Optical artefacts in transfection mode FTIR microspectroscopic images of single cells on a biological support: the effect of back-scattering into collection optics

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Infrared microspectroscopic imaging data of single human prostate cancer cells, on an artificial extracellular matrix (Matrigel) thin-film surface, are presented. The spectral intensity maps, obtained in reflection mode, appear to show that the protein intensity distribution observed at the location of a cell changes dramatically depending on the concentration and/or thickness of the underlying Matrigel layer. Specifically, cells adhered to a low protein concentration or thin surface exhibit a higher protein intensity signal than the surrounding layer whereas those on a high protein concentration or thick surface exhibit a lower protein intensity signal. These results are qualitatively explained by a simple model that takes into account the fact that radiation scattered from cells can enter the collection optics of the microscope without passing through the Matrigel layer. This leads to an *apparent* reduction in absorption at the cell.

Introduction

In recent years there has been a significant increase in the use of infrared microspectroscopy to study biological and biomedical samples.^{1–4} In addition to studying tissue samples at cellular resolution there has been increasing interest in the study of single biological cells.^{5–11} However, after a flurry of activity in this field it has become apparent that spectra obtained from single eukaryotic cells are strongly influenced by factors other than simple biochemical composition. Previous studies have shown that IR spectra acquired from a cell with a condensed nucleus, such as a pyknotic nucleus¹² or a nucleus from a Gap₁ (G₁)-phase cell,¹³ exhibit atypical, non-Beer–Lambert reductions in the $\nu_s(\text{PO}_2)$ and $\nu_{as}(\text{PO}_2)$ peak intensities. This loss of signal was attributed to a significant reduction in the transmission of photons through the condensed chromatin. In addition, Mohlenhoff *et al.*¹² report that the nucleus can give rise to the well known phenomenon of Mie-type scattering, which takes place when the wavelength of incident radiation is similar to the size of the scattering particle. The Mie-type scattering results in a broad sinusoidal oscillation in the baseline of the spectrum and can lead to distortions in both the position and intensity of absorption bands.¹⁴ The situation is complicated further by the fact that biological cells even of a single cell line are not completely uniform in size and shape leading to variations in the extent of

Mie scattering. In this paper, infrared microspectroscopic imaging data of single human prostate cancer cells, on an artificial extracellular matrix, are presented along with a possible explanation of apparent irregularities in the IR spectrum of a eukaryotic cell based upon the existence of such scattering.

As part of a wider investigation into the metastatic behaviour of prostate cancer,^{15–17} we have used FTIR microspectroscopy to study PC-3 cells (prostate cancer cells from bone marrow metastasis) cultured onto a MirrIRTM slide partially coated with Matrigel, an artificial basement membrane consisting mainly of protein. The major components of Matrigel are laminin (56%; a glycoprotein), collagen type IV (31%) and other components including heparin sulfate proteoglycan, entactin (8%), and several growth factors, which are present at ng ml^{−1} and pg ml^{−1} concentrations.¹⁸ The aim of the research is to further understand the biochemical processes associated with cell migration. However, during the course of this research, anomalies in the infrared spectral intensity maps have been observed which need to be understood before meaningful conclusions can be drawn regarding the more subtle biochemical changes that might be occurring. It is these anomalies that are the subject of this paper.

Experimental

The MirrIRTM slides (Kevley technologies, Ohio, USA) are microscope slides with a special ‘low-e’ coating that is infrared reflecting but *ca.* 80% transparent to visible light. These slides were sterilised in 70% ethanol prior to use. Ten microlitres of Matrigel (50 $\mu\text{g ml}^{-1}$) were deposited as a stripe onto MirrIRTM slides at 4 °C using a chilled pipette. The slides were incubated at 37 °C for 2 h to enable the Matrigel to polymerise. Following this, 1.3×10^5 PC-3 cells were introduced in Ham’s F-12 media, which contained 7% v/v FCS and 2 mM

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Fig. 1 Schematic diagram showing the thin stripe of Matrigel on a MirriRTM slide.

L-glutamine (Invitrogen, Paisley, UK). The PC-3 cell line was then cultured upon the slides, Fig. 1, and were fixed in formalin according to standard procedures detailed elsewhere.^{10,11}

FTIR microspectroscopic maps with 6.25 μm pixel resolution were collected in rapid-scan mode using either a Biorad FTS-6000 equipped with a liquid-nitrogen-cooled 64×64 array MCT focal plane array (FPA) detector, or a Perkin-Elmer Spectrum One/Spotlight 300 spectrometer equipped with a liquid-nitrogen-cooled 16×1 MCT linear detector array. Mid-IR spectra were collected in reflection mode for the wavenumber range $4000\text{--}748\text{ cm}^{-1}$. The background scan was recorded at 8 cm^{-1} spectral resolution with 75 scans, whilst the sample scan was recorded at 8 cm^{-1} spectral resolution with 60 scans. SR-FTIR spectral maps of the PC-3 cell lines were collected at the Daresbury Laboratory synchrotron radiation source (SRS) using beamline 11.1 coupled to a Nicolet Continuum microscope equipped with a single point MCT detector.¹¹ FTIR area maps of the PC-3 cell were collected with a $7\text{ }\mu\text{m} \times 7\text{ }\mu\text{m}$ aperture sampling aperture at $3\text{ }\mu\text{m}$ step size. A background was recorded after every ten sample spectra to correct for water vapour differences during the course of the mapping experiment and differences in the SR beam current. Optical images of these cell lines were obtained at $320 \times$ magnification.

Results

Fig. 2A(i) shows an optical image of PC-3 cells on Matrigel. The red-dashed line denotes the Matrigel–MirriRTM substrate boundary. To the right of the red line is the bare MirriRTM surface, to the left is the MirriRTM surface covered in a layer of Matrigel. This area was analysed by FTIR microspectroscopy and the associated IR peak extracted images, depicting the intensity distribution of the lipid hydrocarbon [$\nu_{\text{s/as}}(\text{CH}_2) + \nu_{\text{s/as}}(\text{CH}_3)$] and protein (amide I + amide II) domains, are shown in Fig. 2A(ii) and (iii), respectively. Fig. 2B shows IR spectra acquired from each cell location (Locations 2 and 4) and the surrounding Matrigel (Locations 1 and 3). IR spectra of both Matrigel locations and Cell 2 demonstrate a flatter baseline in the spectral region $2800\text{--}1750\text{ cm}^{-1}$ relative to the same region in the IR spectrum of Cell 1. The irregularities observed within this spectral region do not occur as a result of absorption by functional group vibrations arising from Matrigel or the cell, but have been attributed to a combination of Mie-type scattering and anomalous dispersive effects.^{1,2} As expected, the lipid hydrocarbon signal demonstrates high intensity at the cell locations, relative to the Matrigel surroundings, due to the cumulative absorption of

Fig. 2 A: (i) Optical image of PC-3 cells on Matrigel. The red-dashed line denotes the Matrigel–MirriRTM substrate (right) interface. FTIR spectral maps depicting the intensity distribution of the (ii) lipid hydrocarbon and (iii) protein peak area signals. (iv) A cross-section through the protein intensity map is displayed as a graphical plot depicting the protein peak area values from a region of high concentration of Matrigel (at $0\text{ }\mu\text{m}$) to one of lower concentration (towards $218\text{ }\mu\text{m}$) and bisecting Cells 1 and 2. B: Representative FTIR spectra acquired from Cells 1 and 2 residing on Matrigel of different thickness.

lipid-containing biomolecules in the cells and Matrigel. Fig. 2A(iv) depicts the magnitude of the protein peak area values in the IR spectra acquired from consecutive points along the axis (solid black line) in the protein intensity map, Fig. 2A(iii). Looking first at the right hand side of Fig. 2A(iii) and (iv) it can be seen that the protein intensity at $210\text{ }\mu\text{m}$ is low. This is because the Matrigel layer is thin close to the boundary. The protein intensity rises at Cell 2 relative to the surrounding Matrigel layer. This is as expected since the cell is rich in protein and the data are consistent with those obtained for cells on the Matrigel-free MirriRTM slides.^{10,11} The intensity returns to a minimum again before rising steadily between 140 and $100\text{ }\mu\text{m}$. This rise is due to an increase in the protein concentration in the Matrigel which is a heterogeneous gel layer. (Note that the hydrocarbon signal is slightly lower in this area so the rise in protein signal is not due to a substantial increase in the thickness of the Matrigel layer, although an increase in thickness would give a similar result. Indeed, similar behaviour has been observed for cells on a variable thickness Matrigel layer but for the sake of brevity they have not been included here.) The protein intensity then decreases significantly, reaching a minimum at $80\text{ }\mu\text{m}$ corresponding to the position of Cell 1, before rising again in the area away from the cell. At the position of the cell, the IR beam has an

increased pathlength through protein (Cell + Matrigel) and therefore would be expected to experience an increase in absorption. The observed *decrease* in protein intensity at Cell 1 is completely unexpected.

Fig. 3A shows an optical image of a second sample of PC-3 cells cultured onto a Matrigel surface. This area has been chosen because it is well away from the edge of the Matrigel layer. Fig. 3B shows the infrared intensity map of (i) the protein signal and (ii) the lipid signal of the area shown in Fig. 3A. Again it is noticeable that there is a protein intensity gradient increasing from bottom right to top left of the image. This is mirrored inversely by the lipid intensity, which increases from top left to bottom right. Once again the phenomenon observed in Fig. 2A(iii) is also observed in Fig. 3B(i), *i.e.* where the protein intensity of the Matrigel is low the protein intensity at the cell is higher relative to the Matrigel background. Conversely, when the background Matrigel protein intensity is high the relative protein intensity at the cell is low. In order to verify these data further this sample was taken to the synchrotron facility and data were obtained from exactly the same area. Selected cells within the previously analysed area were carefully mapped point by point, with each cell map taking 3–5 h depending on size. The lipid and protein intensity distributions from these maps are shown in Fig. 3C. Importantly, the intensity maps confirm the data obtained with the FPA detector, for example, contrast the protein map of Cells 2, 3 or 4 with that of Cell 5. Note that Cell 1, located in the region between high and low protein intensity, seems to show an intermediate behaviour between the two extremes. Also note, with the higher quality synchrotron data, it is clear

that the nucleus exhibits more pronounced behaviour compared with the rest of the cell.

FTIR imaging studies combined with time-lapse video microscopy and using matrix metalloprotease (MMP) inhibitors have determined that the low IR protein signals exclusively at the cell-occupied area are not due to local proteolysis of Matrigel through matrix metalloproteases. Cells treated with MMP inhibitors such that they cannot degrade Matrigel still show a lower protein IR absorption signal if they are on a thick Matrigel layer. In addition, we have shown that cells that move from one position to another on the Matrigel do not leave a trail, where the protein intensity is reduced. This is the subject of a separate publication; however, it is clear that the apparent decrease in protein intensity observed in the infrared intensity map does not depict a real decrease in protein concentration at that location.¹⁹ In order to understand this apparent anomaly, a simplified model of the system is produced below that qualitatively reproduces the behaviour observed. Note that implicit in the model is a consideration of both variable protein concentration and variable Matrigel thickness.

The model

The system is modelled as in Fig. 4. The symbols on the left represent thickness t , Napierian absorption coefficient α and (for the nucleus) Napierian turbidity τ (a measure of scattering efficiency over all angles). Thickness t_1 is variable as the scan passes across the cell. We can consider either (i) fixed α_2 and variable t_2 (varying thickness of Matrigel) or (ii) fixed t_2 and varying α_2 (equivalent to varying concentration). The

Fig. 3 A: Optical image of PC-3 cells on Matrigel in a central area away from the Matrigel edge. B: Infrared intensity distributions of (i) protein and (ii) lipid obtained using a FPA detector. C: Infrared intensity maps of lipids and protein from the five cells indicated in A, obtained using a SR-FTIR microscope.

Fig. 4 Schematic diagram of the elements used in the model.

MirrIR™ slide is assumed to have 100% reflectivity. Ignoring any back-scattering from the nucleus into the detector at this stage, the intensity signal at the detector (at a particular wavelength) will be

$$I = I_0 \exp[-2t_1(\alpha_1 + \tau) - 2t_2\alpha_2] \quad (1)$$

This assumes that no scattered radiation enters the detector. It is also a slight approximation in that the incident irradiation is not exactly perpendicular to the slide. Essentially the irradiation passes forward within the volume trapped by two narrow semi-cones with a common apex and axis and returns within the adjacent mirror image of this volume. It is this factor which allows some back-scattering to reach the detector as indicated below. Consider scattering from the nucleus from the initial entry of the radiation, Fig. 5.

Area A , necessarily very small, is illuminated throughout the nucleus. Intensity at t is

$$I_t = I_0 \exp[-t(\alpha_1 + \tau)] \quad (2)$$

Fig. 5 Schematic diagram depicting an incremental volume Adt , of the nucleus for which both absorption and scattering are considered.

Incident energy flux on volume element Adt is $I_t A$. An infinitesimal part of that equal to

$$I_t A \times \tau dt \quad (3)$$

is scattered over all angles; this essentially defines turbidity τ . Alternatively, one can consider the flux scattered by the element in a particular direction, at angle θ to the incidence direction, per (solid angle $\times$ element volume). At one extreme, with scattering particle sizes much smaller than the radiation of light, this is $I_t R_\theta$, where R_θ is called the Rayleigh ratio and is thereby defined; for unpolarised radiation, it is inversely proportional to the fourth power of wavelength and directly proportional to $1 + \cos^2\theta$, so one can write the scattered flux for the infinitesimal element over all angles as

$$I_t R_{90} A dt \int_0^{2\pi} \int_0^\pi (1 + \cos^2\theta) \sin\theta d\theta d\phi = I_t R_{90} A dt \times 2\pi \times \frac{8}{3} \quad (4)$$

where R_{90} is the Rayleigh ratio at $\theta = 90^\circ$ (where $\cos\theta = 0$), ϕ is the second 3D angle and $\sin\theta d\theta d\phi$ is the elemental solid angle. Comparison of (3) and (4) indicates that

$$R_{90} = \frac{3\tau}{16\pi} \quad (5)$$

However, because of the scattering-dependence on the inverse fourth power of wavelength, Rayleigh scattering and consequent turbidity in the mid-IR is very weak. At the other extreme, where scattering particles are of similar size to the wavelength, scattering is substantially greater; this is Mie-type scattering for which the Rayleigh formulae are inapplicable. The mathematics here is much more complicated and indeed feasible only for idealised particle shapes such as spheres or cylinders.²⁰ However, the key qualitative feature is that scattering in forward directions is somewhat greater than that in the backward directions, and this distinction increases as the ratio of particle size to wavelength increases. For the purpose of our model, we require a compromise situation where there is both reasonable Mie-type scattering and reasonable relative scattering in backward directions; in practice, there could be a whole range of scatterer sizes, including Rayleigh scatterers. Importantly, the concept of an effective turbidity τ is still applicable as are (1)–(3).

Recalling the previously described shape of the incidence and detected beams, a significant fraction, say F , of the scattered flux is assumed to reach the detector. By (2) and (3), the scattered energy flux from the element reaching the detector will then be

$$FI_t A \tau dt \times \exp[-t(\alpha_1 + \tau)] = FI_0 A \tau dt \times \exp[-2t(\alpha_1 + \tau)] \quad (6)$$

the additional $\exp[-t(\alpha_1 + \tau)]$ on the left being associated with the fact that the scattered radiation has to pass through distance t of the nucleus before reaching the detector. Integration of (6) over all t (0 to t_1) then gives a scattering addition to intensity (1) of

$$\frac{FI_0 \tau}{2(\alpha_1 + \tau)} \{1 - \exp[-2t_1(\alpha_1 + \tau)]\} \quad (7)$$

There will be some scattering into the detector from the second pass through the nucleus but, because radiation will be substantially attenuated at this stage by both absorption and scattering, this additional scattering will be neglected in the model.

The total detected intensity divided by the incident intensity will then be

$$Q = \exp[-2t_1(\alpha_1 + \tau)] \left[\exp(-2t_2\alpha_2) - \frac{F\tau}{2(\alpha_1 + \tau)} \right] + \frac{F\tau}{2(\alpha_1 + \tau)} \quad (8)$$

One now needs to know whether Q increases or decreases with increasing t_1 – as the nucleus is traversed from edge to centre, *i.e.* what is the sign of $\frac{dQ}{dt_1}$?

$$\frac{dQ}{dt_1} = \underbrace{\exp[-2t_1(\alpha_1 + \tau)]}_{+ve} \left[F\tau - \underbrace{2(\alpha_1 + \tau)\exp(-2t_2\alpha_2)}_{+ve} \right] \quad (9)$$

Since both the first exponential and the sign of the multiplier of the second exponential are positive as indicated, the sign of $\frac{dQ}{dt_1}$ is the sign of

$$\frac{F\tau}{2(\alpha_1 + \tau)} - \exp(-2t_2\alpha_2) \quad (10)$$

Note that when either F (the fraction of scattered radiation reaching the detector) or τ (the turbidity) = 0, the quotient in (10) is zero and therefore the whole of (10) and $\frac{dQ}{dt_1}$ are negative in accordance with normal expectation. Otherwise, the positive quotient could exceed $\exp(-2t_2\alpha_2)$ and thus make $\frac{dQ}{dt_1}$ positive.

(10) will be (positive/negative) if

$$\frac{F\tau}{2(\alpha_1 + \tau)} > / < \exp(-2t_2\alpha_2) \quad \text{or} \quad t_2 > / < \frac{1}{2\alpha_2} \ln \left[\frac{2(\alpha_1 + \tau)}{F\tau} \right] \quad (11)$$

$$\text{or} \quad \alpha_2 > / < \frac{1}{2t_2} \ln \left[\frac{2(\alpha_1 + \tau)}{F\tau} \right]$$

For a particular value of F and α_2 , there will be a value of t_2 (essentially the thickness of the Matrigel) where, as it increases from zero, (10) changes over from negative to positive. Likewise, for a particular value of F and t_2 , there will be a value of α_2 (proportional to the concentration of protein in the Matrigel) where as it increases from zero, (10) changes over from negative to positive. In other words, there will be a particular thickness, or concentration, whereby the (protein) intensity observed at the cell relative to that in the surrounding Matrigel changes as depicted in Fig. 2 and Fig. 3. Thus the model qualitatively explains the observed phenomenon.

Discussion

The model explains the observed behaviour because the fraction of the scattered light that enters the collection optics without passing through the bulk of the sample, thus leading to a decrease in extinction, has been taken into consideration. The opportunity for scattered light to be detected in this way is due to the large solid angles of collection used in typical infrared microscope optics (for a 32 $\times$ objective the half angle

is 40.5° and this means that scattered radiation, admittedly at various intensities, will reach the detector at all angles between about 180° – for adjacent parts of the annular semi-cones – and 180° – 2 $\times$ 40.5° = 99°) and the fact that the data are collected in reflection mode. Fig. 6 shows the two extreme scattering situations. Fig. 6(a) schematically depicts Mie scattering where the total amount of scattering is high. However, Mie scattering is predominantly a forward-scattering process which means although the total amount of scattering is high the proportion back-scattered is low. Conversely, if one considers the other extreme, where Rayleigh scattering from particles much smaller than the wavelength of light occurs, the total amount of scattering is very low but the proportion of this back-scattered into the detector is high Fig. 6(b). A eukaryotic cell contains scatterers covering a wide range of shapes and sizes, *e.g.* nucleoli, mitochondria, vesicles, lysosomes, ribosomes and proteins. In particular, a large number that are of an intermediate size, 0.5–1 μm , lead to scattering with a range of angular/intensity distributions between the two regimes depicted in Fig. 6. Importantly for this model, it is simply the total amount of light scattered directly from the cell into the collection optics that results in an apparent decrease in protein absorption.

What are the implications of these results? In order to obtain spectroscopic data from isolated or groups of cells, where the

(a) Mie Scattering

Mie scattering predominantly in forward direction

(b) Rayleigh Scattering

Significant Rayleigh scattering in all directions

Fig. 6 Schematic diagram showing the different angular distributions of scattered light associated with the (a) Mie and (b) Rayleigh scattering. Note, light back-scattered through angles between 100 and 180° is capable of directly entering the collection optics, see insert.

spectrum of the cell alone is important (for, say, identification purposes) they must be attached to or deposited on an infrared-transparent or infrared-reflecting surface. Using either sampling method, transmission or transflection, scattering of infrared radiation directly from the cell, specifically the nucleus, will alter the appearance of the spectrum. This phenomenon is reasonably well understood and strategies have been developed that help alleviate the problem.¹⁴ Thus, in most cases, qualitative and semi-quantitative results regarding intensities can still be obtained. However, interest is growing in obtaining data from cells in model biological environments where the focus is on the interaction between the cell and its surroundings. The above results show, however, that meaningful data can only be obtained from the cells if the underlying surface is optically very thin. If the underlying surface becomes optically thick, either due to an increase in physical thickness or an increase in concentration of a species of spectral relevance, then the intensity data can be distorted to an extent that they become meaningless. This has serious ramifications for researchers working in the field and needs to be taken into consideration during the experimental design.

Conclusion

In this paper, infrared microspectroscopic intensity images of PC-3 cells on a Matrigel surface have been presented. The data show that on areas of Matrigel where the protein signal intensity in the Matrigel is low, due to low concentration and/or thickness, the protein signal at the cell is high compared with the surrounding area. Conversely, on areas where the protein signal is already high then the protein signal at the cell is lower than the surrounding area. A model has been produced which shows that a switch from a positive to a negative signal, with respect to the surrounding area will occur at a given thickness of or concentration of protein within the Matrigel layer. Importantly, the model includes light that is directly back-scattered into the microscope collection optics which is likely to originate from scattering between the Mie and Rayleigh regimes.

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