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Raman spectroscopy for physiological investigations of tissues and cells☆

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ABSTRACT

Raman micro-spectroscopy provides a convenient non-destructive and location-specific means of probing cellular physiology and tissue physiology at sub-micron length scales. By probing the vibrational signature of molecules and molecular groups, the distribution and metabolic products of small molecules that cannot be labeled with fluorescent dyes can be analyzed. This method works well for molecular concentrations in the micro-molar range and has been demonstrated as a valuable tool for monitoring drug–cell interactions. If the small molecule of interest does not contain groups that would allow for a discrimination against cytoplasmic background signals, "labeling" of the molecule by isotope substitution or by incorporating other unique small groups, e.g. alkynes provides a stable signal even for time-lapse imaging such compounds in living cells. In this review we highlight recent progress in assessing the physiology of cells and tissue by Raman spectroscopy and imaging.

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1. Introduction and background

Optical methods are commonly used to probe the physiological state of cells because of their ability to obtain detailed biochemical information from fixed and living cells without the need for direct physical contact with the cell [1]. While fluorescence is perhaps the most commonly used optical technique for this purpose, it requires the application of exogenous fluorescent tags to identify and detect specific biomolecules. This procedure, however, necessitates careful control studies, because the addition of foreign organic molecules to cells has the potential to alter a cell's biochemical profile or to harm it. This is certainly also true for genetically modified organisms whose cells express fluorescent proteins as fusions with the protein of interest. The inclusion of such exogenous reporters into cells can have a direct effect on a cell's physiology

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[2,3]. Also, fluorescence-based approaches require decisions to be made beforehand about which molecular targets require labeling to enable their monitoring. This process can be quite tedious and it limits the amount of biochemical information that can be obtained during a single experiment. Therefore, alternative optical techniques for probing the physiological state of cells continue to be explored with the hope that these will hold considerable promise for revealing new insights into cellular behavior while minimally affecting cells during the measurement.

Over the past 25 years, Raman spectroscopy has emerged as a highly sensitive and promising analytical technique that has been applied to a number of biomedical problems at the single cell as well as the tissue level, e.g. in cancer, infectious diseases, regenerative medicine, and cardiovascular disease. Raman spectroscopy is a laser-based spectroscopic technique for the direct detection and characterization of molecular bonds. The underlying process is the inelastic scattering of photons on molecular bond vibrations (aka "Raman scattering"), where a small fraction of photons (~1 in 10^8) are inelastically scattered and lose a portion of their energy to the molecular bond vibration. The difference in energy between the incident and scattered photons corresponds to the energy that was required to excite (or de-excite) the molecular vibration. Detection of these scattered photons results in a spectrum of narrow peaks, each of which can be assigned to a specific vibrational resonance of a molecular group. Each Raman peak of a particular molecular vibration occurs at a specific vibrational energy relative to the wavelength of the excitation source, which is displayed as a Raman "shift" in units of "wavenumbers" (in cm^{-1}). Therefore, a Raman spectrum resembles a "molecular fingerprint" of the sample under investigation. When applied to the analysis of single cells, Raman spectroscopy provides information about the biochemical composition of cells that could otherwise only be obtained by destructive techniques, such as chromatography or mass spectrometry [4]. In addition, since a molecular vibration is sensitive to its neighboring molecular bonds and molecular structure, Raman spectroscopy also provides information about the conformation of biomolecules and their interactions. When combined with confocal microscopy, single cell and subcellular chemical information can be obtained [4].

Raman spectroscopy has found extensive applications in biology and biochemistry for the characterization of the structure and interactions of biomolecules [5]. Such assessments are often made based on the presence or absence of one or a few select Raman peaks. The definitive identification of specific peptides or proteins, however, typically requires that the entire spectrum of Raman-active vibrational modes is being evaluated. For example, many amino acids, such as tyrosine, tryptophan, and phenylalanine, have distinct peaks in the 600 to 1700 cm^{-1} spectral fingerprint region. Amide linkages between amino acids give rise to two Raman-active vibrations, the amide I (C=O) stretching vibration, and the amide III (C–N) stretch and (N–H) in-plane bending modes. The relative positions of these two vibrations can provide information about the conformation in which proteins are predominantly present in a sample, e.g. in alpha helix or beta sheet conformation. DNA has several distinct spectral peaks that can either be assigned to the sugar phosphate backbone or to the four DNA bases. The symmetric stretching vibration of two phosphate oxygen groups in the diphosphate ester PO^{2-} group occurs between 1100 and 1150 cm^{-1}. The O–P–O stretching mode of DNA depends on DNA conformation: for DNA in B-form it is located near 835 cm^{-1}, and for A-form (DNA and RNA) it occurs at 800–815 cm^{-1}. The exact position of these peaks provides information about DNA conformation (A, B, C, or Z form) or about subtle changes to the structure of DNA. Phospholipid molecules, which make up the plasma membrane of cells, have spectral markers due to both, the head and tail groups. For example, polar head groups have a C–N stretching vibration at 720 cm^{-1}, while hydrophobic chains have vibrational peaks in the 1000–1150 cm^{-1} region due to C–C skeletal modes. The intensity and location of these peaks are extremely sensitive to the structural conformation of the chains and, therefore, varies depending on whether they are *trans* or gauche configurations, or if the chains are saturated or unsaturated. *Cis* and *trans* structures of the C=C group can be elucidated by identification of lipid-related peaks at 1655 and 1668 cm^{-1}, respectively. These Raman signatures can be very sensitive to the biological state of the cell; and as such, spontaneous Raman spectroscopy can be a powerful approach for studying cell physiology.

Raman spectroscopy is particularly attractive for detecting and imaging the distribution of small molecules, where labeling with fluorescent dyes is not feasible because the molecules of interest are smaller or similar in size as the fluorescent molecule. Here, it is of advantage to use molecules that have one or more distinctly different molecular groups compared to typical biomolecules, so that the compound of interest can be isolated against the background of cells and tissues by the unique Raman-active vibration of this group. This condition is often met for synthetic drugs, the uptake and metabolic reaction products of which are then easy to detect by following the distribution of the Raman-active group. If small molecules do not exhibit unique Raman spectral modes, then their interaction with cells can often still be investigated by probing their effect on the cellular physiology. E.g. if the small molecule is toxic above a certain minimum concentration, then its interaction with cells will often initially lead to the release of cytochrome c from mitochondria, followed by membrane blebbing, and ultimately the disintegration of cells. Another, often utilized way to specifically detect small molecules by Raman spectroscopy is to artificially provide them with a unique Raman signature through substituting some atoms by stable isotopes, where e.g. hydrogen is replaced by deuterium [6]. This leads to a dramatic change in a corresponding peak's wavenumber position because deuterium has twice the atomic mass of hydrogen. Other stable isotopes, e.g. the replacement of ^{12}C by ^{13}C, lead to subtle, but still noticeable shifts in the Raman spectra of molecules containing these elements. A particular benefit is the creation of "new" peaks in areas where there is no Raman activity for most naturally occurring compounds. For biological materials this is true for the spectral range between 1800 and 2800 cm^{-1}. The deuteration of biomolecules, such as lipids, will then lead to a new peak occurring at ~2200 cm^{-1} that can easily be identified in the otherwise flat spectral region. Such peaks are indeed so well isolated that they can also be used for highly selective imaging of the distribution of molecules carrying this signature. The rapid growth of "click chemistry", i.e. the formation of a covalent bond between alkyne and azide groups in the presence of copper through a cycloaddition reaction, has led to increasing commercial availability of compounds carrying these groups, which can now be exploited for Raman-based detection and imaging [7,8]. These "tools" were initially developed for the specific fluorescent labeling e.g. of nucleotides as part of a cell proliferation assay, or to specifically label small molecules, such as sugars and lipids in cells. The triple bonds in alkynes and azides also serve as a unique molecular group giving rise to Raman stretching vibrations around 2100 cm^{-1}, which are also ideal molecular tags for coherent Raman imaging in the form of coherent anti-Stokes Raman scattering (CARS) microscopy or stimulated Raman scattering (SRS) microscopy [9,10]. The combination of stable isotopes together with such unique molecular groups opens up a wide range of possibilities for creating spectrally narrow optical probes.

In this review we discuss how Raman spectroscopy has evolved during the last decades to the point where it is now possible to directly probe the physiological state of isolated cells, to follow changes in their physiology, and to expand these methods to the tissue context for medical diagnostics. We will focus primarily on applications that make use of intrinsic Raman markers to assess the physiological state of cells, their interpretation, and the response of these markers to external stimuli; rather than describing the uptake of drugs by specific drug-related marker modes. Direct imaging of drug interactions and drug uptake, the characterization of microorganisms, and the use of nonlinear Raman techniques are all being discussed by other, specialized review articles in the same issue of Advanced Drug Delivery Reviews.

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