Combining Optoacoustics and Resonance Raman Spectroscopy for Quantification of Biomolecules *in situ*

Merve Meinhardt-Wollweber*[a]
[a]HOT-Hannover Center for Optical Technologies, Leibniz Universität Hannover, Nienburger Str. 17, 30167 Hannover, Germany

ABSTRACT

Resonant Raman spectroscopy is a technique to select and enhance the vibrational fingerprint of target molecules. Amplification of a specific Raman spectrum or specific components therein may be by several orders of magnitude. This allows to detect their molecular fingerprint even at low concentrations or in fluorescent environment without external signal enhancement. We use this approach to identify carotenoids of the antioxidant network. Quantification of the carotenoids relies on knowledge about attenuation of excitation light and Raman signal on their way through a given sample. To calculate this attenuation, the optical properties of the sample have to be known. Optoacoustics is a hybrid technique for non-invasive measurement of optical properties. We present a combined fiber sensor for optoacoustics and Raman spectroscopy allowing to probe optical properties parallel to Raman measurements. To the best of our knowledge, this is the first time that optoacoustics and vibrational spectroscopy have been combined in one sensor. It paves the way for identification and quantification of specific molecules in living tissues.

Keywords: resonant Raman spectroscopy, optoacoustics, quantification, carotenoids, skin antioxidants

1. INTRODUCTION

A complex antioxidant network of enzymatic and non-enzymatic components is constantly at work in our body to maintain the oxidative balance. Oxidative stress may damage cells, contributes to ageing and diseases and may even induce cancer.[1,2] Deliberate use of antioxidants to work against oxidative stress and consequently prevent disease is tempting at first sight but controversial in the light of current knowledge about the effect of high doses of single antioxidant compounds. Looking at only one group of molecules within the antioxidant network – carotenoids – in vitro studies showed pro- or antioxidant effects depending on carotenoid concentration, oxygenation, and other parameters.[3] A meta-analysis of interventional trials even showed a significant increase of mortality for β-carotene supplemented participants.[4] Synergistic effects and a balanced composition of the components of the complex antioxidative network may explain these controversial results.[3,5] Quantitative analysis of all components of the antioxidant network and their kinetics under oxidative stress in vivo could contribute greatly to our understanding of their function and interaction and pave the way for promising strategies in disease prevention based on antioxidants.

So far, blood or tissue samples have to be taken in order to analyze the antioxidant status in humans or animals. Non-invasive quantitative analysis of specific molecules in the living tissue is important in order to follow molecule kinetics and understand molecular responses to environmental influences. However, such in situ measurements are difficult due to low concentration of target molecules and interference from other tissue components. Spectroscopic methods can at least provide non-invasive access to and analysis of tissue that is easily accessible from the outside such as skin, eye, mouth etc. First steps towards spectroscopic in vivo monitoring of antioxidants were done by Gellermann and Lademann et al..[6-9] They used resonant Raman spectroscopy to measure carotenoids in living human skin. Carotenoids (and other antioxidants) show characteristic Raman fingerprints to identify them even in the complex molecular environment of whole skin. Resonant enhancement in Raman spectra of specific molecules is a technique to select and enhance the vibrational fingerprint of target molecules. Amplification of a specific Raman spectrum or specific components therein may be by several orders of magnitude. This allows to detect molecular fingerprints even at low concentrations or in fluorescent environment without external signal enhancement. We follow this approach to identify carotenoids of the antioxidant network.

Quantification of the carotenoids relies on knowledge about attenuation of excitation light and Raman signal on their way through the sample medium, e.g. human skin. The reason for this is that while Raman intensity does scale with the

*merve.wollweber@hot.uni-hannover.de; phone +49 511 762 17013; fax +49 511 762 17908; www.hot.uni-hannover.de

concentration of the scattering molecule, the unknown attenuation of exciting and scattered radiation within the sample medium strongly influences the measured signal intensity. This additional factor makes impossible absolute calculations of antioxidant status in vivo so far but only allows intraindividual comparison. We attempt to overcome this limitation by combining the successfully established resonant Raman spectroscopy with optoacoustics as this technique allows parallel measurement of the individual light attenuation. To the best of our knowledge, this is the first time that optoacoustics and vibrational spectroscopy have been combined in one sensor. Identification and quantification of Raman active molecules in living human skin will be possible with this combination of methods.

2. MATERIALS AND METHODS

2.1 Resonant Raman spectroscopy of carotenoids

Raman spectroscopy allows to identify molecules by their characteristic vibronic states which show in the energy change of inelastically scattered photons. The Raman spectrum of a particular molecule is independent of the excitation wavelength. However, if the energy of the scattered photon matches the energy of an electronic state of the molecules, absorption and scattering cross sections are increased and so is the intensity of specific lines in the respective Raman spectrum. This resonance effect may enhance the Raman spectrum by several orders of magnitude. In this way, molecules can be detected even at low concentrations despite the fact that Raman scattering is an extremely weak process by itself. Besides target molecules may be deliberately selected and enhanced above others.

For the combined Raman and optoacoustic measurements, an OPO laser system (NL303G + PG122/UV, Ekspla, Lithuania) was used, providing wavelength tunable light pulses of 4 ns duration. Excitation-emission-matrices (EEM) were measured by step-by-step tuning the laser over the (pre)resonance range of the respective molecule. All Raman spectra were measured accumulating 200 mJ of excitation light. No other normalization was applied.

Raman spectra are recorded by an Andor Shamrock SR 500i imaging spectrograph equipped with an Andor Newton DU940P-BU CCD-camera. A 100 µm entrance slit and a 1200 l/mm grating blazed at 300 nm were used. Spectral blocking of Rayleigh scatter is provided by Razor Edge longpass filters.

2.2 Optoacoustics

In optoacoustics, a laser pulse is absorbed in a sample inducing a pressure transient within the sample. The profile shape depends on the spatial light distribution within and the absorption properties of the sample. This pressure profile is released as an ultrasonic transient from which the optical properties of the tissue – causing light distribution and absorption – may be deduced.[10-12]

Optoacoustic signals are measured by a piezoelectric 9 µm thin PVDF film. Gold electrodes were custom coated (Precision Acoustics, UK).

2.3 Poly(vinyl alcohol) tissue phantoms

Poly(vinyl alcohol) (PVA) hydrogel was used as matrix material for skin tissue phantoms. Because of their high water content in the order of 80%, PVA hydrogels show acoustical properties that are very similar to those of tissue. Depending on the production protocol, they can be made translucent or even transparent. We used hydrogels stained with β-carotene that showed only little scattering from the PVA matrix. Melanin was used as additional stain to produce PVA hydrogels with various absorption coefficients from a natural absorber.

The optical properties of the PVA hydrogels were measured by a spectrophotometer (Uvikon 931, Kontron Instruments). Measured spectral attenuation coefficients served as reference for the optoacoustically derived optical properties (see section 3.2).

PVA (>99% hydroyzed) was purchased from Sigma Aldrich, Germany. Ethanol (HPLC-grade) and carotenoids were purchased from Roth, Germany.

3. RESULTS AND DISCUSSION

3.1 A combined optoacoustic and Raman detector

To allow parallel optoacoustic and Raman measurements, a fiber sensor head was designed. As shown in Fig. 1, illuminating and collecting fibers are guided through a PVDF block on which a piezoelectric pressure sensor is glued. A

cap from PVDF provides mechanical protection and serves as spacer between ultrasound detector and optical fibers on the one hand and the sample on the other hand. In this geometry, the active area of the pressure sensor is located centrally beneath the illuminated volume of the sample. A fiber bundle consisting of seven 200 µm core fibers is placed next to the illuminating fiber (800 µm core) to collect scattered photons. Several ways of positioning illumination and detection fibers such as 90° or 180° rotation were tested but placing next to each other with a slight tilt (~10°) between them and 10°-20° against the surface normal gave the best results.

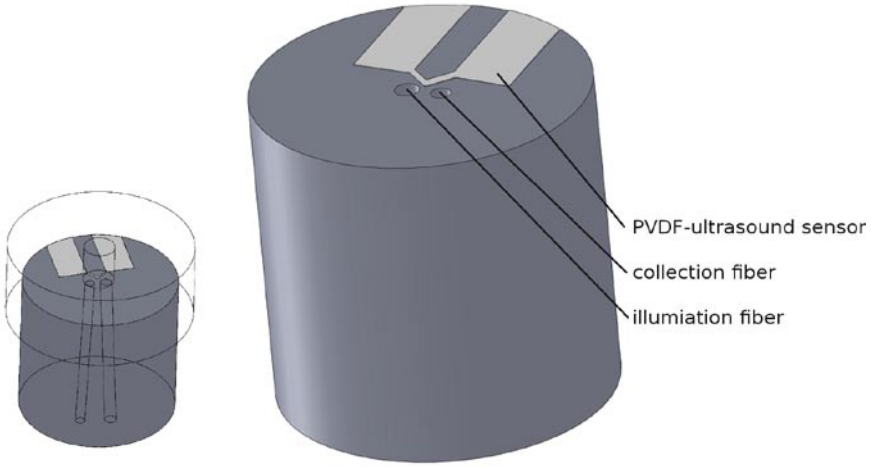

Figure 1. Combined optoacoustic and Raman detector. On the right, the PVDF block with fiber guides and PVDF ultrasound sensor. On the left, the detector is shown with the spacer cap as a grid model. The hole in the cap above the fiber outlets and ultrasound sensor is filled with ultrasound gel during measurement to provide acoustic connection to the samples placed on top of the cap.

Raman spectra are accumulated to provide an acceptable signal to noise ratio. For comparability of measured spectra, we applied the same accumulated energy to each sample during illumination. For in vitro samples, 200 mJ were set as the accumulated energy and less than 100 pulses are needed to apply them. In vivo, pulse energies should rather be lowered to avoid skin irritation of the illuminated skin.

Optoacoustic measurements are single pulse measurements. Results from several applied single pulses may be taken for averaging. For example, all or some of the pulses that are accumulated for the Raman measurement may be taken.

Single pulse energy is measured online by a photodiode at the illumination fiber. This power sensor is synchronized with the optoacoustic measurements so that the applied pulse energy for each optoacoustic measurement is stored together with the pressure signal. Besides, this data is used for online calculation of the accumulated pulse energy for the energy normalized Raman measurements.

3.2 Optoacoustic determination of extinction coefficients

PVA hydrogels stained with different concentrations of β-carotene and melanin were used to verify and check the optoacoustic determination of the optical properties of samples. Figure 2 shows effective attenuation coefficients as determined in a spectrophotometer vs. absorption coefficients as obtained from optoacoustic measurements.

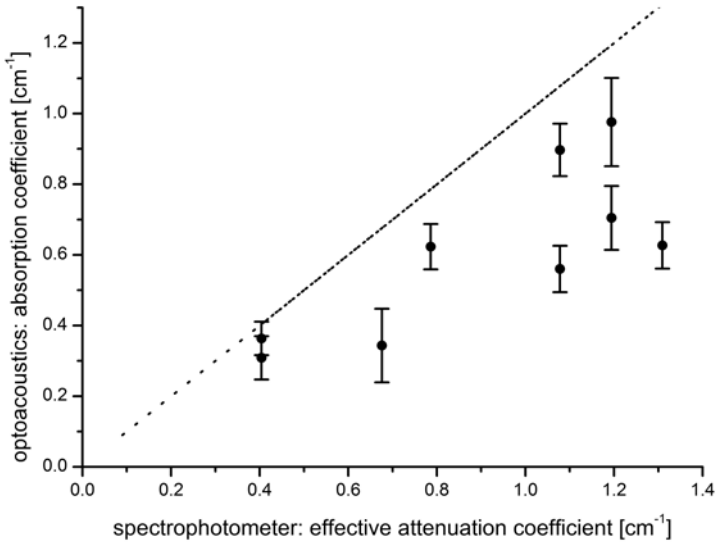

Figure 2. Effective attenuation coefficients of stained PVA hydrogels at 488 nm as determined in a spectrophotometer and the respective data obtained by optoacoustic measurements. Error bars show standard deviations.

These preliminary results show that even though the optoacoustic measurements systematically underestimate the extinction, they yield acceptable results in the correct order of magnitude. The underestimation is probably due to the fact, that only absorption coefficients are calculated from the optoacoustic data so far. It should be noted that the concentration of each dye may vary by a factor of two from sample to sample. So, the scattering coefficients of the different samples are expected to differ accordingly from each other.

3.3 Resonance mapping

Figure 3 shows an excitation-emission-matrix (EEM) of β-carotene solved in ethanol (~10 µg/ml). This set of spectra was obtained by tuning the excitation wavelength in 2 nm steps in the range 446 nm to 514 nm. As outlined before, the same cumulative dosis was used for each spectrum. Each spectrum was background corrected but no post processing normalization of the spectra was carried out. Consequently, all Raman lines show a slight enhancement towards shorter wavelengths as expected due to the λ^{-4} dependence of Rayleigh scattering. The strongest β-carotene Raman lines are seen at 1540 and 1170 cm^{-1} and clearly show resonant enhancement at excitation around 480 nm. The slight changes of the peak wavenumbers with the excitation wavelength are not reproducible but probably caused by irregularities in the grating turret controls.

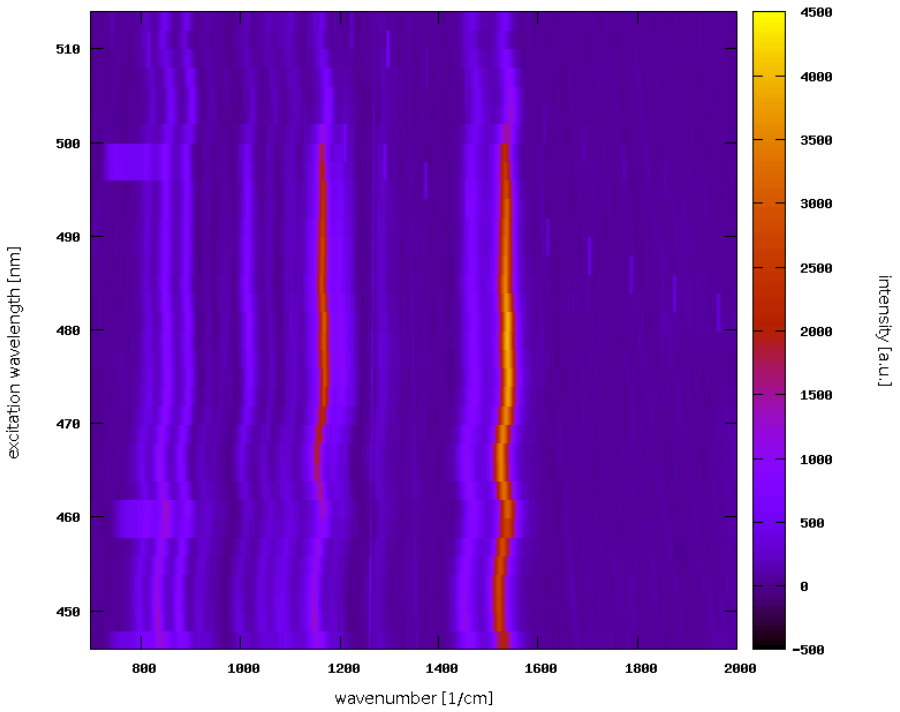

Figure 3. Excitation-emission-matrix of β-carotene solved in ethanol.

First EEM measurements of PVA hydrogels stained with β-carotene in the same excitation range did not show the same pronounced resonance effect (data not shown). β-carotene concentration in the hydrogel was about half that of the stock solution shown in Figure 3. However, the signal to noise ratio was much poorer. One reason might be a significantly lower and possibly spectrally shifted resonance enhancement in the hydrogels. As can be seen in Figure 4, a bathocromic shift and broadening of the β-carotene absorption spectrum occurs in the hydrogel environment. An explanation is difficult at this stage. Solvents may have a significant effect on the spectral position of the main absorption band of carotenoids. Two solvents are present in the hydrogels: ethanol and water. The water content of the hydrogels is very high. If water was responsible for the effect, a marked decrease of the usual absorption band and its replacement by a significant single peak in the near ultraviolet would be expected.[13] However, only a weak *cis*-peak is observed in the ultraviolet range. Ethanol does not cause spectral shifts of carotenoid spectra. Large bathocromic shifts and slight spectral broadening occur for carotenoproteins.[13] Whatever the reason, this change of molecular energy characteristics may also impact Raman scattering cross sections and consequently lower resonance enhancement.

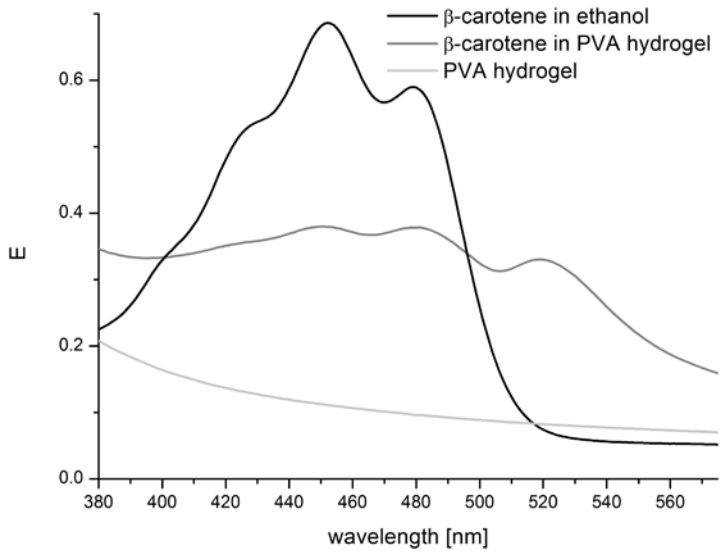

Figure 4. Extinction spectra of β-carotene solved in ethanol or in a poly(vinyl alcohol) hydrogel. The spectrum of an unstained hydrogel is given as reference.

3.4 Quantification

Parallel measurement of resonantly enhanced Raman signals and optical properties by optoacoustics were carried out to test whether this combination of methods allows absolute quantification of target molecules (e.g. carotenoids) within a sample of unknown optical properties. Slight alterations and bleaching effects can be observed during accumulation of one accumulated Raman spectrum. This is reflected in the optoacoustic results for the optoacoustic properties at the beginning and ending of the accumulation and can also be seen in weak blurring of the sample at measurement spots from time to time.

For the in vitro samples, attenuation coefficients were determined by spectrophotometric and optoacoustic measurements. As shown in section 3.2, results for the optical properties differ slightly for the two methods but are in the same order of magnitude. Several approaches from Lambert-Beer-law to Kubelka-Munk-theory were applied to calculate the expected carotenoid Raman signal attenuation from these data in order to conclude carotenoid concentration for a given sample. So far, results from all approaches are not satisfactory. Kubelka-Munk-theory for example predicts only about one quarter of the real concentrations.

4. CONCLUSION

The combination of resonant Raman spectroscopy and optoacoustics in a single detector was shown. In this context, using no post-measurement normalization on the Raman spectra but measuring the accumulated pulse energy during excitation works very well as the resonance Raman profiles (Figure 3) clearly show. This is an important step towards in vivo measurements, where normalization and comparison of Raman spectra is extremely difficult. Still, the sensitivity of the Raman setup should be further enhanced to ensure good signal to noise ratios at even lower concentrations and to further minimize necessary irradiation in order to avoid photobleaching of the samples. For the optoacoustic part of the setup, future work will concentrate on extraction of scattering coefficients from the data - in addition to the absorption properties that have been analyzed so far.

This combination of methods promises to allow quantitative and non-invasive measurements of Raman active molecules in samples of previously unknown optical properties. An appropriate and reliable model for Raman signal attenuation within the sample is prerequisite to benefit from this approach.

5. ACKNOWLEDGEMENTS

This work was kindly supported by the German Research Foundation (DFG, grant no. WO 1641/2-1).

Special thanks to Matthias Koch for his support and software for graphical processing and analysis of spectroscopic data and to Michael Senft for his invaluable technical support.

REFERENCES

1. Kaur, C. and Kapoor, H. C., "Antioxidants in fruits and vegetables," Int J Food Sci Tech 36, 703-725 (2001).
2. Black, H. S. and Lambert, C. R., [Oxidants and Antioxidants in Cutaneous Biology: Current problems in dermatology 29], Karger, Basel, 140-156 (2001).
3. El-Agamey, A., G., Lowe, M., McGarvey, D. J., Mortensen, A., Phillip, D. M., Truscott, T. G. and Young, A. J., "Carotenoid radical chemistry and antioxidant/pro-oxidant properties," Arch Biochem Biophys 430(1), 37-48 (2004).
4. Bjelakovic, G., Nikolova, D., Gluud, L. L., Simonetti, R. G. and Gluud, C., "Mortality in Randomzed Trials of Antioxidant Supplements for Primary and Secondary Prevention: Systematic Review and Meta-analysis," JAMA 297(8),842-857 (2007).
5. Mortensen, A., Skibsted, L. H. and Truscott, T. G., "The interaction of dietary carotenoids with radical species," Arch Biochem Biophys 385(1), 13–19 (2001).
6. Hata, T. R., Scholz, T. A.,Ermakov, I. V., McClane, R. W., Khachik, F., Gellermann, W. and Pershing, L. K., "Non-Invasive Raman Spectroscopic Detection of Carotenoids in Human Skin," J Invest Dermatol 115, 441-448 (2000).
7. Ermakov, I. V., Sharifzadeh, M., Ermakova, M. and Gellermann, W., "Resonance Raman detection of carotenoid antioxidants in living human tissue," J Biomed Opt 10(6) 064028-1 (2005).
8. Darvin, M.E., Gersonde, I., Meinke, M., Sterry, W. and Lademann, J., "Non-invasive in vivo determination of the carotenoids beta-carotene and lycopene concentrations in the human skin using the Raman spectroscopic method. J Phys D: Appl Phys. 38, 2696-2700 (2005).
9. Darvin, M. E., Gersonde, I., Albrecht, H., Sterry, W. and Lademann, J., "In vivo Raman Spectroscopic Analysis of the Influence of UV Radiation on Carotenoid Antioxidant Substance Degradation of the Human Skin," Laser Physics 16(5), 833-837 (2006).
10. Meinhardt, M., Krebs, R., Anders, A., Heinrich, U. and Tronnier, H., "Effect of ultraviolet adaptation on the ultraviolet absorption spectra of human skin in vivo," Photoderm Photoimmunol Photomed 24, 76–82 (2008).
11. Meinhardt, M., Krebs, R., Anders, A., Heinrich, U. and Tronnier, H., "Wavelength dependent penetration depths of ultraviolet radiation in human skin," J Biomed Opt 13(4), 044030 (2008).
12. Meinhardt, M., Krebs, R., Anders, A., Heinrich, U. and Tronnier, H., "Absorption Spectra of Human Skin in Vivo in the Ultraviolet Wavelength Range Measured by Optoacoustics," Photochem Photobiol 85, 70-77 (2009).
13. Britton, G., Liaaen-Jensen, S. and Pfander H. [Carotenoids Volume 1B: Spectroscopy], Birkhäuser, Basel 44-45 (1995).