio-assay. However, it requires multiple steps, each with separate reagents. Each ELISA analysis requires a separate distinct reaction and, in addition, requires a label for detection of the analyte. Advantageously, the nanomechanical assay described here needs no label and can be performed in a single
30 reaction without additional reagents.

[00145] Moreover, a chip functionalized with molecular “springs” can be used to perform multiple assays by, for example, coating each chip with a different antibody. The potential

advantages of a label-free assay that can measure multiple analytes in a single step without addition of other reagents are numerous, and could ultimately translate to much cost-effective tests.

[00146] For example, this could increase the availability of multiple serum tumor markers screening, which is currently cost-prohibitive. The ability to design different sensitive molecular springs can enable both high resolution detection as well as a high dynamic range, as exemplified in this study.

[00147] Despite being able to detect p53 at the current detection limit of ELISA at sub-nanomolar ranges, there is room for further improvement in sensitivity through the use of a new molecular spring. The label-free option makes it particularly attractive for applications such as drug discovery, which requires one to detect specific binding between small molecules with proteins. This technique may also be applied in detecting DNA hybridization, making it a common platform for detecting DNA and proteins, as well as DNA-protein interactions. This technique is sufficiently general to detect many specific biomolecular interactions without the need for labels. Nanomechanical sensing has provided important advances in the detection of biomolecules and other biological targets, such as viruses and bacteria. Although it has not yet been accepted as a practical alternative to well-established techniques, it is believed that the mechanochemical concept disclosed herein is a step forward for nanomechanical sensing in becoming a widely adopted tool in biology laboratories.

[00148] In summary, the theoretical wavenumber shift in osmium carbonyl clusters on gold surface as a result of deformation of structure and electron distribution change were evaluated and confirmed through a series of experiments. The carboxylic acid on the osmium carbonyl clusters provides biological functionality to the structure and enables its use as a biosensor. Most importantly, the phenomena of the wavelength shift matches very well with the predicted DFT data, which is a very reasonable estimate for shortening of bonds and deformation of geometry of osmium carbonyl clusters. This study provides a physical description of the processes in complex nanoscale systems based on the mechano-chemical concept and can serve as a firm foundation for further designs of functional nano-springs.

[00149] **Example 7: Organometallic Carbonyl Coated Chip for Highly Sensitive SERS-based Biosensing of Glycoprotein based Cancer Markers in Biofluids**

[00150] Bladder cancer is the second most common cancer affecting urinary system. Early diagnosis of this cancer is extremely important as this disease may become malignant and

spread outside of bladder to affect other organs. The current gold standard for bladder cancer diagnosis, cystoscopy, is an invasive process that brings discomfort and pain to the patients. Although cystoscopy being the world most accepted method in bladder cancer diagnosis with high specificity, it suffers from low sensitivity of tumors/lesions detection. Accordingly, because of the costly and invasive nature of cystoscopy, much attention has been diverted to explore other possible techniques and methodologies for fast, reliable and non-invasive urine-based assays.

[00151] Raman spectroscopy is an optical method based on inelastic light scattering. Based on the detected spectral changes in cancerous and non-cancerous cells, it emerged in recent years as a promising tool for cancer diagnosis but with low sensitivities. Surface-enhanced Raman Spectroscopy (SERS) overcomes the low sensitivity of traditional Raman spectroscopy through the use of roughened metal surface substrate, providing ultra-high sensitivity in detection and characterization of analytes.

[00152] In the presence of metallic nano particles (usually gold or silver) localized on the substrate, SERS can achieve $>10^{10}$ higher signals as compared to normal Raman scattering. Although techniques like nuclear magnetic resonance and X-ray crystallography are able to provide molecular structure, they required complicated and tedious sample preparation. SERS technology, therefore, provides a potentially quick, accurate and non-invasive approach in label-free molecule identification technique for bladder cancer urine analysis.

[00153] Urine analysis is a direct and reliable approach to gain information about ones urinary system health. Also, it can be collected non-invasively and in large quantities. Because urine is exposed to the epithelium layer of bladder, proteins and peptides expressed by these linings were secreted in the urine. To-date, alpha-1-antitrypsin (A1AT) is one of the biomarkers for bladder cancer that have been identified and investigated. As disclosed herein, use of SERS technology in A1AT spiked urine analysis using organometallic carbonyl coated chip has been demonstrated.

[00154] Example 7.1: Methodology

[00155] Materials and methods in this approach were similar to the previous approach mentioned above, but only difference is that here A1AT antibody was conjugated to the carboxylic acid group of osmium clusters. Briefly, the osmium coated chip was incubated in the anti-A1AT antibody solution together with EDC/NHS for 1 hour. Upon incubation

completion, the chip was thoroughly washed with PBS and incubated with sample (20 μ L) for 5 minutes prior to Raman measurement.

[00156] Example 7.2: Results and Discussion

[00157] Similar to previous study as mentioned above, signals of osmium carbonyl in 2000 cm^{-1} was monitored for protein binding events because it was confirmed in the previous study that upon binding the Raman shift would be changed either (i) in the shift position, or (ii) in the intensity.

[00158] **FIG. 6** suggested that the Raman shifts changes in peak shift when anti-A1AT antibody is binding with A1AT protein (0.01 0.1, 0.5, 1.0 mg/mL) on osmium coated chip. Comparing the highest intensity peaks of carbonyl signal at different concentrations, it was found that the carbonyl peak shift increases as a function of A1AT concentration.

[00159] While the study of osmium coated chip with commercial A1AT protein showed a nice trend, the study was extended to urine samples. To demonstrate the potential practicality of the sensor system disclosed herein, determination of A1AT protein in urine samples spiked with a known A1AT concentration (0.4 mg/mL) was carried out. The concentration of A1AT in the urine sample was determined to be 0.45 mg/mL, in good agreement with the amount of A1AT added.

[00160] In conclusion, use of an osmium coated chip as a sensor in a novel assay for bladder cancer marker A1AT has been demonstrated. Compared with other glucose detection methods reported, the sensor chip disclosed herein exhibits several advantages.

[00161] Firstly, no prior purification of the sample is needed. Secondly, an extremely low sample volume is required. Thirdly, it shows very high specificity for A1AT. Lastly, the spectroscopic handle for proteins quantification is in a spectral window (1800 cm^{-1} to 2200 cm^{-1}) which is relatively devoid of interference from any functional groups of biomolecules. Given this combination of advantages, it is expected that osmium coated chip as biological sensor chip will find wide applicability, and this concept for a bladder cancer marker assay presented here can be developed into a clinical diagnostic tool.

[00162] While the present invention has been particularly shown and described with reference to exemplary embodiments thereof, it will be understood by those of ordinary skill in the art that various changes in form and details may be made therein without departing from the spirit and scope of the present invention as defined by the following claims.

CLAIMS

What is claimed is:

- 5 1. Method for detecting one or more analytes using surface enhanced Raman spectroscopy (SERS), the method comprising
- 10 a) providing a SERS-active substrate having at least one metal carbonyl cluster compound covalently attached thereon;
- b) contacting one or more analytes with the at least one metal carbonyl cluster compound; and
- c) detecting changes in surface enhanced Raman signal from the at least one metal carbonyl cluster compound as an indication of the presence of the one or more analytes.

- 15 2. Method according to claim 1, wherein the at least one metal carbonyl cluster compound has general formula (I)



20 wherein

M at each occurrence denotes a metal selected from Group 6 to Group 11 of the Periodic Table of Elements;

x is an integer from 10 to 12; and

25 each L is independently selected from the group consisting of -H, -SH and -A-(CH₂)_n-COOH, wherein A is selected from the group consisting of S, O, C and N; and n is an integer from 1 to 10.

- 30 3. Method according to claim 2, wherein M is Os.

4. Method according to claim 2, wherein A is S.

5. Method according to claim 1, wherein the metal carbonyl cluster compound is $\text{Os}_3(\text{CO})_{10}(\mu\text{-H})[\mu\text{-S}(\text{CH}_2)_{10}\text{COOH}]$.
6. Method according to claim 1, wherein providing a SERS-active substrate having at least one metal carbonyl cluster compound covalently attached thereon comprises
 - a) attaching a plurality of nanospheres on a support;
 - b) depositing a first metallic layer on the plurality of nanospheres;
 - c) depositing a second metallic layer on the first metallic layer to form a metallic bilayer; and
 - d) covalently attaching at least one metal carbonyl cluster compound to the metallic bilayer.
7. Method according to claim 1, wherein detecting changes in surface enhanced Raman signal from the at least one metal carbonyl cluster compound comprises detecting changes in pattern and/or intensity of SERS signal in the region of 1800 cm^{-1} to 2200 cm^{-1} .
8. Method according to claim 1, wherein the one or more analytes are contacted with the at least one metal carbonyl cluster compound by placing the one or more analytes on the at least one metal carbonyl cluster compound without attaching the one or more analytes to the at least one metal carbonyl cluster compound.
9. Method according to claim 1, wherein the metal carbonyl cluster compound is conjugated or attached to an analyte-binding molecule.
10. Method according to claim 9, wherein the analyte-binding molecule is A1AT antibody.
11. Method according to claim 1, wherein the method is a multiplex method for detecting more than one analyte.

12. Biosensor for the detection of an analyte using surface-enhanced Raman spectroscopy, the biosensor comprising at least one metal carbonyl cluster compound covalently attached to a SERS-active substrate, the at least one metal carbonyl cluster compound having general formula (I)

5



wherein

10

M at each occurrence denotes a metal selected from Group 6 to Group 11 of the Periodic Table of Elements;

x is an integer from 10 to 12; and

each L is independently selected from the group consisting of -H, -SH and -A-(CH₂)_n-COOH, wherein A is selected from the group consisting of S, O, C and N; and n is an integer from 1 to 10.

15

13. Biosensor according to claim 12, wherein M is Os.

14. Biosensor according to claim 12, wherein A is S.

20

15. Biosensor according to claim 12, wherein the metal carbonyl compound is Os₃(CO)₁₀(μ-H)[μ-S(CH₂)₁₀COOH].

25

16. Biosensor according to claim 12, wherein the SERS-active substrate comprises a metallic bilayer deposited on a plurality of nanospheres which are attached on a support.

17. Biosensor according to claim 12, wherein the metal carbonyl cluster compound is conjugated or attached to an analyte-binding molecule.

30

18. Biosensor according to claim 17, wherein the analyte-binding molecule is A1AT antibody.

19. Method of manufacturing a biosensor comprising at least one metal carbonyl cluster compound covalently attached to a SERS-active substrate, the at least one metal carbonyl cluster compound having general formula (I)



wherein

M at each occurrence denotes a metal selected from Group 6 to Group 11 of the Periodic Table of Elements;

x is an integer from 10 to 12; and

each L is independently selected from the group consisting of -H, -SH and -A-(CH₂)_n-COOH, wherein A is selected from the group consisting of S, O, C and N; and n is an integer from 1 to 10,

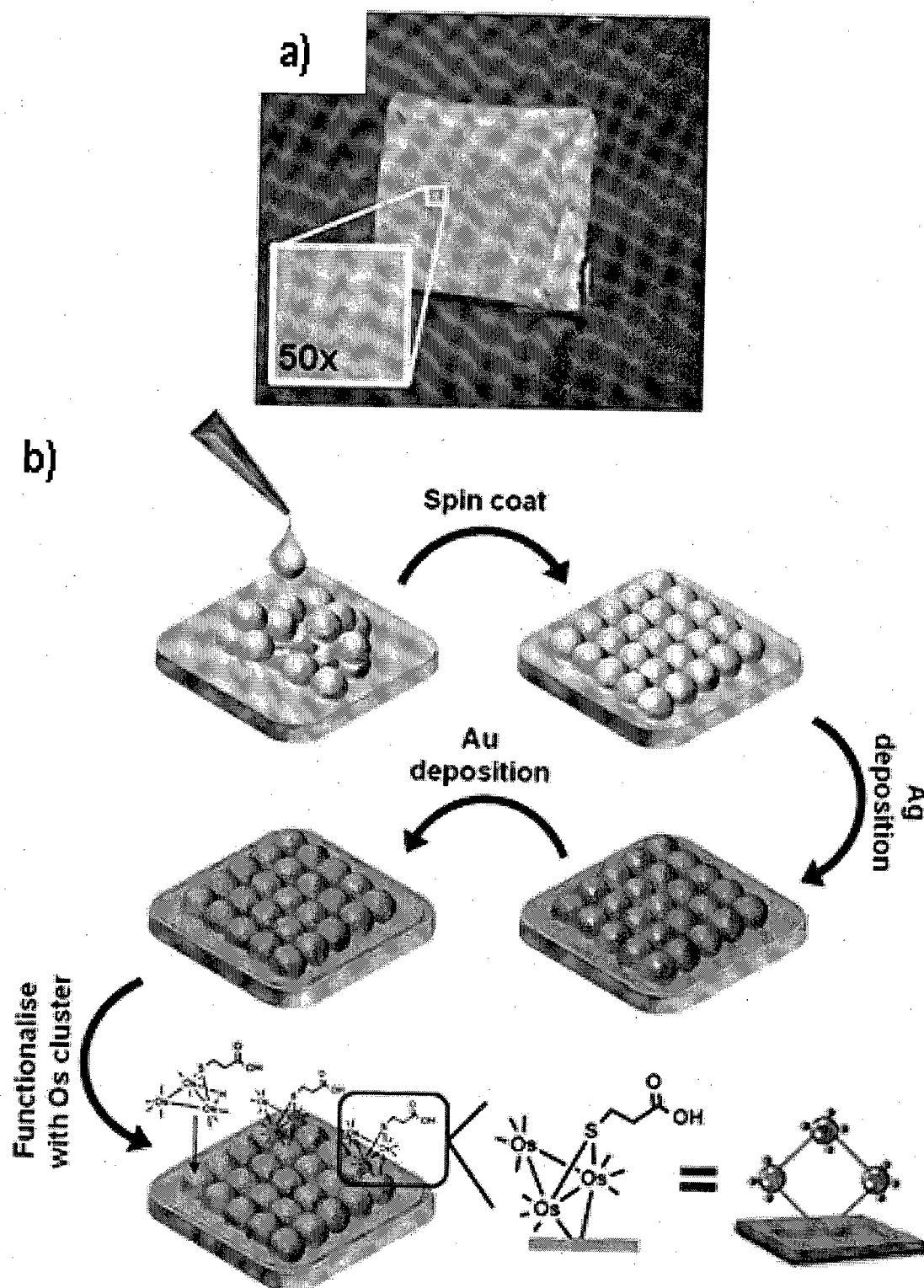
the method comprising

- a) attaching a plurality of nanospheres on a support;
- b) depositing a first metallic layer on the plurality of nanospheres;
- c) depositing a second metallic layer on the first metallic layer to form a metallic bilayer; and
- d) covalently attaching the at least one metal carbonyl cluster compound to the metallic bilayer to form the biosensor.

20. Method according to claim 19, wherein the metal carbonyl compound is Os₃(CO)₁₀(μ-H)[μ-S(CH₂)₁₀COOH].

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FIG. 1



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FIG. 1 (Cont.)

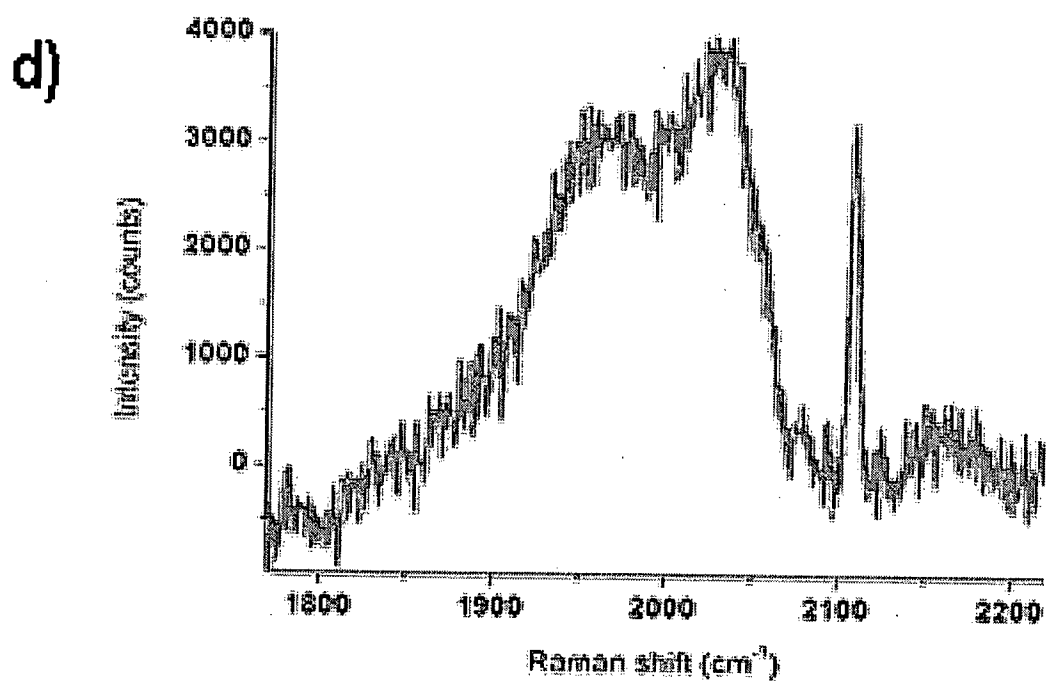
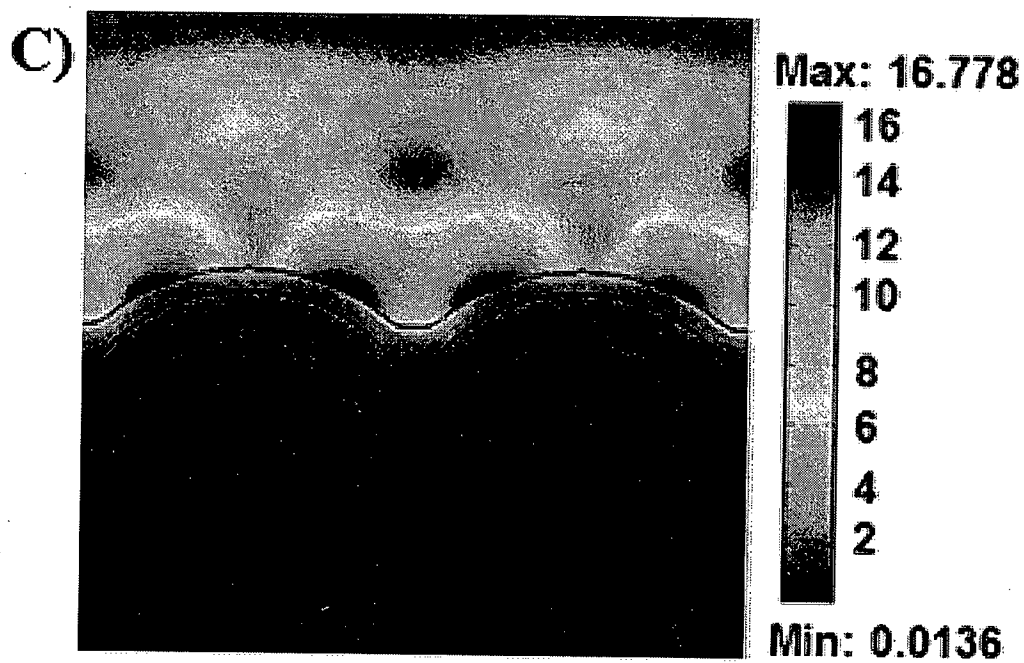


FIG. 2

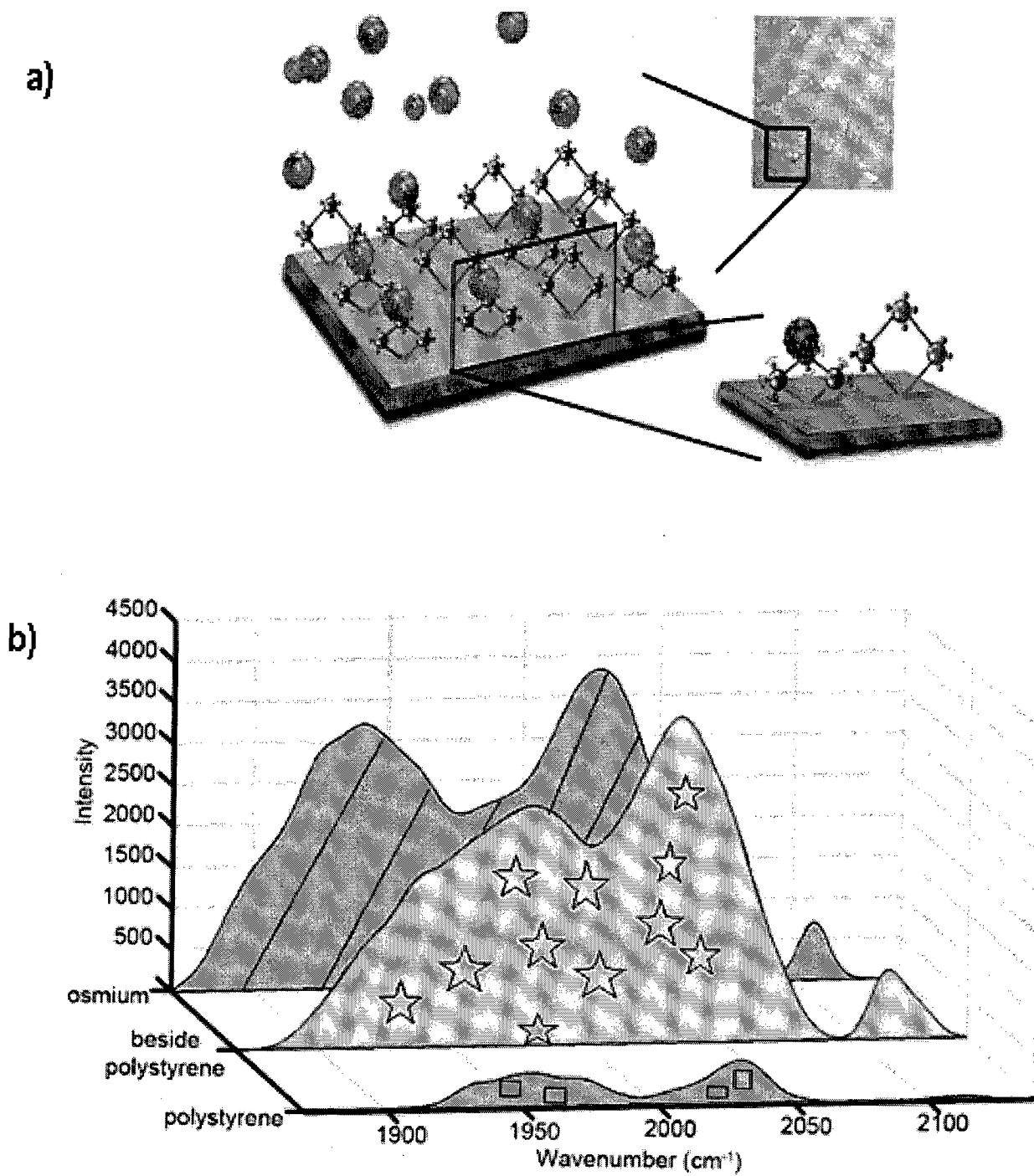


FIG. 2 (Cont.)

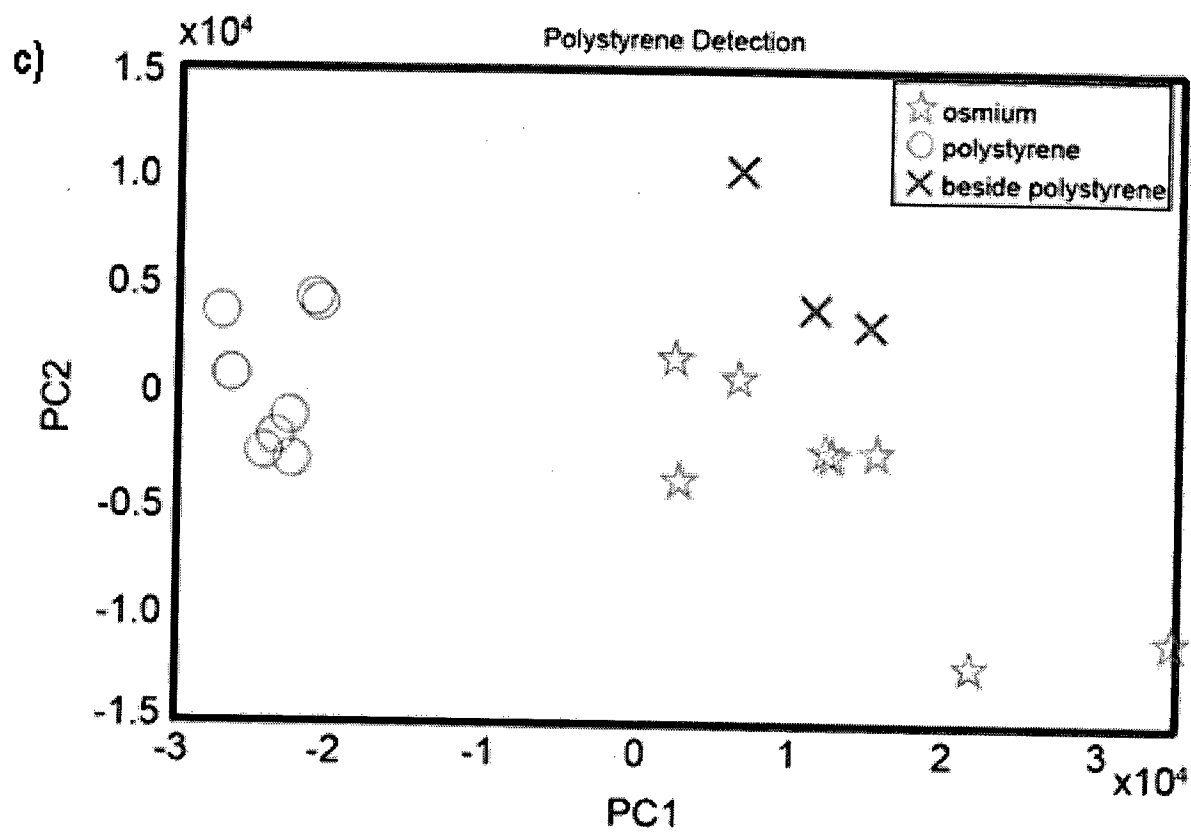


FIG. 3

(A)

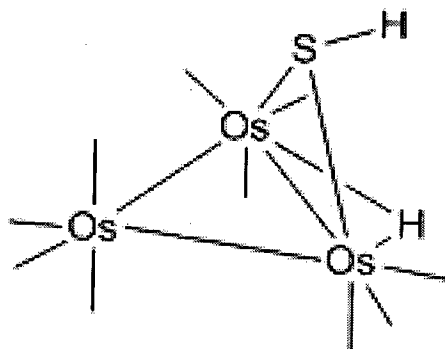
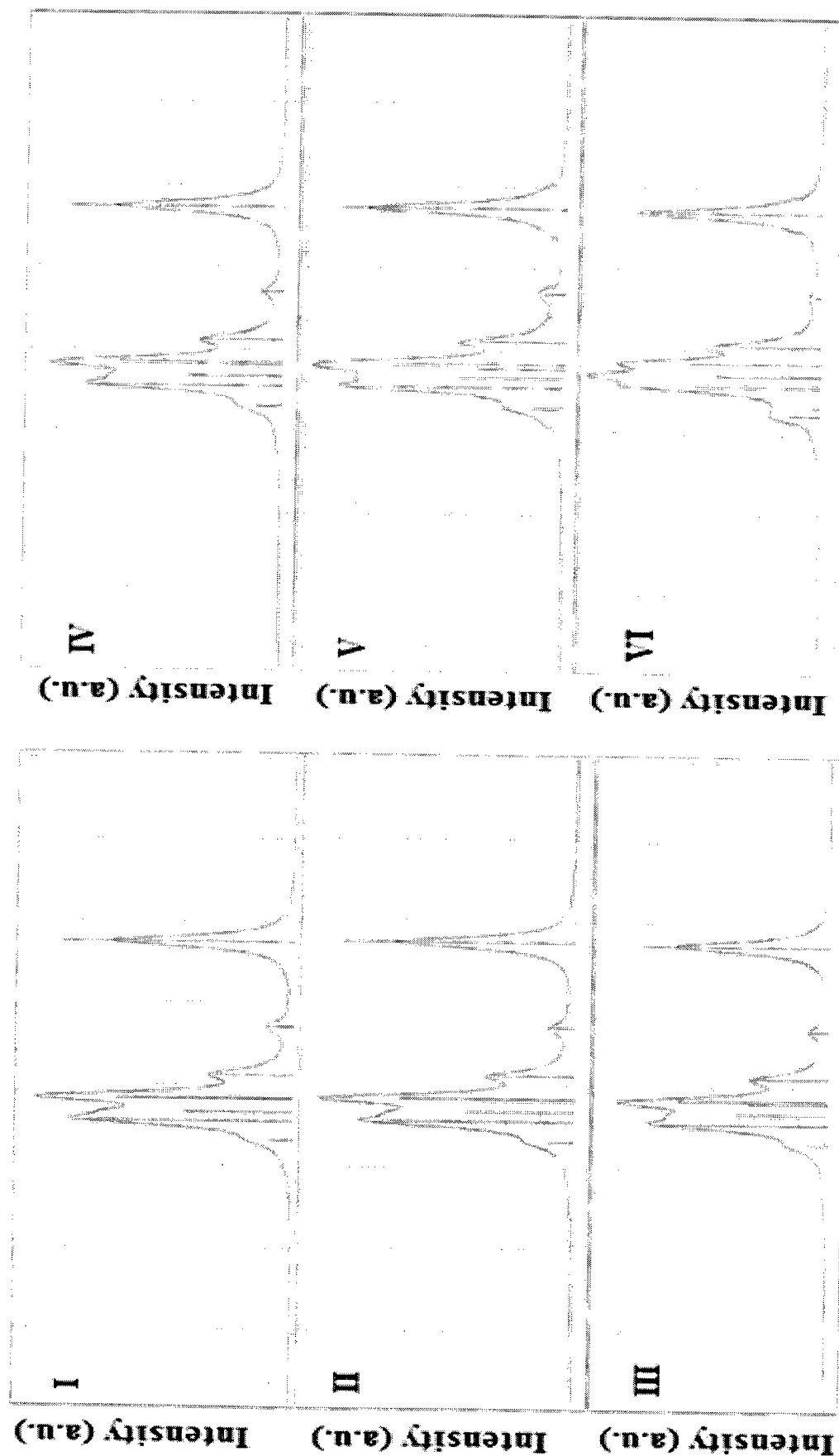


FIG. 3 (Cont.)

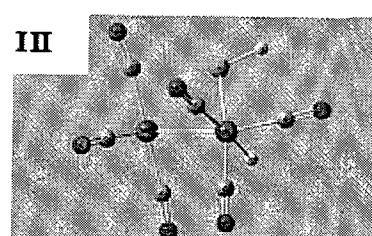
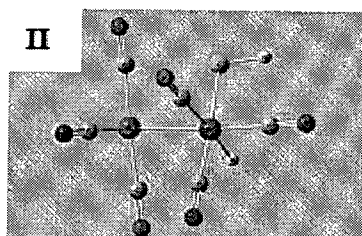
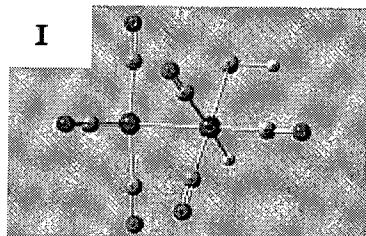
B)



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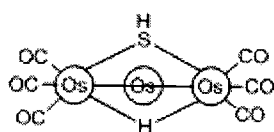
FIG. 3 (Cont.)

c)

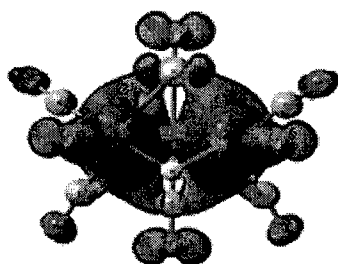


(d)

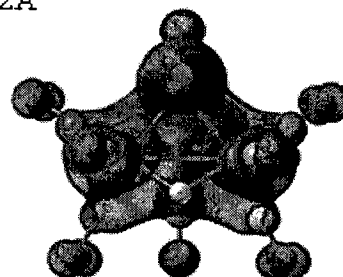
Front view



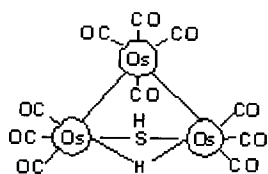
1A



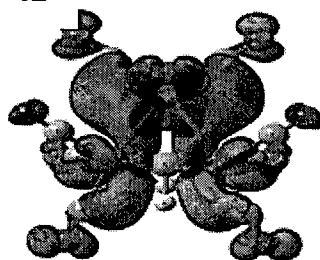
2A



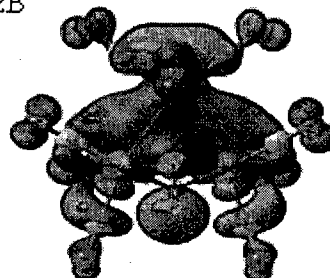
Top view



1B



2B



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FIG. 4

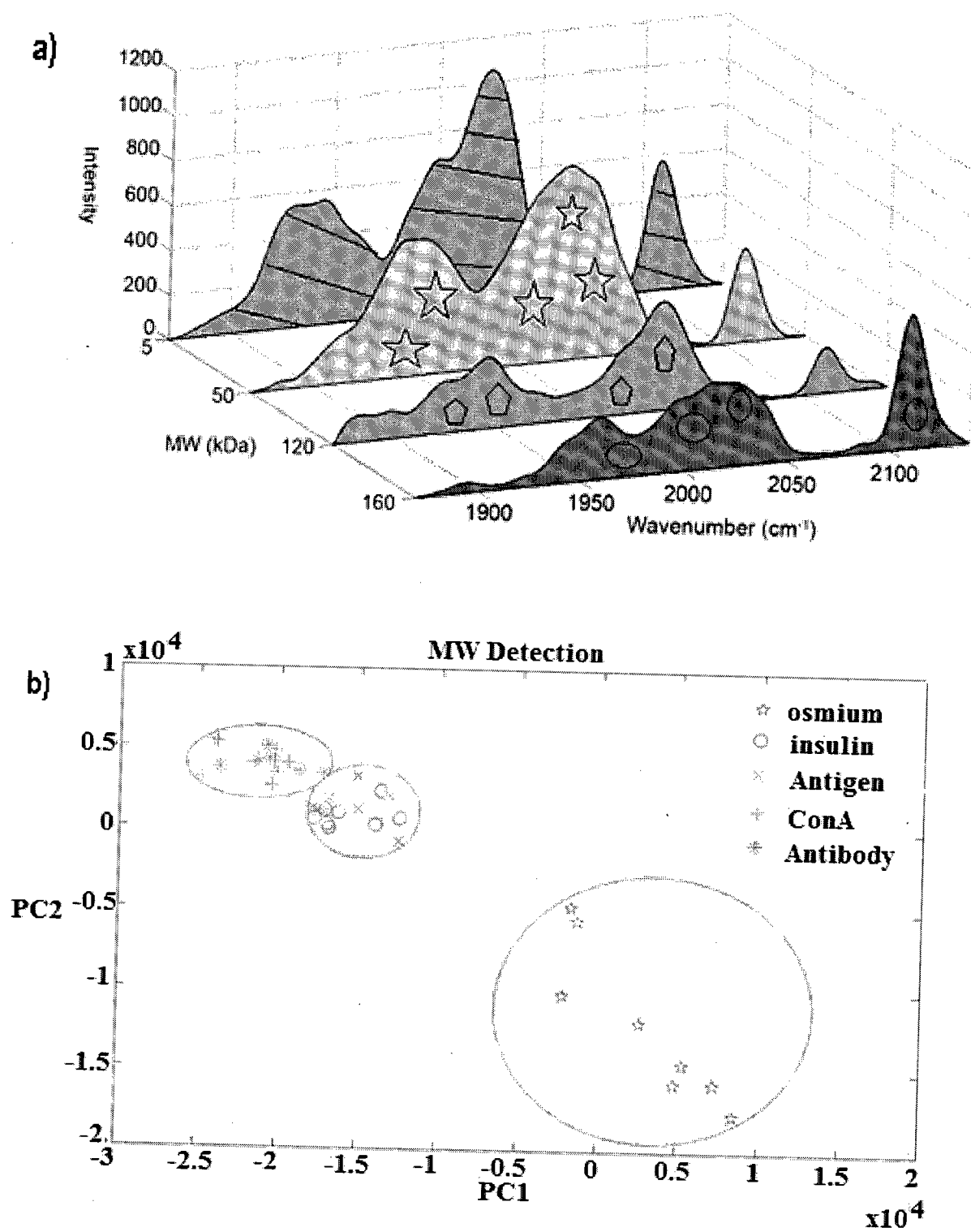
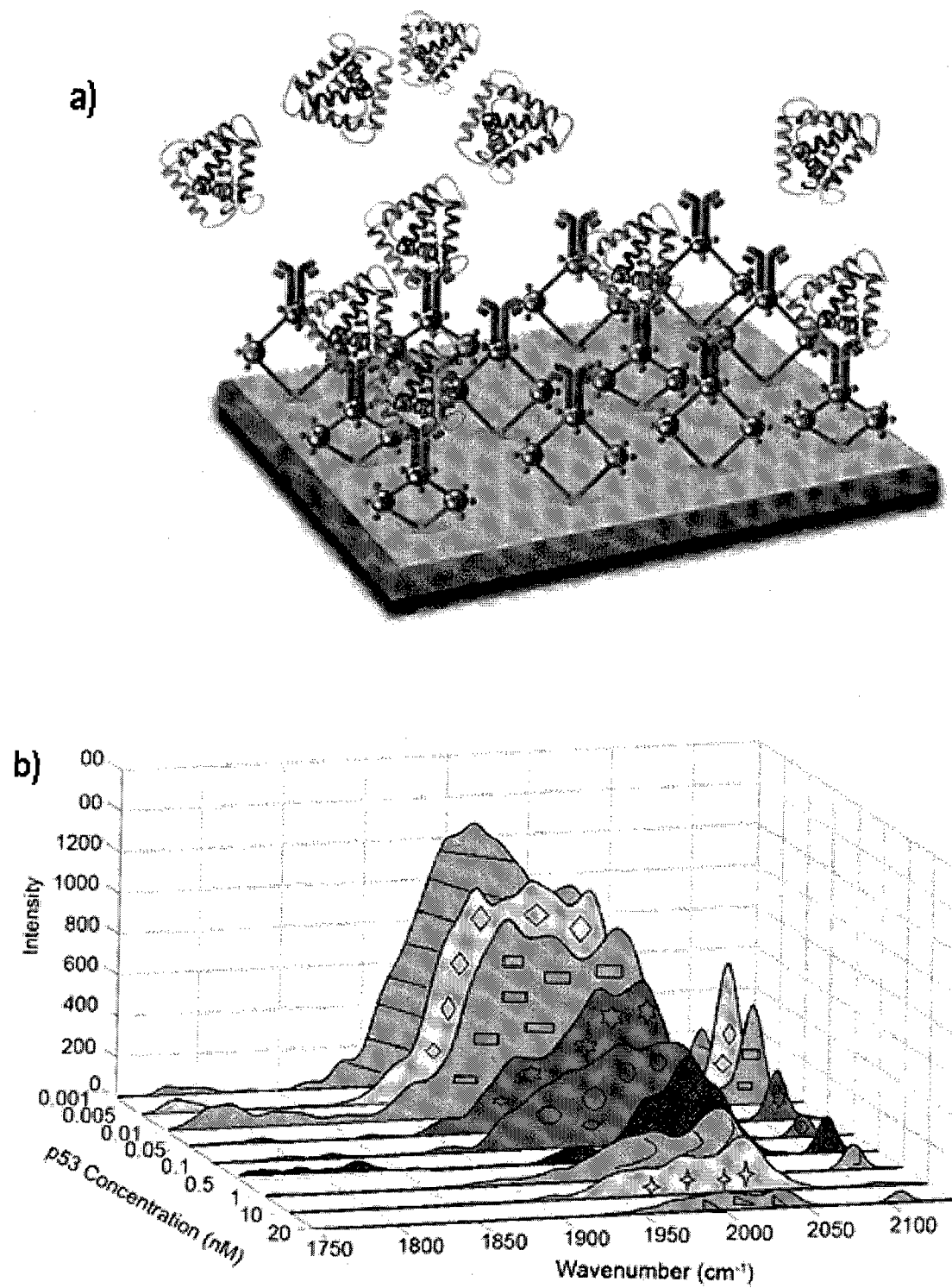


FIG. 5



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FIG. 5 (Cont.)

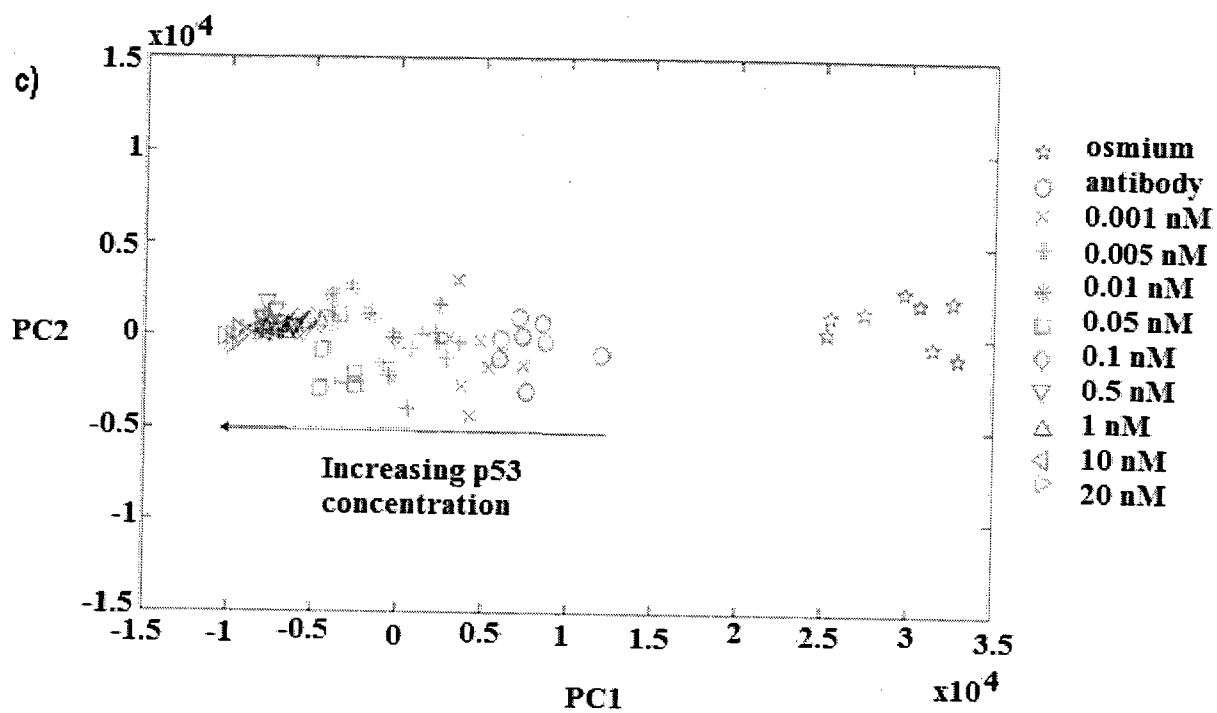


FIG. 6

(A)

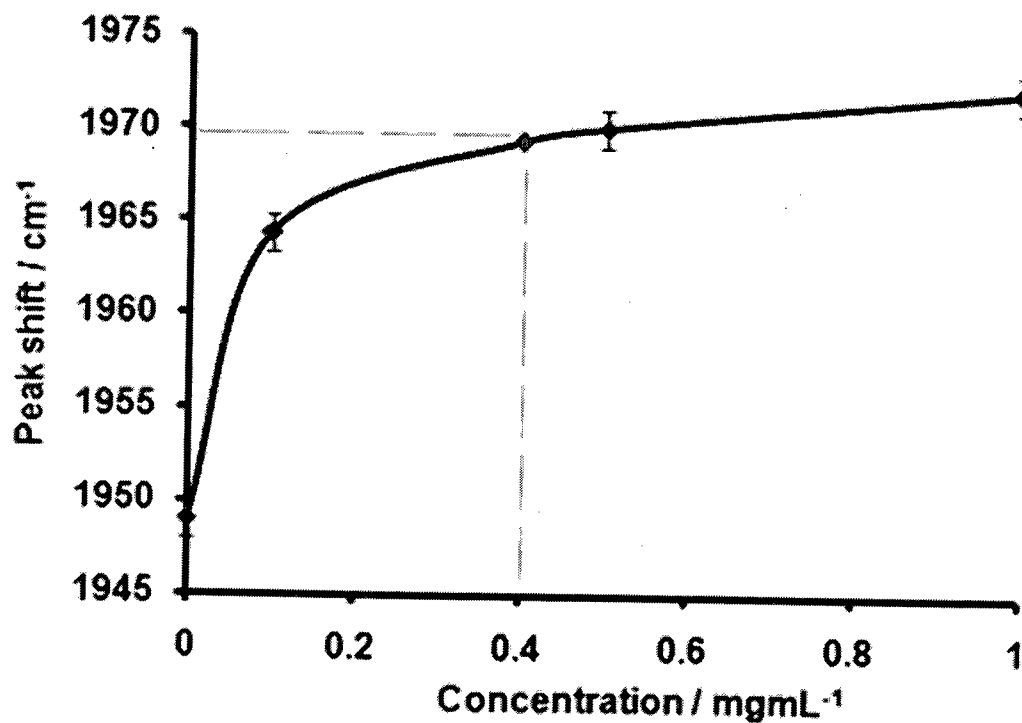
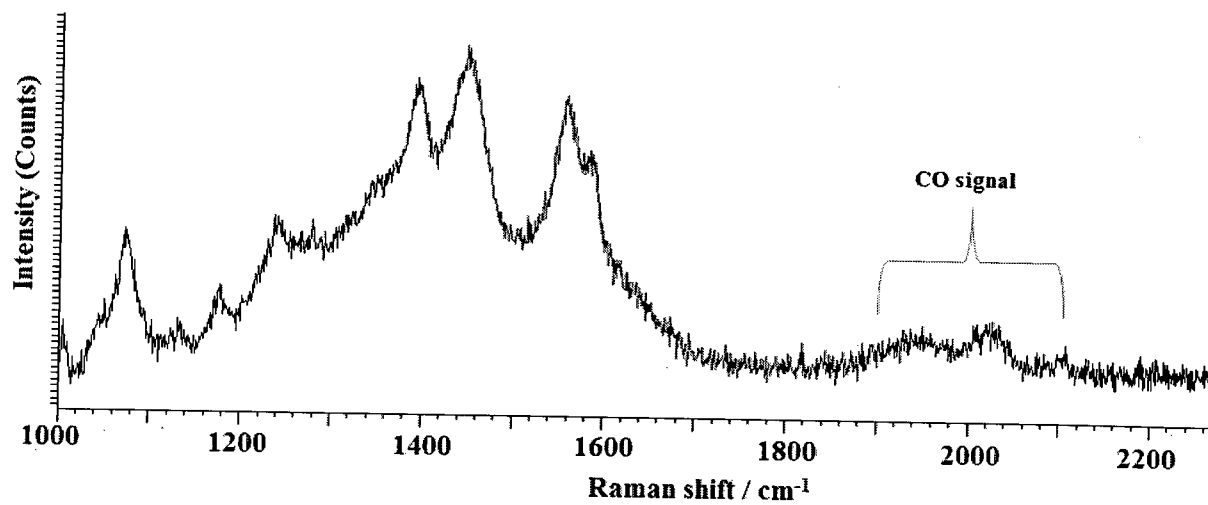


FIG. 6 (Cont.)

(B)



INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2014/000105

A. CLASSIFICATION OF SUBJECT MATTER

G01N 21/65 (2006.01) B82Y 15/00 (2011.01) G01J 3/44 (2006.01) C01G 55/00 (2006.01)

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)

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)

EPODOC, DWPI, INSPEC, GOOGLE: (OR G01J3/ic/cc, G01N21/ic/cc, A61B5/ic/cc, B82B3/ic/cc); (OR ((SURFACE_ENHANC+) 3W RAMAN), SERS, G01J3/44/IC/CC, G01N21/65/IC/CC, B82Y5/CC); (OR (SUBSTRATE? 2D ACTIV+), IMMOBILI[Z,S]+, REPORTER?, ANALYTE_BIND+, ANTIBOD+, RECEPTOR?, MARKER?); (OR (METAL 3W CARBONYL+), +CARBONYL+)); ((OR OS, +OSMIUM+, OS3, (A1AT 2W ANTIBOD+)) and similar terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "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) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed		"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
Date of the actual completion of the international search 20 May 2014	Date of mailing of the international search report 20 May 2014	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au Facsimile No.: +61 2 6283 7999		Authorised officer Firoozeh Rabbani AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262832287

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2014/000105
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KONG KV, et. al.: "Metal carbonyl-gold nanoparticle conjugates for live-cell SERS imaging". Angew. Chem. Int. Ed. Engl. 51 (39), (2012), pg 9796–9799 Refer to the whole document and in particular to Fig. 1 and description at pg. 7978 to 7979; Fig. 5; Fig. 6(b)	1-3, 6-7, 9, 12-13, 16-17, 19
Y	Refer to the whole document and in particular to Fig. 1 and description at pg. 7978 to 7979; Fig. 5; Fig. 6(b)	4-5, 8, 10, 11, 14-15, 18, 20
Y	LI, C. et al.: "Osmium Carbonyl Clusters on Gold and Silver Nanoparticles as Models for Studying the Interaction with the Metallic Surface", J. Phys. Chem. C, Vol. 113, (2009), pg 18562-18569 Refer to the whole document and in particular to abstract; figure 2; experimental section 2.3; results and discussion section 3.1-3.4	4-5, 8, 10, 11, 14-15, 18, 20
A	P. R. SAJANLAL et. al.: "Bimetallic Mesoflowers: Region-Specific Overgrowth and Substrate Dependent Surface-Enhanced Raman Scattering at Single Particle Level", Langmuir, 26 (11), (2010), 8901–8907 Refer to the whole document and in particular to pg 8902	19
P,X	WO 2013/180652 A1 (AGENCY FOR SCIENCE, TECHNOLOGY AND RESEARCH) 05 December 2013 Refer to the whole document and in particular to para [0023]-[0025]; [0055]-[0056]; [0067]-[0069]; [0083], [0086]-[0087]	1-3, 7-9, 11-13, 17

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/SG2014/000105	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2013/180652 A1	05 Dec 2013	None	
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			