Raman Fingerprints in Detection of Breast Cancer

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Abstract

Diagnosing cancer with high specificity, sensitivity and trauma-free procedure is a major challenge. Development of spectroscopic methods for the detection of cancer has met this challenge with reasonably remarkable success. Raman spectroscopy stands out in comparison to other spectroscopic methods due to its flexibility in collection volumes, non-destructiveness and its ability to differentiate between chemical structures, even with extremely small changes in the atomic arrangements. Raman spectroscopy produces a unique fingerprint of a particular biomolecule. This property helps in qualitative and quantitative analysis of the molecules in a cancer tissue, thereby distinguishing a normal cell from that of the one that is cancerous. Different types of Raman spectroscopic techniques such as SERS (Surface-Enhanced Raman Spectroscopy), CARS (Coherent Anti-stokes Raman Spectroscopy), FTRS (Fourier Transform Raman Spectroscopy) and RRS (Resonance Raman Spectroscopy) have been used to intensify Raman signal, and have shown promising results in cancer diagnosis. The availability of a variety of spectroscopes has also broadened the scope for Raman imaging and *in-vivo* diagnosis of cancer lesions. This research paper reviews a number of Raman spectroscopic methods for the detection of breast cancer.

Keywords: Breast cancer; Raman spectroscopy; Raman fingerprints; Diagnosis; Biomarker

Breast cancer is a disease that presents a major threat to women today [1], with nearly half a million deaths attributed mainly to the lack of early diagnosis [2,3]. Common types of breast cancer include Invasive Ductal Carcinoma (IDC), Invasive Lobular Carcinoma (ILC), Ductal Carcinoma *in-situ* (DCIS), Atypical Ductal Hyperplasia (ADH), Fibroadenoma (FD), and Inflammatory Breast Cancer (IBC) [4-6]. Although the exact reason for the cause of cancer is still not clear, scientists have found hormones, proteins, lipids and genes involved in signaling pathways as a cause. Therefore, identification and monitoring of abnormal activity of these biological molecules can significantly reduce the number of deaths [7-12]. Currently, standard techniques employed for breast cancer diagnosis include X-ray Mammography, Ultrasonography, Positron Emission Tomography and Magnetic Resonance Imaging [9,13,14]. However, these techniques reflect poor resolution, sensitivity and specificity, especially in scanning younger women having higher breast density [13]. These tests are expensive and time consuming. Identification of tumor

markers also has a place in cancer diagnosis, but these are rarely used in clinical practice [15]. Histopathologists carry out surgical procedures on cancer lesions for their characterization, and this procedure is known to induce trauma in patients [16].

The demand for highly accurate, sensitive and non-invasive techniques necessitated the scientists to introduce optical methods for cancer diagnosis [16]. The techniques are a) Fluorescence Mediated Tomography, b) Photo Acoustic Imaging, and c) Optical Acoustic Imaging. These are accurate, non-destructive, rapid and affordable [13,17]. These techniques help in early diagnosis of most cancers and also mediate response to cancer treatment. However, one of the widely used optical methods is Fluorescence spectroscopy. This method helps in analyzing gene amplification and expression, and other protein mechanisms [15]. However, Fluorescence spectroscopy tends to poorly identify breast cancer due to photo-bleaching effects. This limitation is due to presence of endogenous fluorophores in a breast tissue [18].

Raman spectroscopy on the other hand is an optical method that is more advantageous over fluorescence spectroscopy [11]. Firstly, it does not require external labeling agents, because contrast is dependent on optical property of the biological tissue obtained due to polarizability in the tissue under irradiation [13]. Secondly, Raman spectroscopy works on the principle of Raman scattering, i.e., a vibrational phenomenon involving inelastic scattering [7,19]. Therefore, it provides better biochemical information of the tissue in comparison to fluorescence microscopy, thus helping us with better understanding of chemical changes that occur in abnormal breasts [15]. Raman spectroscopy can also identify both normal and cancerous tissues based on morphological changes [11], and distinguish them by detecting structural changes in amino acids and concentration level changes in biomolecules [20]. In recent years, Raman spectroscopy has shown better sensitivity, spatial resolution and real-time information making its availability in various fields such as risk assessment of drugs on skin [21], gas masks [22], and paper industries [23]. These properties allow coupling of Raman spectroscope with optical fibers for *in-vivo* cancer detection, replacing conventional biopsy in vogue [13].

Raman Spectroscopy

Raman spectroscopy uses confocal micro-spectroscopy for acquisition of spectrum [15]. The system consists of an inverted research microscope which allows efficient back-scattering of incident radiation and an integrated Raman spectrometer [7]. The Raman spectrometer (see Figure 1) is provided with a Near-IR laser as an excitation source focused onto the sample by collimation of radiation through a 50x objective [24]. The back-scattered radiation passes through a notch filter to eliminate Rayleigh scattering and Raman signal is then dispersed by grating onto a Detector i.e., CCD chip for data acquisition [25]. A 50 μ m pin-hole slit is used after the notch filter to remove stray light from the surroundings. The power of excitation source used is about 20mW and the integration time for spectra acquisition is set to 45s [24].

Raman spectroscopic methods

Raman bands for breast cancer biomarkers (see Figure 2) can be obtained from 300-1800 cm^{-1} known as the fingerprint region. This is a region, where a particular peak is a fingerprint of a specific molecule [9]. Apart from DNA and proteins, the above frequencies provide information about lipids [4]. Raman peaks for biological molecules depicting abnormal concentration in a cancer cell is sharper and stronger, whereas a normal cell concentration would result in a broader spectrum [26]. Therefore, Raman signal is directly proportional to the 'product' concentration [7]. However, there are reports of high intensity peaks for lipids in normal tissue compared to those found in cancer tissue [17], and the readers should be wary of this.

Conventional Raman spectroscopy has low efficiency considering that only a tiny portion of radiation is in-elastically scattered [27], which are in magnitudes ~ 14 times smaller in magnitudes in the order 14 times smaller than that of the fluorescent techniques. However, the magnitudes can be increased up to 10^8 to 10^{11} times to obtain higher sensitivity [13]. Section-3 discusses three Raman Spectroscopic techniques

(Surface-Enhanced Spectroscopy (SERS), Coherent Anti-Stokes Raman Scattering (CARS) and Fourier Transform Raman spectroscopy (FTRS) & Resonance Raman Spectroscopy (RRS)) used in breast cancer detection, and further evaluates their performance in obtaining a Raman signal.

Surface-Enhanced Raman Spectroscopy (SERS)

Nanoparticles of noble metals such as Au, Ag and Cu are introduced into cell to be analyzed in order to increase the sensitivity [28]. These metals are known to display intense band upon absorption in UV-VIS which is not characteristic of its corresponding bulk metal [29]. The intense band formation is due to incident photon frequency being in resonance with the collective oscillation of conducting electrons [28]. This phenomenon is utilized in creating a strong electromagnetic field along the nanoparticle surface, thus amplifying Raman signal of the molecule along the surface (see Figure 3) [25,30]. AuNP, (Silver nanoparticles) have also been used to monitor anti-cancer drug delivery [31].

Colloidal silver nanoparticles which were infused into the breast cancer cells [30], showed enhancement of peaks at 800-900 cm^{-1} , 950-1100 cm^{-1} and 1151-1400 cm^{-1} which are Raman fingerprints of a cancerous cell. They revealed clarity in the region of 1150-1400 cm^{-1} corresponding to amide 3, a protein band closely linked to breast cancer which was not possible using conventional Raman spectroscopy [28]. SERS spectrum at the wave number 1619 cm^{-1} also provided for detailed studies of this protein [2]. Results have suggested SERS as a strong tool for identifying chemicals constituents such as phenylalanine, tryptophan, tyrosine and detecting changes in DNA structure [25]. These were possible due to enhancement of intensities of respective constituent peaks by this technique [28]. The interaction between Gold Nanoparticles (GNP_s) and protein through binding site at phenylalanine was identified through peak at 1030 cm^{-1} in the SERS spectra [25,29]. GNP based SERS could carry out live cell targeting of Epidermal Growth Factor

Figure 1: Raman Spectrometer setup [This figure was prepared in-house by the authors].

Figure 2: Cancer Biomarkers identified using Raman Spectroscopy.

Receptor (EGFR), a biomarker of cancer cell and also perform imaging of EGFR. Research on using SERS to detect EGFR using knock-out cells at cellular level is also being carried out [32]. A novel method to target HER2-positive breast cancer cells (Human Epidermal Growth factor Receptor) with the help of a SERS probe

enabling conjugation of proper targeting ligands unlike anti-HER2 antibody has also been developed [30]. SERS displays significant enhancement in the spectra allowing easy interpretation of data, and this is crucial in diagnosing living tissue where the electron density is minimal and conventional Raman spectroscopy can

Figure 3: This figure illustrates the initial enhancement of electric field near the vicinity of the metallic nanoparticle (E_{Loc}) induced by excitation of Plasmon resonance followed by the Enhancement of Raman Scattering (E_{Scat}), thus providing Enhanced Raman Signal (E_{SERS}) allowing detection of lower concentration molecules through SERS technique. [Reproduced with written permission from Boujday, et al. [38]; Licensee Sensors, 2015].

Figure 4: Experimental setup of CARS depicting usage of pump and Stokes beam lasers (i.e., Ti: sapphire laser source) for simultaneous excitation and therefore, detection of several coherent vibrations providing highly specific and stronger magnitude Raman signal at anti-stokes frequency. [Reproduced with written permission from Li B, et al. [39]; Licensee SPIE 2011].

produce only a very weak signal. Furthermore, the ability of SERS to detect cancer phenotypes and proteins expressed on the surface simultaneously enables early diagnosis of breast cancer.

Coherent Anti-Stokes Raman Spectroscopy (CARS)

Coherent Anti-Stokes Raman Scattering (CARS) (see Figure 4) is a technique based on introduction of Raman active molecular vibrations, which results in enhanced anti-stokes signal [7]. It can be noted that each of the spectra obtained from same point of both the cancer and normal cell were the same (see Figure 5). However, the only difference was that the intensity of the peaks

in cancer cell was stronger and sharper [33]. Raman spectra of a noncancerous tissue reveal evident peaks ranging from 877 - 3009 cm^{-1} . The vibration of C-C and C = C of the polyene chain at 1158 and 1518 cm^{-1} denote presence of carotenoids in normal breast tissue, which are devoid of in a IDC Raman spectra [13]. CARS images depict large number of bright spots representing lipid droplets in the cell. Based on the fact that lipids are building blocks of membrane provide nutrition and store energy, this imaging technique could distinguish between normal cell and cancer cell considering abnormal lipid content [18].

The hormone mediated malignant growth of breast cancer

cells used Medroxyprogesterone Acetate (MPA) and synthetic androgen R1881 to study hormone-mediated lipogenesis. The study indicated increase in size and number of intracellular lipid droplets based on CARS images (see Figure 6) obtained from hormone treated & control breast cancer cells, and also to develop a better method of quantifying lipids [7]. CARS make it possible to differentiate a cancer cell from a normal cell, at a cellular level without the necessity of identifying a biomarker and also use of a fluorescent dye. Furthermore, CARS images help in studying how hormones and lipids play a role in cancer progression.

Fourier Transform Raman Spectroscopy (FTRS) & Raman Resonance Spectroscopy (RRS)

In-vivo and *ex-vivo* studies on the mammary glands of rats have used high frequency Fourier Transform Raman spectroscopy (see Figure 7) to distinguish between normal and cancerous tissues [26]. Optical fibers were used to carry out *in-vivo* experiments [18]. Unlike normal spectrometers which use lower frequencies called as “fingerprint regions” i.e., 500-1800 cm^{-1} (see Figure 8a) [24], FT-Raman spectrometer uses higher frequency i.e., 2400–

3800 cm^{-1} (see Figure 8b) to obtain information for characterizing and differentiating normal and cancer tissues. The study also demonstrated the use of FT-Raman spectrometer to collect information on secondary & tertiary structures and also level of hydration in cancer tissues [26]. Research has shown that bands around 2854 cm^{-1} , 2895 cm^{-1} , and 3010 cm^{-1} associated with lipids are stronger in normal tissues, than for cancer tissues [19]. Also, the higher frequency region allows the calculation of TUFA/ TFA (i.e., ratio of total unsaturated fatty acids and total amount of fatty acids), whose value can be used as a molecular marker to identify normal from cancerous cells. A cancerous cell usually indicates low ratio value depicting presence of high amount saturated fatty acids i.e., higher concentrations of palmitic and stearic fatty acids. Hormones play a major role in converting these unsaturated fatty acids into saturated fatty acids provoking cancer induction [7].

Confocal Micro RR Spectroscopy (see Figure 9) detect better vibrations in biological tissues, provide higher resolution for biological changes at molecular level, and detect more vibrational modes because the excitation wavelength is close to molecular

Figure 5: Typical Raman spectra obtained from the various colored points in non-cancerous tissue, necessary in identifying biochemical composition and studying Raman vibrational pattern behavior. [Reproduced with written permission from Surmacki, et al. [13]; licensee BioMed Central Ltd. 2013].

Figure 6: Autofluorescence and CARS images of treated versus vehicle control breast cancer cells. (a) T47D-MPA autofluorescence image, (b) T47D-MPA CARS image, (c) T47D-V autofluorescence image, and (d) T47D-V CARS image [Reproduced with written permission from Potcova, et al. [7]; Licensee SPIE 2014].

Figure 7: Experimental setup of FTRS [This figure has been prepared in-house by the authors].

absorption wavelength [34]. Since a Fourier Transform Raman spectroscopy produces Raman bands in the higher frequency region, it differentiates a normal and an abnormal breast tissue based on peak intensity. The spectrum is devoid of noise from optical fiber signal, thus it can be used for obtaining accurate results for different altered states of a biomolecule. This paves way for the usage of FTRS as a diagnostic tool for *in-vivo* cancer studies. Raman Resonance Microscope (RRM) reveals high sensitivity in detecting carotenoids and lipids as Raman biomarkers of breast cancer, thereby clearly distinguishing a normal, benign and cancerous breast tissue. Therefore, both FTRS and RRM hold good possibilities in future to carry out real-time *in-vivo* and *ex-vivo* detection of breast cancer.

Raman Spectra Data Processing and Data Analysis

The spectra obtained cannot be directly used to extract required signal, the acquired spectrum contain signals from the glass, PBS solution and cosmic rays, MATLAB is used to nullify this unwanted data [7]. Spectrum processing is also done to simplify computation and to ensure high performance in cancer

detection. Microcal Origin pro 7.0 software is used to remove fluorescence in the background by fitting spectra to a quadratic equation [34]. GRAMS/ AI spectroscopy software removes the background signal of the quartz slide used to hold the sample [15]. Ocean optics QE65Pro Raman spectrometer yields noise and fluorescence in the spectra background, resulting in smaller peaks (see Figure 10). MATLAB software is used to reduce noise by Symmlet-5 wavelet filter and fitting spectra to third-order polynomial to remove fluorescence background to yield evident peaks. Data processing thus allows us to confirm changes that occur during tumor formation i.e., configurationally, qualitative and quantitative changes in proteins, lipids and nucleic acids through revealing evident peaks at 1078, 1305, 1447 cm^{-1} etc., indicating dominance of lipids levels in normal tissue and peaks at 1083, 1278, 1453 cm^{-1} indicating presence of proteins in cancerous tissue. (see Figure 11) [35].

Data analysis involves normalizing the spectra i.e. vector normalization, division by standard deviation and mean subtraction [33]. Principal Component Analysis (PCA) allows unsupervised technique to classify noncancerous and cancerous

Figure 8: Raman spectra of pure substances at (a) Fingerprint region (b) Higher frequency (used in FTRS) [Reproduced with written permission from Potcova, et al. [7]; Licensee SPIE 2014].

Figure 9: Experimental setup of Con-focal RRS [Reproduced with written permission from Casabella, et al. [40]; Licensee RSC 2016].

tissue. The dimensionality of the dataset is reduced by PCA resulting in set called Principal Components (PC_s) [4].

Classification using lipid phenotypes have been done using PCA analysis for spectral region from 2820-3030 cm^{-1} . Raman bands 3014, 2890 and 2848 cm^{-1} associated with lipids and band at 2940 cm^{-1} associated with lipid and protein accounted for PC1 score. Raman bands at 2846 cm^{-1} (i.e., TFA) and 2935 cm^{-1} associated with chain end CH_3 accounted for PC2. This

allowed grouping and interpretation of lipid content in different cancer phenotypes. Also the researchers hypothesized presence of cholesterol corresponding to band at 2940 cm^{-1} , due its large number of terminal $-\text{CH}_3$ groups. They associated role of cholesterol in proliferation and metastatic ability of breast cancer cells (see Figure 12) [11]. This is required because a single spectrum contains large number of information, and PCA helps to extract important components. PCA is followed by K-means algorithm which helps in grouping the data sets [4].

Figure 10: Raman spectra of breast tissue before processing [Reproduced with written permission from Li Q, et al. [41]; licensee Biomedical Optical Express, 2014].

Figure 11: Raman spectra of breast tissues after preprocessing [Reproduced with written permission from Li Q, et al. [41]; licensee Biomedical Optical Express, 2014].

Principal components are then used as inputs for a discriminant analysis called Linear Discriminant analysis (LDA), which is a prediction method to differentiate groups involved (see Figure 13). LDA provides information on sensitivity and specificity of the experiment carried out [36]. Therefore, the multivariate analysis and classification models help in using these datasets for objective diagnosis and quantitative measurements of large number of new patient samples without supervision [37]. Data processing makes optical classification of non-cancerous and cancerous tissue simple, and also prevents the corruption of data from external signals, which play important role during *in-vivo* application. Data analysis method also helps in diminishing

background interference and other noise signals. This method further increases the sensitivity and spatial resolution of different Raman spectroscopic techniques, thus promoting screening of large number of samples for classification especially in clinical studies.

Conclusion

Detection of breast cancer with high specificity and sensitivity has been a major challenge in cancer diagnostics. As has been presented in this review, breast cancer can be diagnosed with Raman spectroscopic methods such as SERS, CARS, FTRS and RRS. These techniques have shown promising results due to their

Figure 12: PCA scores used to develop principal components based on the cell variability present inside each cell line and between the different cell lines. [Reproduced with written permission from Nieva, et al. [11]; licensee Plos One, 2012].

Figure 13: Discriminant analysis model of non-metastatic and metastatic cell lines. [Reproduced with written permission from Nieva, et al. [11]; licensee, Plos One, 2012].

versatility and sensitivity. However, there has been considerable research on other Raman spectroscopic methods in breast cancer detection such as Tip-Enhanced Raman Spectroscopy (TERS), Optical Tweezers Raman Spectroscopy (OTRS), and Laser Tweezers Raman Spectroscopy (LTRS). Several Variations of Raman spectroscopy have also been attempted in order to enhance sensitivity and spatial resolution. The property of Raman spectroscopy that a particular biological molecule forms a peak at a particular wave number irrespective of the wavelength of the excitation source makes these techniques more reliable and accurate. Raman Spectroscopy is also being exploited to study hormones, proteins, lipids and genes involved in signaling pathways in cancer formation.

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