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A Review on the Prospects of Optical Spectroscopic Techniques in the Detection and Treatment of Cancers

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Abstract: Experimental cancer research in laboratory has helped tremendously in understanding the cancer biology. Among the various physical examinations available for cancer detection, optical spectroscopic techniques were widely used in the last few decades owing to their non-invasive modalities. The diagnostic method carried out using the basis of optical spectroscopic techniques is termed as photodynamic diagnosis (PDD) while the treatment modality is known as photodynamic therapy (PDT). PDT has emerged as a novel therapeutic method that uses a combination of light and a chemical agent, photosensitizer (PS). This review article will provide a collection of research insights on these techniques are applied for understanding the photophysical processes and photochemical pathways involved in PDT, physico-chemical properties of PS in association with the cancerous tissues. These are simple and sensitive techniques such as absorption, steady state and time-resolved fluorescence techniques and Raman spectroscopy. An account of fluorescence studies on PS formulation for PDT is presented here, where PS is treated as a fluorescent drug molecule.
Keywords: Photodynamic diagnosis, photodynamic therapy, photosensitizer, fluorescence spectroscopy, fluorescence detection, Raman spectroscopy.
Abbreviation: Photodynamic diagnosis – PDD; Photodynamic therapy – PDT; Photosensitizer – PS; Fluorescence detection – FD; Excited singlet state - S_1; Excited triplet state - T_1; Intersystem crossing – ISC; Förster resonance energy transfer – FRET; 1-anilino-8-napthalene sulfonate – ANS.

Introduction

Experimental cancer research in the laboratory started more than a century ago and our knowledge in understanding the biology of cancer has expanded tremendously in the last few decades (1-6). Among the various physical examinations available for cancer detection, optical spectroscopic techniques were widely used in the last two decades, owing to their non-invasive modalities (7-10). The diagnostic method carried out using the basis of optical spectroscopic techniques is termed as photodynamic diagnosis (PDD) while the treatment modality is known as photodynamic therapy (PDT).

PDT has emerged as a novel therapeutic method that uses a combination of chemical agent, termed as photosensitizer (PS) and light. The PS molecules, localized within the tumor cells, generate reactive molecular species upon activation by photons of appropriate wavelength and thereby induce cytotoxicity to the tumor cells. The PDT treatment is novel due to the selective accumulation of PS within the target cancerous tissues, thus there is minimal or no invasion of the healthy tissues. The number of researchers working in this field of research has increased drastically over the last few decades and significantly many research articles have been published (8-21). During the infancy of PDT research, the major focus of these articles was on the elucidation

of the mechanistic pathways of PDT. However, the current focus is on the utility of imaging techniques in both detection and treatment of cancer, with an emphasis on optical spectroscopic methods. The current review will discuss the salient features of the simple and sensitive techniques such as absorption, steady state and time-resolved fluorescence and Raman spectroscopic techniques used in this field of research. Since the field of research discussed here is multi-disciplinary, the principles of each of the spectroscopic techniques will be provided in this review article for the better understanding of the readers. Along with a mechanistic background of the photophysical processes of photosensitizer with light illumination is also explained here. The examples of the research work cited here will clearly indicate that the absorption and fluorescence techniques can be utilized in the diagnosis of cancer by monitoring the changes in the photophysical properties of PS molecules. Whereas, the biological changes in human body are always associated with chemical changes, Raman spectroscopy serves as a tool in monitoring the chemical changes.

Principles of absorption, fluorescence and Raman spectroscopy

Absorption and fluorescence spectroscopy

Jablonski diagram (figure 1) gives a convenient approach for understanding the photophysical processes that occur as a result of light absorption, namely photon absorption, internal conversion, fluorescence, intersystem crossing, phosphorescence, delayed fluorescence and triplet-triplet transitions. The electronic states involved are S_0 (ground state), S_1, S_2, …. (singlet excited states) and T_1, T_2, …..(triplet excited states). Different vibrational levels are associated with each electronic state.

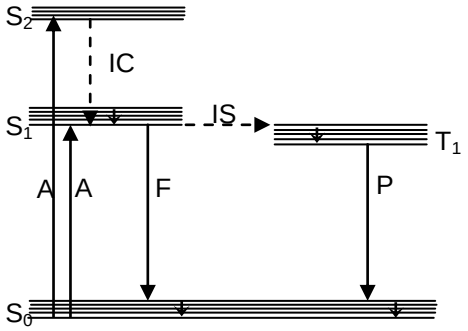

Figure. 1 Jablonski diagram depicting the photophysical processes upon excitation of a molecule.

Absorption (A) of a photon can bring a molecule from the 0 (lowest) vibrational energy level of S_0 to one of the vibrational levels of S_1, S_2, …..The subsequent possible de-excitation processes are:

- Internal conversion (IC) – non-radiative transition between two electronic states of same spin multiplicity
- Fluorescence (F) – emission of photons accompanying $S_1 – S_0$ transition
- Intersystem crossing (ISC) – non-radiative transition between two isoenergetic vibrational levels belonging to electronic states of different spin multiplicities
- Phosphorescence (P) – emission of photons accompanying $T_1 – S_0$ transitions

The excited molecule undergoes many other processes such as vibrational relaxation, delayed fluorescence, triplet-triplet transitions. The time scale of fluorescence is best suitable to understand the processes occurring such as solvent relaxation, dynamic quenching, calculating Förster distance and getting information regarding the dynamics of nano-environment surrounding excited molecule. This time scale includes the most important biologically relevant processes of fast sub molecular motions at the short end of this time scale and the translational and rotational motion of macromolecules at the other extreme of this time scale.

A molecule which is fluorescent is termed as fluorophore or fluorescent probe. The photophysical parameters of a fluorophore gives valuable information in response to its association with micro-heterogeneous media such as micelles, lipid bilayer membranes, proteins, polymeric gels, bile salt aggregates and many other organized media. The information is obtained by correlating the dynamic processes of excited species.

Raman spectroscopy

The Raman Effect is a fundamental process in which energy is exchanged between light and matter (Figure 2). From the energy level point of view, the process of Raman scattering can be viewed as the transition of a molecule from its ground state to an excited vibrational state, accomopanied by the simultaneous absorption of an incident photon and emission of a Raman scattered photon.

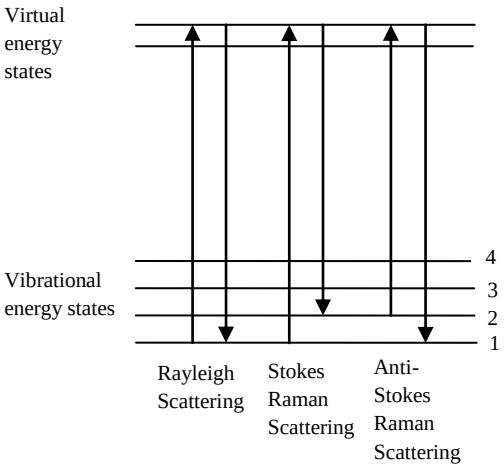

Figure. 2 Phenomenon of Raman scattering

The Raman spectrum is a plot of the scattered intensity as a function of the energy difference between the incident and scattered photons. The loss (or gain) in a photon energy is equal to the energy difference between the final and initial vibrational energy levels of the molecule participating in the interaction. The observed peaks (bands) in the Raman spectrum are relatively narrow, easy to resolve and sensitive to molecular structure, conformation and environment. Generally biological tissues are inhomogeneous in composition and highly scattering. Thus the complete analysis of Raman signal requires an understanding of optical parameters and photon propagation in turbid media. Raman signals are inherently weak, and in addition early diagnosis of disease requires detection of tissue molecular constituents in low concentration. This is accentuated by the fact that lasers with high intensity cannot be used to observe weak signals from the tissues because of the potential sample damage. Also, the complex nature of the tissue composition results in the light absorption throughout the entire uv-visible region and subsequent fluorescence emission interferes with the weak Raman signals strongly. Recently, researchers have been successful in eliminating the background fluorescence of the tissue by using *Near-IR* excitation source for the irradiation of the sample.

Photophysical processes and photochemical pathways in PDT

Photodynamic action results from the interaction of photons emitted from visible light at a particular wavelength with PS, which is localized within the tumor tissues (Figure 3). PS molecule in the singlet ground state electronic configuration absorbs a photon of specific wavelength and it is excited to a higher (singlet) energy state, $PS(S_1)$, whose lifetime is about 10^{-9} s. The PS molecule returns to the ground state by emitting photons, resulting in fluorescence. This process is useful for the diagnosis of cancer, through fluorescence detection (FD) of PS. Alternatively, the excited molecule may convert to the triplet state $PS(T_1)$ *via* intersystem crossing (ISC), which involves a change in the spin of an electron. The triplet state has a longer lifetime ($\sim 10^{-6}$ – 10^{-3}s) and lower energy than the singlet state. The PS in triplet state can relax back to singlet ground state with emission of photons, resulting in phosphorescence. The photophysics of photodynamic therapy undergoes two types of reactions, namely type I and type II reactions. The $PS(S_1)$ molecule undergoes type I reaction while the $PS(T_1)$ molecule undergoes type I or type II reaction. If the triplet state is not quenched by the interaction with oxygen, it relaxes back to the ground singlet state with emission of photons. Phosphorescence can also be used as a diagnostic modality but it is difficult to measure.

In type I reaction, the $PS(S_1)$ molecule reacts directly with a substrate through electron or proton transfer and both become radical ions. These radical ions can cause cell damage. In the presence of oxygen, these radicals may further react and produce oxygenated products. As a result, the sensitizer is consumed.

Alternatively, the sensitizer radical ion may directly transfer its electron to oxygen and produce highly reactive oxygen species, such as superoxide and peroxide anions, which can then attack cellular targets. A type I reaction pathway is more likely for PS(T_1) species than for the PS(S_1) because the lifetime of triplet state is longer than that of the singlet state. The type II reaction consists of energy transfer from PS(T_1) molecule to oxygen in the triplet state, $O_2(T_1)$, producing the highly cytotoxic singlet oxygen $O_2(S_1)$. This species has a short lifetime (~ 10^{-6} s) and induces irreversible cell damage. In this pathway, the sensitizer is not consumed. It returns to its ground state by the process of phosphorescence, without undergoing any chemical alterations and might be able to repeat the process of energy transfer many times. Singlet oxygen is widely believed to be the major damaging species in PDT. Due to its extreme reactivity, singlet oxygen has a short lifetime in cellular environment and limited diffusivity in tissue, allowing it to travel only approximately 0.1µm. Hence, they only affect the tissue where the PS is localized. It is evident that the effects of PDT are oxygen dependent and rely on the oxygen tension within the target tissue (21-25). Both type I and type II reactions can occur simultaneously and the ratio between them depends on the PS, substrate, oxygen concentration and the binding constant of PS to substrate. Most of the PS currently in use as PDT agents exhibits the type II reaction mechanisms (21-24).

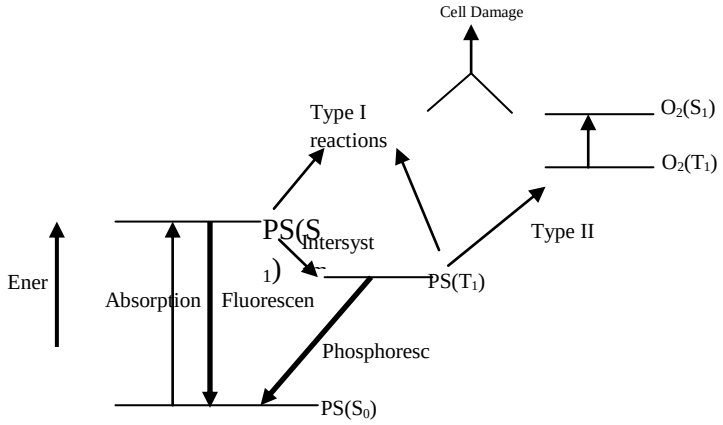

Figure. 3 Schematic diagram of photophysical processes and photochemical pathways in photodynamic therapy

Photosensitizers

Photosensitizers form the core of the PDT method to treat different types of cancer. Research on identifying ideal PS is quite challenging and new compounds are continually being tested for both *in vivo* and *in vitro* applications (8-12). Many researchers have investigated the photophysical and photochemical properties of various types of compounds for PDT action on cancerous tissues (19, 25-30). The outcome of these investigations has led to the proposition of following pre-requisites for an efficient photosensitizer:

- High chemical purity, good chemical and physical stability, low tendency to aggregate in aqueous media and suitable chemical properties for administration to target tissues.
- High extinction co-efficient in the wavelength range of 600 – 900 nm for optimum tissue penetration and high quantum yield.
- Selective uptake and retention by cancer tissues, low toxicity to the healthy normal tissues and short time interval between administration and maximal accumulation within the tumor tissues.

As mentioned previously, most of the PS currently in use as PDT agents undergoes type II reaction. Since PS molecules in the triplet state are capable of yielding singlet oxygen which is highly effective in destroying cancerous tissues, it is essential for the PS molecule to have high efficiency of triplet state generation (31). For the purpose of diagnosis, the PS molecules should be able to estimate the spread of cancerous tissues and hence it is more important for them to have high fluorescence quantum yield than high ability to generate triplet state. When selecting a suitable PS for both the modalities, it is also important to take into account the environmental effects on the photophysical properties of PS. For example, the generation of triplet state is

different in aqueous media and within the cells. Besides lower efficiency of incorporating PS aggregates into cells, the aggregation of PS reduces triplet state generation (19). Hence, aggregation of PS is an unfavorable phenomenon for PDT. An understanding of the photophysical and photochemical properties of prospective PS has to be established to select a proper PS for both FD and PDT. It is also observed that the orientation of the PS molecules incorporated into the tissue with respect to the membrane or proteins is important because it can lead to a difference in their extent of interaction. The four main classes of PS are porphyrin derivatives, chlorins and purpurins, phthalocyanines and porphycenes. They exhibit different photochemical and photophysical properties in terms of mechanisms of action and light activation.

Absorption, fluorescence techniques in PDT

The steady state and time dependent fluorescence spectroscopic techniques are usually employed in characterizing the photophysical properties of the PS in different media (31-43). These studies are useful in establishing the fundamental information regarding the photophysical behavior of PS incorporated within various possible drug delivery systems and their interaction with certain physiological targets. Such data are critical in evaluating the efficiency of the PDT action of a particular PS, both *in vitro* and *in vivo*. Measurement of the quantum efficiency of the triplet state generation of a PS can be carried out by steady state fluorescence spectroscopy, enabling an estimation of the PDT efficiency of the PS. For FD of cancer, the PS should have high fluorescence quantum yield and therefore a high quantum yield of the singlet state generation. In contrast, for PDT action on cancer, the PS should have high quantum yield of the triplet energy state and phosphorescence. The PS molecules have lifetime sufficient for the ISC transition to occur (39). A typical absorption spectrum of most of the PS molecules consists of a very strong band near 400 nm, called the *Soret band* or B band, and four weaker bands between 450 nm and 650 nm, called *Q bands*. The emission spectra scanned with excitation wavelength at 400 nm provide information on the localization of PS within the tumor cells and thereby used for the FD of cancer. On the other hand, the emission spectra scanned at longer wavelength provide information pertinent to the PDT action of the PS under evaluation (32-43). The fluorescence spectroscopic techniques are useful in various fields of research, ranging from material research to biomedical research, owing to their significant advantages (44, 45), such as specificity, sensitivity, quantitation, environmental sensitivity, high temporal resolution and high spatial resolution.

Localization of photosensitizer

Fluorescence spectroscopic techniques are among the most widely used tools for identification of solute location within a substrate, where the solute acts as a fluorescent probe. As most of the fluorescent probes are hydrophobic in nature, they are suitable for investigating the physico-chemical properties of liposomes and macromolecules such as proteins, polymers, dendrimers, cyclodextrins and surfactants, which are used as drug delivery vehicles (32-43). The PS molecules are fluorescent in nature and the fluorescence probing technique has been adapted for analyzing the localization of PS within drug delivery media and within tumor cells. The spectral modifications of the fluorescent probe due to the incorporation of the probe within an organized medium yield useful knowledge on the interaction between the probe and the medium. Similarly, the association of PS with a cancerous cell membrane can be studied by monitoring the changes in fluorescence properties of the PS molecules. A comparison of the fluorescence properties (fluorescence intensity, emission wavelength, fluorescence anisotropy and lifetime) of the PS between a homogeneous (aqueous and different solvents) medium and a heterogeneous (organized) medium offers an understanding of the nature of interaction between the PS and the heterogeneous medium. Using fluorescence probing technique, one can estimate the binding constant of the PS with a particular substrate. Moreover, the concentration of PS required for such studies is relatively small, usually in the order of 1 µM. The advanced fluorescence techniques, such as Förster resonance energy transfer (FRET) and fluorescence quenching studies, can be advantageous in identification of the precise localization of PS (37). Combination of fluorescence studies with the time-resolved spectroscopy can be useful in understanding the heterogeneity of the medium surrounding the fluorescent probe (39,40 and 44).

Self-association of photosensitizer in aqueous media

PS molecules which are hydrophobic or amphiphilic can undergo self-association in an aqueous environment. The aggregates exhibit physico-chemical properties that are different from those of monomer. The absorption spectral features of the aggregates are characterized by a broadened *Soret band* along with a broad

and red-shifted *Q-band* (45-53). The fluorescence intensity of the aggregates is much lower than that of the monomers. A red-shifted weak and broad fluorescence is indicative of the aggregates. The presence of aggregates can be identified by bi-exponential and tri-exponential fluorescence decays, exhibiting a very short decay component which is less than 50 ps. In addition, the fluorescence and singlet oxygen quantum yield of an aggregated species are lower than those of the corresponding monomer. This observation is attributed to the association of PS molecules, leading to the fast non-radiative excitation energy relaxation of the aggregates (45). Consequently, the fluorescence lifetime of the aggregated PS is shorter and thereby reduces the ISC transition from S_1 to T_1 state. The ISC transition is an essential step in the photochemical pathways within the PDT modality. The aggregated species of PS molecules are not advantageous for PDT action because they lack photosensitizing ability. Hence, an understanding of the aggregation pattern and development of methodologies to avoid the aggregation of PS is important.

Kelbauskas and Dietel studied the uptake of photosensitizer aggregates into tumor cells using subcellular time-resolved fluorescence spectroscopy with high temporal resolution and high sensitivity (47). The characteristic spectral features of PS aggregates, such as pyropheophorbide-a derivatives and chlorin e6 trimethyl ester derivatives, in aqueous solutions were elucidated. The dynamic equilibrium between the monomers and dimers of pheophorbide a (Pheo) was studied using absorption and fluorescence spectroscopy (48). It was shown that the monomer–dimer equilibrium state of Pheo in solution provided a good opportunity to study the photophysical properties of tetrapyrrolic dimers. The equilibrium state exhibited good photostability. The amount of dimers in the solution could be controlled by the temperature or solvent composition. Steady state and time-resolved fluorescence measurements present a convenient method to identify and characterize the PS aggregates in aqueous media. With a detailed study on the aggregated species, researchers can look at the possibility of encapsulating aggregates into suitable drug delivery media for effective uptake into tumor cells, where disaggregation ensues to yield monomers for PDT.

Fluorescence studies on photosensitizer formulation

Most of the PS are hydrophobic drugs and the transport of such drugs usually takes place through their dissolution by cell membranes and serum proteins. For better solubilization and specific targeting of hydrophobic drugs, a variety of drug delivery systems, such as emulsions, surfactant, liposomes and polymers are employed (54-57). The specific incorporation of the probe into the hydrophobic or hydrophilic sites of the substrate in accordance with their chemical nature, adds strength in finding the physico-chemical properties of the drug delivery media. The spectral modifications due to changes in the fluorescence properties of the PS molecule while undergoing dissolution within a particular delivery medium provide information on the compaction of both molecules (58-66). With the PS molecule being fluorescent, it is convenient to study the bioavailability and biocompatibility of PS by probing its fluorescence parameters such as fluorescence intensity, emission wavelength, fluorescence anisotropy and time-resolved fluorescence intensity decay. Compared with conventional solutions, lipid-based dye formulations are subjected to specific environmental factors like low polarity, high viscosity and increased local oxygen concentration (67,68). Importantly, a lipophilic sensitizer in a liposomal formulation will be located into the lipid phase at a high concentration. Therefore, spectral modification upon irradiation of lipid-based PS formulations can be used as a tool in understanding the interaction between the drug molecule and the substrate. The network of polymeric gels and their association with PS molecules can be analysed using specific fluorescent probe like 1-anilino-8-napthalene sulfonate (ANS), which has negligible fluorescence in aqueous media and increases drastically when it is incorporated within a gel (69-72). Recent advancements in formulation studies of PS have seen the use of hydrogels, dendrimeric substrates and also quantum dots (73-77).

Raman Spectroscopy

The progression of disease is accompanied by chemical changes in the human body and hence Raman spectroscopy can provide the physician with valuable information for diagnosing disease. And since light can be delivered and collected rapidly via optical fibers, which can be incorporated into catheters, endoscopes, cannulas and needles, as necessary, Raman spectroscopy can be performed *in vivo* in real time (78-80). Diagnostic applications of Raman spectroscopy currently under investigation are wide-ranging. For example, Raman spectroscopy may be used to monitor blood analytes non-invasively. It may be used to perform minimally invasive, real-time, tissue diagnosis *in vivo*, in case where biopsy cannot be performed readily, such as coronary artery disease and Alzheimer's disease, or where a high incidence of false positive screening tests

leads to unnecessary biopsy procedures, as in breast cancer. In general, Raman spectra of tissue are composed of relatively narrow bands, typically of 10-20 cm^{-1} in width, which exhibit the presence of many biochemicals (53). The relative contributions of these biochemicals to the tissue, Raman spectrum are proportional to their relative abundance in the tissue. This is the basis for the quantitative nature of the information Raman spectroscopy can provide for diagnosis. The quantitative nature of Raman spectra, combined with the ability to provide unique fingerprints of the biochemicals present in tissue, illustrate the potential of Raman spectroscopy to provide objective, quantitative diagnostic information for tissue analysis.

Summary and conclusions

Cancer therapy has benefitted the most from the advancements in optical spectroscopic techniques with their fascinating achievements in biomedical research, owing to their non-invasive nature to the surrounding healthy tissues. The development in laser applications and the availability of a wide variety of lasers which can be tuned to detect particular diseases have led to a new research field to address biomedical problems. Such developments have led to the close association of molecular imaging techniques with various diseases, such as cancer and neuronal degenerative disorders. As a result, the simple and sensitive techniques for cancer research, such as absorption, steady state and time resolved fluorescence spectroscopy have been neglected. For example, identification of suitable PS molecules for particular cancer detection and therapy, can be easily made by using fluorescence spectroscopy with a variety of useful fluorescence parameters. Fluorescence spectroscopy can offer sufficient information regarding the singlet state of the PS molecule and makes cancer detection easy. Furthermore, the phosphorescence properties of the PS molecule offer information regarding its triplet state, giving a qualitative assessment of the PS for PDT action against cancer.

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