



(51) International Patent Classification:

G01N 1/28 (2006.01) G01N 21/65 (2006.01)

(21) International Application Number:

PCT/IN2019/050540

(22) International Filing Date:

24 July 2019 (24.07.2019)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

201811028087 26 July 2018 (26.07.2018) IN

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP,

(54) Title: SCREENING KIT FOR DETECTION OF GRADES OF CERVICAL CANCER AND PROCESS FOR THE PREPARATION THEREOF

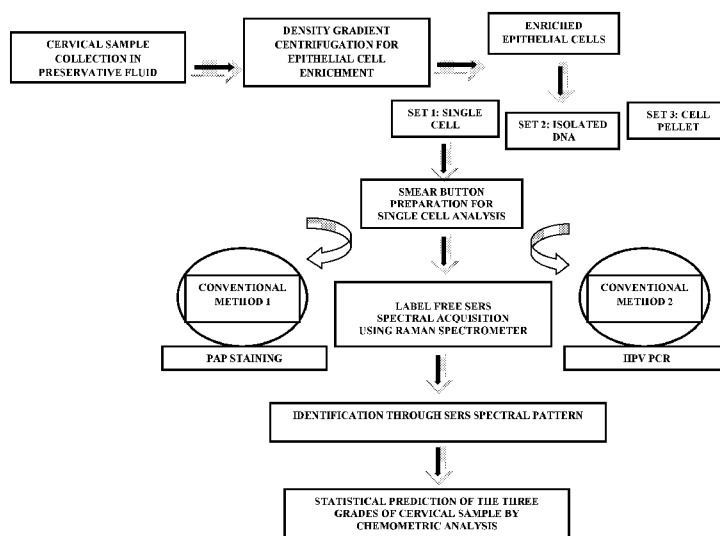


Fig. 6

(57) **Abstract:** The capabilities of using gold nanoparticle as surface-enhanced Raman scattering (SERS) substrate to obtain cervical smear harvested cells biochemical information for non-invasive cervical precancerous detection were presented in this patent document. A SERS reagent and a platform has been developed and optimized for the generation of a differential spectral fingerprinting for cervical cancer detection. SERS measurements were performed on three group's cervical exfoliated cell samples: one group from patients (n = 36) with pathologically confirmed cervical cancer and another group with high-grade squamous intraepithelial lesion (HSIL) (n = 41) and the last group from healthy volunteers (control subjects, n = 47). Tentative assignments of the Raman bands in the measured SERS spectra suggested interesting cancer specific biomolecular changes, including an increase in the relative amounts of amino acids, nucleic acid, carotenoid contents in the cell samples of cervical cancer patients as compared to that of healthy subjects. The results from



KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- *as to the identity of the inventor (Rule 4.17(i))*
- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
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Published:

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
- *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

SCREENING KIT FOR DETECTION OF GRADES OF CERVICAL CANCER AND PROCESS FOR THE PREPARATION THEREOF

FIELD OF INVENTION

The present invention relates to a screening kit for detection of different grades of cervical cancer. More, particularly the present invention relates to efficient platform developed to identify pre-cancerous lesions of the cervix viz.: (high-grade squamous intraepithelial lesion (HSIL) and cervical squamous cell carcinoma (CSCC) in the cells exfoliated from uterine cervix using a differential Raman spectral pattern.

BACKGROUND OF THE INVENTION

Cancer of the uterine cervix is one of the most common cancers among women worldwide and the second most prevalent cancers among women in India. It is considered as a preventable cancer as cervix is an easily amenable organ and occurrence of a heterogeneous spectrum of epithelial abnormalities (precancerous lesions) 10-15 years prior to the occurrence of invasive cancer has been well established (Sujathan et.al). The significance of Pap smear test for the detection and eradication of these precancerous lesions of cervix has been well documented. Based on this, systematically organized screening programmes for cervical cancer have been implemented in many of the developed countries. A rapid reduction in the incidence of this disease has been observed in countries like Sweden, Demark, Finland etc, where women have been covered by organized Pap smear screening programmes. The role of persistent infection with high risk HPV in cervical carcinogenesis is now well established and prophylactic vaccine against two of the high risk HPVs are currently available. Considering the several other high risk strains of this virus prevalent among the women, vaccine plus Pap smear test and HPV DNA test is now advocated for the effective control this disease. However, in India and many other low resource countries none of these measures have been implemented so far and cervical cancer continues to take extraordinary toll on the lives of our women. India contributes one fifth of the global burden of cervical cancer. As per the Globocan report of cervical cancer, around 528,000 estimated new cases with 266,000 death cases have been reported in 2012. Almost 9 out of 10 cervical cancer deaths occur in low resource countries including India. If we could also introduce population screening programmes, we could have saved the lives of our women. The major impediment for implementing screening programme by Pap smear is the lack of trained cytologists for microscopic analysis of the Pap smears of

the eligible women of the community. So there is an urgent need for alternative cost effective and reliable method of screening without the requirement of highly trained cytologists.

Pap smear test is the microscopical analysis of cells which are naturally exfoliating from the squamocolumnar junction of cervix. These cells often stick to the cervical mucus which is collected by scraping with a spatula. These cells are studied for various morphological features of precancerous lesions (LSIL, HSIL etc) or malignancy. Since the precancerous lesions are asymptomatic, only through regular screening programmes it can be picked up. The microscopic analysis of cells require highly trained cytotechnologists. The doctor at the time of examination, visualize the cervix and collect a cell sample from the cervix and preserve in a preservative fluid. These cells are smeared onto a glass slide, stained with Pap stain and studied under microscope by trained cytotechnologists for morphological features and reported as normal or positive for precancerous lesions LSIL/HSIL or positive for malignancy. All positive samples are further confirmed by a cytopathologist. The smears are often reported as per the modified Bethesda system. The precancerous lesions are treated and followed up in a systematic way to ensure that the lesion is not progressing. As per the reports of the developed countries, where there is regular systematic screening program using Pap smear, more than 85% of the smears are within normal limits. So, if these normal smears can be screened out by a cost effective method, without utilizing the skilled cytotechnologists, the other 15% can be subjected to manual methods without over use of the resources.

Because skilled cytotechnologists are less available and as it is a time consuming technique, some other alternative techniques are in high demand. The demand for specific and accurate cervical cancer screening has driven the development of novel diagnostic probes having high selectivity and sensitivity. Raman spectroscopy (RS) based on the inelastic light scattering can provide important biochemical information of macromolecules such as proteins, nucleic acids and lipids, because each molecule has its own pattern of vibrations that can serve as a Raman signature. Raman spectroscopy recently has emerged as a novel non-invasive diagnostic tool for cancer detection and identification of malignancy at different stages of neoplasia in tissues. Some groups have investigated the applications of Raman spectroscopy in differentiating normal and malignant tissues in various body sites, such as lungs, stomach, bladder, breast, parathyroid, prostate and cervix.

However, Raman scattering suffers the disadvantage of extremely poor efficiency due to its inherently small cross-section. Besides, the Raman spectra of biological samples are often superimposed on top of a strong fluorescence background that may be huge and make it difficult to extract the Raman signals.

So in medical diagnosis these disadvantages make it a great challenge for practical applications of conventional Raman spectroscopy. There has been a great interest in Raman spectroscopy technique in the past two decades owing to the discovery of the surface-enhanced Raman scattering (SERS). Surface-enhanced Raman scattering was first reported by Fleischman et al. in 1974. Recent studies show have
5 shown that with SERS technique, Raman signals can be enhanced by 10 to 14 orders of magnitude when the Raman active molecules are attached to nano-textured metallic surfaces, simultaneously with reduced autofluorescence background. SERS technology greatly improves the detection sensitivity of Raman spectroscopy, and has drawn considerable attention due to its great potential in biomedicine. SERS based immunoassay, which relies on a specific interaction between an antigen and a
10 complementary antibody, is the strategy developed for most current oncology applications. Label-free SERS produces huge and complex data sets and it necessitates more refined analytical processes to mine substantial information from the spectral data. A great inconsistency in the intensity and spatial scattering generated due to the variable deposition and orientation of gold nanoparticles reduces the clarity of spectral data obtained. Even though such challenges are existing, introduction of chemometric
15 analysis like Principal Component Analysis (PCA), Linear Discriminant Analysis (LDA), Cluster Analysis, Support Vector Machine Analysis (SVM) have enriched or improved the spectral data sets for classification and characterization.

For cervical cancer, smears made from scraped cervical exfoliated cells is the accepted screening method which can be taken conveniently and even continuously throughout the treatment for diagnosed
20 patient without causing pain in a non-invasive manner and biopsy is done for confirmation of high grade lesions and invasive carcinoma. All studies reported earlier used tissue samples for Raman measurements rather than cell samples. Collection of tissue sample needs surgical procedures and hence cannot be suggested for screening purpose. Gold nanoparticles (AuNPs) are used in this study as SERS substrate because of their favorable physical, chemical properties and biocompatibility.

In this invention, we explored the use of AuNPs for SERS spectral evaluation in cervical exfoliated cells in order to find out the biochemical changes which could able to differentiate between NRML, HSIL and CSCC patients by distinct changes in Raman fingerprints of variable abundance of intracellular amino acids with associated proteins, lipids, nucleic acids, DNAs and variables viz., amide II, amide III etc. To our knowledge, this is the first report on SERS based analysis of cervical exfoliated
30 cells using a label free nanoformulation with AuNPs for cervical precancerous detection. The results showed significant level of differentiation between three classes using single cell, cell pellet and extracted DNA measurements.

Definitions

AuNPs - Gold nano-particles

NRML - Normal

HSIL - high-grade squamous intraepithelial lesion

5 CSCC - cervical squamous cell carcinoma

(iv) BRIEF DESCRIPTION OF THE DRAWINGS

The objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings, which respectively show

- Fig 1 : Representative spectrum for CSCC sample from 400 cm^{-1} to 1800 cm^{-1}
- 10 • Fig 2 : Representative spectrum for HSIL sample from 400 cm^{-1} to 1800 cm^{-1}
- Fig 3 : Representative spectrum for NRML sample from 400 cm^{-1} to 1800 cm^{-1}
- Fig 4: Differential spectra for DNA samples from NRML, HSIL and CSCC (500 cm^{-1} to 1800 cm^{-1}) and its respective peak assignments
- Fig 5: Linear discriminant analysis in a) Single cell, b) Cell pellet, c) Extracted DNA
- 15 • Fig 6: Scheme representing the overall work
- Fig 7: Comparison of mean SERS spectral peak from NRML, HSIL and CSCC samples.

OBJECTIVES OF THE INVENTION

The main objective of the invention relates to a kit for screening of different grades of cervical cancer

Another objective of the present invention is to provide the abundance of the three grades viz. NRML, HSIL, and CSCC by Raman fingerprint analysis.

Yet another objective of the present invention is to provide the validation of the signature spectra viz., presence of amino acids associated proteins, lipids, nucleic acids, DNAs, in terms of peak intensity, peak shift with sufficient number of patient samples.

Still another objective of the present invention is differential recognition, sensitivity, specificity, and prediction accuracy to be validated by chemometric analysis.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides a label free detection kit useful in SERS based platform for three major grades of exfoliated cells obtained from cervix with simple processing and utilization of specified concentration of AuNPs as SERS substrate which designated by significant Raman spectral pattern of biomolecular fingerprint between healthy subjects, high-grade squamous intraepithelial lesion (HSIL) and cervical squamous cell carcinoma (CSCC) patients. Raman intensity difference together with specific peak assignments measured SERS bands make it clear that benign and malignant cervical

tumors gave rise to the structural and specific biomolecular changes of cervical epithelial cells, including the relative amounts of various biomolecules like DNA, protein, lipids etc. Carotenoid peak with significant intensity is high in the case of cancer samples. These variations may be connected to metabolic changes among normal to carcinoma samples. These results from this exploratory study demonstrated the great potential of cervical cancer by SERS based platform as a clinical tool for label-free, noninvasive, and convenient for the diagnosis of early stages of cancer detection and screening.

In an embodiment of the present invention it provides a kit for screening of different grades of cervical cancer comprising of:

- a) Gold (AuNP) Nanoparticle having size in the range of 40-50nm nm as the SERS substrate.
- b) Preservative fluid comprising of > 50% Ethanol, Methanol, Isopropanol, Formaldehyde, Saline solution, Di- potassium hydrogen phosphate.
- c) Density gradient solution comprising of 20-50 % (w/v) of sucrose in MilliQ water
- d) Phosphate Buffered Saline (PBS) composed of Sodium Chloride, Potassium Chloride, Disodium phosphate, Potassium dihydrogen phosphate.
- e) Pre- coated glass slide comprising of Poly-L-lysine, APES [(3- Aminopropyl) triethoxy silane. for the effective attachment of sample. (Surface enhanced Raman scattering PLATFORM).

In an embodiment of the present invention it provides a kit where in the concentration of the nanoparticle is in the range of $8-10 \times 10^{13}$ particles/ml.

In an embodiment of the present invention it provides a kit where in the preservative fluid used for fixing the sample on the slide is selected from the group comprising of > 50% Ethanol, Methanol, Isopropanol, Formaldehyde, Saline solution, Di- potassium hydrogen phosphate.

In an embodiment of the present invention it provides a kit where in the density gradient solution comprises of 20-50 % (w/v) of sucrose in MilliQ water.

In an embodiment of the present invention it provides a kit where in the Phosphate buffered saline comprises of Sodium Chloride, Potassium Chloride, Disodium phosphate, Potassium dihydrogen phosphate.

In an embodiment of the present invention it provides a kit where in the glass slide is coated with compound selected from the group comprising of PolyL-lysine, APES [(3- Aminopropyl) triethoxy silane for adhesion of cells in the glass slide.

In yet another embodiment of the present invention it provides a kit where in the different grades of cervical cancer is selected from the group consisting of NRML (Normal), HSIL (High-grade Squamous Intraepithelial Lesion), CSCC (Cervical Squamous Cell Carcinoma).

In yet another embodiment of the present invention it provides a method for detection of different stages of cervical cancer comprising the following steps:

- a. Providing cell samples in preservative fluid comprising of > 50% Ethanol, Methanol, Isopropanol, Formaldehyde, Saline solution, Di- potassium hydrogen phosphate.
- 5 b. Centrifuging the cell samples obtained in step **a** to obtain a pellet by using density gradient fluid comprising of 20-50% (w/v) of sucrose in MilliQ water to enrich the more denser epithelial cells as a pellet in the bottom of the tube and less denser interfering cells like RBCs, polymorphs, inflammatory cells, mucus will be cleared in the supernatant.
- 10 c. Resuspending the cell pellet obtained in step **b** in PBS buffer comprising of Sodium Chloride, Potassium Chloride, Disodium phosphate, Potassium dihydrogen phosphate.
- d. Providing glass slide pre-coated with compounds comprising of Poly-L-lysine, APES [(3- Aminopropyl) triethoxy silane.
- 15 e. Dropping down the suspension obtained in step **c** onto pre-coated glass slide obtained in step **d**;
- f. Incubation of dropping down cell suspension onto pre-coated glass slide obtained in step **e** with AuNPs approximately 10-30 minutes.
- g. Measuring the surface enhanced Raman scattering from the cell samples and
20 analyzed the Raman spectral pattern to differentiate the three grades i.e. NRML, HSIL and CSCC.

DETAILED DESCRIPTION OF THE INVENTION

A SERS based diagnostic platform extended with a kit including gold nanoparticles, AuNPs (40-50 nm) $8 \text{ to } 10 \times 10^{13}$ particles/ml as the SERS substrate for differentiation of NRML, HSIL and CSCC
25 of the cervical exfoliated cells. Our ultimate aim is to validate the differential spectral pattern utilizing the SERS-nanoformulation which distinctly recognizes the HSIL and CSCC cells from NRML within the exfoliated cells collected from the cervix. This study was approved by the local Ethics Committee. Prior to specimen collection, all patients have signed informed consent forms. Pathologically confirmed cervical smears, NRML, HSIL and CSCC are collected in BD preservative fluid using speculum.
30 Density gradient centrifugation was performed to bring down the epithelial cells as a pellet. The pellet was then re-suspended in PBS buffer and dropped as a smear button in a glass slide. SERS spectral analysis was done using 9:1 ratio of AuNPs and sample ($8 \text{ to } 10 \times 10^{13}$ particles/ml) with diode laser of

633 nm laser excitation source with spectrograph grating 600gr/mm using maximum 10-20 sec integration time and around 10-15 accumulations. Single cells, cell pellet and extracted DNA was investigated in order to differentiate the three categories. NRML has comparatively less intense peaks than HSIL and CSCC samples when normalized to its highest peaks. The significant SERS spectral signatures between NRML, HSIL and CSCC were observed to be 481, 573, 666, 729, 826, 956, 1002, 1080, 1163, 1175, 1286, 1373 and 1558 cm^{-1} . Overall aromatic amino acid peaks like tryptophan, tyrosine and phenyl alanine were prominent in the SERS spectrum showed an incremental increase in intensity from HSIL and CSCC when compared to NRML. A prominent peak at 1080 cm^{-1} corresponding to phosphate backbone of nucleic acids were evident in CSCC samples with a shift in NRML sample at 1066 cm^{-1} . Amide III signal was found to be higher in HSIL and CSCC while Amide II showed a shift between the NRML, HSIL and CSCC samples. Linear discriminant analysis showed a 14nm shift between the NRML and CSCC samples, 11nm shift between NRML and HSIL samples, 3nm shift between HSIL and CSCC samples. Linear discriminant analysis showed a clear discrimination between NRML, HSIL and CSCC samples in whole cells, cell pellet and extracted DNA. In addition, chemometric analysis through Support Vector Machine (SVM) analysis showed a prediction accuracy of > 90% with a standard deviation of < 1 % for single cell, > 75% predication accuracy with a standard deviation of < 1 % for cell pellet and > 90% prediction accuracy with a standard deviation of < 4 % for extracted DNA.

EXAMPLES-

The following examples are given by way of illustration of the present invention and therefore should not be construed to limit the scope of the present invention.

EXAMPLE-1

Preparation of SERS based screening kit:

- (i) Synthesis and Optimization of the concentration of Gold Nanoparticle (AuNPs) for label free detection.

Gold nanoparticles (AuNPs, size around 40-50 nm) was prepared by well-established citrate reduction method. The characterization of the synthesized nanoparticles were performed through UV-vis spectroscopy, Dynamic Light Scattering (DLS) and High Resolution Transmission Electron Microscopy (HRTEM). The size was approximately 40-50 nm which serves as an optimal performing SERS substrate.

(ii) Optimum concentration of AuNPs which will provide maximum Raman signal intensity;

Optimum concentration has been evaluated (AuNPs 8 to 10×10^{13} particles/ml) which provided the maximum SERS intensity.

(iii) A preservative fluid was prepared for preservation and fixation of the sample collected comprising of $> 50\%$ Ethanol, Methanol, Isopropanol, Formaldehyde, Saline solution, Di-potassium hydrogen phosphate.

(iv) A density gradient solution was prepared comprises of 20 - 50% (w/v) of sucrose in MilliQ water for enriching the epithelial cells in the samples.

(v) A phosphate buffered saline buffer was prepared comprises of Sodium Chloride, Potassium Chloride, Disodium phosphate, Potassium dihydrogen phosphate for resuspension of cell pellet.

(vi) SERS platform consist of glass slide coated with comprising of PolyL-lysine, APES [(3-Aminopropyl) triethoxy silane] for adhesion of cells sample in the glass slide.

EXAMPLE -2

SERS Fingerprint from Exfoliated cells

The exfoliated cell samples were collected in the preservative solution comprising of $> 50\%$ Ethanol, Methanol, Isopropanol, Formaldehyde, Saline solution, Di-potassium hydrogen phosphate. Density gradient centrifugation was performed using sucrose as gradient solution to enrich the epithelial cells in the cervical smear samples. The pellet obtained was resuspended in the PBS buffer comprising Sodium Chloride, Potassium Chloride, Disodium phosphate, Potassium dihydrogen phosphate. The resuspended solution was dropped in the glass slide coated with Poly L-lysine, APES [(3-Aminopropyl) triethoxy silane. After 10 - 30 minutes incubation, the excess fluid is discarded and the slide is stored in absolute ethanol till SERS measurement. Single cell has been focused bright field and spectra have been taken from different fields of the cell. A number of spectra have been taken from random locations of the cells and also from nucleus after morphologically suspected normal and abnormal cell identification. The ratio of nuclear size to cytoplasm has been taken as a feature for morphological discrimination parameter for taking the SERS measurements. SERS spectral analysis is done using AuNPs with a concentration of 8 to 10×10^{13} particles/ml) and analyzed through diode laser of 633 nm laser excitation source with spectrograph grating 600 gr/mm setting 10 - 15 sec integration time and 10 - 20 accumulations. The laser power is between 3 - 7 mW. The fingerprint region 400 cm^{-1} to 1800 cm^{-1} was analyzed for the spectral differences between the three classes.

- Signature Raman spectra obtained due to the abundance of amino acids associated proteins, lipids, nucleic acids, DNAs, in terms of peak intensity, peak shift with a sufficient number of patient samples
- Differential recognition : Specificity, specificity and prediction accuracy obtained by chemometric analysis.

EXAMPLE -3

Single cell spectral analysis from cervical squamous cell carcinoma (CSCC)

The CSCC samples were collected in the preservative solution comprising of > 50% Ethanol, Methanol, Isopropanol, Formaldehyde, Saline solution, Di- potassium hydrogen phosphate. Density gradient centrifugation was performed using sucrose as gradient solution to enrich the epithelial cells in the cervical smear samples. The pellet obtained was resuspended in the PBS buffer comprising Sodium Chloride, Potassium Chloride, Disodium phosphate, Potassium dihydrogen phosphate. The resuspended solution was dropped in the glass slide coated with PolyL-lysine, APES [(3- Aminopropyl) triethoxy silane. After 10-30 minutes of incubation, the excess fluid is discarded and the slide is stored in absolute ethanol till SERS measurement.

CSCC cells are having a variety of peaks in the range 400-1800 cm^{-1} including 481, 573, 666, 729, 826, 920, 956, 1002, 1012, 1080, 1156, 1163, 1175, 1286, 1373, 1544 cm^{-1} . The aromatic amino acids, tryptophan, tyrosine and phenyl alanine peak has significant intensity in CSCC samples. Tryptophan abundance were evident from the peak at 573 cm^{-1} peak. Tyrosine peaks at 1163 cm^{-1} were prominent in CSCC samples. Phenyl alanine peak at 1002 cm^{-1} in all samples showed a shoulder peak at 1012 cm^{-1} in HSIL and CSCC samples. Carotenoid peak at 956 and 1156 cm^{-1} is having significant intensity which helps the cancer cells to resist damage and also helps the cancer cells in the synthesis of large amount of glycoproteins. The PO₂ stretching of nucleic acid at 1066 cm^{-1} in NRML samples have a significant increase and shift to 1080 cm^{-1} respectively in HSIL and CSCC sample. The PO₂ stretching peak at 826 cm^{-1} which shows DNA content increase is prominent in HSIL and CSCC samples which is absent in NRML. The nucleic acid bases cytosine, guanine, adenine and thymine peaks at 666, 729, 1175, 1373 cm^{-1} were prominent in the CSCC samples. The Amide III signal were shifted to 1286 cm^{-1} in CSCC samples from 1260 cm^{-1} in NRML samples and found to be prominent in CSCC while Amide II showed a shift from 1558 cm^{-1} in NRML to 1544 cm^{-1} in CSCC samples. Linear discriminant analysis showed a clear discrimination of carcinoma sample from NRML, HSIL and CSCC samples in single cell, cell pellet and extracted DNA.

EXAMPLE -4**Single cell spectral analysis from High-grade Squamous Intraepithelial Lesion (HSIL)**

The HSIL samples were collected in the preservative solution comprising of > 50% Ethanol, Methanol, Isopropanol, Formaldehyde, Saline solution, Di- potassium hydrogen phosphate. Density gradient centrifugation was performed using sucrose gradient solution to enrich the epithelial cells in the cervical smear samples. The pellet obtained was resuspended in the PBS buffer comprising Sodium Chloride, Potassium Chloride, Disodium phosphate, Potassium dihydrogen phosphate. The resuspended solution was dropped in the glass slide coated with either PolyL-lysine, APES [(3- Aminopropyl) triethoxy silane. After 10-30 minutes of incubation, the excess fluid is discarded and the slide is stored in absolute ethanol till SERS measurement.

Cells from HSIL were found to have significant signature peaks in the range 400 -1800 cm^{-1} including 481,573,666,729,826, 956, 1002, 1012, 1080, 1163,1175,1280,1373 and 1547 cm^{-1} . Peaks at 1012 cm^{-1} and 573 cm^{-1} showed the increasing activity of abnormality in HSIL samples which is assigned to phenyl alanine and tryptophan respectively. The nucleic acid peak at 826 cm^{-1} have been shifted in HSIL and CSCC samples. A peak at 1080 cm^{-1} assigned to PO2 stretching has been slightly increased which shows the increasing abundance of nucleic acids, but is lesser than CSCC samples. A peak at 1175 cm^{-1} showed the increase of cytosine, guanine in HSIL samples.1280 cm^{-1} peak was assigned to Amide III which is also shifted when compared with NRML and CSCC.1547 cm^{-1} peak was assigned to Amide II which is also slightly shifted from 1544 cm^{-1} in CSCC samples.

EXAMPLE -5**Single cell spectral analysis from Normal (NRML)**

The NRML samples were collected in the preservative solution comprising of > 50% Ethanol, Methanol, Isopropanol, Formaldehyde, Saline solution, Di- potassium hydrogen phosphate. Density gradient centrifugation was performed using sucrose gradient solution to enrich the epithelial cells in the cervical smear samples. The pellet obtained was resuspended in the PBS buffer comprising Sodium Chloride, Potassium Chloride, Disodium phosphate, Potassium dihydrogen phosphate. The resuspended solution was dropped in the glass slide coated with either PolyL-lysine, APES [(3- Aminopropyl) triethoxy silane. After 10-30 minutes of incubation, the excess fluid is discarded and the slide is stored in absolute ethanol till SERS measurement.

Normal cells showed signature peaks in the range 400-1800 cm^{-1} including 666, 729, 850, 956, 1002, 1066,1163,1260,1373 and 1558 cm^{-1} . Peaks at 1002 cm^{-1} showed the presence of phenyl alanine.

A slight peak at 1066 cm^{-1} was found associated with nucleic acid PO_2 stretching which is shifted to 1080 cm^{-1} prominently in CSCC samples. The 1163 cm^{-1} peak was associated with lipids $\text{C}=\text{C}$ stretch and tyrosine. The amide III peak at 1260 cm^{-1} was prominent in normal samples. The amide II peak showed at 1558 cm^{-1} which was significantly shifted in HSIL and CSCC samples.

5 EXAMPLE -6

Differences between the CSCC, HSIL and NRML exfoliated cervical cells

NRML	HSIL	CSCC
Major signature peaks 666, 729, 850, 956, 1002, 1066, 1163, 1260, 1373 and 1558 cm^{-1}	Major signature peaks 481, 573, 666, 729, 826, 956, 1002, 1012, 1080, 1163, 1175, 1280, 1373 and 1547 cm^{-1}	Major signature peaks 481, 573, 666, 729, 826, 920, 956, 1002, 1012, 1080, 1156, 1163, 1175, 1286, 1373, 1544 cm^{-1}
Nucleic acid PO_2 stretching at 826 cm^{-1} is shifted to 850	826 cm^{-1} is prominent in	826 cm^{-1} is significantly increased.
The nucleobases at 666, 729, 1175, 1373 and 1421 cm^{-1} are not prominent	The nucleobases at 666, 729, 1175 and 1373 is prominent than normal samples	The nucleobases at 666, 729, 1175 and 1373 cm^{-1} is prominent
Tryptophan peak at 573 cm^{-1} is not prominent.	Tryptophan peak prominent than normal samples	Tryptophan peak at 573 cm^{-1} is prominent
Phenyl alanine peak at 1002 cm^{-1}	Phenyl alanine peak shifted to 1012 cm^{-1}	Phenyl alanine peak shifted to at 1012 cm^{-1}
Tyrosine not prominent	Tyrosine not prominent	Tyrosine peak at 1163 cm^{-1} is prominent
No prominent peak	Slight peak corresponding to 956 cm^{-1} carotenoid is present	A carotenoid peak at 956 and 1156 cm^{-1} is prominent
Nucleic acid PO_2 stretching at 1066 cm^{-1}	PO_2 stretching shifted to 1080 cm^{-1}	PO_2 stretching at 1080 cm^{-1} is prominent
Amide III peak at 1260 cm^{-1}	Amide III peak shifted to 1280 cm^{-1}	Amide III peak shifted to 1286 cm^{-1}
Amide II peak at 1558 cm^{-1}	Amide II peak shifted to 1547 cm^{-1}	Amide II peak shifted to 1544 cm^{-1}

EXAMPLE-7**Cell Pellet spectral analysis**

The exfoliated cell samples were collected in the preservative solution comprising of > 50% Ethanol, Methanol, Isopropanol, Formaldehyde, Saline solution, Di- potassium hydrogen phosphate. Density gradient centrifugation was performed using sucrose gradient solution to enrich the epithelial cells in the cervical smear samples. The cell pellet obtained was resuspended in the PBS buffer comprising Sodium Chloride, Potassium Chloride, Disodium phosphate, Potassium dihydrogen phosphate.

The cell pellet is directly mixed with AuNPs and SERS spectral analysis was carried out. Because of the heterogeneous nature of the pellet which comprises of both normal and abnormal cells, a mixture of signature Raman spectra were acquired and nearly 75 % prediction accuracy was obtained through SVM analysis.

EXAMPLE-8**DNA spectral analysis**

DNA was isolated from different NRML, HSIL and CSCC samples collected in the preservative solution comprising of > 50% Ethanol, Methanol, Isopropanol, Formaldehyde, Saline solution, Di-potassium hydrogen phosphate and the SERS spectral analysis using AuNPs were carried out. A number of spectra have been taken with a specified proportion of AuNPs with laser of 633 / 786 nm laser excitation source with spectrograph grating 600gr/mm / 1200gr/mm having 10-15 sec integration time and 10-20 accumulations. The laser power has been used in between 3-7mW. The fingerprint region 400 to 1800 cm^{-1} was analyzed for the spectral differences between the three classes. 729, 1175 and 1421 and 1578 cm^{-1} corresponding to nucleobases were prominent in abnormal DNA samples. The phosphate backbone of nucleic acid is evident significantly at 826 and 1080 cm^{-1} peaks.

EXAMPLE-9**Chemometric analysis**

Validation of spectral differences between NRML, HSIL and CSCC through statistical analysis. All statistical analysis including PCA, LDA and SVM analysis were done using R software. Intra group variations can occur due to noise during acquisition of Raman data from cells which lead to reduction of specificity of the PCA. Hence we further adopted LDA and SVM for further analysis.

Linear Discriminant Analysis (LDA)

For classification, LDA is better by theory and concept. LDA is used for analyzing variables in studied groups which are statistically significant. Clear demarcation between all the three samples was obtained for NRML, HSIL and CSCC.

Support Vector Machine (SVM)

Support Vector Machine are supervised learning models or a machine learning technique with algorithms that analyse data for classification and regression analysis. Analysis were done by randomly selecting 75% of the spectra as the train set and rest 25% were used as the test set. The SVM analysis were repeated with 500 different random samples and measured the average prediction accuracy. The accuracy were found above 90% for single cell, 75% for cell pellet and 90% for extracted DNA respectively.

CELL	NRML	HSIL	SCC
NRML	9	0	322
HSIL	17	49	3
SCC	226	4	2
Prediction accuracy		93.84 %	
Standard deviation		0.73 %	
PELLET	NRML	HSIL	SCC
NRML	5	0	31
HSIL	6	3	7
SCC	24	0	5
Prediction accuracy		74.26 %	
Standard deviation		0.05 %	
DNA	NRML	HSIL	SCC
NRML	19	0	0
HSIL	0	13	0
SCC	1	0	11
Prediction accuracy		92.21%	
Standard deviation		3.84%	

Support Vector Machine analysis in a) Single cell, b) Cell pellet, c) Extracted DNA

ADVANTAGES OF THE INVENTION

The main advantages of the present invention are as follows.

1. It provides a significant difference of SERS spectral pattern of NRML, HSIL and CSCC were found through SERS based label free detection.
- 5 2. As immunostaining is a time consuming and skilled cytotechnologists are required for correct evaluation and HPV, PCR causes nonspecific amplification of abnormal samples irrespective of its grades and expensive, SERS is an accurate, simple and reliable technique which can differentiate normal, HSIL and cancerous samples through its differential spectra.
- 10 3. It provides a diagnostic screening kit which differentiated the grades of cervical cancer exfoliated cells through a label free detection platform using surface enhanced Raman scattering (SERS) technique.
4. The screening kit adopted a new SERS technique which enriched the cervical exfoliated cells in order to get maximum differentiation of three grades.
5. It provides the abundance of aromatic amino acids like tryptophan, phenyl alanine and tyrosine
15 and their specific peak shifts which differentiated significantly between NRML, HSIL and CSCC.
6. It provides the nucleic acid bases i.e. cytosine, guanine, adenine peaks at 666, 729, 1175, 1373 cm^{-1} prominent in the CSCC samples.
7. It provides the major identification of carotenoid peak at 956 and 1156 cm^{-1} with high intensity got in CSCC samples which were not prominent in normal samples.
- 20 8. The identification of PO_2 stretching of nucleic acid which showed the increase in DNA seen at 1070-1090 cm^{-1} range specific only to HSIL and CSCC positive samples.
9. It provides the Amide III peak and Amide II at 1260 and 1558 cm^{-1} showed a prominent shift in HSIL and CSCC samples.
- 25 10. It provides the Raman spectra has been evaluated through chemometric analysis which showed more than 80% sensitivity in cell samples and can be utilized as reference spectra for screening of cervical precancerous lesions.

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WE CLAIM:

1. A kit for screening different grades of cervical cancer comprising of:
 - a) Gold Nanoparticle (AuNPs) having size in the range of 40-50 nm as the SERS substrate
 - b) Preservative fluid.
 - c) Density gradient solution.
 - d) Phosphate Buffered Saline (PBS).
 - d) Pre- coated glass slide. The kit as claimed in claim 1, wherein the concentration of the nanoparticle is in the range of $8-10 \times 10^{13}$ particles/ml.
2. The kit as claimed in claim 1, wherein the preservative fluid used for fixing the sample on the slide is selected from the group comprising of > 50% Ethanol, Methanol, Isopropanol, Formaldehyde, Saline solution, Di- potassium hydrogen phosphate.
3. The kit as claimed in claim 1, where in the density gradient solution is 20-50% (w/v) of sucrose in MilliQ water.
4. The kit as claimed in claim 1, wherein the phosphate buffered saline is composed by Sodium Chloride, Potassium Chloride, Disodium phosphate, Potassium dihydrogen phosphate.
5. The kit as claimed in claim 1, wherein the glass slide is pre-coated with compound selected from the group comprising of Poly-L-lysine, APES [(3- Aminopropyl) triethoxy silane.
6. The kit as claimed in claim 1, wherein the different grades of cervical cancer is selected from the group consisting of NRML (Normal), HSIL (High-grade Squamous Intraepithelial Lesion), CSCC (Cervical Squamous Cell Carcinoma).
7. A method for detection of different grades of cervical cancer comprising the following steps:
 - a. Providing cell samples in preservative fluid comprising of > 50% Ethanol, Methanol, Isopropanol, Formaldehyde, Saline solution, Di- potassium hydrogen phosphate.
 - b. Centrifuging the cell samples obtained in step **a** to obtain a pellet by using density gradient fluid comprising of 20-50% (w/v) of sucrose in MilliQ water.

- c. Resuspending the cell pellet obtained in step **b** in PBS buffer comprising of Sodium Chloride, Potassium Chloride, Disodium phosphate, Potassium dihydrogen phosphate.
- d. Providing glass slide pre-coated with compounds comprising of Poly-L-lysine, APES [(3- Aminopropyl) triethoxy silane.
- 5 e. Dropping down the cell suspension obtained in step **c** onto pre-coated glass slide obtained in step **d**;
- f. Incubating the dropped down cell suspension onto pre-coated glass slide obtained in step **e** with AuNPs for 10-30 minutes.
- 10 g. Measuring the surface enhanced Raman scattering from the cell samples and analyzing the Raman spectral pattern to differentiate the three grades of cervical cancer i.e. NRML, HSIL and CSCC.

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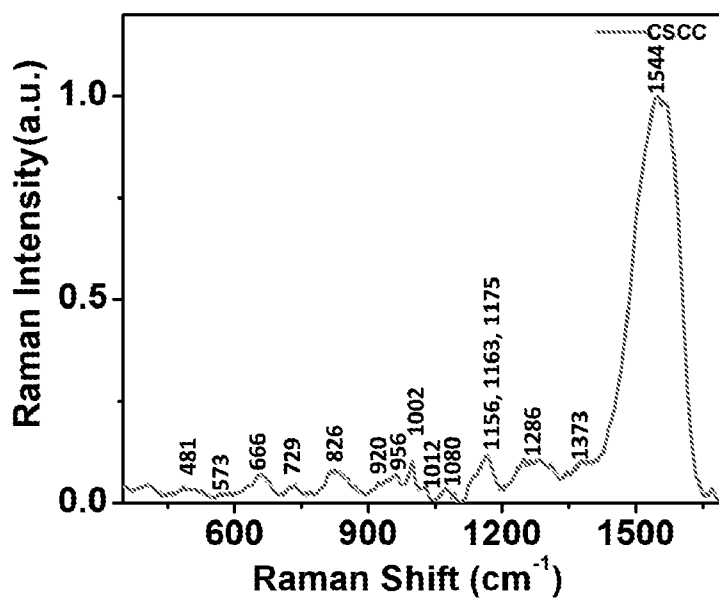


Fig. 1

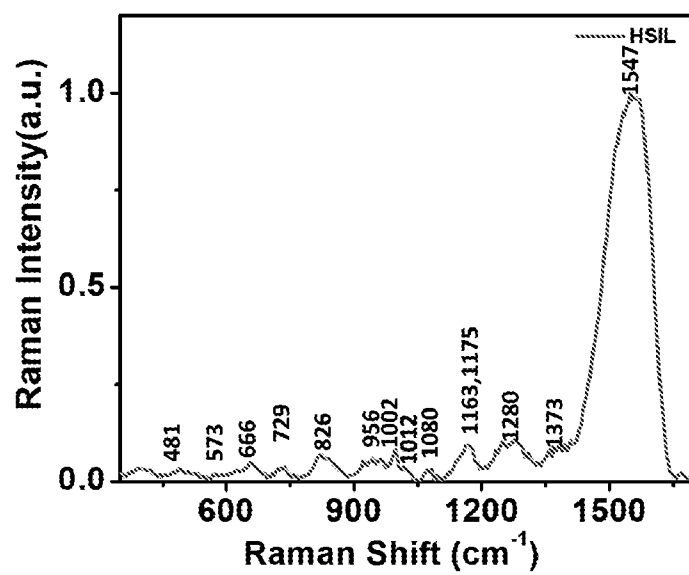


Fig. 2

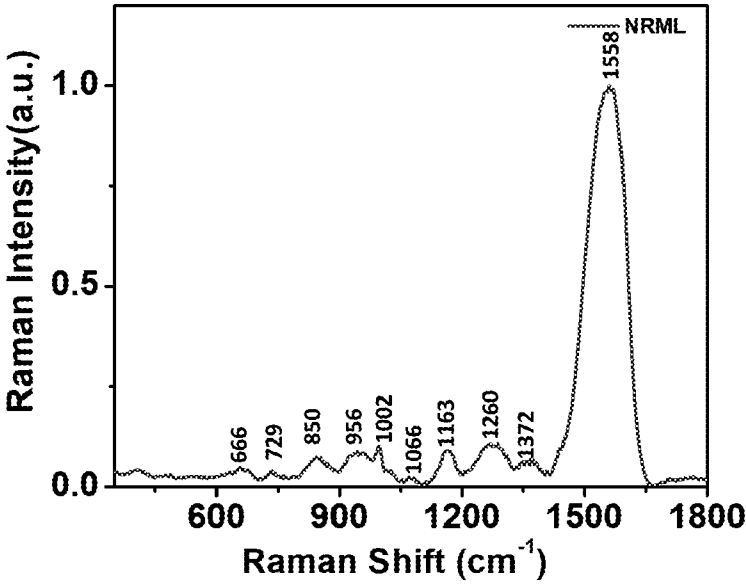


Fig. 3

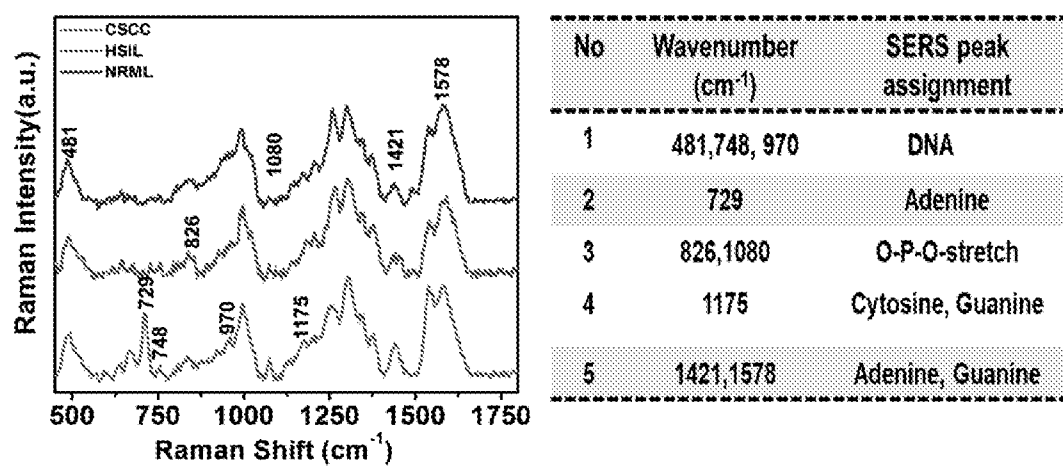


Fig. 4

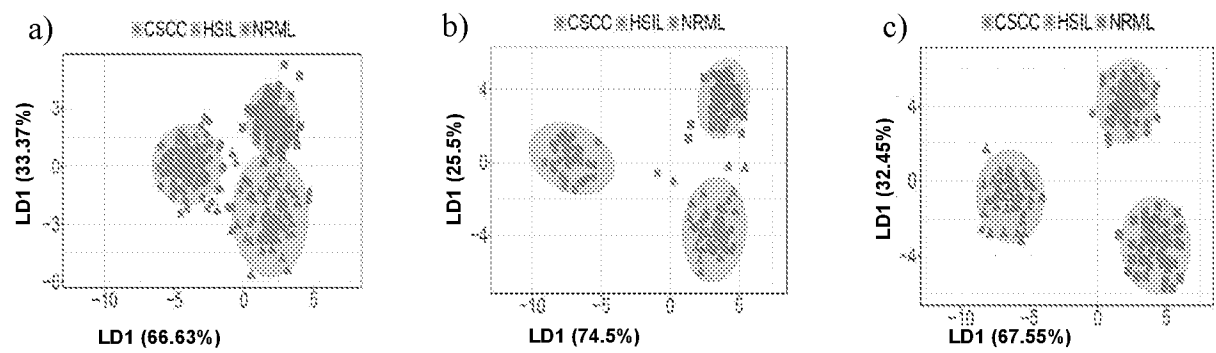


Fig. 5

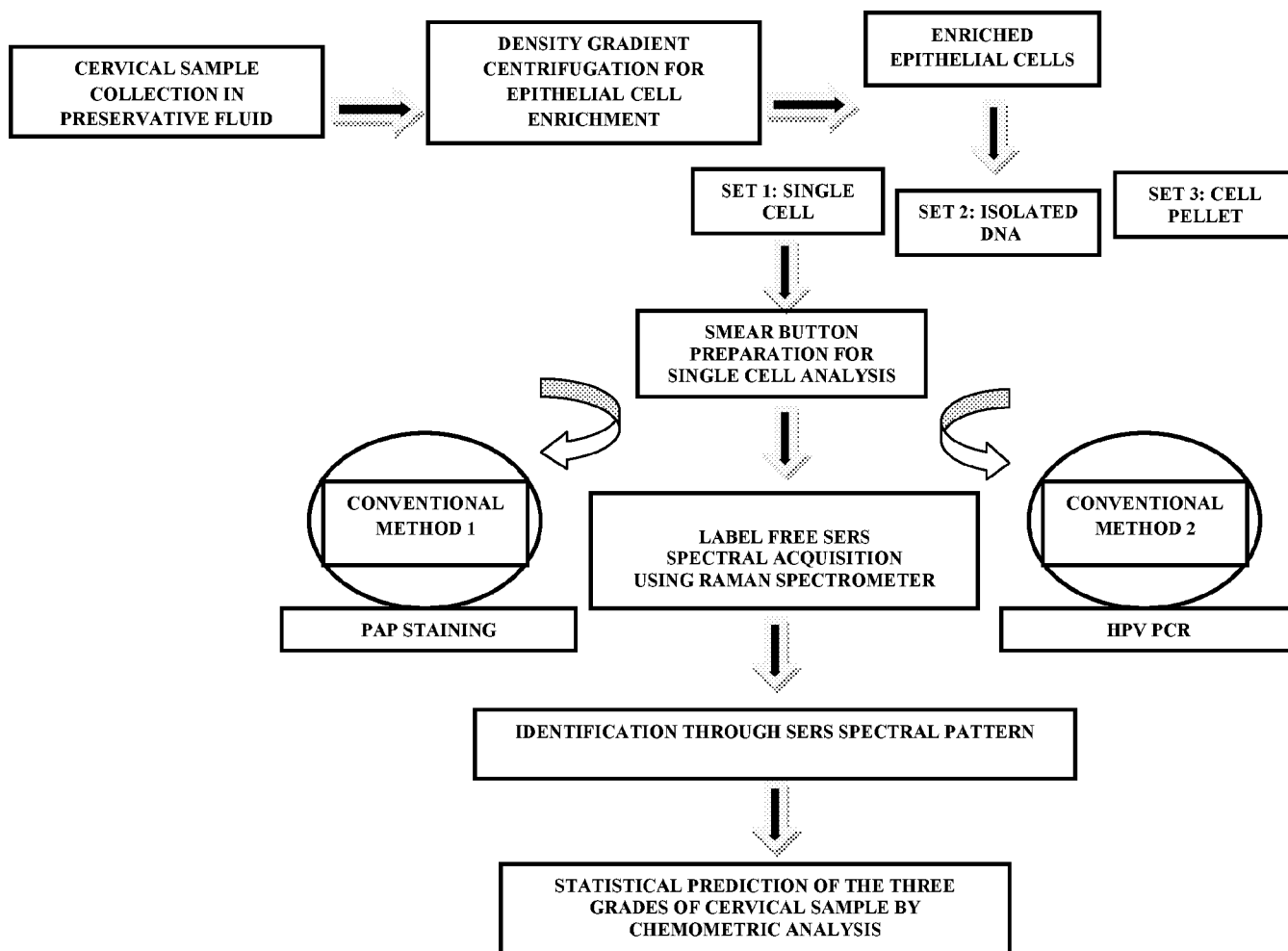


Fig. 6

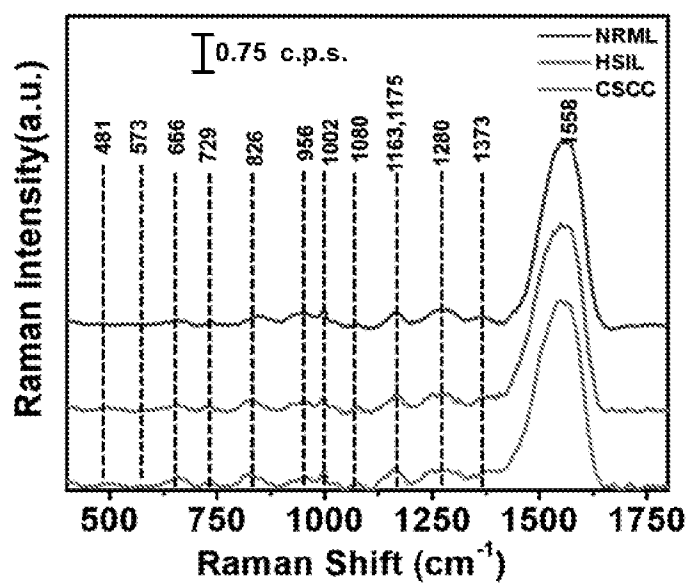


Fig. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2019/050540

A. CLASSIFICATION OF SUBJECT MATTER
G01N1/28, G01N21/65 Version=2019.01

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

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)

TotalPatent One, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Sánchez-Rojo SA, et al. Cervical cancer detection based on serum sample surface enhanced Raman spectroscopy. Revista mexicana de física. 2016 Jun;62(3):213-8. abstract, conclusion	1-7
Y	Kearney, Padraig, et al. "Raman spectral signatures of cervical exfoliated cells from liquid-based cytology samples." Journal of biomedical optics 22.10 (2017): 105008. abstract, Para 3.1-3.2, Figures 3-5, conclusion	1-7
Y	Feng S, et al. Blood plasma surface-enhanced Raman spectroscopy for non-invasive optical detection of cervical cancer. Analyst. 2013;138(14):3967-74. abstract, method, results	1-7
Y	CN106137970A [NINGBO INSTITUTE OF MATERIALS TECHNOLOGY AND ENGINEERING, CHINESE ACADEMY OF SCIENCES] 23 NOVEMBER 2016 (23/11/2016) abstract, claim 1-2 and 7-8	1-7

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"D" document cited by the applicant in the international application	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"E" earlier application or patent but published on or after the international filing date	"&" document member of the same patent family
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 20-11-2019	Date of mailing of the international search report 20-11-2019
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