Rapid Communication

Journal of RAMAN SPECTROSCOPY

Received: 13 February 2009 Accepted: 24 February 2009 Published online in Wiley Interscience: 17 April 2009

(www.interscience.wiley.com) DOI 10.1002/jrs.2269

The intensity of the 1602 cm^{-1} band in human cells is related to mitochondrial activity

Vishnu Vardhan Pully, and Cees Otto*

We report a Raman band at 1602 cm^{-1} in the spectra of human cells, which previously had only been observed in mitochondria of yeast cells. This band, which has not yet been assigned to a particular molecular species, was found to occur in HeLa cells, peripheral blood lymphocytes, human mesenchymal stem cells and bovine chondrocytes. The band is proposed as an indicator of the activity of mitochondria in cells. Cells were cultured with and without serum or temporarily deprived of serum. The band can be observed for all these variations in cell culture methodology. The band intensity decreases under the influence of an increase of the calcium ion concentration in the surrounding medium. **Copyright © 2009 John Wiley & Sons, Ltd.**

Keywords: mitochondria; Ca^{2+} influence; hyperspectral Raman imaging; living human cells

Introduction

Recently, Huang *et al.*[1] detected a band at 1602 cm^{-1} in the Raman spectrum of mitochondria of a yeast cell. The intensity of the band was observed to be dependent on the nutrition status and could be made to decrease in intensity after addition of oxidants,[2] KCN,[1,3] application of stress[2] and variation in atmospheric condition[2] and was defined as 'Raman spectroscopic signature of life'.[1] Raman spectra of isolated mitochondria[4] revealed a band at 1602 cm^{-1}, and made it unequivocally clear that the origin of this band was related to this cell organelle. This was in agreement with an earlier observation[3,5] that this band co-localized with a GFP-tagged mitochondrial protein. A change in the Ca^{2+}-ion concentration in the medium surrounding the isolated mitochondria gave rise to a decrease in the intensity of this band.[4] Metabolic processes in mitochondria are dependent on Ca^{2+} ions and mitochondria are important as Ca^{2+}-storage sites for cellular activity and cell signaling.[6]

Although the intensity of the band gradually reduced when nutrients were removed from the medium, it was shown that the addition of nutrient medium revitalized yeast cells[2] and led to an increase in the intensity of the 1602 cm^{-1} band, thus establishing the reversibility in the intensity of this band. The intensity decrease of the band in yeast cells is accompanied by the occurrence of polyphosphates[7] in the vacuole. The observations described above are of profound importance as they suggest that information can be acquired on mitochondrial activity with label-free Raman imaging methods. The band, so far, has not been reported in Raman spectra[8–10] or Raman hyperspectral images[11–14] of living or fixed cells.

Here, we show the presence of a 1602 cm^{-1} band in other eukaryotic, living cells like the immortal cancer cell line of HeLa cells, bovine chondrocytes, intravenously acquired, fresh peripheral blood lymphocytes (PBL) and human mesenchymal stem cells (hMSCs). We also show the decrease in intensity of this band under the influence of increased $[Ca^{2+}]$ ions in living HeLa cells. This result corresponds qualitatively with earlier observation in isolated mitochondria.[4]

Experimental

A confocal Raman microspectrometer, which has been described before,[15] was used. The system was based on a krypton ion laser light source with an emission band at 647.1 nm. Hyperspectral Raman imaging was performed by stepping the laser beam over the sample in a raster pattern and spectral acquisition at each position with a laser power of 35 mW and dwell time of 0.5 s/pixel. Univariate and multivariate analyses were performed over the hyper spectral Raman data as described earlier.[11,12]

All eukaryotic cells, HeLa cells, bovine chondrocytes, PBLs and hMSCs, were seeded on CaF_2 slide or glass cover slips and cultured overnight in an incubator. The adhered cells were washed twice with phosphate buffer solution (PBS) without Ca^{2+} ions before Raman measurements. The experiments on 'glass coverslides' were a control to test if CaF_2 slides could give rise to a decrease in intensity. HeLa cells were separately cultured overnight over glass cover slips in an incubator under the influence of cell culture medium with serum, without serum and with serum starvation. In serum starvation the medium with serum was added to the cells 6 h before the measurements. In previous procedures cells were prepared for Raman imaging by washing the culture medium away with PBS, which does not contain Ca^{2+} ions. In the present experiments one fraction of the cells was washed with PBS and a second fraction was washed with Hanks balanced salt solution (HBSS). A standard solution of HBSS contains 1.26 mM. Ca^{2+} ions.

Results and Discussions

Figure 1(a) shows the white light micrograph of the nucleus and the perinuclear region of a HeLa cell, whose corresponding hierarchical

* Correspondence to: Cees Otto, Biophysical Engineering Group, MESA+ Institute for Nanotechnology, University of Twente, Enschede, The Netherlands. E-mail: c.otto@utwente.nl

Biophysical Engineering Group, MESA+ Institute for Nanotechnology, University of Twente, Enschede, The Netherlands

Copyright © 2009 John Wiley & Sons, Ltd.

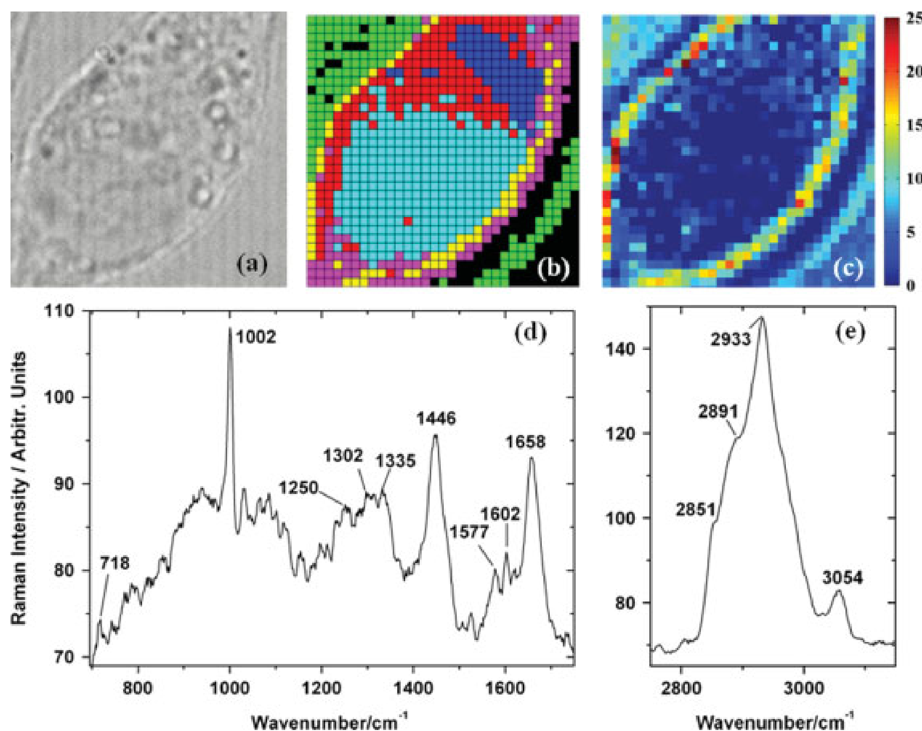

Figure 1. Raman microspectroscopy of HeLa cell. (a) White light micrograph of a HeLa cell measured. The size of the area is 20 × 20 µm. The pixel size is 625 nm, (b) An image from hierarchical cluster analysis (seven clusters) of the HeLa cells shown in (a), (c) Univariate Raman image over the band 1602 cm^{-1} ($\Delta = 22$ cm^{-1}), (d) Raman difference spectra of the average spectrum of the yellow pixels minus the average spectrum of the black pixels, which correspond to the buffer.

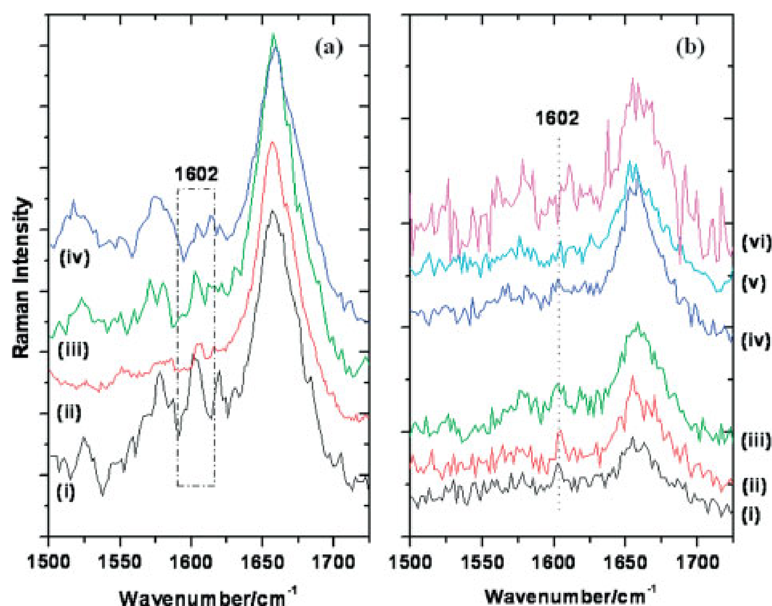

Figure 2. Raman difference spectra (cytoplasm minus buffer). (a) Raman spectroscopy showing the presence of 1602 cm^{-1} band in different eukaryotic cells (i) HeLa cell, (ii) bovine chondrocyte, (iii) hMSC and (iv) PBL cell; and (b) Influence of PBS ($-Ca^{2+}$) and HBSS ($+Ca^{2+}$) buffer media on the intensity of the 1602 cm^{-1} band in HeLa cells. (i) With serum and with PBS wash, (ii) Without serum and with PBS wash, (iii) With serum starvation and with PBS wash, (iv) With serum and with HBSS wash, (v) Without serum and with HBSS wash and (vi) With serum starvation and with HBSS wash.

cluster image (seven levels), is shown in Fig. 1(b). In Fig. 1(c) the univariate Raman image is shown of the band area between 1591 cm^{-1} and 1613 cm^{-1} encompassing the 1602 cm^{-1} band. A high correspondence between the univariate image in Fig. 1(c) and the yellow cluster in Fig. 1(b) was observed. The Raman difference spectrum of the yellow cluster spectrum minus the cluster spectrum corresponding to buffer (black pixels) is shown in Fig. 1(d). The spectrum reveals a clear band at 1602 cm^{-1}. The corresponding high wavenumber spectrum is presented in Fig. 1(e). A band at 2851 cm^{-1} caused by lipids can be observed as a weak shoulder on the strong bands at higher wavenumber, dominated by the partially branched, aliphatic aminoacid residues

Copyright © 2009 John Wiley & Sons, Ltd.

normal to proteins. The band at 1602 cm^{-1} correlates with both lipid bands and protein bands at 1002 cm^{-1} (Phenylalanine), 1250 cm^{-1} (Amide III), 1302 cm^{-1} (CH_3, CH_2 bending and twisting of lipids and proteins), 1446 cm^{-1} (protein and lipid CH_2 bending mode) and 1658 cm^{-1} (Amide I). The 1602 cm^{-1} band also correlates with a band at 718 cm^{-1}. This band can be assigned to the C–N stretching vibration in phosphatidylcholine lipid head groups, which are abundant in the mitochondrial membrane [16].

The band at 1602 cm^{-1} can also be observed in the cytoplasm of other eukaryotic cells like bovine chondrocytes, PBLs and hMSCs as shown in Fig. 2(a). The intensity of the band varied with cell type. The understanding behind this observation awaits an interpretation of the band. However, it may simply be a result of variations in the concentration of mitochondria or differences in the mitochondrial Ca^{2+}-ion concentration.

The Raman difference spectra of cells washed with PBS buffer and with Hank's buffers are shown in Fig. 2(b). The spectra in Fig. 2(b) (i, ii and iii) showed a significant band of 1602 cm^{-1} for the cell culture conditions that were washed with PBS solution. However, the band was much weaker or not observed in Fig. 2(b) (iv, v and vi) when the cells were washed with Ca^{2+}-ion-containing HBSS solution. The influence of Ca^{2+} ions on the intensity of the band at 1602 cm^{-1} corresponds well with results shown by Tang *et al*.[4] The results in Fig. 2(b) show that the occurrence/absence of the 1602 cm^{-1} band is not dependent on the culture methods included in this study, i.e. with serum, without serum or with serum starvation, but depends on the Ca^{2+} ion concentration. The results also show that CaF_2 substrates did not clearly influence the visibility of the 1602 cm^{-1} band.

The assignment of the 1602 cm^{-1} band to a particular molecular species is not clear. So far the band has revealed correlations with lipid-type modes and (vide supra) also with a mixture of phospholipid and protein bands. The interpretation so far was that only a single new band was observed. This could suggest a di-atomic species. The Raman band of O^{16}–O^{16} occurs at 1553 cm^{-1}. Huang *et al*.[5] showed that the 1602 cm^{-1}-band position was not dependent on (isotope) labeled oxygen. This supports the conclusion that the band is not connected to O_2 as a di-atomic molecule. Tang *et al*.[4] observed the band with an excitation wavelength at 785 nm, whereas Hamaguchi *et al*. used 633 nm to excite the Raman spectra. Irrespective of the precise excitation wavelength the intensity of the band was comparable to intense Raman bands at 1446 cm^{-1} and 1658 cm^{-1}. This indicates an absence of a clear dependence on the excitation wavelength. A dependence would be expected if a chromophoric group from (one type of) the mitochondrial proteins would contribute to the band. This observation makes a potential contribution of (pre-) resonant Raman scattering from a molecular species less likely, although it is not completely excluded. Our Raman setup uses 647.1 nm as an excitation wavelength and shows the 1602 cm^{-1} band to be significantly weaker than the 1446 cm^{-1} and 1658 cm^{-1} bands in the same cluster. Again, without a credible assignment, it is purely speculative at this point why this is precisely so. The Raman spectrum (Fig. 1(d)) shows intense correlations with a complex series of other bands of which a phenylalanine mode at 1002 cm^{-1} is the most pronounced. We do not feel however that the 1602 cm^{-1}-band is a phenylalanine mode, as phenylalanine has intense modes at 1586 cm^{-1} and 1605 cm^{-1}. The contribution of these modes can be observed in Fig. 2(a). Tang *et al*.[4] have proposed that the band originates from tyrosine. We believe this to be incorrect. Tyrosine does have a medium to strong band at 1602 cm^{-1}; however, tyrosine also has a band two times more intense at 1616 cm^{-1}, which is at best weak in the spectra. Other correlations with tyrosine bands at 830 cm^{-1} and 850 cm^{-1} are also weak to absent.

In conclusion, we have shown in whole eukaryotic cells from four different origins that the band at 1602 cm^{-1} can be observed. In agreement with Tang *et al*.[4] we have observed that the intensity of the 1602 cm^{-1} band depends on Ca^{2+}-ion uptake. The observation of the band in isolated mitochondria[4] is evidence that the band is not a 'signature of life' as isolated mitochondria could be considered to be functional but not capable of self-reproduction. The fact that the intensity of the band is reversible upon nutrition/malnutrition[2] suggests that malnutrition was not sufficiently advanced to signal the cell into apoptosis, as it is known that apoptosis is an irreversible pathway.[6] The assignment of the band to mitochondria as a cell organelle previously shown by[3,4] is also supported by our results. Apart from co-localization with lipids, our spectra also strongly support a co-localization with proteins.

Acknowledgement

The financial support of the Dutch Program for Tissue Engineering through grant number TGT. 6737 is gratefully acknowledged.

References

[1] Y. S. Huang, T. Karashima, M. Yamamoto, H. Hamaguchi, *J. Raman Spectrosc.* **2003**, *34*, 1.
[2] Y. S. Huang, T. Nakatsuka, H. Hamaguchi, *Appl Spectrosc.* **2007**, *61*, 1290.
[3] Y. S. Huang, T. Karashima, M. Yamamoto, T. Ogura, H. Hamaguchi, *J. Raman Spectrosc.* **2004**, *35*, 525.
[4] H. Tang, H. Yao, G. Wang, Y. Wang, Y. Qing Li, M. Feng, *Opt. Express.* **2007**, *15*, 12708.
[5] Y. S. Huang, T. Karashima, M. Yamamoto, H. Hamaguchi, *Biochemistry* **2005**, *44*, 10009.
[6] D. R. Green, J. C. Reed, *Science.* **1998**, *281*, 1309.
[7] Y. Naito, A. Toh-e, H. Hamaguchi, *J. Raman Spectrosc.* **2005**, *36*, 837.
[8] I. Notingher, S. Verrier, H. Romanska, A. E. Bishop, J. M. Polak, L. L. Hench, *Spectroscopy*. **2002**, *16*, 43.
[9] S. Verrier, I. Notingher, J. M. Polak, L. L. Hench, *Biopolymers* **2004,**, *74*(,), 157.
[10] K. W. Short, S. Carpenter, J. P. Freyer, J. R. Mourant, *Biophys J.* **2005**, *88*, 4274.
[11] N. Uzunbajakava, A. Lenferink, Y. Kraan, B. Willekens, G. Vrensen, J. Greve, C. Otto, *Biophys J.* **2003**, *84*, 3968.
[12] H. J. van Manen, Y. M. Kraan, D. Roos, C. Otto, *Proc. Natl. Acad. Sci.* **2005**, *102*, 10159.
[13] C. Matthäus, S. Boydston-White, M. Miljković, M. Romeo, M. Diem, *Appl. Spectrosc.* **2006**, *60*, 1.
[14] C. Matthäus, T. Chernenko, J. A. Newmark, C. M. Warner, M. Diem, *Biophys. J.* **2007**, *93*, 668.
[15] H. J. van Manen, A. Lenferink, C. Otto, *Anal. Chem.* **2008**, *80*, 9576.
[16] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, *Molecular Biology of the Cell* – Fifth edition, Garland Science: New York, **2007**.

Copyright © 2009 John Wiley & Sons, Ltd.