

Review Article

Review on Application of Raman Spectroscopy in Oral Diseases: A Ray of Hope

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Abstract: Oral cancers are known to have catastrophic effects on individuals and the society by and large. They may have an indolent appearance in the preliminary stages which warrants an even more precision in their diagnosis. One of the major drawbacks in the management of oral cancers is the inability to detect it in the early stages causing failure in prompt treatment. Unfortunately, inspite of voluminous work in this regard there is still search of a dependable diagnostic implement. This hunt is even more amplified in developing countries. Optical diagnostic techniques can give impressive results in clinical set-ups. This review discusses about using Raman principle including raman spectroscopic use in medical and dental field with a special emphasis of Shifted Excitation Raman Difference Spectroscopy (SERDS) and tries to address concerns about this non-invasive technique. Through this paper we wish to focus reader's attention towards this virgin area with an intention to take a step further towards the identification and cure of oral diseases.

Keywords: Bio-Optical; Spectroscopy; Oral Diseases

INTRODUCTION

Mankind has suffered a lot due to the deleterious effects of Tobacco. Oral cancer has made its presence felt with a stronger impact on developing nations due to low socio-economic status. Despite, improvisation in oral cancer treatment, the 5year survival rate has remained 50% for the last 50 years [1]. This percentage had increased (63.2%) for a period between 2005 to 2011 in United States [2]. Tobacco related cancers (TRC) for males in India are estimated to go up from 190,244 in the year 2010 to 225,241 in the year 2020 [3,4]. The problem is multiplied by the fact that there are very few resources to identify malignancy in preliminary stages. Clinical diagnosis has played a vital role in this respect. Diagnostic aids such as Oral brush biopsy, Toluidine blue staining, Chemiluminescence, Tissue Fluorescence Imaging, Tissue Fluorescence Spectroscopy [5] have been intensely investigated. These procedures have drawbacks which can influence the diagnosis and prognosis of patients. Oral brush biopsy is a very superficial investigation and a need for biopsy cannot be ruled out. Fluorescence spectras can be obtained easily from tissues. However, they have a major problem that it is very difficult to obtain biochemical information from the broad based fluorescence bands [6]. Exfoliative cytology is a routinely practiced screening technique for oral cancers. It has its own limitations as it requires extremely trained staff, is

difficult to standardize and moreover it suffers from inter and intra-observer variability [7]. Due to recent advancements in imaging techniques, the sole important goal of identification of oral precancers cancers does not seem farfetched. Bio-optical diagnostic tools are proving their worth in bridging the gap between early diagnosis and treatment planning [8]. Optical imaging technologies are being investigated for improving cancer diagnosis and treatment, as they can provide rapid, real-time tissue evaluation with a high degree of spatial resolution [9,10]. Spectroscopy has emerged as the latest trend in diagnosing potentially malignant disorders in its early stages so as to tackle this deadly disease eventually reducing its mortality [11-15]. Received Raman spectra from biological samples produces a "fingerprint" representing the molecular vibrations specific to chemical bonds, thus yielding information of a samples chemical (or biochemical) composition [16]. Although there is sufficient literature on use of Raman Spectroscopy (RS) for various cancers [17-21] its applicability for oral precancers and cancers is still being explored. This review is written with the intention that readers explore the use of Raman spectroscopy for identifying the oral precancer and cancer thereby eliminating or atleast limiting the use of biopsy which is an invasive technique.

PRINCIPLE OF RAMAN SPECTROSCOPY

RS is a scattering technique. It is based on Raman Effect, i.e., frequency of a small fraction of scattered radiation is different from frequency of monochromatic incident radiation. It is based on the inelastic scattering of incident radiation through its interaction with vibrating molecules. It probes the molecular vibrations [22,23].

When light interacts with matter, the photons which make up the light may be either absorbed, scattered or may not interact with the material and may pass straight through it. The scattered photons can be observed by collecting light at an angle to the incident light beam, and provided there is no absorption from

any electronic transitions which have similar energies to that of the incident light, the efficiency increases as the fourth power of the frequency of the incident light. However, the main scattering technique used for molecular identification is Raman scattering [24]. The fundamental point here is that photons interact with molecules and disturb the electrons leaving it in an unstable stage. This leads to two kinds of scattering, a dominant Rayleigh scattering and less but more informative Raman scattering [25].

History: Since its introduction in 1928 by C.V. Raman, Raman effect has undergone lot of changes in the instrumentation [Table 1].

Table 1: Raman Effect has undergone lot of changes in the instrumentation

S. No	Year	Changes	Implications
1.	1928	Raman and Krishnan used filtered sunlight as a radiation source. They observed the scattering light visually, using a set of compensating colored filters to enhance the optical sensitivity	Raman effect was introduced in its most inexpensive and using natural resources.
2.	1940	Monochromators incorporated in spectrometers were used with 2 or 3 dispersion stages	This reduced the elastic Rayleigh scattering
3.	1952	Mercury Toronto arc lamp was used inevitably	Improved radiation source
4.	1960	Laser was used as a monochromatic source.	This made examination of small size samples with production of excellent spectras.
5.	1970	Introduction of Fourier transform infrared instrumentation	Increased accuracy. Dramatic improvement in achievable signal-to-noise ratio
6.	1986	Methods to reduce fluorescence were employed since many years. This led to introduction of Nd:YAG lasers with FT raman Spectroscopy	This reduced fluorescence to a great extent making raman spectras more usefull in terms of interpretation
7.		Introduction of holographic notch filters	Made use of successive dispersion stages unnecessary thus increasing the luminosity of Raman experiment.
8.	1987	The charge coupled device (CCD) was first used for raman spectroscopy	It reduced the dark current and made raman spectras more
9.	1990s	SERDS technique introduced by Shreve, Cherepy and Mathies	Raman signal could be recovered as a derivative signal even when Raman was not detectable with single excitation.
10.	Early 1990s	Confocal Raman Microscopy was studied extensively	Increased spatial resolution and rejection of background light

Application of Raman Spectroscopy in dental field

Sequential progression in experimental oral carcinogenesis in Hamster buccal pouch model was investigated by Kumar *et al.* They observed that lipids dominated in the early stages of cancer and as it progressed there was protein dominance and protein to lipid ratio increased [26]. Cals FL investigated surgical margins of tumor and were able to distinguish between Oral squamous cell carcinoma(OSCC) and healthy tissues [27]. Schleusener J *et al* applied in vivo application of raman spectroscopy in malignant

Melanoma, Basal Cell carcinoma and Squamous cell carcinoma. Discrimination proved to be unsuccessful between cancerous lesions and suspicious lesions that had been histopathologically verified as benign by dermoscopy [28]. Carvalho LF *et al* applied raman spectroscopic technique to dysplastic cells (oral pre-cancers) and oral squamous cell carcinomas. They collected raman spectras from nucleoli, nuclei and cytoplasm and they were able to discriminate between pre-cancer and cancer. They have proposed it as a rapid screening technique [29]. Although there is an

explosion of raman spectroscopic studies in cancers, its applicability in the diagnosis part of oral pre-cancers and cancers is not explored much. Few studies related to changes between oral pre-cancers, cancers and normal tissues have shown that this optical method can be used as a non-invasive technique [30,31].

Oliveira AP *et al* using Fourier-transform Raman Spectroscopy concluded that the chief difference between normal and malignant spectra arising from respective tissues from the oral cavity is due to its composition, conformational, and structural changes of proteins, and possible increase of its content in malignant epithelia [32].

In a pioneer study carried out by Venkatakrishna K *et al*, they collected raman spectras from normal and malignant oral tissues. They found significant differences between the spectras of the two kinds of tissues. According to them this difference may be because of two reasons:

1. Malignant cells have broad variety of receptor-type molecules (glycoproteins) associated with their external membranes. On the other hand cells of normal tissues are well organized and have bilayer membranes as compared to malignant cells which have lost their orientation when illuminated by laser beams.
2. Many cancer cells induce dense fibrous stroma which will alter the spectrum [33].

Drawbacks in Raman Spectroscopy & Methods to overcome them

The basic and the most fundamental problem with Raman spectroscopy, is its inaccuracy due to the more dominant Fluorescence spectras which interfere with it. There have been documented methods to reduce this interference. These are as follows:

1. Mathematical methods include first-order and second order derivatives, frequency domain filtering, polynomial fitting and wavelet transformation methods. Of these, polynomial curve fitting and advanced subtraction methods have been used successfully [34,35]. Automated methods for polynomial curve fitting have been preferred over manual methods as they can be very laborious and involves lot of time.
2. Changes made in hardware such as wavelength shifting and time gating can be resourceful to reduce fluorescence [36]. This depends on alterations in the instrument and pre-treatment and post-treatment methods for the samples to be tested are necessary.
3. The use of excitation light can be Ultraviolet Light. But this has a grave disadvantage that it has penetration in microns and is mutagenic making it unusable for in-vitro tissues. Near infrared Lasers have shown promising results in this regard [37]. These need to be used with Fourier Transformer

RS. The hindrance with it is that CCD have poor efficiency in Infrared region.

4. Surface enhanced raman scattering is another way. This technique enables amplified Raman signals from Raman-active analyte molecules that have been adsorbed onto certain roughened noble metal surfaces [38].
5. The recent Shifted excitation Raman difference spectroscopy (SERDS) has many advantages to its credit.

SERDS Technique

The possibility of physical fluorescence suppression with mathematical fluorescence suppression approaches has been explored. This physical suppression of fluorescence can be attained by SERDS technique. It was found that the cumulation of both approaches assured the most efficient fluorescence suppression and best reconstruction quality of the Raman spectra [39].

Procedure and Instrumentation

SERDS is based on hypothesis known as Kasha's rule which states that the emitted fluorescence remains constant for a small change in excitation photon energy. The Raman spectra shift for this small alteration. This can be used to subtract 2 different Raman spectras caused by this small change in excitation photon eliminating the fluorescence background [40,41]. In this procedure a tunable diode laser with a wavelength of 830 nm is used. A provision to alter the wavelength at regular intervals of 0.5 nm is made. The laser is guided by optics and the Raman signals are collected using a telescope. The laser spot diameter on the sample should be 200 μm while typical acquisition time 2 seconds. A notch filter is used to reject the Rayleigh scattering. The Raman signal is detected by a spectrometer equipped with a N₂ cooled CCD detector.

The in vivo set up uses an optical probe for both exciting the sample and collecting the Raman signal. In this case a laser at 785 nm is used as excitation source. To eliminate both the signals produced in the fibers and the elastically scattered light that enters into the collection fibers, a set of a long-pass and notch filter are glued on the distal side. The coupling between spectrometer and optical fiber is made using an SMA connector. In all cases the slit of the spectrometer is set to 100 μm . A home-made holder is employed to keep the proximal portion of the optical fiber 2 mm above the skin. To maintain sterile conditions in the oral cavity, proximal portion of the optical fibre can be covered by disposable plastic stops [42].

Application of Raman Spectroscopy in other oral pathologies

Yan B *et al* studied raman spectras obtained from pleomorphic adenoma, warthin's tumor and

normal parotid gland. They observed that the content of DNA in neoplastic tissues increased compared with normal tissues, but the content of proteins and lipids increased in the pleomorphic adenoma and decreased in the Warthin's tumor. The authors were of the opinion that the increasing content of DNA in neoplastic tissues suggests that active proliferation occurs, and the different contents of proteins and lipids in these tumors suggest that the metabolic mechanisms differs [43].

Raman studies on normal and Osteoradionecrosis (ORN) bone and on bone exposed to radiation, but not in the ORN state, showed that irradiation produces immediate structural changes in the inorganic bone matrix with a slight loss in cells. ORN bone, in addition to the structural changes that had already occurred on radiation exposure, shows almost complete loss of cellular components [44].

Numerous studies on the use of this technique to diagnose early dental caries have been done. Raman spectroscopy helps in characterizing hydroxyapatite crystals ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$). Hydroxyapatite crystals undergo dissolution in acidic pH, and phosphate ions, $(\text{PO}_4)^{3-}$ and hydroxyl ions (OH^-) react with the hydrogen ions (H^+) in the tooth-biofilm interface and form $\text{H}_2\text{PO}_4^{2-}$. Several studies strongly recommend Raman spectroscopy as an effective and relatively easy tool for early diagnosis of dental caries because during the caries process the inorganic matter of the tooth is gradually replaced by organic matter, which shows stronger organic peaks than normally seen [45-48].

RS can also be used in developmental disorders of teeth. Raman spectroscopy will be very useful in these conditions to study the mineral content and severity of the disorder and can specifically identify the type of disorder and associated enzyme by studying the extracellular matrix protein [49,50].

CONCLUSION

RS has an abundance of potential in identifying oral precancers and cancers. This is achieved by the transmutates it can detect at molecular levels afore there are any morphological changes. Focused studies regarding its applicability in clinical conditions are need of the hour. This technique has advantages that it is a non-invasive and its results are reproducible and most importantly there is no need for sample preparation. Through this review we desire that RS will be used in future as a non-invasive diagnostic technique for oral cancers and other diseases.

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