Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 152 (2016) 58–63

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Polarized Raman spectroscopy unravels the biomolecular structural changes in cervical cancer

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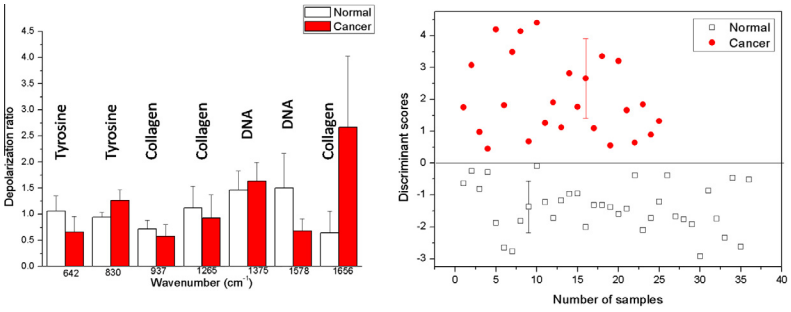
HIGHLIGHTS

- Tissues were characterized by polarized and conventional Raman spectroscopy.
- The spectra were statistically analyzed by Linear Discriminant Analysis.
- Polarized Raman spectroscopy yielded better classification accuracy.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 7 April 2015
Received in revised form 12 June 2015
Accepted 17 June 2015
Available online 3 July 2015

Keywords:
Polarized Raman spectroscopy
Cervical cancer
Cancer diagnosis
Linear Discriminant Analysis

ABSTRACT

Polarized Raman spectroscopy has emerged as a promising technique giving a wealth of information about the orientation and symmetry of bond vibrations in addition to the general chemical information from the conventional Raman spectroscopy. In this regard, polarized Raman Spectroscopic technique was employed to study the changes in the orientation of biomolecules in normal and cancerous conditions. This technique was compared to the conventional Raman spectroscopic technique and was found to yield additional information about the orientation of tyrosine, collagen and DNA. The statistically analyzed depolarization ratios by Linear Discriminant Analysis yielded better accuracy than the statistical results of conventional Raman spectroscopy. Thus, this study reveals that polarized Raman spectroscopy has better diagnostic potential than the conventional Raman spectroscopic technique.

1. Introduction

Cervical cancer is one of the most common gynecological malignancies with the risk factors, including human papillomavirus (HPV) infection, smoking, altered immune status (HIV or AIDS infection), oral contraceptives, exposure to carcinogenic chemicals and multiple parity. Among these, there is overwhelming evidence that the presence of HPV is the most important factor initiating the pathogenesis of cervical cancer [1]. It is the fourth most common cause of cancer death in women worldwide, with 528,000 new cases reported every year, and a mortality of 266,000 in 2012 [2]. However, it is a slow growing cancer and often takes 10–20 years to progress from the neoplastic stage to invasive carcinoma [3]. This slow cell proliferation makes it feasible for early diagnosis and design an appropriate treatment to combat this disease, to improve the survival rate and quality of life. Currently, early diagnosis by periodic screening at cytological level by Pap smear test has a high specificity (~93%) but a low sensitivity (~57%) [4]. On the other hand, screening by colposcopy provides 96% sensitivity with a low specificity of 46% [5]. As the diagnostic accuracy of the currently available diagnostic modalities are not satisfactory, there is an imperative need for an efficient diagnostic modality.

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http://dx.doi.org/10.1016/j.saa.2015.06.053
1386-1425/

In this context, pathologists and oncologists are looking for alternate or complementary screening modalities, in particular at the molecular level.

Various optical methods have been considered to characterize the cells and tissues at the molecular level. Of these modalities, the pioneering work of Alfano and his group opened a new arena for the diagnosis of cancer by Raman spectroscopy [6] and has been increasingly explored since then. Following this, many studies have been carried out for the diagnosis of various cancers, including cervix [7–10], oral [11,12], kidney [13], skin [14], breast [15], esophagus [16], laryngeal [17], bladder [18], prostate [19] cancers, etc. All these studies in conjunction with various statistical techniques or models have achieved appreciable overall accuracy [20].

One of the complementary techniques of Raman spectroscopy is polarized Raman spectroscopy (PRS). PRS is observed as a result of interference of polarized light with vibrating molecules and gives valuable information about the orientation and conformation of the molecules probed and is utilized for the assignment of vibrations. Every vibrational mode has a unique differential polarizability ellipsoid and the Raman scattering intensity depends on the shape of the ellipsoid and the incident polarized light. The shape of the ellipsoid is determined by measuring the depolarization ratio (ρ) and is defined as the ratio of intensities of the perpendicular to the parallel component of the scattered radiation, with the s in parallel direction i.e.,

$$\rho = \frac{I_{\perp}}{I_{\parallel}}$$

where $I_{\perp}$ and $I_{\parallel}$ represents the Raman scattering intensities when the polarization direction of the analyzer is perpendicular and parallel to that of the incident beam, respectively [21,22]. Depolarization ratio, also provides valuable information about the symmetry of normal mode vibrations that are indispensable in making band assignments for the analysis of chain conformation [23] as well as the secondary structure [24,25] and molecular orientation [23,26].

This technique has also been extended for various biomedical applications. For example, PRS has been used to obtain collagen and mineral orientation in bones and cartilage for 3D structure determination and diagnosis of bone disruption [27–31]. It has been also utilized to study the intercalation of ethidium bromide with DNA [32]. Recently, PRS also has gained attention in the field of diagnosis of cancer. Olivier Piot and his co-workers have utilized this technique for discriminating healthy epidermis and basal cell carcinoma [33,34]. To the best of our knowledge, no data is available on the characterization of cervical tissues by PRS.

In this regard, this pilot study is aimed to characterize and compare the PRS signatures with conventional Raman spectral signatures to know whether there exists any additional wealth of information about the tissues during the transformation of normal into cancer cells and tissues. Discriminant analysis of Raman spectra of normal and cancer tissues and depolarization ratio values by Linear Discriminant Analysis (LDA) was carried out. The conventional Raman spectroscopy yielded 82.0% accuracy, whereas PRS yielded 100% accuracy of the original dataset.

2. Materials and methods

2.1. Sample preparation

Cervical tissue samples from both normal ($n = 36$) and malignant ($n = 25$) were obtained from the Aringar Anna Cancer Hospital and Research centre (Kancheepuram, India) with previous consent of the patient and ethical clearance from the hospital. Every tissue was sliced to 20 μm thickness and mounted on a quartz slide. Raman measurements were acquired, following which the same tissue slice was Haematoxylin and Eosin (H&E) stained for histopathology report.

2.2. Raman instrumentation and spectral acquisition

The schematic setup used for polarization Raman spectroscopic measurements is shown in Fig. 1. Polarization-dependant Raman spectra were recorded with a LabRam microspectrometer (Horiba Jobin Yvon, Lille, France). The excitation beam from a diode laser 784.12 nm was focused on the sample using a 50× objective (numerical aperture = 0.75, Olympus). Rayleigh scattering was removed by an edge filter. For these experiments, the confocal hole was set to 400 μm. The Raman Stokes signal was dispersed with a holographic grating (600 grooves/mm) and data was recorded using a Peltier cooled charge-coupled device (CCD) detector (1024 × 256 pixels). As displayed in Fig. 1, an analyzer was placed before the confocal slit of the spectrograph to allow the detection of the polarized scattered light along the directions parallel and perpendicular to the incident polarized light. Three spectra were recorded at each position, one using conventional Raman spectroscopy and the other two using polarized Raman, with the polarizer oriented parallel and the analyzer oriented along the parallel or perpendicular axis. For every sample, the Raman spectra were acquired from four to six positions and the average of them were used for statistical analysis. The acquisition time was set as 30 s for the spectral range of 600–1800 cm^{-1}.

2.3. Data processing

Every Raman instrument has a unique spectral responsiveness; hence, to correct this, an intensity calibration factor was applied to every raw spectrum. Successively, the spectra were baseline corrected and smoothened by Savitzky-Golay filter using the Labspec 5 software provided by Horiba Jobin Yvon.

Depolarization ratios for the bands of tyrosine (642, 830 cm^{-1}), collagen (937, 1265 and 1656 cm^{-1}), DNA bases (1373, 1578 cm^{-1}) were calculated. These bands were identified as potential markers, as collagen and DNA undergo changes due to oncogenesis. Both are highly structured molecules and in malignancy, their orientations are found to change [35,36]. These peaks were analyzed by Linear Discriminant Analysis (LDA) and the intensities of these peaks from conventional Raman spectroscopy was also analyzed by LDA [37].

3. Results

A number of reports [7–10] have been published, using the conventional Raman spectroscopy in conjunction with statistical methods for cervical cancer diagnosis, with appreciable accuracy to the maximum of 97% taking into account the hormonal status of the patient [10]. In the present study, Raman spectra of histologically confirmed normal and carcinoma of cervix were measured in the fingerprint region, i.e., from 600 to 1800 cm^{-1}. Fig. 2 shows the averaged spectrum of normal (Fig. 2a) and cancerous tissue (Fig. 2b). Briefly, the normal and cancerous cervical tissues have shown prominent Raman vibrational peaks at 642 cm^{-1} (Tyrosine in collagen), 814 cm^{-1} (C–C stretch backbone in collagen), 830 cm^{-1} (Tyrosine), 836 cm^{-1} (O–P–O backbone of DNA), 855 cm^{-1} (Collagen), 874 cm^{-1} (C–C stretch of 4 hydroxy-proline in collagen), 920 cm^{-1}(C–C stretch of Proline ring in collagen), 937 cm^{-1} (Collagen), 1002 cm^{-1} (Phenyl ring/tryptophan breathing mode), 1031 cm^{-1} (Proline), 1207 cm^{-1} (Tyrosine & Phenylalanine), 1243 cm^{-1} (Amide III), 1265 cm^{-1} (Amide III), 1373 cm^{-1} (DNA/RNA bases), 1445 cm^{-1} (CH_2/CH_3 deformation),