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

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ABSTRACT

Simultaneous detection of multiple biomarkers is always an obstacle in immunohistochemical (IHC) analysis. Herein, a straightforward spectroscopy-driven histopathologic approach has emerged as a paradigm of Raman-label (RL) nanoparticle probes for multiplex recognition of pertinent biomarkers in heterogeneous breast cancer. The nanoprobes are constructed by sequential incorporation of signature RL and target specific antibodies on gold nanoparticles,

which are coined as Raman-Label surface enhanced Raman scattering (RL-SERS)-nanotags to evaluate simultaneous recognition of clinically relevant breast cancer biomarkers i.e., estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor2 (HER2). As a foot-step assessment, breast cancer cell lines having varied expression levels of the triple biomarkers are investigated. Subsequently, the optimized detection strategy using RL-SERS-nanotags is subjected to clinically confirmed, retrospective formalin-fixed paraffin embedded (FFPE) breast cancer tissue samples to fish out the quick response of singleplex, duplex as well as triplex biomarkers in a single tissue specimen by adopting a ratiometric signature RL-SERS analysis which enabled to minimize the false negative and positive results. Significantly, sensitivity and specificity of 95% and 92% for singleplex, 88% and 85% for duplex, and 75% and 67% for triplex biomarker has been achieved by assessing specific Raman fingerprints of the respective SERS-tags. Furthermore, a semi-quantitative evaluation of HER2 grading between 4+ / 2+ / 1+ tissue samples was also achieved by the Raman intensity profiling of the SERS-tag, which is fully in agreement with the expensive fluorescent in situ hybridization analysis. Additionally, the practical diagnostic applicability of RL-SERS-tags has been achieved by large area SERS imaging of areas covering 0.5 to 5 mm2 within 45 min. These findings unveil an accurate, inexpensive and multiplex diagnostic modality envisaging large-scale multi-centric clinical validation.

1. Introduction

Clinical biomarkers partake an imperative role in breast cancer prognosis and its heterogenous as well as differential expression pattern exhibit the key challenge in choosing the proper treatment modality. Hence, breast cancer biomarker detection prevails the decisive feature which needs a distinct troubleshooting approach (Shah et al., 2014). The three vital prognostic markers widely assessed in breast cancer diagnosis includes hormonal nuclear receptors estrogen (ER) (Russo and Russo, 2006), progesterone (Lange, Carol A, 2008) and cell surface human epidermal growth factor type 2/neu (HER2/neu) receptors (Costa and Czerniecki, 2020). Based on these biomarker expression profiles, four major classification of breast cancer are designated, viz., luminal A, luminal B, HER2 enriched and basal like subtypes (Parise and Caggiano, 2014). The occurrence and extend of cell surface biomarker HER2 is often measured

by a grading system having 4^+, 3^+, 2^+, 1^+ and 0 grades correlating the staining intensity. According to HER2 testing guidelines and samples with a staining score of 2^+ or less is considered as negative (Cornejo et al., 2014). The gold standard method immunohistochemistry (IHC), which is generally employed for the detection of these biomarkers is relatively inexpensive, but the results are often subjective as influenced by inter-observer variability. Besides, evaluation of multiple biomarkers simultaneously in a single tissue specimen is not possible, which turned out as a lengthy process to complete the investigation of all three-biomarker status (Dixon et al., 2015). In the case of HER2 detection, 95 % concordance is mandatory within IHC and Fluorescent in situ hybridization (FISH). FISH is a cytogenic analysis to detect the number of HER2 gene copies per nucleus. Even though FISH is a robust technique for HER2 detection, it necessitates expensive reagents along with laboratory equipment setup (Bogdanovska-Todorovska et al., 2018; Furrer et al., 2015; Wesoła and Jeleń, 2015). Eventhough, techniques like multiplex immunohistochemistry/immunofluorescence (mIHC/mIF) have been introduced as improved methods to detect multiple biomarkers in single tissue section, its applicability has been rescricted due to several disadvantages like, limited dynamic range for intensity of the chromogenic substrates in mIHC. On the other hand, mIF has a large linear dynamic range for many fluorophores for quantitative analysis, however, they must be chosen carefully for preventing bleed through. The highly sensitive tyramide signal amplification (TSA) method is also insubstantial due to the complicated steps involved, tyranide overaction and its time consuming nature (Hernandez et al., 2021; Sheng et al., 2023; Tan et al., 2020). Exploration and validation of alternative simple and relatively rapid techniques are always recommended which can meet acceptable diagnostic quality.

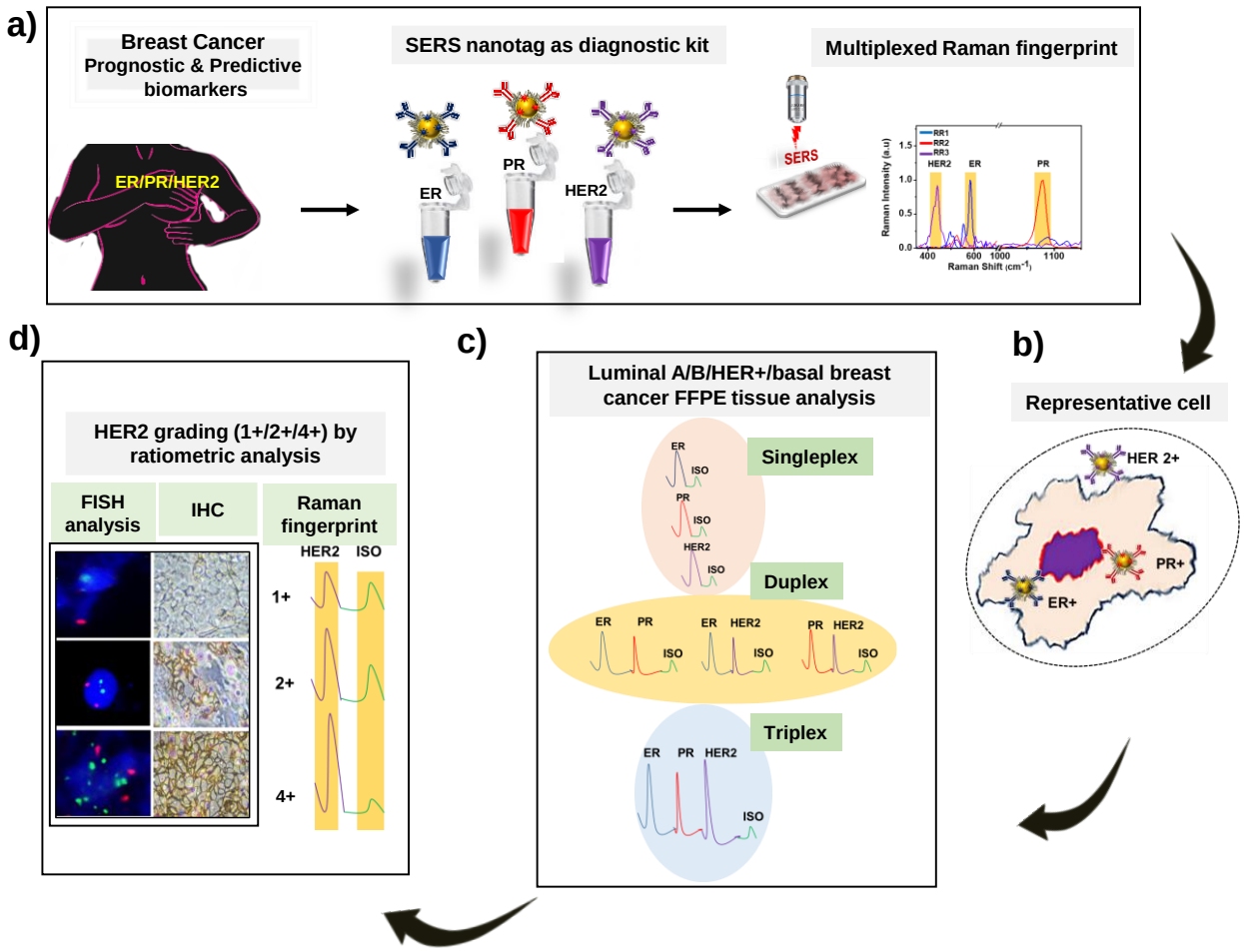
Surface enhanced Raman spectroscopy (SERS), is an advanced and ultrasensitive technique which received immense scientific attention especially in diverse clinical diagnostic applications including cancer in last several years (Haka et al., 2005; Ramya et al., 2021)

(Joseph et al., 2018). In diagnostic scenario, label-free SERS and Raman label -based SERS-nanotags are the two major practices adopted as a detection modality so far. SERS-nanotags or SERS-nanoprobes are prepared by incorporating Raman reporter molecules enabling inherently strong Raman cross-section to plasmon resonant metallic nanoparticles like gold or silver. Subsequently, a protective polymeric layer and a target specific recognition motif like a peptide, antibody or aptamer are functionalized to the SERS-nanotags which exclusively recognize specific biomarkers *in vitro*, *in vivo* and *ex vivo* specimens having moderately high heterogeneity (Wang et al., 2013).

Sensitive singleplex/multiplex detection of clinically relevant cancer biomarkers utilizing SERS-nanotags has shown a very promising alternative strategy, stepping up towards the upcoming technology for clinical diagnostics (Dinish et al., 2014; Nariman et al., 2017; Sun et al., 2021). Hence, SERS-nanotags resemble an ideal SERS-based immunoassay component for the precise detection of multiplex biomarkers in a personalized manner with high sensitivity and specificity (Schlücker et al., 2011).

Even though a few SERS-based multiplexed studies have been demonstrated for other cancer types (Davis et al., 2018; Lin et al., 2021; Maiti et al., 2012; Narayanan et al., 2015; Zavaleta et al., 2013) as well as breast cancer biomarkers in cells (Lee et al., 2014), serum (Li et al., 2015) and fresh tissue (Wang et al., 2017) etc., there is no report so far on multiplex detection of three prevalent prognostic markers *i.e.*, ER, PR and HER2 in a single tissue sample as a complementary system to IHC as well as FISH analysis. Similarly, biomarker detection in fresh tissue samples via topical application of SERS-tags followed by raster scanning to determine surgical margin during lumpectomy has also been investigated (Y. Wang et al., 2016; Y. W. Wang et al., 2016), whereas the present study prevails its distinct feature of biomarker detection in the retrospective formalin fixed paraffin embedded breast tissue specimens as a parallel

platform that envisages to nullify the highly time consuming and subjective nature of immunohistochemistry and expensive FISH technique.



Scheme 1. Schematic illustration of the experimental design for differentiating the clinically relevant triple biomarkers, ER, PR and HER2. a) strategy for the multiplexed detection using ER, PR and HER2 conjugated SERS-tags having AuNPs as substrate, b) representative design for detection of biomarkers in cells/paraffin embedded breast tissue samples using SERS mapping, c) Three phases of biomarker detection executed as single, dual and triple biomarker analysis from the breast tissue specimens, d) HER2 grading of IHC and FISH confirmed 1^{+}, 2^{+} and 4^{+} tissue sample through ratiometric SERS mapping.

Herein, we have evaluated the clinical implementation of the multiplexing capability of the SERS technique using multiplex Raman-label SERS(RL-SERS)-nanotags to determine the breast cancer prognostic biomarkers, ER/PR/HER2 complementary to IHC. The preeminence of the study over the so far reported SERS multiplexing studies includes the clinical relevance of the selection of biomarkers and its detection in FFPE specimens, time efficient approach to analyze the maximum area of tissue samples by ratiometric large area scanning and the analysis

of an ambient number of different tissue subtypes in terms of biomarker status. Moreover, this is the first-ever report on the semi-quantitative evaluation of HER2 grading between 4+ / 2+ / 1+ tissue samples by ratiometric SERS intensity profiling, in concordance with FISH analysis (Scheme 1). Collectively, to establish the utility of SERS-nanotags as a next-generation clinical diagnostic modality enabling a fast, facile, accurate, and reliable spectro-histologic technique for multiplex prognostic analysis of heterogenous breast cancer biomarkers.

2. Results and Discussion

2.1. SERS-nanotags (SERS-nanotags) for multiplex detection:

Gold nanoparticles (AuNPs: 40-45 nm) were selected as a SERS substrate for the preparation of SERS-nanotags to perform the multiplexing recognition of ER, PR and HER2 in breast cancer due to its excellent SERS enhancement reported so far (Njoki et al., 2007). The synthesized SERS substrate was characterized by UV-vis Spectroscopy, Dynamic Light Scattering (DLS) and High-Resolution Transmission Electron Microscopy (HR-TEM) analysis (**Figure S1a, b and c**). Further, SERS-nanotags were fabricated by tagging Raman labels (reporters) having multiplexed Raman peaks which were designated *w.r.t.,* each breast cancer biomarker by the respective Raman signature peak of the reporter molecule. Among three Raman reporters, commercially purchased crystal violet (CV) and 4-mercapto benzoic acid (MBA) were chosen based on multiplex Raman peaks at 440 and 1084 cm^{-1} respectively, along with the in-house synthesized Raman reporter, squaraine di-lipoic acid (SDL) (Ramya et al., 2015) with a non-overlap Raman peak at 580 cm^{-1} *w.r.t* CV and MBA.

The structure and the SERS fingerprint pattern of the three SERS nanotags were portrayed in **Figure S2a, b, c and d.** Isotype antibody conjugated SERS-tags were prepared independently with Dithio nitrobenzoic acid (DTNB) as well as 3,3 -Diethylthiacarbocyanine iodide (DTTC) having signature Raman peak at 1320 cm^{-1} and 505 cm^{-1} respectively (**Figure S2e & f**). The stability and biocompatibility of nanotags were improved by a protective layer of polyethylene glycol (PEG) and the carboxy functionalized PEG which facilitated the conjugation of target-

specific antibodies. UV-Vis spectroscopy, TEM and DLS analysis of the PEGylated nanoparticles were also accomplished to confirm the PEG layer formation (**Figure S1d, e and f**). The stickiness nature and aggregation tendency of nanoparticles were minimized with the employment of Tween 20 (Lin et al., 2010). The biocompatibility of the fabricated RL-SERS-tags was further confirmed by cell viability assay in different breast cancer cell lines (**Figure S3 a, b and c**). The simultaneous detection of breast cancer biomarkers was executed by constructing three multiplexing RL-SERS-nanotags conjugated with the specific monoclonal antibodies for the detection of clinically valid biomarkers i.e., ER, PR and HER2. The successful conjugation of antibodies to AuNPs was confirmed by UV-Vis absorbance (**Figure S4 a,b,c**) which showed a protein absorption peak around 280 nm without much compromise in the SERS activity (**Figure S4 d,e,f**). Besides, polyacrylamide gel electrophoresis (PAGE) analysis **(Figure S5a,b,c)** and 3, 3′ , 5, 5′ Tetramethylbenzidine (TMB) assay **(Figure S5 d)** also confirmed the presence of the tethered antibodies on the SERS-nanotags. The stability of the SERS-nanotags was also monitored up to six months which ensured the stability of the nanotags based on consistant SERS intensity of the signature peaks. **(Figure S6).**

2.2. Multiplex detection by SERS-Nanotags in ER, PR and HER2 overexpressed Breast cancer cells (Phase I):

As a proof-of-principle of multiplex detection, the RL-SERS-nanotags have endeavoured in breast cancer cell lines. To accomplish this, immunophenotyping was performed to ensure the ER and PR abundance in MCF-7 cells as well as HER2 over-expression in SK-BR-3 cells and compared with the triple negative MDA-MB-231 cells (**Figure S7a, b and c**). Further, incubation time of SERS-nanotag was optimized within 2 hours (**Figure S8**). SERS analysis of MCF-7 (luminal A subtype, ER^+/PR^+), after incubating with AuNP@SDL@anti-ER, AuNP@MBA@anti-PR and AuNP@CV@anti HER2 SERS-nanotags identified the

protuberant Raman signature peaks of the Raman labels SDL at 580 and MBA at 1084 cm^{-1} from the nuclear location without any prominent CV peak at 440 cm^{-1} from the whole cell demonstrating the effective recognition of ER and PR (**Figure S9 a-d**). Similarly, upon incubation of all three nanotags with HER2-enriched breast cancer subtype SK-BR-3, resulted the presence of noticeable Raman peak of CV (crystal violet) at 440 cm^{-1} from the cell surface milieu (**Figure S10 a-d**). Similarly, a basal subtype($ER^+/PR^+/HER2^+$triple negative receptors) MDA-MB-231 cell line resembled a negligible expression as indicated from all the three RL-SERS-nanotags ensuring the specificity of the nanotags (**Figure S11a,b and c**). A parallel confirmation has been carried out for the recognition of biomarkers through RL-SERS-nanotags, by dark field microscopic analysis based on the back scattering property of the metallic nanoparticle uptake as detailed in the supporting information (**Figure S12 a, b and c).**

2.3. Multiplex Detection Strategy in Clinically Confirmed Breast tissue samples

After the proof-of-concept confirmation in breast cancer cell lines, analysis in retrospective clinical FFPE tissue sections accumulated from lumpectomy/mastectomy-derived specimens was performed for the SERS detection of ER, PR, and HER2 biomarkers. Ratiometric SERS evaluation was executed to detect the presence of single and multiple biomarkers, with non-targeted IgG isotype antibody conjugated SERS-tags along with the targeted ones as a control to minimize the false positive signal arising due to the non-specific binding. For attaining these goals, three sets of detection modes were executed viz., singleplex i.e., detection of single biomarkers either ER, PR or HER2 individually, then duplex with a combination of any two out of three biomarkers and triplex analysis, which aimed to detect the expression status of all the three biomarkers simultaneously in a single tissue specimen.

2.3.1 Singleplex analysis of tissue biomarkers by SERS-nanotags

The first stage of clinical sample analysis was envisioned to detect a single biomarker either ER/PR/HER2 in FFPE breast cancer tissue specimens. One of the major challenging factors in

the detection technique was the nonspecific binding of nanotags to the specimen, which may lead to false positive results (**Figure S13**), thus reducing the specificity of the detection model. To circumvent this stipulation, we have introduced an isotype antibody (IgG) conjugated SERS-tag as a control with the respective singleplex nanotag (Wang et al., 2014) that provided a ratiometric approach where the ratio of SERS intensity profile of ER / PR/ HER2 conjugated SERS-nanotags and isotype control SERS-nanotags were measured. As shown in **Figure 1a**, IHC confirmed ER positive tissue treated with AuNP@SDL@anti-ER SERS tag provided a high-resolution Raman image and an average Raman intensity (ARI) corresponding to the signature peak of SDL at 580 cm^{-1} demonstrating the sensitivity of ER nanotag. Similarly, from different experiments, PR (**Figure 1b**) and HER2 (**Figure 1c**) positive tissues incubated with SERS-nanotag (AuNP@MBA@anti-PR & AuNP@CV@anti-HER2) afforded distinct Raman images and average spectrum with marker reporter peaks at 1084 (MBA) and 440 (CV) cm^{-1} respectively. A ratio less than or equal to one is considered negative for that particular biomarker where as a value greater than one is considered positive (**Table S1**). Therefore, the formulated methodology by using RL-SERS-nanotags demonstrated a sensitive and specific detection of the corresponding single biomarker in the FFPE breast cancer tissue specimens.

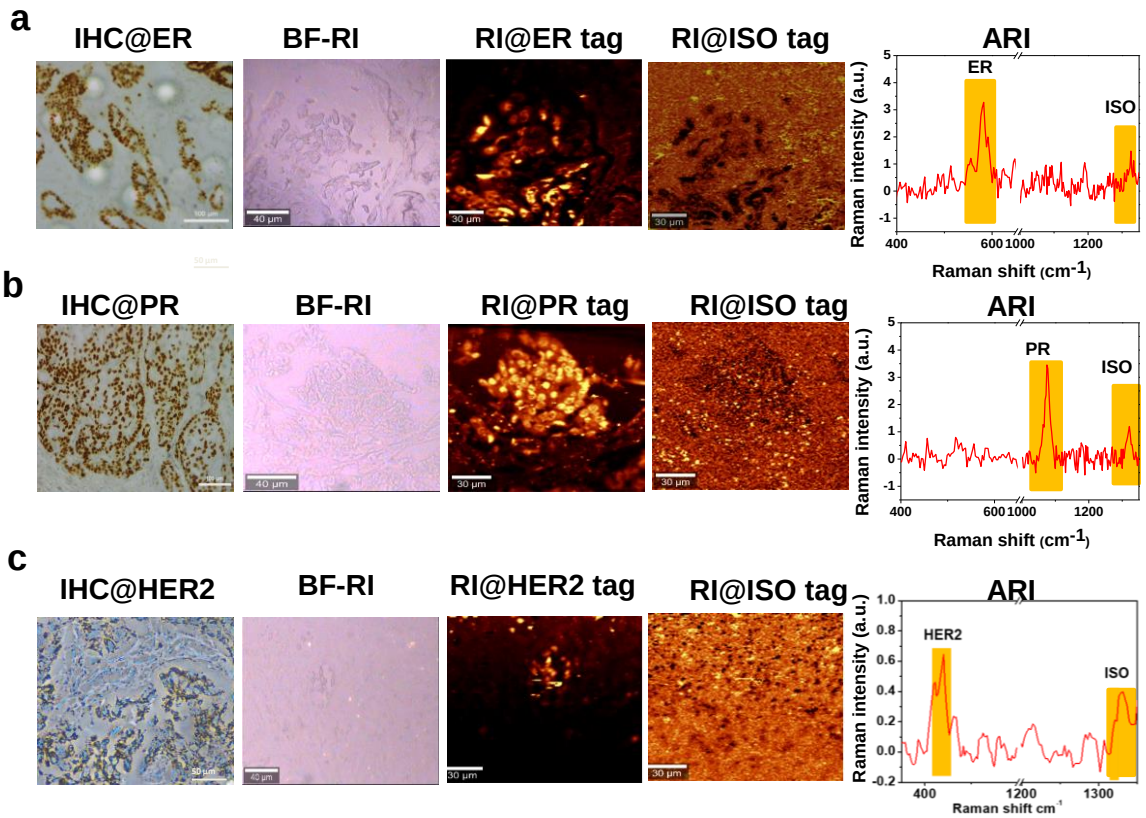


Figure 1. SERS singleplex analysis of a) ER^+ b) PR^+ and c) $HER2^+$ tissue using AuNP@SDL@PEG@anti-ER, AuNP@MBA@PEG@anti-PR and AuNP@CV@PEG@anti-HER2 nanotags along with AuNP@DTNB@PEG@anti-isotype tag. IHC-Immunohistochemistry confirmation for ER, PR and HER2 positivity of the specimens, BF-RI- Bright field images of the tissue area subjected for Raman image scanning, RI-Raman image corresponds to (a) 580 cm^{-1} of AuNP@SDL@PEG@anti-ER, (b) corresponds to 1084 cm^{-1} of AuNP@MBA@PEG@anti-PR and (c) corresponds to AuNP@CV@PEG@anti-HER2, RI@ISO tag corresponds the Raman images of AuNP@DTNB@PEG@anti-isotype., ARI-Average Raman intensity from the average scan spectrum of the ER^+, PR^+ and $HER2^+$ tissue samples respectively.

2.3.2. Duplex analysis for tissue biomarkers by SERS-nanotags

Even though a few kits-based methods persisted, dual biomarker detection is a still challenging aspect to achieve through IHC. Using the current RL-SERS-tags, we have examined combinations of two biomarker analysis at a time, along with isotype control and investigated the differential expression status in a single tissue specimen. As shown in **Figure 2a**, luminal A tissue sample having ER^+ PR^+ expression was treated with SERS-tags conjugated to anti-ER (AuNP@SDL@anti-ER) and anti-PR (AuNP@MBA@anti-PR) antibodies. The average scan spectrum generated from the Raman image symbolized the presence of the signature peaks from SDL at 580 cm^{-1} and MBA at 1084 cm^{-1} with a ratiometric value >1 as compared to

AuNP@DTNB@Isotype confirming the specific recognition of ER and PR. Corresponding receptor negative samples viz., $ER^{-}PR^{+}$ (**Figure 2b**) and $ER^{+}PR^{-}$ tissues (**Figure 2c**) exhibited minimal SERS signals of SDL (580 cm^{-1}) and MBA (1084 cm^{-1}) respectively with a ratiometric value of <1. (**Table S2**). ER /HER2 and PR/HER duplexing were also experimented with to confirm the dual biomarker detection ability of the RL-SERS tags **(Figure S 14, Table S3)**.

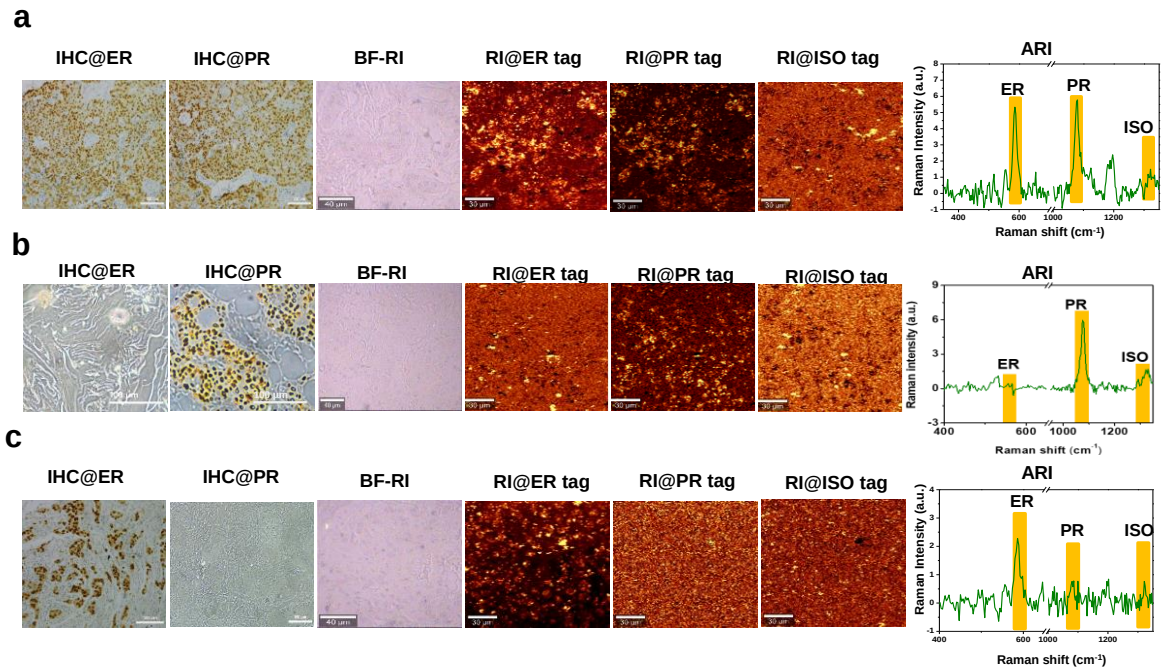


Figure 2. SERS duplex analysis of a) of ER^{+} and PR^{+} tissue, b) ER^{-} and PR^{+} tissue and c) ER^{+} and PR^{-} tissue samples using AuNP@SDL@PEG@anti-ER, AuNP@MBA@PEG@anti-PR and AuNP@CV@PEG@anti-HER2 nanotags along with AuNP@DTNB@PEG@anti-isotype tag. IHC-Immunohistochemistry confirmation for ER, PR and HER2 positivity of the specimens, BF-RI- Bright field images of the tissue area subjected for Raman image scanning, RI-Raman image corresponds to (a) 580 cm^{-1} of AuNP@SDL@PEG@anti-ER, (b) corresponds to 1084 cm^{-1} of AuNP@MBA@PEG@anti-PR and (c) corresponds to AuNP@CV@PEG@anti-HER2, RI@ISO tag corresponds the Raman images of AuNP@DTNB@PEG@anti-isotype, ARI-Average Raman intensity from the scan spectrum.

2.3.3. Triplex analysis for tissue biomarkers in luminal B and basal tissue samples by RL-SERS-nanotags

In the set-up for triplex analysis, IHC confirmed luminal B ($ER^+PR^+HER2^+$) tissue sample was incubated with all the three SERS-nanotags i.e., AuNP@SDL@anti-ER, AuNP@MBA@anti-PR and AuNP@CV@anti-HER2. SERS analysis revealed the recognition of ER, PR and HER2 biomarkers even in a single scan with its average spectrum having corresponding peaks of SERS-nanotags at 580, 1084 and 440 cm^{-1} with scanned images showing the receptor positive and negative areas of the sample corroborating the detection of three biomarkers in a single specimen (**Figure 3a**). Further, in another set, where IHC confirmed ER, PR and HER2 negative tissue (basal type) specimen was analysed with the SERS-tags, it showed negligible Raman peaks for the three biomarkers (**Figure 3b**). A summarized data of tissue analysis is shown in **Table S4.**

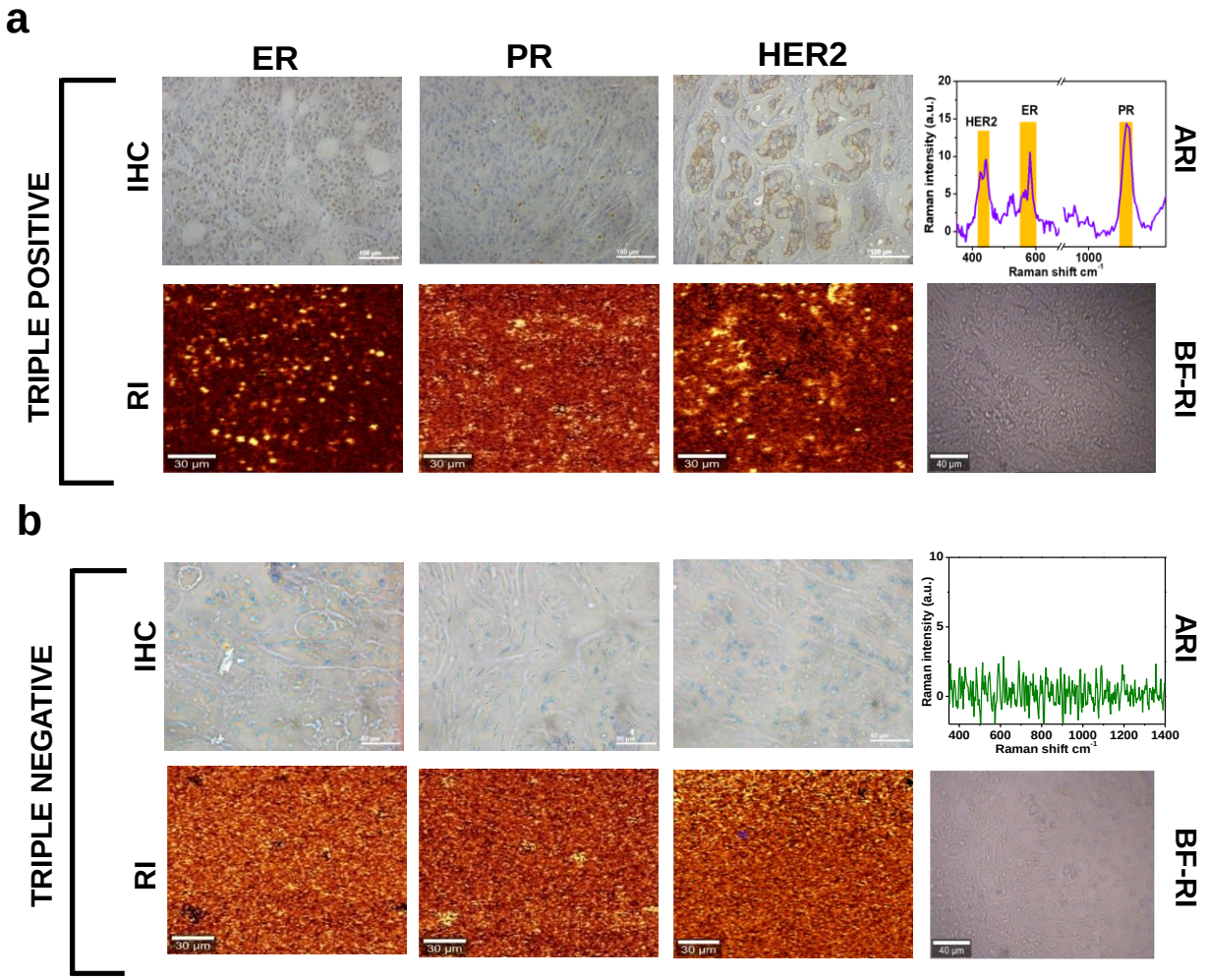
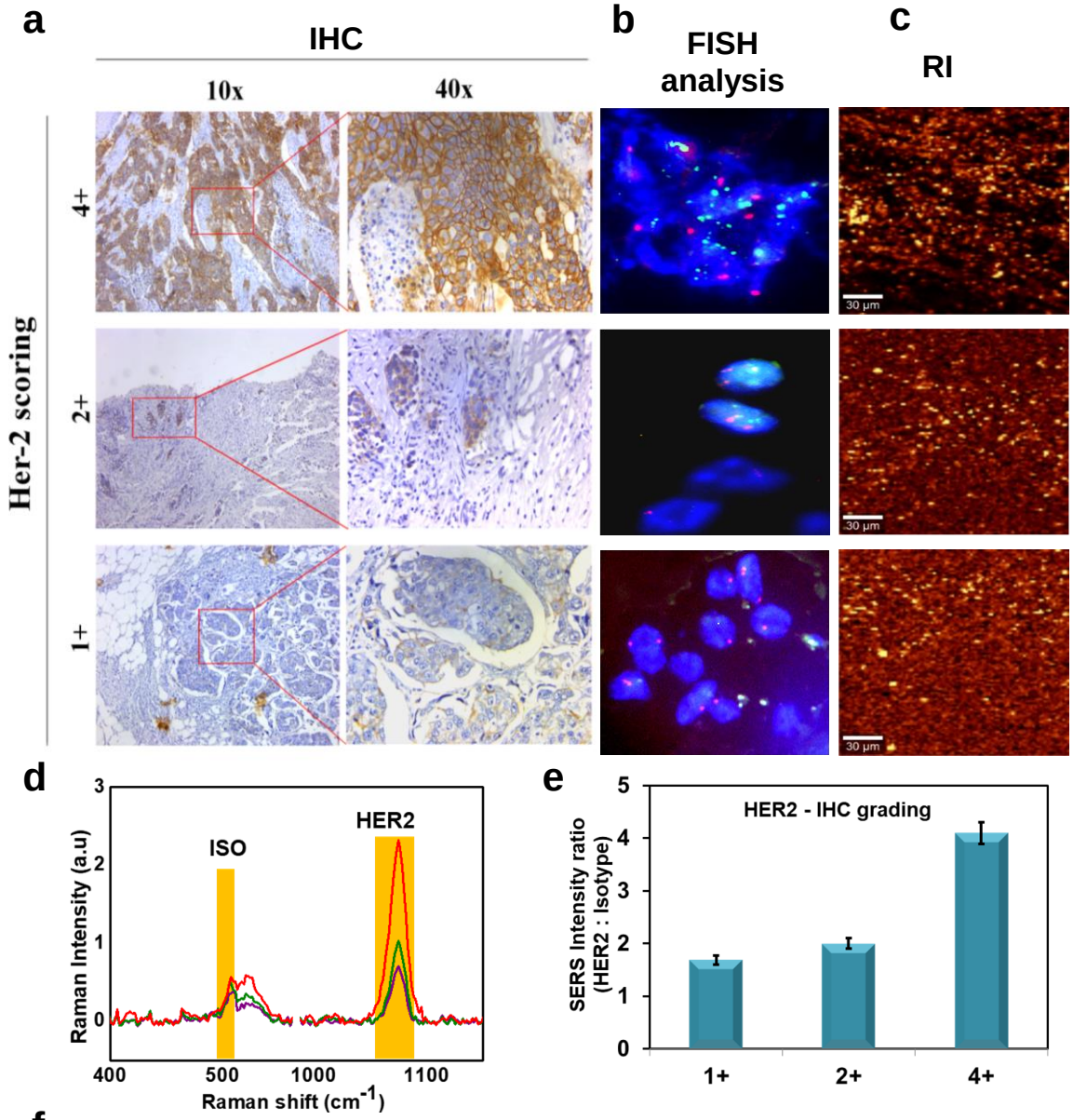


Figure 3. SERS analysis of a) Luminal B ($ER^+PR^+HER2^+$) and b) Basal (ER-PR-HER2-) tissue using AuNP@SDL@anti-ER, AuNP@MBA@anti-PR and AuNP@CV@anti-HER2 nanotags. IHC-Immunohistochemistry confirmation for ER, PR and HER2 positivity of the specimens, BF-RI- Bright field images of the tissue area subjected for Raman image scanning, RI-Raman image corresponds to (a) 580 cm^{-1} of AuNP@SDL@PEG@anti-ER, 1084 cm^{-1} of AuNP@MBA@PEG@anti-PR and 440 cm^{-1} of AuNP@CV@PEG@anti-HER2, ARI-Average Raman intensity denotes the average scan spectrum of the duplex biomarkers in tissue samples respectively.

2.4. HER-2 grading based on signature Raman label

Unlike ER and PR, HER2 overexpression is considered for effective targeted therapy by the treatment of clinically approved trastuzumab. IHC grading of 3^+ and more are judged to be HER2 positive, whereas 2^+ / equivocal expression require further confirmation by FISH (Fluorescent in situ hybridization) analysis in which the number of HER2 gene copies per nucleus is assessed. This method is again a time-consuming and highly expensive technique in cytopathology. We introduced a ratiometric analysis of SERS-nanotag for measuring HER2

grading based on the signature Raman peak which preciously profiled the intensity ratio between HER2-tag and isotype-control-tag. The Raman reporter DTTC was used for the isotype antibody conjugated SERS-nanotag. As indicated in **figure 4**, IHC grades of 1^+, 2^+ and 4^+ tissue samples (**Figure 4a**) parallelly confirmed by FISH analysis (**Figure 4b**) were treated to Raman-labeled SERS-tag (AuNP@MBA@anti-HER2) and isotype control SERS-tag (AuNP@DTTC@Isotype) to assess the ratiometric analysis. **Figure 4c** revealed the Raman scanned image, which indicated the gradation of HER2 expression. The Raman intensity profile of the signature Raman label MBA (1084 cm^{-1}) and DTTC (505 cm^{-1}) designating HER2 -tag and isotype-tag respectively is depicted in **Figure 4d**. The ratio of HER2-tag to isotype-tag obtained from the average scan intensities is plotted in a bar diagram to get a mathematical interpretation of the same (**Figure 4e**). Average SERS intensity from image scanning was found to be higher for 4^+ HER2 tissue with an intensity ratio of 3.67± 0.51 followed by 2^+ HER2 (Ratio: 2.17± 0.2) and 1^+ (Ratio: 1.75± 0.15) in harmony with the IHC staining pattern (**Figure 4 f**).



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HER2 grade	A.R.I HER2	A.R.I Iso	HER2:ISO
1^+	1.19 ± 0.32	0.69 ± 0.20	1.75 ± 0.15
2^+	0.88 ± 0.64	0.40 ± 0.41	2.17 ± 0.20
4^+	3.93 ± 0.82	1.07 ± 0.15	3.67 ± 0.51

Figure 4. SERS analysis showing HER2 grading in $HER2^+$ tissue using HER2 targeted AuNP@MBA@anti-HER2 and Isotype targeted AuNP@DTTC@anti-isotype nanotags. a) IHC analysis, b) FISH analysis (Orange flourochrome-for centromere (*CEP17)* of chromosome 17 as internal control, Green flourochrome - HER 2 gene),c) Raman Imaging (RI), d) Average Raman Intensity (ARI) and color representations, Purple ($HER2^{1+}$), Green ($HER2^{2+}$), Red ($HER2^{4+}$), e) representation of HER2 grading by bar diagram and f) table showing ratiometric signal values of HER2 versus isotype tags. Data, Average ± SD of three different analysis.

2.5. Practical diagnostic applicability of RL-SERS-nanotags by large area SERS imaging

The major bottleneck of our multiplexed SERS analysis in tissue samples was to screen the entire area of the tissue specimen (usually of 5 to 10 mm^2 area) by Raman mapping since it generally allows visibility up to 0.15 mm^2 to 0.2 mm^2 area only in minimal time. To accomplish the total specimen analysis, large area Raman scanning technique was carried out with total areas covering 0.5 as well as 5 mm^2. **Figure S15** demonstrates the large area scanning with 0.5 mm^2 area of the duplexing analysis, which was performed in various combinations like ER /PR, PR / HER2 and ER/HER2 along with TNBC samples (**Figure S16**). The results illustrate the large area scanning as a robust method to cover maximum tissue area to get more accuracy. Owing to the excellent sensitivity of the SERS-nanotags, we have achieved a large area scan of up to 5 mm^2, which provided a clear-cut idea about the prevalence of biomarkers in the sample. This method was able to accurately detect different biomarkers in combination from the scanning spectrum of gratifying resolution within 45 min scan duration (**Figure S17-S20**). Considering the high throughput analysis of samples, still it is required to analyze many samples at that stipulated time, which can be addressed by the advancements of current technologies in future. A comparative analysis of conventional IHC and SERS techniques explaining the pros and cons of both techniques is summarized in **Table 1**.

Table 1: Comparative pros and cons analysis of SERS vs IHC

Criteria	***Pros of SERS over IHC***	
	IHC	**SERS**
Multiplex detection of biomarkers	Difficult to attain due to the deficiency of standard methods	Easily possible
Sample preparation time	7-24 h	5-6 h
Secondary antibody and developing agents	Required	Not required
Analysis time	0.5 h/sample/one biomarker	1h/sample with multiple biomarker
Performance / Precision	Subjective due to the possibility of inter-observer error	Objective in nature and permits semiquantitative measurement
Criteria for biomarker grading	Based on percentage of stained cells and stain intensity	SERS mapping based on average spectral intensity
HER2 receptor grading	2^+/Equivocal samples required FISH confirmation	By ratiometric semi quantitative approach FISH confirmation can be justified
Criteria	***Cons of SERS over IHC***	
	IHC	**SERS**
Instrument cost	Inexpensive bright field histopathology microscope	Expensive Raman microscope is required
Clinical validation	Clinically validated technique	Clinical validation yet to be done
High throughput analysis	Automated systems are available	Advancement in the technique is required
Requirement of artificial intelligence (AI)	Not required	With the support of AI method, the technique can be improved for quantitative detection.

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3. Conclusion

In summary, we have successfully introduced RL-SERS-nanotags based diagnostic platform for the detection of clinically relevant breast cancer biomarkers in singleplex, duplex as well as triplex fashion of IHC-confirmed breast cancer tissue subtypes. Optimized analysis mode of the RL-SERS-nanotags along with untargeted isotype control SERS-nanotag rendered least non-specific binding and ensured to minimize the false positive results. Moreover, HER2 grading by ratiometric profiling of HER2 and isotype control tags eventually confirmed the 1^+, 2^+ and 4^+ tissue samples were perfectly complementing with time-consuming IHC as well as expensive FISH analysis. Finally, we have executed the whole area Raman mapping of a single specimen with the multiplexing RL-SERS tags reflecting the capability of the newly emerged platform to provide rapid results in less than an hour with minimal non-specific binding. The study thus reveals a robust and highly sensitive diagnostic modality with futuristic potential for the detection of tumors as well as tumor recurrence exhibiting differential biomarkers associated with patient-to-patient heterogeneity. The inability of the system to perform high throughput analysis in a fixed time may be improved by the introduction of advancements in instrumentation in near future.

4. Experimental Section

Detailed methodology of nanoparticle synthesis, characterization, RR incorporation, antibody conjugation, biocompatibility analysis, tissue processing methods and SERS analysis parameters are provided in the supporting information.

Supporting Information

Supporting Information is available from the journal site.

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Conflict of Interest

The authors declare no conflict of interest.

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TOC

Raman spectroscopy driven histopathologic approach have been evolved for the clinical diagnostics utilizing targeted Raman-label-SERS (RL-SERS)-nanotags which ensures a rapid, sensitive and accurate multiplexed detection of clinically relevant breast cancer biomarkers, ER, PR and HER2 in single tissue specimen by the marked signature Raman fingerprint resembling the corresponding biomarker.

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

ToC figure