(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 December 2006 (21.12.2006)

PCT

(10) International Publication Number
WO 2006/134376 A2

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/GB2006/002205

(22) International Filing Date: 15 June 2006 (15.06.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0512230.4 15 June 2005 (15.06.2005) GB

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

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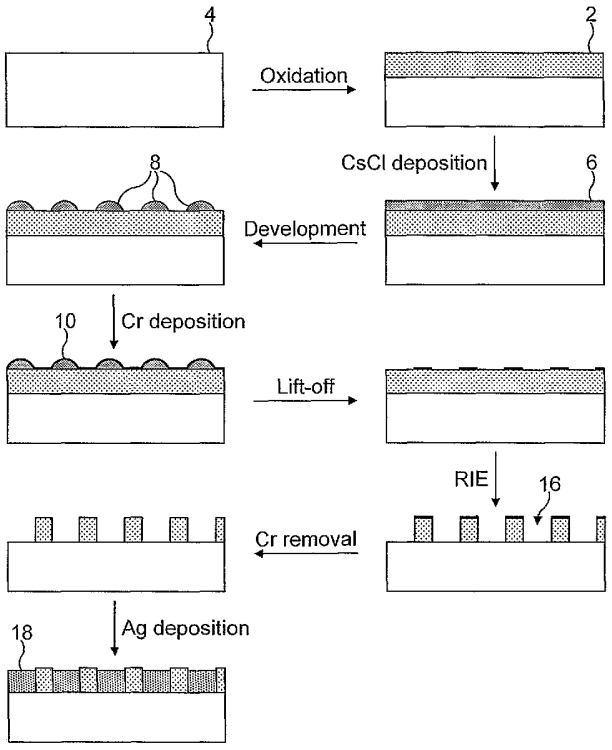
(54) Title: FLAG FREE CHEMICAL CAPTURE DETECTION

(57) Abstract: A method for label-free detection of chemical capture using a SERS substrate is disclosed. Also provided are methods of obtaining a suitably functionalised SERS substrate.



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European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

FLAG FREE CHEMICAL CAPTURE DETECTION

The present disclosure relates to the detection of a target molecule by chemical capture (for example hybridization) between a probe and a target molecule using Surface Enhanced Raman Spectroscopy (SERS). In particular, although not exclusively, the target molecule is a bio-molecule. More particularly, the target molecule is an oligonucleotide.

Surface Enhanced Raman Spectroscopy is a spectroscopic technique which combines laser spectroscopy with the optical properties of metallic nanostructures, resulting in strongly increased Raman signals when the studied molecules are attached to sub-micron sized gold or silver structures. In the present context, SERS is understood to include Surface Enhanced Resonant Raman Spectroscopy (SERRS).

While SERS has been recognized as a powerful analytical tool, previous SERS studies of oligonucleotides have found spectra where adenine related signals dominate to an extent where other bases can barely be distinguished. The present inventors have, for the first time, obtained spectra of oligonucleotides which allow the distinction of spectral features or peaks corresponding to breathing and other modes of nucleotides other than adenine.

In the first aspect of the invention, there is provided a method of detecting chemical capture of target molecules in a sample with probe molecules which are supported on a region of a SERS surface as defined in Claim 1. The molecules may be oligonucleotides. The abovementioned discovery of well resolved SERS spectra for oligonucleotides has allowed the development of a method of detecting hybridisation based on the signature derived from the SERS spectra before and after hybridisation has occurred, thus providing a label or tag free method of detecting hybridization or other probe/target pairs.

The signatures may be derived from a set of predetermined peaks of the spectra using, for example, peak area or peak height. The set of peaks may comprise two peaks and preferably a first peak is used which corresponds to the breathing mode of adenine and a second peak is used which corresponds to the breathing mode of thymine or cytosine. The first peak may then be located at 731cm^{-1} and the second peak may be located at 790cm^{-1}. Preferably, the signatures are computed from the ratio of measures derived from the two peaks.

Advantageously, the method may include taking a calibration reading from a calibration region of the surface which is spatially distinct from the region used for detecting hybridisation. By measuring the SERS spectrum from the calibration region before and after the sample is applied, the analysis can be calibrated for changes in the efficiency of the SERS substrate. The calibration region supports a calibration molecule which preferably does not hybridise with the target molecules. For example, the calibration molecule may be a single strand oligonucleotide comprising exclusively adenine, thiamine, cytosine or guanine nucleotides.

The method may be advantageously applied to an analytical chip including a plurality of hybridisation (probe) regions distributed across the chip and preferably also including a plurality of calibration regions also distributed across the chip.

The invention further extends to analysis systems as defined in Claim 15, a computer program as defined in Claim 16 and a computer readable medium or data signal as defined in Claim 17.

In a further aspect of the invention there is provided a SERS chip including one or more regions of a SERS surface as defined in Claim 18. It can be shown that, (non-obviously), a SERS signal does not always increase with increase of target material when the concentration of probe molecules on the chip is increased. In particular, when the chip is covered with a dense packing of probe oligonucleotides, there may be insufficient space for the target molecules to insert themselves into this close packing. Accordingly, and unexpectedly, an improved signal can be obtained when the probe molecules on the chip are packed less densely than a close packing. In the case of oligonucleotides, given the similar size of the probe and target molecules, the packing of probe molecules is preferably substantially one half of the close packing, providing sufficient space for the target oligonucleotides while ensuring that the surface of the chip is at optimal coverage.

According to a further aspect of the invention, a SERS chip is provided as defined in Claim 21. By providing the probe and calibration molecules in distinct regions of the chip, a calibration procedure as described above is enabled. The probe molecules and/or the calibration molecules are preferably oligonucleotides.

A further aspect of the invention relates to the use of a SERS chip as defined in Claim 24. In conventional hybridisation chip arrangements using, for example, a fluorescent label attached to the target molecule to detect hybridisation, it may be advantageous for the number of probe molecules to exceed the number of target molecules such that the likelihood of a target molecule binding a probe molecule is maximised. In the label-free approach of the present invention, counter intuitively, the converse is true and a number of probe molecules which is smaller than the number of target molecules may be more advantageous. This is because any probe molecule which does not hybridise

when the sample is applied will result in an undesired background signal potentially affecting the results of the analysis adversely. Preferably, the number of probe molecules is sufficiently small with respect to the number of target molecules to ensure that substantially all the probe molecules are hybridized. Preferably, a SERS chip as defined above is used.

In yet a further aspect of the invention, there is provided a SERS chip as defined in Claim 27. The irregular array of metallic structures is thought to enhance the SERS characteristics of the substrate because, effectively, it provides a relatively wide sample of structure dimensions and geometry as compared to an array which is manufactured to be regular. Because SERS is thought to be related to the shape and dimensions of the SERS surface, a random sample of structures is thought to be advantageous because it will ensure that at least a sub-range of the structures result in good enhancement. By contrast, a regular array of structures may produce a better signal if tuned exactly to the optimum parameters for SERS, but the optimum parameters are more likely to be missed completely for a regular spectra than for an irregular spectra sampling the space of structures and dimensions. Preferably, the surface includes silver structures supported on a silicon substrate and the structures may advantageously be torroids or pillars.

In a further aspect of the invention, a method of determining suitable probe surface concentrations as defined in Claim 31 is provided.

In a further aspect of the invention, a method of applying probe/target molecules as claimed in Claim 32 is provided. Because the electrolyte in the buffer does not adsorb onto the SERS surface, the structural integrity of the surface is better maintained.

In a further aspect of the invention, a reactive substrate as claimed in claim 34 is provided,

Specific embodiments of the invention are now discussed, by way of example only and with reference to the accompanying figures in which like reference numerals refer to like features and in which:

Figure 1 illustrates the manufacture of a SERS substrate;

Figure 2 is a schematic depiction of a SERS substrate with attached oligonucleotide probes;

Figure 3 shows a block diagram of a system implementing a method of detecting hybridisation;

Figure 4 shows SERS spectra for two oligonucleotides before and after hybridisation;

Figure 5 is a flow diagram of the method for detecting hybridisation; and

Figure 6 is a schematic depiction of a SERS substrate including calibration regions.

Details of fabrication of SERS substrates including an irregular array of structures can be found in M. Green and F.M Liu, *J. Phys. Chem. B,* 2003, **107**, 13015 and F. M. Liu and M. Green, *J. Mater. Chem.,* 2004, **14** 1526, herewith incorporated herein by reference. Further details are contained in Mino Green, Feng-Ming Liu, Lesley Cohen, Peter Köllensperger and Tony Cass, "SERS Platforms for High Density DNA Arrays", Faraday Discussions, **132**, 269-280 (2005)., incorporated herein by reference (and referred to as Green *et al* in the remainder). As the steps will therefore be apparent to the skilled reader they are described here only in brief, with reference to Figure 1.

In a first step an oxide layer 2 typically 300 nm thick, is grown on a piece of silicon wafer.4 The oxidized silicon is then etched with H_2O_2 (27.5 wt%) : NH_4OH (0.88): water (1:1:1) to render it clean and hydrophilic. A thin layer 6, thickness L (typically 10nm), of CsCl is evaporated on the clean surface. The CsCl thin film is then placed in an atmosphere of controlled relative humidity, whence it self-reorganizes into hemispherical islands 8. The mean island diameter, <d>, and their coverage of the surface, F, can be adjusted by the humidity, the original thickness, L, and development time. The relation $3L \approx$ <d>F is found to be a good guide. A thin layer of Cr 10, thickness about 10% of <d>, is then thermally deposited over the entire surface. The Cr/CsCl coated substrate is then placed in water and ultrasonically agitated for 3 minutes. This causes the CsCl and its chromium cap to lift-off, leaving a Cr-patterned SiO_2 surface with holes 12 in the Cr film 14 where previously there had been CsCl. The patterned substrate is placed in a plasma etching apparatus (Plasmalab, 80 Plus®, Oxford Instruments) to etch the exposed SiO_2, and adjusted to give vertical sidewalls to the wells 16 that are formed. The remnant Cr mask film is then stripped off, using a commercial Chrome Etchant (Rockwood Electronic Materials, UK). This structure is then chemically etched to increase the average diameter of wells and to narrow the diameter distribution using an SiO_2 (not shown in Figure 1). Thus, before silver deposition, the substrates is washed in 69% HNO_3, then NH_4OH solution (7%~8%NH_3 in water) for 2 min respectively, and then pre-etched in 1%HF solution for 1 min. After a final wash in water, the substrate is transferred to a solution of 6mM $AgNO_3$+0.5%HF for silver deposition: exposure is for 20s (no stirring), then 20s (60rpm stirring). Silver deposition occurs on the patterned silicon by a process of galvanic exchange, viz:

$$Si + 4Ag^{+} + 6F^{-} = SiF_6^{2-} + 4Ag$$

Silver structures 18 in the form of tori are formed at the bottom of the etched wells: a typical substrate used throughout the paper is as shown on Fig. 1. The average characteristics of the torus structures are, for example: outer diameter, d_o=450 nm, inner diameter, d_i=190 nm, the fractional area covered in silver 0.36.

If pillars, rather than toroids, are required, the concentration of HF can be decreased to 0.12%, changing the dynamics of silver deposition to result in pillars of silver supported on a silicon substrate.

In order to avoid the silver torus nanostructures becoming unstable when subjected to extensive treatment in various solutions, a suitable stabilization agent can be used (e.g. a surfactant) especially when very diluteDNA solutions ($<10^{-9}$ M) are used.

In a second step, probes are deposited on the substrate. Immobilisation of oligonucleotide probes on a SERS surface can be achieved in any way known to a skilled person. Typically, 30µl of stock 0.05mM of disulphide linked aqueous DNA solution (DNA-C_6-S-S-C_6-DNA) are mixed with 10µl of a solution that was 10 mM with respect to DTT in 0.1 M TEAA buffer; and kept for 2 h. The reducing agent, DTT, causes the rupture of the disulphide bonds, yielding DNA with the thiol group at the 3' end of the DNA strand, (HS-C_6-DNA). The solutions are then applied to an Amersham G-25 MicroSpin column to remove the excess DTT and the percolate extracted twice with 700 µl ethyl acetate. Aliquots of this solution may then be diluted down with, for example, 0.1M TEAA to the desired concentration. The functionalised oligonucleotides attach to the SERS structures via S-Ag bonds.

The solution of desired concentration is then applied to the SERS surface of the substrate using a commercially available micro printing device (a so called "mircro spotter") for putting down drops of solution on a surface in specific areas. Typically, the solution can be deposited in drops of a diameter down to 60 microns, theoretically allowing more than 10^4 different drops to be applied to a chip having a surface area of half a square centimetre. By applying a drop of solution containing oligonucleotide probes functionalised for attachment to the SERS surface, a sample applied to a chip prepared as described above can be tested for the presence of a large number of different target molecules corresponding to different probe molecules.

An alternative technique for functionalizing a clip uniformly over a larger area (e.g. 0.5 x 0.5cm^2) is derscribed in Green *et al.*

The irregular array of structures obtained as described above has been found to result in SERS enhancements greater than known SERS substrates. Because SERS is thought to be related to the shape and dimensions of the SERS surface, a random sample of structures is thought to be advantageous because it will ensure that at least a sub-range of the structures result in good enhancement. By contrast, a regular array of structures may produce a better signal if tuned exactly to the optimum parameters for SERS, but the optimum parameters are more likely to be missed completely for a regular spectra than for an irregular spectra sampling the space of structures and dimensions.

Two factors influence the desired surface coverage of the SERS surface: on the one hand increasing the number of probe molecules per unit area should increase the Raman signal because there are more signal sources per unit area, but on the other hand, sufficiently large gaps must be provided between the probe molecules such that the steric constraints associated with hybridisation

with a target molecule can be satisfied. Therefore, the concentration of the probe molecules in the probe solution should be adjusted such as to obtain a surface coverage on the SERS surface which is less than complete, that is less than close packing (close packing being understood to refer to the packing of molecules resulting in the highest surface coverage). In the case of oligonucleotide probes and targets, the probe and its complementary target will have roughly the same size and a surface coverage of about one half of the closed packing coverage is therefore desirable. This is illustrated in Figure 2, showing schematically a SERS substrate chip 20 having probe oligonucleotides 22 attached thereto in an upright configuration such that there is sufficient space between the probes 22 for a target 24 to bind.

The required concentration may be calculated using a suitable steric model or may be determined experimentally. In practice, the latter may be more effective and an optimal concentration of the probe solution can be determined by examining the overall intensity of the Raman spectrum, the intensity of certain features thereof (such as the intensity or area of one or more peaks), the ability to discriminate between the hybridised and non-hybridised state (see below) or a combination of the above as a function of probe concentration. Conveniently, this may be done efficiently by using a chip as described above to which drops of varying concentration are applied. The chip can then be scanned, as described below, before and after application of a solution containing target molecules and the results are analysed to select the probe concentration resulting in the best signal characteristics.

Further details on the influence of probe concentration on hybridisation are disclosed in Green *et al.*

A further consideration in the design of a SERS chip for detecting hybridisation between a target and probe results from the fact that un-hybridised probe molecules will result in an undesirable background signal. Accordingly, it is desirable that all probe molecules in a given region of the chip should hybridise with target molecules if the appropriate target molecule is present in the sample applied to the chip. Conversely, this means that, contrary to what can be expected for conventional hybridisation chips, the number of probe molecules should be small compared to the number of target molecules applied to the chip. Thus, for use with samples of certain concentrations of target molecules, the number of probe molecules on the chip for a given target should be tuned such that all probe molecules hybridise when the target is present. This can be achieved by reducing the cross sectional area of the drops printed on the chip to an area not much larger than the area of a laser beam used for obtaining the Raman spectra, for example, twice as large.

If the probe and/or target molecules are provided in a buffered solution, it is preferred that the supporting electrolyte does not adsorb onto the silver (or other metal) structures in order to avoid destabilising the structures. For example, F^- ions do not adsorb onto the silver structures and a NaF buffer solution may be used.

With reference to Figure 3, a Raman spectroscopy system according to an embodiment of the invention is now described. The controller 26 controls a Raman spectrometer 28, for example, a Raman microscope and a sample handler 30. The sample handler comprises a Raman cell accepting a chip to be analysed and, optionally, means for applying a sample containing target molecules to the chip. The controller 26 controls the relative motion of the laser beam of the Raman spectrometer and the SERS surface of the chip inside the Raman cell. This can be achieved, for example, by deflecting the laser

beam or by moving the chip on a translational stage, for example, by means of a piezo-electric manipulator. The controller 26 further controls analyser 38 which includes electronic circuits, for example a micro computer, for analysing data obtained by the Raman spectrometer 28, as described in more detail below.

A method of detecting hybridisation of target molecule nucleotides in the sample applied to the chip with probe oligonucleotides supported on a region of the chip is now described with reference to Figures 4 and 5. Figure 4 shows spectra obtained for oligonucleotide probes (lower traces) and probe-target hybridised complexes (upper traces) for bacterial DNA glnH and mammalian DNA OBP (thick and thin traces, respectively). The respective sequences are set out in the table below:

Name	Sequence 5' to 3'
glnH Probe	AGA AGA AAC CGC AAA AGC---C_6---SH
glnH Target	GCT TTT GCG GTT TCT TCT
OBP Probe	ATC CCC GCC ATT CCATTT TCA TCG CTT CAA AGA TGC CGC CCA GCA---C_6---SH 3'
OBP Target	TGC TGG GCG GCA TCT TTG AAG CGA TGA AAA TGG AAT GGC GGG GAT

As can be seen from comparing the traces before and after hybridisation, the ratio of the peak height of the peaks at 731 and 790cm^{-1} changes notably on hybridisation. Thus by defining a signature of the spectrum as, for example, the ratio of the 731 cm^{-1} peak (corresponding to the breathing mode of cytosine and thymine) to the peak at 790cm^{-1} (corresponding to the breathing mode of adenine) one can detect the occurrence of hybridisation by a corresponding change in the signature. This forms the basis of the detection method described below. If the result obtained from these peaks is ambiguous, further peaks (either of the same or other nucleotides can be analysed analogously). Further

details of SERS measurements and experimental results can be found in Green *et al.*

With reference to Figure 5, a chip, functionised as described above, is placed in the Raman cell and is scanned to obtain a baseline spectrum for each region or dot of probe molecules at step 40. The obtained data is then used to calculate a baseline signature for each region of probe molecules at step at 42. At step 44 a sample is applied to the chip for a sufficient time to allow hybridisation to occur and the chip is washed at step 46. Both steps 44 and 46 can be carried out in any suitable way known to a skilled person and can either be integrated within the sample handler or be carried out off-line by removing the chip following the baseline scan and replacing it after step 46.

At step 48, following application and washing of the sample, a sample scan is obtained analogously to the baseline scan at step 40. Following the sample scan, a sample signature is calculated for each region at step 50. Finally, at step 52, the sample signature is compared to the baseline signature for each region to determine whether or not hybridisation has occurred in a given region. The identity of the target molecule will then be inferred from the identity of the probe molecule of regions where hybridisation occurred.

As mentioned above, the baseline and sample signatures can be calculated as the ratio of the peaks (measured by peak height or peak area) of the breathing modes of adenine and cytosine/thymine. Other peaks may also be included in the analysis to resolve ambiguities or increase accuracy. However, other measures can be used to derive the signature, provided that a change between probe and probe/target complex molecules can be detected. Another possibility would be to regard the whole spectrum, suitably discretised, as the signature

and detect a change of the signature by a suitable measure of similarity, for example, a dot product between spectra.

Because hybridisation is detected from inherent characteristics (the Raman Spectrum) of the probe/target, no labels are required.

Given the handling of the chip and its exposure to sample solution, a change in the efficiency of the SERS surface of the chip, and a resulting change in the detected SERS signal may well occur (see above). This may be less problematic where the signature is calculated as a ratio of features of the spectrum such that overall changes in intensity do not result in changes in this ratio, but it would clearly be desirable to be able to calibrate for any such changes of SERS efficiency. This will allow SERS spectra to be compared visually between before and after exposure to the sample, as well as between different chips. Of course, where the signature depends on the absolute magnitudes, as in the case of the product, it is vital that the signal is calibrated in some way.

A SERS chip 20 including probe regions 54 and calibration regions 56 is depicted schematically in Figure 6. The probe regions are distributed evenly across the chip and contain probe molecules attached to the surface of the chip, as described above. Interspersed with the probe regions, one or more calibration regions 56 are provided on the chip. These regions contain calibration molecules attached to the surface of the chip and can be used for calibration as described below. A baseline scan 40 and a sample scan 48 are carried out in the same manner as described with reference to Figure 5, with the additional step of identifying the spectra obtained at each scan for the calibration regions. These calibration spectra are then used to calculate a normalisation factor which can be used to calibrate the measurement for any

changes in overall SERS efficiency during application of the sample. If a plurality of calibration regions distributed across the chip are used, this calibration can be derived locally for probe regions adjacent to each calibration region, or a global normalisation factor can be calculated. In the latter case, a single suitably placed calibration region may be sufficient. The normalisation factor may be derived in various ways, for example, using the peak intensity or peak area of a certain peak, and averaged over a number of peaks or the whole spectrum.

A number of considerations apply to the choice of an appropriate calibration molecule. On the one hand, the calibrating molecule should be similar to the class of probe molecules used on the chip such that any change in the SERS efficiency of the substrate is comparable for the calibration molecule and the probe molecule. On the other hand, it must be ensured that the calibration molecule does not hybridise with any of the target molecules corresponding to the probe molecules on the chip so that any changes in the calibration signal are attributed to changes in the SERS efficiency of the chip rather than hybridisation. In the case of oligonucleotide probes, this can be conveniently achieved by using a calibration molecule consisting of an oligonucleotide having a sequence which does not (or is highly unlikely to) occur naturally. For example, an oligonucleotide sequence of only adenine, cytosine, guanine or thymine can be used. Preferably, an adenine only oligonucleotide is used because of its superior signal strength as compared to the other nucleotides. Furthermore, the length of the calibration oligonucleotide can be matched to the length of probe oligonucleotides adjacent to the calibration region in question or can be chosen to correspond to the average sequence length of the probe nucleotides.

An alternative or additional approach to addressing the issue of deterioration of the chip with handling and exposure to sample solution is to stabilise the chip surface.

The above issue is overcome by coating the functionalized substrate with a thin layer of a water gel such as agarose or polyacrylamide. The gel material, before the formation of the gel, is placed on the functionalized substrate (which has a hydrophilic surface) in the form of a thin liquid film, ca. 1mm thick. The properties of the gel (typically 98% water) are such as to allow virtually unimpeded passage of water, electrolytes, oligonuclitedes and small proteins, while at the same time retaining silver toroides in their initial position.

This stablization of a SERS surface using gel can be applied to structures other than those described in disclosure. It can be used anywhere there are structures that can be destabilized by chemical treatment and agitation. It is understood that the gel need not be water based and that for a hydrophobic surface a non-water based gel would be appropriate as long as it is lyophilic with the surface in question.

The two gel materials mentioned above are the most common materials used in bio-chemical laboratories, but there are many other gel materials that will prove suitable. The requirements are passage of the requisite chemicals through a water medium. The method of placing the gel on the surface can be simply by putting a drop of material on the surface, or placing material on the surface and spinning or draining excess material away or by placing material on the surface followed by photo-setting of a projected area of gel in the required place.

It will be appreciated that individual features of the embodiments described above can be varied, interchanged or juxtaposed as necessary. It is further

understood that the invention extends to embodiments in which probe and target molecules are not oligonucleotides. The probe/target molecules can be other bio-molecules such as antibodies/antigens for detecting the presence of certain proteins or any other molecule which can be hybridised or otherwise chemically captured with a probe molecule on the SERS chip such that a change in the corresponding Raman spectrum occurs. Equally, the methods described above are applicable to detectors for only one target molecule. In this case, chip production can be simplified in that the probe molecules can be applied to the chip surface simply by submerging the chip in a suitable probe solution. Finally, it is understood that the method steps described above can be carried out in any suitable order. For example, the spectra resulting from baseline and probe scans can be stored on a suitable recording media and the calculation of the signatures and subsequent analysis can be carried out off line once all of the data is acquired.

It is understood that the embodiments described above are exemplary only and can be extended and generalised in ways apparent to the skilled person and that the scope of the invention is defined by the appending claims.

CLAIMS

1. A method of detecting chemical capture of target molecules in a sample with probe molecules supported on a region of a SERS surface, the method including
    a. measuring a first Raman spectrum from the region before the sample is applied thereto and computing a first signature from the first spectrum
    b. measuring a second Raman spectrum from the region after the sample is applied thereto and computing a second signature from the second spectrum
    c. comparing the first and the second signatures, a change from the first to the second signature being indicative of chemical capture having occurred.

2. A method as claimed in claim 1, the chemical capture being hybridization.

3. A method as claimed in Claim 1 or claim 2, the probe and target molecules being oligonucleotides.

4. A method as claimed in Claim 3, wherein the first and second signatures are computed as a function of one or more measures representative of respective predetermined peaks of the spectra.

5. A method as claimed in Claim 4 wherein the signatures are computed as a function of a first measure corresponding to a first peak and a second measure corresponding to a second peak.

6. A method as claimed in Claim 5, the first peak corresponding to the breathing mode of adenine and the second peak corresponding to the breathing mode of thymine or cytosine.

7. A method as claimed in Claim 5, the first peak being located substantially at 790cm^{-1} and the second peak being located at substantially 731cm^{-1}.

8. A method as claimed in Claim 5, Claim 6 or Claim 7, the signatures being computed from the ratio of the respective first measure and the respective second measure.

9. A method as claimed in any one of Claims 4 to 8, the measures being representative of peak area or peak intensity.

10. A method as claimed in any one of the preceding claims, the method including

aa. measuring a third Raman spectrum before the sample is applied from a calibration region of the SERS surface spatially distinct from the said region, the calibration region supporting calibration molecules giving rise to a calibration Raman spectrum;

bb. measuring a fourth Raman spectrum from the calibration region after the sample has been applied;

cc. using the third and fourth Raman spectrum to calibrate the signatures, verify the integrity of the SERS surface or both.

11. A method as claimed in Claim 10, the calibration molecule being selected not to bind with the target molecules.

12. A method as claimed in Claim 11, the calibration molecule being a single stranded oligonucleotide comprising only one of adenine, thymine, cytosine or guanine.

13. A method as claimed in any one of the preceding claims, the SERS surface being part of an analytical chip including a plurality of said regions, distributed across the chip.

14. A method as claimed in 13, when dependent on Claims 10, 11 or 12, the analytical chip including a plurality of calibration regions distributed across the chip.

15. An analysis system comprising a sample holder for holding a SERS chip, a Raman spectrometer and an analyser configured to implement a method as claimed in any one of the preceding claims.

16. A computer program including code instructions implementing a method as claimed in any one of Claims 1 to 14.

17. A computer readable medium or data signal carrying a computer program as claimed in Claim 16.

18. A SERS chip including one or more regions of a SERS surface, each region supporting a plurality of probe oligonucleotides, wherein the ratio of the number of probe oligonucleotides to the number corresponding to a close packing of probe oligonucleaotides is less than one.

19. A SERS chip as claimed in Claim 18, wherein the said ratio is substantially equal to one half.

20. A SERS chip as claimed in Claim 19, wherein the said ratio is substantially equal to or within a range formed from the group of 0.1. 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9.

21. A SERS chip including one or more regions of a SERS surface supporting a respective plurality of probe molecules and one or more calibration regions of the SERS surface supporting a plurality of calibration molecules; wherein the probe molecules bind respective target molecules which are not bound by the calibration molecules.

22. A SERS chip as claimed in Claim 21, the probe molecules being oligonucleotides.

23. A SERS chip as claimed in Claim 22, the calibration molecules being oligonucleotides.

24. A use of a SERS chip having a SERS surface region supporting a first number of probe molecules, the use including applying a sample containing a second number of target molecules to the region, wherein the second number exceeds the first number.

25. A use as claimed in Claim 24, wherein the second number is sufficiently large to ensure substantially all probe molecules are hybridised.

26. A use as claimed in Claims in 24 or 25, the SERS chip being a chip as claimed in any one of Claims 17-22.

27. A SERS chip as claimed in any one of Claims 18 to 23, the SERS surface including an irregular array of metallic structures supported on a substrate.

28. A SERS chip as claimed in Claim 27, including silver structures supported on a silicon substrate.

29. A SERS chip as claimed in Claim 27 or 28, the structures including torroids.

30. A SERS chip as claimed in Claim 27 or 28, the structures including pillars.

31. A method of determining a suitable probe surface concentration for a SERS chip, the method including calculating a first signature from a spectrum obtained from a region of a given probe surface concentration before a target containing samples is applied to the said region, calculating a second signature from a spectrum measured from the said region after a target containing sample is applied to the said region; repeatedly calculating first and second signatures for regions of varying probe surface concentrations; and choosing the probe surface concentration for which the difference between the first and second signatures is largest as the suitable probe surface concentration.

32. A method of applying probe or target molecules in a buffer solution to a SERS surface, the buffer solution comprising an electrolyte which does not adsorb onto the SERS surface.

33. A method as claimed in Claim 32, the SERS surface including silver structures and the electrolyte including fluorine ions.

34. A reactive substrate in particular a SERS chip as claimed in any one of claims 18 to 23 and 27 to 29 in which structures are supported on a surface of the substrate including a layer of a lyophilic gel disposed on the substrate to stabilize the structures on the substrates.

35. A reactive substrate as claimed in claim 34 in which the gel is water based.

36. A reactive substrate as claimed in claim 35 in which the gel includes agarose or polyacrylamide.

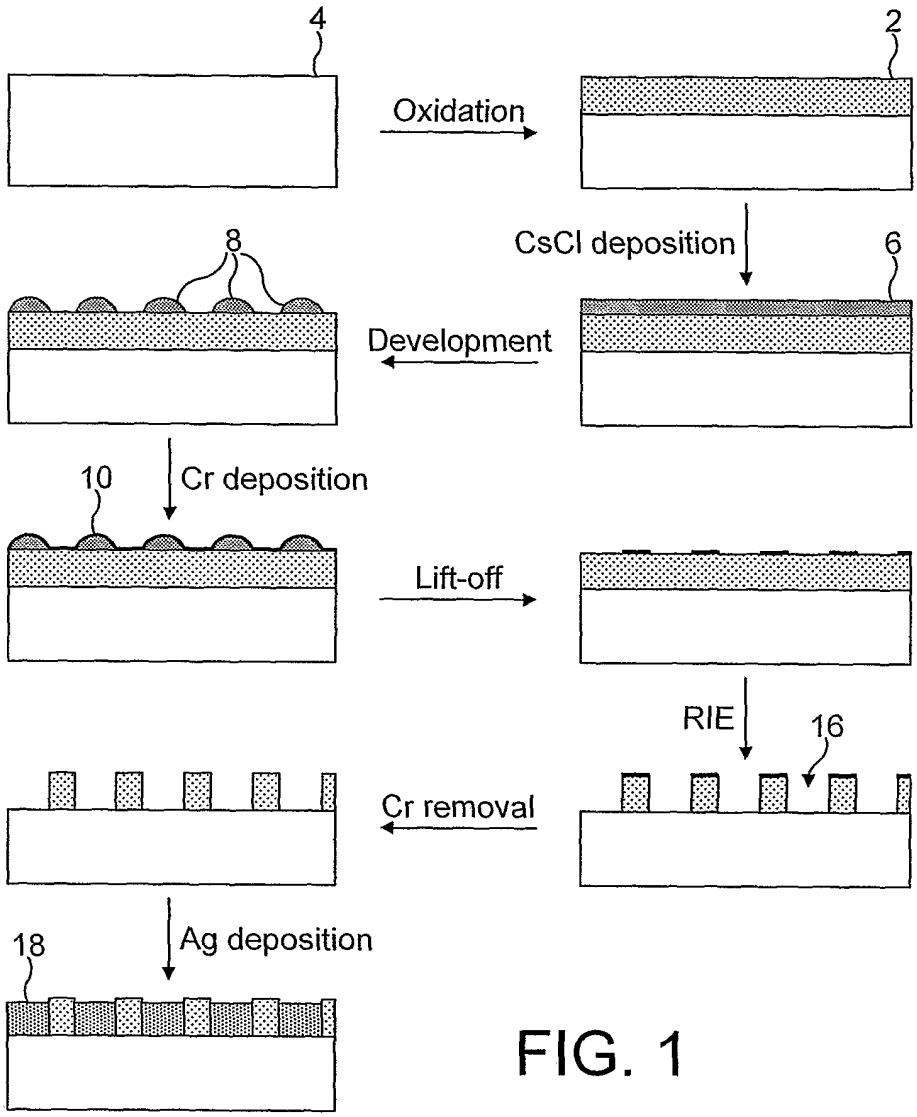
4
2
Oxidation
CsCl deposition
8
6
Development
10
Cr deposition
Lift-off
RIE
16
Cr removal
18
Ag deposition

FIG. 1

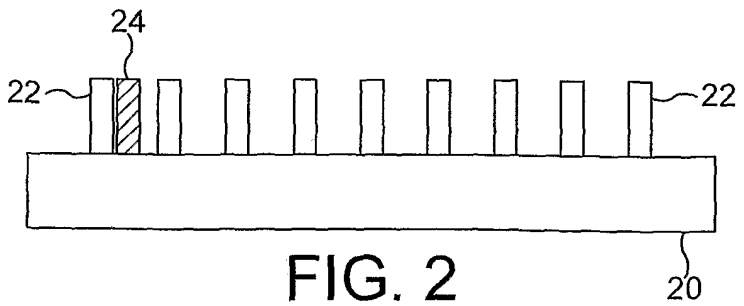
24
22
22
20

FIG. 2

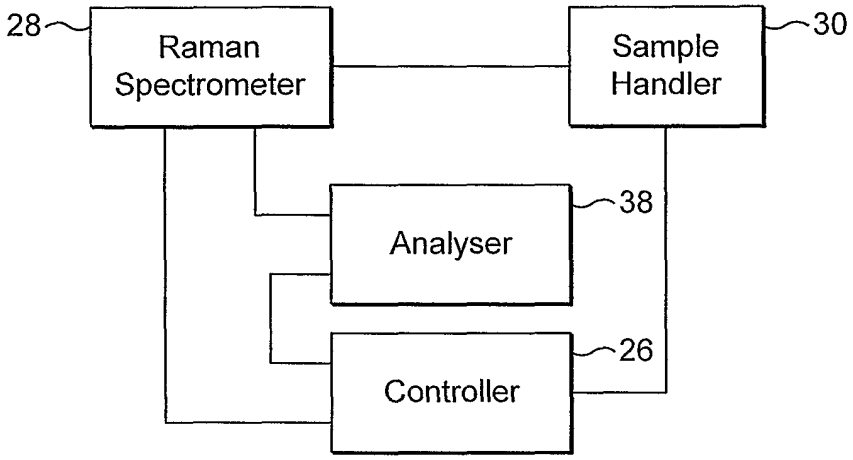
28
Raman Spectrometer
Sample Handler
30
Analyser
38
Controller
26

FIG. 3

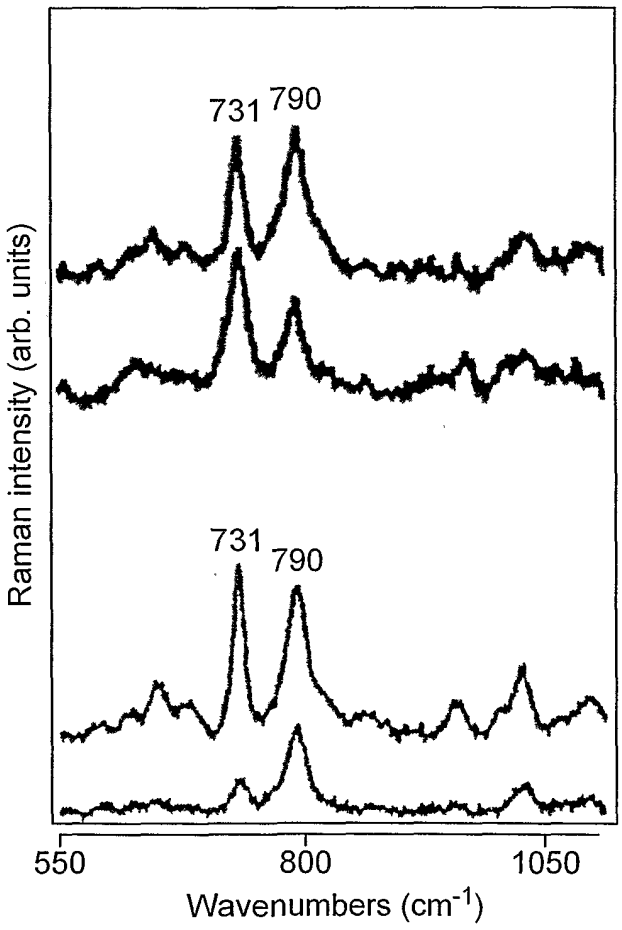
731
790
731
790
Raman intensity (arb. units)
550
800
1050
Wavenumbers (cm-1)

FIG. 4

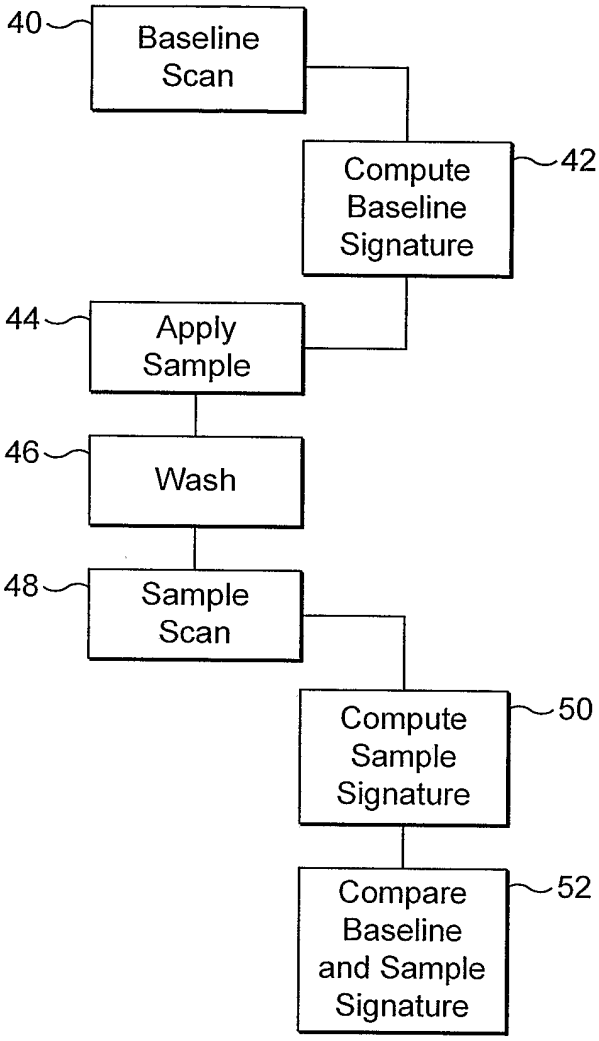
40
Baseline Scan
42
Compute Baseline Signature
44
Apply Sample
46
Wash
48
Sample Scan
50
Compute Sample Signature
52
Compare Baseline and Sample Signature

FIG. 5

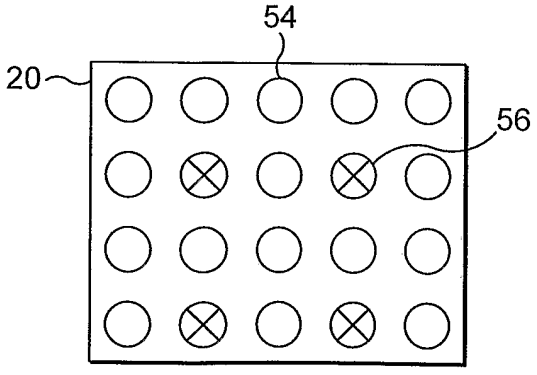
54
20
56

FIG. 6