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Progress in Raman spectroscopy in the fields of tissue engineering, diagnostics and toxicological testing

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Abstract This review summarises progress in Raman spectroscopy and its application in diagnostics, toxicological testing and tissue engineering. Applications of Raman spectroscopy in cell biology are in the early stages of development, however, recent publications have demonstrated its utilisation as a diagnostic and development tool with the key advantage that investigations of living cells can be performed non-invasively.

Some of the research highlighted here demonstrates the ability of Raman spectroscopy to accurately characterise cancer cells and distinguish between similar cell types. Many groups have used Raman spectroscopy to study tissues, but recently increased effort has gone into single cell analysis of cell lines; the advantages being that cell lines offer ease of handling and increased reproducibility over tissue studies and primary cells. The main goals of bio-Raman spectroscopy at this stage are twofold. Firstly, the aim is to further develop the diagnostic ability of Raman spectroscopy so it can be implemented in a clinical environment, producing accurate and rapid diagnoses. Secondly, the aim is to optimise the technique as a research tool for the non-invasive real time investigation of cell/material interactions in the fields of tissue engineering and toxicology testing.

1 Introduction

Although the Raman effect was first observed by the Indian scientists Raman and Krishnan in 1926, it was not until the early 1990's that Raman spectroscopy was applied in the successful analysis of living cells and tissues [1]. Raman spectroscopy is an analytical technique that can produce a fingerprint representing different chemical bonds present in a sample, from which information about molecular composition can be determined [2]. A typical Raman set-up is shown in Fig. 1. Typically, a NIR (near-infrared) laser is directed via a series of mirrors through a microscope and onto a sample resulting in light scattering. A small proportion of these scattered photons have a shifted frequency (known as Raman shift) as a result of the interaction. By filtering out the photons that have not had altered frequencies via a notch filter and directing the Raman shifted photons onto a detector, information regarding the chemical bonds present within the sample is obtained. Each chemical bond results in certain frequency shifts of the photons, for example a Raman shift of 1005 cm^{-1} corresponds to phenylalanine; the intensity of this peak is relative to the concentration.

There are several key advantages of Raman spectroscopy over other analytical techniques for studying living cells: it is non-invasive (no probes (e.g. antibodies) are used), spectra can be obtained in a few minutes and automated chemical analysis can be performed. Infrared (IR) spectroscopy has the drawback that since water is a strong IR absorber, it can obstruct the peaks corresponding to cellular components. In Raman spectroscopy, water does not affect the relevant area of the spectrum, allowing living cells to be analysed in near-physiological conditions (at 37°C in media). Fluorescence spectroscopy yields complementary information from cells by using fluorescent antibodies for specific molecules [3]. In fluorescence experiments, cells may need to be fixed, whereas Raman spectroscopy is widely applicable to live cells.

The first Raman based cellular results were obtained in 1990–91 when Puppels et al. developed a highly sensitive

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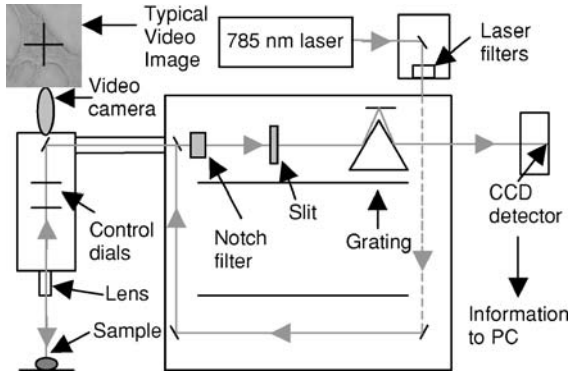

Fig. 1 A typical Raman spectroscopy experimental set-up. Additional features such as an automated XYZ stage and a heated stage for cell work may also be present

confocal Raman spectrometer enabling high resolution single-cell studies. They obtained spectra of the cell nucleus and cytoplasm without degradation of the sample using a 660 nm wavelength laser [1, 4]. Wavelengths in the near infrared (NIR) are most often used for living cells. The relative amount of fluorescence is lower when using wavelengths in the NIR, although wavelengths longer than 850 nm suffer from reduced efficiency of the charge-coupled device (CCD) detectors resulting in poor spectra [5]. Using markers for apoptosis such as sytox green and caspase-3 antibodies, our group has demonstrated that cells can survive many hours exposure to constant NIR laser light even when applied to the nucleus (unpublished data).

Lasers with large spot-sizes (10 μm and larger) are ideal for investigating entire cells and tissues, while diffraction limited spot-size lasers can be used for the purpose of Raman spectroscopic imaging, a technique that produces maps of chemical distribution within a cell. Cellular processes such as mitosis, apoptosis and phagocytosis incur large-scale material relocation within the cell. Protein analysis by Raman spectroscopy can provide information to give clues as to how these processes proceed and what factors are involved. Uzunbajakava et al. used a laser with a 550 nm lateral spacial resolution in order to map the distribution of DNA, RNA and proteins within HeLa cells [6].

Statistical methods are applied to extract important information from the collected spectra. Principal Component Analysis (PCA), can be used to determine the most significant variation between groups of spectra [7]. Principal components between cell spectra have been successfully used for the discrimination of various cancer tissues [8, 9]. The principal components are linear combinations of variables that describe the major sources of variance between the spectra. The first principal component (PC1) accounts for the most variance, and subsequent PCs account for decreasing amounts of different sources of variance. By plotting the scores of PCs (amounts of each PC) against each other, it is often possible to discriminate between groups of spectra measured from different samples. Linear Discriminant Analysis (LDA) can group the data according to where the variation occurs and Classical Least Squares (CLS) modelling can be used to evaluate how relative amounts of specific cellular biopolymers change over time [10].

2 Applications

2.1 Diagnostics

All diseases ultimately result in fundamental changes within the biochemistry of the cell or tissue. There is huge potential in the diagnostic ability of Raman spectroscopy. It has advantages over "gold standard" histopathology in the field of cancer detection as spectra can be produced in seconds and analysis can be automated yielding clinically useful quantitative data in minutes. A typical cell spectrum is shown in Fig. 2. Since Raman spectroscopy is also non-destructive and painless, research into its application as a diagnostic tool has increased steadily with a corresponding increase in participation by clinicians [9].

Many Raman spectroscopy diagnosis studies have been conducted on tissues, for example the detection of skin cancer basal cell carcinoma, with 95.8% $\pm$ 2.7% correct classification [11], and melanoma diagnosis [12]. The detection of lung tumours due to differing peak intensities of, for example, nucleic acids and phospholipids [13] and the ability to differentiate between 2 differing lung cancers [14] has also been demonstrated as have measurements to distinguish between normal and cancerous breast tissue [15]. Novel Raman spectroscopy equipment based on fibre-optic probes has also been developed to create a quick and reliable method of identifying breast cancers in vivo. Spectral maps of breast tissues have been constructed by Kneipp et al. [16], where ducts and

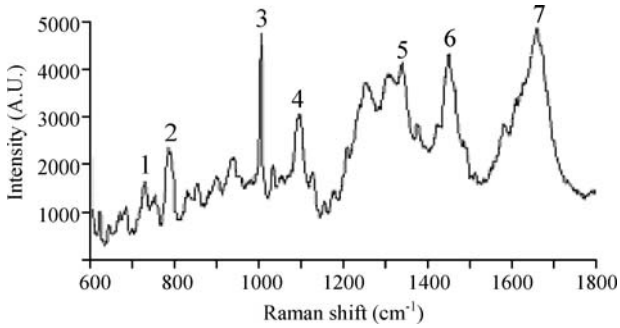

Fig. 2 A cell Raman spectrum indicating a few of the peaks typically found. Different cell types have slightly different spectra enabling classification based on Raman spectroscopy. 1: 729 cm^{-1}, Adenine, 2: 788 cm^{-1}, O-P-O DNA backbone, 3: 1005 cm^{-1}, Phenylalanine, 4: 1094 cm^{-1}, O-P-O DNA backbone, 5: 1340 cm^{-1}, Amide III, 6: 1449 cm^{-1}, CH_2 deformation, 7: 1659 cm^{-1}, Amide I

other tissue features can clearly be identified by differences in the Raman spectra and confirmed by conventional haematoxylin and eosin histological staining.

Investigations into the suitability of Raman spectroscopy as a diagnostic tool operating at the single cell level have been conducted in our group [17–19]. The ability to distinguish cancer cells from related primary cells has been demonstrated. We have also used Raman spectroscopy in conjunction with PCA analysis to distinguish between osteoblasts and osteosarcoma cells, showing the diagnostic potential of the technique [18].

Another method used for distinguishing between cancer cell types, as used by Krishna et al. [20], is to first grow the cells in standard culture flasks and then centrifuge them into a pellet composed of one cell line, or 50% of one cell line and 50% of another. This approach is more realistic as patients are likely to have a mix of healthy and cancerous cells in the area being tested so it is important to know whether Raman can detect differences between cancerous and mixed cancerous/non-cancerous cells. Raman spectroscopy can identify a particular cell type within a mixture of human cancer cell types (human breast cancer MCF7 cell line and either human promyelocytic leukaemia sensitive cell line HL60 or human uterine sarcoma cell line (Mes-sa)). The mean spectral differences between the groups indicate differences in relative amounts of DNA, proteins, lipids and carotenoids; these differences can be used for classification of the cells via multivariate analysis. Not only do the pure cell lines form distinct groups in the PCA analysis, but a distinct group can also be observed with a mixture of the same 2 cell types. This implies that Raman spectra of a heterogeneous tissue comprising cancer and non-cancer cells would still allow positive identification.

Crow et al. [21], used Raman spectroscopy to identify and grade immortalised prostate cancer cells. They studied four cell lines (LNCaP, MDApca2b, PC 3, DU145) representing various stages and aggressiveness of androgen-independent prostate cancer. LNCaP and MDApca2b can be considered to be well-differentiated and retain androgen sensitivity, whereas PC 3 and DU145 are less differentiated, have no androgen sensitivity and show increased aggressive ability such as higher migration and invasiveness. PCA and three linear discriminant functions were applied to each of the data in order to increase the separation between cell lines and thus diagnostic ability of the algorithm. The diagnostic ability was then tested for each individual spectrum using the 'leave one out' cross-validation method. Sensitivity of at least 96% between the androgen-sensitive and insensitive cell lines was attained in the LDA analysis demonstrating the diagnostic potential of Raman spectroscopy.

2.2 Chemical detection and testing

Raman spectroscopy could prove a useful tool in determining the hazard and exposure assessment of chemicals in specific relation to human cells or tissues. There is a high demand for new testing methods for chemicals due to both financial and ethical pressures [22]. Raman spectroscopy has already been investigated for the purpose of building rapid biosensors for toxic chemicals [23]. The use of living cells in a biosensor may be of benefit in the case of an unknown chemical attack or a suspected chemical leak as the exact chemical may not be known and many other sensors only respond to specific chemicals. The toxins sulphur mustard and ricin were applied to A549 cells at various concentrations and statistical methods were used to characterise the differences between the interaction of cells and toxins [23]. The leave-one-out cross-validation LDA method showed that damaged cells could be detected with a sensitivity of 98.9% and specificity of 87.7%. A high degree of accuracy was achieved in identifying the nature of the toxic agent (88.6% in the case of sulphur mustard and 71.4% in the case of ricin), with most of the error occurring when testing low concentrations of the toxins, an aspect that will improve as more sensitive Raman spectroscopy systems are developed. The biosensor was also able to detect changes in the cell death mechanism between low concentrations of sulphur mustard (200 μM and 500 μM) and high concentrations (1000 μM) via specific spectral peak changes, with low concentrations inducing apoptosis of the cells and high concentrations inducing necrosis.

2.3 Pharmaceutical testing

Raman spectroscopy has extensively been used to characterise active groups and different polymorphs and isomers of pharmaceuticals [24], but little has been done to develop this technique for drug testing on human cells or tissues. Our group has recently been developing techniques to assess the potential of Raman spectroscopy in this field, specifically the action of the anti-cancer drug etoposide on the human lung epithelial cell line A549 has been investigated [25]. The drug causes double-stranded breaks in the DNA of replicating cells, thus targeting cancer cells much more readily than healthy cells. By constructing a CLS model from representative cellular biopolymers such as DNA, RNA and various lipids, proteins and carbohydrates, cellular changes over a period of 48 h exposure to etoposide were monitored. As expected, the predicted levels of DNA decreased with exposure time to etoposide (Fig. 3).

Uzunbajakava et al. have devised a method of investigating whether a cell responds to the addition of a drug [26]. An inactive cell has compacted DNA in the nucleus, whereas an active cell has more uniformly distributed DNA around the

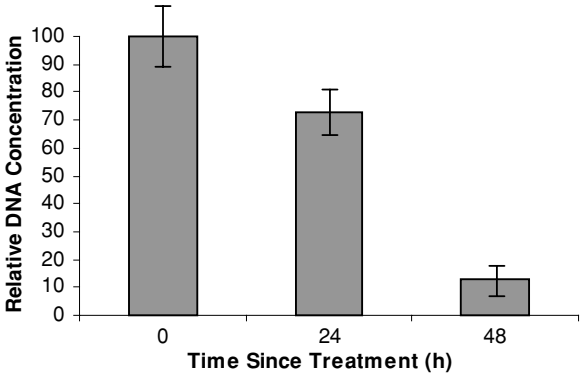

Fig. 3 DNA levels in A549 cells after treatment with the anti-cancer drug etoposide as determined using a CLS model applied to Raman spectroscopic data

nucleus. They could identify this by mapping DNA distribution using small step sizes of 1 μm or less through active lens epithelial cells and inactive peripheral blood lymphocytes.

2.4 Applications in tissue engineering

Initial work in our group showed spectral differences between healthy and apoptotic cells. Changes in DNA backbone bonding correspond to DNA fragmentation in apoptotic cells and were indicated by reduced peaks corresponding to the O-P-O backbone of DNA (e.g. at 788 cm^{-1}) [27]. Raman spectroscopy also confirmed that mitotic cells have higher levels of DNA. Evaluating these basic differences in health and status of cells provided the impetus for continued research into cellular behaviour that should prove invaluable in the field of tissue engineering.

Embryonic stem (ES) cells, with unique properties such as the ability to differentiate into any adult cell type, offer huge potential in the field of tissue engineering. One of the key difficulties in working with ES cells is to keep them in the undifferentiated state and maintain their pluripotency. Work by Notingher et al. has identified a means of characterising the degree of differentiation of murine embryonic stem cells (mES) [17]. RNA and protein levels were determined by calculating the area under the Raman spectral shift peaks at 813 cm^{-1} and 1005 cm^{-1} that correspond to RNA and phenylalanine (an amino acid that was used to indicate total protein levels). This ratio was used as a measure of mRNA translation and a marker of the degree of differentiation between undifferentiated mES cells and spontaneously differentiated cells (4 days). Cluster analysis showed that clear differences were present between undifferentiated mES cells, cells allowed to differentiate for 4 days and cells allowed to differentiate for 20 days. PCA analysis showed that 49% of the variance between the spectra was accounted for in PC1, the loading of which was remarkably similar to RNA, indicating that RNA concentration is the key difference determined by Raman in mES cells as they differentiate (Fig. 4).

Recent work in our group has used Raman spectroscopy in combination with gene and protein expression studies to investigate the effect of 45S5 Bioglass® (BG) dissolution ions on foetal osteoblasts (FOBs). BG is an important biomaterial with applications in tissue engineering including bone restoration and middle ear repair. Evaluating the effect of the extracts and dissolution products of BG on various cell types and in conjunction with various materials can be conducted non-invasively using Raman spectroscopy. PCA was used to analyse the Raman spectra and identified differences between FOBs exposed to BG for 1, 3, 7 and 14 days and between cells that were grown in media containing BG dissolution ions or not. PC 2 enabled the best separation between the groups, the loading of which had an appearance similar to the pure RNA spectrum suggesting that the level of RNA is a key difference between the groups. This was confirmed by a least squares evaluation that showed that RNA levels decreased over time.

RNA levels are associated with differentiation, suggesting that BG ions accelerated FOB differentiation. Raman spectroscopy can therefore be used to evaluate cellular behaviour non-invasively. This should be a huge benefit to investigators in the field of tissue engineering as it enables the rapid screening of real-time cellular interactions with bioactive materials.

Raman spectroscopy was successfully used to distinguish between various bone cells that are commonly used in bone tissue engineering [18]. Raman spectroscopy can be of great benefit in this field as bone cell phenotypic classification can be obtained at different time points throughout the same experiment without compromising cell viability. Raman spectroscopy combined with PCA and LDA analysis was used to differentiate between human osteosarcoma-derived MG63 cells and both primary osteoblasts (HOB) and retroviral

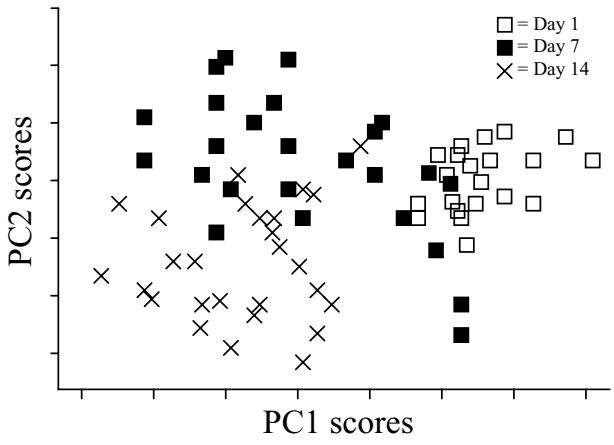

Fig. 4 A plot of PC 1 versus PC 2 for undifferentiated ES cells (day 1) and ES cells differentiated within embryoid bodies for 7 or 14 days. Variance in RNA levels has enabled unsupervised principal component analysis to distinguish between ES cells at different stages of differentiation

transfected human alveolar bone cells with SV40 large T antigen (SV40 AB). HOBs and SV40 AB are both non-tumour cell types. The classification of the cells based on the PCA-LDA analysis of the Raman spectra was tested with the cross-validation method. All the MG63 cells were classified correctly, showing that Raman spectroscopy and the statistical methods employed could identify key differences between cell types. Overall correct classification was 70%, the highest error being between HOB and SV40 AB cells which are the 2 non-tumour cell types.

3 Summary

The wide-ranging potential for Raman spectroscopy has been demonstrated. The technique has the key advantages of being non-invasive, rapid and able to detect even subtle changes or differences between cells. The ability to image and investigate more intricate mechanisms in cells and the diagnostic ability of the technique are constantly improving. Currently our group is working on further developing novel diagnostic and drug testing applications by improving the predictive ability of Raman spectroscopy through novel statistical models and sampling techniques.

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