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Diagnostic spectro-cytology revealing differential recognition of cervical cancer lesions by label-free surface enhanced Raman fingerprints and chemometrics☆

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Abstract

Herein we have stepped-up on a strategic spectroscopic modality by utilizing label free ultrasensitive surface enhanced Raman scattering (SERS) technique to generate a differential spectral fingerprint for the prediction of normal (NRML), high-grade intraepithelial lesion (HSIL) and cervical squamous cell carcinoma (CSCC) from exfoliated cell samples of cervix. Three different approaches i.e. single-cell, cell-pellet and extracted DNA from oncology clinic as confirmed by Pap test and HPV PCR were employed. Gold nanoparticles as the SERS substrate favored the increment of Raman intensity exhibited signature identity for Amide III/Nucleobases and carotenoid/glycogen respectively for establishing the empirical discrimination. Moreover, all the spectral invention was subjected to chemometrics including Support Vector Machine (SVM) which furnished an average diagnostic accuracy of 94%, 74% and 92% of the three grades. Combined SERS read-out and machine learning technique in field trial promises its potential to reduce the incidence in low resource countries.

Key words: Surface enhanced Raman spectroscopy; Cervix; Label-free; Gold nanoparticle; Human papilloma virus

Cervical cancer is the 4th common cancer in women worldwide leading to malignancy related death.[1] In India, the second most populous country in the world, cervical cancer accounts for 22.86% of all cancers among women. The incidence of this disease has been reduced dramatically by the implementation of screening programs in developed countries including a consolidated tactics inclusive of early detection, prevention, screening and proper treatment programs. The predisposing

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factor for the cause of cervical cancer is the persistent infection with high risk human papilloma virus (HPV).[2] The Bethesda system refers different stages of cervical dysplasia which can be identified by a Pap smear test,[3] a well-established cytological staining technique practiced in clinics for identifying cancerous lesions by gently collecting exfoliated cells from the cervix. The chronological occurrence of epithelial abnormalities leads to the development of invasive cancer starting from healthy to low grade squamous intraepithelial lesion (LSIL/ CINI/ mild dysplasia), then to high grade squamous intraepithelial lesion (HSIL/ CIN II & III / moderate) and finally to invasive squamous cell carcinoma (severe dysplasia/ in situ carcinoma).[4] Even though Pap staining is a well-established routine test, some recent reports suggest its limitations owing to low sensitivity, high false negative rates, and inter-observer variability. The sensitivity was found to be 51% only with 98% specificity and 35.5% false negative rate.[5,6] These limitations have forced to re-evaluate the significance of cytology as a primary screening test. HPV DNA analysis has also been included in the cervical cancer detection strategy using polymerase chain reaction (PCR), but many of the HPV infections are transient without causing any clinically significant lesions and get cleared without any intervention, upon time. Hence, the specificity and positive predictive value of HPV test in a population is considerably low. Therefore, Pap smear plus HPV testing and vaccination against high risk HPV is considered as a forefront overall strategy for the effective control of cervical cancer in developed countries. Lack of adequate trained manpower[7] to screen all eligible women in the community is an additional impediment for implementing such population screening programs in a systematic manner. Therefore, there is an urgent need for low cost alternative techniques which should ideally be rapid, non-destructive and objective to emerge as a population screening program.

A molecular level study of cellular and biological processes are essential for understanding the mechanism and detection of diseases together with its therapeutic outcomes. The quest for specific and accurate cancer screening has driven the development of novel diagnostic methods including spectroscopic techniques to improve detection of target biomarkers, especially to distinguish early malignant lesions.[8] Non-invasive diagnosis of malignancies has been well-studied by optical spectroscopic methods, mostly by Fourier-transform infrared spectroscopy,[9] Raman[10] fluorescence[11] etc. and out of which Raman spectroscopy offers some distinctive benefits over other techniques. It is based on inelastic scattering of monochromatic light possessing high spatial resolution which can pretend near-infrared (NIR) radiation with less sample preparation and minimal influence of water bands. A large number of studies employing Raman scattering together with an accurate database for each characteristic peak frequencies to analyze non-cancerous or cancerous tissues,[12] classification of normal, malignant and benign samples for different cancers like breast,[13] cervical[14] oral,[15] gastric[16] etc. using various multivariate analysis[17] has been developed. The challenges associated with large data sets has been resolved extensively by applying various chemometric methods,[18] such as principal component analysis (PCA),[19] linear discriminant analysis (LDA),[20] least-squares regression as well as cluster analysis and machine learning techniques like Support Vector Machine (SVM).[21] However, a major issue of Raman scattering is its inherently weak signal which led to a handful of improvisations over typical Raman spectroscopy out of which Surface Enhanced Raman Scattering (SERS),[22,23] received huge acceptance allowing the ultrasensitive detection of analytes with low concentration through the amplified signals around 10^8–10^{14} folds by the excitation of surface plasmons rendered by metallic nanoparticles. The potential of ultrasensitive SERS analysis in single living cells has been demonstrated using colloidal gold nano particles (AuNPs) deposited inside cells which resulted in strong enhancement of Raman signals of biomolecules opening up exciting opportunities for biomedical studies.[24] Subsequently, several studies based on SERS platform was explored including the precise tracking of the biomolecular changes in different stages of mitosis,[25] apoptosis,[26] classification of healthy and cancerous samples in breast cancer,[27] bladder cancer,[28] gastric cancer,[29] colorectal cancer[30] parotid cancer,[31] kidney tumor staging[32] etc.

Cervical cancer has been well studied by conventional Raman spectroscopy in the past several years for differentiating normal and abnormal subjects using blood serum,[33] cells,[34–36] tissues,[37] in vivo[38] etc. When compared to the conventional Raman spectroscopy, SERS is an ultrasensitive technique requiring very less acquisition time, better signal to noise ratio, increased fold intensity, specificity and Raman cross section. A few SERS studies on cervical cancer has been reported using blood plasma-[39] and serum[40] samples but all these cases involves invasive sample collection procedures. Also, blood based biomolecular changes like leakage of circulating tumor cells, DNA, proteins, exosomes etc. will be prevalent in the metastatic stage only, so early stage detection or classification using blood is not practicable. However, employing SERS in exfoliated cell samples for cervical cancer detection will provide direct insight into the origin of abnormality. Considering the existing diagnostic modalities and upcoming fundamental issues, we report for the first time, a label free SERS based spectroscopic approach in exfoliated cells for generating a sensitive differential spectral fingerprint for straight forward detection of squamous cell carcinoma and its precursor lesions during the progression of cervical cancer. The analysis has been performed for distinguishing three groups i.e. NRML, HSIL and CSCC using three different forms of analytes viz. single cell, cell-pellet, extracted DNA (Figure 1). Significant peak variations, shifts and spectral differences were evaluated within and between the groups using chemometric interpretation viz., PCA, LDA and SVM in order to generate the prediction accuracy showing its potential to emerge as a screening method to monitor the progression of cervical cancer. Along with SERS based spectro-cytology as a new insight, comparative conventional cytological and HPV PCR analysis were also carried out. Another hallmark of cancer progression is the altered metabolism to satisfy the increasing need of energy. Intracellular concentration of amino acids is a diagnostic parameter for cancer as the abnormal proliferation requires more amino acids for protein synthesis. Thus, amino acid profiling by ultra-fast liquid chromatography (UFLC) technique[41] was attempted as an alternative confirmatory approach for identifying the progression of cancer related amino acid metabolites along with SERS.

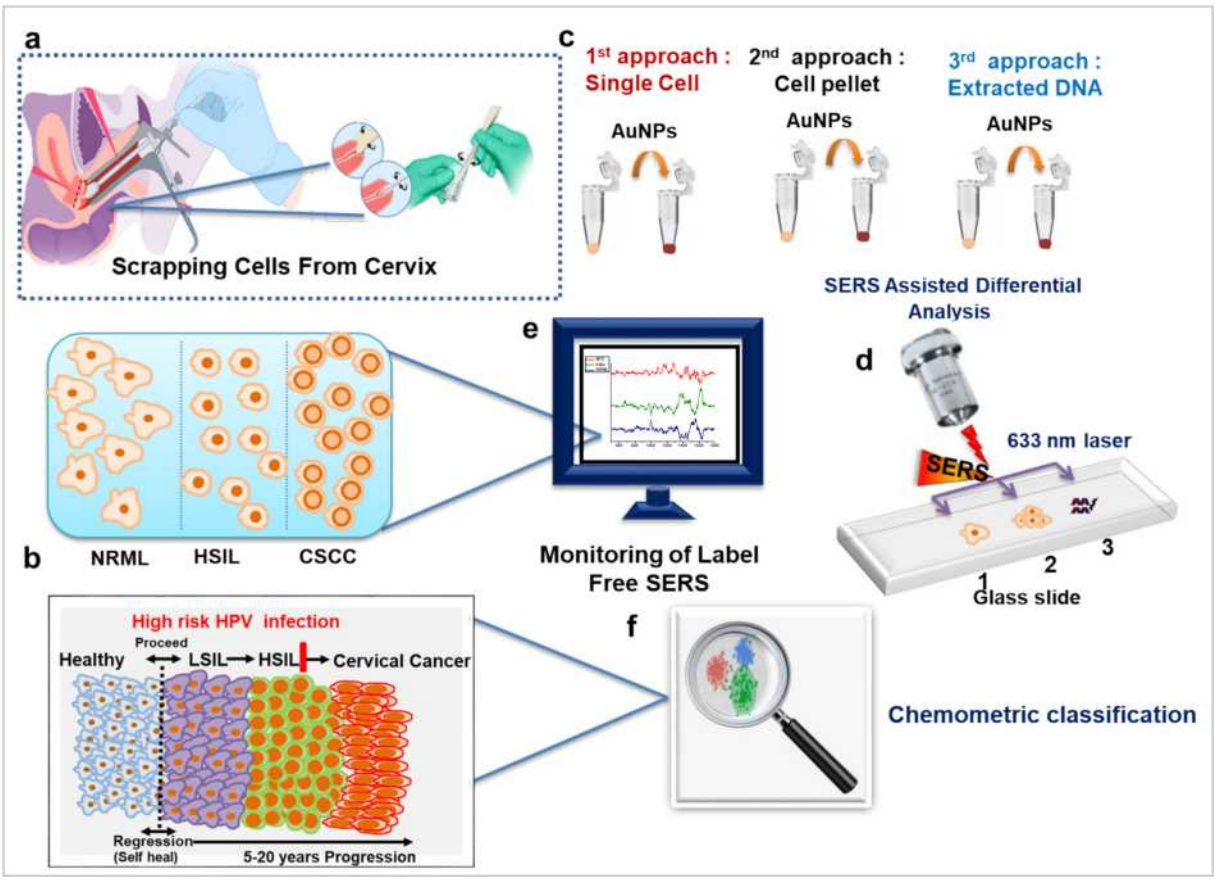


Figure 1. Schematic illustration of experimental design for differentiating three grades viz. normal (NRML), high grade intraepithelial lesion (HSIL), cervical squamous cell carcinoma (CSCC) using SERS., a) Scrapping cells from the cervix using cytobrush, b) progression pattern of cervical cancer c) Set 1: single cell, Set 2: cell pellet, Set 3: extracted DNA (mixed with AuNPs), d) independent SERS analysis of 1) single cell,2) cell pellet, 3) extracted DNA in glass slide, d) empirical signal monitoring of the three grades f) chemometric analysis.

Methods

Chemicals and materials

Gold (III) chloride hydrate, trisodium citrate dehydrate were purchased from Sigma-Aldrich. Ethyl alcohol (99.9% purity) was purchased from Changshu Yangyuan Chemical, China. Detailed description of experiments is provided in the supporting information.

SERS substrate preparation

Gold nanoparticles (AuNPs, 40–45 nm) were synthesized by well-known citrate reduction method.

SERS analysis and data analysis

Spectral analysis using confocal Raman analysis was performed with the aid of a confocal Raman microscope (WITec, Inc., Germany, alpha 300R) with a laser beam directed to the sample through 20× objective with a Peltier cooled CCD detector. Detailed procedure is provided in the supporting information. The raw spectral data were pre-processed by Project FOUR 4.0 (WITec, Germany) before the data statistical analysis in order to remove the interference noises, cosmic rays and oversaturated spectra. PCA, LDA and SVM statistical chemometric tools were utilized for the classification.

Results

Fabrication of colloidal SERS substrate

We have employed colloidal spherical AuNPs (absorbance 530 nm) within a size range of 40–45 nm emphasizing its best SERS activity[42,43] reported so far. AuNPs synthesized as per the standard method[44] with optimized size, shape and monodispersity were confirmed by Ultraviolet–visible (UV–Vis) absorbance, Dynamic Light Scattering (DLS) and High Resolution Transmission Electron Microscopy (HR-TEM) (**Supplementary Figure S1**). Colloidal AuNPs concentrations were obtained nearly 8–10 × 10^{10} particles/ml.

Biomolecular fingerprinting of cervical squamous cell carcinoma model

Label-free biomolecular SERS fingerprints were evaluated with an epithelial type cell line, SiHa derived from grade II squamous cell carcinoma of the cervix which can be considered

as the best in vitro model prior to clinical investigation. SERS analysis showed the presence of a range of molecular fingerprints out of which Raman peaks at 481, 1330 and 1456 cm^{-1} corresponds to the -C-N-C-bending, -P=O-stretching vibration and -CH2 vibration of DNA signal, 572 cm^{-1} designates the -O-C=O for tryptophan abundance, 621 and 1002 cm^{-1} relates to signals from -O-C=O and aromatic ring chain vibration in phenyl alanine, 643 and 1211 cm^{-1} represents -O-C=O bending and -C-C-N- vibration in tyrosine, 735 and 746 cm^{-1} represents -O-C=O bending vibration in adenine and thymine respectively (**Supplementary Figure S2**). We also observed Raman peaks at 826 and 1085 cm^{-1} corresponds to DNA phosphate backbone (-O-P-O-) stretching vibration, 957 and 1154 cm^{-1} indicated the presence of -C-C- aliphatic alicyclic chain vibration in carotenoids and peak at 1545 cm^{-1} resembled the presence of Amide II, nucleic acid and tryptophan, 1697 cm^{-1} corresponds to Amide I (**Supplementary Table S1**).[12] The distinct well resolved biomolecular fingerprints from SiHa cells provided a blue-print of the major molecular fingerprint viz., aromatic amino acid, DNA, Amides (I & II), which were benchmarked as a model for clinical investigations.

SERS-aided grading with cervical exfoliated single cells

Pathologically confirmed cervical smears of major three grades i.e. NRML, HSIL and CSCC were processed using liquid based cytology procedure. The details of patient samples is depicted in **Supplementary Table S2**. A monolayer of diagnostically relevant cells upon incubation with AuNPs was evaluated for the single cell analysis. In a preliminary analysis, the SERS mapping of the single cell showed the variations in the morphology of three different types of cells, viz. NRML, HSIL and CSCC (Figure 2, *a*). In single cells, 5 μm × 5 μm nuclear area was preferred for image scanning to avoid variability arising from the cytoplasmic area. Since the analysis involved addition of AuNPs, Pap stained slides were subjected to de-staining followed by SERS analysis to select the diagnostically relevant cells (**Supplementary Figure S3**).

Initially, individual SERS fingerprint was evaluated empirically followed by chemometric statistical analysis to group the three classes. The mean spectra accumulated from the average of the collected spectrum excluding the outliers were normalized to its highest peak (Amide II at 1550 cm^{-1}) for observing the spectral variations. The analysis in exfoliated single cell samples,

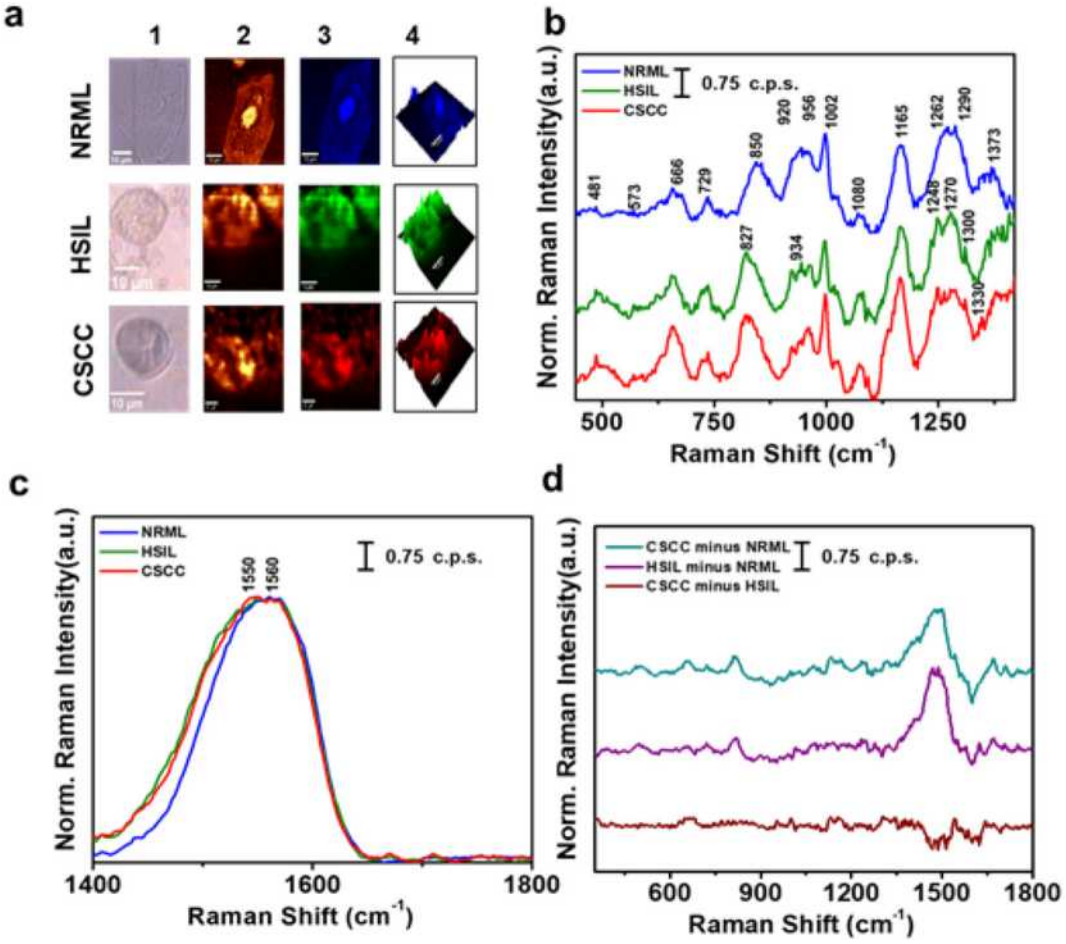


Figure 2. SERS analysis from exfoliated single cell samples**,** a) SERS imaging of single cell, 1) Bright field, 2) Raman imaging, 3) cluster mapping and 4) 3D cluster mapping (Scale bar: 10 μm, Laser power density 3–7 mW power density), b) Mean SERS spectra acquired from cervical exfoliated single cell samples (region 400–1400 cm^{-1}), c) Mean SERS spectra from range 1400–1800 cm^{-1}, d) Difference SERS spectra for NRML, HSIL and CSCC samples.

NRML, HSIL and CSCC showed distinctive peaks correlating to the model cell line signals. Distinct Raman peak at 481 cm^{-1} associated with -C-N-C bending vibration of DNA was found to be increased in HSIL and CSCC samples which showed an indication of high nuclear content. The -O-C=O- bending vibration of amino acid tryptophan corresponding to 573 cm^{-1} was prominent in HSIL and CSCC samples displaying the presence of high protein content which may be mostly from the histone protein and nuclear regulatory proteins inside the nucleus. The -O-C=O- bending vibration peak at 643 and 666 cm^{-1} are indicative to tyrosine, thymine and guanine ring vibrations present in all grades whereas adenine ring vibration at 729 cm^{-1} identified prominently in the abnormal grades i.e. HSIL and CSCC predicting the increase in nuclear elements. On the other hand, O-P-O stretching at 826 cm^{-1} and 1080 cm^{-1} favored the indirect existence of nucleic acid. A -C-O-C- bending vibration peak at 920 cm^{-1} is slightly prominent in abnormal samples which correspond to the amino acid, proline. C-C aliphatic, alicyclic peaks identified at 956 and 1165 cm^{-1} attributed to carotenoid showed higher intensity in CSCC compared with NRML. Amide III signal from proteins at 1262 cm^{-1} in NRML is shifted in HSIL and CSCC samples to 1270 cm^{-1}. Similarly, amide II signal arising at 1560 cm^{-1} from the protein counterparts inside the nucleus showed a clear shift of around 10 nm between normal and abnormal samples (Figure 2, *b* and *c*).[12] Interestingly, the ratio between 1270 and -O-C=O symmetric stretching at 1373 cm^{-1} were well resolved for the prediction of abnormality from the mean spectra. The ratio value was found to be 1.55, 1.2 and 1.02 for NRML, HSIL and CSCC respectively in single cells. The ratio is decreasing because the peak corresponding to 1373 cm^{-1} is increasing in the abnormal samples. The variations existing between the three groups were acquired by subtracting the mean spectra of NRML from CSCC, NRML from HSIL and HSIL from CSCC. The positive peaks in the difference spectra obtained showed the presence of bio-molecular activity and negative peaks showed the absence of the same (Figure 2, *d*). The differentiating peak were visualized by box plot analysis performed in R software (**Supplementary Figure S4**). All the peak assignments from the single cell analysis is depicted in **Supplementary Table S3**.

SERS-aided grading with cervical exfoliated cell pellets

In the pellet form of exfoliated cells, it exhibited a prominent SERS peak representing -C-O-C bending vibration at 490 and 1022 cm^{-1} corresponding to the presence of glycogen prevalent in NRML cells compare to abnormal cells in cervical smears. Identified -O-C=O Raman peaks at 667, 729 and 1373 cm^{-1} indicated the increase in the DNA material in HSIL, CSCC samples specific to thymine, guanine and adenine respectively. Again, sharp peak showing -C-C-N- at 1170 cm^{-1} corresponding to tyrosine was found in abnormal samples. Visible Raman peak shift was obtained at the Amide II region in the CSCC samples when compared to NRML samples as specified a peak at around 1568 cm^{-1} in NRML shifted to 1547 cm^{-1} in CSCC specimen (Figure 3, *a* and *b*). We have evaluated the ratio between P-O-C anti symmetric stretch at 970 and 1022 cm^{-1} which is considered as a predictive indicator of abnormality from the mean spectra leading to the values of 0.61, 1.13 and 1.34 for NRML, HSIL and CSCC respectively. The pellet comprising cervical exfoliated cells was a mixture of normal and abnormal cells with its mean signature Raman spectra contained some common features.

The variations existing between the three groups were attained by subtracting NRML from CSCC, NRML from HSIL and HSIL from CSCC mean spectra. The positive peaks in the difference spectra illustrated the presence of bio-molecular activity and negative peaks showed the absence of its fingerprints (Figure 3, *c*). Standard deviation and mean spectra is shown in Figure 3, *d*. The prominent peak differences between NRML, HSIL and CSCC samples were visualized in box plot analysis performed in statistical R software (**Supplementary Figure S5**). All the peak assignments from the cell pellet analysis is depicted in **Supplementary Table S4**.

SERS-aided grading with cervical exfoliated cell DNA

In the progression of Raman fingerprinting obtained from single cell and cell pellet analysis for the differential diagnosis of cervical precancerous and cancerous lesions, cellular DNA was extracted to re-investigate nucleotide profiling. Majority of the SERS peaks obtained from extracted DNA correlated with the peaks obtained from the single cell and cell pellet. The specific peaks at 729 cm^{-1} corresponded to -O-C=O- adenine ring vibration, 826 and 1080 cm^{-1} related to the O-P-O stretching vibration in DNA, 1172 cm^{-1} attributed to -C-C-N- bending vibration in cytosine and guanine, 1421 and 1578 cm^{-1} were related to -C-N- stretching vibration and NH_2 deformation in Amide II in adenine and guanine (Figure 4, *a*). Difference spectra were assessed by subtracting NRML from CSCC, NRML from HSIL and HSIL from CSCC mean spectra from the DNA samples (Figure 4, *b*). The mean spectra and standard deviation is shown in Figure 4, *c*. The peak variations significant between NRML, HSIL and CSCC DNA samples were measured in box plot analysis employing R software (**Supplementary Figure S6**). All the peak assignments from the extracted DNA analysis is depicted in Figure 4, *d*.

UFLC analysis of amino acid metabolites in cervical exfoliated cells

In the course of complementary validation, we have investigated the presence of amino acids contributing to the differential spectra obtained from single cell and cell pellet analysis by UFLC. A peak at retention time of 13.65 min in HSIL and CSCC samples were exactly correlating with the standard amino acid tryptophan. Similarly, the retention times of 15.45 min for tyrosine, 17.8 min for proline, and 18.6 min for phenyl alanine were identified in HSIL and CSCC with their respective standards. Interestingly, all the four amino acid peaks were negligible in NRML samples **(Supplementary Figure S7).** The presence of tyrosine, phenyl alanine, tryptophan and proline reflected in the progression stage in HSIL and CSCC samples leading to the increased protein content.

Chemometric analysis

As we obtained a large spectral data set in all the three groups NRML, HSIL and CSCC modeling and prediction of the same was very much essential to discriminate between them. Initially, we adopted chemometric analysis using PCA to

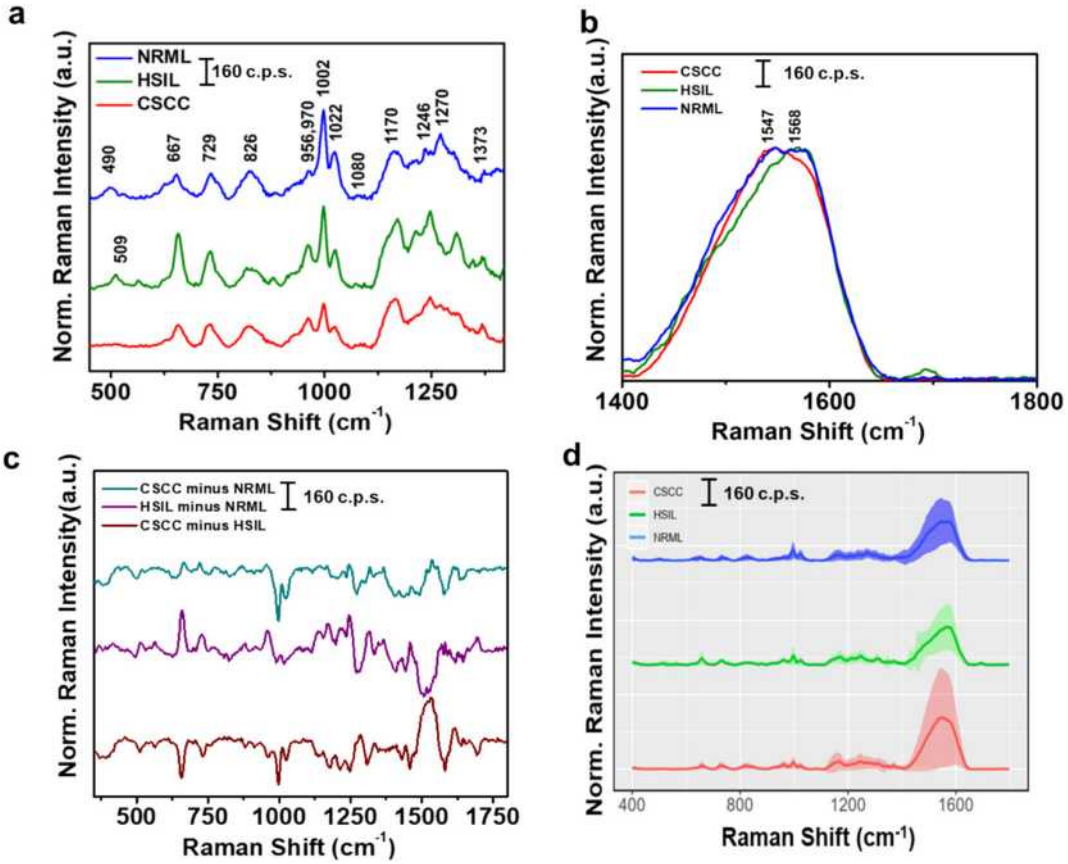
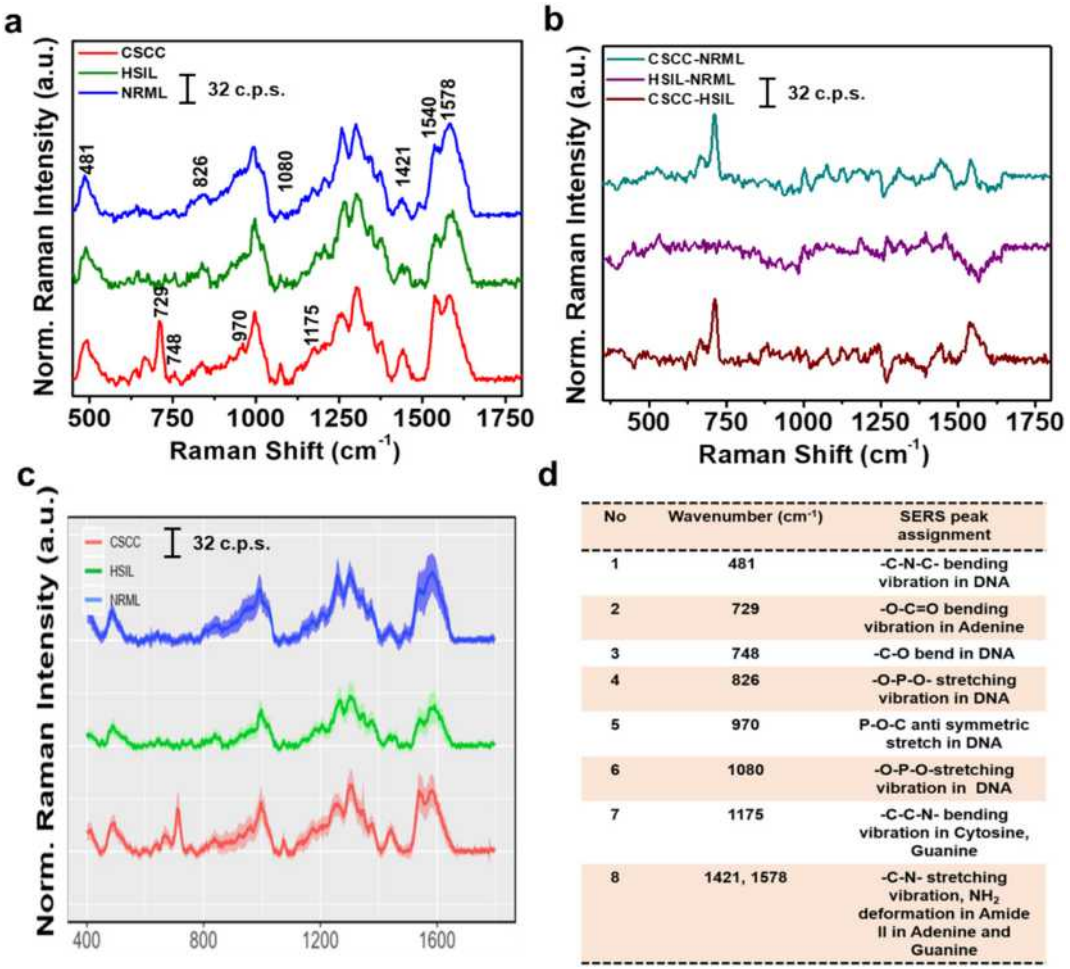


Figure 3. SERS analysis from exfoliated cell pellet samples, a) Mean SERS spectra acquired from cervical exfoliated cell pellet (region 400–1400 cm^{-1}), [Laser power density 3–7 mW power density], b) Mean SERS spectra of cell pellet from range 1400–1800 cm^{-1}, c) Difference SERS spectra, d) Tentative SERS peak assignments from cervical cell pellet samples; NRML, HSIL and CSCC.

classify them using MATLAB software. A predictive classification was obtained using PCA analysis in single cell, cell pellet and extracted DNA respectively (Figure 5, *a*, *c* and *e*). It was observed that the increase of noise in Raman spectra using PCA. Subsequently, the intra-group variability was enhanced which reflected to the reduction of specificity in PCA. Therefore, to improve the specificity we adopted LDA analysis as the next trial. LDA is a stereotype of Fisher's linear discriminate used in statistics, machine learning and pattern recognition into linear combination of features that characterizes or separates two or more classes and attempts to amplify the difference between the classes of data which is difficult to get from PCA. We observed a clear demarcation between single cell, cell pellet and extracted DNA from LDA analysis (Figure 5, *b*, *d*, and *f*). In the course of gradual improvement of prediction accuracy, SVM analysis was attempted by randomly selecting 75% of the spectra as the train set and rest 25% were used as the test set. The SVM analysis was repeated with 500 different random samples and measured the average prediction accuracy. The accuracy was found to be 94% for single cell, 74% for cell pellet and 92% for extracted DNA with 0.73%, 5.04% and 3.84% standard deviation respectively (Figure 6, *a–c*). Thus based on the created reference spectral module an unknown sample can be predicted. The percentage of prediction accuracy was generated along with ROC curve (Figure 6, *d–f*). ROC curve is a graphical plot which shows the diagnostic ability of a classifier system by varying the discrimination threshold. At different threshold setting, the ROC curve is plotted by true positive rate against false positive rate. True positive is termed sensitivity and false negative is termed 1-specificity. The accuracy of the analysis depends on how good the test separates the group got tested into those with and without the disease. Accuracy is measured by calculating the area under the curve. The ROC curve of single cell, cell pellet and extracted DNA showed that SVM is an incremental diagnostic model for classifying the groups (Figure 6, *g–i*). The sensitivity and prediction accuracy of the technique was calculated (Table 1).

Cytopathological Pap staining analysis

In order to get a clear discrimination between normal vs abnormal exfoliated cells, both bright field images from Raman microscope and Pap staining of NRML, HSIL and CSCC were evaluated for morphological analysis (Figure 7, *a* and *b*). All the abnormal samples were further confirmed by colposcopic biopsy. In Pap staining, the superficial NRML cells were stained pink with pyknotic nucleus, intermediate cells stained light blue



No	Wavenumber (cm^{-1})	SERS peak assignment
1	481	-C-N-C- bending vibration in DNA
2	729	-O-C=O bending vibration in Adenine
3	748	-C-O bend in DNA
4	826	-O-P-O- stretching vibration in DNA
5	970	P-O-C anti symmetric stretch in DNA
6	1080	-O-P-O-stretching vibration in DNA
7	1175	-C-C-N- bending vibration in Cytosine, Guanine
8	1421, 1578	-C-N- stretching vibration, NH_2 deformation in Amide II in Adenine and Guanine

Figure 4. SERS analysis from extracted DNA samples from exfoliated cells**,** a) Mean SERS spectra [Laser power density 3–7 mW power density], b) the difference spectrum, c) the standard deviation and d) tentative SERS peak assignments from cervical clinical DNA samples; NRML, HSIL and CSCC**.**

to green color where as in HSIL, the enlarged nucleus reflecting high DNA content with minimal cytoplasm showed purple color nucleus. In CSCC, the cells started to show invasive nature with a slender shape formation to the nucleus. To correctly identify the desired classes of cells, initially the Pap staining was performed to mark the cell position and the same cells were subjected to SERS analysis (**Figure S3**).

Human papillomavirus analysis

To identify the HPV specific DNA integrated in the infected samples, HPV PCR were employed. To evaluate and compare the Pap smear test, NRML, HSIL and CSCC samples were loaded after DNA isolation from the respective samples out of which HSIL and CSCC showed a band corresponding to HPV DNA. A PCR amplification product of size varying between 230 and 270 bp was obtained in 1.5% agarose gel indicative of an infection with oncogenic HPV in HSIL and CSCC whereas no observable band was identified for NRML samples (Figure 7, *c* and *d*). The PCR product obtained was indicative of oncogenic HPV types 16, 18, 31, 33, 35, 45, 52 and 58.

Discussion

As cervical cancer progresses from normal to abnormal in a grade dependent way, the identification of precancerous lesions can be beneficial for the early diagnosis. We have utilized AuNPs which is a well explored SERS substrate with tunable optical property and is chemically inert when mixed with a wide range of analytes or biomolecules providing characteristic Raman fingerprints. The analysis has been approached by the modulation of three different samples i.e. single cell, cell pellet and extracted DNA to arrive at a conclusion for adopting the most simplest and accurate results to uplift the technique as a diagnostic modality. Liquid based cytology (LBC) fixation were performed which improved preservation of the cell sample eliminating artifacts and degenerative changes developed in the course of air-drying. In addition, LBC will reduce the background caused by blood contamination in the cervical scrape samples which usually interferes in the sample interpretation.[45] SERS substrate incubation in fixed cells and living cells are the two widely used methods to study single cells using SERS. Reports suggests that valuable information of real

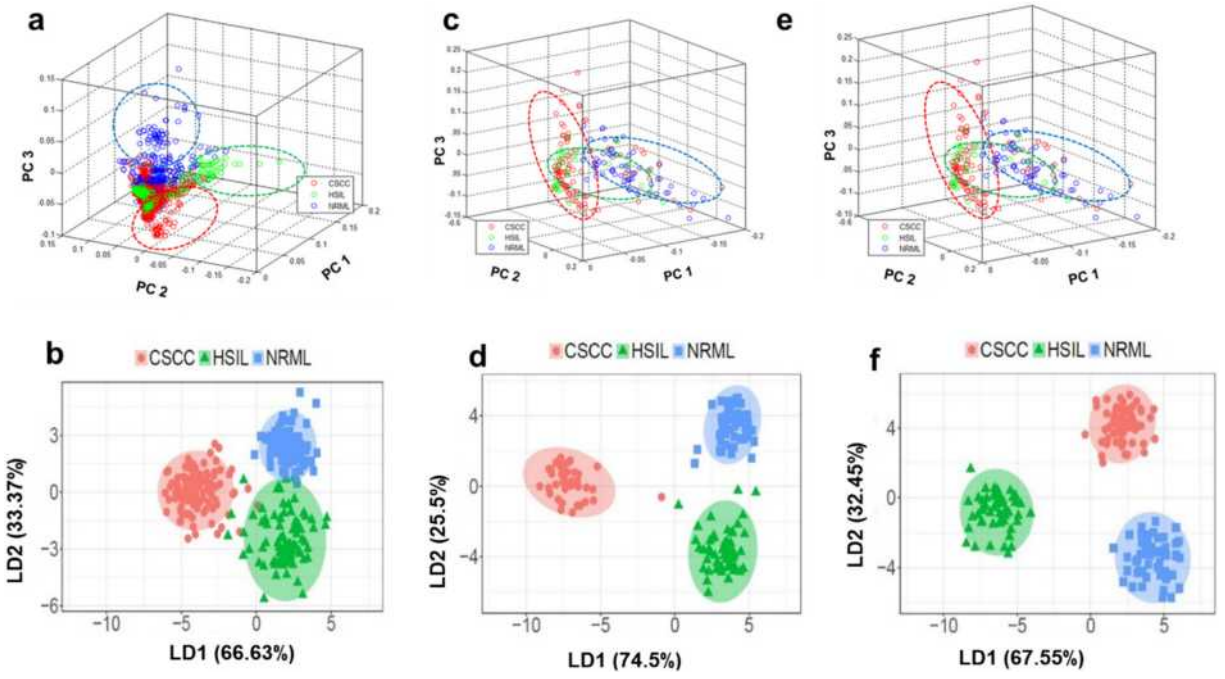
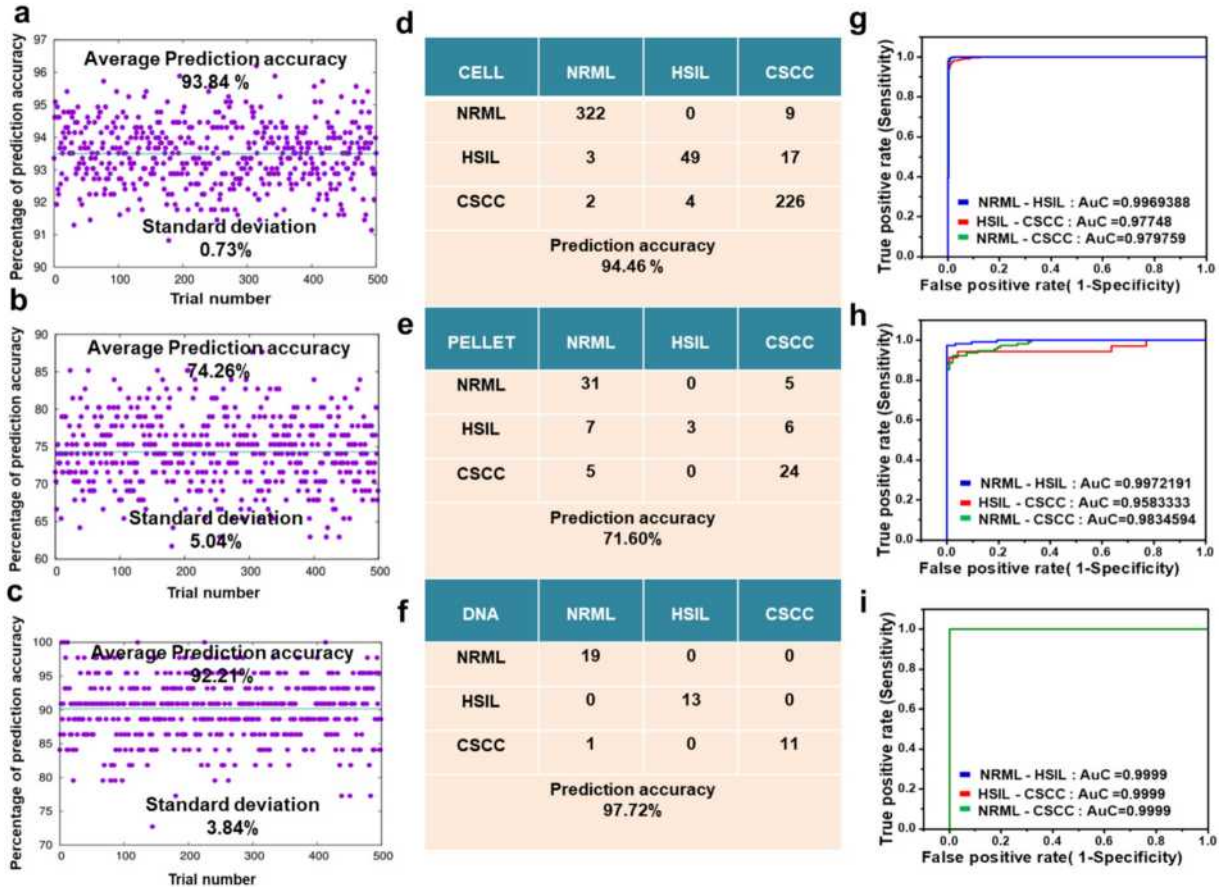


Figure 5. Chemometric discrimination between PCA and LDA scatter plots: (a, b) single cell, (c, d) pellet and (e, f) extracted DNA.



CELL	NRML	HSIL	CSCC
NRML	322	0	9
HSIL	3	49	17
CSCC	2	4	226
Prediction accuracy 94.46 %			

PELLET	NRML	HSIL	CSCC
NRML	31	0	5
HSIL	7	3	6
CSCC	5	0	24
Prediction accuracy 71.60%			

DNA	NRML	HSIL	CSCC
NRML	19	0	0
HSIL	0	13	0
CSCC	1	0	11
Prediction accuracy 97.72%			

Figure 6. Percentage of average prediction accuracy chart in a) single cell, b) cell pellet and c) extracted DNA, Representative classification based on SVM analysis of d) single cell, e) cell pellet and f) extracted DNA, SVM ROC curve in g) cell, h) pellet and i) DNA.

Table 1
Sensitivity and prediction accuracy of SERS-based cervical precancerous lesions detection.

	Sample	Total	True prediction	False prediction	Prediction accuracy
Single cell	NRML	331	322/331	9/331	94.46% ± 0.73
	HSIL	69	49/69	20/69	
	CSCC	232	226/232	6/232	
Cell pellet	NRML	36	31/36	5/36	71.60% ± 5.04
	HSIL	16	3/16	13/25	
	CSCC	29	24/29	5/29	
Extracted DNA	NRML	19	19/19	0/19	97.72% ± 3.84
	HSIL	13	13/13	0/13	
	CSCC	12	11/12	1/12	

time cellular processes is obtained from live cells than fixed cells as fixation can lead to changes in the molecular vibrations leading to variations in the spectral pattern.[46] Our group has already demonstrated the accurate evaluation of surface charge of AuNPs for live cell uptake using positive, negative and neutral nanoparticles.[47] In the present study, as the cervical scrape cells are already fixed during collection, the variations thus arising is commonly assessed during the differential spectral evaluation in all the three groups, i.e. NRML, HSIL and CSCC spectra and finally such variations can be nullified.

Single cells were analyzed after morphologically identifying the ratio of nucleus to cytoplasm features. Cell pellet analysis were performed in a blinded manner without any morphological evaluation of the cells which may be the most ideal option for conducting a screening study. Extracted DNA analysis were performed to confirm the identity of Raman fingerprints of nucleic acids and UFLC analysis to profile the differential amino acids in the cervical cancer progression. The preliminary studies conducted in fixed SiHa cell line showed fingerprint peaks corresponding to amino acids, nucleic acid bases, phosphate stretchings, carotenoids etc. which formed a basis for the peak analysis in real patient samples. The O-P-O stretching at 826 cm^{-1} showed the increased nuclear content in HSIL and CSCC samples, which is identified as a shifted position in normal at 850 cm^{-1}. Such a peak shift is visible in IR-FT Raman spectra in cancerous cervix comparing benign and normal.[48] Another distinguishing factor between the three groups were the presence of -C-O-C- stretching of the amino acid proline.

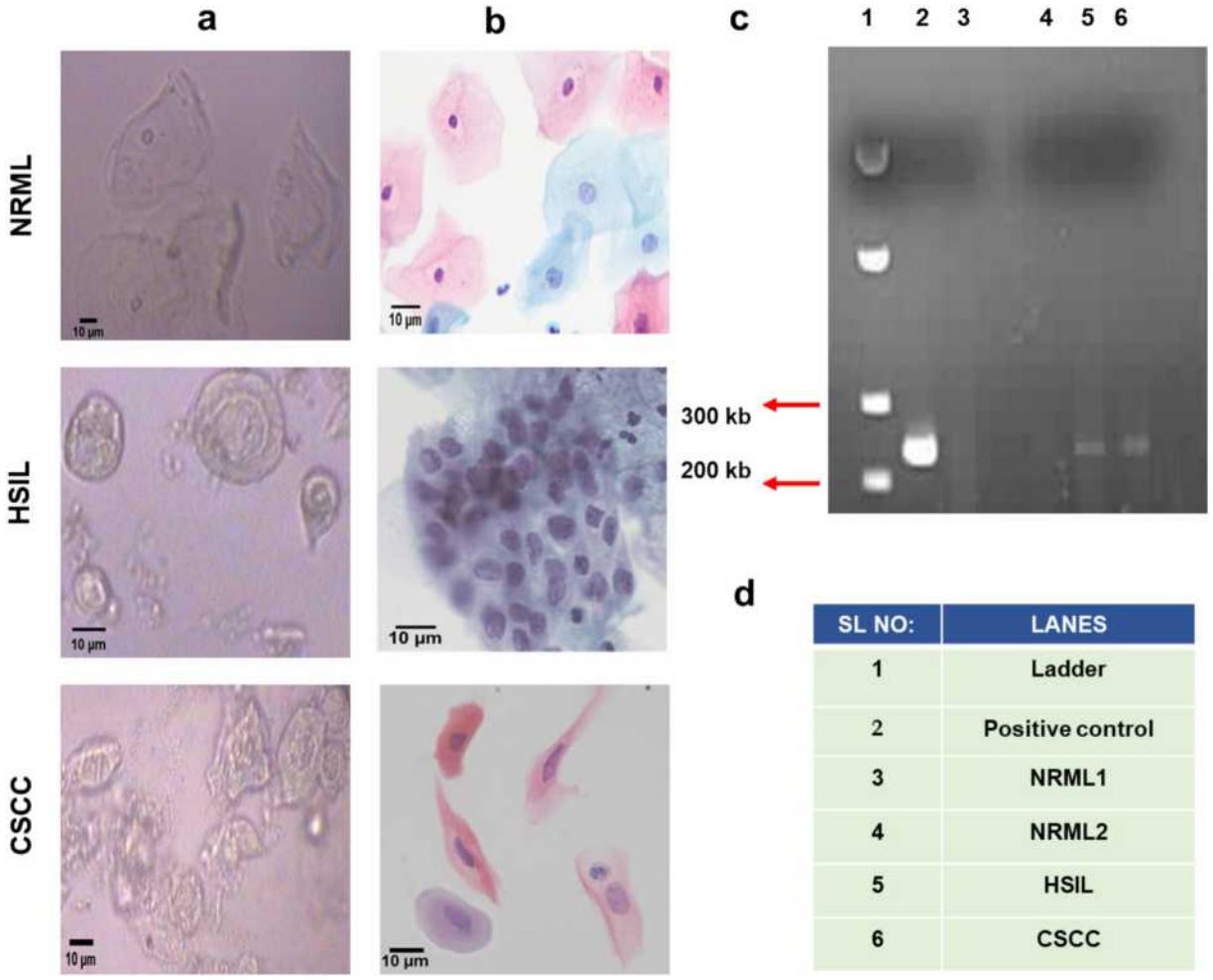


Figure 7. Conventional clinical cytopathological analysis of cervical exfoliated cells, a) Bright field and b) Pap stained images of NRML, HSIL and CSCC exfoliated cells, Scale bar corresponds to 10 µm, c) HPV PCR of clinical DNA samples and d) Sample loading order of HPV PCR ladder (1), positive control (2), NRML1 (3), NRML2 (4), HSIL(5), CSCC(6).

It has been proven that proline rich tyrosine kinase 2 (Pyk2) plays an important role in tumor progression in various human cancers.[49] Pyk2 is a non-receptor tyrosine kinase which controls tumor survival, its proliferation, migration, invasion properties, metastasis and resistance to chemotherapy.[50,51] In addition, there exists a correlation of a biomarker known as c-myc over expression distinct in cervical cancer which increases proline biosynthesis from glutamine and is a prognostic marker useful in guiding treatment decisions in cervical cancer. Pyk2 is also found to be essential for the intracellular trafficking of HPV 16 in human keratinocyte cells.[52–54] Thus the increase in proline may be indicative of tumor progression. The increase of carotenoid signal at 956 cm^{-1} and 1165 cm^{-1} in single cells might be due to the reason that cancer cells tends to accumulate carotenoids to resist damage.[55] Considering cell pellet, in positive sample it is said to be heterogeneous, i.e., all the three types of cells; viz. NRML, HSIL and CSCC may be present. Even though all cell types are present, there occurs a presence of premalignant changes in the normal looking cells[36] of the positive smears. The presence of glycogen is usually considered as a parameter for classifying normal and abnormal cells in cytological specimens of the cervix.[56,57] Extracted DNA analysis were performed to re-confirm the signals obtained from single cell and pellet sample analysis. Increase in DNA content was evident between the groups when compared with normal counterparts. For confirming the presence of amino acids playing differentially between the three classes, UFLC analysis were performed. The analysis confirmed the results obtained in SERS analysis by signals obtained from amino acids, tryptophan, tyrosine, phenyl alanine and proline. In addition to the real positive patient samples, SiHa cell also showed the presence of the above mentioned amino acids. As SiHa contains 1–2 copies[58] of HPV 16 and since Pyk2 is essential for the intracellular trafficking of HPV 16 in human keratinocyte cells, increase in proline may be the indicative for the tumor progression as mentioned earlier. Also, it is evident that cancer cells require more metabolic requirements for uncontrolled proliferation when compared to normal cells which is assessed by the specific amino acids for the metabolic reprogramming resulting in the deregulated replication. Chemometry was further utilized for the classification and prediction within the dataset employed for extracting data or information comprising complex data set from a chemical or biological source based on multivariate analysis, mathematical or computational models, to find the relationship between two or more closely related entities, structural analysis etc. PCA, LDA and SVM were performed out of which SVM showed good classification between the classes. The main advantage of PCA technique enabled to reduce the huge dimension of data-set without losing much information. But as the specificity was less, LDA and SVM analysis was attempted which increased the classification much better. Conventional analysis like Pap smear were performed to correctly identify the desired cells for SERS analysis. HPV PCR was also done which shows the presence of HPV DNA in the samples as HPV is considered as the causative factor behind cervical cancer. But the disadvantage of HPV PCR technique is that as HPV infection can be get cleared with time and a few percentage of normal samples may be predicted as positive for HPV DNA, also the sensitivity from HPV PCR is considered significantly low.

In conclusion, a label free ultrasensitive Raman fingerprint has been benchmarked to generate a well resolved biomolecular variation between the samples. Nucleic acids variations were confirmed by SERS analysis of extracted DNA while amino acid metabolites were validated by UFLC analysis. The results indicated the prevalence of nucleobases adenine, guanine, cytosine, phosphate backbone and amino acids tryptophan, phenyl alanine and proline which are the major metabolites evolved during tumorigenesis. Further spectral differentiation was validated by statistical analysis which included chemometric interpretations mainly by PCA, LDA and SVM. This is the first representation of SERS spectroscopic analysis in exfoliated cell samples. Moreover, the SERS based spectro-cytology was found to be minimally invasive and more sensitive than those employing serum or plasma as exfoliated cells represents a better source of sample for analyzing progression of cervical cancer. Taken together, the present strategy represents an accurate, simple and reliable method for the differential diagnosis of cervical cancer which might serve as a clinical detection technique in the near future. Also, we envision to store the reference data in a chip model (**Figure S8**) within a futuristic hand held Raman spectrometer for the screening of cervical cancer in large population.

Author contributions

V.K. and S.V.N. undertook all the synthesis and experimental studies. K.S.K and M.M.J helped in all chemometric analysis. G. S. and J.B.N helped in result interpretation and suggestions. K.G. R. contributed in intellectual content. All authors discussed the results and commented on the manuscript. K.K.M., K.S. and K. S.K. was responsible for the overall direction and coordination of the project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2020.102276.

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

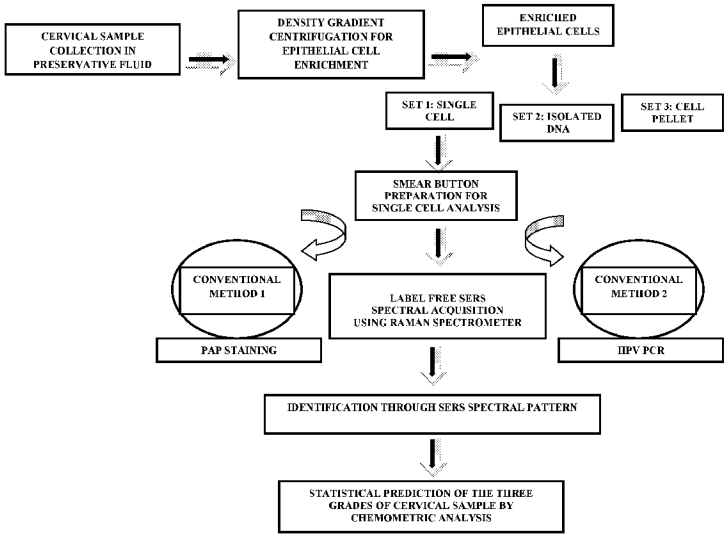

Fig. 6

(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

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[Continued on next page]

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.

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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))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

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this study demonstrated that gold nanoparticle based SERS substrate harvested exfoliated cervical smear cell analysis has tremendous potential for the non-invasive detection of cervical precancerous lesions.

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 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 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 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 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 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

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}
- 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
- 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.

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.

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.

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 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 to 10 x 10^{13} particles/ml as the SERS substrate for differentiation of NRML, HSIL and CSCC 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. 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 to 10 x 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 1080cm^{-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 x 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 x 10^{13} particles/ml) and analyzed through diode laser of 633 nm laser excitation source with spectrograph grating 600gr/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 1012cm^{-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 PO_2 stretching peak at 826cm-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 1012cm^{-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.1547cm^{-1} peak was assigned to Amide II which is also slightly shifted from 1544cm^{-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 C=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.

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 NMRL, 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.

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.

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 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.

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.

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.

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.

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.

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.

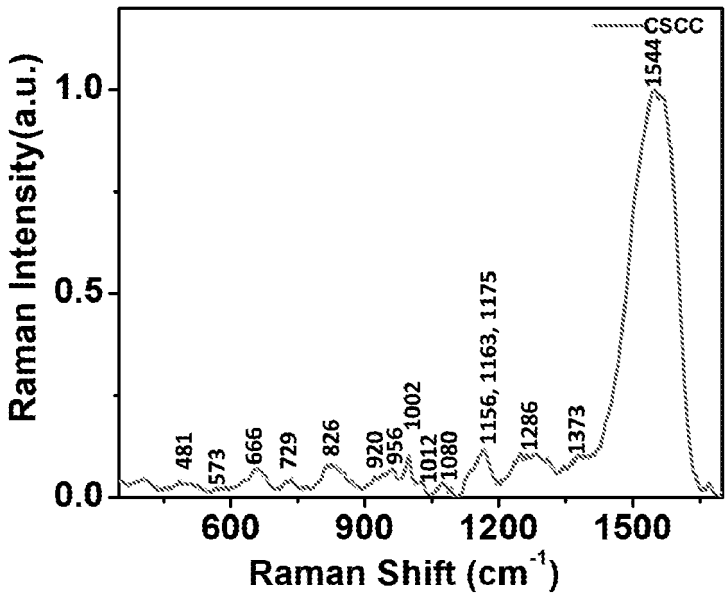
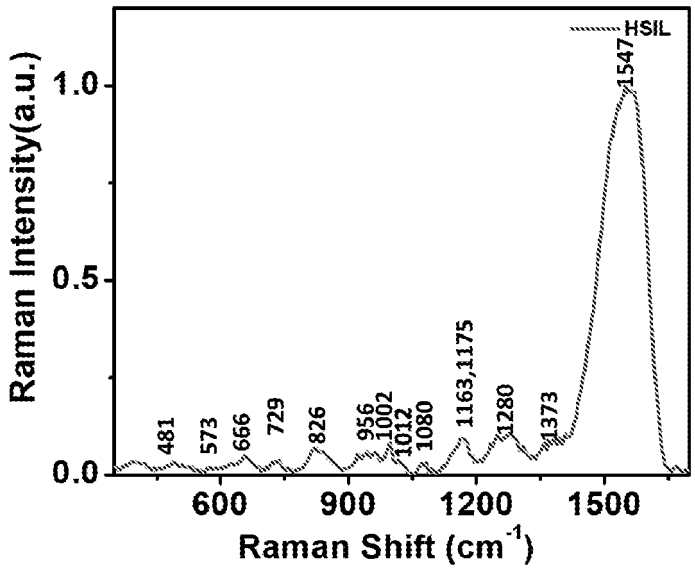

CSCC
1544
Raman Intensity(a.u.)
1.0
0.5
0.0
481
573
666
729
826
920
956
1002
1012
1080
1156, 1163, 1175
1286
1373
600
900
1200
1500
Raman Shift (cm-1)

Fig. 1


HSIL
1547
Raman Intensity(a.u.)
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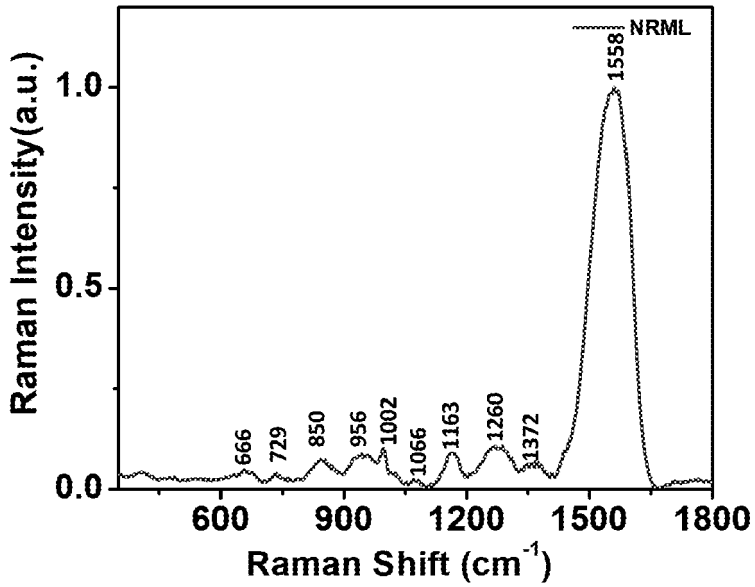
0.5
0.0
481
573
666
729
826
956
1002
1012
1080
1163,1175
1280
1373
600
900
1200
1500
Raman Shift (cm-1)

Fig. 2


NRML
1558
Raman Intensity(a.u.)
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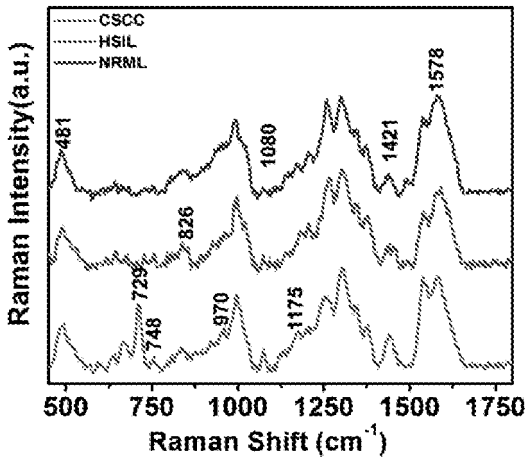
0.5
0.0
666
729
850
956
1002
1066
1163
1260
1372
600
900
1200
1500
1800
Raman Shift (cm-1)

Fig. 3



No	Wavenumber (cm^{-1})	SERS peak assignment
1	481,748, 970	DNA
2	729	Adenine
3	826,1080	O-P-O-stretch
4	1175	Cytosine, Guanine
5	1421,1578	Adenine, Guanine

Fig. 4

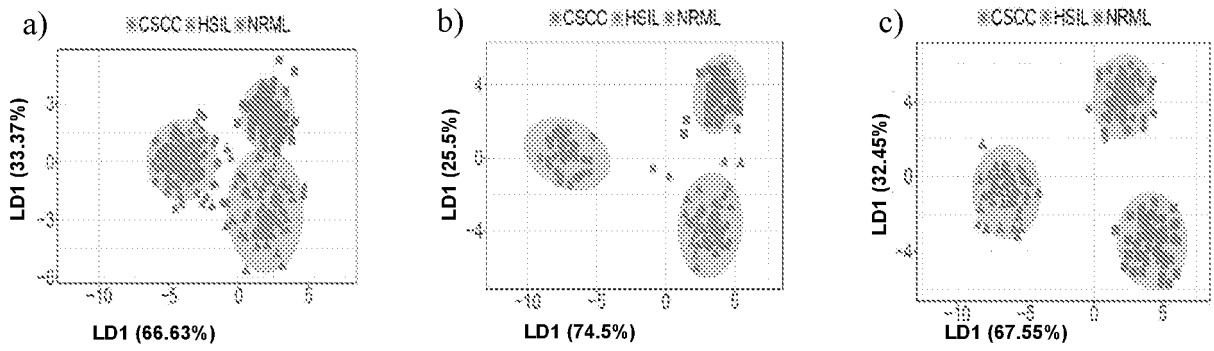

a)
CSCC HSIL NRML
LD1 (33.37%)
LD1 (66.63%)
b)
CSCC HSIL NRML
LD1 (25.5%)
LD1 (74.5%)
c)
CSCC HSIL NRML
LD1 (32.45%)
LD1 (67.55%)

Fig. 5

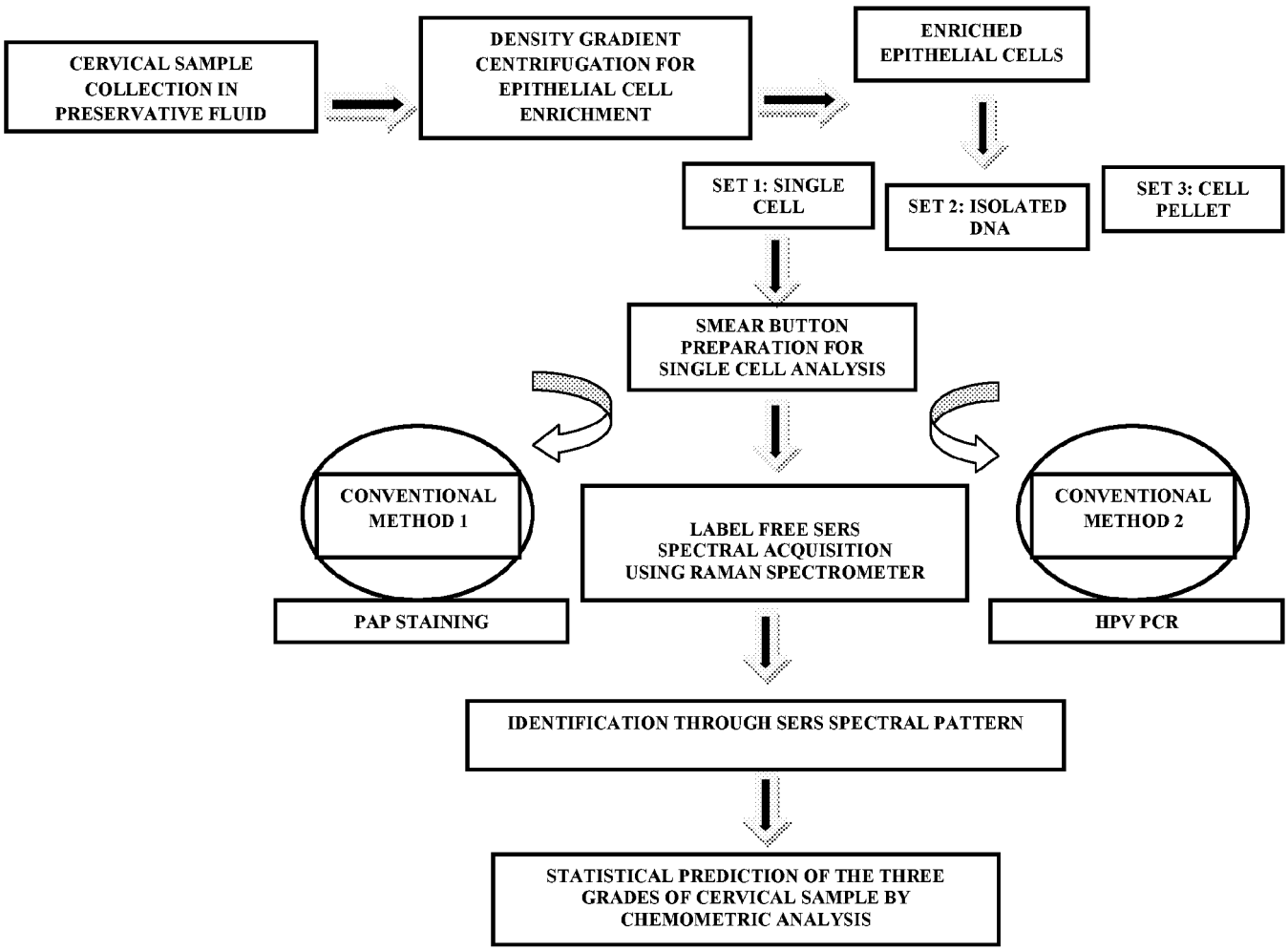

CERVICAL SAMPLE COLLECTION IN PRESERVATIVE FLUID
DENSITY GRADIENT CENTRIFUGATION FOR EPITHELIAL CELL ENRICHMENT
ENRICHED EPITHELIAL CELLS
SET 1: SINGLE CELL
SET 2: ISOLATED DNA
SET 3: CELL PELLET
SMEAR BUTTON PREPARATION FOR SINGLE CELL ANALYSIS
CONVENTIONAL METHOD 1
LABEL FREE SERS SPECTRAL ACQUISITION USING RAMAN SPECTROMETER
CONVENTIONAL METHOD 2
PAP STAINING
HPV PCR
IDENTIFICATION THROUGH SERS SPECTRAL PATTERN
STATISTICAL PREDICTION OF THE THREE GRADES OF CERVICAL SAMPLE BY CHEMOMETRIC ANALYSIS

Fig. 6

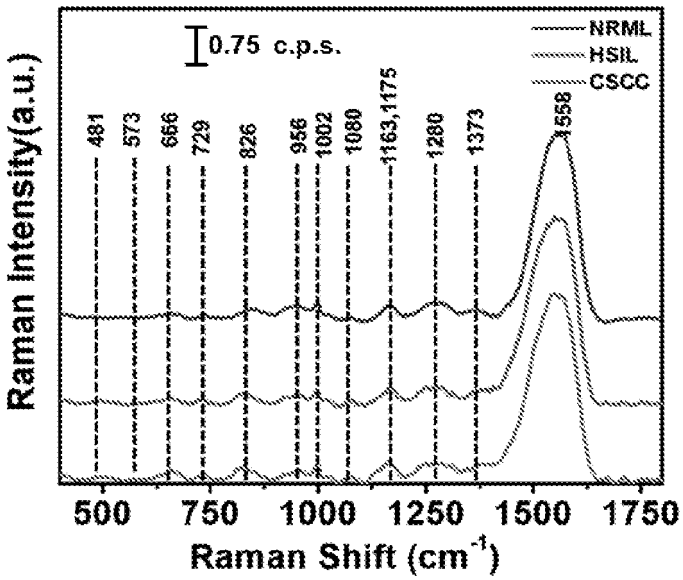

0.75 c.p.s.
NRML
HSIL
CSCC
481
573
656
729
826
956
1002
1080
1163,1175
1280
1373
1558
Raman Intensity(a.u.)
500
750
1000
1250
1500
1750
Raman Shift (cm-1)

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

Date of the actual completion of the international search	Date of mailing of the international search report
20-11-2019	20-11-2019
Name and mailing address of the ISA/ Indian Patent Office Plot No.32, Sector 14,Dwarka,New Delhi-110075 Facsimile No.	Authorized officer Akash Kumar Telephone No. +91-1125300200

INTELLECTUAL PROPERTY INDIA
Patents | Designs | Trademarks
Geographical Indications

Receipt of Electronic Submission

The Receiving Office (RO/IN) acknowledges the receipt of a PCT International Application filed using ePCT-Filing. An Application Number and Date of Receipt have been automatically assigned (Administrative Instructions, Part 7).

Submission Number:	050577	
Application Number:	PCT/IN2021/050577	
Date of Receipt:	14 June 2021	
Receiving Office:	Indian Patent Office	
Your Reference:	P-WO100569	
Applicant:	COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH	
Number of Applicants:	1	
Title:	A SERS-NANOTAG AND A DIAGNOSTIC KIT FOR DETECTING BREAST CANCER BIOMARKERS	
Documents Submitted:	PWO100569-appb-000004.pdf (P_WO100569 Specification.pdf)	1249855
	PWO100569-appb.xml	975
	PWO100569-decl.xml	12662
	PWO100569-fees.xml	1705
	PWO100569-requ.xml	15427
	PWO100569-vlog.xml	747
Submitted by:	sunaina koul (Customer ID: user_IN_KOUL_SUNAINA_2820)	
Timestamp of Receipt:	14 June 2021 11:49 UTC+5:30 (IST)	
Official Digest of Submission:	06:75:D5:27:04:F9:EB:7C:17:04:AE:4D:2D:64:7B:0C:09:33:67:23	

/RO/IN/

PCT

(Original in Electronic Form)

VIII-4-1	**Declaration: Inventorship (only for the purposes of the designation of the United States of America)** Declaration of Inventorship (Rules 4.17(iv) and 51bis.1(a)(iv)) for the purposes of the designation of the United States of America:	**I hereby declare that I believe I am the original inventor or an original joint inventor of a claimed invention in the application.** **This declaration is directed to the international application of which it forms a part.** **I hereby declare that the above-identified international application was made or authorized to be made by me.** **I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.**
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VIII-4-1-1-4	Inventor's Signature: (The signature must be that of the inventor, not that of the agent)	**/MAITI, Kaustabh Kumar/**
VIII-4-1-1-5	Date:	**14 June 2021 (14.06.2021)**
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VIII-4-1-2-2	Residence: (city and either US state, if applicable, or country)	**Trivandrum, Kerala, India**
VIII-4-1-2-3	Mailing Address:	**CSIR-National Institute for Interdisciplinary Science and Technology, (Formerly RRL)** **695019 Trivandrum, Kerala** **India**
VIII-4-1-2-4	Inventor's Signature: (The signature must be that of the inventor, not that of the agent)	**/MURALI, Vishnu Priya/**
VIII-4-1-2-5	Date:	**14 June 2021 (14.06.2021)**
VIII-4-1-3-1	Name (LAST, First)	**KARUNAKARAN, Varsha**
VIII-4-1-3-2	Residence: (city and either US state, if applicable, or country)	**Trivandrum, Kerala, India**
VIII-4-1-3-3	Mailing Address:	**CSIR-National Institute for Interdisciplinary Science and Technology, (Formerly RRL)** **695019 Trivandrum, Kerala** **India**
VIII-4-1-3-4	Inventor's Signature: (The signature must be that of the inventor, not that of the agent)	**/KARUNAKARAN, Varsha/**
VIII-4-1-3-5	Date:	**14 June 2021 (14.06.2021)**

VIII-4-1-4-1	Name (LAST, First)	**SELVAKUMAR, Deepika**
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VIII-4-1-4-3	Mailing Address:	**CSIR-National Institute for Interdisciplinary Science and Technology, (Formerly RRL) 695019 Trivandrum, Kerala India**
VIII-4-1-4-4	Inventor's Signature: (The signature must be that of the inventor, not that of the agent)	**/SELVAKUMAR, Deepika/**
VIII-4-1-4-5	Date:	**14 June 2021 (14.06.2021)**
VIII-4-1-5-1	Name (LAST, First)	**MURALI, Madhukrishnan**
VIII-4-1-5-2	Residence: (city and either US state, if applicable, or country)	**Trivandrum, Kerala, India**
VIII-4-1-5-3	Mailing Address:	**CSIR-National Institute for Interdisciplinary Science and Technology, (Formerly RRL) 695019 Trivandrum, Kerala India**
VIII-4-1-5-4	Inventor's Signature: (The signature must be that of the inventor, not that of the agent)	**/MURALI, Madhukrishnan/**
VIII-4-1-5-5	Date:	**14 June 2021 (14.06.2021)**
VIII-4-1-6-1	Name (LAST, First)	**KUNJURAMAN, Sujathan**
VIII-4-1-6-2	Residence: (city and either US state, if applicable, or country)	**Trivandrum, Kerala, India**
VIII-4-1-6-3	Mailing Address:	**Regional Cancer Centre (RCC), Medical College Campus 695011 Trivandrum, Kerala India**
VIII-4-1-6-4	Inventor's Signature: (The signature must be that of the inventor, not that of the agent)	**/KUNJURAMAN, Sujathan/**
VIII-4-1-6-5	Date:	**14 June 2021 (14.06.2021)**
VIII-4-1-7-1	Name (LAST, First)	**VALLIAMMA NEELAKANTAPILLAI, Saritha**
VIII-4-1-7-2	Residence: (city and either US state, if applicable, or country)	**Trivandrum, Kerala, India**
VIII-4-1-7-3	Mailing Address:	**Regional Cancer Centre (RCC), Medical College Campus 695011 Trivandrum, Kerala India**
VIII-4-1-7-4	Inventor's Signature: (The signature must be that of the inventor, not that of the agent)	**/VALLIAMMA NEELAKANTAPILLAI, Saritha/**
VIII-4-1-7-5	Date:	**14 June 2021 (14.06.2021)**

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(Original in Electronic Form)

VIII-4-1-8-1	Name (LAST, First)	**LEKSHMI, Asha**
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VIII-4-1-8-3	Mailing Address:	**Regional Cancer Centre (RCC), Medical College Campus 695011 Trivandrum, Kerala India**
VIII-4-1-8-4	Inventor's Signature: (The signature must be that of the inventor, not that of the agent)	**/LEKSHMI, Asha/**
VIII-4-1-8-5	Date:	**14 June 2021 (14.06.2021)**

A SERS-NANOTAG AND A DIAGNOSTIC KIT FOR DETECTING BREAST CANCER BIOMARKERS

FIELD OF THE INVENTION

The present invention relates to a SERS-nanotag comprising gold nanoparticle, an encapsulating agent, a Raman reporter and an antibody. The present invention also relates to a diagnostic kit having the SERS-nanotag for simultaneous detection of multiple breast cancer biomarkers selected from the group consisting of Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2) and Ki67 in a paraffin embedded breast tissue sample.

BACKGROUND OF THE INVENTION

Development of diagnostic SERS-nanoprobes for early and accurate detection of a disease is a challenging task in biomedical research. In the field of bio imaging, diagnostics and drug delivery, many optical imaging technologies are flourished, out of which SERS has emerged as a promising technique for detection of biological and chemical molecules adsorbed on nano roughened metallic surfaces like gold, silver etc. SERS employs the principle of Raman spectroscopy which is based on the inelastic scattering of incident radiation. It allows capturing of unique signatures corresponding to vibrations of molecules and provides signal enhancement upto10^{8}- 10^{14} folds than the normal Raman spectroscopy which enabled for minute chemical changes in biological samples even in cells and tissues.

Breast cancer is the most common cancer among women. Hormone receptors including Estrogen receptor (ER) and Progesterone receptor (PR) status are key biomolecules in breast cancer. Over-expression of HER2/Neu gene is associated breast cancer patient's prognosis and therapy and Ki67 is a proliferative marker. ER, PR, HER2 and Ki67 panel

is essential in an estimation process of breast cancer prognosis which plays a significant role in treatment choice for breast cancer worldwide.

Presence of different biomarkers needs different modes of treatment strategy. Hence, it is very useful to detect the biomarkers quickly in real time and simultaneously. There are few reports on biomarker detection in clinical samples using SERS platform, but no such SERS based biomarker detection kit has been formulated yet especially for HER-2 grading. Salehi et al., in 2014 [Salehi, M., Schneider, L., Strobel, P., Marx, A., Packeisen, J., Schlucker, S. 2014. Two-Color SERS Microscopy for Protein Co-localization in Prostate Tissue with Primary Antibody-Protein A/G-Gold Nanocluster Conjugates. Nanoscale,6(4), pp. 2361-7] reported a formulation in which silica coated gold nanoclusters were used as SERS substrate for the detection of PSA and p63 on non-neoplastic prostrate tissue samples. Whereas, Wang et al., in 2017 [Wang, Y.W., Reder, N.P., Kang, S et al., 2017. Raman-encoded molecular imaging (REMI) with topically applied SERS nanoparticles for intraoperative guidance of lumpectomy. Cancer Research, 77(16), pp. 4506–16] reported a Raman-encoded molecular imaging (REMI) technique where the targeted nanoparticles are topically applied on excised tissues to enable rapid visualization of a panel of cell surface biomarkers at surgical margin on clinical samples. Currently immune-histochemical analysis is followed by pathologists to determine the multiple breast cancer biomarkers. To surmount the disadvantages associated with conventional immunohistochemistry technique such as being highly subjective and time consuming, there is a need for a technique for fast detection of multiple breast cancer biomarkers.

OBJECTIVES OF THE INVENTION

The main objective of the present invention is to provide a SERS-nanotag comprising colloidal AuNPs, a Raman reporter molecule, a biocompatible polymer and an antibody raised against a biomarker selected from the group consisting of Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2) and Ki67 for specific simultaneous detection.

Another objective of the present invention is to provide a diagnostic kit for simultaneous detection of multiple biomarkers selected from the group consisting of Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2) and Ki67 in a breast tissue sample by surface enhanced Raman scattering (SERS) modality where each biomarkers is identified by Raman fingerprint of the respective SERS-nanotags of the kit.

Still another objective of the present invention is to provide a tissue processing step and an antigen retrieval step to remove paraffin wax and unmask the antigens from the paraffin embedded breast cancer tissue.

Another objective of the present invention is to provide a SERS analysis i.e. scanning, and imaging to gather information from maximum locations in order to know the abundance of the biomarkers.

Yet another objective of the present invention is to provide a SERS intensity based semi-quantitative system for HER-2 gradation, since an over expression of HER-2 (2+ and above from immunohistochemistry grading) is considered by the clinicians to judge the samples as positive.

SUMMARY OF THE INVENTION

An aspect of the present invention provides a SERS-nanotag comprising:

i. gold nanoparticles having size in the range of 40-50 nm;
ii. an encapsulating agent;
iii. a Raman reporter molecule; and
iv. an antibody

Another aspect of the present invention provides a process for synthesis of the SERS-nanotag comprising the steps of:

a. providing gold nanoparticle having size in the range of 40- 50 nm in a solution;

b. concentrating the gold nanoparticles of step (a) by centrifugation at 6000 rpm for 30 minutes followed by addition of 0.05% TWEEN 20 to obtain a stabilized concentrated gold nanoparticle solution;

c. adding a Raman reporter molecule to the concentrated gold nanoparticle solution obtained in step(b) and incubating for 30 minutes followed by addition of an encapsulating agent and incubating for 3 hours to obtain a biocompatible gold nanoparticle solution;

d. concentrating the biocompatible gold nanoparticle solution obtained in step (c) by centrifugation at 10,000 rpm for 10 minutes and removing excess encapsulating agent to obtain a solution;

e. re-suspending the solution obtained in step (d) in a buffer and adding (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and sulfo-NHS to obtain a reaction mixture;

f. incubating the reaction mixture obtained in step (e) for 30 minutes, centrifuging and re-suspending in the buffer;

g. adding an antibody to the reaction mixture of step (f) and incubating in a shaker incubator;

h. centrifuging the reaction mixture after incubation and re-suspending in the buffer to obtain the SERS-nanotag.

Yet another aspect of the present invention provides a diagnostic kit for detection of breast cancer biomarker comprising:

I. the SERS-nanotag;

II. xylene;

III. absolute ethanol;

IV. citrate buffer (pH 6.1);

V. phosphate buffer saline;

VI. bovine serum albumin; and

VII. an instructions manual.

Still another aspect of the present invention provides a method for detecting breast cancer biomarker in a tissue sample comprising the steps of:

(i) taking a paraffin embedded formalin fixed tissue sample;

(ii) washing the sample with xylene;

(iii) washing the sample of step (ii) with absolute ethanol followed by washing with 95% ethanol followed by washing with 70% ethanol and then with 50% ethanol to obtain a washed tissue sample;

(iv) treating the washed tissue sample of step (iii) with citrate buffer to obtain a treated tissue sample;

(v) incubating the treated tissue sample of step (iv) with bovine serum albumin and washing with phosphate buffer saline;

(vi) incubating the tissue sample of step (v) with the SERS-nanotag for 30 minutes and washing;

(vii) performing Raman spectroscopy on the tissue sample of step (vi) to take signature peaks; and

(viii) analyzing the peaks to confirm the presence of breast cancer biomarker.

BRIEF DESCRIPTION OF THE ACCOMPANYING 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.

Fig. 1: Flowchart representing the working of screening method

Fig. 2: Synthesis and characterization of AuNPs/AgNPs

Fig. 3: Gold nanoparticle with CV as the Raman reporter: A) Raman spectrum of PEGylated AuNP with CV as the reporter and B) Structure of CV

Fig. 4: Gold nanoparticle with SDL as the Raman reporter: A) Raman spectrum of PEGylated AuNP@SDL and B) structure of SDL

Fig 5: Gold nanoparticle with MBA as the Raman reporter: A) Raman spectrum of AuNP@PEG@MBA and B) Structure of MBA

Fig 6: Gold nanoparticle with Py L Et as the Raman reporter: A) Raman spectrum of AuNP@PEG@Py L Et and B) Structure of Py L Et

Fig 7. SERS single spectral analysis of one biomarker: (A) Bright field image of ER/PR negative HER-2 positive tissue, (B) SERS finger print from the same tissue incubated with AuNP@PEG@CV@Anti-HER-2 + AuNP@PEG@SDL@Anti-ER, (C) Immunohistogram of the same tissue stained for HER-2, (D) Bright field image of ER/PR positive HER-2 negative tissue, (E) SERS spectra from the same tissue incubated with AuNP@PEG@CV@Anti-HER-2 + AuNP@PEG@SDL@Anti-ER, (F) Immunohistogram of the same tissue stained for ER.

Fig. 8: SERS imaging of breast cancer tissues

Fig. 8a. Simultaneous detection of two biomarkers: SERS images from the breast cancer tissues incubated with AuNP@SDL@PEG@antiER and AuNP@CV@PEG@antiHER2 were taken. (A) Bright field image of ER+/HER2 + tissue, (B) Raman image *w.r.t* 440 nm, spectral fingerprint, (C) Raman image *w.r.t* 580 nm spectral fingerprint, (D) Average spectrum from the scan [B, C, D are from the same area of bright field image (A)], (E) Bright field image of TNBC tissue, (F & G) Raman image of the same area *w.r.t* 440 and 580 nm spectral fingerprint, (H) Average spectrum from the scan.

Fig. 8b. Simultaneous detection of three biomarkers**:** SERS images from the breast cancer tissues incubated with AuNP@SDL@PEG@antiER and AuNP@CV@PEG@antiHER2 and AuNP@MBA@PEG@antiPR were collected. **(**A) Bright field image of ER+/PR+/HER2 + tissue, (B) Raman spectrum from image scan showing simultaneous peaks for HER2, ER and PR, (C) Raman image *w.r.t* 440 cm-1, spectral fingerprint, (D) Raman image *w.r.t* 580 cm-1 spectral fingerprint, (E) Raman image *w.r.t* 1080 cm-1, spectral fingerprint [C, D, E are from the same area of bright field image (A)].

Fig. 9. SERS analysis for Ki67 expression in TNBC tissue sample**:** TNBC samples were incubated with AuNP@Py L Et@antiKi67 for 30 min and SERS analysis was performed after thorough washing.

Fig. 10. HER-2 Grading of tissue samples with SERS analysis**:** a) Immunohistochemical analysis, b) SERS images from image scan and c) representative spectra from different HER2 grades.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is focused on simultaneous detection of multiple biomarkers in a breast tissue sample based on SERS-nanotags using Raman fingerprint analysis. A nanoparticle probe comprising gold or silver nanoparticles having size in the range of 40-50 nm anchored with a Raman reporter molecule and encapsulated with a biocompatible polymer is conjugated with a breast cancer specific antibody which is transformed into a SERS-nanotag. Further, these SERS-nanotag conjugated to target specific antibodies raised against a biomarker selected from the group consisting of Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2) and Ki67are used to validate the simultaneous recognition capabilities in paraffin embedded breast cancer tissue samples.

A diagnostic kit comprising the SERS-nanotag enables simultaneous detection of multiple biomarkers in a breast tissue sample with a highly sensitive and specific Raman peak of the Raman reporter attached to the nanoparticle which corresponds to the presence of respective antibody attached to the same nanoparticle.

An embodiment of the present invention provides a SERS-nanotagcomprising:

i. gold nanoparticles having size in the range of 40-50 nm;
ii. an encapsulating agent;
iii. a Raman reporter molecule; and
iv. an antibody

In another embodiment of the present invention, there is provided a SERS-nanotag, wherein the encapsulating agent is selected from the group consisting of a polysaccharide, polyethylene glycol, and serum albumin.

In still another embodiment of the present invention, there is provided a SERS-nanotag, wherein the polysaccharide is selected from the group consisting of chitosan, and hyaluronic acid.

In yet another embodiment of the present invention, there is provided a SERS-nanotag, wherein the encapsulating agent is polyethylene glycol

In another embodiment of the present invention, there is provided a SERS-nanotag, wherein the Raman reporter molecule is selected from the group consisting of cyanine dilipoic acid (Cy7DLA), hemicyaninecarbaldehyde (HCC), Pyryliniumhexylamine (PHA), Squaraine di-lipoic acid (SDL), Pyrenelipidene ethyl quartanised (Py L Et), crystal violet (CV) and Mercapto benzoic acid (MBA).

In still another embodiment of the present invention, there is provided a SERS-nanotag, wherein the antibody is a monoclonal or a polyclonal antibody.

In yet another embodiment of the present invention, there is provided a SERS-nanotag, wherein the antibody is a monoclonal antibody.

In still another embodiment of the present invention, there is provided a SERS-nanotag, wherein the antibody is a polyclonal antibody.

In an embodiment of the present invention, there is provided a SERS-nanotag, wherein the antibody is raised against a biomarker selected from the group consisting of Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2) and Ki67.

An embodiment of the present invention provides a process for synthesis of the SERS-nanotag comprising the steps of:

a. providing gold nanoparticles having size in the range of 40-50 nm in a solution;

b. concentrating the gold nanoparticles of step (a) by centrifugation at 6000 rpm for 30 minutes followed by addition of 0.05% TWEEN 20 to obtain a stabilized concentrated gold nanoparticle solution;

c. adding a Raman reporter molecule to the concentrated gold nanoparticle solution obtained in step (b) and incubating for 30 minutes followed by addition of an encapsulating agent and incubating for 3 hours to obtain a biocompatible gold nanoparticle solution;

d. concentrating the biocompatible gold nanoparticle solution obtained in step (c) by centrifugation at 10,000 rpm for 10 minutes and removing excess encapsulating agent to obtain a solution;

e. re-suspending the solution obtained in step (d) in a buffer and adding (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and sulfo-NHS to obtain a reaction mixture;

f. incubating the reaction mixture obtained in step (e) for 30 minutes, centrifuging and re-suspending in the buffer;

g. adding an antibody to the reaction mixture of step (f) and incubating in a shaker incubator;

h. centrifuging the reaction mixture after incubation and re-suspending in the buffer to obtain the SERS-nanotag.

In an embodiment of the present invention, there is provided a process for synthesis of the SERS-nanotag, wherein the gold nanoparticles are in a concentration in the range of 7×10^9 to 4×10^{10} particles/mL.

In another embodiment of the present invention, there is provided a process for synthesis of the SERS-nanotag, wherein the Raman reporter molecule is in a concentration in the range of 0.5 to 100µM.

In yet another embodiment of the present invention, there is provided a process for synthesis of the SERS-nanotag, wherein the antibody is in a concentration in the range of 2 to 20 µg.

In an embodiment of the present invention, there is provided a process for synthesis of the SERS-nanotag, wherein the Raman reporter molecule is selected from the group consisting

of cyanine dilipoic acid (Cy7DLA), hemicyaninecarbaldehyde (HCC), Pyryliniumhexylamine (PHA), Squaraine di-lipoic acid (SDL), Pyrenelipidene ethyl quartanised (Py L Et), crystal violet (CV) and Mercapto benzoic acid (MBA).

In another embodiment of the present invention, there is provided a process for synthesis of the SERS-nanotag, wherein the encapsulating agent is selected from the group consisting of a polysaccharide, polyethylene glycol, and serum albumin.

In yet another embodiment of the present invention, there is provided a process for synthesis of the SERS-nanotag, wherein the buffer is selected from the group consisting of MES buffer, Phosphate buffer and Tris buffer.

In an embodiment of the present invention, there is provided a process for synthesis of the SERS-nanotag, wherein the antibody is raised against a biomarker selected from the group consisting of Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2) and Ki67.

Another embodiment of the present invention provides a diagnostic kit for detection of breast cancer biomarker comprising:

I. the SERS-nanotag;
II. xylene;
III. absolute ethanol;
IV. citrate buffer;
V. phosphate buffer saline;
VI. bovine serum albumin; and
VII. instructions manual.

Yet another embodiment of the present invention provides a method for detecting breast cancer biomarker in a tissue sample comprising the steps of:

(i) taking a paraffin embedded formalin fixed tissue sample;
(ii) washing the sample with xylene;

(iii) washing the sample of step (ii) with absolute ethanol followed by washing with 95% ethanol followed by washing with 70% ethanol and then with 50% ethanol to obtain a washed tissue sample;

(iv) treating the washed tissue sample of step (iii) with citrate buffer to obtain a treated tissue sample;

(v) incubating the treated tissue sample of step (iv) with bovine serum albumin and washing with phosphate buffer saline;

(vi) incubating the tissue sample of step (v) with the SERS-nanotag for 30 minutes and washing;

(vii) performing Raman spectroscopy on the tissue sample of step (vi) to take signature peaks; and

(viii) analyzing the peaks to confirm the presence of breast cancer biomarker.

In still another embodiment of the present invention, there is provided a method for detecting breast cancer biomarker in a tissue sample, wherein the breast cancer biomarker is selected from the group consisting of Estrogen receptor (ER), Progesterone receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2) and Ki67.

The aim of the present invention is to detect multiple biomarkers in a breast tissue sample by SERS based diagnostic platform in a simultaneous detection mode using SERS-nanotags and confirm the presence or absence of the biomarkers much faster with high precision level as compared to analysis with current gold standards.The present invention provides a SERS based simultaneous diagnostic kit having a SERS-nanotag comprising a nanoparticle, a biocompatible polymer, a Raman reporter (RR) molecule and an antibody raised against breast cancer biomarkers selected from the group consisting of Estrogen receptor (ER), Progesterone receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2) and Ki67.

The nanoparticle can be colloidal gold nanoparticles (AuNPs), colloidal silver nanoparticles (AgNPs), gold coated silver nanoparticles (Au@AgNPs) or gold (Au)/silver

(Ag) coated glass slide. In a preferred embodiment of the present invention, the nanoparticles are AuNPs having size in the range of 40-50nm. These gold nanoparticles are then encapsulated in a biocompatible polymer which is selected from the group consisting of a polysaccharide, polyethylene glycol, and serum albumin. Both, in-house synthesized and commercially available Raman reporters are used as a signature Raman peaks to obtain simultaneous detection. The in-house Raman reporter (RR) molecules used in the present invention are Cy7DLA (cyanine dilipoic acid), HCC (hemicyaninecarbaldehyde), PHA (Pyryliniumhexylamine), SDL (Squaraine di-lipoic acid), Py L Et (Pyrenelipidene ethyl quartanised), and commercially available Raman reporters include CV (crystal violet) and MBA (Mercapto benzoic acid).The Raman reporter molecules are coupled to the commercially purchased antibodies raised against biomarkers i.e. ER, PR, HER2, and Ki67, to formulate the SERS-nanotags for detection of breast cancer biomarkers i.e. ER, PR, HER2, and Ki67.

The present invention also co-relates and validates the Raman fingerprint from the Raman reporter molecule with-respect-to biomarker in a breast tissue sample in a simultaneous Raman fingerprint. This programme was approved by the local Ethics Committee and prior to specimen collection; all patients had signed informed consent forms. Pathologically confirmed breast cancer tissues with different ER, PR, HER2, Ki67 status were collected from Regional Cancer Centre (RCC), Trivandrum, Kerala, India. A separate bit of tumor tissue from each subject was paraffin embedded and 4 micron sections were obtained. Sections were selected after confirming the presence of tumor in Haematoxylin and eosin stained samples. Paraffin wax was removed by rinsing in xylene followed by different grades of alcohol and antigen retrieval was performed prior to the incubation with SERS-nanotag. SERS spectral analysis was carried out using with diode laser of 633 / 785 nm laser excitation source with spectrograph grating 600gr/mm using maximum 1-20 sec integration time and around 1-15 accumulations.

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 substrate

Colloidal gold nanoparticles (AuNPs, size around 40-50 nm) were prepared by citrate reduction method [Kim Ling, J., Maier, M., Okenve, B., Kotaidis, V., Ballot, H. and Plech, A., 2006. Turkevich method for gold nanoparticle synthesis revisited. The Journal of Physical Chemistry B, 110(32), pp.15700-15707]. The characterization of the synthesized nanoparticles was done through UV-VIS spectroscopy, Dynamic Light Scattering (DLS) and High Resolution Transmission Electron Microscopy (HRTEM). Figure 2 shows the synthesis and characterization of AuNPs/AgNPs. The size was approximately 40-50 nm which served as an optimal SERS substrate in order to get maximum enhancement.

EXAMPLE 2

Synthesis of nanotag: AuNP@PEG@CV

The gold nanoparticles of size 40-45 nm were concentrated from 25 mL to 3.6 mL by centrifugation at 6000 rpm, for 30 minutes. To this, 0.05% of TWEEN 20 was added for stabilizing the gold nanoparticles and vortexed for few minutes. Then, ~ 400 µL of 80µM Raman reporter1 (crystal violet (CV) in dimethyl sulfoxide (DMSO)) was added and incubated for half an hour. For making the tag biocompatible, 45µL SH-PEG-COOH was added and incubated for 10 minutes. To this solution, 275 µL SH-PEG-OCH_3 was added and further incubated for 3 hrs. Then, the solution was concentrated to 1 mL by centrifugation at 10,000 rpm for 10 minutes. Excess PEG was removed by centrifuging

the solution again at 10,000 rpm for 10 minutes. Finally, the solution was re-suspended in milliQ water to obtain AuNP@PEG@CV. Figure 3 shows gold nanoparticle with CV as the Raman reporter: A) Raman spectrum of PEGylated AuNP with CV as the reporter and B) Structure of CV.

EXAMPLE 3

Synthesis of SERS-nanotag: AuNP@PEG@CV@AntiHER2

Anti-HER2 (Rabbit Monoclonal antibody; ABCAM) for the biomarker HER2 was purified using 3 KDa centrifugal filters. PEG encapsulated nanoparticles obtained in example 2 (1-1.5 mL) were centrifuged at 8000 rpm for 15 minutes and re-suspended in ~500 µL MES buffer (HIMEDIA) (50 mM, pH 6.1). 5 µL of freshly prepared EDC (SIGMA-ALDRICH) (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, 250 mM) was added and after a few minutes gap, 6 µL of freshly prepared sulfo-NHS (ALDRICH CHEMISTRY) (N-hydroxysuccinimide, 250 mM) was also added. After incubating the reaction mixture for 30 minutes, the reaction mixture was centrifuged at 10000 rpm for 10 minutes and re-suspended in 500 µL MES buffer. Then, 4 µg antibody was added to that and incubated in a shaker incubator for 2 hrs at room temperature and kept for overnight incubation at 4°C. After that, the mixture was centrifuged at 10000 rpm for 10 minutes and finally re-suspended in fresh 500 µLMES to obtain the antibody conjugated SER-nanotag AuNP@PEG@CV@Anti HER2.

EXAMPLE 4

Synthesis of nanotag: AuNP@PEG@SDL

The gold nanoparticles of size 40-45 nm were concentrated from 7.2 mL to 1 mL by centrifugation at 6000 rpm, for 30 minutes). 0.05% of TWEEN 20 was added for stabilizing the gold nanoparticles and vortexed for few minutes. Then, 37 µL of 100µM Raman reporter 2 (Squaraine di-lipoic acid (SDL) in dimethyl sulfoxide) and 262.5µL

milliQ water was added and incubated for 10 minutes. For making the tag biocompatible, 62µL SH-PEG-COOH was added and incubated for 10 minutes. To this solution, 368 µL SH-PEG-OCH_3 was added and further incubated for 3 hrs. Then, the solution was concentrated to 1 mL by centrifugation at 10,000 rpm for 10 minutes. Excess PEG was removed by centrifuging the solution again at 10,000 rpm for 10 minutes. Finally, the solution was re-suspended in milliQ water to obtain AuNP@PEG@SDL. Figure 4 shows gold nanoparticle with SDL as the Raman reporter: A) Raman spectrum of PEGylated AuNP@SDL and B) structure of SDL.

EXAMPLE 5

Synthesis of SERS-nanotag: AuNP@PEG@SDL@Anti ER

Anti-ER (Rabbit Monoclonal antibody; ABCAM) for the biomarker ER was purified and conjugated by EDC-NHS coupling as described in example 3 (procedure for HER2 conjugation). Here also, 4 µg antibody was added to the PEGylated AuNPs obtained in example 4 to obtain antibody conjugated SERS-nanotag: AuNP@PEG@SDL@Anti ER.

EXAMPLE 6

Synthesis of nanotag: AuNP@PEG@MBA

The gold nanoparticles of size 40-45 nm were concentrated from 25 mL to 3.6 mL by centrifugation at 6000 rpm, for 30 minutes. To this, 0.05% of TWEEN 20 was added for stabilizing the gold nanoparticles and vortexed for few minutes. Then, 400 µL of 200µM Raman reporter 3 Mercapto benzoic acid (MBA) was added and incubated for half an hour. For making the tag biocompatible, 45µL SH-PEG-COOH was added and incubated for 10 minutes. To this solution, 275 µL SH-PEG-OCH_3 was added and further incubated for 3 hrs. Then, the solution was concentrated to 1 mL by centrifugation at 10,000 rpm for 10 minutes. Excess PEG was removed by centrifuging the solution again at 10,000 rpm for 10 minutes. Finally, the solution was re-suspended in milliQ water to obtain

SERS-nanotag: AuNP@PEG@MBA. Figure 5 shows gold nanoparticle with MBA as the Raman reporter: A) Raman spectrum of AuNP@PEG@MBA and B) Structure of MBA.

EXAMPLE 7

Synthesis of SERS-nanotag: AuNP@PEG@MBA@Anti-PR

Anti-PR (Rabbit Monoclonal antibody; ABCAM) for the biomarker PR was purified and conjugated to the AuNP@PEG@MBA obtained in example 6 by the procedure described in example 3 to obtain antibody conjugated SERS-nanotag: AuNP@PEG@MBA@Anti-PR.

EXAMPLE 8

Synthesis of nanotag: AuNP@PEG@Py L Et

The gold nanoparticles of size 40-45 nm were concentrated from 25 mL to 3.6 mL by centrifugation at 6000 rpm, for 30 minutes. To this, 0.05% of TWEEN 20 was added for stabilizing the gold nanoparticles and vortexed for few minutes. Then, 400 µL of 100µM Raman reporter 4 Pyrenelipidene ethyl quartanised (Py L Et) was added and incubated for half an hour. For making the tag biocompatible, 45µL SH-PEG-COOH was added and incubated for 10 minutes. To this solution, 275 µL SH-PEG-OCH_3 was added and further incubated for 3 hrs. Then, the solution was concentrated to 1 mL by centrifugation at 10,000 rpm for 10 minutes. Excess PEG was removed by centrifuging the solution again at 10,000 rpm for 10 minutes. Finally, the solution was re-suspended in milliQ water to obtain SERS nanotag: AuNP@PEG@Py L Et. Figure 6 shows gold nanoparticle with Py L Et as the Raman reporter: A) Raman spectrum of AuNP@PEG@Py L Et and B) Structure of Py L Et.

EXAMPLE 9

Synthesis of SERS-nanotag AuNP@PEG@Py L Et@Anti-Ki67

Anti-Ki67(ABCAM, Mouse monoclonal antibody) for the biomarker Ki67 was purified and conjugated to the AuNP@PEG@Py L Et (4 µg) obtained in example 8 by the procedure described in example 3 to obtain antibody conjugated SERS-nanotag AuNP@PEG@Py L Et@Anti-Ki67.

EXAMPLE 10

Single cell spectral analysis from Paraffin embedded tissue sample

Breast cancer tissue samples having various biomarker expression status were collected from Regional Cancer Centre, Trivandrum, Kerala, India for SERS analysis. Ethical approval for the same was obtained from the consigned authorities prior to the experiments.

Optimized processing of tissue

Prior to spectral analysis, tissue processing was carried out by the following standardized steps for the paraffin embedded formalin fixed tissues.

A) Deparaffinization in Xylene: The paraffin embedded formalin fixed tissues were washed with xylene for 3 times, 8 minutes each.

B) Hydration By Graded Alcohol : Then the formalin fixed tissues were washed with absolute ethanol for 2 times, 5 minutes each; Then the formalin fixed tissues were washed with 95 % Ethanol for 3 minutes, then washed with 70 % Ethanol for 3 Minutes, then washed with 50 % Ethanol for 3 Minutes, then washed with distilled water for 5minutes.

C) Antigen Retrieval: The washed formalin fixed tissues were treated with 10 Mm citrate buffer (pH 6.1) at 500-700 W for 10 minutes in a microwave oven and then kept for 1- 5 minutes rest. Then, more volume of citrate buffer was added to the tissues and again heated for 5-10 minutes at 500-700 W. The slides of formalin

fixed tissues were allowed to cool at room temperature for 15-20 minutes and then immersed in de-ionized water for 15-20 minutes at room temperature.

D) Blocking With BSA : The fixed tissues were incubated with 3% BSA in PBS at room temperature and then washed three times with PBS.

E) Incubation With SERS Nanotag : The fixed tissues were incubated with antibody conjugated SERS nanotags for 30 minutes in a humid chamber and then washed 3-5 times with PBS. Raman spectra and Raman images were acquired under 633 / 785 nm laser by placing the samples under 10X or 20 X on objective of Raman microscope. Confocal Raman microscope (WITec, Germany) with Peltier cooled charge-coupled device detector unit was used for the analysis. The samples were excited using a 633/785 nm laser with 10 mW-30 mW power, and Raman spectra were collected in the region of 300 −2000 cm $^{-1}$ with a resolution of 1 cm $^{-1}$ and an integration time of 2-10 seconds and 3-5 accumulations. Prior to each analysis, calibration was done with a silicon standard (Raman peak at 520 cm^{-1}). Data processing was performed using WITec Project Plus (v5.2) software package. Raman imaging was performed for an area of 150 -175 µm x 150 -175 µm area with an integration time of 0.01 to 0.1 seconds integration time and 100-150 lines and points per image.

Figure 7 shows SERS single spectral analysis of one biomarker : (A) Bright field image of ER/PR negative HER-2 positive tissue; (B) SERS finger print from the same tissue incubated with AuNP@PEG@CV@Anti-HER-2 + AuNP@PEG@SDL@Anti-ER, shows the 440 cm^{-1} marker peak of CV which was used as the corresponding Raman reporter for HER-2 biomarker detection. As the sample analyzed was HER-2 positive and ER negative tissue, the spectral pattern obtained was also lacking the 580 cm^{-1} marker peak of SDL correspond to ER biomarker ; (C) Immunohistogram of the same tissue stained for HER-2 showing the deeply stained HER-2 positive cells; (D) Bright field image of ER/PR positive HER-2

negative tissue; (E) SERS spectra from the same tissue incubated with AuNP@PEG@CV@Anti-HER-2 + AuNP@PEG@SDL@Anti-ER, spectral pattern from tissue sample showing the 580 cm^{-1} marker peak of SDL but not the 440 cm^{-1} of CV denotes the ER positive and HER-2 negative expression status.; (F) Immunohistogram of the same tissue stained for ER showing the ER positive cells.

Figure 8 shows the SERS imaging of breast cancer tissues. Figure 8a shows simultaneous detection of two biomarkers: SERS images from the breast cancer tissues incubated with AuNP@SDL@PEG@antiER and AuNP@CV@PEG@antiHER2 were taken; (A) Bright field image of ER+/HER2 + tissue; (B) Raman image w.r.t440 nm, spectral fingerprint with bright spots showing the HER-2 positive cells; (C) Raman image w.r.t 580 nm spectral fingerprint with bright spots denoting the ER positive cells ; (D) Average spectrum from the scan presenting the 440 cm^{-1} and 580 cm^{-1} peaks of CV and SDL corresponding to HER-2 and ER biomarkers respectively portraying the ER and HER-2 positivity [B, C, and D, are from the same area of bright field image (A)]; (E) Bright field image of TNBC tissue; (F & G) Raman image of the same area w.r.t 440 and 580 nm spectral fingerprint; (H) Average spectrum from the scan without CV and SDL peaks depicts the absence of biomarkers .

Figure 8b shows the simultaneous detection of three biomarkers: SERS images from the breast cancer tissues incubated with AuNP@SDL@PEG@antiER and AuNP@CV@PEG@antiHER2 and AuNP@MBA@PEG@antiPR were collected: (A) bright field image of ER+/PR+/HER2 + tissue; (B) Raman spectrum from image scan showing simultaneous peaks for HER2 (440 cm^{-1} peak of CV), ER (580 cm^{-1} peak of SDL) and PR (1080 cm^{-1} peak of MBA) showing the presence of all the three biomarkers; (C) Raman image w.r.t 440 cm-1, spectral fingerprint; (D) Raman image w.r.t 580 cm-1 spectral fingerprint; (E) Raman image w.r.t 1080 cm-1, spectral fingerprint [C, D, E are from the same area of bright field image (A)].

Figure 9 shows the SERS analysis for Ki67 expression in TNBC tissue sample: TNBC samples were incubated with AuNP@Py L Et@antiKi67 for 30 min and SERS analysis was performed after thorough washing. Spectral data shows the Py L Et peak and the Raman

image with bright spots shows the Ki67 positive cells denoting the Ki67 biomarker expression in the tissue sample.

The present diagnostic strategy is able to detect single, duel and triple biomarkers in breast cancer tissue sample accurately. Figure 7 shows precisely the presence of single biomarker i.e. HER-2 and ER in respective tissue sample. In figure 8, dual biomarker i.e. HER-2 and ER were detected in a single tissue sample whereas in TNBC sample, absence of any reporter peaks were observed. Similarly, ER, PR and HER-2 biomarkers were simultaneously detected from ER+/PR+/HER-2+ tissue sample and Ki67 abundance in TNBC sample.

EXAMPLE 11

HER-2 grading by SERS

Breast cancer tissue samples with different levels of HER-2 expression were collected from Regional Cancer Centre (RCC), Trivandrum, Kerala, India. Tissues were analyzed by immunohistochemistry to confirm the grading by a pathologist. Samples were processed as mentioned in above examples. For HER-2 grading, AuNP@CV@HER-2 was added to the tissue samples and incubated for 30 min and washed thoroughly to take the signature peaks from SERS analysis. Spectra were collected by spectral accumulation and also through image scanning. All the tissue processing procedure was as early described and the SERS analysis was performed with 633 nm laser, under 10 X objective of confocal Raman microscope. For single spectral analysis, integration time used was 3-5 seconds with 2-5 accumulations using 10 mW power. Image scanning was performed for 150 x 150 µm area with 0.01 seconds integration time. Average CCD counts were taken for comparison of different HER-2 graded samples.

Figure 10 shows the HER-2 Grading of tissue samples with SERS analysis: (a) Immunohistochemical analysis showing the increased expression of HER-2 from 1+ to 4+ sample; (b) SERS images from image scan with increased number of bright spots in the images as the HER-2 expression increases; and (c) representative spectra from

different HER2 grades. Table 1 provides the average CCD counts for the signature peaks acquired from different HER2 grades.

Table 1

HER-2 Grade	Avrg CCD @ 440	Avrg CCD@1615
1+	3	18
2+	15	32
3+	24	65
4+	40	1150

HER-2 grading is an important aspect during the selection of treatment regimens for breast cancer patients as HER-2 over expression, i.e., 3+ and above are considered as HER-2 positive whereas 2+ expression is considered as borderline. Here, tissue samples with varying HER-2 expression levels (1+ to 4+) were analyzed by single spectral obtained from the Raman mapping providing the average spectra. Both the analysis showed an increase in spectral intensity of Raman reporter CV in accordance with the Her-2 expression levels. Table 1 denotes the CCD counts obtained from the Raman image analysis corresponding to the 440 and 1615 cm^{-1} peaks of CV where both the peak intensities were found to be increasing from 3 to 40 *w.r.t* to peak at 440 cm^{-1} and 18 to 150 *w.r.t.* peak at 1615 cm^{-1} respectively as the HER-2 expression levels increases from 1+ to 4+. Thus, the present Her-2 grading by Raman spectral analysis can be utilized as a complementary technique to IHC to confirm the HER-2 grading.

Comparison of the technique of the present invention with IHC

Immunohistochemistry (IHC) is the existing gold standard method for detection of breast cancer biomarkers in formalin fixed paraffin embedded tissue samples. Table 2 provided

below compares both the techniques in terms of specificity, easiness and time required for sample processing and analysis.

Table 2

Parameters	**IHC**	**SERS Diagnostic kit of the present invention**
Mutiplexing Analysis	Very difficult. No standard method.	Easily to analyze in single tissue sample
Type of analysis	Highly Subjective (Inter observer variation)	Objective; semi-quantitative analysis is possible for Her-2 grading
Time required for sample preparation	4-27 hrs	4-5 hrs
Time required for analysis	0.5 hr for single biomarker	0.5-1 hr irrespective of single or more biomarkers
Specificity	>95%	Nearly 90 % for single biomarker., 80-85 % multiplexing analysis
False positive results	0-36 % may happen due to nonspecific binding of horse radish peroxidase (HRP) conjugated secondary antibody (Nuovo, G., 2016. False-positive results in diagnostic immunohistochemistry are related to horseradish peroxidase conjugates in commercially	10-30 % may happen due to the nonspecific binding of nanoparticles

	available assays.Annals of Diagnostic Pathology, 25, pp.. 54–59.)	
False negative result	0-10 % due to low levels of biomarker in the sample, poor tissue fixation, problems with the tissue processing and antigen retrieval steps etc (True, L.D. 2008. Quality control in molecular immunohistochemistry. Histochemistry and Cell Biolo,130, pp. 473–480.)	More sensitive technique, Approximately 0-8 % may happen due to poor fixation, tissue processing and antigen retrieval steps

Advantages of the invention

1. Development of diagnostic screening kit for accurate detection of a diseases is a challenging task in biomedical research. In the field of bio-imaging and diagnostics, SERS has emerged as a highly sensitive and promising technique for detection of biological and chemical molecules which are adsorbed on nano roughened metallic surfaces like gold or silver.
2. The present invention provides a SERS-nanotag comprising colloidal AuNPs, a Raman reporter molecule, a biocompatible polymer and an antibody raised against a biomarkerselected from the group consisting of ER, PR, HER2 and Ki67 for specific simultaneous detection of ER, PR, HER2, and Ki67. The preparative steps are critically optimized for highly selective detection of clinically relevant biomarkers only from breast tissue samples.

3. In the present invention, a tissue processing step and an antigen retrieval has been incorporated with an easy and straight forward way to remove the paraffin wax and unmask the antigens from the paraffin embedded breast tissue.
4. In the present invention, SERS analysis i.e. scanning, and imaging of the SERS-nanotag is performed to gather the information from maximum locations in order to know the abundance of biomarkers in the breast tissue sample.
5. In the present invention, a SERS intensity based semi-quantitative system for HER-2 gradation has been provided using the SERS-nanotag since the over expression of HER-2 (2+ and above from immunohistochemistry grading) is considered by the clinicians to judge the samples as positive.
6. The present invention demonstrates an ultrasensitive simultaneous detection modality based on SERS-nanotags which aims novelty technical advancement over the existing technology.
7. Simultaneous recognition of breast cancer biomarkers ER, PR, HER2, and Ki67 expression in a single detection mode with a single laser utilizing respective antibody conjugated SERS-nanotag of the present invention is termed as SERS based immunoassays.
8. Simultaneous detection modality of the present invention is achieved by initial validation in paraffin embedded breast cancer tissue samples. By evaluation of SERS spectral analysis of the emission from SERS-nanotag, ER, PR, HER2 and Ki67 status from the tissue sample is confirmed which definitely propagates into treatment management with high precision, minimum assay time, and in a cost effective manner.
9. The nanotag of the present invention is highly accurate and there is very low possibility of false positive and false negative results.

We claim:

1. A SERS-nanotag comprising:
    i. gold nanoparticles having size in the range of 40-50 nm;
    ii. an encapsulating agent;
    iii. a Raman reporter molecule; and
    iv. an antibody.
2. The SERS-nanotagas claimed in claim 1, wherein the encapsulating agent is selected from the group consisting of a polysaccharide, polyethylene glycol, and serum albumin.
3. The SERS-nanotag as claimed in claim 2, wherein the polysaccharide is selected from the group consisting of chitosan, and hyaluronic acid.
4. The SERS-nanotag as claimed in claim 2, wherein the encapsulating agent is polyethyleneglycol.
5. The SERS-nanotag as claimed in claim 1, wherein the Raman reporter molecule is selected from the group consisting of cyanine dilipoic acid (Cy7DLA), hemicyaninecarbaldehyde(HCC), Pyryliniumhexylamine(PHA), Squaraine di-lipoic acid(SDL), Pyrenelipidene ethyl quartanised(Py L Et), crystal violet (CV) and Mercapto benzoic acid (MBA).
6. The SERS-nanotag as claimed in claim 1, wherein the antibody is a monoclonal or a polyclonal antibody.
7. The SERS-nanotagas claimed in claim 1, wherein the antibody is raised against a biomarker selected from the group consisting of Estrogenreceptor (ER), Progesterone receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2) and Ki67.
8. A process for synthesis of the SERS-nanotagas claimed in claim 1 comprising the steps of:
    a. providing gold nanoparticle having size in the range of 40- 50 nm in a solution;

b. concentrating the gold nanoparticles of step (a) by centrifugation at 6000rpm for 30 minutes followed by addition of 0.05% TWEEN 20 to obtain a stabilized concentrated gold nanoparticle solution;

c. adding a Raman reporter molecule to the concentrated gold nanoparticle solution obtained in step (b) and incubating for 30 minutes followed by addition of an encapsulating agent and incubating for 3-4 hours to obtain a biocompatible gold nanoparticle solution;

d. concentrating the biocompatible gold nanoparticle solution obtained in step (c) mL by centrifugation at 10,000rpm for 10 minutes and removing excess encapsulating agent to obtain a solution;

e. re-suspending the solution obtained in step (d) in a buffer and adding (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and sulfo-NHS to obtain a reaction mixture;

f. incubating the reaction mixture obtained in step (e) for 30 minutes, centrifuging and re-suspending in the buffer;

g. adding an antibody to the reaction mixture of step (f) and incubating in a shaker incubator;

h. centrifuging the reaction mixture after incubation and re-suspending in the buffer to obtain the SERS-nanotag.

9. The process as claimed in claim 8, wherein the gold nanoparticle are in a concentration in the range of 7×10^9 - 4×10^{10} particles/mL.

10. The process as claimed in claim 8, wherein the Raman reporter molecule is in a concentration in the range of 0.5 to 100µM

11. The process as claimed in claim 8, wherein the antibody is in a concentration in the range of 2 to 20 µg.

12. The process as claimed in claim 8, wherein the Raman reporter molecule is selected from the group consisting of cyanine dilipoic acid (Cy7DLA), hemicyaninecarbaldehyde(HCC), Pyryliniumhexylamine (PHA), Squaraine di-lipoic acid(SDL), Pyrenelipidene ethyl quartanised(Py L Et), crystal violet (CV) and Mercapto benzoic acid (MBA).

13. The process as claimed in claim 8, wherein the encapsulating agent is selected from the group consisting of a polysaccharide, polyethylene glycol, and serum albumin.
14. The process as claimed in claim 8, wherein the buffer is selected from the group consisting of MES buffer, phosphate buffer and Tris buffer.
15. The process as claimed in claim 8, wherein the antibody is raised against a biomarker selected from the group consisting of Estrogen receptor (ER), Progesterone receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2) and Ki67.
16. A diagnostic kit for detection of breast cancer biomarker comprising:
    I. the SERS-nanotagas claimed in claim 1;
    II. xylene;
    III. absolute ethanol;
    IV. citrate buffer;
    V. phosphate buffer saline;
    VI. bovine serum albumin; and
    VII. instructions manual.
17. A method for detecting breast cancer biomarker in a tissue sample comprising the steps of:
    (i) taking a paraffin embedded formalin fixed tissue sample;
    (ii) washing the sample with xylene;
    (iii) washing the sample of step (ii) with absolute ethanol followed by washing with 95% ethanol followed by washing with 70% ethanol and then with 50% ethanol to obtain a washed tissue sample;
    (iv) treating the washed tissue sample of step (iii) with citrate buffer to obtain a treated tissue sample;
    (v) incubating the treated tissue sample of step (iv) with bovine serum albumin and washing with phosphate buffer saline;
    (vi) incubating the tissue sample of step (v) with the SERS-nanotag as claimed in claim 1 for 30 minutes and washing;
    (vii) performing Raman spectroscopy on the tissue sample of step (vi) to take signature peaks; and

(viii) analyzing the peaks to confirm the presence of breast cancer biomarker.

18. The method as claimed in claim 17, wherein the breast cancer biomarker is selected from the group consisting of Estrogen receptor (ER), Progesterone receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2) and Ki67.

ABSTRACT

The present invention discloses a SERS-nanotag comprising gold nanoparticle, an encapsulating agent, a Raman reporter and an antibody. The present invention also discloses a diagnostic kit consisting of SERS-nanotags for identification of breast cancer biomarker selected from the group consisting of Estrogen Receptor (ER), Progesterone Receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki67, simultaneously in a breast cancer tissue sample using a surface enhanced Raman scattering signature peaks. The multiplexing Raman peak pattern provides the presence of multiple biomarkers at a time in heterogeneous paraffin embedded breast cancer tissue samples with a concentration level of the SERS-nanotags by applying single laser (532nm /633nm /785 nm) revealing simultaneous Raman peaks for the respective biomarkers.