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(71) Applicant: THE UNIVERSITY OF BIRMINGHAM
[GB/GB]; Edgbaston, Birmingham B15 2TT (GB).

(72) Inventors: GOLDBERG-OPPENHEIMER, Pola; The University of Birmingham, Edgbaston, Birmingham West Midlands B15 2TT (GB). RICKARD, Jonathan James Stanley; The University of Birmingham, Edgbaston, Birmingham West Midlands B15 2TT (GB). DI PIETRO, Valentina; The University of Birmingham, Edgbaston, Birmingham West Midlands B15 2TT (GB). BELLI, Antonio; The University of Birmingham, Edgbaston, Birmingham West Midlands B15 2TT (GB).

(74) Agent: SOMERVELL, Thomas; Marks & Clerk LLP, Alpha Tower, Suffolk Street Queensway, Birmingham B1 1TT (GB).

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(54) Title: SURFACE ENHANCED RAMAN SCATTERING APPARATUS AND METHOD

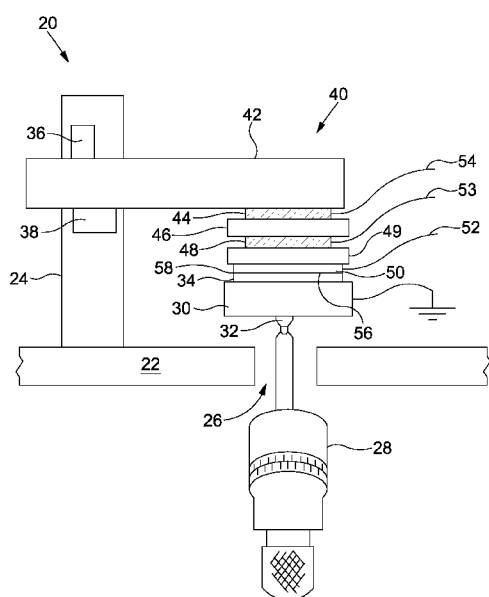


Figure 2

(57) Abstract: The present invention relates to an apparatus and method for use in Surface Enhanced Raman Scattering (SERS) techniques. There is provided an apparatus for fabricating a platform for Surface Enhanced Raman Scattering, SERS, the apparatus comprising a first support for a first substrate that has a film on a first surface thereof; a second support supporting a second substrate, wherein the second substrate has a surface facing the first surface of the first substrate, when supported, with a gap between the facing surfaces; a first gap adjustment means for adjusting the gap between the first and second substrates to a micrometre level of precision; a second gap adjustment means for further adjusting the gap to a nanometre level of precision; a connection to a source of electrical power for applying an electric field across the gap between the facing surfaces of the first and second substrates; a sensor for sensing a current flowing through the first and second substrates; a controller for controlling the current, and means for heating the first and second substrates to an elevated temperature. Also provided is a platform for Surface Enhanced Raman Scattering and a method of analysing the composition of a sample.

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Surface Enhanced Raman Scattering Apparatus and Method

Field of the invention

- 5 The present invention relates to an apparatus and method for use in Surface Enhanced Raman Scattering (SERS) techniques.

Background to the invention

- 10 Point-of-care diagnostics have created an urgent need for rapid, early-stage detection of disease-indicative biomarkers including cancer, immune-deficiencies and neurodisorders. While demonstrating correlation with disease severity, these biomarkers are released into the body fluids in miniscule amounts and are undetectable at early stages with available biochemical techniques. The levels of
- 15 molecular constituents in blood are directly associated with the physiological state of the body and therefore detection of these molecules in serum is often used for prevention, identification, and treatment selection for a variety of diseases. Currently, no technique exists to measure these compounds with sufficient sensitivity and timeliness at the point-of-care.

20

- Surface-enhanced Raman scattering (SERS) is an extremely sensitive molecular finger-printing technique which can detect chemical substances down to the single molecule level. SERS techniques find application in forensics, healthcare and diagnostics. SERS is based on the electromagnetic field enhancement by localised
- 25 optical fields (surface plasmon, polaritons or plasmons) on nanostructured surfaces. Localised plasmon resonances can be tuned by manipulation of the surface architecture at the sub-micrometre level. This gives rise to the huge Raman signal enhancements SERS is known for. The platform (substrate) on which SERS is performed (i.e. onto which a sample to be analysed is placed) is critical for successful
- 30 early stage detection. Any imperfections in platforms have a significant effect on the ultimate response.

- Carefully designed and optimised SERS-based devices could enable the next-generation sensing technologies. The substrate on which SERS is performed is often
- 35 the critical component for successful detection. However, reliable and consistent

fabrication of highly-sensitive and reproducible SERS-active structures still remains a considerable challenge. SERS-active platforms are often plagued by irreproducibility, instability and lack of tuneability. Any imperfections in substrates have a significant effect on the ultimate response and therefore SERS requires constant developments of ways of controlling surface architecture. The high enhancement is usually observed with random metallic nanoparticles, from which only a *minute* fraction exhibits SERS-activity, substantially affecting signal, exhibiting high-sample variability, aggregation, and poor batch-to-batch repeatability.

In “Hierarchical Electrohydrodynamic Structures for Surface-Enhanced Raman Scattering” (Advanced Optical Materials – Adv. Mater. 2012, 24, OP175-OP180), Goldberg-Oppenheimer et al. describe a Hierarchical Electrohydrodynamic (HEHD) process for forming nanostructure SERS platforms, which may provide a SERS enhancement of the order of 1.0×10^7 . However, this would not be sufficient for reliable detection of certain biomarkers, such as Traumatic Brain Injury (TBI) biomarkers, that will be discussed further below.

The present invention seeks to provide an improved SERS apparatus.

Summary

A first aspect of the present invention provides an apparatus for fabricating a platform for Surface Enhanced Raman Scattering, SERS. The apparatus comprises a first support for a first substrate that has a film coating on a first surface thereof, and a second support supporting a second substrate. The second substrate has a surface facing the first surface of the first substrate, when supported, with a gap between the facing surfaces. A first gap adjustment means is provided for adjusting the gap between the first and second substrates to a micrometre level of precision. A second gap adjustment means is provided for further adjusting the gap to a nanometre level of precision. A connection is provided to a source of electrical power for applying an electric field across the gap between the facing surfaces of the first and second substrates. A sensor senses a current flowing through the first and second substrates, and a controller is provided for controlling the current. The apparatus also includes means for heating the first and second substrates to an elevated temperature.

By “on” it will be understood that the film coating is applied as a layer to the substrate, such that the film coating covers a surface of the substrate.

5 The means for heating the substrates may comprise a heated chamber surrounding the substrates. A heated chamber has the advantage of providing a homogenous temperature all around the substrates without any temperature gradient, allowing for more accurate control of the gap between the substrates. Alternative means may be provided. For example, the means for heating may comprise a heating element disposed adjacent to the substrates.

10

Heating the substrates to an elevated temperature, for example above a glass transition temperature of the film coating leads to destabilisation of the coating, which enables the generation of the surface form.

15 The first gap adjustment means may comprise a micrometer. The second gap adjustment means may comprises one or more piezo actuators.

The present inventors have found that by using a first and a second actuating means to adjust the gap between the upper and lower substrates, in which the second actuating means allows adjustment to a nano-scale of precision, and which may be achieved with use of one or more piezo actuators, a significant improvement in the reproducibility and enhancement factor of the surface form can be achieved. The use of this surface form in SERS can result in improved sensitivity and consistency of detection.

20 The surface of the second substrate may be a flat (planar) surface. Alternatively, the surface of the second substrate may comprise a template pattern of pillars that protrude towards the surface of said first substrate when supported. An advantage of using a patterned second substrate is that fabrication of the structures is more controllable and repeatable.

30

The pillars preferably have an aspect ratio of approximately 0.8. The pillars preferably have a height in the range 475-500nm. The pillars preferably have a diameter of approximately 625nm.

35 The first and/or second substrate may comprise silicon or indium tin oxide.

A second aspect of the invention provides a method of fabricating a platform for Surface Enhanced Raman Scattering. The method comprises providing a first and a second substrate. The first substrate comprises at least one film coating on a surface of the substrate. The method also includes supporting the first and second substrates such that said surfaces face one another with a gap therebetween, adjusting the gap between the first and second substrates to a micrometre level of precision, and further adjusting the gap to a nanometre level of precision. The first and second substrates are heated to an elevated temperature so as to fluidify the film coating. The method also includes applying an electric field across the gap between the facing surfaces of the first and second substrates and controlling the electric field so that the film coating adopts a profile of pillars at locations on the first substrate that correspond to the pillars protruding from the second substrate to thereby generate a surface form of said first substrate.

As used herein, fluidifying of the film coating means that the coating can flow, but is not melted.

The elevated temperature is preferably above a transition temperature for fluidifying of the film coating. The transition temperature may be a glass transition temperature. The elevated temperature may be 170°C or above.

Controlling the current is preferably used to control electrohydrodynamic instabilities in the film coating to provide a patterning of the pillars spanning the gap between the substrates.

The gap may be adjusted to a micrometre level of precision using a micrometer. The gap may be adjusted to a nanometre level of precision using one or more piezo actuators. The gap may be adjusted to a micrometre level of precision by adjusting the position of the second substrate. The gap may be adjusted to a nanometre level of precision by adjusting the position of the first substrate.

As used herein, the term “gap” will be understood as referring to an interval or space between the upper and lower substrate. The gap is generated from the positioning of the upper and lower substrates when mounted onto the attachment means.

By “adjust the gap”, as used herein, it will be understood that the actuating means can increase or decrease the size of the gap between the upper and lower substrate. The gap may be adjusted by adjusting a position of the attachment means. Advantageously, the adjustment of the gap by the actuating means reduces the variation in the size of the gap between the upper and lower substrates across the surface area of the substrates.

Following adjustment, the size of the gap may be nanoscale. For the purposes of the present invention, nanoscale will be understood to refer to a gap of at least 1nm and no more than 100nm. Typically, following adjustment the size of the gap may be in the range 20nm to 250nm.

The second substrate may comprise a surface having a template pattern of protruding pillars with the pillars protruding from the second substrate surface towards said surface of the first substrate. The step of applying an electric field across the gap between the facing surfaces generates the pattern of pillars at locations on the first substrate that correspond to the pillars protruding from the second substrate.

The template form of the second substrate will be understood to refer to an arrangement or pattern which serves as a guide pattern for the fabrication of the surface form. Thus, by “corresponds to”, the surface form will be understood to comprise a pattern or arrangement which correlates to the pattern or arrangement of the template. The protrusions advantageously result in a laterally varying electric field between the upper and the lower substrate, which may cause increased electrostatic pressure in the vicinity of the protrusions. The speed at which the film coating is destabilised may be increased in the vicinity of the protrusions. The destabilised film coating may thus be drawn towards the protrusions so that the surface form corresponding to the template is generated.

It will be appreciated that the pillars of the first substrate, once adopted, are spaced from one another. The step of adjusting the gap between the first and second substrates to a micrometre level of precision, and further adjusting the gap to a nanometre level of precision may be used to control the spacing between adjacent pillars.

The spacing between adjacent pillars may additionally or alternatively be controlled by the strength of the applied electric field.

- 5 The method may further comprise an additional step of applying a metal nano-layer to the film coating. The metal may be a plasmon-active metal, and may comprise or consist of one or more noble metals, such as silver, platinum, indium, aluminium, copper, lithium, sodium, potassium or gold. The metal may comprise or consist of gold. Gold is a particularly suitable choice as it is much more stable than, for instance, silver, resulting in substrates that provide more consistent signals.
- 10

- The film coating is preferably at least 50nm thick. The film coating may have a thickness in the range 100nm to 500nm. The film coating is preferably no more than 700nm thick. The film coating may comprise or consist of polystyrene. The thickness of the at least one film coating on the surface of the substrate may control the spacing of the pillars of the first substrate.
- 15

- In some embodiments the ratio of the thickness of the at least one film coating on the surface of the substrate to the gap between the first and second substrates is at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8.
- 20

- The ratio of the thickness of the at least one film coating on the surface of the substrate to the gap between the first and second substrates may be at least 0.2 and no more than 0.8.
- 25

- In some embodiments the ratio of the thickness of the at least one film coating on the surface of the substrate to the gap between the first and second substrates is at least 0.3 and no more than 0.8.

- 30 In some embodiments the ratio of the thickness of the at least one film coating on the surface of the substrate to the gap between the first and second substrates is 0.5.

Before the substrate is released, it is preferable to maintain the electric field while the temperature is allowed to drop to below the fluidifying transition temperature - i.e., the

glass transition temperature of the polymer. This helps to preserve the final structure intact and to avoid fluctuations in the polymer and distortion of the patterns.

5 After generating the surface form, the method preferably comprise an additional step of releasing the first substrate. The method may further comprise the step of applying octadecyltrichlorosilane (OTS) between the upper and lower substrate to facilitate the release of the first substrate. The OTS may be applied by surface grafting a self-assembled monolayer. The monolayer may be grafted before assembling the substrates in the apparatus. The method may further comprise a step of rendering the
10 surface apolar by, for example, silanization. The substrate is released manually. It is easily disassembled due to the non-sickly self-assembled layer as described above.

By improving the consistency of the pattern, the method of the present invention thus provides a method for the fabrication of a platform for use in SERS with improved
15 reproducibility and enhancement factor.

As the skilled person will appreciate, "aspect ratio", as used herein, refers to the proportional relationship between the width and the height of a feature. In the context of the present invention, aspect ratio thus refers to the proportional relationship between
20 the width and the height of a surface form, such as a pillar.

By "enhancement factor", as used herein, this will be understood to refer to the magnitude of enhancement of signal in SERS. This is a known term to the skilled person.

25 The at least one film coating may comprise one or more polymers. In some embodiments the polymer(s) may comprise or consist of one or more of ethyl cellulose, polyvinyl alcohol, polyvinyl acetate or polystyrene. Such materials are suitable because they can be spin-coated or drop-cast (e.g., deposited) into a homogeneous
30 thin film (without undergoing phase separation or dewetting), and can be fluidified (can flow but are not melted) and therefore have a transition temperature such as a glass transition, softening point or crystallisation temperature. In some embodiments the polymers may comprise or consist of polystyrene. Polystyrene has the advantage of being inexpensive and has been found to perform well. Other suitable materials may

include PMMA, block-copolymers, hybrid materials (e.g., incorporating nanomaterials such as CNTs, nanoparticles etc. in a polymer matrix) and glassy materials.

5 In some embodiments the one of the upper and lower substrates comprises a plurality of film coatings, for example at least two, at least three, at least four or at least five film coatings. In some embodiments the one of the upper and lower substrates comprises at least three film coatings. Where the substrate comprises a plurality of film coatings, each film coating may comprise a different polymer.

10 The method may further comprise a preliminary step of depositing the at least one film coating onto one of the upper and lower substrates. Deposition may comprise spin-coating the one of the upper and lower substrates. Spin-coating is a method of coating known to those in the art. Briefly, spin-coating may comprise coating the substrate in a precursor solution to the film coating, followed by rotation at high speed, for example
15 100-3000 rotations per minute.

The precursor solution may comprise one or more polymers. In some embodiments the solution comprises the polymers at a concentration in the range 1wt% up to 10wt%. Prior to spin-coating, the precursor solution may be filtered.

20

In some embodiments, prior to the deposition of the film coating onto the one substrate, one or both of the upper and lower substrates are cleaned. Cleaning may comprise application of a solution comprising acid, for example sulphuric acid. Cleaning may further comprise an additional step of rinsing the substrate in water. The water may be
25 deionised.

In other embodiments cleaning comprises application of water comprising detergent. The cleaning step may be carried out at a temperature of at least 50°C, or up to 80°C or above. Cleaning may further comprise sonic cleaning and/or irradiation.

30

The cleaning of the one or both of the upper and lower substrates may further comprise applying a snow jet to the one or both substrates.

35

In embodiments where a pattern of pillars is generated, the method may comprise a further step of checking the homogeneity of the electrohydrodynamically generated

pillars. For instance, pillars having the same aspect ratio will appear as one colour – e.g. a plurality of blue dots. Pillars having different aspect ratios will appear as different colours e.g. as a plurality of different coloured dots.

- 5 A third aspect of the invention provides the platform for Surface Enhanced Raman Scattering producible by the method of the second aspect.

A fourth aspect of the invention provides a platform for Surface Enhanced Raman Scattering. The platform comprises a substrate with a polymer surface form on the
10 substrate. The surface form comprises an array of pillars protruding from the surface, each of the pillars having a diameter in the range 500-850nm and an aspect ratio in the range 0.6-1.0.

In some embodiments each of the pillars has a diameter in the range 500-750nm.
15

Without wishing to be bound by theory, the present inventors believe that the uniform size and, optionally, spacing of the pillars means that the adjacent pillars of the present invention are not plasmonically coupled to each other, unlike some other submicrometer SERS structures. Due to the uniform size and optionally, spacing of the
20 pillars, plasmon-polariton and plasmon resonance enhancement can be localised to the top surface of each pillar. Thus, advantageously, SERS enhancement can arise from a single pillar.

It is therefore envisaged that each individual pillar can function as an isolated detection
25 centre.

Thus, in some embodiments, each pillar is an individual detection centre. Since each of the pillars in an array of pillars can act as an individual detection centre, it is envisaged that the array may enable the detection of more than one target molecule in a sample.
30 This may advantageously provide a platform for multiplex SERS.

It will be appreciated that the pillars are spaced from one another. In some embodiments, the spacing between adjacent pillars is at least 1µm and no more than 10µm. The spacing between adjacent pillars may be at least 1µm and no more than
35 5µm. In some embodiments, the spacing between adjacent pillars is at least 1.5µm and

no more than 2um. Without wishing to be bound by theory, the present inventors believe that the spacing of the pillars assists in each pillar acting as an individual detection centre.

5 In some embodiments the platform comprises a substrate with a polymer surface form on the substrate, wherein the surface form comprises an array of uniformly sized pillars protruding from the surface, each of the pillars having a diameter in the range 500-850nm and an aspect ratio in the range 0.6-1.0.

10 In some embodiments the platform comprises a substrate with a polymer surface form on the substrate, wherein the surface form comprises an array of uniformly sized pillars protruding from the surface, each of the pillars having a diameter in the range 500-750nm and an aspect ratio in the range 0.6-1.0.

15 By "uniform" it will be understood that the protrusions match each other. For example, the diameter of a protrusion may substantially match the diameter of another protrusion. The height of a pillar may substantially match the height of another pillar.

20 The platform may further comprise a metal nano-layer coating on the surface form. The metal is preferably a plasmon-active metal. The metal may comprise or consist of gold.

The surface form may comprise or consists of at least two polymer layers.

25 Preferably the pillars have an average height of 475-500nm. Preferably the pillars have diameters of 550-625nm. The pillars may have an aspect ratio of approximately 0.8. The pillars may each have a diameter of approximately 625nm.

30 Preferably the area of the surface formed with these pillars is at least 0.25 mm². The substrates placed in the apparatus may be of any suitable size, for example 1cm x 1cm or 0.5cm x 0.5cm. The patterned area for SERS may typically be 0.5mm².

It will be appreciated that each pillar has a top surface. In some embodiments the top surface of each pillar is concave i.e. a portion of the top surface curves inwards towards the substrate.

In some embodiments the top surface of each pillar is roughened. Without wishing to be bound by theory, the inventors believe that the provision of a roughened top surface provides a further level of signal enhancement to each pillar.

5 The polymer surface form may have a blue colour.

Another aspect of the invention relates to use of the platform according to the third aspect in a method of analysing the composition of a sample. The method comprises: depositing the sample on the metal nano-layer coat of the platform; directing a laser
10 beam at the sample on the platform to promote Surface Enhanced Raman Scattering of the laser beam; collecting electromagnetic radiation scattered by the sample; and analysing the collected electromagnetic radiation scattered by the sample. A Raman scattered spectrum prepared from the collected electromagnetic radiation can be used to analyse the composition of the sample.

15 Another aspect of the present invention provides a method of analysing the composition of a sample. The method comprises depositing the sample onto a metal nano-layer coat on a polymer form on a substrate. The polymer form comprises an array of uniformly sized pillars protruding from the surface, each of the pillars having a
20 diameter in the range 500-850 nm and an aspect ratio in the range 0.6-1.0. The sample is subjected to Surface Enhanced Raman Scattering by directing a laser beam at the sample and detecting electromagnetic radiation emitted from the sample. A Raman scattered spectrum obtained from the detected electromagnetic radiation is then used to analyse the composition of the sample.

25 In some embodiments, each of the pillars has a diameter in the range 500-750nm.

It is envisaged that the sample may be directly deposited onto the metal nano-layer coat. Advantageously, this means that the sample does not require dilution in a carrier
30 fluid or any processing before being placed onto the metal nano-layer coat, even when the sample is solid. Without wishing to be bound by theory, the inventors believe that the same signal enhancement is achieved regardless of whether the sample is directly deposited onto the metal nano-layer coat or diluted in a carrier fluid before being deposited.

35

In some embodiments, analysing the composition of the sample may comprise detecting a change in the composition of the sample. In some embodiments, analysing the composition of the sample comprises detecting the presence or absence of one or more target molecules in a sample, or detecting an increase or decrease in the level of one or more target molecules in a sample.

In some embodiments, the method may be used to detect changes in the conformation of one or more target molecules within a sample. For example, since SERS surfaces are highly sensitive and specific, small changes in the vibrational or rotational modes of molecules enabled very small changes in the conformation of molecules (such as polysaccharides) to be detected via the shifts in the Raman signal.

In some embodiments, the method further comprises applying a label. The label may be specific for one or more target molecules within the sample. For example, the label may comprise an antibody or aptamer. The label may be conjugated. The label may be applied to the sample prior to deposition of the sample onto the metal nano-layer coat. Alternatively, the label may be applied to the metal nano-layer coat prior to deposition of the sample onto the metal nano-layer coat.

In other embodiments, the method does not comprise applying a label to the sample.

In embodiments comprising applying a label, the method may comprise subjecting the label to Surface Enhanced Raman Scattering prior to subjecting the sample + label to Surface Enhanced Raman Scattering. In this way, the label signal can be obtained and subtracted from the sample + label signal so as to avoid background signal.

In some embodiments, analysing the composition of the sample comprises comparing the Raman scattered spectrum of the sample with a reference spectrum. The reference spectrum may be, for example, a signature or 'fingerprint' spectrum of a target molecule. In some embodiments, the reference spectrum is obtained from a control sample of which the composition is known. However, it will be appreciated that the reference spectrum can be any spectrum which is useful for comparison. In some embodiments, the sample spectrum is compared with the spectra of one or more samples obtained at different time points, or at different locations. For example, in healthcare applications, a reference spectrum may be spectrum of a sample obtained

from a healthy subject, or the spectrum of a sample obtained before or after treatment. In some embodiments, the sample spectrum is compared with at least two reference spectra. For example, a patient sample spectrum may be compared with a signature spectrum of a biomarker of interest and also with a spectrum of a control sample
5 obtained from a healthy subject (i.e. a subject who is known not to be suffering from the disease or injury which the patient is suspected of having).

The one or more target molecules may have a concentration in the sample of less than $10 \times 10^{-12} \text{ mol/dm}^3$.

10

The platform and the method described above may be used for detecting the presence, absence, or change in the level of any target molecule. Target molecules may be, for example, proteins, peptides, antibodies, enzymes, amino acids, nucleic acids, polysaccharides or fatty acids. It will be appreciated that the invention is not restricted
15 to the analysis of a single target molecule, but it may be used to detect multiple target molecules simultaneously. For example, the total chemical or biochemical composition of a sample (e.g. a plasma sample) can be analysed.

The invention thus finds utility in a range of applications such as diagnosis and
20 monitoring of disease (in human and animal health), food and safety (e.g. the detection of pathogens and toxins), environmental analysis (e.g. water purity, contamination) and security (e.g. chemical and biological agents).

The composition of the sample may be analysed to detect the presence or absence of
25 one or more target molecules in a sample, and/or detect an increase or decrease in the level of one or more target molecules in a sample. In some embodiments the one or more target molecules are biomarkers. The biomarkers may be indicative of a disease or injury.

The present invention thus provides a method and a platform for the diagnosis and/or
30 monitoring of a disease or injury in a subject, and/or for determining the severity of disease or injury. The disease or injury may be cancer, immune disease or a neurodisorder. In some embodiments the injury is traumatic brain injury (TBI).

In some embodiments the biomarkers are selected from the group consisting of lipoprotein (REF), glial-astrofibrillary protein (GFAP), neuron-specific enolase (NSE), N-acetyl aspartate (NAA), Interleukin-1 (INT-1), S100B calcium-binding protein B and ubiquitin C-terminal hydrolase (UCH-L1). These biomarkers have been shown to correlate with severity of TBI.

The sample may be a sample of biological fluid. In some embodiments, the sample is or comprises blood, serum, plasma, saliva, cerebrospinal fluid or urine.

In some embodiments, the biomarker is NAA. The sample may be (whole) blood, serum or plasma. It has been found for the first time that NAA levels increase in the blood of TBI patients following injury.

Thus, according to a further aspect of the invention, there is provided a method of diagnosing and/or monitoring traumatic brain injury (TBI) in a subject, the method comprising detecting NAA in a blood sample obtained from the subject.

Traumatic brain injury occurs when an external force traumatically injures the brain. There are different systems for classifying TBI based on, for example, severity, type of injury and prognosis. The most commonly used system for classifying TBI is the Glasgow Coma Scale (GCS), which grades a person's level of consciousness on a scale of 3–15 based on verbal, motor, and eye-opening reactions to stimuli. In general, a TBI with a GCS score of 13 or above is defined as mild, 9–12 as moderate and 8 or below as severe. Another system, the Mayo Classification System, has three main classifications including definite moderate-severe TBI, probable mild TBI, and possible TBI. Multiple criteria are used in each diagnosis including loss of consciousness, post-traumatic amnesia, skull fracture, and evidence of neuroradiological abnormalities including subdural haematoma, cerebral contusion, and hemorrhagic contusion. The classification of TBI using the GCS or Mayo systems will be known to those skilled in the art.

The TBI may be mild TBI (mTBI), moderate TBI or severe TBI (sTBI). In some embodiments, the TBI is moderate-to-severe TBI (m-sTBI). As used herein, references to “mild”, “moderate” and “severe” TBI are made in accordance with the GCS.

References herein to “moderate-to-severe” TBI encompass both moderate and severe TBI in accordance with the GCS.

In some embodiments, the subject is human.

5

In some embodiments, “detecting NAA”, as used herein, comprises determining a level of NAA in the sample. In some embodiments “detecting NAA” comprises determining whether the level of NAA in the sample is increased or decreased, or substantially unchanged, relative to a reference. Suitable methods of detecting a change in the level of a target molecule, are described hereinabove. In some embodiments, the NAA is detected using SERS. The NAA may be detected using a platform or method as described herein.

10

15 Brief description of the drawings

Embodiments of the invention will be described below by way of example and with reference to the accompanying Figures:

20 Figure 1a illustrates schematically production of a platform form using a known hierarchical electro-hydrodynamic (HEHD) process;

Figure 1b illustrates schematically a platform having a surface form with pillars having a 0.8 aspect ratio;

25 Figure 1c illustrates schematically production of a platform with a surface form, in accordance with embodiments of the invention.

Figure 2 illustrates an apparatus for forming a platform in accordance with embodiments of the invention.

30 Figure 3 shows: dilution and SERS measurements curves for (a) Benzenthionol (BT) and (b) N-acetyl aspartate (NAA); a calibration graph (c) for BT and the corresponding regression curve (d).

Figure 4 shows: (a) representative SERS spectra of BT measured from various (n=17) RED fabricated substrates at 3 different locations; (b) a reproducibility histogram of relative enhancement factor of RED-SERS substrates showing nearly 90% reproducibility across the various RED substrates.

Figure 5 shows (a) a Finger Print Spectrum of NAA in solution and (inset) as a solid, and (b) representative spectra of an NAA spiked blood sample in comparison to the whole blood only.

Figure 6 shows Raman Shift spectra demonstrating detection of NAA from samples of (a) TBI only and (b) TBI + EC injuries patients compared to a reference (non-TBI patients) and showing the characteristic NAA finger print from fluid.

Figure 7 shows first and second principal component scores for each of a TBI and non-TBI patient, detecting NAA from plasma.

Figure 8 shows a series of uniform pillars of a RED fabricated substrate in accordance with embodiments of the invention.

Detailed description

Embodiments of the present invention focus on an improved method of fabrication of substrates that make up the SERS platform. The objective is to provide cost-effective, highly-reproducible enhancing platforms for rapid SERS detection based on a single-step reproducible electro-hydrodynamic (RED) process. These RED substrates are preferably tailored for highly-sensitive biomarker sensing and optimised for high SERS-enhancement. The RED-SERS sensitivity and specificity may be demonstrated by the detection of traumatic brain injury (TBI) biomarkers down to a pico-molar range (less than $10 \times 10^{-12} \text{ mol/dm}^3$) from complex biofluids including blood and serum. Detection of TBI-indicative biomarkers will find diagnostic applications in brain-injury point-of-care scenarios. However, the versatile nature of the substrates and method of fabrication will enable extension for timely-diagnostics of a range of major diseases.

The invention is based on controllable, reproducible electro-hydrodynamic (RED) sub-microstructures engineered for rapid-SERS detection. These RED-SERS active micro-substrates are fabricated using an innovative apparatus combining a micromanipulator and piezo actuators for finest adjustments, enabling highly parallel, capacitor-like EHD patterning for homogeneous fabrication of pillars. The tuneability of the pillar aspect ratio and its effect on strength of plasmon resonances and SERS allows the optimization of substrates for different laser excitation wavelengths. Pillars are then covered by a thin nano-layer of a plasmon-active material, such as gold, and enable detection of analytes, e.g. bio-analytes, of interest within a sample in contact with the prepared surfaces using rapid Raman measurements. N-acetyl aspartate (NAA),

S100B – a calcium-binding protein B of the S-100 protein family and a validated TBI biomarker – and glial-astrofibrillary protein (GFAP) are TBI-indicative biomarkers that have been detected using these fabricated substrates directly from blood and serum down to pico-molar range. Combined computational analysis including the principal component analysis (PCA) allows further discrimination of the minute quantities within the biofluid samples. (PCA) is a well-known and established method in the field of spectroscopy, particularly Raman spectroscopy. Importantly, technological versatility of these substrates is not limited to TBI and can be applicable in many clinical areas which will accelerate the successful early-diagnosis of major diseases.

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The fabrication of substrates using an electro-hydrodynamic (EHD) process involves subjecting initially homogeneous dielectric fluidified films to an electric field. This gives rise to a destabilizing electrostatic pressure at the interface between the fluidified dielectric and air, which arises from the coupling of the interfacial displacement charges to the electric field. This pressure couples to the capillary wave spectrum of the fluidified dielectric interface, overcoming the compensating surface tension, and amplifying interface undulations with a characteristic wavelength. This instability develops over time, and the amplification of a narrow band of wavelengths draws in material from the surrounding film, eventually detaching into individual surface structures, or pillars, thus forming an array of pillars across the substrate.

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The fabrication of EHD substrates is typically performed by applying a voltage between a top plate and a bottom plate, or substrate, onto which a thin film of dielectric has been deposited.

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Typically a solution of the relevant material being patterned is prepared at concentrations of 2–3wt%, although other concentrations between about 1wt% and 10wt% may be used, followed by filtering through a poly-tetra-fluoro ethylene (PTFE) membrane with a pore diameter of 100 nm. Subsequently, thin films with thicknesses on the order of 100-500nm are deposited via spin-coating an adequate amount of solution onto a substrate (e.g. silicon or ITO glass) used as bottom electrode. This may be accomplished by wetting the substrate, held in a spinning vacuum chuck, with a certain amount of solution and subsequently rotating it at a high speed, e.g. 100–3000 rotations per minute.

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The thin films may be spin-cast onto the substrates from, for example, toluene or chloroform solutions. Prior to spin-coating, the substrates may be cleaned in a 'Piranha' solution consisting of 3:1 H₂SO₄ (98%):H₂O₂ (30%), followed by thorough rinsing with deionised water and drying under nitrogen. Where the ITO glass is used as a substrate, the ITO-coated glass slides may be cleaned by scrubbing in soap water at 75°C, washing in an ultrasonic bath with acetone and isopropanol, followed by irradiation for 20-30 minutes in an UV-ozone cleaner. Immediately before device assembly, all substrates and electrodes may be subjected to snow-jet cleaning.

In the apparatus used for electro-hydrodynamic patterning (see, for example, "Hierarchical Electro-hydrodynamic Structures for Surface-Enhanced Raman Scattering" (Advanced Optical Materials – Adv. Mater. 2012, 24, OP175-OP180), Goldberg-Oppenheim et al.) a slight misalignment of the capacitor plates results in a wedge geometry with a variation in electrode spacing ranging from 100 nm to 1 µm across a 1 cm wide substrate. This produces a variation across the substrate in the aspect ratio of the pillars formed, as shown in Figure 1a. The present inventors have constructed an apparatus that can be used to produce a much more uniform aspect ratio of pillars across a substrate in a highly repeatable and controllable manner. This is described further below.

The present inventors have also established that the highest enhancement factor (EF) of gold covered SERS active pillars is achieved when the colour of the pillars themselves is blue, corresponding to a pillar aspect ratio of 0.8, as shown in Figure 1b (see further discussion below). They therefore designed and fabricated a dedicated top electrode having downward protruding pillars (typically of 625nm in diameter). When a voltage is applied across the plates, this top electrode generates a laterally varying electric field. The liquid film material is drawn towards the protrusions of the top plate where the electrostatic pressure is the highest, and the destabilisation process is the fastest, faithfully reproducing the imposed patterns as shown in Figure 1c.

In order to carry out the RED patterning process, a rig was designed that allows for several degrees of freedom of movement, and therefore, well-aligned positioning of the top electrode parallel to the bottom thin film. As shown in Figure 2, the rig 20 consists of a base 22 made, for example, of aluminium. Extending upwards from the base is a pillar 24, which in the depicted embodiment is a rectangular glass pillar glued to the

base 22. Attached through an opening 26 in the base is a micrometer 28, which has a non-rotating head 32 with a copper block 30 mounted on the micrometer spindle. A bottom substrate 34 is mounted on the copper block 30. A spring clamp (e.g. a thin beryllium copper spring clamp, not shown), may be used to hold the bottom substrate 34 in place on the copper block 30, also ensuring electrical connection of the bottom substrate to ground.

Spars 36, 38, which extend from the pillar 24, support a top substrate assembly 40 suspended over the bottom substrate 34. The assembly 40 includes a support arm 42 underneath which is mounted a first piezo-actuator 44, an upper block 46, a second piezo-actuator 48, a lower block 49 and the top substrate 50. The top substrate 50 is preferably clamped to the assembly 40 using a spring clamp (not shown), for example a thin beryllium copper spring clamp. The beryllium copper spring clamps are preferred because they are thinner than the height of the silicon substrates and hold the substrates at their edges to allow a bias voltage to be applied to the top and bottom substrates.

Where the substrates 34, 50 comprise a non-conductive material such as silicon wafers, these may be adapted to become electrodes by vapour depositing a metallic layer on the unpolished backside of the wafer – for example a 10 nm chromium layer, followed by a 100 nm gold layer. Wires 52, 53, 54 provide connection to a power source for applying an electrical bias voltage across the top and bottom electrodes/substrates 34, 50, for example via the thin beryllium copper spring clamps referred to above, and for actuating the first and second piezo-actuators 44, 48.

The pillar 24, spars 36, 38 and blocks 46, 49 are preferably made of glass and attached using a suitable adhesive. Glass is particularly suitable because it has a low coefficient of thermal expansion, which is important for controlling the gap (and therefore, the alignment of the electrodes) on such a small nanoscale because any minute change in the size of the substrate supports due to heat will impact the gap. Seemingly minor changes can considerably influence the gap and the alignment of the electrodes. Glass ensures and that there is very little movement due to thermal expansion.

The assembled components form a capacitor device in which the lower substrate 34 acts as the lower electrode, and onto which is applied a thin film of dielectric 56 (e.g. a suitable polymer). Facing it, is a planar or topographically structured top substrate 50, typically comprising a silicon wafer, and mounted leaving a thin nano-gap 58 between the electrode/substrates 34, 50. The micrometer 28 allows 'coarse' micro-adjustment of the gap between the top and bottom electrode within a few microns. The piezo-actuators 44, 48 are wired in parallel and allow a very fine adjustment of the gap (on the nanometre scale).

The whole rig 20 can be heated to an elevated temperature, for example 170°C or above (above the glass transition temperature of the polymers to be patterned) by placing the rig inside a heated chamber (not shown). Preferably the base 22 of the rig 20 rests on the floor of the heated chamber with the micrometer 28 protruding downwards below and outside the chamber. This allows the micrometer 28 to be adjusted while the rig is hot to give a relatively rough adjustment for setting the gap 56 between the top and bottom electrodes/substrates 34, 50. Fine adjustment of the gap 58 is achieved using the piezo actuators 53, 54, which enable the gap to be set with nanometre accuracy.

Alternatively the assembly 40 may be provided with a heating element to heat the electrodes/substrates to an elevated temperature. The heating element may be placed adjacent to or even inside the copper block 30. The heating element may be an electrical heating element with wires for providing power from an electrical supply.

A temperature sensor such as a thermocouple or resistance thermometer may be used to sense the temperature to which the electrodes/substrates are heated.

A sensor, for example an in-built voltammeter, enables in-situ monitoring of the current that is being drawn by the rig during the RED process. This allows control of the electrohydrodynamic instabilities, which results in patterning of the pillars spanning the capacitor gap with very precise and controllable dimensions. Parameters can be set and tuned to provide highly accurate and reproducible dimensions. These parameters may include the initial film thickness, the inter-electrode gap, the generated electric field inside the capacitor device, the patterning and termination times and surface

tension. All of these enable fine tuning of the final morphologies to fabricate optimal SERS active platforms.

In use, the bottom substrate 34 will typically be the “first” substrate onto which the thin film of dielectric has been deposited, the top substrate 50 being the “second” substrate. To ensure that the substrates 34, 50 are as parallel as possible, the two substrates are brought together by adjusting the micrometer 28 until the substrates are pressing tightly together, and then the copper block is secured by a screw clamp (not shown) onto the micrometer head 29. Then, when the micrometer 28 is backed away, the gap 58 remains parallel. When the whole device is heated up the gap 58 can be coarsely adjusted using the micrometer 28 and finely adjusted using the piezo actuators 44, 48. The size of the gap 58 can be gauged by measuring the current being drawn between the top and bottom electrode/substrates 34, 50 and adjusted using the piezo actuators 44, 48 while the process completes.

The top electrode/substrate 50 (i.e. the electrode/substrate that does not have the dielectric film on it) may have a planar surface. However a further option is to use a pre-patterned surface, which advantageously can allow greater control and repeatability in the formation of a pattern of uniform pillars of the dielectric on the facing (lower) substrate 50. The top (second substrate may comprise a surface having a template pattern of protruding pillars with the pillars protruding from the second substrate surface towards the surface of the first substrate. When an electric field is applied across the gap between the facing surfaces, this generates a pattern of pillars at locations on the bottom (first) substrate 34 that correspond to the pillars protruding from the top (second) substrate 50. The template form of the top (second) substrate 50 has an arrangement, or pattern, of protrusions which serve as a guide pattern for the fabrication of the surface form of the bottom (first) substrate 34. The protrusions advantageously result in a laterally varying electric field between the upper and the lower substrate, which cause increased electrostatic pressure in the vicinity of the protrusions. The speed at which the film coating is destabilised is increased in the vicinity of the protrusions. The destabilised film coating is drawn towards the protrusions so that the surface form corresponding to the template is generated.

The shape and the dimensions of the pillars are controlled and determined by the experimental parameters. The spacing between the two electrodes, the initial film

thickness, the surface tension of the polymer being patterned, the voltage applied and the time of patterning. The typical shapes generated are pillars with average height of 475-500nm and 550-625nm diameters, dimensions which can be easily tuned via the above listed experimental parameters.

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Accordingly, an exemplary method of forming a substrate starts with spin coating a thin polystyrene film on a Silicon substrate. The appearance of the film may have various colours depending on the thickness. A dark navy blue colour typically corresponds to 100nm thickness of the initial homogeneous film. The effect of a wedge geometry as occurs in the prior methods referred to above, will typically result in a continuous variation of the pillar aspect ratios resulting in a "rainbow" of different colours (typically from purple, pink, though brownish to green and blue). The various colours of the electrohydrodynamically generated pillars originate from their physical dimensions i.e., height and diameter, or aspect ratio. There can be various dimensions and therefore, various aspect ratios. The inventors have found out that when coating the various aspect ratio pillars with a thin gold nano-layer for SERS purposes, the substrates give various degrees of enhancements. For instance, pillars with an aspect ratio 0.75 (green ones) give nearly 30-times enhanced SERS signal compared to the lowest aspect ratio of 0.61 (purple/pink ones). Green and blue give the highest enhancement for SERS. The blue pillars typically have average heights of 475-500nm and diameter of 625nm.

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Thus, the colour of the platform may be used to check the homogeneity of the electrohydrodynamically generated pillars. For instance, pillars having the same aspect ratio will appear as one colour – e.g. a plurality of blue dots. Pillars having different aspect ratios will appear as different colours e.g. as a plurality of different coloured dots.

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To successfully replicate only the blue pillars so as to provide consistently reproducible structures and signals, a patterned top electrode with a protruding structure of pillars having a diameter of 625nm may be used to form a positive replica from the polymer film by guiding the material towards the protruding pillars where the electrostatic pressure is the highest and the patterning is the fastest.

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Moreover, the most dominant factor in the aspect ratio affecting the performance of the formed structures has been found to be the height (and not the width). Many structures

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have been produced that are around 475-500nm in height, and the overall surfaces appear blue.

Before releasing the substrate with the newly formed patterned template surface, it is greatly preferred to keep the electric field on until the temperature has reached below the softening point - i.e., the glass transition temperature of the polymer - to preserve the final structure intact and to avoid fluctuations in the polymer and distortion of the patterns. If the electric field is disconnected before the structures are quenched and solidified, even slight fluctuations or gradients in temperature can impact the final morphology.

To facilitate the release of the patterned template surface, the surface was rendered apolar by surface grafting an octadecyltrichlorosilane (OTS) self-assembled monolayer. The monolayer is grafted before assembling the substrates in the apparatus. A monolayer can be prepared by a simple immersion of a substrate in a solution containing alkane chains such as octadecyltrichlorosilane (OTS) with actively binding end-groups. OTS forms covalent bonds with the oxygen groups of a silicon oxide surface and self-assembles into a highly-ordered monolayer. This chemical reaction of silanization renders the oxidized Si surface apolar (low surface energy), and thus, provides a route to reduce adhesion of the patterned material to the silicon surface. For the present purpose, a non-stick SAM on SiO_x was deposited from a liquid OTS phase. In order to remove all organic contaminations and hydroxylate the surface, SiO_x covered substrates were first cleaned using snow-jet followed by immersion in Piranha solution (a mixture of sulfuric acid and hydrogen peroxide) rinsing with ultra-pure (MilliporeTM) water and drying in a stream of a dry nitrogen gas. Silanization was performed by immersion of the substrate in a freshly prepared silane solution (0.25% OTS in hexadecane). Physisorption of the OTS monolayer on the substrate surface proceeds via silanes binding to hydroxyl-terminated SiO_x layer. After drying in dry nitrogen gas, hydrophobic water contact angles, typically above 100° , were obtained, which are sufficiently high for these experiments.

These RED generated surfaces comprise pillars, each of which acts as an individual enhancing/detection centre. The pillars are preferably then coated with a thin metallic nano-layer, for example a gold layer, to generate EHD-SERS enhancing surfaces that can enable detection of concentrations down to a sub-picoMolar (pM) level.

The limit of detection (LoD) of the RED substrates was established experimentally to be $6.7 \times 10^{-14} \text{M}$ (mol/litre) for standard Benzenethiol (BT) molecules (Fig. 3a) and $1.2 \times 10^{-13} \text{M}$ for NAA from blood samples (Fig. 3b), which for a biomarker with a molecular weight of 175.139 kg/mol is equivalent to 0.021pg/mL. Limits of quantification (LoQ) were found to be $2 \times 10^{-13} \text{M}$ and $3.8 \times 10^{-13} \text{M}$, correspondingly (Fig. 3c-d).

Reproducibility of the enhanced signal of fingerprint signatures was established by making measurements at three random locations on each substrate for 17 sample substrates, each fabricated individually using the described above rig. The Raman Shift spectra of the samples are shown in Fig. 4a. The estimated reproducibility coefficient was found to be 5%, meaning that the absolute difference between any three future measurements made on a particular substrate is estimated to be no greater than 5% on 95% of occasions. Furthermore, the correlated RED-SERS based EF calculation results from 17 individual substrates show that most EF values (89%) are narrowly distributed in the range from 7.3×10^9 to 7.8×10^9 , and a small population of the EF values were observed to be above 8×10^9 (Fig. 4b).

Spectra of blood or plasma samples of patients with severe TBI (sTBI) were compared with a developed library of Raman spectroscopic signatures of biomarkers of interest. Initially the study focussed on the N-acetyl aspartate (NAA) biomarker, which is one of the most abundant molecules present in the central nervous system and due to its exclusive localization in neurons, reflects the functional status of neurons and axons in the brain. NAA is thus considered as a marker for neuronal health and viability and is a sensitive measure of neuronal compromise. First, NAA fingerprint spectra (in both solid and liquid states) were acquired and the characteristic peak signatures corresponding to specific structural and composition information were identified (Figure 5(a)). The spectrum of blood spiked with the NAA biomarker was also obtained (Figure 5(b)). Following this, blood and plasma samples were obtained from 25 healthy individuals (control group) and 37 sTBI subjects. Based on visual inspection, Raman spectra of blood plasma originating from the different groups revealed specific differences at certain peaks. Figure 6a shows a typical blood plasma SERS spectrum of sTBI patients (middle line), the average spectrum for healthy control cohort (bottom line) and the SERS signature of the NAA biomarker (top line) for comparison. The same comparison is made in Figure 6b for sTBI patients with extracranial (EC) injury.

The mean spectra show prominent variability between sTBI and the non-TBI (healthy control group). These areas are indicated with vertical lines in Figures 6a-b. Several consistent differences can be observed, including for instance, (a) the heights of the peak at 930cm^{-1} and 1245cm^{-1} , which are significantly elevated in the blood plasma samples derived from sTBI patients, (b) the peaks of 682cm^{-1} , 717cm^{-1} , 1011cm^{-1} , 1168cm^{-1} and 1517cm^{-1} regions, which distinguish the sTBI and sTBI+EC lines from the controls. The intensity ratios of the peaks correspond to selected features of the NAA compound, including the amides corresponding to $\text{CH}_3\text{-NH-C=O}$: N-acetyl asymmetric CH_3 deformations *vib* (where *vib* stands for 'vibrations' - there will be a different vibration frequency for any give Raman active bond) with medium intensity at 1420cm^{-1} , wagging of C=O and twisting of -OH bonds at 682cm^{-1} , N-H deformations *vib* in the NH-CO-CH_3 composition at 1517cm^{-1} , characteristic of N-acetyl groups. NAA levels are therefore shown to increase in sTBI in the early stages. This biomarker has been detected within minutes from injury and for this reason represents a marker of the primary injury.

Following data collection, spectra were imported into MATLAB for subsequent data processing. Figure 7 shows the resulting clusters, with triangles being the reference (non-TBI) samples and the diamonds the blood plasma samples from TBI patients. Clusters are based on a linkage analysis. Performing the PCA analysis on the full data set reveals principal component loadings that recapitulate the differences that are apparent by visual inspection, as well as other, subtler differences, as shown in Figure. 6. In the sTBI cluster, 15 subjects were assigned to the sTBI only sub-group, while the other 21 subjects belonged to the sTBI+EC subgroup. The subtle spectral differences drawn out via PCA yielded scores plots effectively further discriminated the sTBI and sTBI+EC sub-groups versus healthy volunteers.

An atomic force microscopy (AFM) cross-section of a series of pillars of a RED fabricated substrate in accordance with embodiments of the invention is shown in Figure 8. As is evident from the Figure, the pillars are uniformly sized. In this particular embodiment the height of the series of pillars (as shown in relation to the y axis) is consistently around 780nm. This demonstrates the reproducibility and consistency of RED fabricated substrates fabricated by and in accordance with embodiments of the present invention.

CLAIMS:

1. Apparatus for fabricating a platform for Surface Enhanced Raman Scattering, SERS, the apparatus comprising:
 - 5 a first support for a first substrate that has a film on a first surface thereof;
 - a second support supporting a second substrate, wherein the second substrate has a surface facing the first surface of the first substrate, when supported, with a gap between the facing surfaces;
 - a first gap adjustment means for adjusting the gap between the first and second
 - 10 substrates to a micrometre level of precision;
 - a second gap adjustment means for further adjusting the gap to a nanometre level of precision;
 - a connection to a source of electrical power for applying an electric field across the gap between the facing surfaces of the first and second substrates;
 - 15 a sensor for sensing a current flowing through the first and second substrates;
 - a controller for controlling the current, and
 - means for heating the first and second substrates to an elevated temperature.
2. The apparatus of claim 1 wherein the means for heating comprises a heated
- 20 chamber surrounding the substrates.
3. The apparatus of claim 1 wherein the means for heating comprises a heating element disposed adjacent to the substrates.
- 25 4. The apparatus of any preceding claim wherein the first gap adjustment means comprises a micrometer.
5. The apparatus of any preceding claim wherein the second adjustment means comprises one or more piezo actuators.
- 30 6. The apparatus of any preceding claim wherein said surface of said second substrate comprises a pattern of pillars that protrudes towards said surface of said first substrate when supported.

7. The apparatus of claim 6 wherein said pillars have an aspect ratio of approximately 0.8.

5 8. The apparatus of claim 6 or claim 7 wherein said pillars have a diameter of approximately 625nm.

9. The apparatus of any preceding claim, wherein the first and/or second substrate comprises silicon or indium tin oxide.

10 10. The apparatus of any preceding claim wherein the first and/or second substrates comprise a layer of conductive material forming an electrode for application of the electric field.

11. The apparatus of claim 10 wherein the first support comprises an electrically
15 conductive block onto which the first substrate is mounted.

12. The apparatus of claim 6 wherein the first substrate is mounted to the electrically conductive block to provide an electrical connection to the first substrate.

20 13. The apparatus of any preceding claim wherein the first and/or second substrate is mounted using a metallic spring clamp that is thinner than the substrate.

14. A method of fabricating a platform for Surface Enhanced Raman Scattering, the method comprising:

25 providing a first and a second substrate;

wherein the first substrate comprises at least one film coating on a surface of the substrate;

supporting the first and second substrates such that said surfaces face one another with a gap therebetween;

30 adjusting the gap between the first and second substrates to a micrometre level of precision;

further adjusting the gap to a nanometre level of precision;

heating the first and second substrates to an elevated temperature so as to fluidify the film coating; and

applying an electric field across the gap between the facing surfaces of the first and second substrates so that the film coating adopts a profile of pillars to thereby generate a surface form of said first substrate.

5 15. The method of claim 14 wherein the elevated temperature is above a glass transition temperature of the film coating.

16. The method of claim 14 or claim 15 wherein the elevated temperature is 170°C or above.

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17. The method of any of claims 14 to 16 wherein controlling the current is used to control electrohydrodynamic instabilities in the film coating to provide a patterning of the pillars spanning the gap between the substrates.

15 18. The method of any of claims 14 to 17, wherein the gap is adjusted to a micrometre level of precision using a micrometer.

19. The method of any of claims 14 to 18, wherein the gap is adjusted to a nanometre level of precision using one or more piezo actuators.

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20. The method of any of claims 14 to 19, wherein the gap is adjusted to a micrometre level of precision by adjusting the position of the first substrate.

21. The method of any of claims 14 to 20, wherein the gap is adjusted to a
25 nanometre level of precision by adjusting the position of the second substrate.

22. The method of any of claims 14 to 21, wherein the second substrate comprises a surface having a template pattern of protruding pillars with said pattern of pillars protruding from said second substrate surface towards said surface of the first
30 substrate, and wherein the step of applying an electric field across the gap between the facing surfaces generates the pattern of pillars at locations on the first substrate that correspond to the pillars protruding from the second substrate.

23. The method of any of claims 14 to 22 further comprising an additional step of
35 applying a metal nano-layer to the film coating.

24. The method of claim 23 wherein the metal comprises or consists of gold.

5 25. The method of any of claims 14 to 24 wherein the film coating is at least 50nm thick.

26. The method of any of claims 14 to 24 wherein the film coating has a thickness in the range 100nm to 500nm.

10 27. The method of any of claims 14 to 24 wherein the film coating is no more than 700nm thick.

15 28. The method according to any of claims 14 to 27, wherein the film coating comprises a material that is flowable without being melted at an elevated temperature.

29. The method of claim 28 wherein the film coating comprises or consists of polystyrene.

20 30. The method according to of claims 14 to 29, wherein after generating the surface form, the method comprises an additional step of releasing the first substrate.

25 31. The method of claim 30 further comprising the step of applying octadecyltrichlorosilane between the upper and lower substrate to facilitate the release of the first substrate.

32. The platform for Surface Enhanced Raman Scattering producible by the method according to any of claims 14 to 31.

30 33. A platform for Surface Enhanced Raman Scattering, the platform comprising a substrate with a polymer surface form on the substrate, wherein the surface form comprises an array of uniformly sized pillars protruding from the surface, each of the pillars having a diameter in the range 500-850nm and an aspect ratio in the range 0.6-1.0.

34. The platform of claim 33, wherein each of the pillars has a diameter in the range 500-750nm.

5 35. The platform of claim 33 or 34, wherein each pillar is an individual detection centre.

36. The platform of any one of claims 33 to 35 wherein the platform further comprises a metal nano-layer coating on the surface form.

10 37. The platform of claim 36 wherein the metal is a plasmon-active metal.

38. The platform of claim 37 wherein the metal comprises or consists of gold.

15 39. The platform of any one of claims 33 to 38 wherein the surface form comprises or consists of at least two polymer layers.

40. The platform of any one of claims 33 to 39 wherein the uniform pillars have an aspect ratio of approximately 0.8.

20 41. The platform of any one of claims 33 to 40 wherein the uniform pillars each have a diameter of approximately 625nm.

42. The platform of any one of claims 33 to 41 wherein the uniform pillars each have a top surface which is concave.

25 43. The platform of any one of claims 33 to 42 wherein the uniform pillars each have a roughened top surface.

30 44. The platform of any one of claims 33 to 43 wherein the polymer surface form has a blue colour.

45. Use of the platform according to claim 36 or any one of claims 37 to 44 when dependent on claim 36 in a method of analysing the composition of a sample, the method comprising:

35 depositing the sample on the metal nano-layer coat of the platform;

directing a laser beam at the sample on the platform to promote Surface Enhanced Raman Scattering of the laser beam;

collecting electromagnetic radiation scattered by the sample; and

5 using a Raman scattered spectrum obtained from the detected electromagnetic radiation to analyse the composition of the sample.

46. A method of analysing the composition of a sample, the method comprising:

10 depositing the sample onto a metal nano-layer coat on a polymer form on a substrate, wherein the polymer form comprises an array of uniformly sized pillars protruding from the surface, each of the pillars having a diameter in the range 500-850nm and an aspect ratio in the range 0.6-1.0;

15 subjecting the sample to Surface Enhanced Raman Scattering by directing a laser beam at the sample and detecting electromagnetic radiation emitted from the sample; and

using a Raman scattered spectrum obtained from the detected electromagnetic radiation to analyse the composition of the sample.

20 47. The method of claim 46, wherein each of the pillars has a diameter in the range 500-750nm.

48. The method of claim 46 or claim 47, wherein analysing the composition of the sample comprises detecting a change in the composition of the sample.

25 49. The method of any one of claims 46 to 48, wherein analysing the composition of the sample comprises detecting the presence or absence of one or more target molecules in a sample, or detecting an increase or decrease in the level of one or more target molecules in a sample.

30 50. The method of claim 46, comprising detecting changes in the conformation of one or more target molecules within a sample.

35 51. The method of claim 50, wherein detecting changes in conformation of one or more target molecules comprises detecting changes in the vibrational or rotational modes of molecules via the shifts in the Raman signal.

52. The method of any one of claims 49 to 51 wherein the one or more target molecules are biomarkers.

5 53. The method of claim 52 wherein the biomarkers are indicative of a disease or injury.

10 54. The method of claim 52 or claim 53, wherein the biomarkers are selected from the group consisting of lipoprotein (REF), glial-astrofibrillary protein (GFAP), neuron-specific enolase (NSE), N-acetyl aspartate (NAA), Interleukin-1 (INT-1), S100B calcium-binding protein B and ubiquitin C-terminal hydrolase (UCH-L1).

15 55. The method of any one of claims 46 to 54, wherein the sample comprises blood, serum, plasma, saliva, cerebrospinal fluid or urine.

56. The method of any one of claims 48 to 55, wherein analysing the composition of the sample comprises comparing the Raman scattered spectrum of the sample with a reference spectrum.

20 57. The method of claim 56, wherein the reference spectrum comprises a signature or 'fingerprint' spectrum of a target molecule.

58. The method of claim 56 or claim 57, wherein the reference spectrum is obtained from a control sample.

25 59. The method of claim 58, wherein the composition of the control sample is known.

30 60. The method of claim 58 or 59, wherein the control sample is obtained from a healthy subject.

61. The method of any of claims 46 to 60, wherein the spectrum obtained from the sample is compared with the spectra of one or more further samples obtained at different time points, or at different locations.

62. The method of claim 61, wherein the spectrum obtained from the sample is compared with at least two reference spectra.

5 63. The method of claim 62, wherein a spectrum obtained from a patient sample is compared with a signature spectrum of a biomarker of interest and also with a spectrum of a control sample obtained from a healthy subject.

10 64. The method of any of claims 46 to 63, wherein the one or more target molecules have a concentration in the sample of less than 10×10^{-12} Moles/dm³.

65. Use of the platform in accordance with claim 45, or of the method according to any of claims 46 to 64 for analysing the composition of a sample.

15 66. The use according to claim 65, wherein the target molecules are proteins, peptides, antibodies, enzymes, amino acids, nucleic acids, polysaccharides or fatty acids.

20 67. The use according to claim 65 or claim 66 for detecting multiple target molecules simultaneously.

68. The use according to claim 67 in the analysis of the total chemical or biochemical composition of a sample.

25 69. The use according to any of claims 65 to 68 for one or more of: diagnosis and monitoring of disease; the detection of pathogens and/or toxins; environmental analysis including water purity and/or contamination; and the detection of chemical and/or biological agents.

30 70. Use of the platform in accordance with claim 45, or of the method according to any of claims 46 to 64 for the diagnosis and/or monitoring of a disease or injury in a subject, and/or for determining the severity of disease or injury.

35 71. The use according to claim 70 wherein the disease or injury is one of cancer, immune disease, a neurodisorder, or traumatic brain injury (TBI).

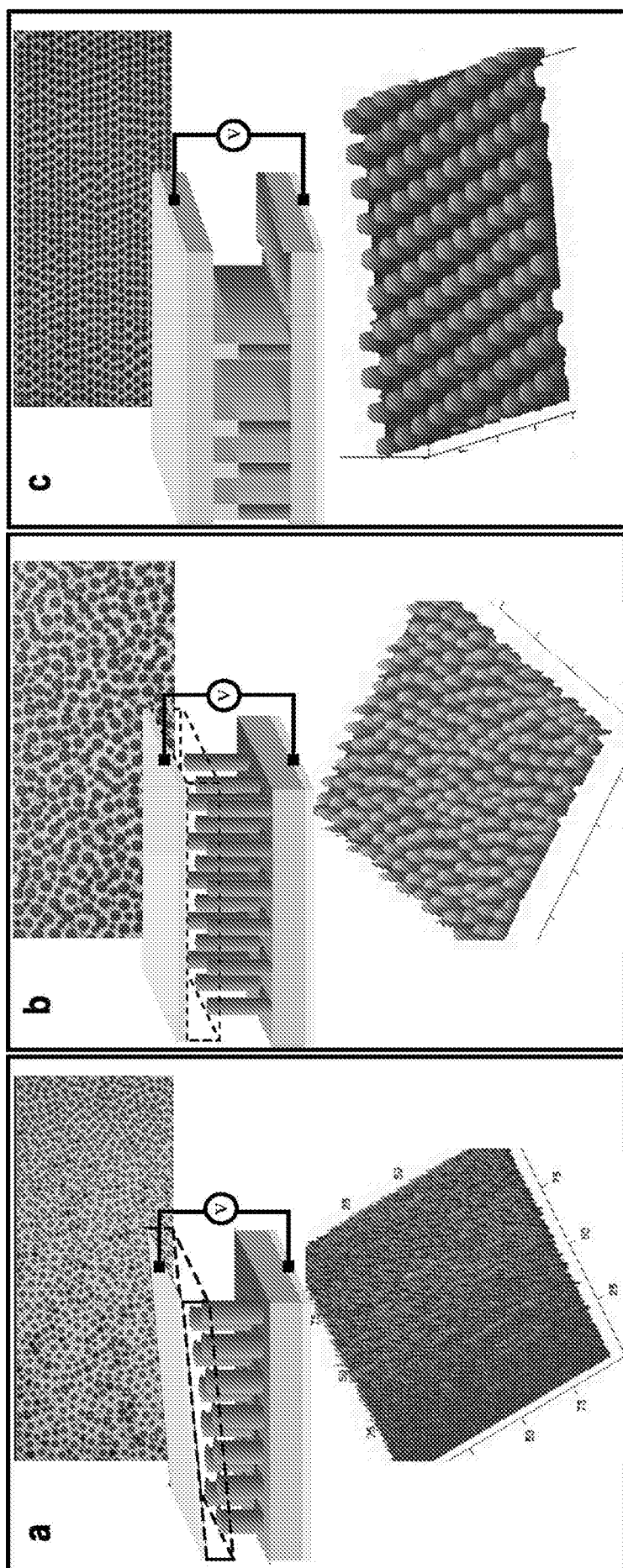


Figure 1

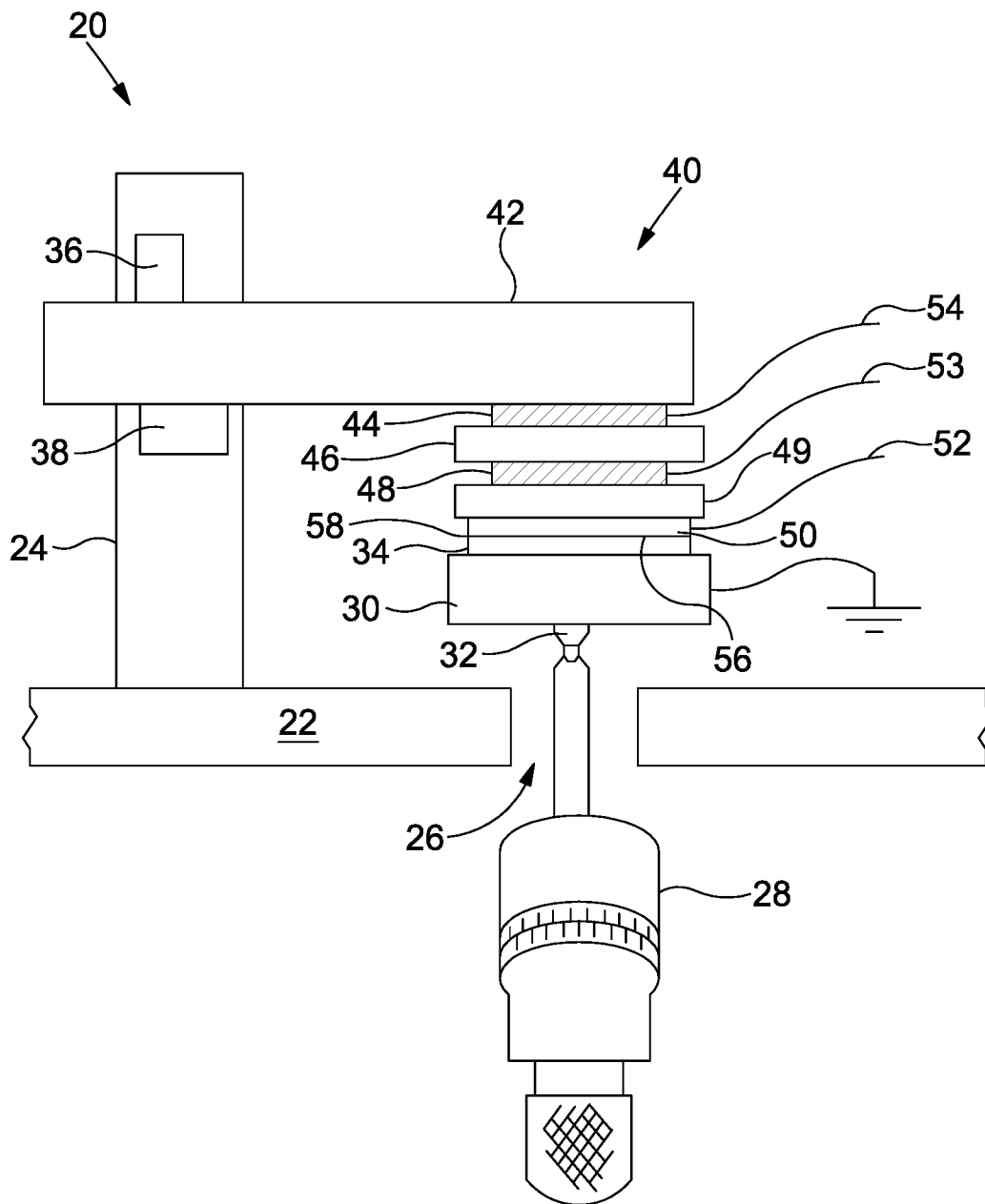


Figure 2

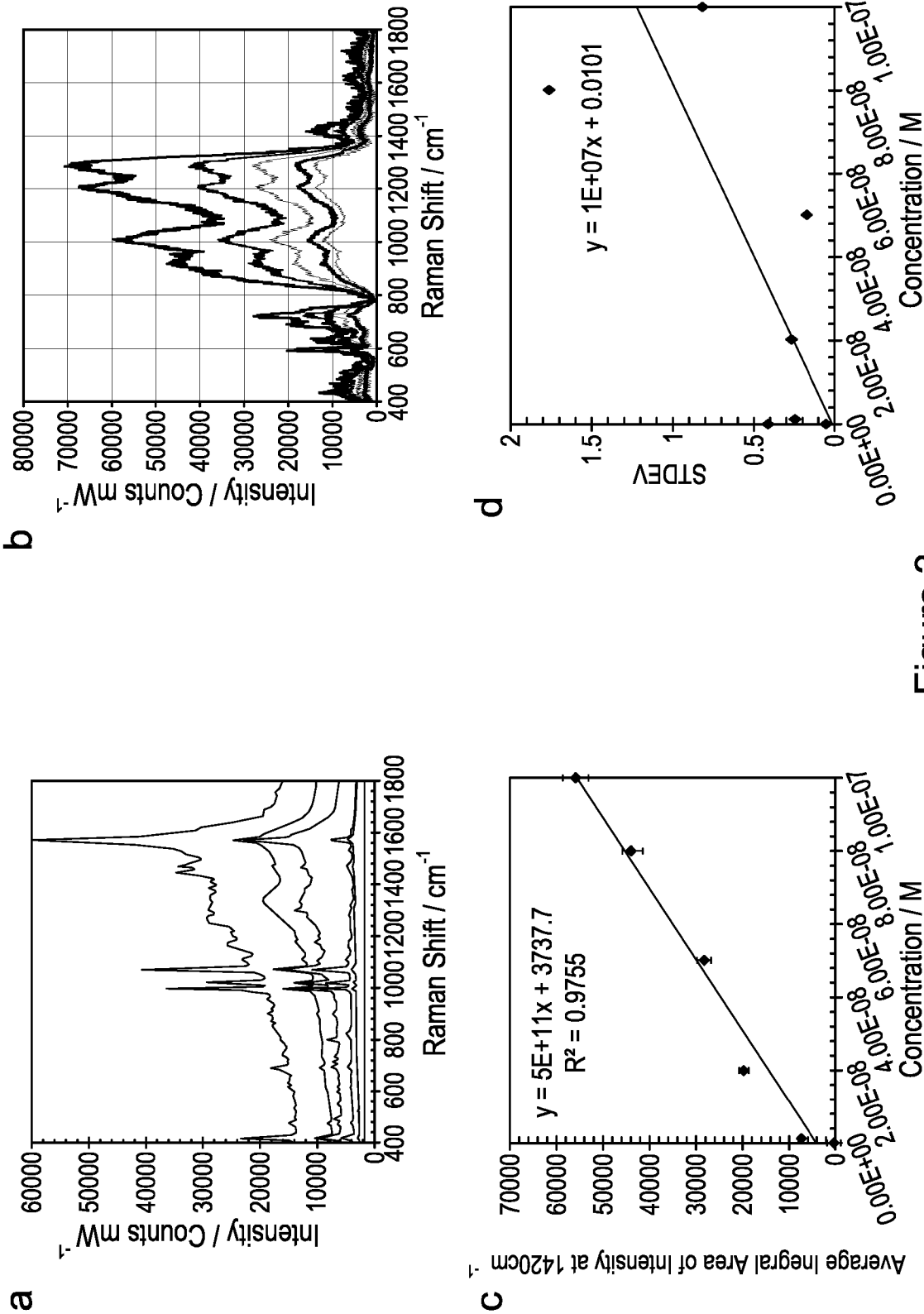


Figure 3

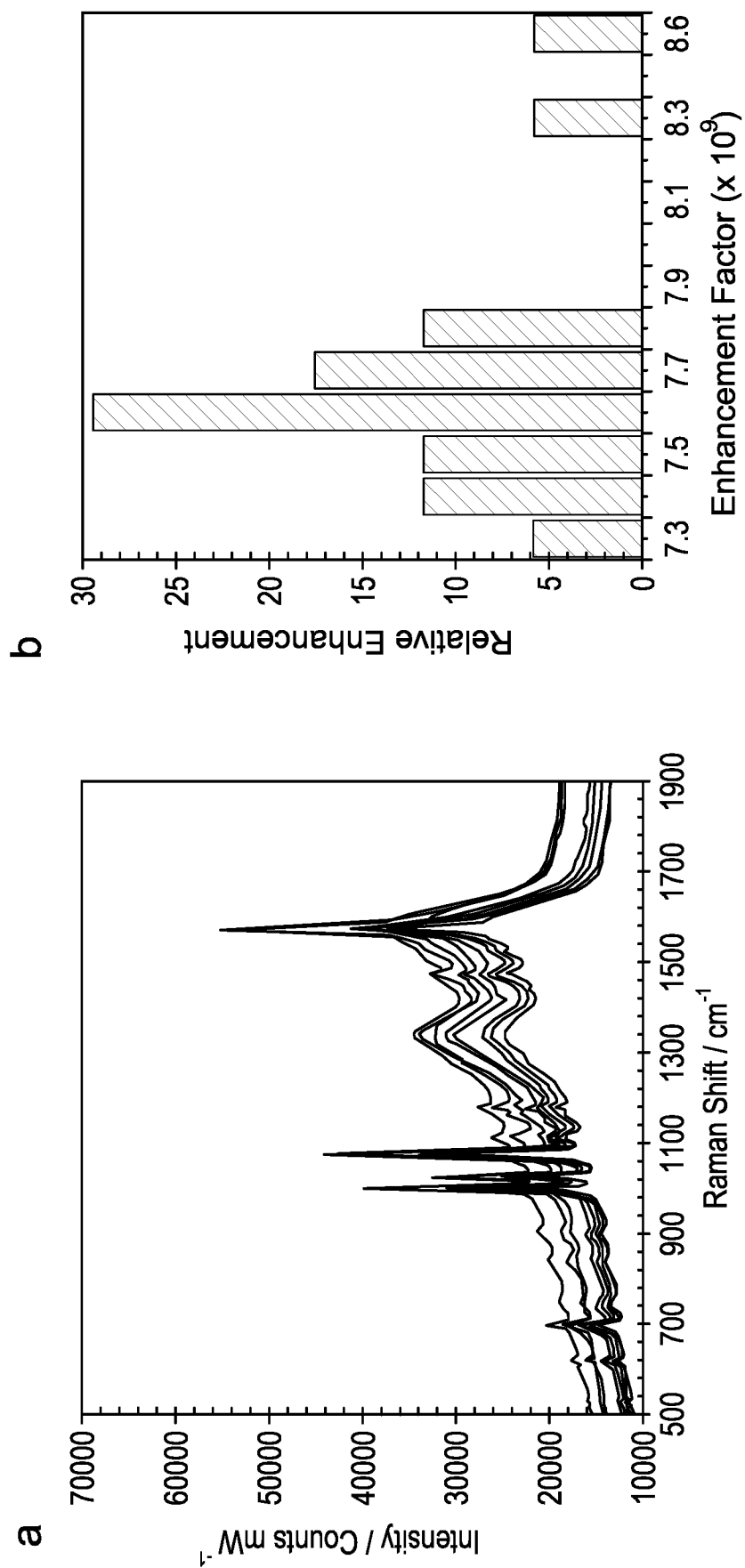


Figure 4

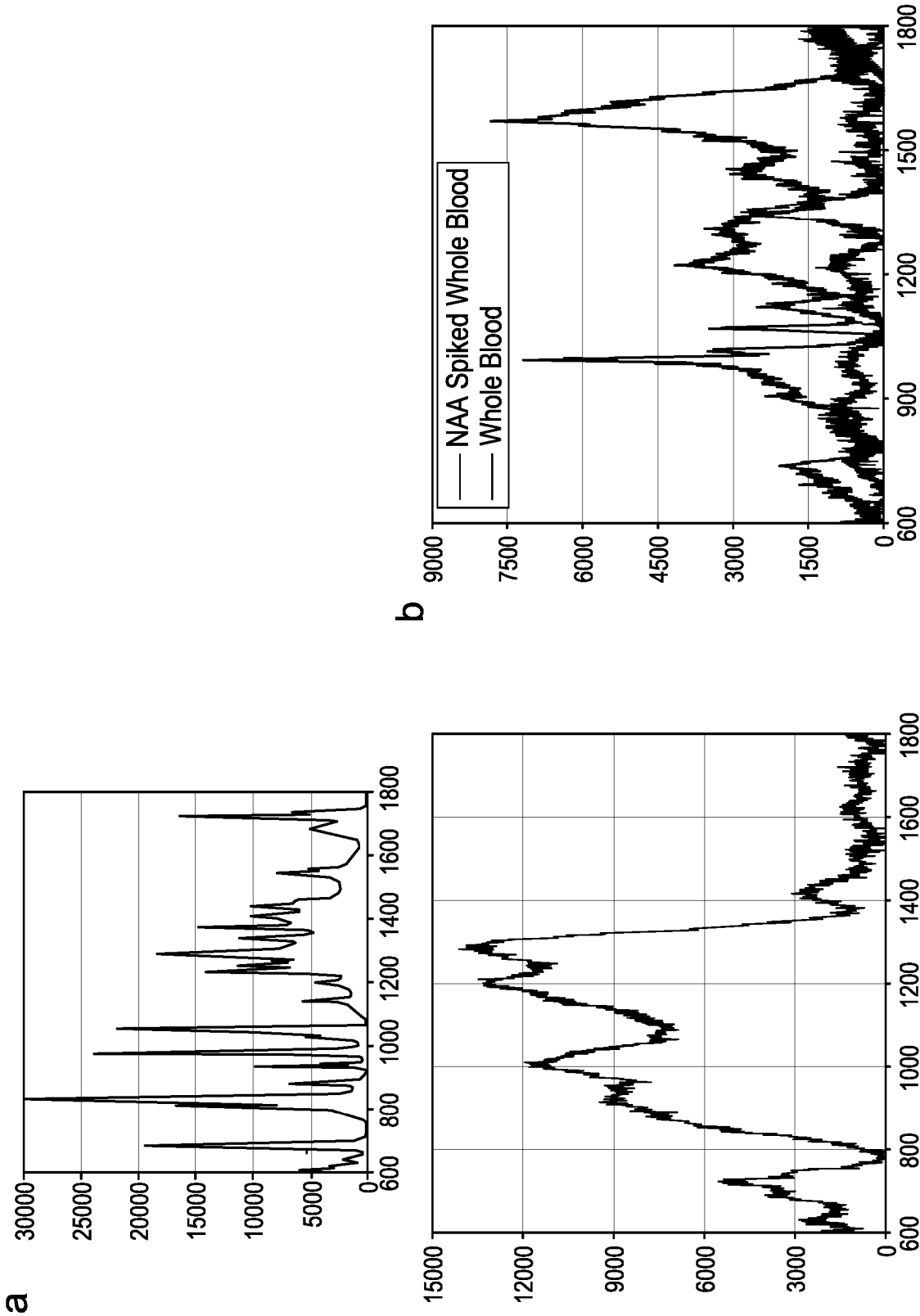


Figure 5

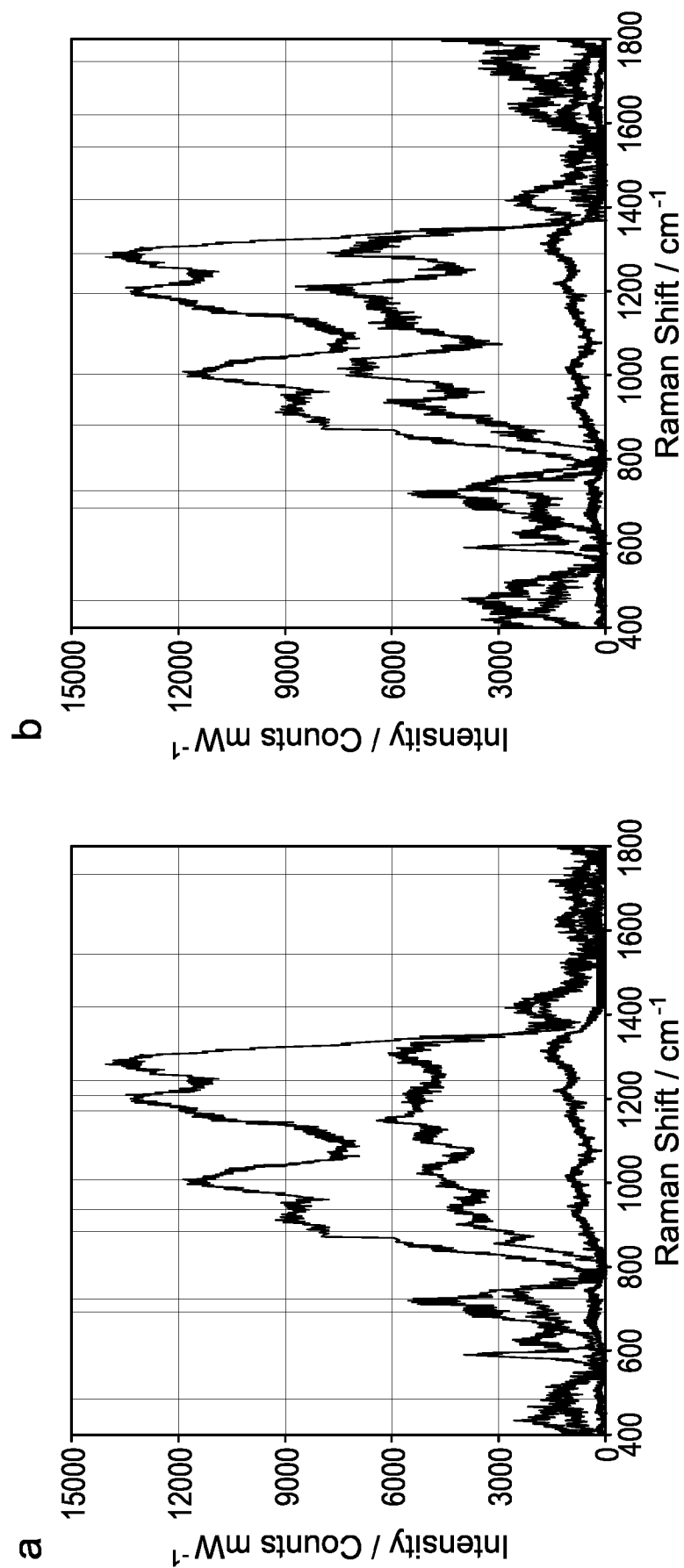


Figure 6

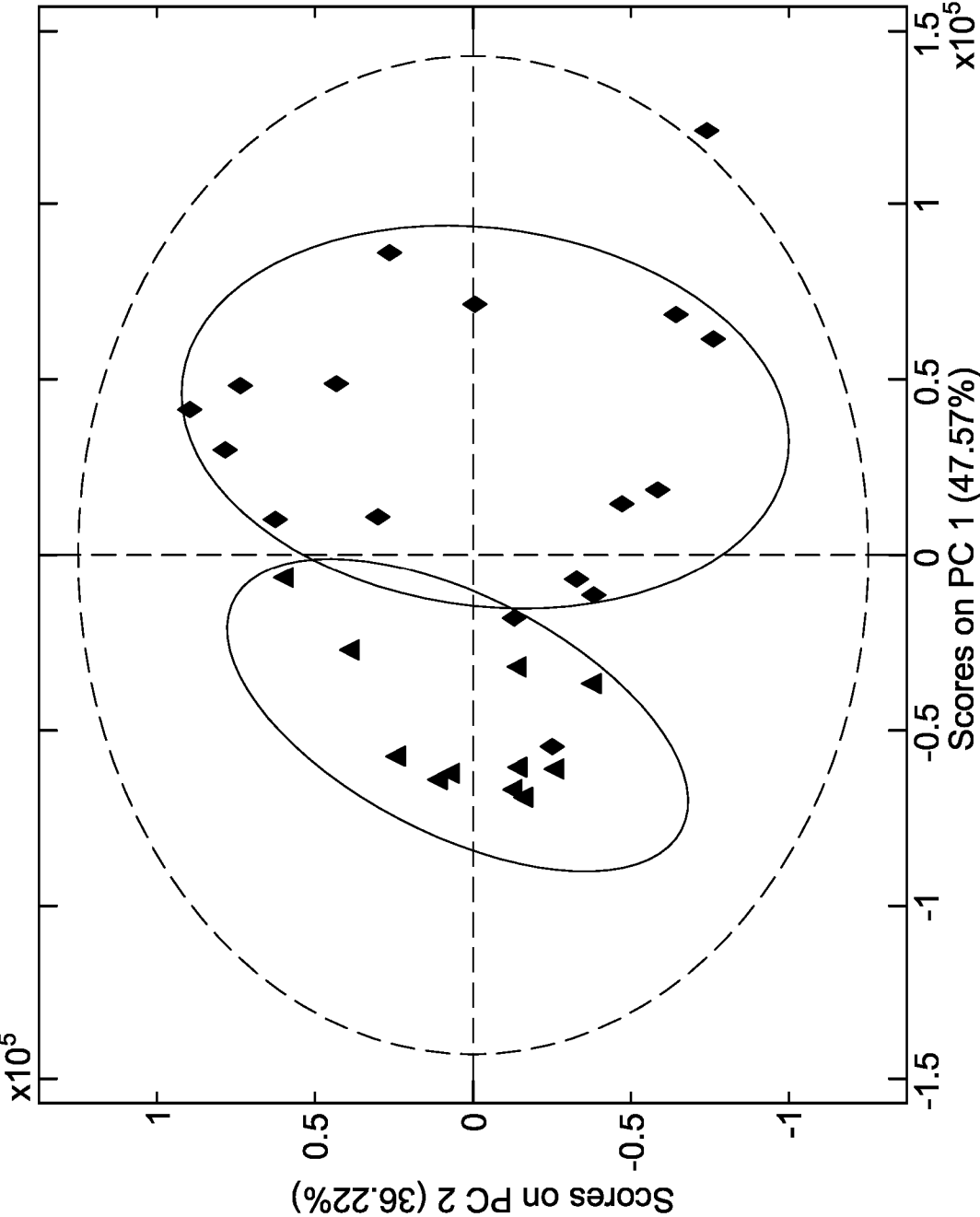


Figure 7

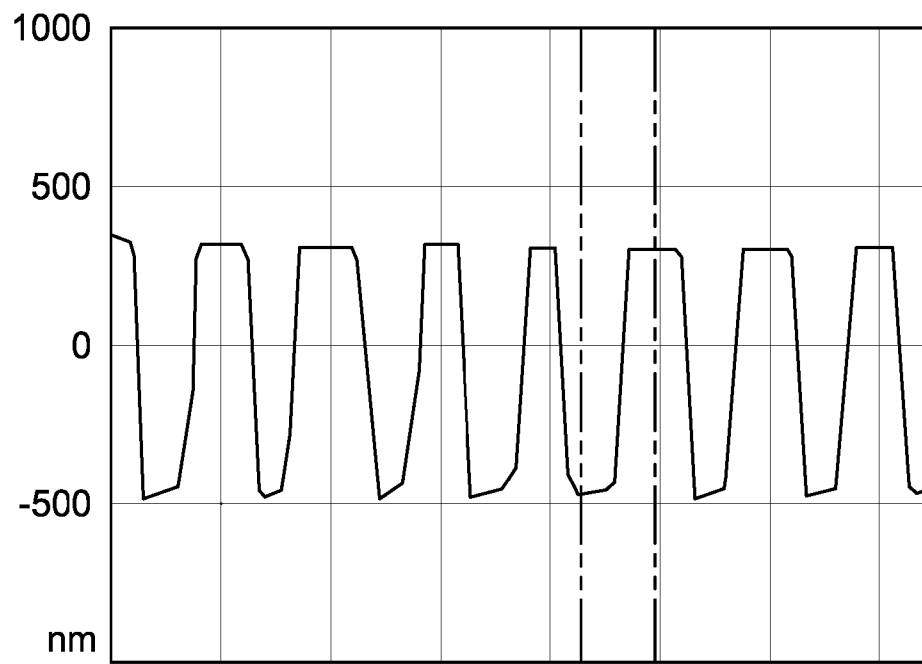


Figure 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2018/050193

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01J3/44 G01N21/65 G03F7/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01J G01N G03F

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

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

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Allen Lee Raines: "Nano-Scale Large Area Gap Control for High Throughput Electrically Induced Micro-Patterning", December 2007 (2007-12), XP055459944, Retrieved from the Internet: URL:https://repositories.lib.utexas.edu/bitstream/handle/2152/3656/rainesal4631.pdf;sequence=2 [retrieved on 2018-03-15] figures 1.6, 7.7 - 7.9 pages v-vii, 18-23, 93-95, 103-125 ----- -/-	1-31



Further documents are listed in the continuation of Box C.



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Date of the actual completion of the international search

19 March 2018

Date of mailing of the international search report

26/03/2018

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Flentje, Farida

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2018/050193

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	figures 4.5, 6.1 - 6.6, 8.2C pages 31-36, 48-50, 56-60, 78-88, 109-111 table 3.1	1-31
X	----- POLA GOLDBERG-OPPENHEIMER ET AL: "Hierarchical Electrohydrodynamic Structures for Surface-Enhanced Raman Scattering", ADVANCED MATERIALS, vol. 24, no. 23, 4 April 2012 (2012-04-04) , pages OP175-OP180, XP055459936, DE ISSN: 0935-9648, DOI: 10.1002/adma.201104159 the whole document	32-71
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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2018/050193

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUMEET MAHAJAN ET AL: "Tunable Microstructured Surface-Enhanced Raman Scattering Substrates via Electrohydrodynamic Lithography", JOURNAL OF PHYSICAL CHEMISTRY LETTERS, vol. 4, no. 23, 19 November 2013 (2013-11-19), pages 4153-4159, XP055459919, US	32
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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