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RAMAN SPECTROSCOPY: A BIOCHEMICAL FINGERPRINT

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ABSTRACT

Oral cancer is one of the most common cancer. Its control and management is very important. Early detection is the most efficient way to reduce high mortality and minimize morbidity. Current practice for differentiating tissue lesions are based on histo pathological criteria. This process is subject to error. Over last several years significant advancement has been successfully carried out with Raman spectroscopy imaging. It can provide molecular and chemical information of the tissue of interest, which makes it a competitive contender in the molecular imaging arena. Raman spectroscopy has shown tremendous promise for the analysis of biological process within living cells, such as cell dynamics, cell differentiation and cell death. This helps physician to diagnose the disease in real time. Raman spectroscopy uses visible or near infra-red rays to measure a spectrum of vibrational bonds in seconds. This article reviews imaging technique and applications of Raman spectroscopy in dentistry.

KEYWORDS: Raman Spectroscopy, Oral Cancer, Raman Imaging.

INTRODUCTION

Oral cancer is the 6th most common cancer in the world with an annual incidence of over 3, 00, 000 cases.[1] Hence, its control and management has become a global health priority. Despite aggressive treatment, the overall prognosis of oral cancer is still poor in advanced cases.[2] Henceforth early detection is the most efficient way to reduce its high mortality and minimize morbidity.[3] Although at present, the usual method of cancer detection is histological evaluation, there has been interest in the use of optical diagnostic in the early detection of cancer, since early diagnosis in the key for better prognosis. Its ability to detect early biochemical changes associated with carcinogenesis prior to the identification by a pathologists would revolutionize cancer diagnostics.[4]

Raman spectroscopy is a structural characterization technique that can assess the change in polarization of molecule.[5] Diseased or malignant tissue cause changes in cellular orientation and biochemical changes in the body and these changes cause variation in the vibrational spectra. This can be compared with baseline spectrum of normal tissue. These spectral changes are like fingerprints; which are invaluable information for diagnosing certain diseases in real time.[6] This makes Raman spectroscopy a competitive contender in the molecular imaging arena.

BACKGROUND

The Raman Effect was first published in 1928 by Professor Raman of Calcutta University for which he was awarded the Nobel prize in 1930.[7] Developments in Raman spectroscopy occurred slowly during the period from 1930 to 1950. Early experimental work was directed towards improving the radiation sources; from filtered sunlight to Nd: YAG in 1986. Raman spectroscopy always had problem of high level of Rayleigh scattering, which was overwhelmed with the introduction of photomultipliers.[8] Signal processing was done with the help of array detectors and later on with CCD in 1987.[9] With the introduction of FT Raman spectroscope and optical fibers, Raman spectroscopy became a boon to the field of biomedicine.

PRINCIPLE

Raman spectroscopy is a spectroscopic technique based on inelastic scattering of monochromatic light, emitted by an excitation source.[10] The excitation source which can be ultra violet, visible or infrared excites the molecule into vibrational motion it.[11] When photons are directed towards the target, most of the light will pass through unchanged and

only some photons will interact with molecules in the matter. These molecules are excited to a partial quantum state, with the emission of a photon at the same frequency as the incident photon, called elastic scattering.[13] But some photons will be scattered with different wavelength, which is called inelastic scattering which is due to the interaction with the sample and it is this wavelength shift which is recorded by the Raman spectroscope.

RAMAN SPECTROSCOPE

A Raman system basically consists of four major components[10]:

1. Excitation source (Laser).
2. Sample illumination system and light collection optics.
3. Wavelength selector (Filter or Spectrophotometer).
4. Detector (Photodiode array, CCD or PMT).

1. Excitation laser source

Basically lasers of three different wave length are used of which UV is the most accepted one. The other sources include visible laser and near infra-red laser.

2. Light Collection Optics

They restrict the data collection to an area or volume which is small enough to eliminate acquisition of unwanted spectral data of adjacent substance.

3. Optic Filters

The scattered light, which are one million times more intense than the Raman spectrum, must be removed from the detected signal. Holographic filters serves this purpose and thus has eliminated the need for cumbersome, multi stage scanning spectrometers. This also enables for rapid data acquisition.[7]

4. Optical probe

Optical probe with suitable dimension is provided for incorporating needles, endoscopes, angioscopes and other clinical devices.[7]

5. Spectrograph

It is used for clinical application, where high quality spectral data can be t rapidly collected.

6. CCD

It is a rectangular array of photosensitive elements or pixels arranged in horizontal and vertical columns, called Focal Plane Array (FPA). Different types of CCDs are used; front illuminated, thinned back illuminated and front or back illuminated deep depletion(best for near infra- red region).[7]

RESULT INTERPRETATION

Technique of Raman spectroscopy involves illumination of sample with a laser with a laser beam within the range of 532-785nm, elimination of the Rayleigh scattering with holographic filter[13] and the use of di choric mirror to reflect the required photons. These photons are then collected with a light collection optics and is sent through interference filter or spectrophotometer to obtain Raman spectrum of a the sample.[10] Raman spectroscope coupled with diode adjust data algorithm for result analysis[4] and baseline correction and smoothening and calibration of spectra is done. The pre proposed spectra of the similar tissue is subjected to Principle Component Analysis (PCA), a known data reduction technique where huge spectral data are decomposed into small independent variables. Spectral data Analysis is carried out over entire region as well as adjacent areas for standardization purpose. Total percentage variance, is employed for standardization of PCA and further analysis is carried out using data discrimination parameters.[13] The specific peaks in the spectrum, are used as fingerprints to differentiate diseased tissues from normal tissues. But the interpretation of these spectral bands can be challenging. Therefore, a thorough knowledge is extremely important to understand the spectral bands related to specific chemical structures.

ADVANTAGES AND DISADVANTAGES

Advantages of Raman spectroscopy include

- It is highly specific.
- Only a very small sample size is required because each molecule has unique fingerprint..[14]
- Early tissue analysis of solid tumor is made possible by detecting the peripheral biochemical changes.[4]
- Minimally interferes with water.[14] So this can be applied to encounter the details about biological hard and soft tissue which contain significant amount of water.

- Comparing to other diagnostic methods, it is labor free and doesn't require any sample preparations.[15]
- Both in vivo and in vitro study can be done.
- It use less harmful near infrared rays.[13]
- It is a non- invasive method which doesn't heat up the cells under investigation.
- It is less time consuming and convenient method compared to biopsy.
- Remote analysis of tissue, (for example, esophageal cancer) is made possible in adjuvant with optical fiber.
- It gives much high resolution than other medical diagnostic techniques like ultrasound, Magnetic Resonance Imaging, Positron emission tomography or x ray imaging.[16]

Disadvantage includes,

- But it cannot be used to study metal and alloy.
- Florescence of organic material will dominate the weak Raman signals. So, its study in tooth is mainly limited to enamel where the organic content is least.
- Rayleigh scattering hinders Raman spectra.[17] So filtration of unwanted rays should be done.
- Raman spectrum is also hindered by auto fluorescence which can be reduced by using lasers (Fourier transform and Nd:YAG) with a greater photon flux intensity.[4]
- Short wavelength photons give better results and quicker acquisition, but this may cause heating up of the sample. So in case of in vivo studies acquisition time will be more.

ADVANCEMENTS

In order to overcome the drawbacks, some modifications were made in the basic set up and they include,

1. Slit scanning raman spectroscopy

This was introduced to reduce the time required for acquisition of image to few minutes. This is done by parallel acquisition of spectra.[16]

2. *Surface Enhanced Raman Spectroscopy*(*SERS*)

The weak Raman signaling can overcome by SERS.[18] The surface enhancers in the form of gold particles in spherical or rod shaped can be used. The geometry and the material gives a massive enhancement in signaling.

3. Tip Enhanced Raman Spectroscopy

Enhancement of weak Raman signaling can be achieved by improving the tip of microscope with sharp gold coated atomic tips. This gives a better resolution.[18]

4. *Coherent Anti Stroke Raman Spectroscopy*(CARS)

Here two different laser frequencies are used. The difference between these laser frequencies is matched to the vibrational frequency of the target molecule. This resonant excitation produces more signal than normal in around five orders of magnitude. But the limitation is that the excitation of one vibrational frequency is only obtained rather than that of full spectrum. CARS has primarily been used to detect molecules that are abundant. Foe eg: CH_2 groups, which have high density is present in particular lipids.[19]

5. *Spectroscopy With Single Walled Carbon Nano Tubes*(*SWINTS*)

SWNT has an intense Raman peak at 1593 cm−1 (the G band) [20], This narrow Raman peak of SWNTs, easily differentiated from the auto fluorescence background, was used to study the bio distribution of functionalized SWNTs in mice [21, 22]. SWNTs are inherently Raman active and do not need a metal surface enhancer to improve their Raman detection. This is highly desirable for biological applications. Excitation and scattering of photons by SWNTs are both in the near-infrared region, which make it the most transparent optical window for in vivo imaging.

APPLICATION IN DENTISTRY

With the advancement in instrumentation and development of fiber optic probes the use of Raman spectroscopy for biomedical applications including dentistry has significantly increased.[23–26] Vibrational spectroscopic techniques such as Raman and Fourier transform infrared (FTIR) are now emerging as alternatives to traditional diagnostic techniques for the analysis of biological tissue.[27] Its applications have also been extended into the analysis of biomaterials, structural chemistry, and surface analysis.[14] Mineral-phase in synthetic compounds and natural tissues is characterized by Raman spectroscopy and imaging. Raman spectroscopy also has the ability to discriminate premalignant, malignant and normal tissue based on surface finding[28] which is of immense importance for the early diagnosis of cancer.

Saliva

Many macromolecules such as proteins and nucleic acids gives a Raman signature to saliva.[29] For instance, in case of oral squamous cell carcinoma survival rate is poor,

particularly 5 year, due to its late presentation.[30] Raman analysis of saliva helps in its early detection. However, inherently weak Raman spectra from saliva makes the interpretation difficult.[31] In order to overcome this drawback Raman signals are enhanced using a surface enhanced approach with gold particles.[31]

Hard Tissue Pathology

Raman spectroscopy can be used for the early detection of most commonly encountered oral hard tissue pathologies like dental caries and periodontitis.[17]

Dental caries

Etiology of dental caries is multifactorial, such as dental plaque[32-34], dietary carbohydrates, saliva, tooth morphology, and low salivary pH and cariogenic potential of the dental plaque.[35] Dental caries is clinically characterized by progressive demineralization of inorganic structures and destruction of the organic structure of the tooth.[36] It remain asymptomatic until it reaches dentin and pulp. By the time a patient experiences symptoms, there will be a large cavity with 30 to 40% demineralization of inorganic matter and also radiographically evident.[37] Even though there are various techniques to diagnose dental caries[38] they can identify dental caries only in advanced stages.[24, 39] This helps to stop the progression but cannot revert the destruction.

Hydroxyapatite crystal undergoes dissolution in acidic pH and forms $H_2PO_4^{-2}$. But PO_4^{-3} forms dissolved crystals, which shows increased Raman's peak at 1043, 590, and 431 cm−1[24, 52, 53], 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. Hence, early detection of dental caries and its prevention are necessary and is possible with Raman spectroscopy.

Developmental disorder of enamel and dentin

In addition to early diagnosis of dental caries, Raman spectroscopy can be used to demonstrate the developmental defects affecting enamel, dentin, and bone.[40] Structural characteristics of amelogenin was studied using Raman spectroscopy and FTIR for detecting, Amelogenesis Imperfecta which is a rare developmental disorder affecting enamel characterized by hypomineralization and/or hypoplasia of enamel and may be associated with morphological and biochemical changes in the teeth and other structures in the body.[41] Early diagnosis of these genetic disorder enables a dentist to take measures to restore affected teeth

and also to diagnose other defects affecting elsewhere in the body.[17] With help of Raman spectroscopy these conditions can be studied to know 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.[17]

Fluorosis

The molecular structure of human enamel affected by Fluorosis was studied by Zavala-Alonso et al.[42] Mild, moderate and severe cases of fluorosis were studied using micro-Raman spectroscopy and the structural difference in phosphate and B-type carbonate peaks was compared with healthy enamel. There was a significant difference in B-type carbonate peaks of the fluorosis group when compared with healthy enamel, suggesting that carbonate ions are easily dissolved in the presence of fluoride.[17]

Oral microbial flora

Oral cavity harbors 1×10^{14} microorganism. For providing better treatment, the identification of causative microorganism is very essential.[43] Comparing to histopathological studies for identification of microorganism the procedure became wieldier with introduction of Raman spectroscopy by making it labor free, rapid and precise. Also, it require only very small sample size ($1\mu m^{3)}$ and that too without tissue removal and dyes, labels and contrast enhancing agents. For instance, Raman and FTIR spectroscopy yield valuable information by identifying microorganism within plaque , so that the treatment can be targeted and made better in case of gingival and periodontal infections. It is also have significance in the field of microbiological research, food safety, industrial cleanroom maintenance, and water contamination control purposes.[44-46]

Dental material

Raman spectroscopy can be applied to characterize the functional group of organic compounds and their interaction with human enamel and dentin. By knowing the composition of dental materials their properties can be modified to suit the intra oral conditions.[47]

Oral soft tissue

Many oral cancer are not identified until lesions are advanced. The lack of profound clinical presentation, patient reluctance to undergo biopsy and sampling errors, may hinders the timely diagnosis.[48-50] Minimal changes in microanatomy of the cell such as nuclear and cytoplasmic ratio, redox status of the cell, expression of biomarkers, composition and tissue

architecture, chemical changes, the vascularity or neo angiogenesis, and cellular perfusion can be implicated in the detection of the early changes that occur in the cell periphery of the lesion and the presence of subclinical abnormalities can there by detected. This can be achieved by a non- invasive or minimally invasive approach with Raman spectroscopy for which oral cavity can serve as a natural orifice to gain accessibility.

Implants

Raman spectroscopy can be a valuable diagnostic aid in patient selection for dental implant by assessing bone quality and quantity with available advanced intraoral probes. Study of Osseo integration provides idea on how to better modify the surface characteristics of the implant to achieve better bone bonding properties.

Others

Measurement of bone composition would serve as an early aid in osteomyelitis and osteoradionecrosis. Spectral analysis of the blood supply of the free flap through transcutaneous approach could also pave its way for reconstruction and analysis of the proximity of the blood vessels in cases of infections involving the fascial spaces. It can be also used to check the efficacy of dental treatments like, proper irrigation in root canal treatment, efficacy of bonding of dental materials with tooth and standard of bleaching.

CONCLUSION

Raman spectroscopy is a convenient, noninvasive, accurate and rapid method for diagnosis of any pathologies. This imaging modality has advanced significantly and many critical proof-of principle experiments have been successfully carried out. Once the probe delivery system is resolved, 'see and treat' method can craft miracle in the field of biomedicine. The combination of Raman diagnostic techniques with other technologies should also be explored. No single imaging modality can have all the desirable features such as high sensitivity, good spatial/temporal resolution, multiplexing capacity, low cost, and high-throughput, combination of Raman imaging with other techniques such as fluorescence, photo acoustic[51], or PET imaging may work wonder and may become a fertile area for future research. As a future trend ex vivo sensing and in vivo imaging for personalized patient will arise. This combination of sensing and diagnosis may provide a synergistic approach in predicting which patient will respond to specific molecular therapy and can monitor their response. In the coming decades let us wait and witness rapid expansion in field of biomedicine with Raman spectroscopy.

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