Raman Spectroscopy for the characterization of algal cells

Ota Samek [*a], Alexandr Jonáš [a], Zdeněk Pilát [a], Pavel Zemánek [a], Ladislav Nedbal [b], Jan Tříska [b], Petr Kotas [b], Martin Trtílek [c]
[a]Institute of Scientific Instruments of the AS CR, v.v.i., Academy of Sciences of the Czech Republic, Kralovopolska 147, 61264 Brno, Czech Republic;
[b]Institute of Systems Biology and Ecology of the AS CR, v.v.i., Academy of Sciences of the Czech Republic, Zámek 136, 37333 Nové Hrady, Czech Republic;
[c]Photon Systems Instruments, Drásov 470, 664 24 Drásov, Czech Republic

ABSTRACT

Raman spectroscopy can elucidate fundamental questions about intercellular variability and what governs it. Moreover, knowing the metabolic response on single cell level this can significantly contribute to the study and use of microalgae in systems biology and biofuel technology. Raman spectroscopy is capable to measure nutrient dynamics and metabolism *in vivo, in real-time*, label free making it possible to monitor/evaluate population variability. Also, degree of unsaturation of the algae oil (iodine value) can be measured using Raman spectra obtained from single microalgae. The iodine value is the determination of the amount of unsaturation contained in fatty acids (in the form of double bonds). Here we demonstrate the capacity of the spatially resolved Raman microspectroscopy to determine the effective iodine value in lipid storage bodies of individual living algal cells. We employed the characteristic peaks in the Raman scattering spectra at 1,656 cm^{-1} (cis C=C stretching mode) and 1,445 cm^{-1} (CH_2 scissoring mode) as the markers defining the ratio of unsaturated-to-saturated carbon-carbon bonds of the fatty acids in the algal lipids.

Keywords: Raman spectroscopy; algal cells; lipids; iodine value

1. INTRODUCTION

Photosynthetic organisms transform the energy of solar photons into the free energy of chemical bonds that provide for nearly the entire energy supply of Earth's biosphere. Annually, photosynthesis accounts for net primary production of ca. 56.4×10^{15} g of carbon assimilated from the atmosphere on land and of ca. 48.5×10^{15} g in the ocean[1]. Most of the ocean photosynthesis occurs in planktonic algae.

The most often considered products from algae for the fuel industry are algal lipids[2]. Typical storage lipids in algae are triacylglycerols: tri-esters of glycerol with saturated or unsaturated fatty acids. Here, we focus on the degree of fatty acid unsaturation which is the main parameter that determines the application potential for fuels or as dietary supplements or for pharmaceutical raw materials.

For the purpose of selection and generation of production strains, one needs to characterize the lipids non-invasively. This is in contrast to gas chromatography-mass spectrometry (GC-MS) measurements on algal species[3,4]. Vital staining by BODIPY 505/515[5] or by Nile Red[6] are currently considered for fluorescence-activated cell sorting of lipid-rich algal cells.

Raman spectroscopy offers an attractive alternative for lipid detection that has not yet been sufficiently exploited in algae. So far, Raman applications in microbiology have aimed mostly at detecting medically relevant organisms[7-9]. There is a database of Raman spectral features of biologically relevant molecules that facilitates assignment of the most prominent Raman bands observed in living cells[10]. Also, quite a few recent reviews summarize the use of Raman spectroscopy for the detection and identification of important molecules in biological samples[11-13]. Because Raman spectroscopy of algae is complicated by autofluorescence of pigments applications which can be found in the scientific literature are restricted to only a small number of algal species[14-16].

Here, we present Raman spectra of lipid bodies measured with Raman microspectroscopy for the three algal species: *Botryococcus sudeticus*, *Chlamydomonas sp*., and *Trachydiscus minutus*. Our goal was to estimate the applicability of

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the Raman measurements for lipid characterization in species with significantly different unsaturation levels. An example is *Trachydiscus minutus* which contains a high amount of highly unsaturated fatty acids[17]. The other two algal species have been for a long time in the focus of an intense research.

The intensities of the Raman spectral peaks that correspond to the saturated and unsaturated carbon bonds in lipid molecules were used to estimate the degree of unsaturation (iodine value) in the lipid bodies similarly to the references[18-21]. The degree of unsaturation was quantified using the iodine value that is widely applied in biofuel and food industry[22]. We believe that the detection of the iodine value based on Raman microspectroscopy has a great potential for testing/production of higher generation biofuels (fuel-making algae) and dietary supplements.

2. MATERIALS AND METHODS

2.1. Organisms and cultivation conditions

Botryococcus sudeticus Lemmermann, CCALA 780 (VAZQUEZ-DUHALT/UTEX 2629), *Chlamydomonas sp*. CCALA, and *Trachydiscus minutus* (Bourrelly) Ettl, CCALA, were obtained from the Culture Collection of Autotrophic Organisms, CCALA (Institute of Botany, Academy of Sciences of the Czech Republic). *T. minutus* was cultivated in 50% Šetlík-Simmer medium[23] in 100 mL air-bubbled batch cultures. The cells were harvested in early stationary phase. *Chlamydomonas sp*. and *Botryococcus sudeticus* were cultivated in 150 mL Erlenmeyer flasks in BBM medium at room temperature in daylight at a laboratory window with occasional manual mixing. The cells were harvested at late stationary phase. The long term cultivation in the stationary phase was observed to induce the deposition of storage lipids in algal cells.

2.2. Nile Red staining and fluorescence microscopy

The technique of vital Nile Red staining was used in our study in order to visualize lipid bodies within the algal cells. This allowed us to identify the lipid bodies in the studied cells according to their morphology and size (see Figure 1). Nile Red (9-diethylamino-5*H*-benzo[α]phenoxazine-5-one) was prepared according to Greenspan *et al.*[24].

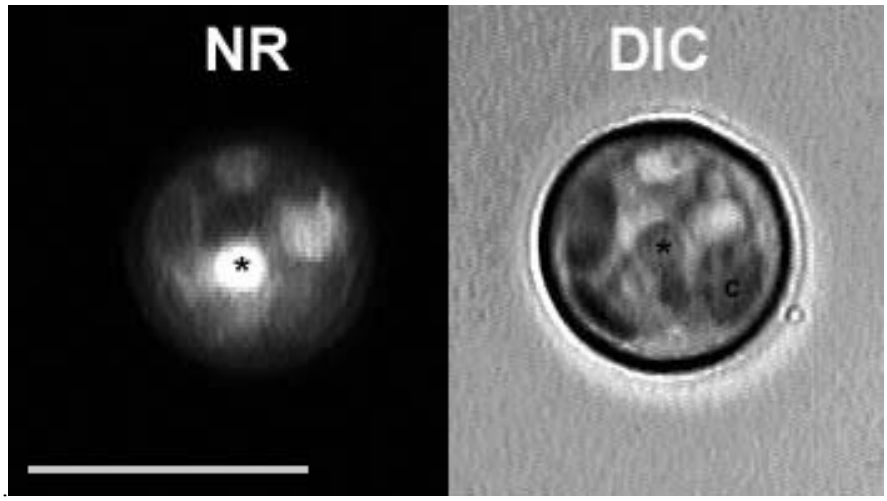

Figure 1. Visualization of the lipid bodies in algal cells. Nile Red (NR) fluorescence image of the lipid bodies (left) and Differential Interference Contrast (DIC) image (right) of the same living cell are compared. The structures corresponding to the lipid bodies can be clearly identified. The scale bar is 10 μm.

2.3. Sample preparation

For *in vivo* microspectroscopic experiments with spatially immobilized algal cells, 2–4% w/v solution of low temperature melting agarose (Sigma, TypeX1) in deionized water was mixed with 10–30% v/v of algal suspension directly on a microscope coverslip.

2.4. Raman microspectroscopy

Raman microspectroscopic experiments with living algal cells were carried out using a home-built experimental system based on a custom-made inverted microscope frame[25]. The full axial extent (depth) Δz of the excitation region was approximately 4 μm[25]. In our setup, Raman scattering spectra from the target cellular compartment were collected by the objective lens and subsequently focused into the entrance slit of an imaging spectrograph. Two edge filters were placed in the detection light path to prevent the excitation light from reaching the spectrograph. The Raman scattered light was dispersed with a 600 gr/mm diffraction grating, imaged on the chip of a high-sensitivity liquid-nitrogen-cooled spectroscopic CCD camera (Spec-10:100BR/LN, Princeton Instruments), and recorded using the camera control software (WinSpec). Recorded spectra were processed off-line using custom-written routines implemented in Matlab software (MathWorks).

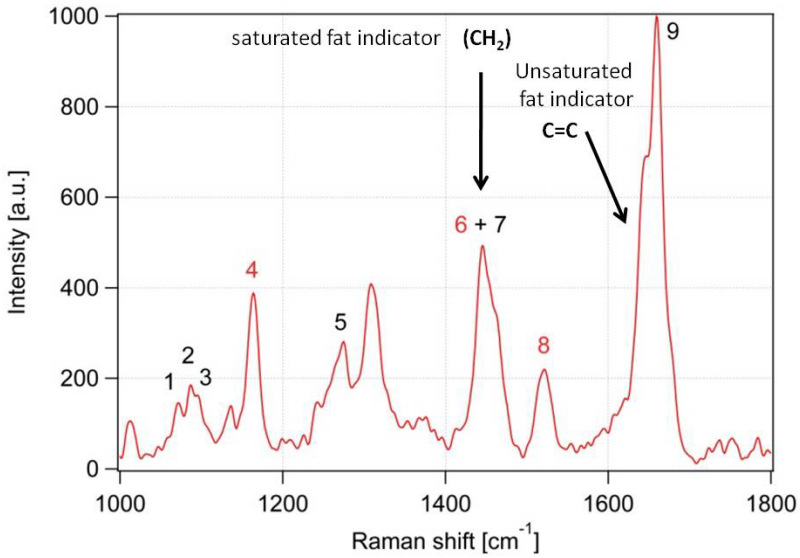

Figure 2. Typical Raman scattering spectrum of Trachydiscus minutus. Individual numbered bands are assigned in Table 1. Raman bands 7 and 9 are used to calculate the degree of lipid unsaturation (iodine value). Spectrum acquisition parameters: integration time 20 s, laser power at the specimen 13 mW.

2.5. Spectrum processing and analysis

In our experiments, we determine the ratio of unsaturated-to-saturated carbon-carbon bonds in algal lipid molecules. We have selected two spectral peaks at 1,656 cm^{-1} (*cis* C=C stretching mode proportional to the amount of unsaturated C=C bonds, peak No.9 in Table 1 and Figure 2) and at 1,445 cm^{-1} (CH_2 scissoring mode proportional to the amount of saturated C-C bonds, peak No.7 in Table 1 and Figure 2). We found these peaks free of any significant interference or overlaps with Raman signals of other cellular components. Both of these peaks are well documented as being very strong

in Raman spectra of lipids[19]. From the ratio C=C/CH_2, the average ratio– specimen mass unsaturation - can be estimated using the calibration curve plotted on the basis of the published iodine values for the fatty acids[26]. Thus, it is possible to directly convert the measured values of C=C/CH_2 to the iodine values for a given sample.

In order to extract quantitative information from the experimentally obtained spectral data, we adopted the Rolling Circle Filter (RCF) technique for background removal[27]. In principle, RCF is a high-pass signal filter that allows separating the narrow Raman spectral peaks from the background whose radius of curvature is significantly higher. With an appropriate choice of the filter parameters background can be effectively removed without causing a significant distortion of the signal peaks. After the background removal, the actual analysis of the Raman spectra can be carried out. Figure 2 shows a typical background-corrected Raman spectrum obtained from a lipid body inside a *Trachydiscus minutus* cell. The most prominent Raman spectral features observed in the spectrum are summarized in Table 1.

Table 1. Summary of the most prominent peaks / bands observed in the Raman spectra of algae. Peak numbers of the table are used to identify features in the spectra shown in Figure 2.

Peak #	Raman feature (in cm^{-1})	Suggested assignment
1	1,060	C-C skeletal stretching vibration,
2	1,085	C-C skeletal stretching vibration;
3	1,125	C-C skeletal stretching vibration;
4	1,157	β—carotene
5	1,267	*cis* double bond =C-H bend
6	1,442	β—carotene
7	1,445	CH_2 bend, scissoring deformation;
8	1,525	B—carotene
9	1,656	*cis* C=C stretching vibration;

3. RESULTS AND DISCUSSION

3.1. Calibration of Iodine Value against spectroscopic data

In order to link the experimentally observed values of C=C/CH_2 to the values of the actual iodine values (IV), we performed a series of Raman spectroscopic measurements of pure fatty acids of varied degree of unsaturation (see Table 2 for the summary of the samples used). The results of these measurements are shown in Figure 3. The calibration curve presented in Figure 3 can be employed to relate the Raman spectroscopic data the corresponding iodine values of the studied sample.

3.2. Determination of iodine value of lipids in living algal cells

As was already mentioned, we investigated the fatty acid composition of lipid storage bodies in three algal species—*Trachydiscus minutus, Botryococcus sudeticus*, and *Chlamydomonas sp.* Before the spectroscopic experiments, the living cells were immobilized in agarose (as mentioned above). Raman scattering spectra were recorded over the spectral range of 300–2,000 cm^{-1}. For the analysis of the algal fatty acid composition, we focused on the spectral range from 1,000 cm^{-1} to 1,800 cm^{-1} that contained the most relevant information.

Table 2. Fatty acids used for the calibration of Raman spectral data. Raman scattering spectra of fatty acids were acquired with 10 s integration time and excitation power of ~13 mW at the specimen.

	Number of double bonds per molecule	**IV Iodine values**	**Raman intensity at 1656 $cm^{-1(C=C)}$/1445 $cm^{-1(CH2)}$**
Palmitic **16:0**	0	0	<0.03
Oleic **18:1**	1	90	0.65
Linoleic **18:2**	2	180	1.46
Arachidonic **20:4**	4	330	4.18
EPA (Eicosapentaenoic acid) **20:5**	5	420	5.61

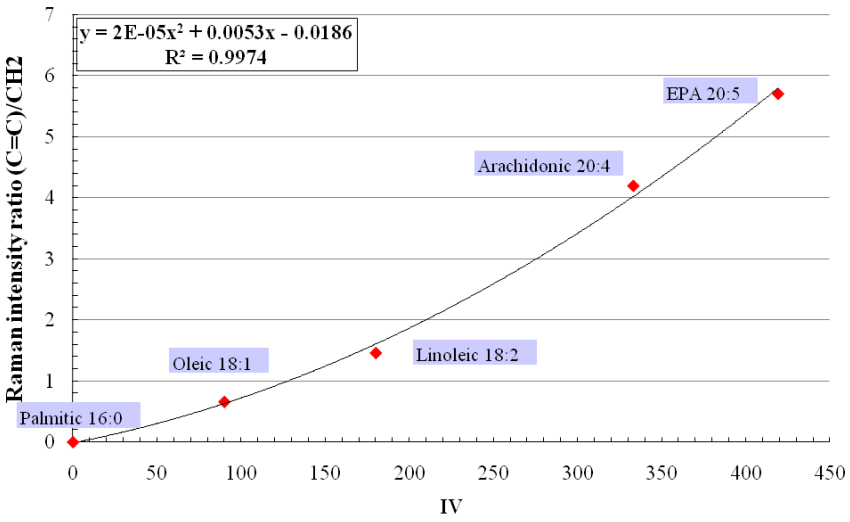

Figure 3. Calibration curve for estimating the iodine value (IV) from the Raman spectral data - the IV range of 0–430 is covered. Formula given in the top left corner of the graph was used for calculating IV.

In order to obtain Raman scattering spectra with a good signal-to-noise ratio, we used acquisition times in the range of 10–20 s and excitation power approximately equal to 15 mW at the specimen plane. Assuming the laser beam was focused to a diffraction limited spot of ~0.8 µm in diameter, the corresponding photon flux density was 2×10^{11} µmol(photons)·m^{-2}·s^{-1}. Raman beam was focused selectively into a lipid body, meaning that the observed fluorescence background is significantly lower compare to the whole algal cell. Also, this selective targeting of lipid bodies with spatially resolved Raman microspectroscopy can minimize the impact of the spectroscopic measurement on the physiological state of cells under investigation.

We have performed a large number of the repeated spectroscopic measurements carried out with the three algal species together with the estimates of their iodine values obtained from the calibration curve of Figure 3. We have observed cell-to-cell variability within each studied species which might indicate differences in the composition and concentration of fatty substances[28-30]. The average iodine values and their standard deviations for the three algal species included in our study were estimated: (216 ± 7) for *Trachydiscus minutus*, (88 ± 5) for *Botryococcus sudeticus*, and (93 ± 2) for *Chlamydomonas sp*.

Systematic studies are still required to establish Raman spectroscopz technique as a method for a rapid and robust investigations of an entire culture. Such studies exploiting the limitations of the Raman spectroscopy technique are currently under way in our laboratories. Here we show the feasibility study which justifies more extensive investigations involving the effect of the growth conditions on the observed spectral signatures and spectroscopic analysis of additional algal species.

4. CONCLUSIONS

We have demonstrated the potential of Raman microspectroscopy for the fast and spatially resolved characterization of the composition of selected intracellular regions in individual living algal cells. In particular, we have focused on lipid storage bodies and quantified the degree of unsaturation of algal lipids (iodine value) which is an important parameter for bio-fuel production and food industry using the calibration curve obtained with pure fatty acids of varied degree of unsaturation. We have found Raman microspectroscopy to be a fast, versatile, and virtually non-invasive tool for *in vivo,* applications *in real-time* for algal lipid engineering and industry.

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