Real-time molecular assessment on oxidative injury of single cells using Raman spectroscopy

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Oxidative stress is encountered in many biological systems; the resultant oxidative injury plays a significant role in the pathogenesis of diverse diseases. Conventional measurements on oxidative injury are employed almost exclusively on a large population of cells either by counting the fraction of cell death or by observing the fluorometric change resulting from exogenous reagents, thereby lacking in molecular detail and temporal specificity. In this work we combine laser tweezers and Raman spectroscopy to observe the response of single cells to oxidative stress. By measuring the temporal changes of vibrational spectra of single optically trapped cells, we demonstrate a molecular-level assessment of cellular oxidative injury in real time, both qualitatively and quantitatively, without the introduction of exogenous reagents. The main experimental findings are supported by the observation of Raman spectra of intermediates and downstream products. The abrogation of the above changes by ascorbic acid further illustrates the therapeutic effect of antioxidants against cellular oxidative injury. This approach is extensible to studies exploring the biochemical transformation of single cells or intracellular organelles in response to various chemical or physical stimuli. With the aid of 'molecular fingerprints', single-cell Raman spectroscopy exhibits a great potential for accessing the chemical aspects of cellular bioactivity, yielding insight into pathophysiological processes and assisting the development of novel therapeutic interventions against diseases. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: oxidative stress; reactive oxygen species; lipid peroxidation; laser tweezers; single cells

Introduction

Oxidative stress, a condition referring to an elevated level of reactive oxidative species (ROS), is commonly seen in various physiological and pathological conditions such as ageing and ischemia-reperfusion injury. When an oxidative stress transcends the capacity of endogenous antioxidant systems, oxidative injury occurs.[1] Implicated in more than 100 acute, chronic and degenerative diseases such as hypertension,[2] atherosclerosis[2,3] and neurodegenerative disorders,[4] oxidative injury has attracted much interest in both fundamental and clinical researches.[5–8] Among all ROS in biological systems, hydroxyl radical is one of the most injurious.[9] Superoxide, generated either by oxidation of xanthine or as a result of electron leak from the mitochondrial electron transfer chain, is readily dismutated by superoxide dismutase to hydrogen peroxide (H_2O_2), which subsequently yields the hydroxyl radical through catalysis by Fe^{2+} (the Fenton reaction).[9] This highly reactive radical is detrimental to any intracellular molecule (lipid, protein, DNA, etc.) that it encounters, resulting in structural and functional derangements.[10,11] Several assays have been developed for the study of oxidative injury,[6,8,12] but these measurements employed almost exclusively the use of a large population of cells owing to limited sensitivity; the results are regarded at best as the averaged behaviour of an ensemble of cells. Furthermore, most conclusions were drawn on observing a pattern of cell death, thereby lacking both molecular-level details and temporal specificity. Fluorometric assays have been used to detect the intermediate or downstream products generated during cellular oxidative injury,[5] but these assays require the introduction of exogenous reagents to produce fluorescent signal. Interference with the cellular physiology under study by these reagents almost inevitably exists and is typically difficult to evaluate.

Advances in laser technologies have stimulated the investigation of biomedical processes at the level of a single cell. Laser tweezers enable one to manipulate a single suspended cell.[13,14] Raman spectroscopy offers structural and quantitative information on the molecules by direct measurements of the vibration characteristics of the chemical bonds. Each characteristic vibration frequency is associated with a specific functional group, and the relative intensity of that vibrational band reflects the variation in concentration of the particular functional group. Through serial recording of the Raman spectra, the transformation of a biochemical reaction can thus be dynamically monitored.[15] By using confocal Raman spectroscopy, the chemical transformation of cellular bioactivity has been studied.[16,17] Single fission yeasts under different nutrients, stresses, and atmospheric conditions have also been examined to elucidate the role of a metabolic activity-related Raman band.[18]

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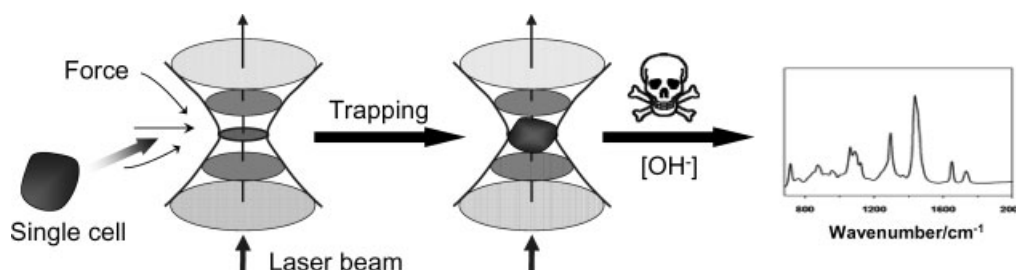
Figure 1. Cartoon illustration of the experimental procedures.

In this study we employed laser tweezers-assisted single-cell Raman spectroscopy to monitor the biochemical transformation of a single yeast cell under oxidative stress (Fig. 1). The integration of these two techniques extends the potentials of both and allows the probing of molecular-level information of a single cell in response to physical or chemical perturbation in real time on a label-free basis.[19–21] By means of sequential spectral measurements, we identified lipid peroxidation occurring during oxidative injury and demonstrated the therapeutic effect of ascorbic acid to such injury at the molecular level.

Materials and Methods

Experiments

All Raman spectroscopic measurements of single biological cells were carried using a home-built system that integrates the capability of optically manipulating a single cell using 'laser tweezers' and Raman spectroscopic measurements on the optically trapped cell (Fig. 2). A 532-nm diode-pumped solid-state laser (532-25, DPSS Inc., USA) was employed to capture individual cell (or liposome hereafter) and produce Raman scattering from the optically trapped cell. The laser-beam was collimated and expanded to about 1 cm in diameter with a spatial filter comprising two lenses and a 100-µm pinhole. The beam was reflected by

a long-pass filter (LP03-532RS-25, Semrock Inc., USA), directed towards to an inverted optical microscope (IX71, Olympus Co., Japan) and focused onto the sample with a high numerical aperture objective (UPLSAPO 100× Oil, Olympus Co., Japan). The waist of the laser beam was adjusted such that the back aperture of the microscope objective was slightly overfilled in order to create a sufficiently large gradient force for tight trapping with a relatively low laser power. The spot size at the focal plan was estimated to be about 0.3 µm in diameter. The back-scattered Stokes Raman signal was collected by the same objective and transmitted through the long-pass filter with the anti-Stokes and the Rayleigh scattering suppressed. The transmitted Stokes Raman scattering was spatially filtered with a 100-µm confocal pinhole aperture. Since the focal spot of the laser is smaller than the size of yeasts (3–5 µm in diameter), the confocal aperture effectively rejected most of the off-focusing contribution from the medium and the substrate. The Stokes Raman-scattered light was focused onto a fiber-coupled spectrograph (Shamrock SR-303i, Andor Technology PLC., U.K.) that was equipped with a 600 groves/mm grating and a thermoelectrically cooled CCD detector (iXon, Andor, England). A video camera system was used to facilitate the trapping of single cells. Throughout this study, the power of the laser was carefully adjusted to be 1.6 mW, measured after the objective, in order to stably hold the suspended cell and to produce Raman spectra with a satisfactory signal-to-noise ratio
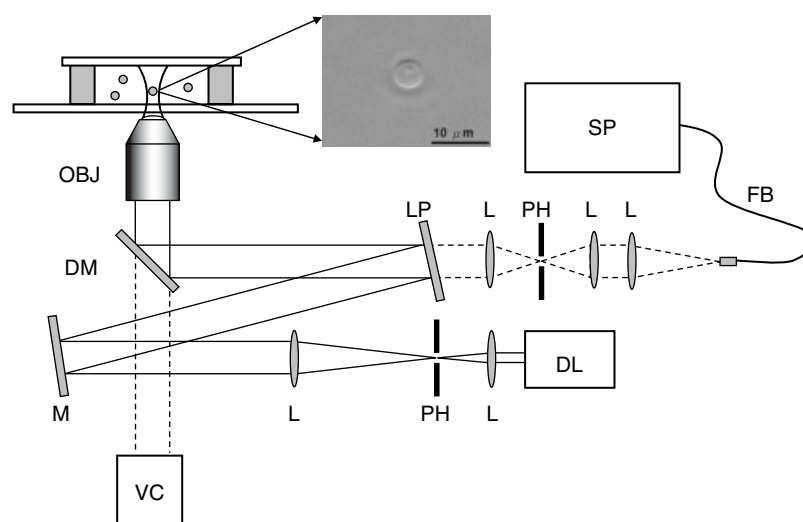

Figure 2. Experimental setup. The beam of a diode-pumped solid-state laser (DL) was expanded and collimated by a spatial filter that comprises two lenses (L) and a pinhole (PH). The beam was reflected by a mirror and a long-pass filter (LP), and was directed to a microscope objective (OBJ). The Raman signal collected by the same objective was transmitted through the long-pass filter and another spatial filter and was focused onto a fiber (FB) coupled spectrograph (SP). A video camera (VC) was installed to facilitate the observation of optically trapped single cells. The bright-field optical image captured by the video camera shows an optically trapped yeast in solution.

without introducing photodamage to the sample. The acquisition time for each spectrum was 60 s. All spectra shown here have been subtracted from a background that was obtained by taking a measurement on the medium without capturing any cells under the same conditions of illumination and acquisition. The serial changes of each band were expressed as the fold of change compared to the baseline. Suspended polystyrene beads (2 μm in diameter) were used to optimize and calibrate the system. The spectral resolution of the our system was estimated to 8 cm^{-1} from the full width at half-maximum (FWHM) of the 1001 cm^{-1} band of polystyrene.

Yeasts, liposomes, and reagents

The yeast culture (*Pichia pastoris*) was kindly provided by Prof. Y.K. Li and was prepared according to standard protocols.[22] All chemicals were purchased from Sigma-Aldrich unless otherwise specified. The simple liposome was made from mono-unsaturated lipids (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, POPC) purchased from Avanti Polar Lipids using protocols that have been described in the literature.[23] The oxidative stress was introduced by adding a solution of hydroxyl radical prepared by mixing equal amounts of solutions of H_2O_2 and $FeSO_4$. The final concentration of hydroxyl radicals was estimated to be 0.19 mM, assuming that the reaction between Fe^{2+} and H_2O_2 was carried to completion. The thiobarbituric acid-reactive substance (TBARS) kit was purchased from ZeptoMetrix and was used according to the protocol provided by the manufacturer.

Statistics

Comparison was made between groups by two-way repeated measures analysis of variance (ANOVA). If the main group effect was statistically significant ($P < 0.05$), subsequent comparisons were made at each time point using a two-sample *t*-test. All data are presented as means ± s.d.

Results and Discussion

The sequential Raman spectra of a normal yeast solution (control) are shown in the left panel of Fig. 3a. We identify four separate spectral bands (1266, 1300, 1441 and 1651 cm^{-1}) and assign these bands to =CH bending, CH_2 twisting, CH_2 bending and C=C stretching modes, respectively. On prolonged exposure to a laser beam, no change is detected, indicating no significant thermal or photochemical damage to that single cell. When the yeast solution is treated with hydroxyl radicals, as seen in the right panel of Fig. 3a, the bands at 1651 and 1266 cm^{-1} diminish progressively ($P < 0.001$ and $P = 0.023$ compared to control, respectively). After exposure to hydroxyl radicals for 40 min, the intensities of these two bands decrease by 67% ($P < 0.001$) and 42% ($P < 0.001$), respectively. In contrast, the bands at 1300 and 1441 cm^{-1} remain without significant alteration ($P = 0.16$). These observations indicate that the functional groups involving cis C=C stretching and =CH deformation disappear gradually during the oxidative process, whereas the twisting and bending modes of CH_2 remain unaltered, a result consistent with the peroxidation of C=C bonds induced by the hydroxyl radical.[24]

To verify this deduction and to acquire mechanistic insight, we prepared liposomes exclusively from mono-unsaturated lipids, POPC, and undertook the same protocol as in the experiments on a single yeast cell. As shown in Figs. 4a and b, the band at 1651 cm^{-1} decreases progressively by treating the single liposome with hydroxyl radical ($P < 0.001$) compared to liposome control (60% decrease after 40 min, Fig. 4b), whereas the band at 1300 cm^{-1} exhibits no significant alteration ($P = 0.60$, Fig. 4b). Results from this liposome system made of pure lipids support the conclusion that the decrease in intensity of the band at 1651 cm^{-1} in the yeast is, at least in a major part, caused by peroxidation of the C=C bonds.

To further consolidate a correlation between the decreased Raman signal associated with a cis C=C stretching mode
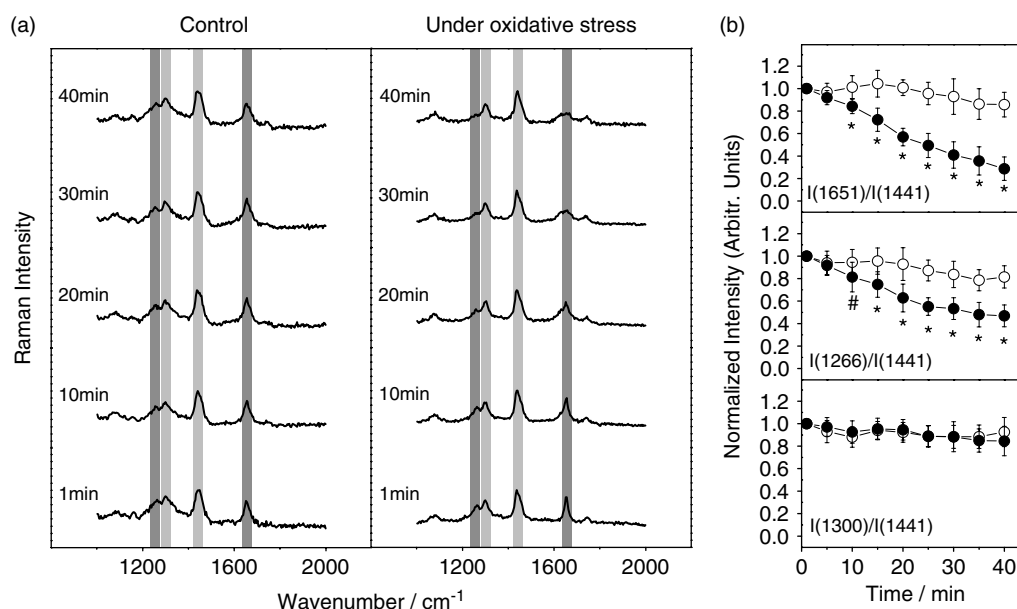

Figure 3. Effect of hydroxyl radical on single optically trapped yeasts. (a) Temporally varying Raman spectra of a single yeast cell. All spectra are background-subtracted and normalized with respect to the most intense 1441 cm^{-1} band, which shows little change over time to account for heterogeneity from cell to cell. For clarity, the two bands decreasing gradually under oxidative stress are highlighted in dark grey, whereas the other two that remained unaltered are highlighted in light grey. (b) Temporal evolution of relative intensities of Raman bands (empty circles: control, solid circles: single yeast cell under oxidative stress; $n = 10$). *$P < 0.001$, #$P < 0.01$.
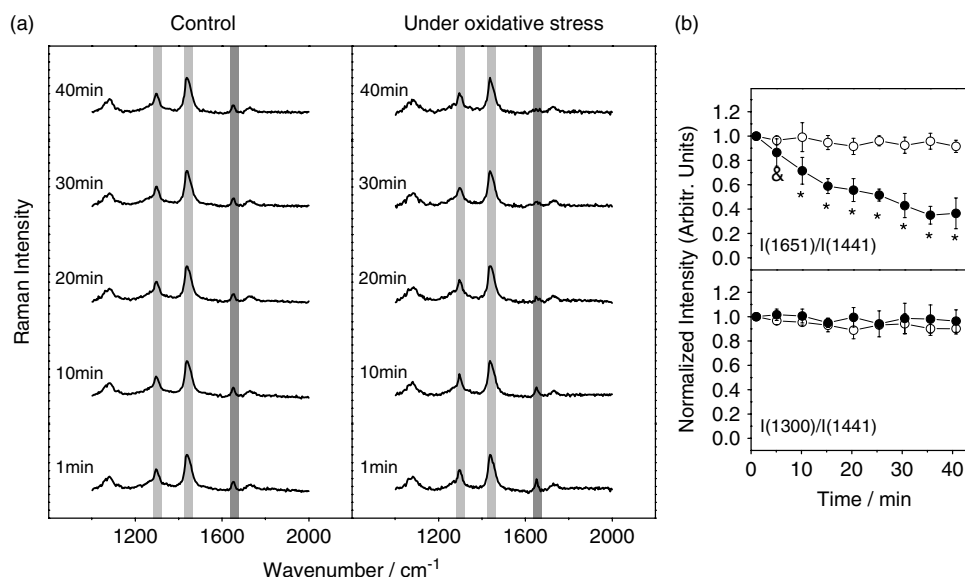
Figure 4. Effect of hydroxyl radical on single optically trapped liposomes. (a) Temporally varying Raman spectra of a single liposome made of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). (b) Temporal evolution of relative intensities of Raman bands (empty circles: control, solid circles: single liposomes treated with hydroxyl radical; n = 5). *$P < 0.001$, &$P < 0.05$.

(1651 cm^{-1}) and the oxidation of C=C bonds by hydroxyl radicals, we added ascorbic acid to the yeast system to test whether such an effect would be inhibited by the scavenging of ROS with an antioxidant. As seen in Fig. 5a, co-treatment of hydroxyl radicals with ascorbic acid (1 mM) results in a smaller decline in the intensity of the bands at 1651 and 1266 cm^{-1} (23% and 14% decrease after 40 min, respectively, both $P < 0.001$ compared to the hydroxyl radical group, Fig. 5b). These results indicate that, by scavenging hydroxyl radicals, ascorbic acid prevents the C=C bond from being oxidized.

As peroxidation of unsaturated fatty acids produces lipid peroxide, which in turn undergoes hydrolysis to become malondialdehyde,[24] we measured also this downstream product as an indicator of lipid peroxidation. As seen in Fig. 6a, the amount of malondialdehyde, measured with TBARS assay,[25] increases significantly after a 40-min exposure to the hydroxyl radical. Meanwhile, the Raman band at 1740 cm^{-1}, characteristic of C=O stretching vibration, increases slightly as the peroxidation proceeded (Fig. 6b), consistent with increased production of malondialdehyde that contains C=O bonds. Both these findings help explain the progressive diminution of C=C bonds and formation
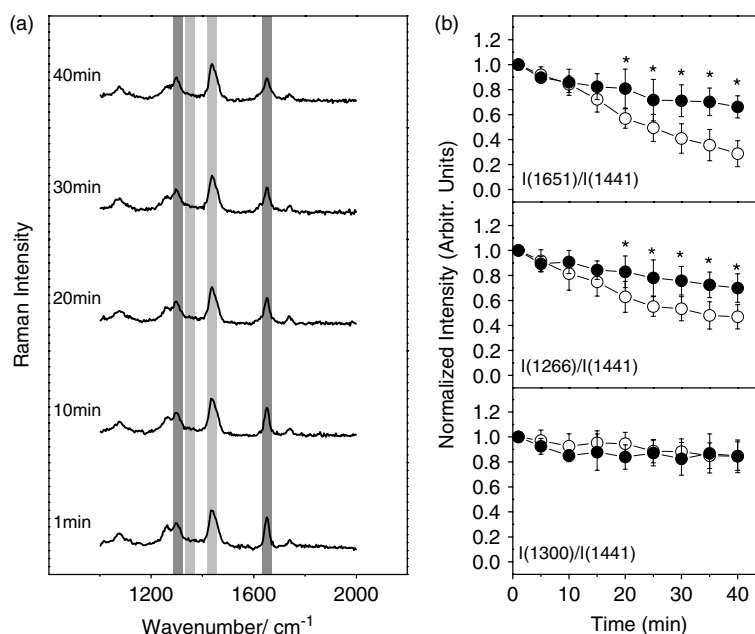

Figure 5. Effect of ascorbic acid on single yeast cells under oxidative stress. (a) Temporally varying Raman spectra of a single yeast cell under oxidative stress and co-treatment with ascorbic acid. (b) Comparison of temporally varying Raman intensities of a single cell under oxidative stress with (solid circles, n = 5) and without (empty circles, n = 10) co-treatment of ascorbic acid. *$P < 0.001$.
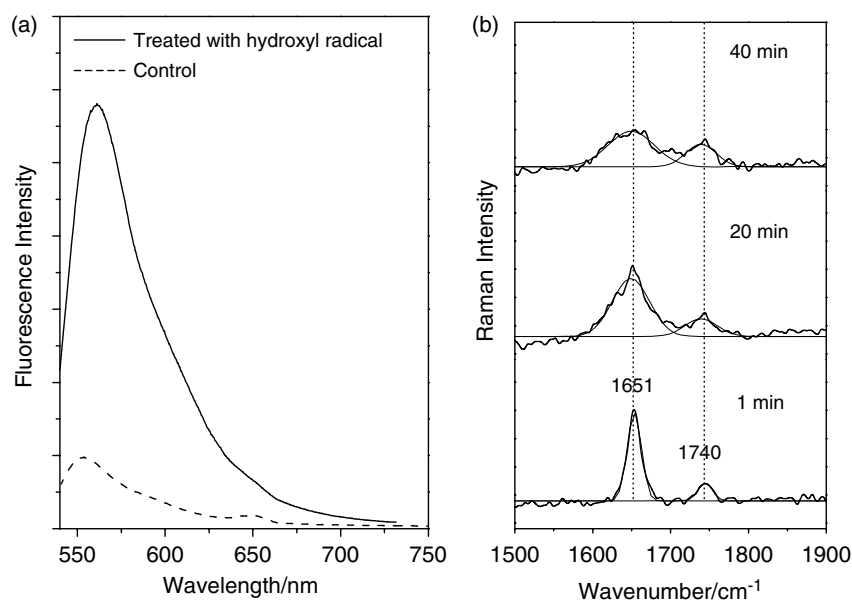
Figure 6. Confirmation of the generation of malondialdehyde. (a) Result of TBARS assay. Fluorescence signal measured from the solution of yeast culture after exposure (40 min) to hydroxyl radical confirmed the generation of malondialdehyde. (b) Temporally varying Raman spectra of a single yeast cell under oxidative stress shown between 1500 and 1900 cm^{-1} demonstrate gradually increasing intensity of the band at 1740 cm^{-1}.

of C=O bonds, delicately demonstrating the lipid peroxidation process by continuous monitoring of the Raman spectra.

In addition to intermediates and downstream products, the subtle changes arising from transient intermediates, which are otherwise difficult to observe in conventional biochemical measurements, can be detected with Raman spectra. For example, the cis C=C bonds in unsaturated fatty acids may undergo a conformational modification during peroxidation to become trans C=C bonds.[24] This, in part, supports the observation of the gradual broadening of the base of the band at 1651 cm^{-1} (Fig. 6b). This interpretation is further supported by results consistent with the inhibition of such effect by ascorbic acid (Fig. 5a). Such a subtle modification not only explains the main findings in Raman spectra but also helps in identifying the prospective chemical intermediates or byproducts in a pathophysiological process.

As poly-unsaturated fatty acid (PUFA) is an important component of the lipid bilayers in cell membranes, and as oxidation of the C=C bond in PUFA plays a major role in lipid peroxidation,[26] spectral measurement of the chemical changes related to C=C bonds allows real-time assessment of lipid peroxidation during cellular oxidative injury. This approach is of great significance in pathophysiology since the generation of ROS during oxidative stress is rapid and, in specific pathogenic processes such as ischemia-reperfusion injury, dynamic and phase dependent.[9] In contrast to measurements of the downstream products (e.g. malondialdehyde) as indirect indicators of lipid peroxidation in conventional fluorometric or colorimetric methods,[9,12,24] single-cell Raman spectroscopy offers direct mechanistic insight by demonstrating target biochemical modifications in the absence of exogenous labeling agents. In addition to demonstrating the lipid peroxidation response of a single cell to oxidative stress and radical scavenging effect of ascorbic acid, the same approach can be applied to a wide variety of biomedical research fields such as immunology, developmental biology, hemato-oncology, neuroscience and stem cell research. The immune response of leukocytes to pathogens, interaction of cancer cells with bioenvironment or drugs, electro-

physiological responses of neurons or cardiomyocytes to stimuli and directed differentiation of stem cells by trophic factors are all examples of targets to which single-cell Raman spectroscopy can be used for fundamental study of cell biology. Entrapment of intracellular organelles (such as mitochondria) for detailed biochemical exploration at the subcellular level is also envisaged.

A potential limitation of Raman spectroscopy to access the biochemical transformation in real time is the detection limit set forth by the low cross section of Raman scattering. This is particularly obvious for detection of the less abundant target chemicals or small-scale chemical changes during the reaction. Before further improvement in the sensitivity of Raman spectroscopy, this technique is best applied in selected biological systems in which the target Raman spectroscopic change is significant enough for detection. Lipid peroxidation serves as a good system for Raman spectroscopic measurement since lipid is one of the main constituents of cytoplasmic membrane and the membrane of intracellular organelles (mitochondria, endoplasmic reticulum, nuclear membrane, etc.). Meanwhile, oxidation of the C=C bonds within unsaturated fatty acids is universal during oxidative stress. The chemical transformation is thus expected to be suitable for single-cell Raman spectroscopic detection. For other biological activities occurring in a much smaller scale, the amount of the biochemical change may be beyond the sensitivity of Raman spectroscopy. However, improvement might be potentially achieved with the introduction of coherent or resonant enhancement techniques that have been well demonstrated in selected biological systems.[27]

Conclusions

In conclusion, we demonstrate that single-cell Raman spectroscopy exhibits an unprecedented capability for real-time characterization of the cellular response to oxidative stress and the therapeutic effect of ascorbic acid. With inherent capability to follow the course of chemical reactions by monitoring

the temporal variation of 'molecular fingerprints', single-cell Raman spectroscopy will be applicable in diverse biological and medical investigations to provide the molecular basis of important pathophysiological bioactivities at a cellular or subcellular level.

Acknowledgments

References

[1] J. A. Knight, *Free Radicals, Antioxidants, Aging, and Diseases*, Ameriacan Association for Clinical Chemistry: Washington, DC, **1999**.

[2] C. Bernal-Mizrachi, A. C. Gates, S. Weng, T. Imamura, R. H. Knutsen, P. DeSantis, T. Coleman, R. R. Townsend, L. J. Muglia, C. F. Semenkovich, *Nature* **2005**, *435*, 502.

[3] L. Y. Wu, M. H. N. Ashraf, M. Facci, R. Wang, P. G. Paterson, A. Ferrie, B. H. J. Juurlink, *Proc. Natl. Acad. Sci. U S A* **2004**, *101*, 7094.

[4] K. J. Barnham, C. L. Masters, A. I. Bush, *Nat. Rev. Drug Discov.* **2004**, *3*, 205.

[5] A. B. AlMehdi, H. Shuman, A. B. Fisher, *Am. J. Physiol.: Lung Cell. Mol. Physiol.* **1997**, *16*, L294.

[6] M. J. Jackson, *Proc. Nutr. Soc.* **1999**, *58*, 1001.

[7] M. T. Lin, M. F. Beal, *Nature* **2006**, *443*, 787.

[8] W. T. Chang, Z. H. Shao, J. J. Yin, S. Mehendale, C. Z. Wang, Y. M. Qin, J. Li, W. J. Chen, C. T. Chien, L. B. Becker, T. L. V. Hoek, C. S. Yuan, *Eur. J. Pharmacol.* **2007**, *566*, 58.

[9] L. B. Becker, *Cardiovasc. Res.* **2004**, *61*, 461.

[10] K. J. Davies, *J. Biol. Chem.* **1987**, *262*, 9895.

[11] O. I. Aruoma, B. Halliwell, E. Gajewski, M. Dizdaroglu, *J. Biol. Chem.* **1989**, *264*, 20509.

[12] J. M. Gutteridge, B. Halliwell, *Trends Biochem. Sci.* **1990**, *15*, 129.

[13] A. Ashkin, *Proc. Natl. Acad. Sci. U S A* **1997**, *94*, 4853.

[14] D. G. Grier, *Nature* **2003**, *424*, 810.

[15] M. P. Houlne, C. M. Sjostrom, R. H. Uibel, J. A. Kleimeyer, J. M. Harris, *Anal. Chem.* **2002**, *74*, 4311.

[16] G. J. Puppels, F. F. M. de Mul, C. Otto, J. Greve, M. Robert-Nicoud, D. J. Arndt-Jovin, T. M. Jovin, *Nature* **1990**, *347*, 301.

[17] Y. S. Huang, T. Karashima, M. Yamamoto, H. Hamaguchi, *Biochemistry* **2005**, *44*, 10009.

[18] Y. S. Huang, T. Nakatsuka, H. Hamaguchi, *Appl. Spectrosc.* **2007**, *61*, 1290.

[19] C. G. Xie, M. A. Dinno, Y. Q. Li, *Opt. Lett.* **2002**, *27*, 249.

[20] K. Ramser, K. Logg, M. Goksor, J. Enger, M. Kall, D. Hanstorp, *J. Biomed. Opt.* **2004**, *9*, 593.

[21] J. W. Chan, A. P. Esposito, C. E. Talley, C. W. Hollars, S. M. Lane, T. Huser, *Anal. Chem.* **2004**, *76*, 599.

[22] A. Edelmann, J. Bar, *Yeast* **2002**, *19*, 949.

[23] M. I. Angelova, D. S. Dimitrov, *Faraday Discuss. Chem. Soc.* **1986**, *81*, 303.

[24] B. Halliwell, S. Chirico, *Am. J. Clin. Nutr.* **1993**, *57*, S715.

[25] S. D. Yan, A. M. Schmidt, G. M. Anderson, J. H. Zhang, J. Brett, Y. S. Zou, D. Pinsky, D. Stern, *J. Biol. Chem.* **1994**, *269*, 9889.

[26] N. A. Porter, S. E. Caldwell, K. A. Mills, *Lipids* **1995**, *30*, 277.

[27] H. A. Rinia, M. Bonn, E. M. Vartiainen, C. B. Schaffer, M. Muller, *J. Biomed. Opt.* **2006**, *11*, 5021.