Hydrohalite spatial distribution in frozen cell cultures measured using confocal Raman microscopy

Asger Kreiner-Møller\textsuperscript{a}, Frank Stracke\textsuperscript{a}, Heiko Zimmermann\textsuperscript{a,b,*}

\textsuperscript{a}Department of Biophysics and Cryotechnology, Fraunhofer Institute for Biomedical Engineering, 66386 St. Ingbert, Germany
\textsuperscript{b}Department of Molecular and Cellular Biotechnology, Saarland University, 66123 Saarbrücken, Germany

A B S T R A C T

Hydrohalite, a crystalline rock salt hydrate, ($\text{NaCl \cdot 2H}_2\text{O}$), can form in cryopreservation samples under certain circumstances changing the local chemical environment of the preserved cells. Evidence of this crystalline phase was recently found by microspectroscopy measurements, and believed to form exclusively extracellularly. We have studied the spatial distribution of hydrohalite in frozen mouse fibroblast cell samples by means of confocal Raman scanning microscopy (CRM). Hydrohalite has a unique Raman spectrum with several bands in the high frequency tail of the OH-stretching band which can be used for unambiguous identification. Hydrohalite can only form through eutectic crystallization in saline solutions without any cryoprotective agents and the spatial distribution thus gives a more detailed view on this crystallization process. This is important since eutectic crystallization has been empirically correlated to cell death, but the exact injury mechanism is unclear. By the means of colocalization of Raman bands we show that hydrohalite can indeed form intracellularly and is not a strictly extracellular phenomenon. We furthermore found that intracellular ice and intracellular hydrohalite very often coincide. Finally we show that the addition of 0.5 wt.% dimethyl sulfoxide ($\text{Me}_2\text{SO}$) inhibits formation of hydrohalite. This study shows how Raman microscopy and successive analysis can be employed non-invasively within cryobiology to give additional chemical and structural information compared to conventional imaging techniques.

© 2014 The Authors. Published by Elsevier Inc. This is an open access article under the CCBY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

Introduction

Crystalline salt hydrates, like hydrohalite ($\text{NaCl \cdot 2H}_2\text{O}$), were recently discovered in cryopreserved biological samples and storage media by means of Raman microspectroscopy [11] and confocal Raman microscopy (CRM) [10]. Hydrohalite can form under continuous precipitation during the cooling process and by eutectic crystallization depending on the medium composition. Up to now it is not clear, if hydrohalite formation is a strictly extracellular phenomenon or if it also forms in the cytoplasm of cells at sub-zero temperatures. An intracellular formation of a second crystalline phase in addition to ice could be a new aspect to understand cellular cryoinjury by both mechanical forces and chemical imbalances.

From their Raman microspectroscopic study, Okotrub et al. [11] deduced a purely extracellular spatial distribution of hydrohalite around the cell membrane. But since the lipid bilayer is only approximately 6 nm thick it is difficult to exactly determine the spatial position of small crystals at the membrane due to the diffraction limit of optical imaging techniques. Raman microscopy however has the potential to discriminate intra- and extra-cellular compounds by image analysis techniques as shown in this work.

Raman scattering is a well understood optical phenomenon [13,15] and has been employed in a wide range of imaging techniques in cell biology, where it is used to distinguish compounds in cell samples or even cell types [3,12,14]. Raman microscopy has recently been introduced to cryobiology [4] and has been shown to be a powerful non-invasive tool to investigate the local chemical environment of cells. It is thus a suitable experimental technique to distinguish all solid phases formed in samples containing the most common compounds in cryopreservation, including phosphate buffered saline (PBS), intracellular salts, $\text{Me}_2\text{SO}$, glycerol and biological material.

The recent introduction of Raman microscopy to cryobiology [4] also had the first direct measurement of hydrohalite, although it was initially not identified or commented. This study showed...
Raman spectra with a characteristic unidentified peak at 3425 cm\(^{-1}\), which turns out to originate from hydrohalite. Hydrohalite formation in absence of cryoprotective agents can be used as a marker for eutectic crystallization, which empirically has been identified as a major cryoinjury mechanism [8].

In the present study we investigate a large set of L929 cells in PBS with and without Me\(_2\)SO using CRM in order to determine whether hydrohalite formation is a strictly extracellular phenomenon or also occur intracellular under certain conditions. There is a linear correlation between the Raman signal and the quantity of the corresponding matter, in our case hydrohalite and cellular matter. The recorded image data of our study consist of a complete Raman spectrum per pixel. From these data chemical maps of the contained compounds can be extracted. Subsequently, color coded overlay images can be prepared and utilized to determine the spatial distribution of hydrohalite and cellular matter. In some cases the overlay images are ambiguous with respect to the hydrohalite localization – mostly due to the limited axial resolution – and specific characteristics in colocalization plots are found to be helpful in the further interpretation of the data. Spatial correlation between hydrohalite and cellular matter will show up in colocalization plots and can be used to determine whether the hydrohalite is located within or outside the cell. It is indeed shown, that hydrohalite can form inside cells under certain conditions, though it seems less serious in established cryopreservation protocols in vital biobanking. However, it has to be considered in the study of cryoinjury mechanisms.

**Methods and materials**

**Experimental setup**

The experimental setup consists of three elements: A confocal Raman microscope, a temperature controlled chamber and a scanning stage. We measured the point spread function giving a radial and axial FWHM of 0.8 μm and 2.5 μm for the optical setup. Further details on the experimental setup can be found in [10].

For the example Raman spectra of Me\(_2\)SO and cellular matter shown in Fig. 1a two samples at room temperature containing either pure Me\(_2\)SO (WAK-Chemie GmbH, Germany) or mouse fibroblasts in PBS (PAN Biotech GmbH, Germany) were used. Two additional samples were used for the Raman spectra of ice and hydrohalite, which was recorded at a temperature of approximately −20 °C using solutions of 25 wt.% NaCl saline solution or demineralised water. The integration time for these Raman spectra is 2 s. The Raman images are recorded using adherent mouse fibroblasts in PBS (PAN Biotech GmbH, Germany) and are cooled to −50 °C at a cooling rate of −1 °C/min. The integration time for each pixel is 100 ms and the images have a scan area of 50 μm × 50 μm. The investigated samples were equilibrated a few minutes in either PBS without Me\(_2\)SO or with 0.5 wt.% Me\(_2\)SO at room temperature before the cooling protocol were applied. The sample volume was approximately 10 μL, which corresponds to a sample height of ≈40 μm.

**Cell preparation**

The investigated cell line is the L929 mouse fibroblast from ATCC (United States). The cells were incubated at 37 °C and a 5% CO\(_2\) atmosphere in Gibco® Dulbecco's modified Eagle medium (Life Technologies, United States) with 10% fetal calf serum on glass cover slips (VWR, United States). The cells were handled using standard procedures.

**Confocal Raman microscopy studies**

We use confocal Raman microscopy to investigate the solid states that form in cryopreservation samples upon cooling. The Raman spectra of the compounds encountered in this study are shown in Fig. 1a. The Raman spectra are sufficiently diverse to distinguish between the different formed solid phases. When no Me\(_2\)SO is present the pronounced CH-stretching band around 2900 cm\(^{-1}\) can be used to identify cellular matter consisting of all biological structures containing CH-groups such as cell nuclei, cytoplasm etc. The hydrohalite Raman spectrum consists of several bands located in the high frequency tail of the OH-stretching band [1,6]. These bands arise due to the crystal structure of hydrohalite where water molecules are situated at specific positions in the crystal grid. Only two bands are visible in our spectra due to a limited spectral resolution. The ratio between these two bands depends on the orientation of the hydrohalite crystal with respect to the polarization of the optical excitation [2]. The band at 3425 cm\(^{-1}\) is the most pronounced and will be used to identify the hydrohalite crystals. Raster scanning the laser over the sample will result in an image where each pixel (i, j) has a corresponding Raman spectrum \(I(o, i, j)\) and thus a chemical fingerprint. An integral over specific bands, corresponding to different molecule bonds, in the Raman spectrum is a representation of the amount of that molecule in the focal volume. By integrating

\[
I_c(i, j) = \int_{3030 \text{ cm}^{-1}}^{3420 \text{ cm}^{-1}} I(o, i, j) \, do - I_{\text{back}}(i, j)
\]

for each pixel position (i, j) a spatial distribution of cellular matter \(I_c(i, j)\) and hydrohalite crystals \(I_{\text{HH}}(i, j)\) can be imaged. The background correction \(I_{\text{back}}(i, j)\) is defined as

\[
I_{\text{back}}(i, j) = 0.5 \cdot (I_{\text{end}} - I_{\text{start}}) \cdot \left( I(o_{\text{end}}, i, j) - I(o_{\text{start}}, i, j) \right)
\]

where the integration limits are denoted \(I_{\text{start}}\) and \(I_{\text{end}}\). An example of such an integration and chosen background is shown in Fig. 2. Background subtraction by linear interpolation was chosen to account for the interference of spectral bands, in particular in case of the broad OH stretching band.

It is preferable to use the CH-band to identify cellular matter to get the highest possible signal-to-noise ratio. This is however not possible when Me\(_2\)SO is present in the sample, since Me\(_2\)SO also contains CH-groups and thus has a significant contribution at this frequency. In samples containing Me\(_2\)SO we will thus use the CO-stretching band located at 1655 cm\(^{-1}\) previously shown to be correlated to the Amide I protein structure [16] and contains no overlap with the Me\(_2\)SO Raman spectrum [5], see inset of Fig. 1a. Using a section of the Raman image shown in Fig. 1e we find the Pearson correlation coefficient to be 0.91 between the CH stretching band and the CO-stretching band. The integration limits in this case are thus

\[
I_c(i, j) = \int_{1700 \text{ cm}^{-1}}^{1530 \text{ cm}^{-1}} I(o, i, j) \, do - I_{\text{back}}(i, j)
\]

In order to further analyze the data a color coded image can be prepared by assigning the cellular band (see Fig. 1c) and the hydrohalite band (see Fig. 1d) to the red and green channels, respectively, then merged into a common RGB-image as shown in Fig. 1e. Such an overlay allow for direct visual comparison of the spatial distribution of cellular matter and hydrohalite crystals. It is not possible to quantify the amount of hydrohalite in the focal...
volume without an internal standard due to varying experimental conditions. However, an absolute measure of the hydrohalite volume fraction in the confocal volume is not essential for the localization study.

In addition to the visual inspection of color coded images colocalization maps are utilized to analyze the measured Raman microscopy images. Colocalization is a tool used in biology to investigate spatial correlation between different types of fluorophores [7,17]. Colocalization is normally investigated by plotting the intensities of two fluorophores against each other for each spatial point in the investigated area. When fluorophores are spatially correlated then the fluorescence intensities are also correlated, and patterns appear in the colocalization plot instead of random distributions. Here we use the same principle, but using Raman scattering intensity instead of fluorescence intensity. We have chosen to plot \( \log_{10}(\rho) \), where \( \rho \) is the normalized density of the data points \( \{I_C(i,j), I_{HH}(i,j)\} \), instead of a scatter plot. Fig. 1f shows a plot of \( \log_{10}(\rho) \) of the data in Fig. 1e. The \( \log_{10}(\rho) \) has been chosen to emphasize the relatively low number of data points containing either cellular matter or hydrohalite compared to the vast majority of data points corresponding to ice. A background of 1 has been added to \( \rho \) to avoid problems with logarithmic scaling. Such colocalization maps can be used to categorize the data and help determine whether the hydrohalite found is either intra- or extracellular. If the hydrohalite has formed strictly extracellular and far away from the cell membrane the colocalization maps show no correlation. Most data points appear along the axes in such cases. This situation is easy to identify by visual inspection of the overlay images. In contrast, hydrohalite found along with cellular matter is almost impossible to localize as intra- or extracellular by visual inspection. This is where the colocalization maps are most beneficial. It was found from the CRM data that cellular matter and hydrohalite crystals from eutectic formation were very fine grained compared to the dimension of the confocal probing volume.
volume. In addition the distribution of compounds in the eutectic phase texture turned out to be virtually uniform. As a consequence cellular matter and eutectically crystallized hydrohalite within the cell appear in a fixed Raman band intensity ratio. In the colocalization map this manifests as a linear correlation, which is finally truncated when the volume fraction of the eutectic mixture in the confocal volume becomes unity. A linear correlation is a clear indication that the hydrohalite is located in the cytoplasm. Another case where colocalization maps proves very useful is when the hydrohalite is formed as a shell outside the cellular membrane (or along parts of the membrane), as proposed by Okotrub et al. [11]. Let us imagine the confocal volume scanning from the medium (no cellular matter, no hydrohalite) over the membrane and the extracellular crystal shell (increasing cellular matter, maximum hydrohalite) into the cellular body (maximum cellular matter, decreasing hydrohalite). This will lead to an inverted “U”-shape, which was observed along with extracellular hydrohalite shells as opposed to the linear correlation in case of intracellular hydrohalite formation.

Results and discussion

We recorded 24 confocal Raman images as the one shown in Fig. 1e distributed on four different samples containing L929 mouse fibroblast cells without Me2SO. All images except one contain hydrohalite found over the entire sample. The last image does not contain hydrohalite. We also investigated 6 samples with Me2SO, but only found a significant amount of hydrohalite in one, of which we recorded 6 Raman images. Each Raman image contained primarily one cell, but images with up to three cells were also recorded. All samples were subjected to identical freezing protocols.

Confocal Raman microscopy images

A typical transmission (TM) image and the corresponding Raman responses from cellular matter and hydrohalite are shown in Fig. 1b–d. These images contain one cell and an interdendritic channel. This can however not directly be concluded from the TM image alone. The Raman images reveal that the dendritic channel contains a high amount of hydrohalite and no cellular matter, whereas the hydrohalite phase overlaps the Raman response from the cellular matter. It can furthermore be concluded from the Raman images that the investigated cell contains a large intracellular ice crystal, since most of the cellular matter is displaced towards the rim of the cell, and this displacement can only be attributed to intracellular ice crystals. These features cannot readily be seen from the TM image and clearly demonstrates how Raman imaging gives both more structural and chemical information compared to conventional imaging techniques.

We found that the recorded Raman images of the samples without Me2SO can be roughly divided into three different classes, exemplified by the Raman images in Fig. 3a–c. Overlaps between the groups do however exist and some images are attributed to multiple classes. The first class, denoted Class A, contains images with very little or no overlap between the hydrohalite phase and cellular matter. This can readily be seen in the Raman images as in Fig. 3a. The hydrohalite in these images are thus clearly extracellular, although in close proximity to the cell. We found that 6 images out of the 24 contained extracellular hydrohalite.

The two remaining classes, denoted Class B and Class C, contain Raman images with overlapping hydrohalite phase and cellular matter, i.e., data points where the focal volume contains both hydrohalite and cellular matter. Class B is defined to contain intracellular hydrohalite whereas the hydrohalite is located outside the cell for Class C. Two more examples of recorded Raman images are shown in Fig. 3b and c. The hydrohalite in the remaining Raman images seem to be rather non-uniformly distributed, which contrasts the study of Okotrub et al., where it is hypothesized from point measurements that the hydrohalite form a uniform shell around the cell, since a higher Raman response was measured at the border of the cell.

We cannot directly conclude from our Raman images whether the hydrohalite detected in the confocal probing volume is within the cell or outside, due to the limited axial resolution of our setup and the small thickness of the lipid membrane of the cell. This knowledge is critical to the understanding of the injury mechanisms of eutectic crystallization. In order to determine the location of the hydrohalite we will employ colocalization image analysis.

Colocalization image analysis

Through the use of colocalization image analysis we can determine whether two phases in a Raman image are spatially correlated. Many of the features found in the Raman images can be found in their corresponding colocalization map. We will use the colocalization map Fig. 1f as an example. The high density of data points in the lower left corner corresponds to data points containing no cellular matter or hydrohalite crystals, and thus describes the dominant ice phase of the Raman image. Any clearly extracellular hydrohalite will result in a vertical branch from the ice region in the colocalization map, which can be seen in Fig. 1f and corresponds to the hydrohalite located in the dendritic channel. Data points containing cellular matter but no hydrohalite are similarly located along the horizontal axis. Data points containing both cellular matter and hydrohalite in the focal volume are located in the remaining of the colocalization map. In the example shown in Fig. 1f the data points are approximately located along a line, meaning that these data points show a spatial correlation between the hydrohalite phase and cellular matter.

Fig. 3d shows the colocalization map from Class A where the hydrohalite are primarily located in dendritic channels around the cell. This results in two rather distinct lines along the cellular and hydrohalite axes in the colocalization map. The Raman spectra measured at the edge of the cell will contain contributions from both cellular matter and hydrohalite which leads to the data points slightly centered in colocalization map. The most distinct feature of extracellular hydrohalite is however the branch located close to and along the vertical axis.

The main characteristic of colocalization maps of images with intracellular hydrohalite (Class B) is that a significant amount of
data points are located along a line towards the top right corner of the colocalization map, such as in the colocalization map shown in Fig. 3e. This shows a spatial correlation between the amount of hydrohalite and cellular matter in the focal volume, which is a clear indication of intracellular hydrohalite. The Raman image in Fig. 3b can thus be attributed to Class B. We would not expect any correlation if the hydrohalite were situated outside the cell. Extracellular hydrohalite would also to some extent show overlap with intracellular ice crystals. This is not observed in both the Raman images and their corresponding colocalization maps supporting the conclusion that the hydrohalite must be situated within the cell. It can be concluded from these observations that an eutectic crystallization has taken place within the cytoplasm. This is to our knowledge the first study to directly show such an incident. 12 of the 24 Raman images could be attributed to Class B.

We observed a single Raman image, shown in Fig. 4 that differed significantly from the other in Class B. The Raman image contains two cells with an overlap of cellular matter and hydrohalite crystals, and no intracellular ice crystals are observed. The colocalization map does however indicate a correlation between the hydrohalite crystals and the cellular matter. The hydrohalite crystals are thus likely to be located within the cells. It is curious that intracellular hydrohalite formation is more likely to occur when also intracellular ice is present (11 images). Only one of the acquired CRM images revealed intracellular hydrohalite without intracellular ice (1 image). This is either due to (a) the chemical and thermal conditions favoring intracellular ice formation also favors hydrohalite formation or (b) that ice acts as a promoter for hydrohalite crystal nucleation.

The images belonging to Class C contain a significant amount of data points with both hydrohalite and cellular matter in the focal volume. A first inspection of the colocalization maps, such as the one in Fig. 3f, does not reveal any clear correlations between the hydrohalite phase and the cellular matter. The colocalization map shows typically in this case an inverted ‘U’. The lack of colocalization between the hydrohalite phase and cellular matter is an indication that the hydrohalite is located outside the cell since intracellular hydrohalite will correlate linearly with the amount of cellular matter.
cytoplasm compounds as previously shown. The Raman image in Fig. 3c can thus be attributed to Class C and in total 9 images out of 24 where found with this characteristic. If we assume that hydrohalite forms as a shell around the cell as suggested by Okotrub et al. we expect a colocalization map with similar characteristics as in Fig. 3f. The highest amount of hydrohalite will be measured at the boundary of the cell, but these data points will also contain a Raman response from the cell. This will lead to the inverted “U”-shape in the colocalization map. Our data thus suggests that hydrohalite indeed can form a rather non-uniform shell around the cell and as such supports the conclusions of Okotrub et al. in parts. Yet furthermore we could detect hydrohalite also inside the cellular cytoplasm as well as in interdendritic channels of the simple preservation media distant to any cell.

Influence of Me2SO on hydrohalite formation

We have repeated the experiments where 0.5 wt% Me2SO has been added to the cell medium. This dramatically changes the equilibrium phase diagram since Me2SO also will be concentrated in the unfrozen interdendritic channels [9]. Hydrohalite was only observed in two samples out of six, where one only contained a very limited amount of hydrohalite, which is in stark contrast to the experiments not using Me2SO. The lack of hydrohalite is unexpected since the phase diagram and earlier studies show that hydrohalite can form in hypertonic solutions with a higher Me2SO to NaCl ratio as a continuous precipitation process [10]. This study is done on an isotonic solution, which in equilibrium would form hydrohalite at these temperatures, but has much narrower interdendritic channels compared to a hypertonic solution. Two kinetic factors can limit the formation of hydrohalite; viscosity and impeded diffusion due to narrow interdendritic channels. The viscosity in the unfrozen solution is high due the presence of Me2SO and the low temperatures. Diffusion afflux to any hydrohalite crystal embryos is furthermore limited due to the very low interdendritic cross sections. We believe that a combination of these two factors prevented hydrohalite formation in the majority of the investigated samples.

Three of the recorded Raman images for the one sample containing a significant amount of hydrohalite are shown in Fig. 5. The recorded images can be divided into classes using the categorization method presented earlier. Fig. 5a show cells where there is no overlap between cellular matter and the hydrohalite phase, i.e. Class A. In total 3 out of 6 images contained clearly extracellular hydrohalite. Fig. 5b and c does on the other hand show a certain spatial overlap of compound distributions, but not in a significant manner that we would correlate to intracellular hydrohalite. The distribution of hydrohalite in these Raman images can be best classified to Class C for Fig. 5c and a superposition of Class A and C for Fig. 5b using the colocalization method.

Conclusion

We have shown that confocal Raman microscopy can be utilized to extract detailed chemical information of frozen biological samples. In samples without Me2SO we used this method to determine the distribution of hydrohalite and thus indirectly conclude if eutectic formation has occurred. It turns out that hydrohalite can either form in the very close proximity of cells as non-uniform shell or even intracellularly. Hydrohalite is thus not a strictly extracellular phenomenon. Furthermore, we showed that hydrohalite has a higher probability of forming within the cytoplasm when ice is also present.

Eutectic formation in general has been shown to lead to cell death [8], but the exact injury mechanism has not been determined. We have shown that hydrohalite formation, and thus eutectic formation, can occur both within and outside cells, which can bring a more detailed view on the mortality of eutectic formation.

When small amounts of Me2SO were added to the investigated solution hydrohalite appeared only in limited quantities. This
indicates that Me₂SO inhibits the formation of hydrohalite due to a kinetic limitation of hydrohalite crystal formation and growth.

Acknowledgment

We would like to thank Iris Riemann for cultivation of cells.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cryobiol.2014.04.018.

References


