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(54) **Title**: SERS-BASED ANALYTE DETECTION

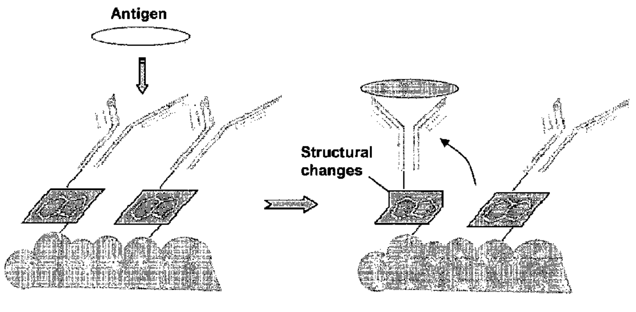


Figure 1 (b)

(57) **Abstract**: The present invention relates to method detecting analytes by surface enhanced Raman spectroscopy (SERS), comprising contacting the analytes with at least one analyte binding molecule attached to a metal substrate surface that enhances Raman scattering via a Raman-active molecular linker; and detecting a surface enhanced Raman signal from said compound. In a further aspect, this invention relates to a conjugate and a biosensor suitable for the invented SERS-based analyte detection method.

SERS-Based Analyte Detection

Cross-Reference to Related Applications

This application claims the benefit of priority of US provisional application No. 61/289,053, filed 22 December 2009, the content of it being hereby incorporated by reference in its entirety for all purposes.

Field of the invention

The present invention lies in the field of spectroscopy and molecular diagnostics and relates to method detecting analytes by surface enhanced Raman spectroscopy (SERS) by spectroscopically detecting antigen/antibody binding events. In particular, the invention is directed to a method for the detection of analytes using surface enhanced Raman spectroscopy (SERS), comprising contacting the analytes with at least one analyte binding molecule attached to a metal substrate surface that enhances Raman scattering via a Raman-active molecular linker; and detecting a surface enhanced Raman signal from said compound. In a further aspect, this invention relates to a conjugate and a biosensor suitable for the invented SERS-based analyte detection method.

Background

In medical practice, identification of a disease requires not just recognition of the symptoms but also detecting specific features that would unambiguously indicate its presence. Furthermore, an early detection in asymptomatic populations is of utmost importance not only to facilitate early treatment but also to reduce health-care costs.

Usually, screening for signs of disease developments, biomarkers, is only conceivable through an analysis of biological fluids, such as blood, urine and cerebral spinal fluid, for circulating disease-related biomarkers. An accurate diagnostic can rarely be accomplished through the detection of just one single biomarker and a panel of markers has to be analyzed for a reliable results, such as in a multiplexed assay. Furthermore, monitoring the expression patterns of a variety of biomarkers at various stages of a disease could not only assist prognosis, but also allow one to follow disease progression.

Today, most protein biomarker assays are based on immunoassays. These usually provide a platform, made of either polymer or glass, bearing several immobilized antibodies spotted on different well-defined locations. 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 the specificity of the assay results. Detection is usually via fluorescence detection, chromophoric absorption or a colorimetric readout. Importantly, conventional immunoassays (i.e. ELISA and fluorescent immunoassay) have limited expandability in terms of the number of proteins that can be detected per assay. This is attributed to the limited number of sensing area that can be incorporated within a single assay platform, due to the minimum laser spot-size achievable in the read-out system because of diffraction-limit, which impose a lower-limit to the useful size of a sensing area to a value not smaller than 200 nm, though in practice the size is usually in the range of 1 μm. Although one may argue that it is possible to modify a fluorescent immunoassay to allow multiple analytes (i.e. proteins/biomarkers) to be simultaneously detected by incorporating more than one fluorophore into each sensing area - for example, by expanding the number of protein-capturing fluorescent beacons used per sensing area, the broad fluorescence bandwidths (60 – 90 nm) unfortunately limit the maximum number of detectable fluorophores per sensing area to about 3. In other words, the maximum number of proteins detectable for each sensing area in a conventional immunoassay cannot exceed 3. Although, many immunosensor arrays have been developed in recent years, a truly rapid, accurate and miniaturizable system is still non-existing.

Vibrational spectroscopic techniques namely infra red (IR), normal Raman and Surface Enhanced Raman (SER) have been considered for analyte detection. Since near IR and mid IR technique suffers with the limitation of competing absorption from aqueous media, Raman spectroscopic techniques have evolved as the methods of choice. One important aspect of the Raman scattering is the correlation between the amount of the frequency shifts and the vibrational modes of the molecules. Since vibrational modes are sensitive to the chemical nature of the molecule, probing molecular vibrations can thus reveal information regarding its chemical geometry and interaction with other molecules. While a plethora of techniques, such as nuclear magnetic resonance (NMR) and X-ray crystallography, can also provide access to chemical structures, optical measurements of vibrational states via Raman scattering offer, owing to the ease of sample preparation, a much more convenient approach,. For this reason, the Raman spectrum, which is unique to each molecule, has been utilized as a "fingerprint" in identifying unknown species, and in a more interesting aspect, Raman scattering is utilized for elucidating conformational changes.

However, under biological conditions the applications have been limited mainly due to the poor sensitivity and the need for high laser power and complicated instrumentation. Most of these drawbacks were overcome by the development of Surface Enhanced Raman spectroscopy (SERS) where the spectral intensity is enhanced tremendously by the interaction of the SERS active analyte molecules with a substrate surface, e.g., a nanoparticle surface of copper, gold or silver. There are many cases where these enhancement factors are up to the level of single molecule detection (Nicholas & Ricardo, *Chem. Soc. Rev.*, **2008**, *37*, 946–954). However, the detection of molecules with such extraordinary sensitivity still depends on the properties of the molecule-nanoparticle ensemble and is currently limited to certain classes of SERS active molecules.

Summary of the Invention

The present invention allows the expansion of an SERS-based detection to a highly multiplexed system capable of detecting multiple proteins per sensing area. In contrast to immunoassays, no secondary or tertiary antibodies are required for the detection, hence minimizing material wastage. Furthermore, the invention requires neither colored substance nor extrinsic labeler as it is based on the detection of a molecular fingerprint.

In an additional aspect, the inventive assay design does not require multiple washing steps. In fact, no or only one washing step is sufficient to achieve detection

In a first aspect, the present invention thus relates to a method for detecting one or more analytes using surface enhanced Raman spectroscopy (SERS), the method including:

- contacting the one or more analytes with at least one analyte binding molecule attached to a metal substrate surface that enhances Raman scattering via a Raman-active molecular linker; and
- detecting a surface enhanced Raman signal from said compound.

In various embodiments of this method the surface enhanced Raman signal of the compound is correlated with the amount of the analytes. The analytes may be contained in a sample and the detection may be *in vitro*. In one embodiment of the invented method, the analytes are detected in a bodily fluid comprising said analyte. The bodily fluid may be selected from the group consisting of plasma, serum, blood, lymph, liquor and urine.

In various embodiments of the claimed methods, the analyte is a protein, peptide, nucleic acid, carbohydrate, lipid, cell, virus, small molecule, or hapten.

In one embodiment, the analyte binding molecule specifically binds the analyte. The analyte binding molecule may be selected from the group consisting of an antibody, antibody fragment or antibody like molecules. If the analyte binding molecule is an antibody, the antibody may be a monoclonal or polyclonal antibody.

The method may also be a multiplex method for detecting more than one analyte, wherein in the contacting step more than one analyte binding molecules are used.

In various embodiments of the method, the analyte binding molecule is covalently coupled to a Raman-active molecular linker that is attached to the substrate surface via covalent interactions. The Raman-active molecular linker compound may be selected from the group consisting of 6-Mercaptopurine, 8-Aza-adenine, N-Benzoyladenine, 2-

Mercapto-benzimidazole, 4-Amino-pyrazole[3,4-d]pyrimidine, Zeatin, Methylene Blue, 9-Amino-acridine, Ethidium Bromide, Bismarck Brown Y, N-Benzyl-aminopurine, Thionin acetate, 3,6-Diaminoacridine, 6-Cyanopurine, 4-Amino-5-imidazole-carboxamidehydrochloride, 1,3-Diiminoisoindoline, Rhodamine 6G, Crystal Violet, Basic Fuchsin, Aniline Blue Diammonium salt, N-[(3-(Anilinomethylene)-2-chloro-1-cyclohexen-1-yl)methylene]anilinemonohydrochloride, O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate, 9-Aminofluorene hydrochloride, Basic Blue, 1,8-Diamino-4,5-dihydroxyanthraquinone, Proflavine hemisulfate salt hydrate, 2-Amino-1,1,3-propenetricarbonitrile, Variamine Blue RT salt, 4,5,6-Triaminopyrimidine sulfate salt, 2-Amino-benzothiazole, Melamine, 3-(3-Pyridylmethylamino)Propionitrile, Silver(I) Sulfadiazine, Acriflavine, 4-Amino-6-mercaptopyrazole[3,4-d]pyrimidine, 2-Aminopurine, Adenine Thiol FAD Fluoroadenine, 4-Amino-6-mercapyopyrazole[3,4-d]pyrimidine, Rhodamine 110, Adenine, 5-Amino-2-mercaptobenzimidazole, Acridine Orange Hydrochloride, Cresyl Violate Acetate, Acriflavine Neutral, Dimidium Bromide, 5,10,15,20-Tetrakis(N-methyl-4-pyridinio)porphyrin Tetra(p-toluenesulfonate), 5,10,15,20-Tetrakis(4-trimethylaminophenyl)porphyrin Tetra(p-toluenesulfonate), 3,5-Diaminoacridine Hydrochloride, Propidium Iodide (3,8-diamino-5-(3-diethylaminopropyl)-6-phenylphenanthridinium iodidemethiodide), Trans-4-[4-(dimethylamino)styryl]-1-methylpyridinium iodide, and 4-((4-(dimehtylamino)phenyl)azo)benzoic acid, succinimidyl ester or derivatives thereof. Preferably, the Raman-active molecular linker is a thiol-group containing compound. In one embodiment, the Raman-active linker molecule is 6-Mercaptopurine.

In various embodiments of the inventive methods, the analyte binding molecule is covalently coupled to the Raman-active molecular linker by amide bond formation. For this coupling a carbodiimide, such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, can be used as a coupling agent.

In various embodiments, the metal substrate surface is made of a noble metal or copper. The noble metal may be selected from the group consisting of silver and gold.

In some embodiments, the substrate is a nanoparticle. The nanoparticle may be coated with or consisting of a noble metal. The noble metal can, for example, be selected from gold and silver. In one specific embodiment, the nanoparticle is coated with a silver film. In another specific embodiment, the nanoparticle is a citrate-stabilized gold nanoparticle.

In another aspect, the present invention relates to a conjugate for the detection of an analyte using surface-enhanced Raman spectroscopy comprising an analyte binding molecule, a Raman-active linker molecule and a metal substrate, wherein the analyte binding molecule is covalently coupled to the Raman-active linker molecule and the Raman-active linker molecule is covalently attached to the metal substrate. In the conjugate, the analyte binding molecule and/or the Raman-active linker molecule and/or the metal substrate can be as defined above in relation with the invented method.

In still another aspect, the invention is directed to a biosensor for the detection of an analyte using surface-enhanced Raman spectroscopy, comprising one or more conjugates according to the invention. The biosensor may further comprise a substrate, wherein the nanoparticles are adherent to the substrate. In various embodiments, the biosensor is configured for *in vivo* and/or *in vitro* use. The analyte may be a protein, peptide, nucleic acid, carbohydrate, lipid, cell, virus, small molecule, or hapten.

In a still further aspect, the invention relates to the use of the biosensor of the invention for the detection of an analyte. The detection may be *in vivo* or *in vitro*.

Brief Description of the Drawings

Figure 1 schematically illustrates the principle of the SERS-based nanoscale sensor.

Figure 2 schematically illustrates the principle of a multiplexed SERS-based nanoscale sensor.

Figure 3 is a schematic drawing of the experimental setup for SERS measurements.

Figure 4 shows SERS spectra derived from a sensor according to the invention using 6-mercaptopurine (6-MP) as a Raman-active linker molecule.

Figure 5 shows response curves of the SERS-based sensor versus antigen concentrations.

Figure 6 shows spectra of clean substrate taken at 100% power, 10s acquisition and 1 accumulation. The three curves represent the spectra of three measurements A, B and C.

Figure 7 shows spectra of 6-MP coated Au IME substrate taken at 10% power, 30s acquisition and 2 accumulations. The three curves represent the spectra of three measurements A, B and C.

Figure 8 shows spectra of 6-MP-anti-p53 coated Au IME substrate taken at 10% power, 30s acquisition and 2 accumulations. The three curves represent the spectra of three measurements A, B and C.

Figure 9 shows spectra of 6-MP-anti-p53 coated Au IME substrate in presence of p53 taken at 10% power, 30s acquisition and 2 accumulations. The three curves represent the spectra of three measurements A, B and C.

Figure 10 shows spectra of 6-MP-anti-p53 coated Au IME substrate after rinsing taken at 10% power, 30s acquisition and 2 accumulations. The three curves represent the spectra of three measurements A, B and C.

Figure 11 shows a stacking of all "A" spectra of Figures 7, 8, 9 and 10. No new peaks are observed upon addition of anti-p53 and p53.

Figure 12 shows the individual spectra of the anti-p53 conjugated biosensor of measurements a) A, b) B and c) C.

Figure 13 shows the individual spectra of the anti-p53 conjugated and p53 incubated biosensor of measurements a) A, b) B and c) C.

Figure 14 shows the individual spectra of the anti-p53 conjugated and p53 incubated biosensor of measurements a) A, b) B and c) C after rinsing the biosensor with PBS.

Figure 15 shows the spectra of Figures 12, 13 and 14 superimposed.

Detailed Description

When a molecule interacts with a monochromatic light carrying a photon-energy less than its first electronic transition, two optical processes can occur. In the first, and the dominant, process, a large portion of the incident light is elastically scattered with no photon energy being absorbed; this is known as the Rayleigh scattering. A second, and the relatively weaker, process involves the adsorption of a small amount of the incident photon energy by the molecular system that then undergoes a transition from a one vibrational state to another, followed by a subsequent re-emission of light at a frequency "shifted" from that of the incidence, and such an optical effect is conventionally known as Raman scattering.

One important aspect of the Raman scattering is the correlation between the amount of the frequency shifts and the vibrational modes of the molecules - here, vibrational modes refer to the "manner" in which the molecule vibrates. Since vibrational modes are sensitive to the chemical nature of the molecule, probing molecular vibrations can thus reveal information regarding its chemical geometry. While a plethora of techniques, such as the nuclei magnetic resonance (NMR) and X-ray crystallography, can also provide access to chemical structures, optical measurements of vibrational states via Raman scattering offer a much more convenient approach, owing to the ease of sample preparation (Garman E & Sweet RM, *Methods Mol. Biol.*, **2007**, 364, 63-94; Chen J & Brooks CL, *Prot.*, **2007**, 67(4), 922-930). For this reason, the Raman spectrum, which is unique to each molecule, was utilized as a "fingerprint" in identifying unknown species, and in a more interesting aspect, Raman scattering is utilized for elucidating conformational changes.

Despite of its high specificity, Raman spectroscopy has limited use due to poor efficiency of Raman scattering. It is estimated that only 1 in every 10^6-10^8 scattered photons is Raman-scattered and this results in a very weak Raman signal.

Surface-enhanced Raman spectroscopy (SERS) was first observed by Fleischman et al in 1974 when remarkably strong Raman signals were obtained for pyridine adsorbed on an electrochemically roughened silver electrode (Fleischman M et al., *Chem. Phys. Lett.*, **1974**, 26, 123). Two mechanisms have been widely accepted for bringing about this enhancement in Raman scattering (which can be as high as 10^{14} times the unenhanced signal) (Kneipp K et al., *Chem. Rev.*, **1999**, 99(10), 2957-2976). They are electromagnetic enhancement and chemical enhancement.

Electromagnetic enhancement accounts for the majority of the enhancement (factor of 10^4-10^7) and arises from the interaction between the analyte that is absorbed or brought in close proximity to the metal surface and the surface plasmon fields excited in the metal by a laser beam (Moskovits M, *J. Raman Spectro.*, **2005**, 36(6-7), 485-496). Conduction electrons that reside on the surface of a metal exhibit lateral freedom of motion as they are constricted only by the positive charges on the 'bulk' metal side. When light interacts with these electrons, they oscillate collectively and this oscillation is known as surface plasmon. On a roughened surface, the oscillations are localized and perpendicular to the surface plane, generating a locally amplified electromagnetic fields responsible for the SERS effect.

The localized surface plasmons (LSP) have a resonant frequency at which the absorption and scattering of light occurs most efficiently. This frequency is dependent upon the metal and the nature of the surface (size, roughness, shape, interparticle spacing and dielectric environment) (Kelly KL et al., *J. Phys. Chem. B*, 2003, 107(3), 668-677). This is of importance in the fabrication of SERS substrates as one may want to manipulate the resonant frequency to be close to the excitation frequency used to ensure maximal enhancements (Haynes CL & Van Duyne RP, *J. Phys. Chem. B* , **2003**, 107(30), 7426-7433).

Chemical enhancement is argued to contribute only an order of $10\text{-}10^2$ order to the overall enhancement (Liang EJ & Kiefer W, *J. Raman Spectro.*, **1996**, 27(12), 879-885). It involves electron coupling between the analyte and metal surface that changes the polarizability of the molecule and forming a surface species that act as resonant intermediates in the Raman scattering. A charge transfer mechanism between the analyte and metal has also been proposed.

A SERS substrate normally refers to a well-engineered metallic nanostructure on which the analytes are absorbed for SERS acquisitions. There are three classes of SERS substrates: roughened metal surfaces, colloidal metal nanoparticles, and planar metallic structures such as arrays of metal nanoparticles supported on a planar substrate like glass (Vo-Dinh T, *Trac-Trends. Anal. Chem.*, **1998**, 17(8-9), 557-582; Baker GA & Moore DS, *Anal. Bioanal. Chem.*, **2005**, 382(8), 1751-1770). As mentioned earlier, the LSP responsible for SERS enhancement is highly dependent upon the surface characteristics of the SERS substrate, making SERS a surface-sensitive technique.

As proteins are weak Raman scatterers their binding to a substrate cannot be easily detected using SERS. However, the inventors of the present invention have found that proteins can be detected using a SERS-based nanoscale stress sensor. In this setup, an analyte binding molecule, such as an antibody, is coupled to a substrate immobilized SERS active substance. By stressing, e.g. stretching and compressing the bonds of the SERS active substance to the substrate and/or antibody as a result of the analyte binding event detectable shifts in the SERS spectrum are induced. Surprisingly, this allows highly sensitive and specific detection of analyte binding.

Thus, in a first aspect, the present invention is directed to a method for detecting one or more analytes using surface enhanced Raman spectroscopy (SERS), comprising

- contacting the one or more analytes with at least one analyte binding molecule attached to a metal substrate surface that enhances Raman scattering via a Raman-active molecular linker; and
- detecting a surface enhanced Raman signal from said compound.

The terms “at least one” or “one or more” as used interchangeably herein in connection with molecules relates to 1, 2, 3 or more, for example at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 15, 20, 25 or a plurality of molecules. In this connection, the term “plurality” means more than two, preferably 3-100.

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. Preferably, this binding is specific so that a specific complex between analyte and analyte binding molecule is formed.

“Specifically binding" and "specific binding" as used herein mean that the analyte 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 of the invention, "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.

The analyte binding molecule may be 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 or fragments (e.g., Fab, $F(ab')_2$, scFv, Fv diabodies and linear antibodies), so long as they exhibit the desired binding activity.

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.

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 hybridoma technique of Koehler and Milstein (U. S. Patent No. 4,376,110), the human B-cell hybridoma technique, and the EBV-hybridoma technique. 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.

"Polyclonal antibodies" are 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.

Alternatively, techniques described for the production of single chain antibodies (U. S. Patent No. 4,946,778) can be used to produce suitable single chain antibodies. Single chain antibodies are typically formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragments that can be produced by pepsin digestion of the antibody molecule and the Fab fragments that can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

The analyte binding molecule may also be any other proteinaceous scaffold that has been adapted or mutated to bind a given ligand with sufficient binding affinity.

Examples of useful scaffolds include those scaffolds described in US patent application 2005/0089932 or US Patent 6,682,736. Another example of suitable scaffolds are members of the lipocalin protein family as described in the international patent applications WO 99/16873, WO 00/75308, WO 03/029471, WO 03/029462, WO 03/029463, WO 2005/019254, WO 2005/019255 or WO 2005/019256, for instance.

In accordance with the above, scaffolds besides members of the lipocalin family include, but are not limited to, a EGF-like domain, a Kringle-domain, a fibronectin type

I domain, a fibronectin type II domain, a fibronectin type III domain, a PAN domain, a G1a domain, a SRCR domain, a Kunitz/Bovine pancreatic trypsin inhibitor domain, tendamistat, a Kazal-type serine protease inhibitor domain, a Trefoil (P-type) domain, a von Willebrand factor type C domain, an Anaphylatoxin-like domain, a CUB domain, a thyroglobulin type I repeat, LDL-receptor class A domain, a Sushi domain, a Link domain, a Thrombospondin type I domain, an immunoglobulin domain or a an immunoglobulin-like domain (for example, domain antibodies or camel heavy chain antibodies), a C-type lectin domain, a MAM domain, a von Willebrand factor type A domain, a Somatomedin B domain, a WAP-type four disulfide core domain, a F5/8 type C domain, a Hemopexin domain, an SH2 domain, an SH3 domain, a Laminin-type EGF-like domain, a C2 domain, Kappabodies, Minibodies, Janusins, a nanobody, a adnectin, a tetranectin, a microbody, an affilin, an affibody or an ankyrin, a crystallin, a knottin, ubiquitin, a zinc-finger protein, an ankyrin or ankyrin repeat protein or a leucine-rich repeat protein, an avimer; as well as multivalent avimer proteins evolved by exon shuffling of a family of human receptor domains.

As mentioned above, in certain embodiments of the invention the analyte binding molecule may be a mutein of the member of the lipocalin protein family. In some of these embodiments, the open end of the β-barrel structure of the lipocalin fold (which encompasses the natural ligand binding site of the lipocalin family) is used to form the target analyte binding site. Members of the lipocalin family of proteins include, but are not limited to the bilin binding protein of *Pieris brassicae* (SWISS-PROT Data Bank Accession Number P09464), human tear lipocalin (SWISS-PROT Data Bank Accession Number M90424), human apolipoprotein D (SWISS-PROT Data Bank Accession Number P05090), the retinol binding protein (RBP) (for example of human or porcine origin, SWISS-PROT Data Bank Accession Number of the human RBP: P02753, SWISS-PROT Data Bank Accession Number of the porcine RBP P27485), human neutrophil gelatinase-associated lipocalin (hNGAL, SWISS-PROT Data Bank Accession Number P80188), rat α_2-microglobulin-related protein (A2m, (SWISS-PROT Data Bank Accession Number P31052), and mouse 24p3/uterocalin (24p3, (SWISS-PROT Data Bank Accession Number P11672), Von Ebners gland protein 2 of *Rattus norvegicus* (VEG protein 2; SWISS-PROT Data Bank Accession Number P41244), Von Ebners gland protein 2 of *Sus scrofra* (pig) (LCN1; SWISS-PROT Data Bank Accession Number P53715), the Major allergen Can fl precursor of dog (ALL 1, SWISS-PROT Data Bank Accession Number O18873), and insecticyanin A or insecticyanin B of the tobacco hawkmoth *Manducta sexta* (SWISS-PROT Data Bank Accession Number P00305 and Q00630, respectively).

The analyte binding molecule may also be a binding protein, receptor or extracellular domain (ECD) thereof capable of forming a binding complex with a ligand, typically a polypeptide or glycopeptide ligand.

Those skilled in the art will recognized that the non-limiting examples given above describing various forms of antibodies as analyte binding molecules can also be extended to other proteinaceous receptors such as recombinant, chimeric, hybrid, truncated etc., forms of non-antibody receptors.

The analyte-binding molecule can also be a non-proteinaceous receptor, such as for example a nucleic acid based molecule, such as an Aptamer or Spiegelmer (Aptamer made of L-ribonucleotides).

In case the analyte binding molecule is a proteinaceous molecule it can be covalently coupled to the Raman-active molecular linker by amide bond formation. This covalent coupling can be achieved by carbodiimide coupling, for example using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as a coupling agent. The coupling can be via the N- or C-terminus or via one or more side chains of the amino acids of the protein. Side chains that can be used for this covalent coupling include, but are not limited to lysine, histidine, cysteine, tyrosine, serine, threonine, aspartic acid and glutamic acid side chains.

The term “linker” or “linker molecule” refers to a Raman-active molecule that links the analyte binding molecule to the substrate surface and facilitates detection of an analyte specifically bound by the analyte binding molecule via a change in the SERS signal. The Raman-active molecule interacts with the substrate surface and thus provides for a

SERS signal. In principle any molecule that can generate a SERS signal upon interaction with a Raman-active surface and that produces a change in the SERS signal as a result of molecular stresses caused by analyte binding to the analyte binding protein can be used. The Raman-active molecular linker can be selected from a variety of known Raman-active compounds that include, but are not limited to 6-Mercaptopurine, 8-Aza-adenine, N-Benzoyladenine, 2-Mercapto-benzimidazole, 4-Amino-pyrazole[3,4-d]pyrimidine, Zeatin, Methylene Blue, 9-Amino-acridine, Ethidium Bromide, Bismarck Brown Y, N-Benzyl-aminopurine, Thionin acetate, 3,6-Diaminoacridine, 6-Cyanopurine, 4-Amino-5-imidazole-carboxamidehydrochloride, 1,3-Diiminoisoindoline, Rhodamine 6G, Crystal Violet, Basic Fuchsin, Aniline Blue Diammonium salt, N-[(3-(Anilinomethylene)-2-chloro-1-cyclohexen-1-yl)methylene]anilinemonohydrochloride, O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate, 9-Aminofluorene hydrochloride, Basic Blue, 1,8-Diamino-4,5-dihydroxyanthraquinone, Proflavine hemisulfate salt hydrate, 2-Amino-1,1,3-propenetricarbonitrile, Variamine Blue RT salt, 4,5,6-Triaminopyrimidine sulfate salt, 2-Amino-benzothiazole, Melamine, 3-(3-Pyridylmethylamino)Propionitrile, Silver(I) Sulfadiazine, Acriflavine, 4-Amino-6-mercaptopyrazole[3,4-d]pyrimidine, 2-Aminopurine, Adenine Thiol FAD Fluoroadenine, 4-Amino-6-mercapyopyrazole[3,4-d]pyrimidine, Rhodamine 110, Adenine, 5-Amino-2-mercaptobenzimidazole, Acridine Orange Hydrochloride, Cresyl Violate Acetate, Acriflavine Neutral, Dimidium Bromide, 5,10,15,20-Tetrakis(N-methyl-4-pyridinio)porphyrin Tetra(p-toluenesulfonate), 5,10,15,20-Tetrakis(4-trimethylaminophenyl)porphyrin Tetra(p-toluenesulfonate), 3,5-Diaminoacridine Hydrochloride, Propidium Iodide (3,8-diamino-5-(3-diethylaminopropyl)-6-phenylphenanthridinium iodidemethiodide), Trans-4-[4-(dimethylamino)styryl]-1-methylpyridinium iodide, and 4-((4-(dimehtylamino)phenyl)azo)benzoic acid, succinimidyl ester and derivatives thereof. "Derivatives" refers to modified compounds that are structurally closely related to the parent compound. Preferred derivatives are compounds that have been modified such that they comprise a thiol (SH) group. The thiol group allows covalent coupling of the linker molecule to a metal surface. The inventive methods thus comprise embodiments, where the Raman-active molecular linker is attached to the substrate surface via covalent interactions.

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.

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 by the methods of the present invention include, for example, cells, tissues, homogenates, lysates, extracts, and purified or partially purified proteins and other biological molecules and mixtures thereof.

Non-limiting examples of samples typically used in the methods of the invention 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 in the methods of the present invention will 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 is compatible with the methods of the invention. Detection in a body fluid can also be *in vivo*, i.e. without first collecting a sample.

"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.

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.

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.

The terms "contacting" or “incubating” as used interchangeably herein refer generally to providing access of one component, reagent, analyte or sample to another. For example, contacting can involve mixing a solution comprising an analyte binding protein or conjugate thereof with a sample. The solution comprising one component, reagent, 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 components, reagents, analytes and/or samples.

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.

The term "hapten" as used herein, refers to a small proteinaceous or non-protein antigenic determinant which is capable of being recognized by an antibody. Typically, haptens do not elicit antibody formation in an animal unless part of a larger species. For example, small peptide haptens are frequently coupled to a carrier protein such as keyhole limpet hemocyanin in order to generate an anti-hapten antibody response.

"Antigens" are macromolecules capable of generating an antibody response in an animal and being recognized by the resulting antibody. Both antigens and haptens comprise at least one antigenic determinant or "epitope", which is the region of the antigen or hapten which binds to the antibody. Typically, the epitope on a hapten is the entire molecule.

The method of the invention can 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. The signal obtained from a multitude of different analyte binding molecule:analyte complexes can be resolved by using different Raman-active linker molecules that produce distinct SERS signals.

The metal substrate surface may be made of a noble metal or copper. "Noble metal", as used herein, relates to a metal selected from the group consisting of ruthenium, rhodium, silver, palladium, osmium, iridium, platinum, and gold, preferably silver and gold.

The substrate may be a nanoparticle, for example a nanoparticle coated with or consisting of a noble metal, as defined above, or copper. The nanoparticle can be coated with a silver film or can be a citrate-stabilized gold nanoparticle. "Nanoparticle" as used herein relates to a particle sized between 1 and 100 nanometers.

The invention also encompasses conjugates for the detection of an analyte using surface-enhanced Raman spectroscopy, wherein these conjugates comprising an analyte binding molecule, a Raman-active linker molecule and a metal substrate, all defined as above. The term "conjugate" as used herein refers to two or more molecules which have been linked together. The linkage to each other may be covalent or non-covalent, but preferably is covalent. In one embodiment of such a conjugate, the analyte binding molecule is covalently coupled to the Raman-active linker molecule and the Raman-active linker molecule is covalently attached to the metal substrate.

These conjugates can be part of a kit for the detection of a given analyte or the conjugate components can, together with coupling agents, form part of a kit, requiring that before use, the conjugate is formed.

The invention also relates to a biosensor for the detection of an analyte using surface-enhanced Raman spectroscopy, comprising one or more of the above conjugates, in particular nanoparticle conjugates. The biosensor may further comprise a substrate with the nanoparticles being attached to or adherent to the substrate. The biosensor can be configured for *in vivo* and/or *in vitro* use. The use of such a biosensor is a further aspect of the present invention. This use can be *in vivo* or *in vitro* and may comprise contacting the biosensor with the analyte containing medium, for example a sample or body fluid, and detecting the SERS signal from the sensor. In a preferred embodiment, the biosensor is configured for a multiplex method that allows the detection of more than one analyte.

One embodiment of the invention is illustrated in Figure 1a. In this particular embodiment, an antibody chosen to target a specific analyte of interest, which can be as defined above, is anchored onto a SERS-active metallic nanostructured surface via a Raman-active molecular linker. The linker schematically displayed in the Figure merely serves as an example and is not intended to be limiting. Linker molecule containing no aromatic ring or a plurality of aromatic rings in some suitable arrangement can also be used. To ensure a firm attachment of the antibody to the nanostructures, the antibody is covalently connected to one end of the linker molecule through an appropriate chemical reaction, e.g. through an amide-bond formation by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) zero-length coupler, while the other end of the linker molecule is covalently attached to the metallic nanostructure through a thiol moiety, thereby anchoring the antibody to the nanostructure.

While the different components can be replaced by other suitable compounds, such molecular arrangement is crucial for the operation of the current sensor system.

The inventors of the present invention have observed and shown that binding of an antigen molecule to its antibody could induce structural changes in both members, i.e. the antibody and the linker molecule, due to binding-related stress. Without wishing to be bound to any particular theory, it is hypothesized that a multitude of factors could give rise to such a binding-related stress. For instance, electrostatic repulsion as well as steric interactions between two dipole like molecules can give rise to stress. It has also been suggested that although electrostatic and steric repulsion may play a role, configurational entropy may supersede these forces and lead to molecular re-orientation/configuration which subsequently brings about stress. In addition, hydration forces between neighboring bound molecules may also result in stress. The inventors have now unexpectedly found that these forces can be exploited for the detection of antigen/antibody binding events. A more elaborated picture of this inventive concept is schematically illustrated in Figure 1b and 1c.

In the example given in Figure 1b, the antibody/linker construct is initially oriented at a specific angle. Upon binding of an antigen to the antibody, repulsion forces, which could be steric, electrostatic, hydration, entropic in nature, are induced, bringing about a re-orientation of the antibody/linker construct. This re-orientation subsequently produces bending stress and results in changes in the internal structure of the linker molecule. Due to the proximity of the linker molecule to the SERS-active surface, such a re-orientation induced structural changes within the molecule becomes detectable via SERS spectrometric analysis. In another example depicted in Figure 1c, the antibody/linker construct is initially oriented normally with respect to the SERS-active surface. Upon binding to antigens, repulsion forces between neighboring antigen molecules result in a re-orientation of the antibody/linker constructs, and in turn bending stresses within the linker's structure. As in the first example, such stress can also be indirectly measured through SERS spectrometric analysis.

With the current embodiment, it is apparent that no washing step may be required for the detection of the binding events since the unbound antigens or contaminants within the sample medium are not within the reach of the plasmon near-field on the SERS-active surface, and thus their Raman signals are not amplified and therefore negligible as compared to the linker signals. Another advantage offered by the current design is the uniqueness of the Raman spectrum of the linker, which allows for discrimination against Raman background, thereby improving overall system's sensitivity.

Another embodiment of the current invention is illustrated in Figure 2a. This embodiment concerns a highly-multiplexed SERS-based nanoscale stress sensor for protein detection. The inventors found out that this sensor can be much more miniaturized than previous fluorescent detection methods, as it is devoid of significant spectral overlaps. The narrow peak-width in the SERS spectra means that signal cross-talk can be minimized, thereby allowing simultaneous measurement of multiple binding events to be performed within a single laser spot. Thus, the current design is expandable to a highly-multiplexed sensing platform.

In this particular embodiment, the sensing area is sub-divided into several regions, each of which bears a specific antibody/linker construct. As an example, three different antibody/linker constructs are shown in Figure 2a. It should be noted that each of the three antibody/linker constructs is comprised of a different antibody and linker molecule so that up to 3 antigens can be targeted simultaneously per sensing area. In one aspect of this particular embodiment, it is not a requirement that each sub-region within the sensing area be comparable in size with the laser spot. In fact, each sub-region can be smaller than the laser spot, so that more than one binding events can be simultaneously probed within a single laser spot (see drawing on the right of Figure 2a). This is made possible by the fact that SERS spectra of different linker molecules are generally not significantly overlapped and thus can be computationally separated. In fact, more than three binding events can be probed simultaneously by one single laser spot, so long as the SERS spectra of the linker are separable. This embodiment thus offers the possibility of a highly-multiplexed protein sensing system.

Other embodiments are within the following non-limiting examples.

Examples

Example 1: Chemicals

6-mercaptopurine (6-MP) monohydrate and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were obtained from Sigma Aldrich. Glycine and phosphate buffer saline (PBS, pH7.4) were obtained from Invitrogen and 1st BASE respectively. Purified mouse anti-human p53 (0.5mg/ml) and recombinant human p53 (10μg in 100μl) were procured from BD Biosciences Pharmingen. The Gold SERS substrates used in this experiment were fabricated by e-beam lithography.

Example 2: Coupling of 6-MP to the SERS substrate

15.2 mg of 6-mercaptopurine (6-MP) were dissolved in PBS to make an approximately 10mM solution. A clean Au IME substrate was immersed in this solution for an hour before it was rinsed with demonized water and left to dry. A Raman spectrum was taken before and after treatment with 6-MP.

Example 3: Immobilization of anti-human p53 onto 6-MP-coated SERS substrates

4μl of anti-human p53 was added to 0.5ml PBS. 650μM of EDC solution was prepared by dissolving 2.4mg EDC in 20ml PBS. 5μl of the EDC solution was added to the anti-human p53 in PBS. The 6-MP coated SERS substrate was immersed in this solution for

2 hours at room temperature after which, it was washed thoroughly with PBS and briefly dried.

Example 4: SERS measurements

2μl of p53 was added to 20μl of PBS. This solution was added to the substrate and incubated for 30 minutes at room temperature. A SERS measurement was then taken. Afterwards, the substrate was rinsed thoroughly with deionized water and a SERS measurement was taken in PBS.

A scheme of the experimental setup is shown in Figure 3. Briefly, a 10 mW He-Ne 632.8 nm laser was attenuated to about 5 mW using a neutral density filter (Edmund Inc.). A set of lenses which acts as both a beam expander and spatial filter was used to produce a 7 mm (Ø) beam of uniform profile. The so obtained beam was focused onto the sensing area of the SERS stress sensor via a dichroic mirror and through an Olympus 40 ×, 0.90 NA microscope objective. The substrate was affixed onto a glass slide via double-sided tape. 20μl of a p53 solution was dropped onto the substrate and covered with a cover slip before placing onto the microscope stage of the Raman system. The laser power at the sample was measured to be 6 mW. The acquisition time used in the experiment was 10 s with all Raman spectra collected from 200-2000cm^{-1}. The Raman signals generated at the sensor were collected by the same objective and focused into a 400 μm optical fiber (Ocean Optics, Inc.) which delivered the signals to a single-stage monochromator (DoongWo, Inc.). The grating used in this study was 600 g/mm, and the CCD detector (ANDOR Inc.) operating temperature was set to -60 °C. An ANDOR software was used to acquire the Raman spectra as well as to control the spectrometer.

The raw SERS spectra were processed using the Wire 3.0 software provided by manufacturer of the Raman system. A straight line baseline subtraction was first performed to remove any background fluorescence. Curve fitting of prominent peaks was carried out using 50% Gaussian curves to locate the centre peak position and determine the peak width and peak intensity.

Exemplary SERS spectra derived from the 6-MP stress sensor are shown in Figure 4. It is obvious that the shapes of the three spectra are generally similar and no new peaks are formed due to antibody coupling to the sensor surface and analyte binding. This is because the proteins are weak Raman scatterers and the enhancement effect in SERS decays rapidly as the separation of the analyte and metallic nanostructures increases. Therefore, a contribution from the anti-p53 antibody and the p53 antigen would not be significant alongside the spectrum of 6-MP.

The SERS peak positions before and after the addition of p53 were curve fitted and the center wavenumbers tabulated in Table 1. The peaks at ~433, ~620, ~866, ~1000 and 1290 cm^{-1} show significant positive shifts upon addition of p53 with ~866 and ~1000 cm^{-1} peaks displaying the greatest percent shift of ~0.45%. Significant negative shifts were also observed for ~1536 and ~1571cm^{-1} peaks. To understand the spectral shifts, it is necessary to revisit the theory of Raman scattering as well as understand the interaction of light with a molecule as determined by its energy levels. There are two types of energy levels: electronic energy level, associated with movement of electrons and vibrational, rotational or translational energy level, associated with movement of atoms in the molecule. Each electronic level will have its subset of vibrational levels. Raman scattering, unlike optical absorption, does not require that the incident photon energy coincide with energy transition to the next electronic level. Instead, an incident photon is usually much lower in energy and excites the molecule to an intermediate virtual state from a ground vibrational level. The virtual state is unsteady and a photon is simultaneously scattered with the molecule returning to a lower vibrational level other than ground. The energy of the scattered photon corresponds to the difference between the energy of the incident photon and the energy transition between the vibrational levels (Raman shift). Therefore, vibrations in molecules are the origin of the Raman Effect. These vibrations are constrained in a molecule by the chemical bonds between the constituent atoms. The observed peak shifts in the current experiment are thus suggestive of compression or stretching of certain bonds within 6-MP upon binding of the p53 to the immobilized anti-p53 antibody. When a bond is compressed or constrained, the vibration frequency increases, resulting in a higher Raman shift. The reverse is true for stretched or un-constrained bond. From Table 1, it can be observed that the bonds that have an upshift are bonds that bind 6-MP to the gold substrate (S-Au) and that to anti-p53 (N9). Also, the peaks with the most upshift (~865 and `1000cm^{-1}) correspond to S-Au vibration. This can be explained by being 6-MP's main linkage to the substrate, and thus the S-Au bond experiences the greatest compression upon p53 binding.

Table 1: Band assignment of shifted peaks

Shift	Peak (cm^{-1})	Assignment
Upshift	433.54	(v)S-C6, (br)pyrim
	620.42	(δ)C8-H(op), (δ)N9-H
	865.99	(br)pyrim, (δ)S-Au
	1000.04	(δ)S-Au
	1290	(v)N1-C2-N3
Downshift	1536.02	(v)N7-C8, (δ)C8-H, (δ)N9-H
	1571.92	(v)C2-N1, (v)C6-C5-C4, (δ)N9-H

Abbreviations: v: stretching vibration; br: ring breathing vibration; δ: deformation vibration; op: out of plane; pyrim: pyrimidine.

Response curves for the current sensor were constructed by plotting the peak shifts at 865, 1000 and 1290 cm^{-1} against the p53 concentrations. The curves are shown in Figure 5. These three peaks were arbitrarily chosen to monitor the p53/anti-p53 binding event simply because of having the most significant upshifts and a relatively large intensity. It should be understood that other binding-sensitive peaks can also be used. From Figure 5, it can be seen that the current sensor is responsive to p53 antigen even at concentrations as low as 10 nM.

The SERS measurements were repeated for clean substrate, 6-MP coated substrate, anti-p53 6-MP coated substrate in the absence and presence of p53 before and after rinsing. The clean Au IME substrate was analyzed at three spots on the sensor surface (A, B and C) using 100% power, 10s acquisition and 1 accumulation as settings. The results are shown in Figure 6. Afterwards, the substrate was coated with 6MP and a SERS measurement carried out with 10% power, 30s acquisition and 2 accumulations (cf. Figure 7). In the next step, anti-p53 antibodies were conjugated to the surface via 6-MP using the EDC crosslinker. A further SERS spectral analysis of the biosensor followed, at 10% power, 30s acquisition and 2 accumulations. The results are shown in Figures 8 and 12, wherein Figure 8 shows a spectra overlay plot for three measurements, whereas Figures 12 a)-c) show each spectrum separately and include the peak wavenumbers. Hereafter, p53 was added to the sensor and SERS spectra recoded at 10% power, 30s acquisition and 2 accumulations (cf. Figures 9 and 13). Then, the surface was thoroughly rinsed with deionized water and PBS was added. SERS spectra were acquired using the following parameters: 10% power, 30s acquisition and 2 accumulations. Figures 10 and 14 show the results of these recordings. It becomes apparent that no new peaks are observed upon addition of anti-p53 and p53 to the sensor surface. This is particularly evident in Figure 11, which represents a cumulative plot of the spectra obtained throughout the procedure from the analysis of location A. Figure 15 represents a cumulative plot of the spectra obtained from three measurements after antibody conjugation, addition of p53 and rinsing the sensor surface.

A large Raman shift is observed when p53 binds to the anti-p53 antibody that has been immobilized on the Au IME substrate (cf. Figure 15). The ~866, ~947, ~1000 and ~1292 peaks, corresponding to vC8-H+vN7-C8+vN9-C8, αC5-N7-C8+δN9-C8+αC6-S, αS-H and δC2-H+δC8-H+αN1-C2-N3+δN9-H, show the most prominent shifts of ~4-5cm^{-1}. These represent bonds between 6-MP and the substrate and/or the antibody.

When the substrate is rinsed with deionized water and the Raman spectrum is taken in PBS, the spectrum closely resembles the initial spectrum before incubation with p53 (cf. Figures 12, 14 and 15).

The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed 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 within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by specific 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.

The content of all documents cited herein is incorporated by reference in their entirety.

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.

Other embodiments are in the following claims. 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

Claims

What is claimed is:

1. A method for detecting one or more analytes using surface enhanced Raman spectroscopy (SERS), comprising
   - contacting the one or more analytes with at least one analyte binding molecule attached to a metal substrate surface that enhances Raman scattering via a Raman-active molecular linker; and
   - detecting a surface enhanced Raman signal from said compound.

2. The method of claim 1, wherein the surface enhanced Raman signal of the compound is correlated with the amount of the analytes.

3. The method of claim 1 or 2, wherein the one or more analytes are contained in a sample and the detection is *in vitro*.

4. The method of any one of claims 1-3, wherein the one or more analytes are detected in a bodily fluid comprising said analyte.

5. The method of claim 4, wherein said bodily fluid is selected from the group consisting of plasma, serum, blood, lymph, liquor and urine.

6. The method of any one of claims 1-5, wherein the one or more analytes are selected from the group consisting of proteins, peptides, nucleic acids, carbohydrates, lipids, cells, viruses, small molecules, or haptens.

7. The method of any one of claims 1-6, wherein the at least one analyte binding molecule specifically binds the one or more analytes.

8. The method of claim 7, wherein the at least one analyte binding molecule is selected from the group consisting of an antibody, antibody fragment or antibody like molecules.

9. The method of claim 7, wherein the at least one analyte binding molecule is a monoclonal or polyclonal antibody.

10. The method of any one of claims 1-9, wherein the method is a multiplex method for detecting more than one analyte, wherein in the contacting step more than one analyte binding molecules are used.

11. The method of any one of claims 1-10, wherein the at least one analyte binding molecule is covalently coupled to a Raman-active molecular linker that is attached to the substrate surface via covalent interactions.

12. The method of any one of claims 1-11, wherein the Raman-active molecular linker compound is selected from the group consisting of 6-Mercaptopurine, 8-Aza-adenine, N-Benzoyladenine, 2-Mercapto-benzimidazole, 4-Amino-pyrazole[3,4-d]pyrimidine, Zeatin, Methylene Blue, 9-Amino-acridine, Ethidium Bromide, Bismarck Brown Y, N-Benzyl-aminopurine, Thionin acetate, 3,6-Diaminoacridine, 6-Cyanopurine, 4-Amino-5-imidazole-carboxamidehydrochloride, 1,3-Diiminoisoindoline, Rhodamine 6G, Crystal Violet, Basic Fuchsin, Aniline Blue Diammonium salt, N-[(3-(Anilinomethylene)-2-chloro-1-cyclohexen-1-yl)methylene]anilinemonohydrochloride, O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate, 9-Aminofluorene hydrochloride, Basic Blue, 1,8-Diamino-4,5-dihydroxyanthraquinone, Proflavine hemisulfate salt hydrate, 2-Amino-1,1,3-propenetricarbonitrile, Variamine Blue RT salt, 4,5,6-Triaminopyrimidine sulfate salt, 2-Amino-benzothiazole, Melamine, 3-(3-Pyridylmethylamino)Propionitrile, Silver(I) Sulfadiazine, Acriflavine, 4-Amino-6-mercaptopyrazole[3,4-d]pyrimidine, 2-Aminopurine, Adenine Thiol FAD Fluoroadenine, 4-Amino-6-mercapyopyrazole[3,4-d]pyrimidine, Rhodamine 110, Adenine, 5-Amino-2-mercaptobenzimidazole, Acridine Orange Hydrochloride, Cresyl Violate Acetate, Acriflavine Neutral, Dimidium

Bromide, 5,10,15,20-Tetrakis(N-methyl-4-pyridinio)porphyrin Tetra(p-toluenesulfonate), 5,10,15,20-Tetrakis(4-trimethylaminophenyl)porphyrin Tetra(p-toluenesulfonate), 3,5-Diaminoacridine Hydrochloride, Propidium Iodide (3,8-diamino-5-(3-diethylaminopropyl)-6-phenylphenanthridinium iodidemethiodide), Trans-4-[4-(dimethylamino)styryl]-1-methylpyridinium iodide, and 4-((4-(dimehtylamino)phenyl)azo)benzoic acid, succinimidyl ester or derivatives thereof.

13. The method of claim 12, wherein the Raman-active molecular linker is a thiol-group containing compound.

14. The method of claim 13, wherein the Raman-active molecular linker is 6-Mercaptopurine.

15. The method of any one of claims 1-14, wherein the at least one analyte binding molecule is covalently coupled to the Raman-active molecular linker by amide bond formation.

16. The method of claim 15, wherein 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide is used as a coupling agent.

17. The method of any one of claims 1-16, wherein the metal substrate surface is made of a noble metal or copper.

18. The method of claim 17, wherein the noble metal is selected from the group consisting of silver and gold.

19. The method of any one of claims 1-18, wherein the substrate is a nanoparticle.

20. The method of claim 19, wherein the nanoparticle is coated with or consisting of a noble metal.

21. The method of claim 20, wherein the noble metal is selected from the group consisting of silver and gold.

22. The method of any one of claims 20-21, wherein the nanoparticle is coated with a silver film.

23. The method of any one of claims 20-22, wherein the nanoparticle is a citrate-stabilized gold nanoparticle.

24. A conjugate for the detection of an analyte using surface-enhanced Raman spectroscopy comprising an analyte binding molecule, a Raman-active linker molecule and a metal substrate, wherein the analyte binding molecule is covalently coupled to the Raman-active linker molecule and the Raman-active linker molecule is covalently attached to the metal substrate.

25. The conjugate of claim 24, wherein the analyte binding molecule is selected from the group consisting of an antibody, antibody fragment or antibody like molecules.

26. The conjugate of claim 25, wherein the analyte binding molecule is a monoclonal or polyclonal antibody.

27. The conjugate of any one of claims 24-26, wherein the Raman-active molecular linker compound is selected from the group consisting of 6-Mercaptopurine, 8-Aza-adenine, N-Benzoyladenine, 2-Mercapto-benzimidazole, 4-Amino-pyrazole[3,4-d]pyrimidine, Zeatin, Methylene Blue, 9-Amino-acridine, Ethidium Bromide, Bismarck Brown Y, N-Benzyl-aminopurine, Thionin acetate, 3,6-Diaminoacridine, 6-Cyanopurine, 4-Amino-5-imidazole-carboxamidehydrochloride, 1,3-Diiminoisoindoline, Rhodamine 6G, Crystal Violet, Basic Fuchsin, Aniline Blue Diammonium salt, N-[(3-(Anilinomethylene)-2-chloro-1-cyclohexen-1-yl)methylene]anilinemonohydrochloride, O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate, 9-Aminofluorene hydrochloride, Basic Blue, 1,8-Diamino-4,5-dihydroxyanthraquinone, Proflavine hemisulfate salt hydrate, 2-

Amino-1,1,3-propenetricarbonitrile, Variamine Blue RT salt, 4,5,6-Triaminopyrimidine sulfate salt, 2-Amino-benzothiazole, Melamine, 3-(3-Pyridylmethylamino)Propionitrile, Silver(I) Sulfadiazine, Acriflavine, 4-Amino-6-mercaptopyrazole[3,4-d]pyrimidine, 2-Aminopurine, Adenine Thiol FAD Fluoroadenine, 4-Amino-6-mercapyopyrazole[3,4-d]pyrimidine, Rhodamine 110, Adenine, 5-Amino-2-mercaptobenzimidazole, Acridine Orange Hydrochloride, Cresyl Violate Acetate, Acriflavine Neutral, Dimidium Bromide, 5,10,15,20-Tetrakis(N-methyl-4-pyridinio)porphyrin Tetra(p-toluenesulfonate), 5,10,15,20-Tetrakis(4-trimethylaminophenyl)porphyrin Tetra(p-toluenesulfonate), 3,5-Diaminoacridine Hydrochloride, Propidium Iodide (3,8-diamino-5-(3-diethylaminopropyl)-6-phenylphenanthridinium iodidemethiodide), Trans-4-[4-(dimethylamino)styryl]-1-methylpyridinium iodide, and 4-((4-(dimehtylamino)phenyl)azo)benzoic acid, succinimidyl ester or derivatives thereof.

28. The conjugate of claim 27, wherein the Raman-active molecular linker is a thiol-group containing compound.

29. The conjugate of any one of claims 24-28, wherein the analyte binding molecule is covalently coupled to the Raman-active molecular linker by amide bond formation.

30. The conjugate of claim 29, wherein 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide is used as a coupling agent.

31. The conjugate of any one of claims 24-30, wherein the metal substrate is made of or coated with a noble metal or copper.

32. The conjugate of claim 31, wherein the noble metal is selected from the group consisting of silver and gold.

33. The conjugate of any one of claims 24-32, wherein the metal substrate is a nanoparticle.

34. The conjugate of claim 33, wherein the nanoparticle is coated with a silver film.

35. The conjugate of claim 33, wherein the nanoparticle is a citrate-stabilized gold nanoparticle.

36. A biosensor for the detection of an analyte using surface-enhanced Raman spectroscopy, comprising one or more conjugates according to claims 24-35.

37. The biosensor of claim 36, further comprising a substrate, wherein the nanoparticles are adherent to the substrate.

38. The biosensor of claim 36 or 37, wherein the biosensor is configured for *in vivo* and/or *in vitro* use.

39. The biosensor of any one of claims 36-38, wherein the analyte is a protein, peptide, nucleic acid, small molecule or hapten.

40. Use of the biosensor of any one of claims 36-39 for the detection of an analyte.

41. The use of claim 40, wherein the detection is *in vivo*.

42. The use of claim 40, wherein the detection is *in vitro*.

Figures

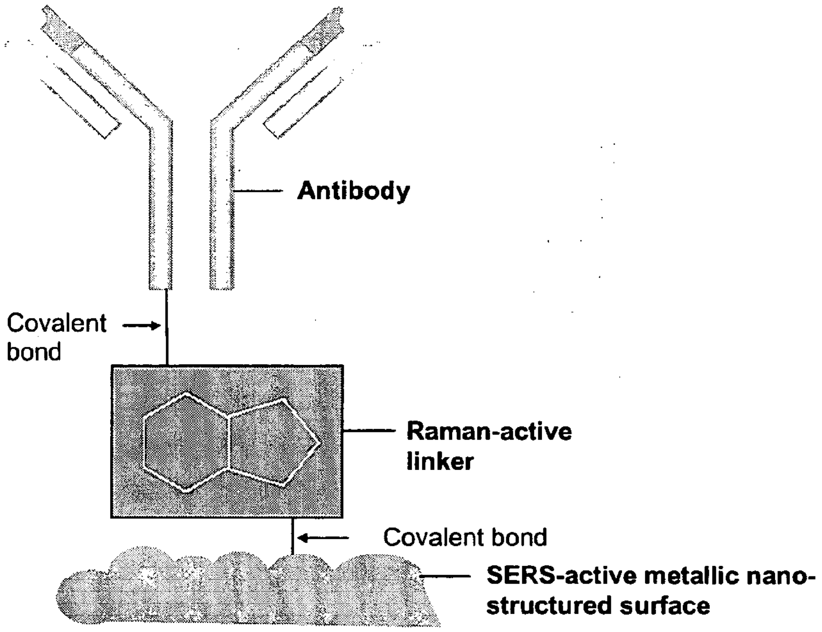


Figure 1 (a)

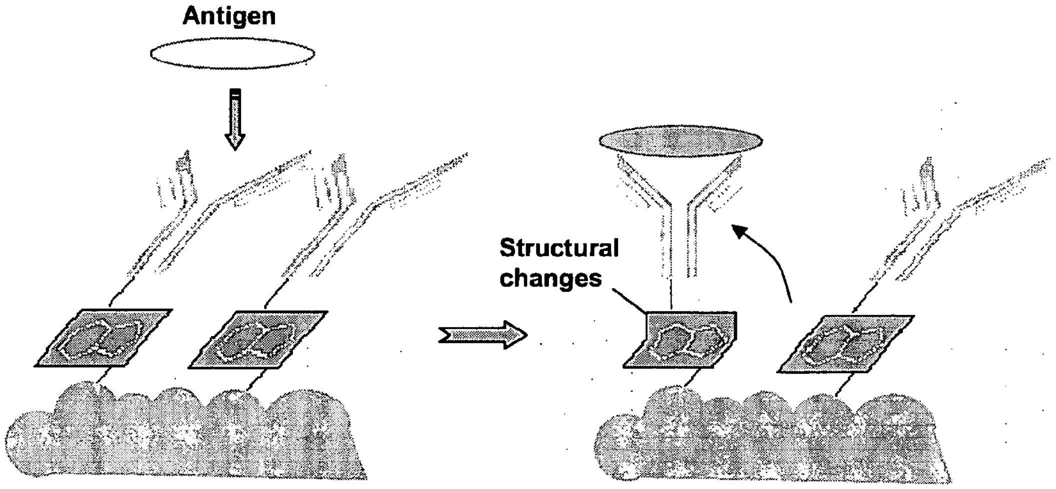


Figure 1 (b)

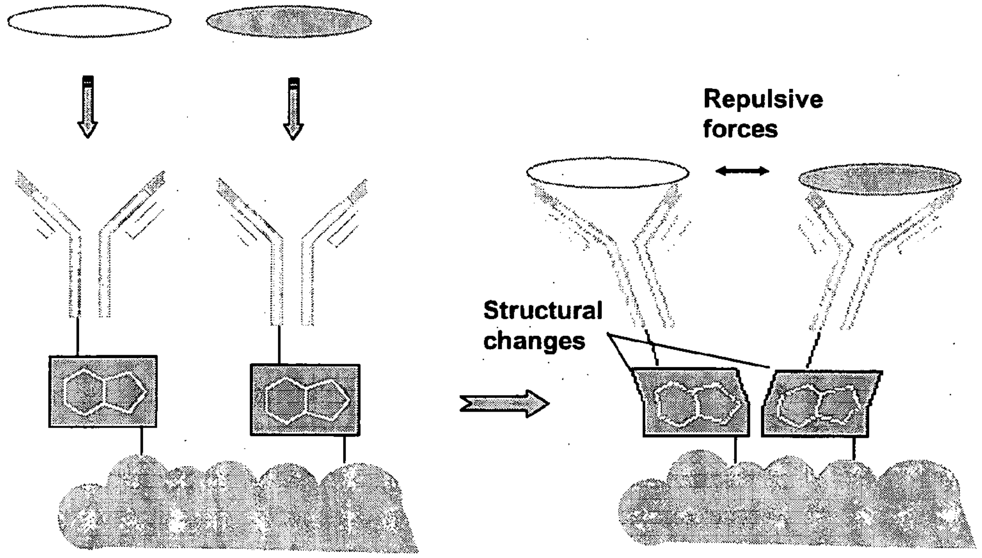


Figure 1 (c)

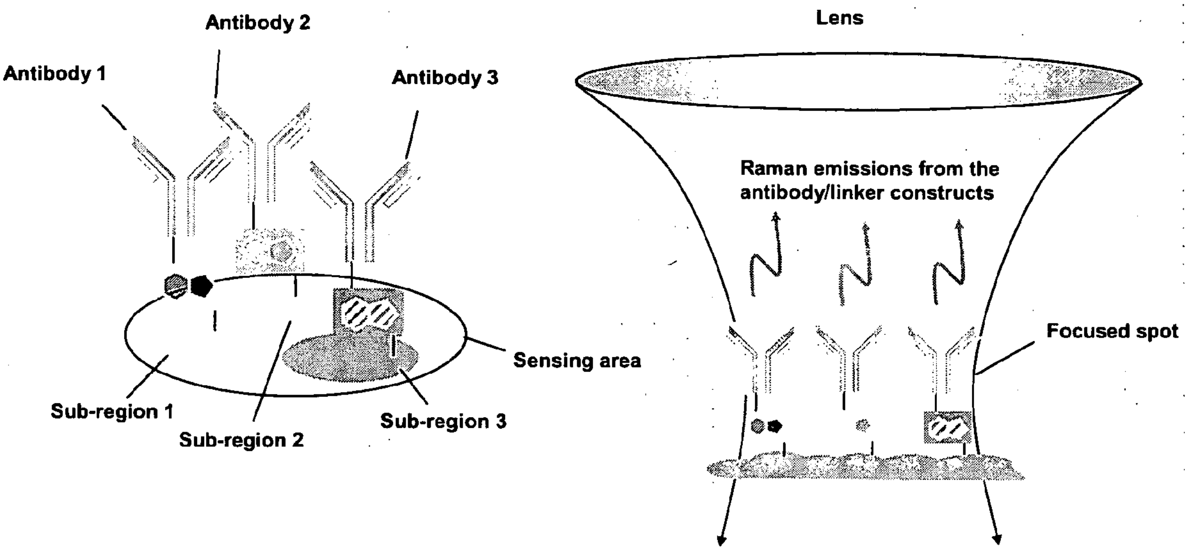


Figure 2

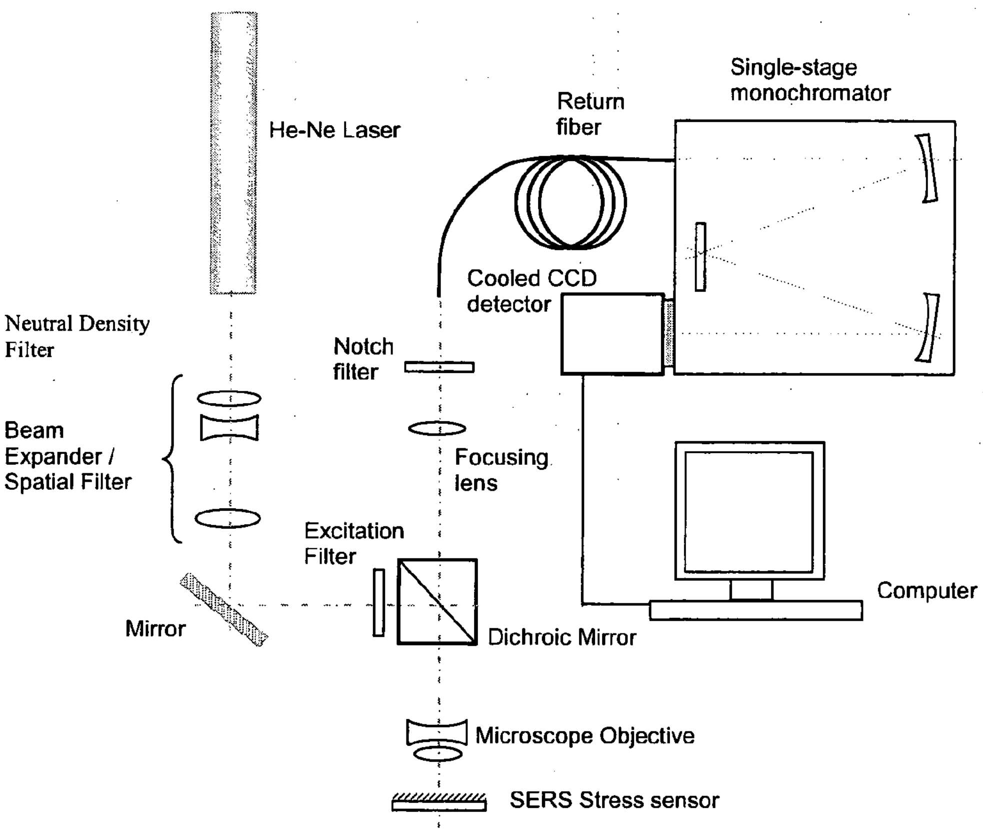
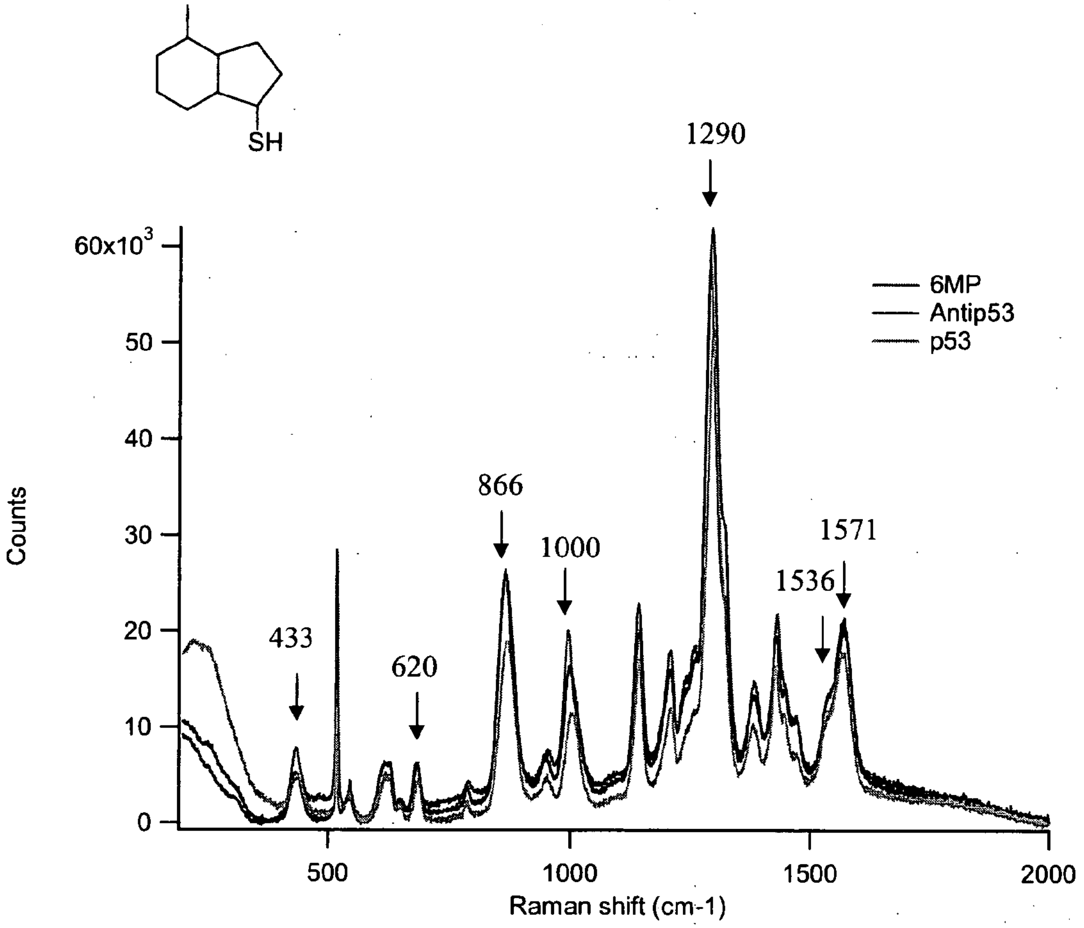

He-Ne Laser
Return fiber
Single-stage monochromator
Cooled CCD detector
Neutral Density Filter
Notch filter
Beam Expander / Spatial Filter
Focusing lens
Excitation Filter
Computer
Mirror
Dichroic Mirror
Microscope Objective
SERS Stress sensor

Figure 3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1290
60x10^3
50
40
30
20
10
0
Counts
6MP
Antip53
p53
866
1000
1571
1536
433
620
500
1000
1500
2000
Raman shift (cm-1)

Figure 4

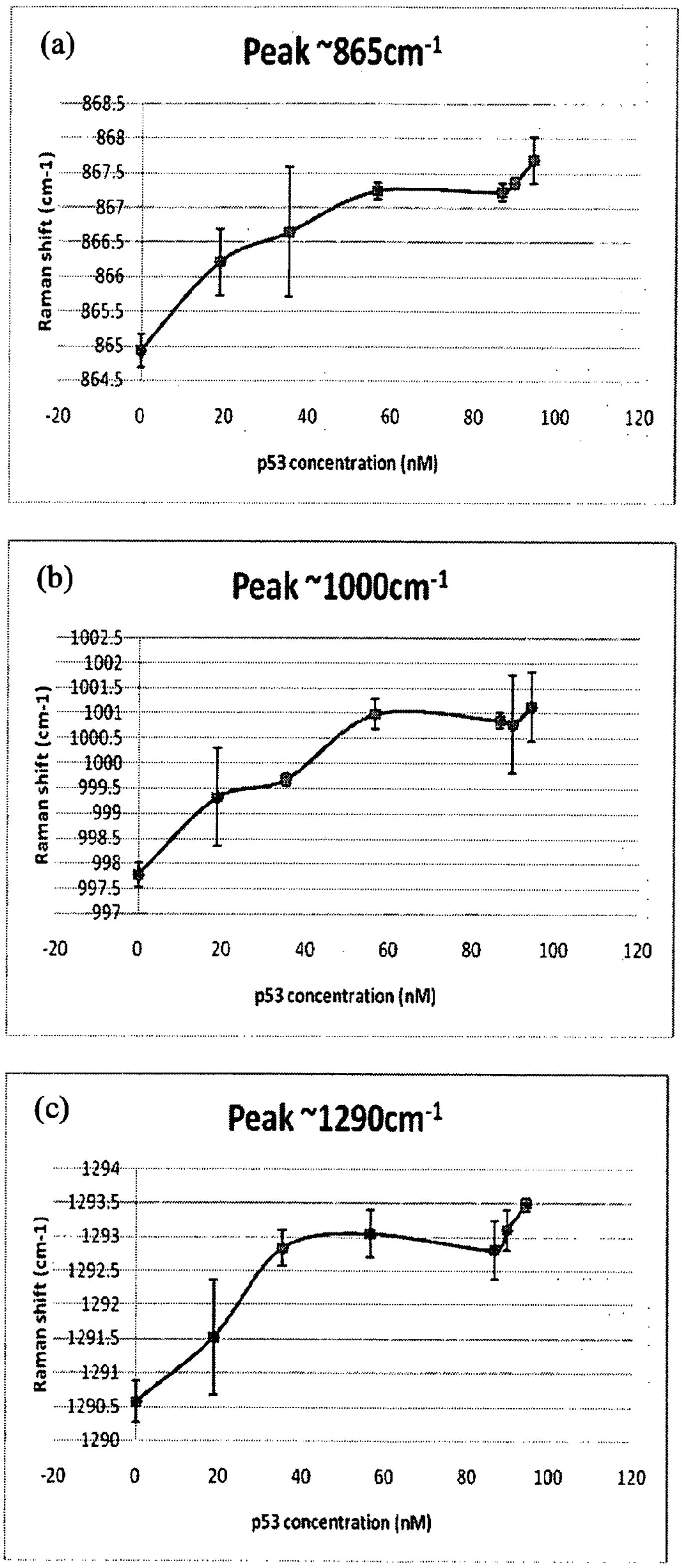

(a)
Peak ~865cm^{-1}
Raman shift (cm-1)
868.5
868
867.5
867
866.5
866
865.5
865
864.5
-20
0
20
40
60
80
100
120
p53 concentration (nM)
(b)
Peak ~1000cm^{-1}
Raman shift (cm-1)
1002.5
1002
1001.5
1001
1000.5
1000
999.5
999
998.5
998
997.5
997
-20
0
20
40
60
80
100
120
p53 concentration (nM)
(c)
Peak ~1290cm^{-1}
Raman shift (cm-1)
1294
1293.5
1293
1292.5
1292
1291.5
1291
1290.5
1290
-20
0
20
40
60
80
100
120
p53 concentration (nM)

Figure 5

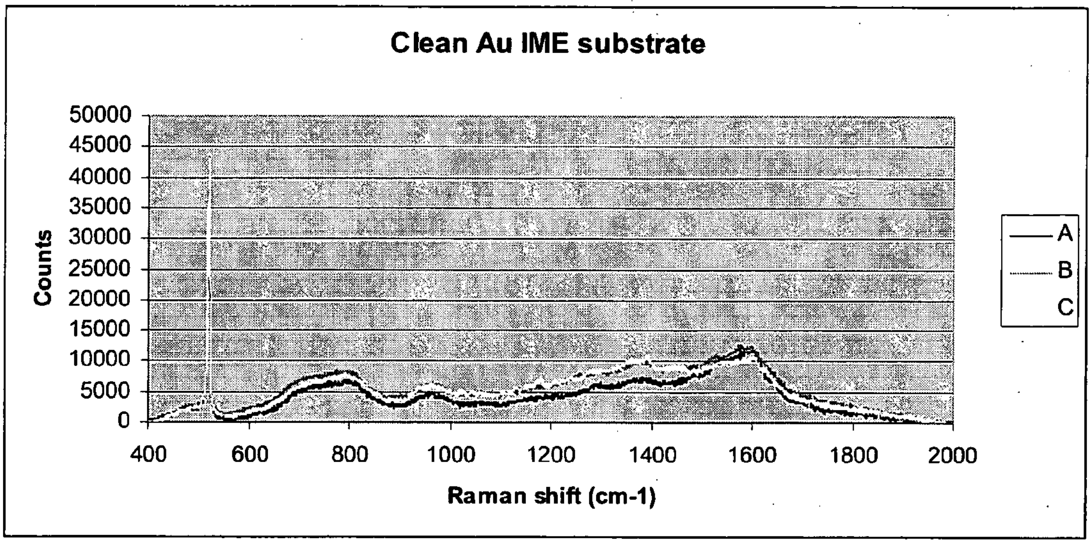
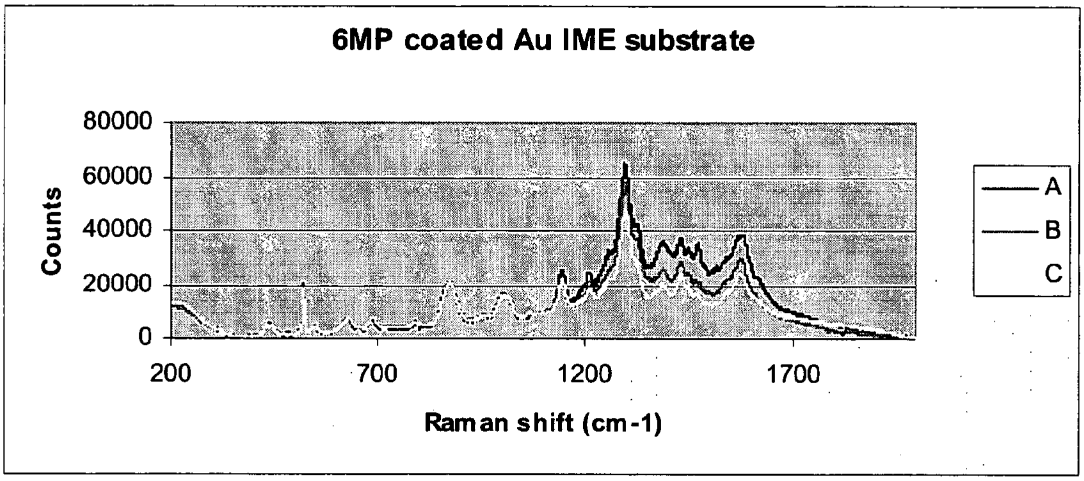


Figure 6



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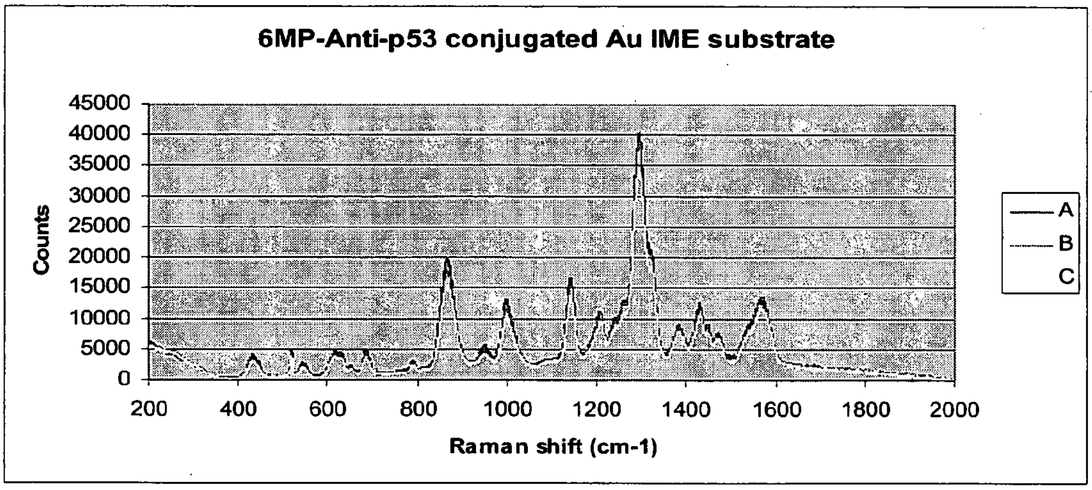


Figure 8

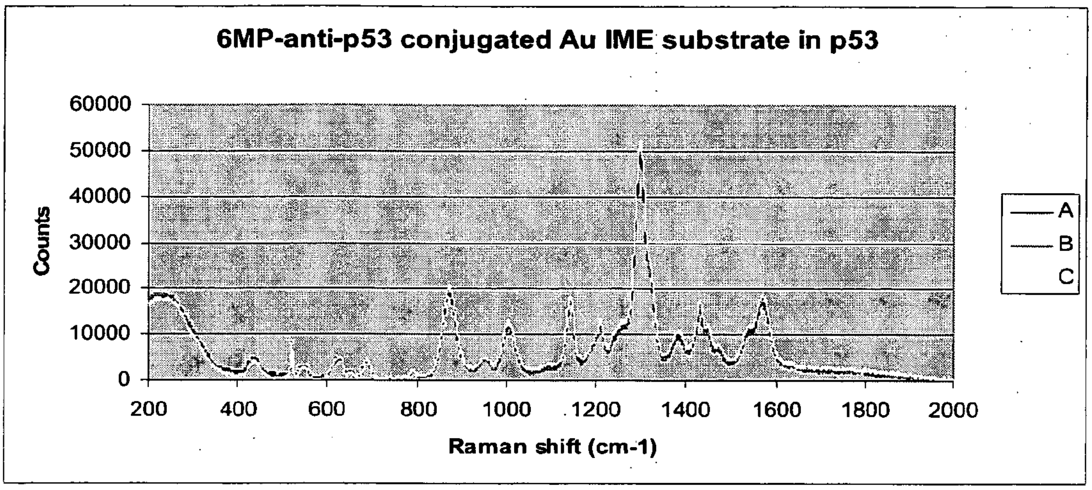


Figure 9

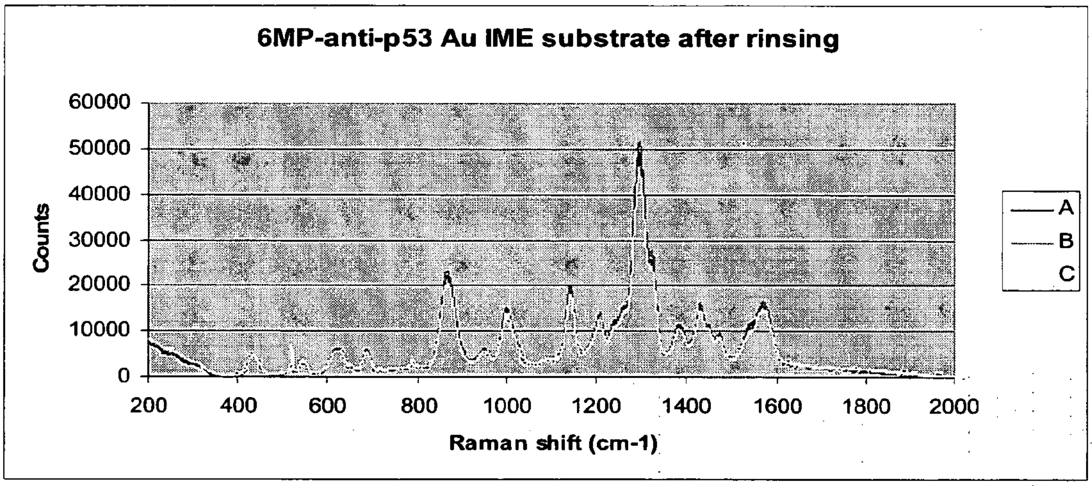


Figure 10

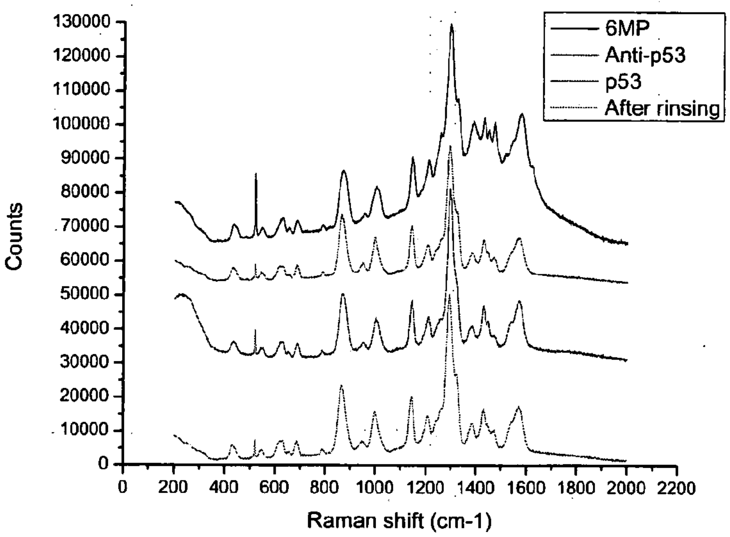

6MP
Anti-p53
p53
After rinsing
Counts
130000
120000
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
Raman shift (cm-1)

Figure 11

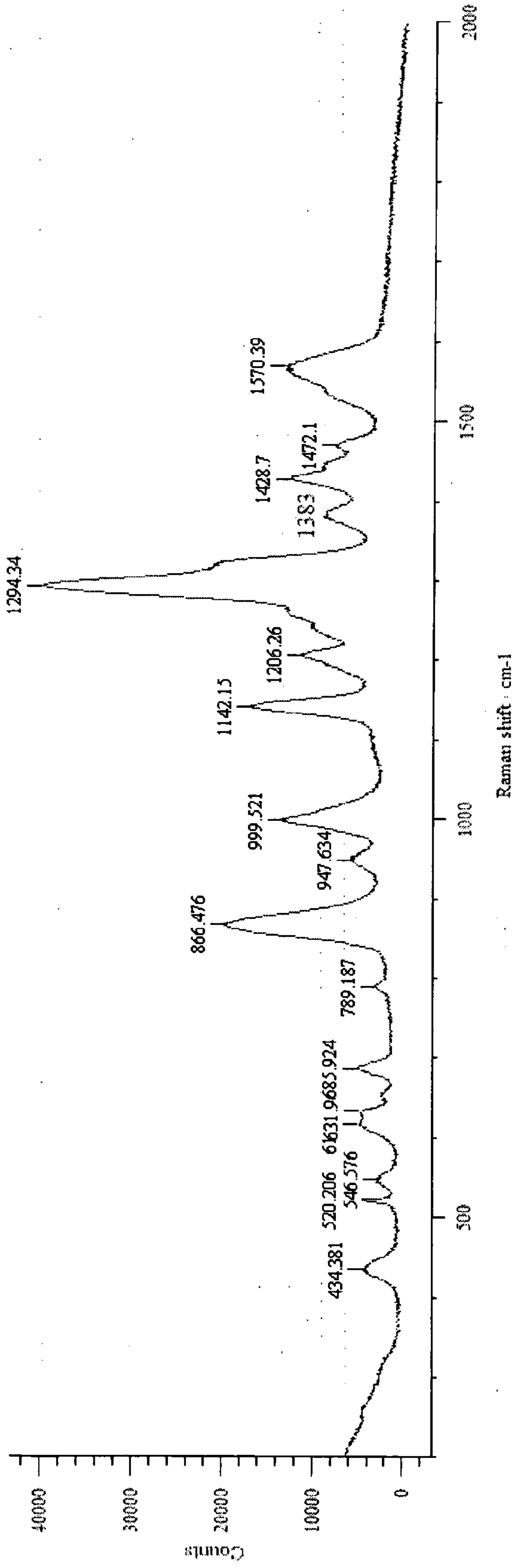

1570.39
1472.1
1428.7
1383
1294.34
1206.26
1142.15
999.521
947.634
866.476
789.187
61631.9685.924
520.206
546.576
434.381
0
500
1000
1500
2000
Raman shift / cm-1
0
10000
20000
30000
40000
Counts

Figure 12 a)

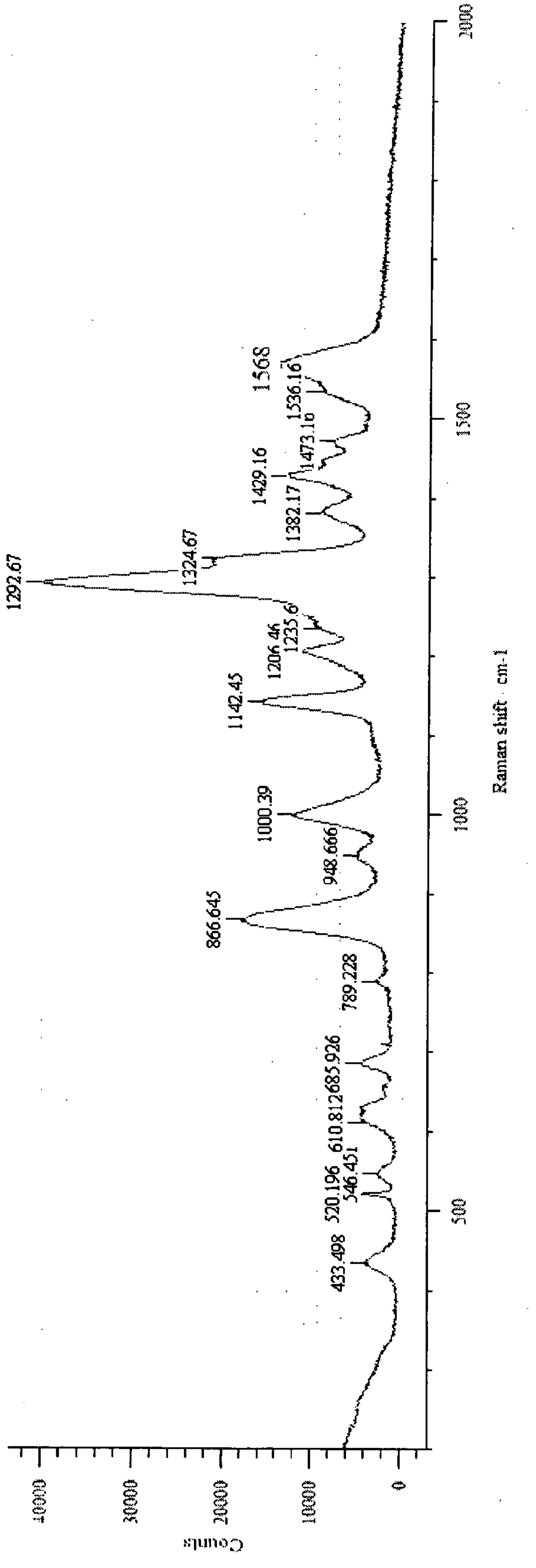

1292.67
1324.67
1568
1536.16
1473.16
1429.16
1382.17
1235.6
1206.46
1142.45
1000.39
948.666
866.645
789.228
685.926
610.812
546.451
520.196
433.498
500
1000
1500
2000
Raman shift · cm-1
0
10000
20000
30000
40000
Counts

Figure 12 b)

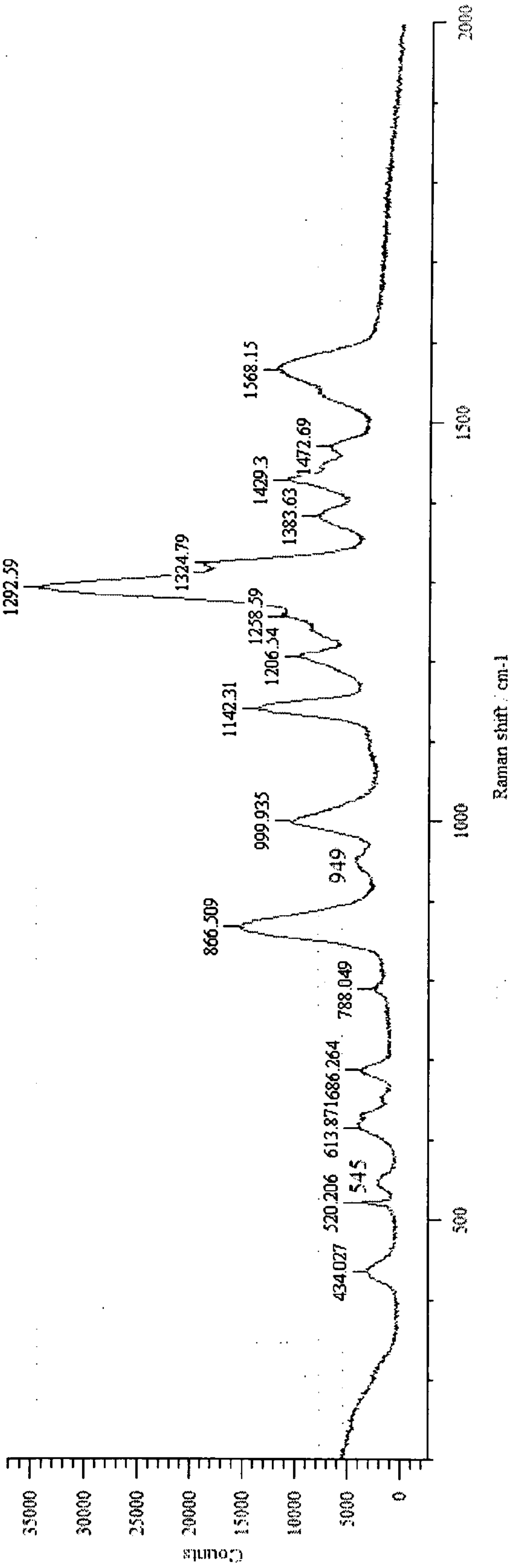

Counts
0
5000
10000
15000
20000
25000
30000
35000
Raman shift : cm-1
500
1000
1500
2000
434.027
520.206
545
613.871
686.264
788.049
866.509
949
999.935
1142.31
1206.54
1258.59
1292.59
1324.79
1383.63
1429.3
1472.69
1568.15

Figure 12 c)

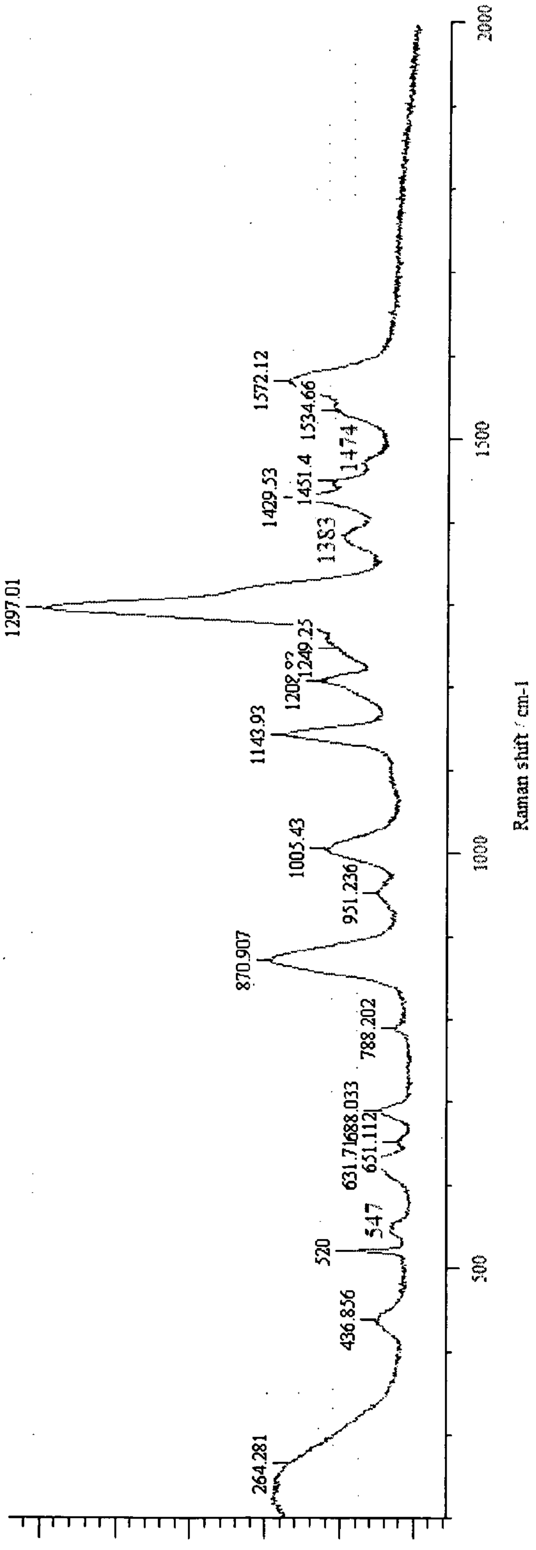

Counts
50000
40000
30000
20000
10000
0
Raman shift / cm-1
500
1000
1500
2000
264.281
436.856
520
547
631.71
651.112
688.033
788.202
870.907
951.236
1005.43
1143.93
1208.92
1249.25
1297.01
1383
1429.53
1451.4
1474
1534.66
1572.12

Figure 13 a)

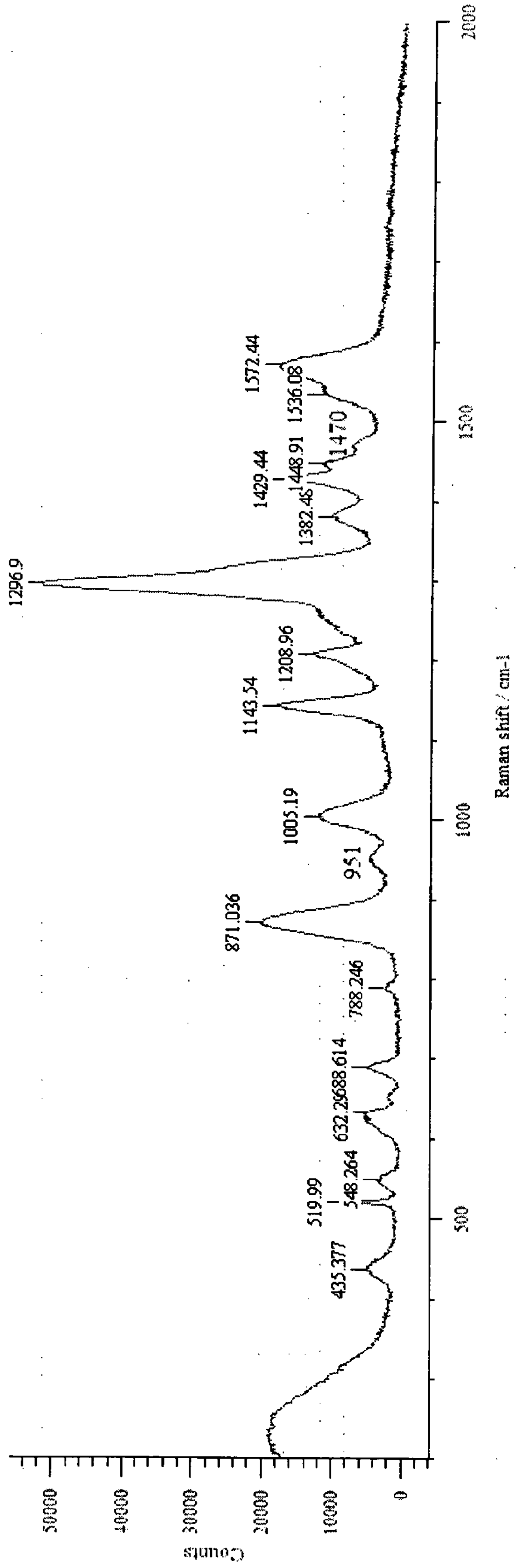
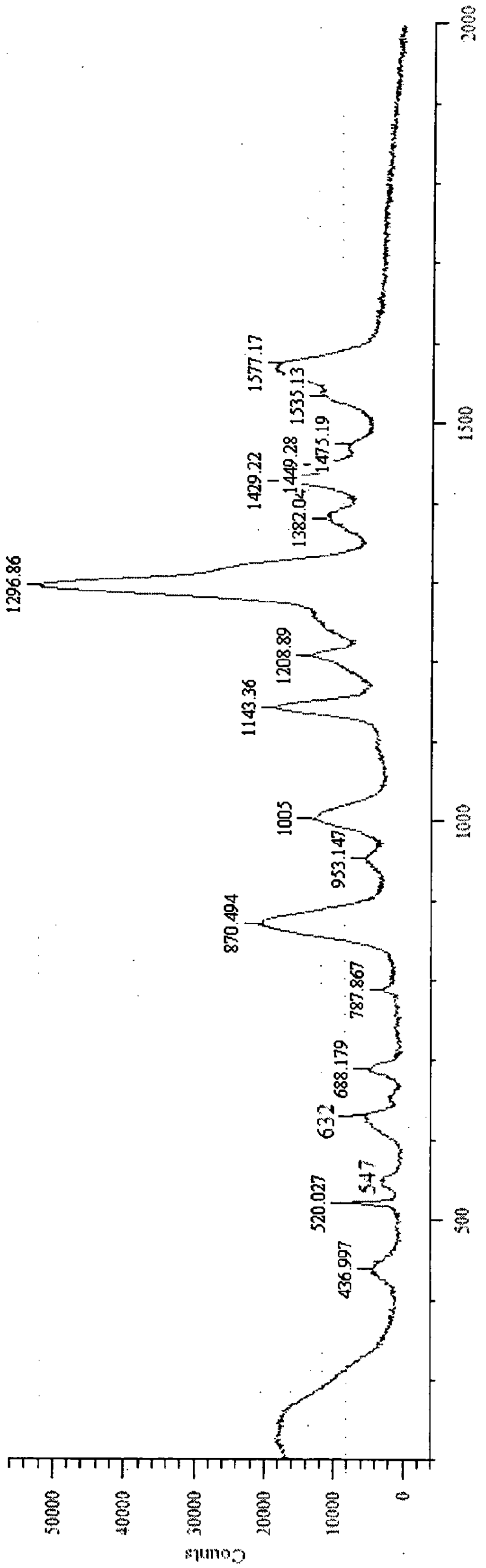

1296.9
1572.44
1536.08
1470
1429.44
1448.91
1382.45
1208.96
1143.54
1005.19
951
871.036
788.246
688.614
632.29
548.264
519.99
435.377
Counts
50000
40000
30000
20000
10000
0
500
1000
1500
2000
Raman shift / cm-1

Figure 13 b)


Counts
0
10000
20000
30000
40000
50000
Raman shift / cm-1
500
1000
1500
2000
436.997
520.027
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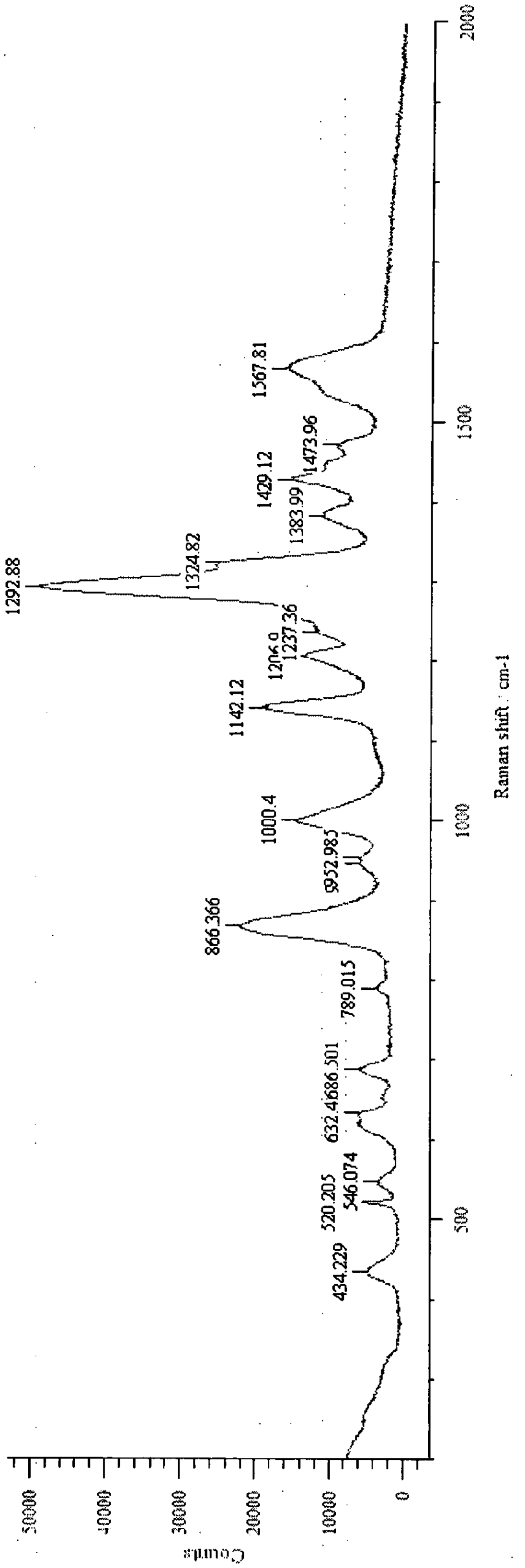
632
688.179
787.867
870.494
953.147
1005
1143.36
1208.89
1296.86
1382.04
1429.22
1449.28
1475.19
1535.13
1577.17

Figure 13 c)


1292.88
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1473.96
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1237.36
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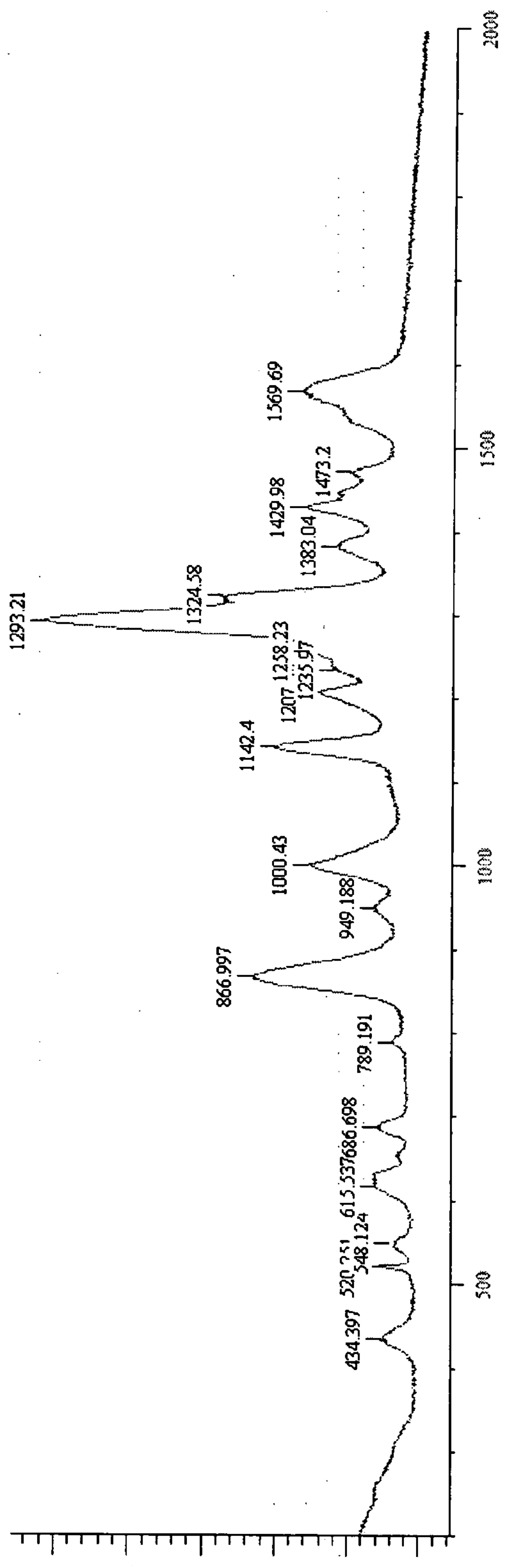
1142.12
1000.4
952.985
866.366
789.015
686.501
632.468
546.074
520.205
434.229
500
1000
1500
2000
Raman shift · cm-1
0
10000
20000
30000
40000
50000
Counts

Figure 14 a)


1293.21
1324.58
1258.23
1235.97
1207
1142.4
1000.43
949.188
866.997
789.191
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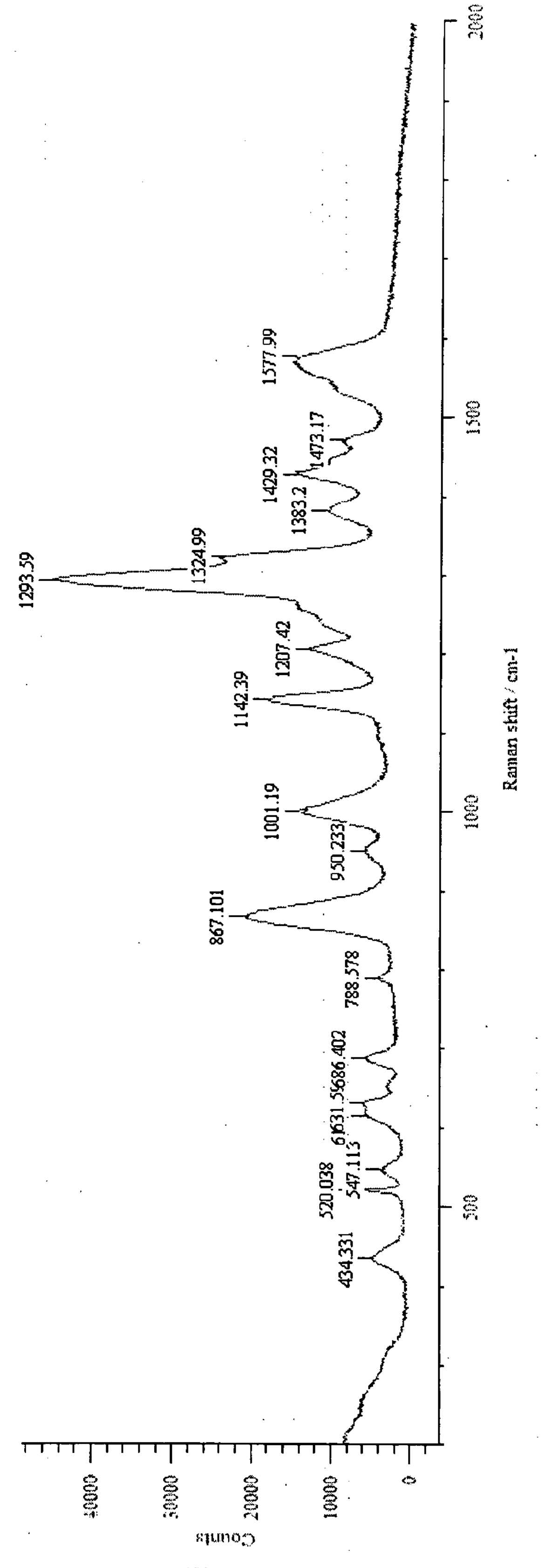
615.537
548.124
520.251
434.397
1383.04
1429.98
1473.2
1569.69
50000
40000
30000
20000
10000
0
Counts
500
1000
1500
2000
Raman shift cm-1

Figure 14 b)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1324.99
1142.39
1207.42
1383.2
1429.32
1473.17
1577.99
1001.19
950.233
867.101
788.578
61631.55686.402
520.038
547.113
434.331
Counts
0
10000
20000
30000
40000
500
1000
1500
2000
Raman shift / cm-1

Figure 14 c)

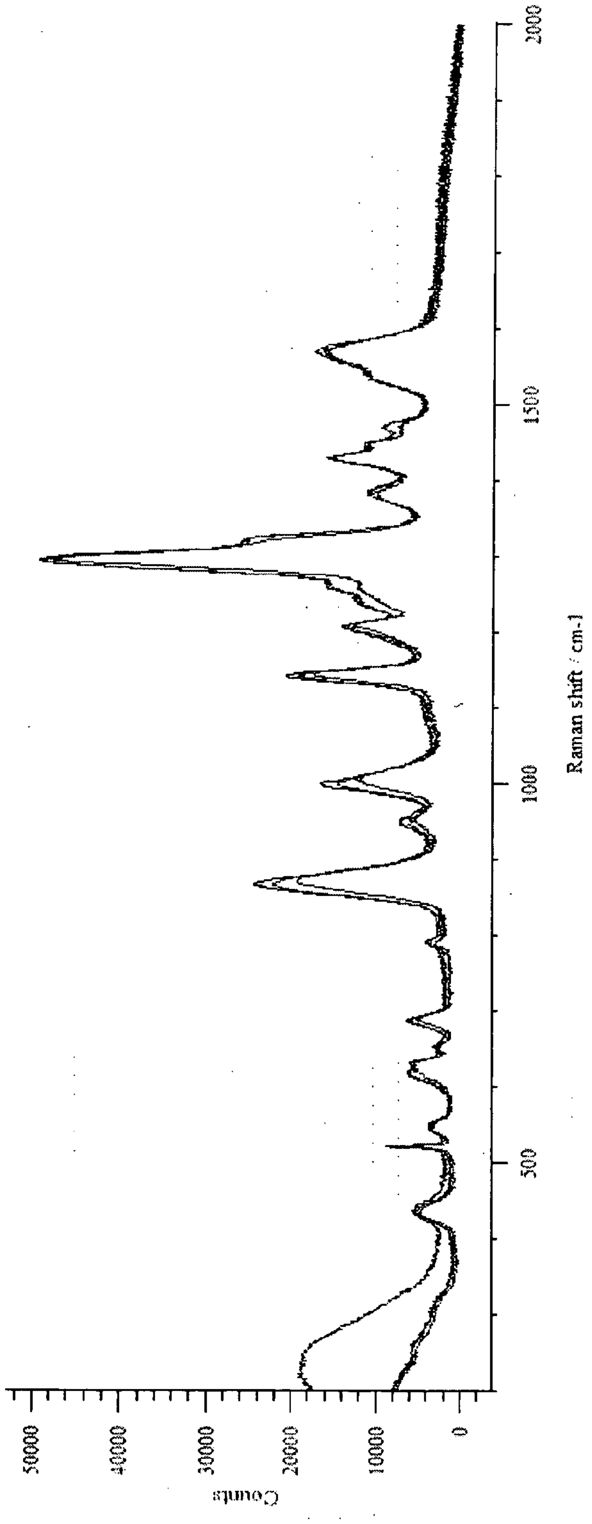
Counts
50000
40000
30000
20000
10000
0
Raman shift / cm-1
500
1000
1500
2000

Figure 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2010/000472

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

G01N 21/65 (2006.01) · *G01N 33/53* (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)
WPI, EPODOC, MEDLINE, HCAPLUS & keywords: Surface Enhanced Raman Spectroscopy, linker, antibody and like terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2005/089901 A1 (PORTER et al.) 28 April 2005 See paragraphs [0011], [0042]-[0046], [0062], [0081], [0082], [0090], [0108]; Figure 1B; Claim 22	1-42
X	EP 2040075 A1 (JULIUS-MAXIMILIANS-UNIVERSITAT WURZBURG) 25 March 2009 See paragraphs [0025]-[0028], [0094], [0095], [0110], [0142]-[0144], [0165], [0194]; Figure 2	1-42
X	YONZON, C. R. et al., "Localized surface plasmon resonance immunoassay and verification using surface-enhanced Raman spectroscopy", Proceedings of SPIE, 2003, Vol. 5224, pages 78-85 See Abstract; Figure 1	1-42

☒ 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 21 February 2011	Date of mailing of the international search report 28 FEB 2011
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **RICHARD FILMER** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 2735

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHLUCKER, S. et al., "Immuno-Raman microspectroscopy: *In situ* detection of antigens in tissue specimens by surface-enhanced Raman scattering", Journal of Raman Spectroscopy, 2006, Vol. 37, pages 719-721 See Abstract; Figure 1	1-42

This Annex lists the known "A" publication level 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 Cited in Search Report		Patent Family Member					
US	2005089901	US	7824926	US	7829348		
EP	2040075	CA	2698430	EP	2193374	US	2010284917
		WO	2009040114				

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX

专利名称(译)	基于SERS的分析物检测		
公开(公告)号	EP2516995A1	公开(公告)日	2012-10-31
申请号	EP2010839909	申请日	2010-12-16
[标]申请(专利权)人(译)	新加坡科技研究局		
申请(专利权)人(译)	机构科学，技术和研究		
当前申请(专利权)人(译)	机构科学，技术和研究		
[标]发明人	KHO KIANG WEI MALINI OLIVO		
发明人	KHO, KIANG, WEI MALINI, OLIVO		
IPC分类号	G01N21/65 G01N33/53 G01N33/532 G01N33/543 G01N33/553 G01N33/58		
CPC分类号	G01N21/658 G01N33/532 G01N33/54373 G01N33/553 G01N33/58		
代理机构(译)	庆祝活动，JENTSCHURA & PARTNER		
优先权	61/289053 2009-12-22 US		
其他公开文献	EP2516995B1 EP2516995A4		
外部链接	Espacenet		

摘要(译)

本发明涉及通过表面增强拉曼光谱（SERS）检测分析物的方法，包括使分析物与至少一种附着于金属基质表面的分析物结合分子接触，所述金属基质表面通过拉曼活性分子接头增强拉曼散射;并检测来自所述化合物的表面增强拉曼信号。在另一方面，本发明涉及适用于本发明的基于SERS的分析物检测方法的缀合物和生物传感器。