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Raman microscopy shows that nitrogen-rich cellular inclusions in microalgae are microcrystalline guanine

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ABSTRACT

Microalgal cells possess a vast diversity of subcellular structures and cytoplasmic inclusions differing in their morphology, functionality, and composition, some of them giving rise to distinct Raman spectral signatures allowing their identification, localization, and visualization *in situ*. Here, we show that certain Raman features observed in Raman spectra of microalgae can be unambiguously attributed to guanine microcrystals because they are clearly distinct from Raman fingerprints of closely related purine species. Using confocal Raman microscopy, we have localized crystalline guanine as a part of cellular inclusions in the chlorophyte *Desmodesmus quadricauda* and in the eustigmatophyte *Trachydiscus minutus*. We propose that this finding also explains the chemical nature of similar nitrogen-rich crystalline structures recently documented in a number of other chlorophyte species by energy-dispersive X-ray spectroscopy. To the best of our knowledge, this is the first Raman microscopy-based direct evidence of the presence of guanine microcrystalline inclusions within microalgal cells. We tentatively propose that the crystalline guanine serves as a very compact, long-term depot of nitrogen in microalgae. Simplicity of specimen preparation requiring no fixation, labeling, or staining of the cells predetermines Raman microscopy as a method of choice for more advanced studies of the physiological role of guanine particles, as well as other crystalline inclusions *in situ* within intact cells.

1. Introduction

Microalgae use sunlight to produce depots of high free energy content such, as lipid, starch, or polyphosphate bodies, as well as to accumulate reserves of nutrients, such as carbon, phosphorus, sulfur, or nitrogen. Tapping these depots offers a great biotechnological potential [1–3]. Their assessment currently relies largely on extraction and chemical analysis of bulk algal biomass by gravimetry [4,5], spectrophotometry and colorimetry [6], mass spectrometry [7,8], gas and high-pressure liquid chromatography [8,9], or nuclear magnetic resonance [9,10]. On a more refined level, these methods can be targeted to a certain compound and/or cellular compartment to track down the origin of the investigated chemical within the cell [11–13].

Further improvement is available from methods allowing rapid, and simultaneous, identification and quantification of various biomolecules at the single-cell or even subcellular level. This is particularly important when working with heterogeneous cultures composed by cells in different physiological states and/or in different phases of their cell cycle. For imaging morphology and elemental/molecular composition of individual cells, microfluorescence [14,15] and analytical electron microscopy [14] are the most exploited methods. Recently, analytical capabilities of (electron) microscopy complemented by energy-dispersive X-ray spectroscopy (EDX) [14,16], secondary ion mass spectrometry (SIMS) [17], confocal Raman microscopy (micro-RS) [18], or combinations thereof, started to be used in algal studies because of their rich information content. Each method has its advantages, limitations, and information costs. Nanoscale SIMS (nano-SIMS) is an extremely sensitive mass spectrometric technique for determining the elemental, isotopic, or even molecular composition of a solid sample surface with a lateral resolution <50 nm [19]. Similarly, analytical electron microscopy, including EDX, provides invaluable information on morphology and elemental composition. However, for imaging by nano-SIMS or EDX, the cells must be laboriously fixed, dried, and immobilized on

Abbreviations: EDX, energy-dispersive X-ray spectroscopy; FWHM, full width at half maximum; micro-RS, confocal Raman microscopy; nano-SIMS, nanoscale secondary ion mass spectrometry; polyP, polyphosphate(s); TEM, transmission electron microscopy.

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a conducting surface or embedded in conducting resin. The complex sample preparation makes these low-throughput methods prone to artifacts arising from cell fixation, washing, staining and/or section preparation. Finally, elemental analysis *per se* is often insufficient for molecular identification.

Raman microscopy is a contactless, rapid, non-invasive, and often non-destructive method combining the advantage of *molecular* specificity of vibrational spectroscopy with a spatial resolution reaching the diffraction limit provided by confocal optical microscopy [20]. The application of micro-RS in biology is rapidly increasing [21] because it can provide biochemical and structural information without extensive sample preparation (*e.g.,* fixation, staining, drying, cutting) and, in some cases, even on living cells [22]. Regarding microalgae, micro-RS was recently shown to be particularly interesting for the simultaneous detection and imaging of dense sub-cellular structures with a relatively homogenous chemical composition, such as starch grains, lipid bodies, or polyphosphate (polyP) granules [18], avoiding their staining or extraction.

In a similar way, confocal micro-RS might be useful for *in situ* express identification and imaging of crystal-like inclusions, profiting from the high local concentration and highly ordered structure of the molecular species of interest in such crystalline inclusions resulting in strong, sharp, and distinct Raman fingerprints facilitating spectral assignment. The crystal-like structures suitable for identification with micro-RS are exemplified by the vacuolar inclusions in a symbiotic dinoflagellate, *Symbiodinium microadriaticum*, which were long misidentified as calcium oxalate [23]. Only recently, using advanced imaging and analytical techniques (energy-filtered TEM analysis and mass spectrometry), these crystals have been shown to be composed of uric acid [24]. The microcrystals laboriously isolated from the cells of a free-living dinoflagellate *Gonyaulax polyedra* have been identified as guanine [25] according to the ultraviolet absorption spectra of their solution in hydrochloric acid, while vacuolar crystal-like particles found in calcareous dinophyte cells have been assumed to be calcite based simply on their solubility in acids [26]. In spite of a long research history, precise molecular composition of diverse crystalline inclusions was rarely investigated by analytical methods. It is possible that some of them have been assigned erroneously, and that re-examination of their true nature is warranted [24].

The physiological significance of crystalline inclusions consisting of purine derivatives also remains elusive although a number of their potential functions were suggested, including phototaxis [27], detoxification [28], bioluminescence [25], and nitrogen storage [29–31]. Insight into their physiological role is obviously limited by the lack of rapid and reliable high-throughput methods for their unambiguous identification and documentation *in situ*, which would not require extraction or complicated sample preparation.

Here, we demonstrate the simplicity and straightforwardness of micro-RS-based identification of guanine as a principal component of the crystal-like inclusions in the cells of the chlorophyte *Desmodesmus quadricauda* and the eustigmatophyte *Trachydiscus minutus*. We also compare our results with recent independent observations of nitrogen-rich crystalline vacuolar inclusions by analytical electron microscopy in a number of other chlorophyte species [16].

2. Experimental section

2.1. Chemicals

All chemicals used for the preparation of cultivation media, proxies of biomolecules used as references of pure species (mixture of neutral lipids, β-carotene, starch, bovine albumin, RNA, DNA, sodium hexamethaphosphate), purine bases (guanine, adenine, xanthine, hypoxanthine, uric acid), and a related nucleoside, guanosine, have been obtained from Sigma-Aldrich in the highest purity grades. For cell immobilization, 1% aqueous solution of low-gelling agarose (Type VII, melting $T = 65$ °C, gelling $T = 28$ °C, Sigma-Aldrich) was used.

2.2. Algal cultivation

Algal cultures of a chlorophyte *D. quadricauda* and a eustigmatophyte *T. minutus* were obtained from the Culture Collection of Autotrophic Organisms, Institute of Botany (CCALA, Czech Academy of Sciences, Třeboň, Czech Republic). Both species have been studied as model organisms with an interesting cell cycle during which a significant accumulation of starch or lipid reserves occurs [32–36]. Their cells are also large enough to allow good resolution of different cellular compartments.

D. quadricauda was cultivated in 100-mL glass tubes bubbled with air enriched to 2% CO_2 (flow rate 350 mL·min^{-1}) as the sole source of carbon, at 30 °C. The nutrient medium, according to Zachleder and Šetlík [37], contained KNO_3 as the sole source of nitrogen. The glass tubes with the algal suspension were exposed to 200 μmol (photons)·m^{-2}·s^{-1} from a panel of warm-white light-emitting diodes in a 15 h/9 h light/dark cycle. Optionally, an aliquot of the cell culture was kept without aeration at room temperature either in darkness or in light for up to several hours prior to the Raman measurement with regular manual shaking.

T. minutus was batch-cultivated in 50-mL BBM medium [38] buffered to pH 7.5 with 15 mM HEPES and modified to contain 1 mg·L^{-1} molybdenum at 22 °C and air bubbling in a 12 h/12 h light/dark cycle under irradiance of 400 μmol (photons)·m^{-2}·s^{-1} from white light-emitting diodes. The cells were harvested in the early stationary phase.

2.3. Raman microscopy

For Raman measurements, 1 mL aliquots of the culture suspension were taken and algal cells were harvested by centrifugation (2000 ×*g* for 30 s). Excess medium was discarded and the cell pellet was resuspended in an appropriate amount of 1% solution of low-gelling agarose ($T = 35$ °C) to obtain cell density of around 10^8–10^9 cells·mL^{-1}. Three μL of this dense suspension were spread as a thin layer between a quartz slide and a round quartz coverslip (diameter 20 mm, thickness 180 μm). Wet edges were dried with blotting paper and sealed with a CoverGrip sealant (Biotium). Such an immobilization ensures that the cells do not move during the measurement. The contribution of 1% agarose to the Raman spectra of the immobilized cells was negligible (Fig. 4).

Raman spectra of *D. quadricauda* were acquired with a confocal Raman microscope WITec alpha300 RSA (WITec) equipped with an oil-immersion objective UPlanFLN 100×, NA 1.30 (Olympus). The spectra were excited by a 532 nm laser, typically with excitation power of approximately 20 mW at the sample (photon flux densities of 4.5×10^{11} μmol (photons)·m^{-2}·s^{-1} in the focus) with an integration time of 80 ms per point. Thanks to the high confocality of the WITec Raman microscope (FWHM 0.86 μm of a standard depth scan test on silicon as a conventional measure of confocality of Raman microscopes) and 125 nm scanning step (in *x* and *y* directions), the Raman images were spatially resolved up to the diffraction limit. Spectral dispersion of the spectrograph allowed coverage of both characteristic (200–1850 cm^{-1}) as well as C—H/O—H stretching (2700–3900 cm^{-1}) regions simultaneously. Prior to the mapping, a wide-area photobleaching of the entire algal cell by a defocused laser beam (wavelength 532 nm, photon flux densities of 4.1×10^6 μmol (photons)·m^{-2}·s^{-1}) was applied as described previously [18], to remove the strong autofluorescence of chlorophyll. Raman maps of several dozens of algal cells have been acquired and treated with the program WITec Project *Plus* (WITec) and our own Matlab (MathWorks) codes that are based on singular value decomposition, as described elsewhere [18,39]. The reference spectra of the pure chemical