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Raman scattering evidence of hydrohalite formation on frozen yeast cells

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ABSTRACT

We studied yeast cells in physiological solution during freezing by Raman microspectroscopy technique. The purpose was to find out the origin of a sharp peak near ~3430 cm^{-1} in Raman spectrum of frozen mammalian cells, observed earlier (J. Dong et al., Biophys. J. 99 (2010) 2453), which presumably could be used as an indicator of intracellar ice appearance. We have shown that this line (actually doublet of 3408 and 3425 cm^{-1}) corresponds to Raman spectrum of hydrohalite ($NaCl{\cdot}2H_2O$), which is formed as the result of the eutectic crystallization of the liquid solution around the cells. We also show that the spatial distribution of hydrohalite in the sample significantly depends on the cooling rate. At lower cooling rate (1 °C/min), products of eutectic crystallization form layer on the cell surface which thickness varies for different cells and can reach ~1 μm in thickness. At higher cooling rate (20 °C/min), the hydrohalite distribution appears more homogeneous, in the sample, and the eutectic crystallization layer around the cells was estimated to be less than ~20 nm. These experimental results are consistent with scenarios predicted by the two-factor hypothesis for freezing induced cell injury. This work demonstrates a potential of Raman microspectroscopy to study peculiarities of the eutectic crystallization around single cells *in vivo* with the high spatial resolution.

Introduction

Various biological and medical applications involve cell preservation technology ([31] and references therein). Cryopreservation of living cells is used for long term culture fixation to suppress genetic and phenotypic drifts. Cryobanking is used for domestic livestock, endangered species genetic resources storage and artificial insemination. Notably, multiple damaging factors arise during cooling cells to cryogenic temperatures, such as ice formation (both extra- and intracellular ones), dehydratation of cells and their membranes, phase transitions of membranes, the toxic effects of cryoprotectants, and eutectic crystallization. For example, the so-called two-factor hypothesis interprets the cell viability as the result of the competition between the intracellular ice formation and the hypertonic solution injuries [15]. But, how multiple factors affect the survival ability of cells remains unanswered. While most cryopreservation protocols have been developed empirically [9], our understanding of physical processes underneath of the cryoprocesses is still obscure. That is why understanding of the cells cryoinjury mechanisms [7,12,16,17,31] is an important task, attracting attention of many research groups.

Several nondestructive techniques have been adapted to investigation of cells freeze/thaw processes investigations *in vivo*, including cryomicroscopy [1,4,25,27,30], infrared spectroscopy [8,21,29], and differential scanning calorimetry [12,14]. However, since some of these techniques provide indirect information or require ambiguous interpretations (see, for example, Ref. [27], where the distinction between intercellular ice formation and darkening effects in cryomicroscopy is discussed), it is of great importance to develop other methods to study the cryopreservation process. Raman scattering spectroscopy is a non-invasive, nondestructive technique, which is sensitive to chemical composition and phase state, and, thus, is exceptionally well suited for these kinds of problems. Raman spectroscopy is actually used with rapidly growing popularity to study various biological problems (e.g. [6,13,20,22,23,26,32] and others). However, until very recently there have been no Raman scattering studies on cryobiology of the cells. To the best of our knowledge, a recent work [10] was the pioneering one in this direction, and this work is an excellent illustration of the Raman technique capabilities for identification of extra- and intracellular ice, spatial distribution of a cryoprotectant and the responses of subcellular structures at the frozen state.

Among others results, authors of the work [10] reported an observation of a sharp peak near ~3430 cm^{-1} in intracellular ice (Fig. 1B of [10]). Origin of this line was not discussed in the work except for the notion that it could be due to trace amounts of organic matter. It is important to find out which substance is

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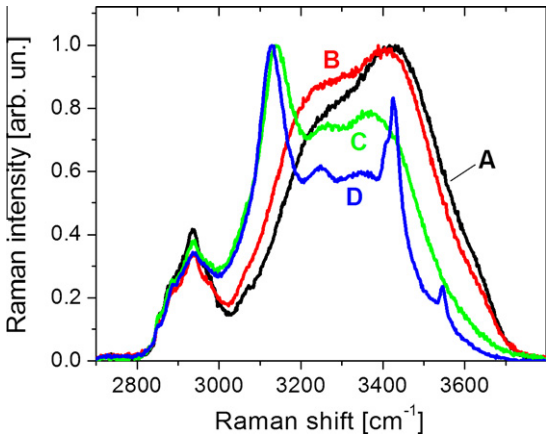

Fig. 1. Raman spectra of yeast cells. Curve A – at room temperature (+25 °C); curve B – supercooled water sample (−10 °C); curve C – after extracellular ice formation (−20 °C); B – after eutectic crystallization (−46 °C).

responsible for the line at 3430 cm^{-1} and whether this line is related to the intracellular ice [10]. If the last is true, the line could serve as an excellent indicator of intracellular ice appearance during cell freezing. Here, we describe a detailed study of the freezing process of *Saccharomyces cerevisiae* yeast cells by Raman microspectroscopy. We demonstrate that the line at 3425 cm^{-1} in Raman spectrum is due to hydrohalite ($NaCl{\cdot}2H_2O$) formation on the cell surface. Measuring Raman spectra, we estimate the thickness of the eutectic crystalline layer, and demonstrate that the thickness depends on the cooling rate.

Materials and methods

Sample preparation

Commercially available *S. cerevisiae* instant yeast cells pellets (20 mg) were added to 5 ml of isotonic saline solution (0.9 wt.% NaCl). For each Raman experiment, 1 μl of the cell suspension was placed into a hermetic chamber with silica window (280 μm in thickness).

Cryostat and temperature control

Raman scattering experiments were carried out in a home-made cryostat, which is continuously cooled by the flow of liquid nitrogen vapor. The cryostat has a good thermal isolation, resulted in low vapor flow, and a split system for vapor pumping, where a pump is placed on a separate vibration isolated surface. This system provides good stability against vibrations, no vibration effects were detected. The cryostat offers an operating temperature range of 100–320 K with the sample chamber in vacuum. The cryostat window is made of silica glass (280 μm in thickness). The cryostat allows us to use microscope objectives with working distance 4 mm and above. Calibrated thermocouple was installed close to the sample chamber. The sample was kept at constant temperature when Raman spectra were acquired. The temperature in the laser illuminated area at low temperatures was additionally controlled by the measurement of the position of the ice Raman line (~3100 cm^{-1}) [28], which is temperature dependent. An optical closed-cycle helium cryostat (DE-204; Advanced Research Systems, Allenton, PA) was used in a calibration experiment in which temperature dependence of Raman line of ice was measured. The additional temperature control allowed us to take into account possible effects of laser heating of the sample, which did not exceed ~2 °C.

Confocal Raman microspectroscopy

Our set-up for Raman experiment with the lateral resolution of ~1 μm includes a confocal microscope, based on a modified Orthoplan microscope (Leitz, Wetzlar, Germany), and an SP2500i monochromator (Princeton Instruments, Acton, MA), equipped with a Spec-10:256E/LN charge-coupled device detector (Princeton Instruments, Trenton, NJ). Elastic scattering contribution was additionally suppressed by a notch filter with OD > 6 (GIPO, Kazan, Russia). Wavelength calibration of the spectrometer was done by a neon-discharge lamp. Raman scattering was excited by a solid-state laser (Millennia; Spectra Physics, Santa Clara, CA) at a wavelength of 532.1 nm and 8 mW of power (the power entering to the cryostat). A 100× air objective with NA = 0.75 and working distance 4.6 mm (Leica, Wetzlar, Germany) was used in all measurements. The diameter of a confocal pinhole was 120 μm, which corresponds to a spot of 1.2 μm (diameter) in the focal plane. The optical path from the objective to the sample includes two silica windows (sample chamber and cryostat windows). Longitudinal aberrations, caused by the windows, reduced the longitudinal resolution to ~12 μm (FWHM), while the lateral resolution was about ~1 μm. Point spread function (PSF) was estimated by numerical calculations (PSFlab [19]). Since the size of yeast cells is ~8 μm or less, extracellular matter contributed to acquired spectra.

As it is seen, the use of vacuum cryostat causes the inevitable reduction of the longitudinal resolution. Advantage of the vacuum cryostat is lack of thermal gradients, and it is paid by the reduction of the longitudinal resolution. Nevertheless, our choice was in favor of the vacuum cryostat, since the lateral resolution rests high, which allows to get information about spatial distribution of 3430 cm^{-1} line by study of its lateral distribution. Moreover, as it will be shown below, namely the reduced longitudinal resolution allowed us to suggest an estimation procedure for the hydrohalite thickness.

Raman experiment with saline solution

An aqueous solution with 25 wt.% NaCl was used to study the Raman spectrum of ice and hydrohalite after eutectic crystallization. The solution was sealed into a glassy tube. An optical closed-cycle helium cryostat was used for sample cooling/heating. Raman spectra of the opaque white sample (after freezing) were measured in nominally right-angle scattering by a TriVista 777 triple-grating spectrometer (Princeton Instruments, Acton, MA) equipped with a Spec-10:400BR charge-coupled device detector (Princeton Instruments, Trenton, NJ). An Ar-ion laser Stabilite 2017 (Spectra Physics, Santa Clara, CA) at a wavelength of 488 nm and 200 mW of power was used for excitation. Raman scattering was collected from a volume of about 1 mm^3.

Results

We studied Raman spectrum of the cells during cooling. The most of the experiments was carried out with a cooling rate of 1 °C/min, which is typical for a number of cryopreservation protocols. Representative Raman spectra of single yeast cells at different temperatures are shown in Fig. 1. The spectrum at room temperature consists of the CH band (2840–3000 cm^{-1}) of organic matter and the broad OH band of the liquid water (2900–3600 cm^{-1}). Extracellular ice spontaneous nucleation took place in the temperature range −15 to −20 °C (Fig. 1, curve C), which is manifested by the prominent peak centered at 3140 cm^{-1} in Raman spectra. The contribution of the liquid water to Raman spectra vanished at temperatures below −40 °C, and new crystalline lines appeared at