Details of Research Work: Dr Kaustabh Kumar Maiti

Significant Contribution on Cancer Diagnostics and Nano-delivery System Based on Surface-enhanced Raman Spectroscopy (SERS)

In the area of cancer diagnostics, Dr. Maiti developed functionalized nano-particle probes for ultrasensitive detection and grading of various human cancer biomarkers viz., breast, cervical, lung, and prostate using advanced Raman scattering (surface-enhanced Raman scattering: SERS) and Imaging as a diagnostic modality.

Two major patented inventions for breast and cervical cancer have been emphasized as follows:

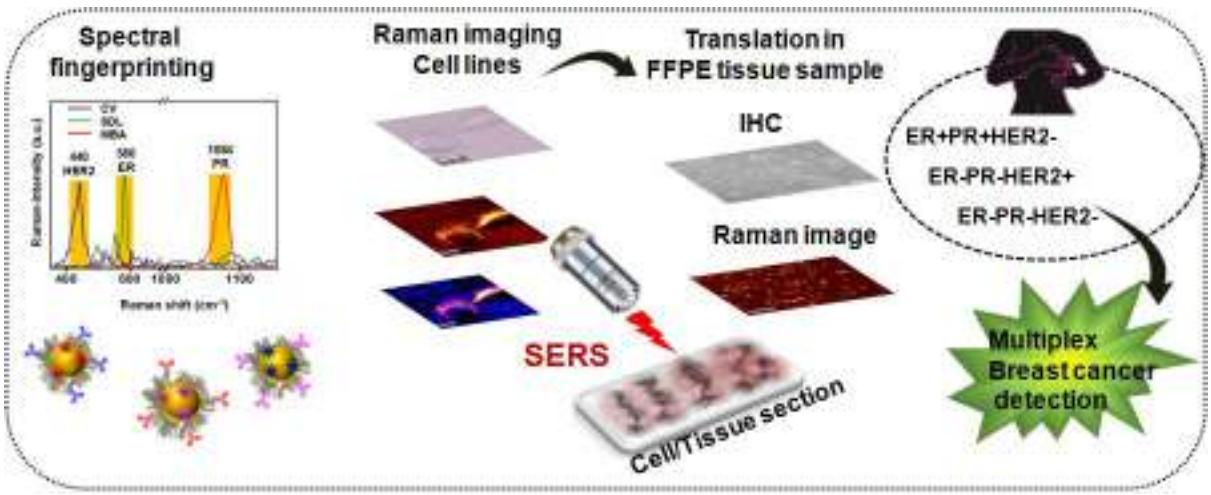
A. Clinically Feasible Diagnostic Spectro-Histology Built on SERS-Nanotags for Multiplex Detection and Grading of Breast Cancer Biomarkers

In the area of breast cancer, Dr. Maiti developed a diagnostic SERS kit for concomitant detection of multiple breast cancer biomarkers from single breast tissue samples using antibody-conjugated SERS-nanotags. The kit can be used for real-time detection of the biomarkers, as and when the sample tissue is extracted from the source. Hence, this kit has immense potential to develop immediate treatment strategies in heterogeneous breast cancer cases.

Background of the diagnostic kit (SERS-Nanotags) for Multiplex detection of Breast Cancer Biomarkers

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. Multiplexed detection is an attractive strategy in cancer diagnosis where multiple biomarkers can be evaluated simultaneously at a particular time. Recognition of pertinent biomarkers in heterogenous breast cancer facilitates clinicians in improving treatment strategies. Although current gold standard immunohistochemistry is capable of detecting single biomarker at a time, its subjective nature, inability of multiplexing and time-consuming nature makes it a hurdle leading to delayed results. In the current strategy, an alternative multiplex-detection technique has been developed for conventional IHC and FISH analysis by sequential addition of diagnostic SERS-nanoprobes with unique Raman reporters having multiplexing capability for the ultrasensitive and rapid detection of differential biomarkers, ER, PR and HER2 in breast

cancer. Initially the SERS-tags were validated in differential biomarker expressed cell line models and later the study was extended for diagnosis in clinically confirmed retrospective formalin fixed paraffin embedded (FFPE) breast cancer tissue samples in single-plex, duplex as well triplex manner. Multiplexing SERS was compared critically in terms of time required for the analysis and found to be an excellent technique which minimizes the processing and experiment time to around 5-6 hr unlike IHC for covering a 5 x 5 mm tissue area. Additionally, HER2 biomarker grading which is executed conventionally using time consuming IHC and expensive FISH analysis was also proved by SERS spectral analysis showing the potential of SERS to be applied in clinics. **Simultaneous detection of these biomarkers enabled to achieve a sensitivity of 95% and a specificity of 92% for single-plex analysis, 88% and 85% for duplex analysis, 75% and 67% for triplex analysis r**espectively. Combined diagnosis of these biomarkers enabled SERS-tags based detection to be turned out as an accurate, inexpensive, reliable and facile technique which can simultaneously identify and the biomarkers variations in different breast cancer subtypes in a semi-quantitative manner.



Technical Details and Results

Fabrication of SERS-Nanotags (Diagnostic SERS kit)

Gold nanoparticles (AuNPs) were chosen as the SERS substrate with around 40-45 nm size for the design of multiplexed SERS-nanotags for the differential recognition of clinically relevant biomarkers viz. ER, PR and HER2 in breast cancer as this size reported to have the best SERS activity so far. SERS nanotags were fabricated by tagging three representative Raman reporter dyes having precise non-overlapping signature fingerprint peaks representative of each biomarker (ER, PR and Her2). The Raman reporters selected for the multiplexing analysis were commercially procured crystal violet (CV), 4-mercapto benzoic acid (MBA) and in-house

synthesized squaraine based Raman reporter, Squaraine Di-lipoic acid (SDL) having distinctive non-overlapping multiplexing Raman peaks at 440, 1084 and 580 cm^{-1} respectively. For facilitating stability and biocompatibility of the SERS nanoprobe, PEG coating was performed in the nanoparticle incorporated Raman reporter which also reduces toxicity of the Raman reporter as well as renders functional groups for antibody conjugation as well. Stickiness nature of nanoparticles to the vial was a limiting factor during the preparation of SERS tags leading to a reduced signal intensity due to particle loss which was efficiently tackled by the use of tween20. Finally, the corresponding antibodies for ER, PR and HER2 biomarkers were conjugated to the pegylated nanotags by modified version of the standard protocol for antibody conjugation, wherein we used Sulfo-NHS in MES buffer instead of NHS for better reactivity. The UV-Vis absorbance exhibited a 260 nm protein absorption peak with a small 1-2 nm shift from the 530 nm plasmon peak confirming successful antibody conjugation. SDS-PAGE gel analysis by silver staining also confirmed efficient antibody conjugation where the conjugated nanotag displayed a slight upshift as shown along with pure antibody (Figure 1a).

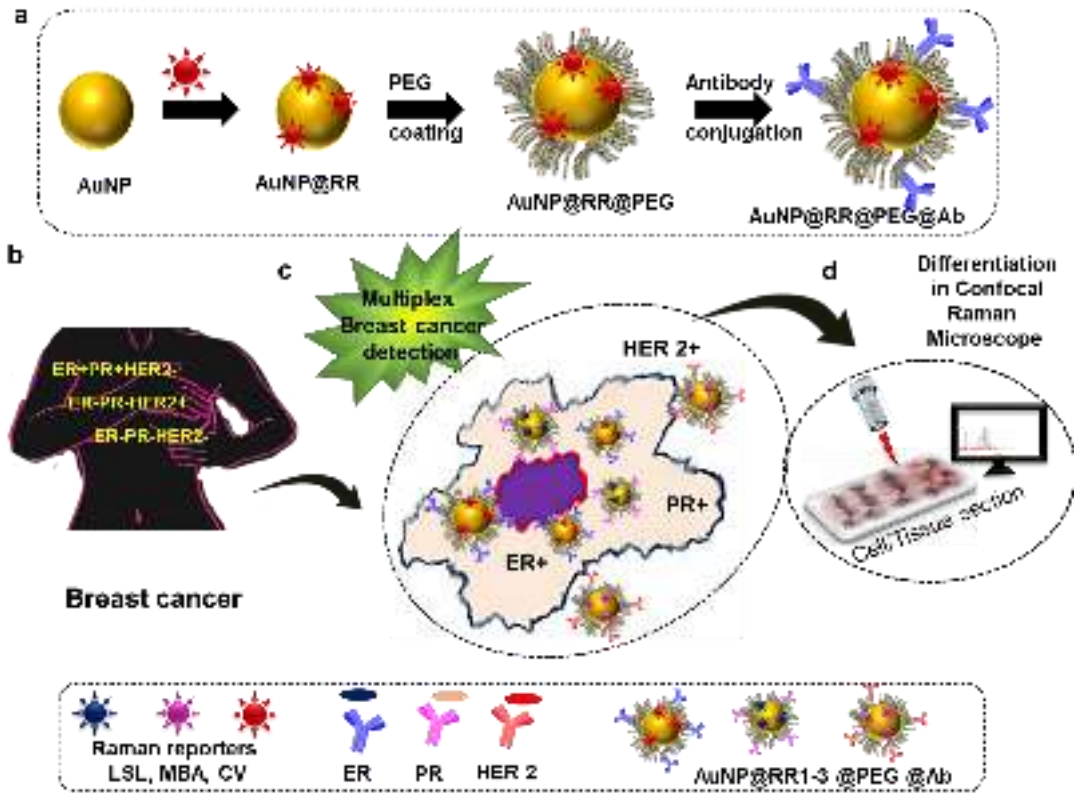


Figure 1: Illustration for experimental design for differentiating the clinically relevant triple biomarkers, ER, PR and HER2. a) Preparation strategy for the multiplexed SERS-tags by using AuNPs based substrate, b) biomarker detection of ER, PR and HER2 biomarker in three cell lines (MCF7, MDA-MB-231and SK-BR-3) having differential expression using confocal Raman microscope c) representative design for detection of biomarkers in cells/tissues by SERS-tags, d) SERS analysis and mapping in retrospective paraffin embedded differential biomarker expressed breast tissue samples after antigen retrieval using confocal microRaman system.

Multiplex Breast Cancer biomarker detection using SERS-nanotags

The SERS-based detection in paraffin removed tissues was compared with the gold standard conventional immunohistochemistry (IHC) analysis. Three sets of tissue biomarker analysis were carried out viz., single plex analysis for either ER, PR or HER2, duplex analysis with a combination of any two out of these and triplex analysis with all three biomarkers. During SERS analysis of tissue samples, sequential addition of nanotags as well as SERS nanotag cocktails experimented out of which sequential addition showed impressive efficacy in terms of SERS signals intensity and accuracy. Similarly, MES buffer wash to remove excess unbound SERS tags was also found to be beneficial over PBST wash in terms of signal strength. IgG isotype antibody conjugated to SERS nanoparticles with DTNB as the Raman reporter was used as a control to achieve a ratiometric calculation of the prevalence of biomarker expression in the samples and also to eliminate the false positive results arising due to the nonspecific binding of the nanoparticles to the tissue samples.

Duplex analysis for tissue biomarkers

Even though a few kits-based methods are there, dual biomarker detection is a challenging thing to attain in IHC, especially for breast cancer biomarkers. Using the current SERS-based platform, various combinations of duplex biomarker analysis were performed in breast tissue samples having differential biomarker expression status. In an IHC confirmed ER^+ $HER2^-$ breast cancer tissue samples, sequential addition of AuNP@SDL@PEG@anti-ER followed by AuNP@CV@PEG@anti-HER2, led to the generation of only 580 cm^{-1} peaks from SDL specifying the overexpression of ER biomarker. The lack of noteworthy peak from 440 cm^{-1} of CV confirmed the minimal expression of HER2 in the tissue sample (Figure 2).

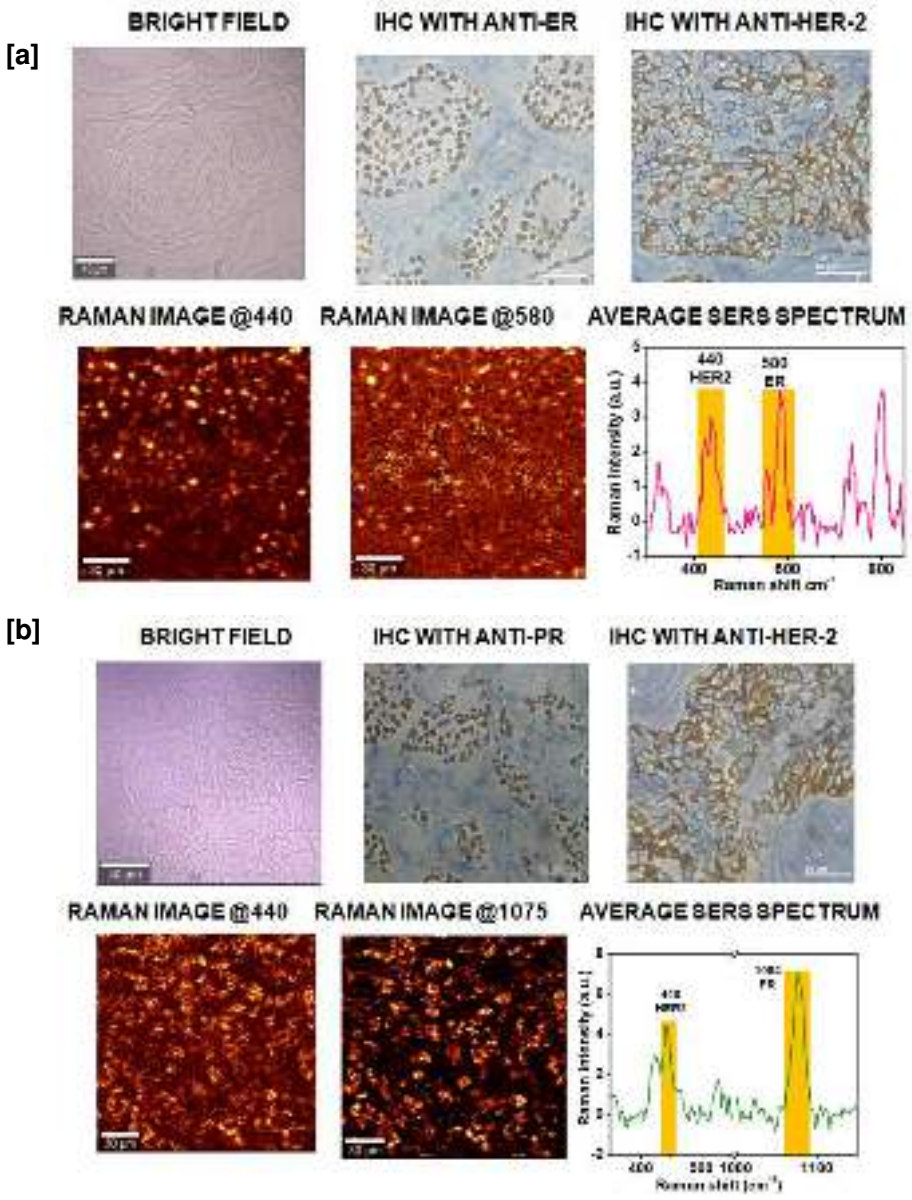


Figure 2: *Bright field images, Immunohistochemistry analysis and Raman imaging of a) ER^+HER2^+tissue using AuNP@SDL@PEG@anti-ER and AuNP@CV@PEG@anti-HER2 nanotags, b) PR^+HER2^+tissue using AuNP@MBA@PEG@anti-PR and AuNP@CV@PEG@anti-HER2 nanotags.*

Triplex analysis for tissue biomarkers

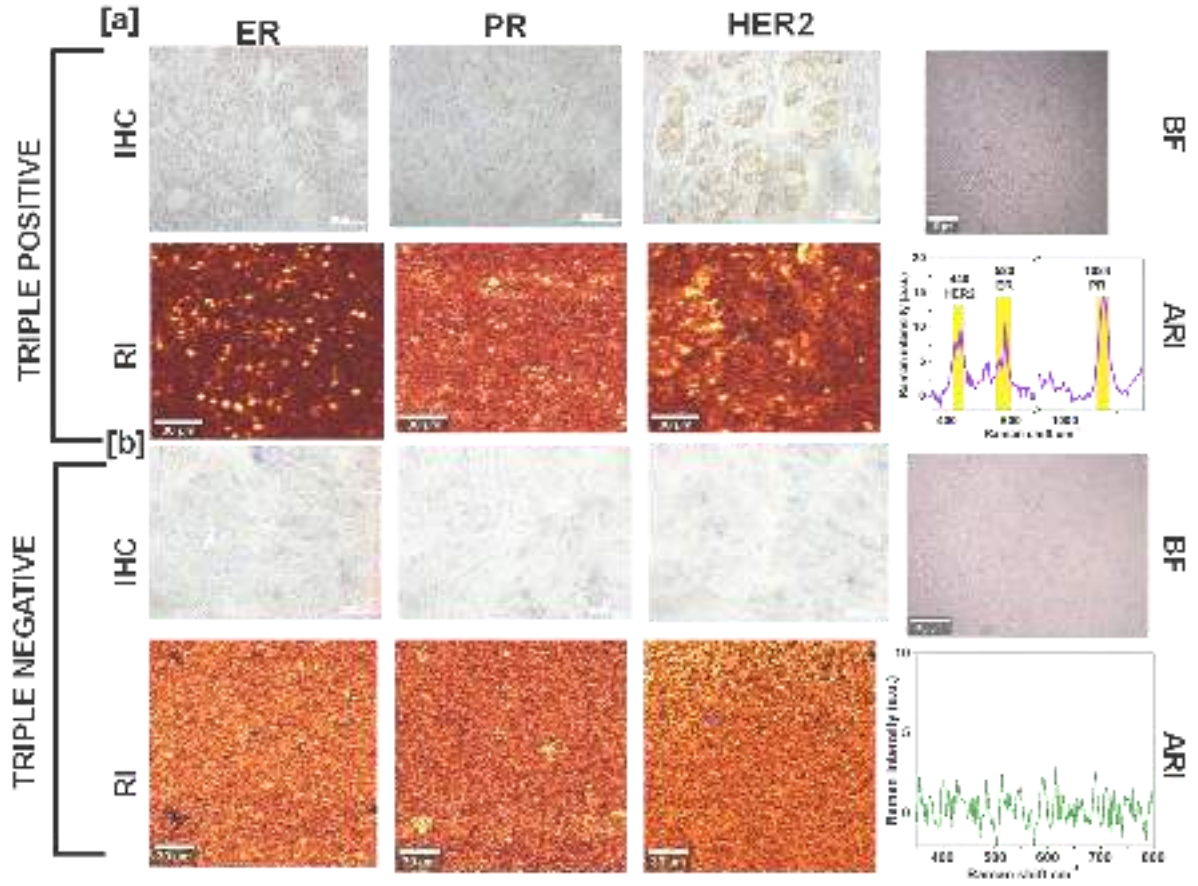
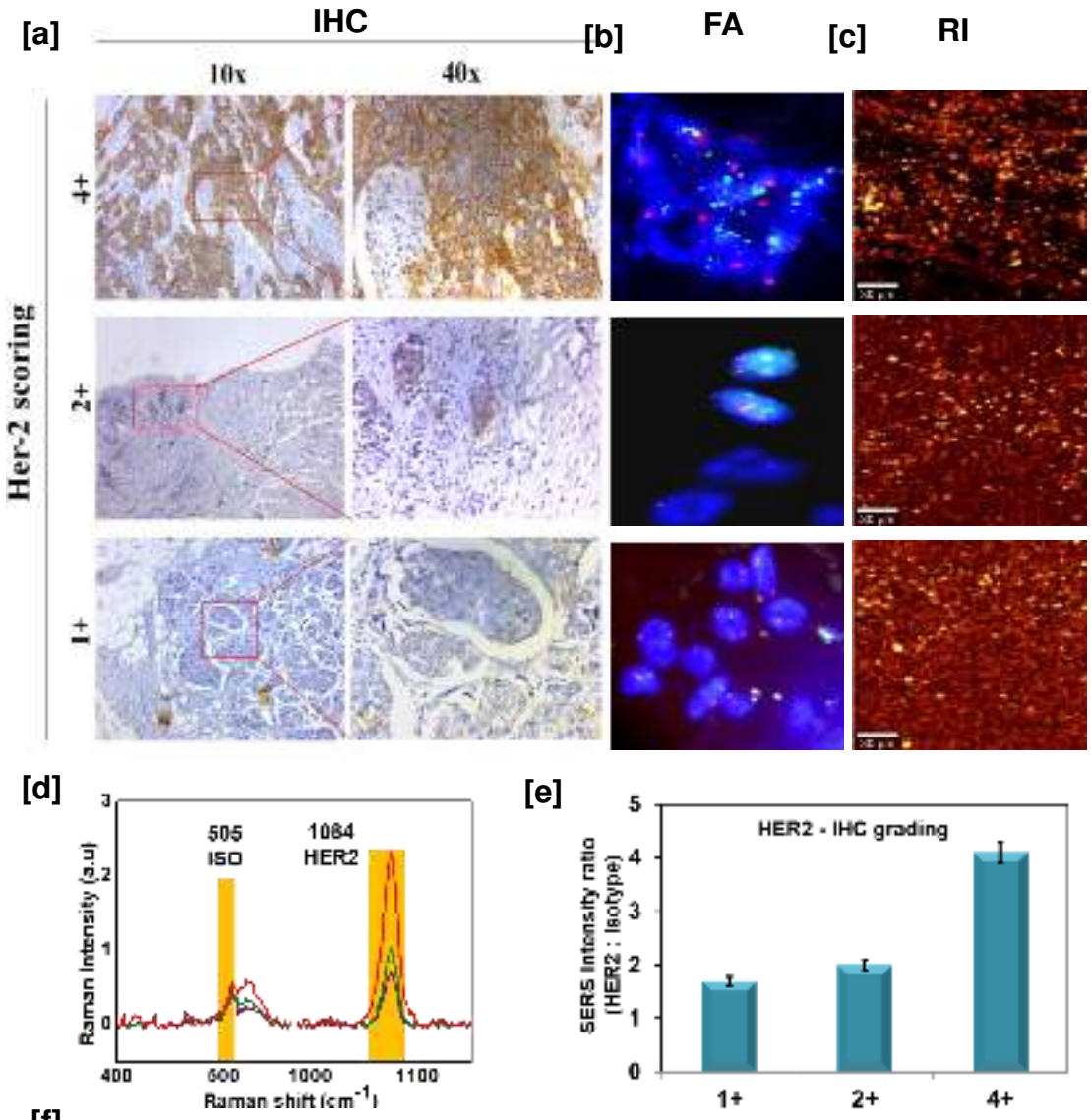


Figure 3: SERS analysis of a) $ER^{+}PR^{+}HER2^{+}$ and b) $ER^{-}PR^{-}HER2^{-}$ tissue using AuNP@SDL@PEG@anti-ER, AuNP@MBA@PEG@anti-PR and AuNP@CV@PEG@anti-HER2 nanotags. (BF-Bright field, RI-Raman imaging, ARI-Average Raman intensity, IHC-Immunohistochemistry)

HER-2 grading by SERS analysis.

Unlike ER and PR, HER2 overexpression is considered for effective targeted therapy against the receptors like Herceptin treatment. IHC grading of 3+ and more are judged to be HER2 positive, whereas 2+ / equivocal expression has to be confirmed by FISH analysis in which the number of HER2 gene copies per nucleus is assessed. This method is again time-consuming and highly expensive. Here we put forward a HER-2 grading system based on the SERS intensity profile that can complement the IHC grading technique. For this, IHC grades of 1+, 2+, and 4+ HER2 tissue samples were incubated with AuNP@MBA@HER2 and AuNP@DTTC@Isotype antibody succeeded by SERS analysis as described earlier. DTTC was used as the Raman reporter for the isotype antibody with a 785 nm laser system and the same experiment was also executed in a 633 nm laser with DTNB as the Raman reporter for the isotype antibody. The ratio of HER2 tag to isotype tag obtained from the average scan intensities was plotted to get a mathematical interpretation of the same. Average SERS intensity from image scanning was of course higher for HER2 4+ tissue with an intensity ratio

of 4.1 followed by HER2 2+ (Ratio 2) and 1+ (Ratio 1.79) in harmony with the IHC staining pattern, which was again confirmed by FISH analysis.



[f]

HER2 grade	A.R.I HER2	A.R.I ISO	HER2:ISO
1+	0.70	0.39	1.79
2+	1.01	0.52	2
4+	2.3	0.56	4.1

Figure 4: *SERS analysis showing HER2 grading in HER2+ tissue using HER2 targeted AuNP@CV@PEG@anti-HER2and Isotype targeted AuNP@DTTC@PEG@anti-isotype nanotags. a) IHC analysis, b) FISH analysis, c) Raman Imaging (RI), d) Average Raman Intensity (ARI), e)*

representation of HER2 grading by bar diagram and f) table showing ratiometric signal values of HER2 versus isotype tags.

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 1: Comparison of the features and main steps of conventional method IHC and SERS

Sl . No	Parameters	IHC	SERS
1	Multiplexing	Very difficult and absence of a standard method.	Easily Possible
2	Time required for sample preparation	7-27 hrs	4-6 hrs
3	Secondary antibody and developing agents	Required	Not required
4	Time required for analysis	0.5 hr/sample /one marker	1 hr/sample
5	Type of analysis	Highly Subjective(Inter observer variation)	Objective semi-quantitative
6	Grading	Based on percentage of stained cells and stain intensity	Based on spectral intensity based mapping
7	HER2 grading	2+/Equivocal samples required FISH confirmation	By ratiometric semi quantitative approach FISH confirmation may be mitigated

Major Advantages

A rapid multiplexed detection of clinically relevant biomarkers in breast tissue is an essential criterion for enabling efficient treatment strategies in heterogeneous breast cancer.

The study emphasized a new diagnostic modality with huge potential for the detection of tumors as well as tumor recurrence exhibiting differential biomarkers associated with patient-to-patient heterogeneity.

Key Advantages

1. In the present invention, SERS analysis i.e., spectral fingerprint, and imaging of the SERS-nanotags were performed to acquire the information with high accuracy for the abundance of multiple biomarkers in a single breast tissue sample.

2. It is the first spectroscopic demonstration where SERS intensity enabled semi-quantitative evaluation of HER-2 gradation 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.

3. 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-nanotags of the present invention is turned out as SERS-immunoassays in clinical cytology.

4. Simultaneous detection modality is achieved by initial validation in paraffin embedded breast cancer tissue samples. By evaluation of SERS signature peaks from the respective nanotags, 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.

5. The nanotag of the present invention is highly accurate and there is a very low possibility of false positive and false negative results.

Patent:

SERS-Nanotag and a Diagnostic kit for the Detection of Breast Cancer Biomarkers; Maiti, Kaustabh Kumar, K. Sujathan, Vishnu Priya Murali, Varsha K, Deepika S, Madhukrishnan M ; **Indian Patent Application ref. No. 202011034768, dated 11.08.2020; PCT Application No. PCT/IN2021/050577, dated 14.06.2021; US Appl. No. 18041283; dated 28.09.2023**

Publication

A clinically feasible diagnostic spectro-histology built on SERS-nanotags for multiplex detection and grading of breast cancer biomarkers; Vishnu Priya Murali, Varsha Karunakaran,

Madhukrishnan Murali, Asha Lekshmi, Shamna Kottarathi, Selvakumar Deepik, Valliamma N. Sarith, Adukkadan N. Ramya, Kozhiparambil G. Raghu, Kunjuraman Sujathan*, Kaustabh Kumar Maiti*;
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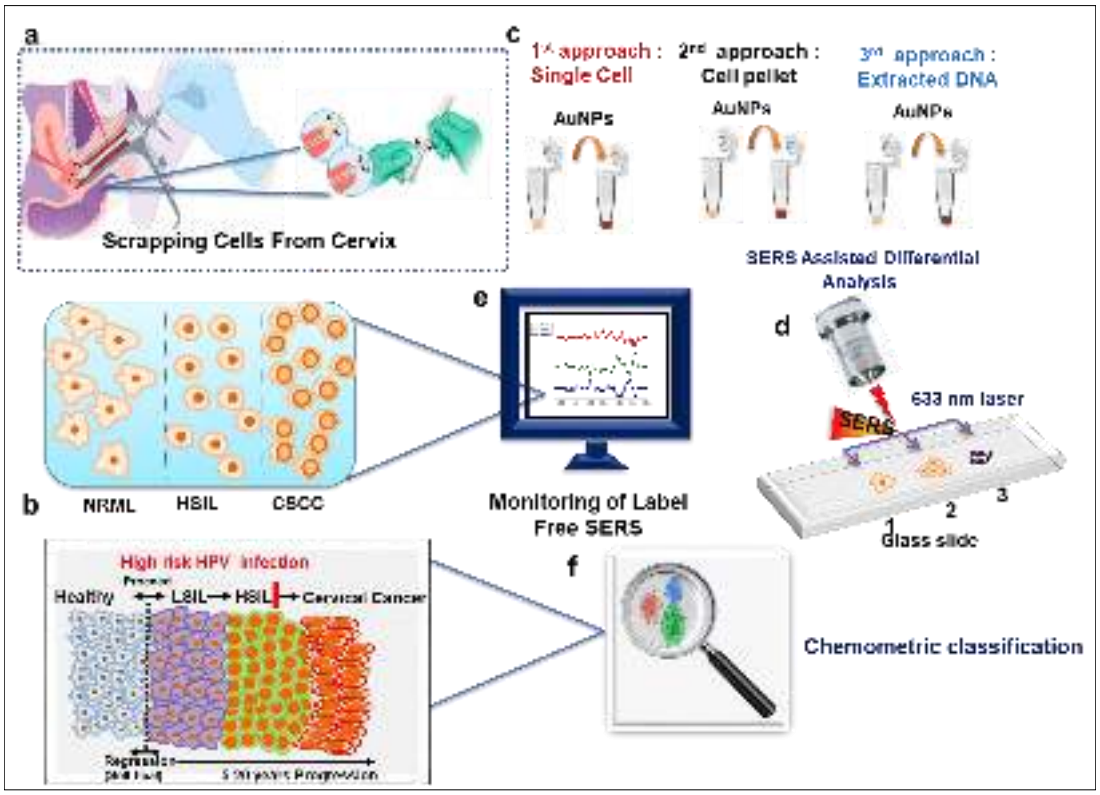
B. Diagnostic Spectro-Cytology for Differential Recognition of Cervical Cancer Lesions by Label-free Surface Enhanced Raman Fingerprints and Artificial Intelligence (AI)

Background of the diagnostic Spectro-cytology for Cervical Cancer

Cancer of the uterine cervix is one of the most common cancers among women worldwide and the second most prevalent cancer among women in India. It is considered a preventable cancer as the cervix is an easily amenable organ and the occurrence of a heterogeneous spectrum of epithelial abnormalities (precancerous lesions) 10-15 years prior to the occurrence of invasive cancer has been well established. The significance of Pap smear tests 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. 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 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 an extraordinary toll on 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.

Herein, an efficient diagnostic platform has been developed to identify precancerous lesions (high-grade squamous intraepithelial lesion (HSIL) and cervical squamous cell carcinoma (CSCC) in the cells exfoliated from the uterine cervix using a differential Raman spectral pattern based on label-free surface enhanced Raman scattering (SERS) technique. A differential spectral fingerprint for the prediction of normal NRML, HSIL and CSCC has been generated by comparing three different approaches i.e., single-cell, cell-pellet and extracted DNA. The tunable plasmonic properties of the gold nanoparticles as the SERS substrate favored the increment of Raman intensity in minimal time in an ultrasensitive manner. The

ratio of SERS signal intensities between 1270 /1370 cm^{-1} in single cell and 956 /1022 cm^{-1} in cell pellet exhibiting the signature identity for Amide III/Nucleobases and carotenoid/glycogen respectively seemed proficient for establishing the empirical discrimination. All the spectral invention was subjected to chemometrics (Machine learning algorithm) including Support Vector Machine (SVM) which furnished an average diagnostic accuracy of 93.84 %, 74.26 % and 92.21 % for single cell, cell pellet and extracted DNA respectively.



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.

Technical Details & Results

Label-free SERS Spectral Analysis

Pathologically confirmed cervical smears of major three grades i.e., NRML, HSIL and CSCC were collected and processed using liquid-based cytology procedure. A smear button was prepared, which concentrated diagnostically relevant cells upon incubation with AuNPs (as Raman signal enhancer) by removing the mucus and blood by repeated density gradient centrifugation. The fingerprint spectral information was extracted mostly in the nuclear region

of the single cells as the variation arising from the cytoplasmic region can be minimized. 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. 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 of each group were normalized to its highest peak (Amide II at 1550 cm^{-1}) in which clear spectral variations were observed. The analysis in exfoliated single cell samples, NRML, HSIL and CSCC showed distinctive peaks correlating to the cell line signals. Distinct Raman peak at 481cm^{-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 of 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. The O-P-O stretching at 826 cm^{-1} showed the increased nuclear content in abnormal samples. Interestingly the peak at 826 cm^{-1} in HSIL and CSCC samples is identified as a shifted position in comparison to normal samples which showed a prominent peak at 850 cm^{-1}. Another distinguishing factor between the three groups were the presence of -C-O-C- stretching of the amino acid proline. It has been proven that proline rich tyrosine kinase 2 (Pyk2) plays an important role in tumor progression in various human cancers. Pyk2 is a non-receptor tyrosine kinase which controls tumor survival, its proliferation, migration, invasion properties, metastasis and resistance to chemotherapy. 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. 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 tend to accumulate carotenoids to resist damage. 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. 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.

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. An increase in DNA content was

evident between the groups when compared with normal counterparts. The 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. Difference spectra were assessed by subtracting NRML from CSCC, NRML from HSIL and HSIL from CSCC mean spectra from the DNA samples (Figure 1).

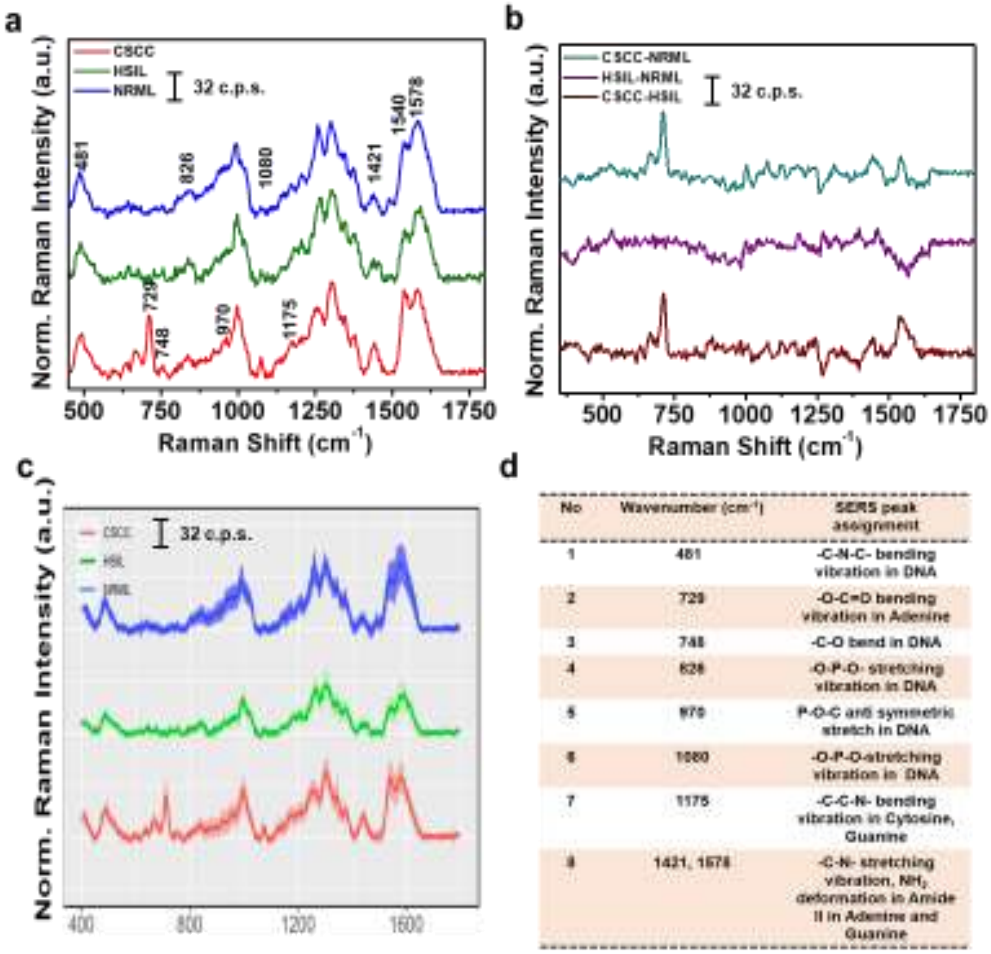


Figure:1 *a) Mean SERS spectra [Laser power density 3-7 mW power density] b) the difference spectrum c) the standard deviation and d) tentative SERS peak assignments from cervical clinical DNA samples; NRML, HSIL and CSCC.*

Chemometric evaluation of differential SERS spectra of NRML, HSIL and CSCC by PCA, LDA and SVM

A huge spectral data set in all the three groups NRML, HSIL and CSCC were discriminated by chemometric modelling and prediction within the dataset employed for extracting information comprising complex data set from a chemical or biological source based on multivariate,

mathematical or computational models. Initially, adopted chemometric analysis named PCA & LDA to classify them using MATLAB software. A predictive classification was obtained using PCA analysis in single cell, cell pellet and extracted DNA respectively. 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 93.84 % for single cell, 74.26 % for cell pellet and 92.21 % for extracted DNA** with 0.73 %, 5.04 % and 3.84 % standard deviation respectively (Figure 2). 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 2)**. 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. The ROC curve of single cell, cell pellet and extracted DNA showed that SVM is an incremental diagnostic model for classifying the groups **(Figure 2g, h, i).** The sensitivity and prediction accuracy of the technique was calculated (**Table 1**). Out of the performed methods, SVM showed a prominent classification between the grades.

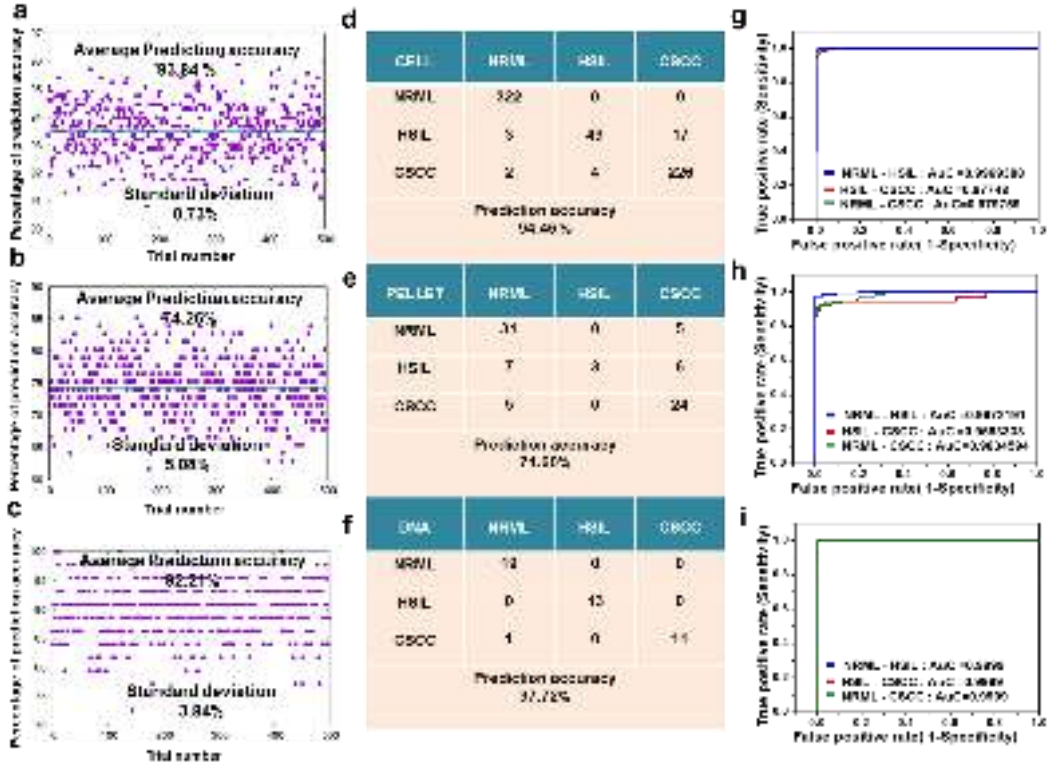


Figure 2. *Percentage of average prediction accuracy chart in a) single cell, b) cell pellet and c) extracted DNA, Classification based on SVM analysis of d) single cell, e) cell pellet and f) extracted DNA, SVM ROC curve in g) cell, h) pellet, i) DNA*

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**	

Investigation of cytopathological evaluation of cervical exfoliated cells by Papanicolaou (Pap) staining:

Conventional analysis like PAP smear were performed to correctly identify the desired cells for SERS-based analysis and subsequent validation of the technique. Normally, cervical cancer is diagnosed using cytopathology analysis commonly known as Pap smear test where single cells are tested. As our sample source of interest was exfoliated cells, Pap staining was performed to identify the pathologically relevant cells. In order to get a clear discrimination between normal vs abnormal exfoliated cells, both bright field images from confocal Raman microscope and Pap staining of NRML, HSIL and CSCC were evaluated for morphological analysis. 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 to green colour where as in HSIL, the enlarged nucleus reflecting high DNA content with minimal cytoplasm showed purple colour 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. If only normal cells are present, the Pap tests predict as normal but in case of abnormal or atypical cells, it turned out as atypical squamous cells of undetermined significance (ASCUS), precancerous squamous intraepithelial lesion, atypical glandular cells, squamous cell cancer or adenocarcinoma cells etc. The primarily focus of the work was to establish a successful discrimination modality of NRML, HSIL and CSCC cells by SERS analysis after pathologically confirmed by Pap test.
The PCR product obtained was indicative of oncogenic HPV types 16, 18, 31, 33, 35, 45, 52 and 58.

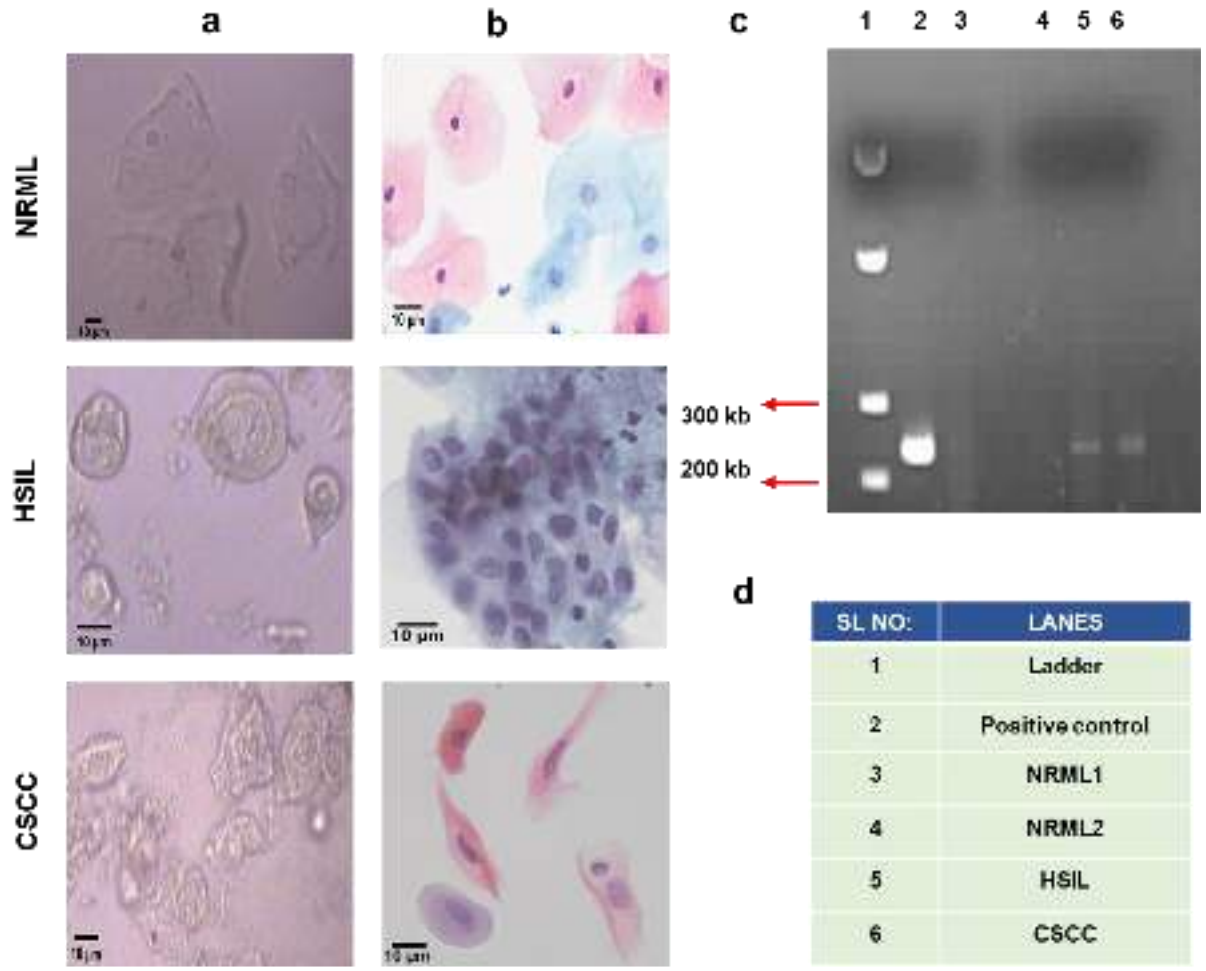


Figure 3. *a) Bright field and b) Pap stained images of NRML, HSIL and CSCC exfoliated cells, Scale bar corresponds to 10µm, c) HPV PCR of Clinical DNA samples, d) Sample loading order of HPV PCR Ladder (1), Positive control (2), NRML1 (3), NRML2 (4), HSIL(5), CSCC(6).*

Major Advantages:

- A label-free ultrasensitive Raman spectral fingerprint has been identified to generate a differential screening of major three grades of cervical exfoliated cells i.e., normal (NRML), intermediate (HSIL) and squamous cell carcinoma (CSCC) of the cervix by employing a strategic platform based on surface-enhanced Raman scattering (SERS).

- A clear identification of SERS fingerprint of the well-resolved biomolecular variations ranging from nucleic acid, amino acids, and protein backbone were established between NRML, HSIL and CSCC of clinical samples. Therefore, SERS fingerprints indicated the prevalence of nucleobases adenine, guanine,

cytosine, phosphate backbone and amino acids tryptophan, phenylalanine and proline which are the major metabolites evolved during tumorigenesis.

- The 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.

- The SERS-based spectro-cytology was found to be minimally invasive and more sensitive than those employing serum or plasma as exfoliated cells represent a better source of samples for analyzing the progression of cervical cancer.

Patent:

- Screening kit for detection of grades of cervical cancer and process for the preparation thereof; Maiti, Kaustabh Kumar, Varsha Karunakaran, K.Sujathan; ***PCT Int. Appl. (2020), WO 2020021568 A1 20200130. Language: English, Database: CAPLUS, Date: 30th January, 2020***

- Screening kit for detection of grades of cervical cancer and process for the preparation thereof; Maiti, Kaustabh Kumar, Varsha Karunakaran, K.Sujathan; **US Patent No. 11313860; Appl. No. 17046183; Date: 26/04/2022**

Publication:

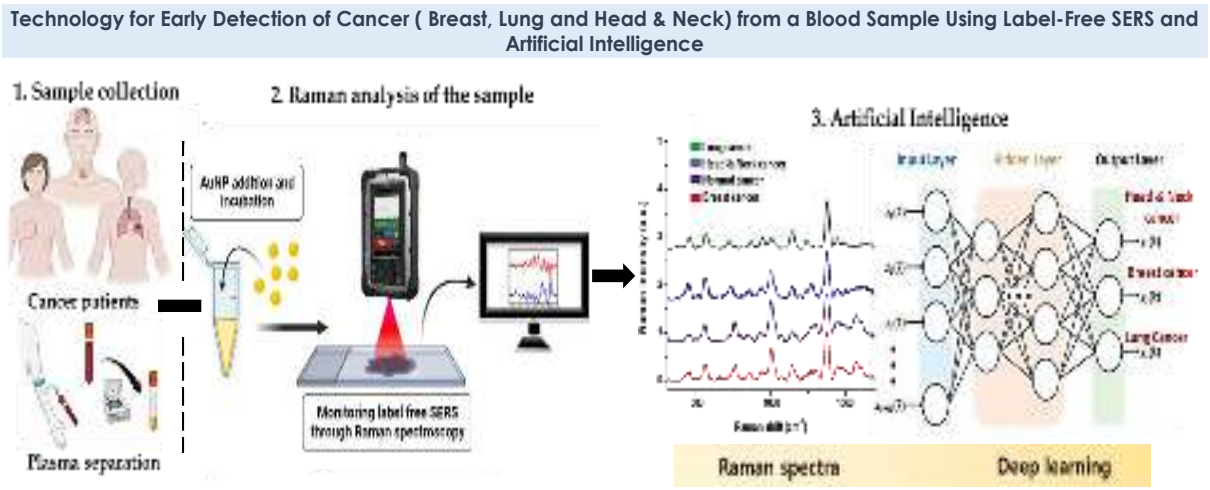
Diagnostic Spectro-cytology revealing differential recognition of cervical Cancer lesions by label-free surface enhanced Raman fingerprints and Chemomssetrics; Varsha Karunakaran, Valliamma N. Saritha, Manu M.Joseph, Jyothi B. Nair, Giridharan Saranya, Kozhiparambil G. Raghu, Kunjuraman Sujathan*, Krishnan Nair S. Kumar*, Kaustabh K. Maiti*

Nanomedicine: Nanotechnology, Biology and Medicine, 2020, 29, 102276 (IF: 6.45)

C. **Technology for Early Detection of Cancer (Breast, Lung, and Head & Neck) from a Blood sample using label-free SERS and Artificial Intelligence**

An innovative technology has been developed to identify the prevalence of cancer (breast, lung, larynx) vs healthy individuals from blood samples. Plasma separated from the subject's blood is used for the acquisition of the Raman spectra. An enhanced Raman fingerprint has been achieved using a newly developed nano-Ag-FA-impregnated solid paper substrate as the surface-enhanced Raman scattering (SERS) substrate for

distinguishing the metabolic Raman spectral pattern from plasma samples of breast, lung, and larynx cancer from the control. The non-invasive and label-free SERS spectra were subjected to an Embedded Artificial Intelligence (EAI) algorithm for classification and prevalence between cancer types vs control samples. The integration of an EAI model-based system enables automated analysis and generation of prevalence scores based on Raman spectral patterns that resembled an overall accuracy of 85-90% streamlining the efficiency of the developed technology.



Schematic illustration for experimental design for (1) blood sample collection and plasma separation; (2) SERS spectral acquisition of plasma samples; (3) Artificial Intelligence algorithm for classification of cancer sub-types

Advantages:

Minute differences in the SERS spectral pattern resembling the metabolic Raman fingerprints of control and three different types of cancer were classified by a label-free SERS-EAI-based technique. As, the range of techniques that are currently used for the diagnosis and subsequent treatment of cancer include fluorescence, optical bioluminescence, optical imaging, MRI (magnetic resonance imaging), X-ray imaging, and ultrasound have low sensitivity and resolution, and X-ray employs potentially harmful radiation which can be very risky for patients. Moreover, when visible on X-ray or MRI, the cancer is already at an advanced stage. SERS is an accurate, simple, reliable, and non-destructive technique that can differentiate healthy, and cancer

samples through its differential spectra and successfully predict through EAI interpretation.

Major advantages are:

- Label-free approach
- Non-Invasive / Less-invasive
- Minimal cost
- No prior preparation is needed
- Portable and easy to handle
- Can be performed as often as necessary

Patent:

A Nano-Silver-fatty acid impregnated hydrophobic paper-based substrate for enhancing Raman scattering; Maiti, Kaustabh Kumar, Radhakrishnan Kokkuvayil Vasu, Selvakumar Deepika, Mohanan Biji, Murali Vishnu Priya, Murali Madhukrishnan, Vijayakumar Selva Rani Renjith, & Littleflowern Arun,

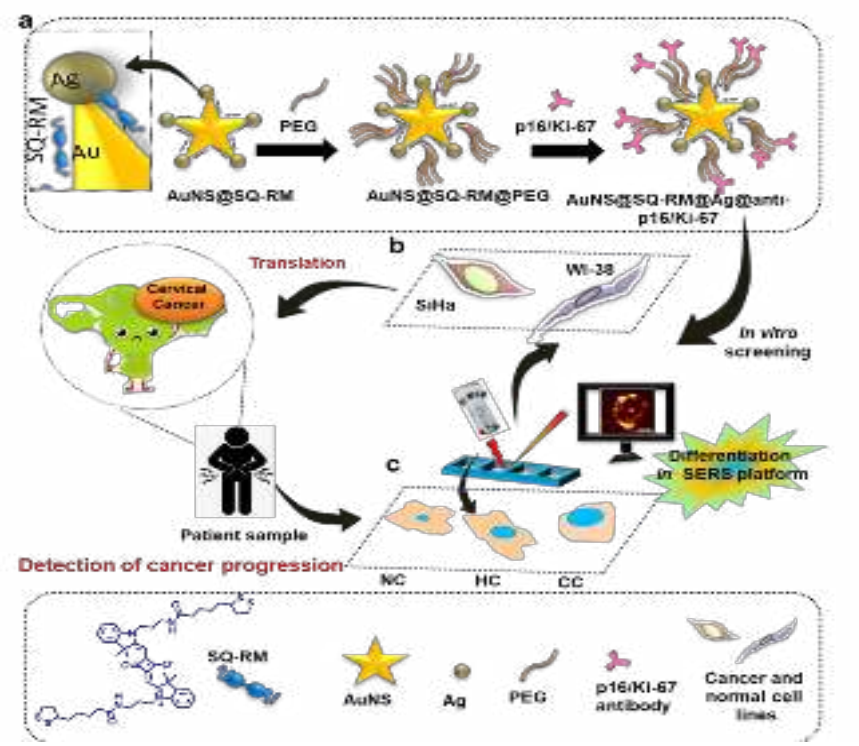
Indian Patent, File no. 0102NF2004, dated 14.08.2024

D. **Highly relevant other Explored SERS-nanoprobe-based Cancer Diagnostics :**

1. **Raman Image-Guided Differential Recognition of Clinically Confirmed Grades of Cervical Exfoliated Cells by Dual Biomarker-Appended SERS-Tag**

Ultrasensitive detection of cancer biomarkers via single-cell analysis through Raman imaging is an impending approach that modulates the possibility of early diagnosis. Cervical cancer is one such type that can be monitored for a sufficiently long period towards invasive cancer phenotype. In this study, surface-enhanced Raman scattering (SERS) nanotag (SERS-tag) has been successfully implemented for the simultaneous detection of p16/K-i67, a dual biomarker persisting in the progression of squamous cell carcinoma of the human cervix. The recognition by the SERS-tag was implemented to different grades of clinically confirmed exfoliated cells including normal cell (NC), high-grade intraepithelial lesion (HC) and squamous cell carcinoma (CC) samples of the cervix. It has been observed a distinct intensity hike of around ten-fold in the single dysplastic HC and CC samples in comparison to NC specimen which clearly justify the prevalence of p16/Ki-67. Amidst the challenges in Raman image guided modality, the technique was further complemented with the gold standard immunocytochemistry dual staining analysis. The synthesized probe is able to map the abnormal cells within 20 min with high reproducibility and stability after the antigen retrieval step for 1mm x 1mm mapping area with good contrast. The tedious time-consuming steps can be avoided and real-time read-out can be achieved using the SERS mapping, unlike immunocytochemistry technique. Therefore, the newly developed Raman image-guided SERS

imaging emphasizes the approach of uplifting SERS in practical utility with further improvement for clinical applications for cervical cancer detection in the future.



Schematic illustration for experimental design for differentiating three grades viz. normal cell (NC), high grade intraepithelial lesion (HC), cervical squamous cell carcinoma (CC) using SERS nanotags, a) synthetic scheme of AuNS@SQ-RM@Ag@PEG@anti-p16/Ki-67, b) SERS mapping in cell lines, SiHa and WI-38, c) SERS mapping in clinical samples, Normal cell (NC), b) High grade squamous intra epithelial lesion cell (HC) and c) cervical squamous cell carcinoma (CC) samples.

Publication

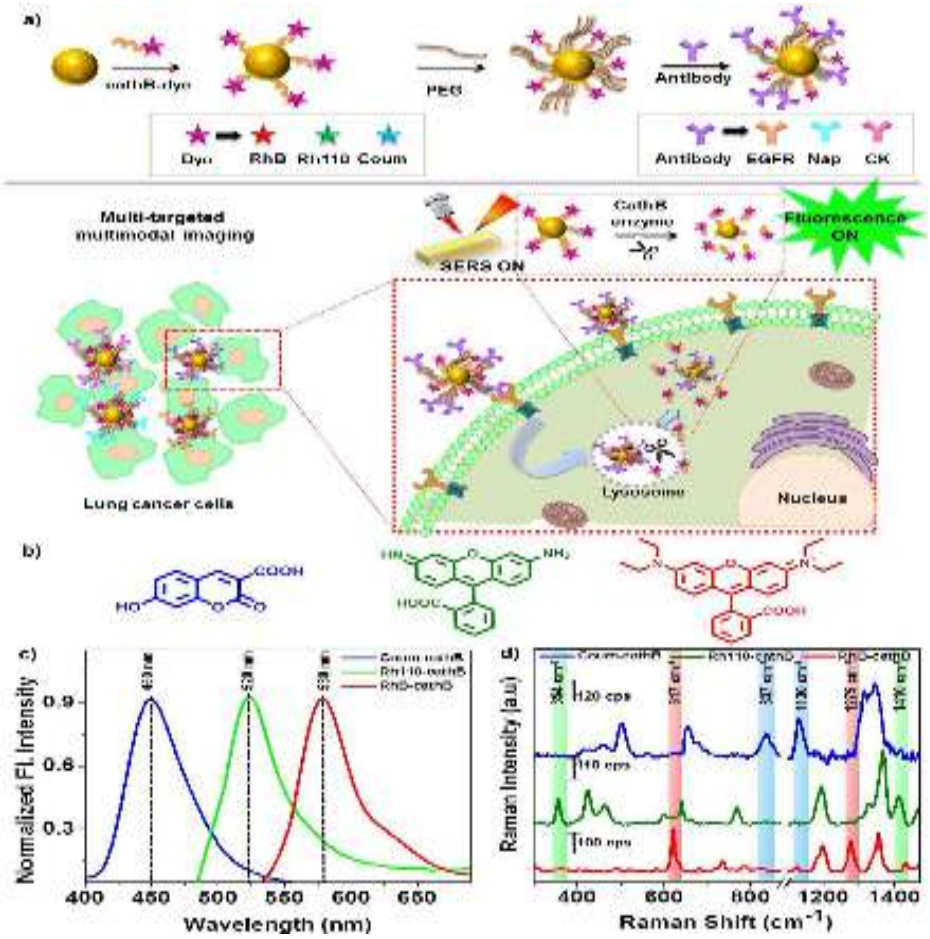
Elucidating Raman Image-Guided Differential Recognition of Clinically Confirmed Grades of Cervical Exfoliated Cells by Dual Biomarker-Appended SERS-Tag; Varsha Karunakaran, Valliamma N. Saritha, Adukkadan N. Ramya, Vishnu Priya Murali, Kozhiparambil G. Raghu, Kunjuraman Sujathan,* and Kaustabh Kumar Maiti*,

Analytical Chemistry, 2021, 93, 32, 11140–11150, (Impact Factor: 8.00)

2. Enzyme-Driven Switchable Fluorescence-SERS Diagnostic Nanococktail for the Multiplex Detection of Lung Cancer Biomarkers

A modular fabrication of smart programmable nanoarchitectures has been explored which could integrate clinically relevant multiplexed detection of the most prevalent panel of

biomarkers present in lung cancer. The multiplex nanoprobes were prepared by attaching dual-functional Raman active fluorogens onto spherical gold nanoparticles through a peptide linker, Phe-Lys-Cys (FKC) which is engineered with a cathepsin B (cathB) enzyme cleavage site. The presence of the cathB induces the scission of FKC upon homing into the cancer cells, resulting in the release of the initially latent fluorophores with a concomitant quenching of the surface-enhanced Raman signal intensity, thereby realizing an on-off switching between the fluorescence and Raman modalities. The enzyme-triggered switchable nanoprobes were utilized for the simultaneous detection of pathologically relevant lung cancer targets by tethering with specific antibody units. The multiplex-targeted multi-color-coded detection capability of the antitags was successfully developed as a valid protein screening methodology which can address the unmet challenges in the conventional clinical scenario for the precise and early diagnosis of lung cancer.



Schematic illustration of enzyme triggered switching of antibody functionalized FSENPs for the multiplexed detection of lung cancer biomarkers. b) Chemical structures of 7-hydroxy-3-carboxycoumarin (blue), rhodamine 110 (green) and rhodamine B (red). c) Fluorescence and d) SERS spectral analysis

Publication

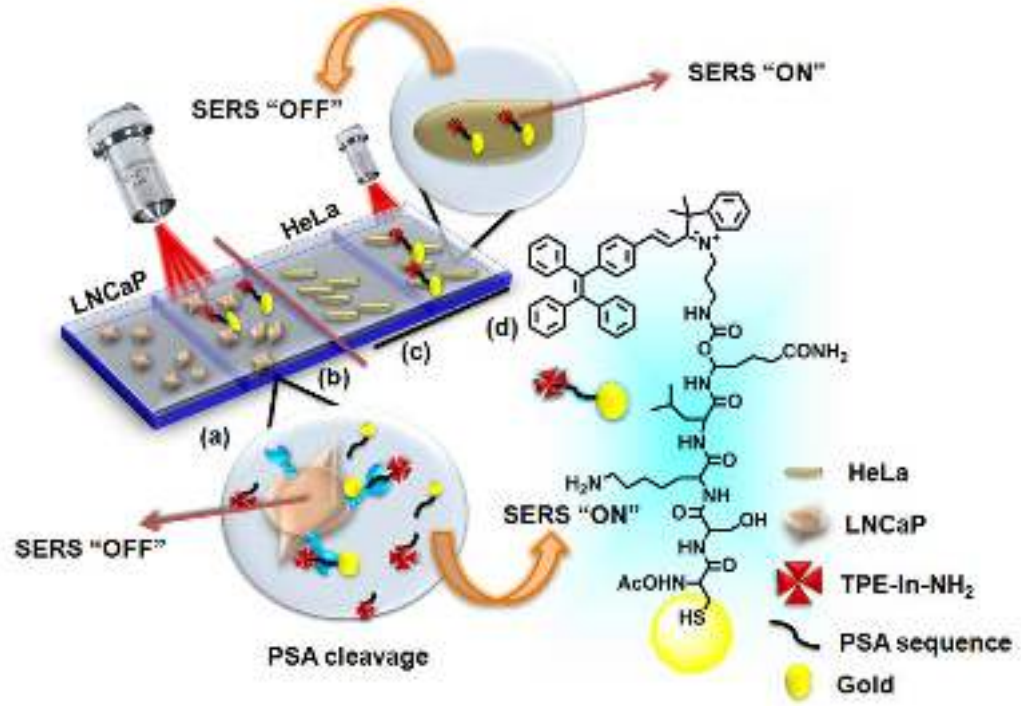
Enzyme-Driven Switchable Fluorescence-SERS Diagnostic Nanococktail for the Multiplex Detection of Lung Cancer Biomarkers; Giridharan Saranya,Manu M. Joseph, Varsha

Karunakaran, Jyothi B. Nair, Valliamma N. Saritha, Vamadevan S. Veena, Kunjuraman Sujathan, Ayyappanpillai Ajayaghosh*, and Kaustabh K. Maiti*,

ACS Applied Materials and Interfaces, 2018, 10 (45), pp 38807–38818 (Impact Factor: 10.38).

3. New Insight of Tetraphenylethylene-based Raman Signatures for Targeted SERS Nanoprobe Construction Toward Prostate Cancer Cell Detection

Newly designed and synthesized molecular probe with a molecular entity of tetraphenylethylene (TPE) which showed unique Raman fingerprints reflected by surface-enhanced Raman scattering (SERS) upon adsorption on nano-roughened gold surface. Out of several TPE-analogues, TPE-In-Boc showed a significant increase in signal intensity in the fingerprint region. An efficient SERS nanoprobe has been constructed using gold nanoparticles as SERS substrate, and the TPE-In as the Raman reporter, which was subsequently conjugated with a specific peptide substrate, Cys-Ser-Lys-Leu-Gln-OH, well known for the recognition of prostate-specific antigen (PSA). The designated nanoprobe TPE-In-PSA@Au acted as SERS "ON/OFF" probe in peace with the vicinity of PSA protease which distinctly recognizes PSA expression with a limit of detection (LOD) of 0.5 ng in SERS platform. Furthermore, TPE-In-PSA@Au nanoprobe efficiently recognized the over-expressed PSA in human LNCaP cell which can be visualized through SERS spectral analysis and SERS mapping.



Thematic representation of PSA recognition by TPE-In-PSA@Au nanoprobe by SERS; (a) LNCaP cells without nanoprobe (b) with nanoprobe, HeLa cells (c) without nanoprobe (d) with nanoprobe.

Publication

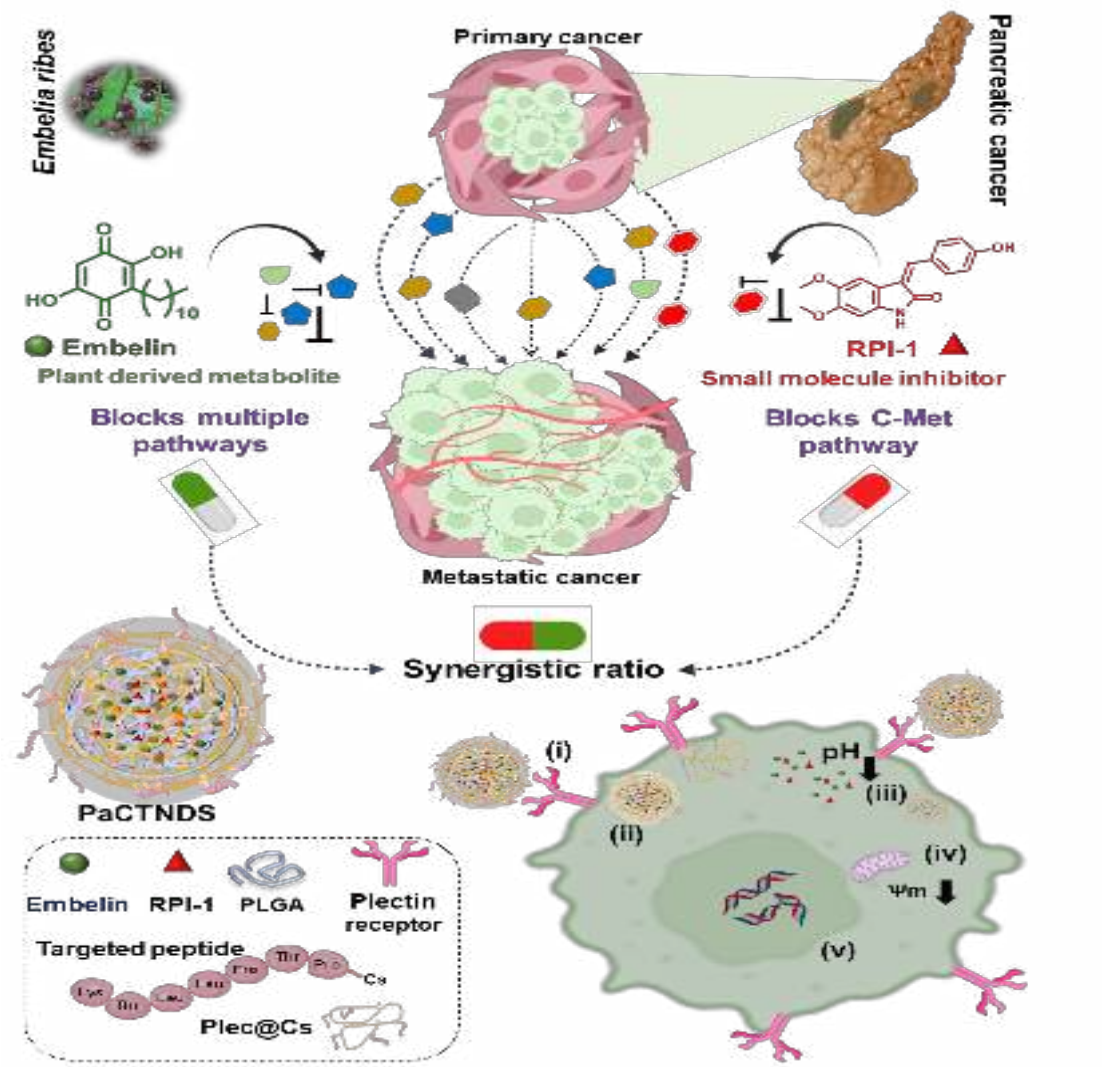
New Insight of Tetraphenylethylene-based Raman Signatures for Targeted SERS Nanoprobe Construction Toward Prostate Cancer Cell Detection;Adukkadan N. Ramya, Manu M. Joseph, Jyothi B. Nair, Varsha Karunakaran,Nisha Narayanan, and Kaustabh Kumar Maiti*,

ACS Appl. Mater. Interfaces., 2016, 8, 10220-10225 (Impact Factor: 10.38).

E. Cancer nano theranostics/nanomedicine utilizing surface-enhanced Raman spectroscopy (SERS)

1. Exploring Targeted Nano-Delivery System by Synergism of Embilin and RPI-1 for Therapeutics of Pancreatic Cancer

Pancreatic cancer (PC) is an aggressive form of malignancy with a poor prognosis and feeble survival benefits. A personalized nanomedicine constituted of the synergistic therapeutic benefit of two agents, namely, embelin and RPI-1, coloaded in a biodegradable nano-delivery system that has been tethered with a targeting peptide substrate for plectin-1, a surface biomarker of PC. The phytomolecule embelin, an alkyl-substituted hydroxyl benzoquinone isolated from the seeds of Embelia ribes, is introduced in this combination therapy and is known as a natural inhibitor of X-linked inhibitor of apoptosis protein and executes anticancer activity via the NF-kB pathway. On the other hand, tyrosine-protein kinase Met (c-MET) is a biomarker of PC wherein the molecule RPI-1, an indolinone derivative, is selective to the c-MET inhibitor. After conducting a series of systematic cytotoxicity evaluations followed by enumeration of combination index, a standalone synergic ratio of embelin and RPI-1 (1: 4.7) was evolved that executed a benchmarked PC-selective toxicity profile. This composition was precisely incorporated within a PC-targeted PLGA−chitosan core− shell nanoparticle delivery system to avoid collateral damages. Appealing features of the nanoconstruct including biocompatibility, PC-targeted uptake, and subsequent execution of cytotoxicity and antimetastatic properties have been systematically evaluated on the PC cell line PANC-1 and later on the xenograft PC model of zebrafish. Finally, the unique metabolic changes associated with the therapeutic action of embelin and RPI-1 were scrutinized by surface-enhanced Raman spectroscopy and liquid chromatography−mass spectrometry analysis wherein almost 25 metabolites associated with the cell death pathway deciphered a significant variation. Therefore, this proof-of-concept personalized nano-delivery warrants further preclinical and clinical evaluations for the management of PC



Schematic representation of the fabrication and mechanistic action of pancreatic cancer targeted nano-delivery system (PaCTNDS, (i) PaCTNDS will get selectively recognized by the Plectin receptor of pancreatic cancer cells and will undergo (ii) targeted endocytosis to gain rapid cell entry. Later, (iii) the intracellular acidic pH causes the chitosan layer to swell, facilitating the release of trapped cargo and biodegradation of the carrier. Subsequently a cascade of biochemical and metabolic changes including (iv) reduction in ΔΨm, release of cyt C and (v) execution of programmed cell death

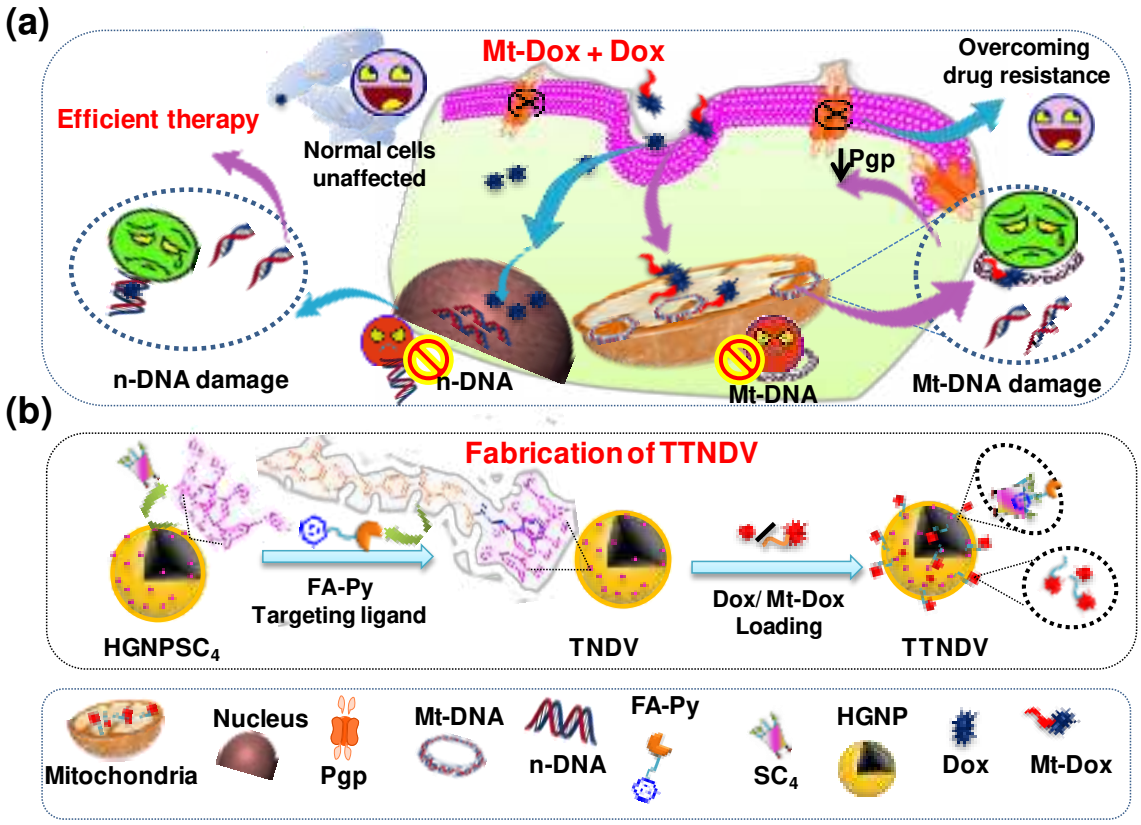
Publication:

Targeted Delivery Polymeric Nanosystem Reinforced by Synergism of Embilin and RPI-1 for Therapeutics of Pancreatic Cancer; Jayadev S. Arya, Manu M. Joseph,* Vishnu Priya Murali, Murukan S. Vidyalekshmi, and Kaustabh Kumar Maiti*

ACS Appl. Nano Mater., (2022); 2022, 5, 12, 18622–18636 (Impact Factor: 6.14)

2. Thermo-responsive Multimodal Photo-Chemotherapeutic Nano-delivery System to Overcome the Barriers of Doxorubicin Therapy

In an attempt to circumvent the major pitfalls associated with conventional chemotherapy including drug resistance and off-target toxicity, a newly evolved strategy have been adopted to simultaneously target both mitochondrial DNA (Mt-DNA) and nuclear DNA (n-DNA) with the aid of a targeted theranostic nano-delivery vehicle (TTNDV). Folic acid anchored *p*-sulfo-calix[4]arene (SC_4) capped hollow gold nanoparticles (HGNP) were meticulously loaded with a pre-tuned ratio (1:100) of antineoplastic doxorubicin (Dox) and its mitochondria-targeted analog, Mt-Dox for sustained thermo-responsive release of cargo. This therapeutic intervention was enabled to eradicate both n-DNA and Mt-DNA leaving no space to develop drug resistance. The SC4-capped HGNPs ($HGNPSC_4$) were experimented with for the first time as a photothermal (PTT) agent with 61.6% photothermal conversion efficiency, to generate tunable localized heat more efficiently than bare HGNPs. Moreover, the cavity of SC_4 facilitated the formation of an inclusion complex with folic acid to target folate receptor-expressing cancer cells and imparted enhanced biocompatibility. The as-synthesized TTNDV was demonstrated to be an ideal substrate for surface-enhanced Raman scattering (SERS) to monitor the molecular level therapeutic progression in cells and spheroidal model. A significant reduction in the tumor mass with marked survival benefits was archived in syngraft murine models through this synergistic photo-chemotherapy. Collectively, this multifunctional nanoplatform offers a robust approach to treating cancer without any scope of generating Dox resistance and off-target toxicity.



A schematic illustration with the mechanistic action of a combination of Dox and Mt-Dox on cancer cells. Mt-Dox facilitates the damage of mitochondrial DNA thereby helping to overcome the troubles associated with Mt-DNA escape viz drug resistance, metastasis, etc. Dox will execute the damage of nuclear DNA for an efficient chemotherapy. (b) Various synthetic steps involved in the fabrication of the TTNDV, viz, tethering of folic acid as a targeting ligand for selective recognition of folate receptors on HGNPSCs, loading of pre-tuned ratio of Dox: Mt-Dox (1:100) into the targeted nano delivery vector to yield TTNDV.

Publication:

Elucidating a Thermo-responsive Multimodal Photo Chemotherapeutic Nano- delivery Vehicle to Overcome the Barriers of Doxorubicin Therapy; Jyothi B Nair, Manu M Joseph*, Jayadev S Arya, Padincharapad Sreedevi, Palasseri T Sujai, and Kaustabh Kumar Maiti*;

ACS Applied Materials and Interfaces, 2020, 12, 39, 43365–43379 (Impact factor: 10.38)

Kaustabh Kumar Maiti
20/08/2024