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SCANNING ELECTRON MICROSCOPY AND ITS APPLICATIONS FOR SENSITIVE SAMPLES

RASTROVACÍ ELEKTRONOVÁ MIKROSKOPIE A JEJÍ APLIKACE PRO SENSITIVNÍ VZORKY

Shortened version of PhD Thesis

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CONTENTS

CONTRIBUTION OF THE THESIS

The research merit of this doctoral thesis is supported by authoring and co-authoring 34 scientific publications. Most of these are directly linked with basic and applied research in the field of microbiology and scanning electron microscopy, especially with the use of low temperatures during sample preparation as well as imaging. Out of the 34 publications, eight were published in impacted journals, one in a non-impacted journal and 25 were published in several international conference proceedings. The engineering contribution related to cryogenic scanning electron microscopy (cryo-SEM) is resulted in the valid utility model no. 2018-35410 and two functional samples:

- (RIV/68081731:_/18:00498266 and RIV/68081731:_/17:00483558).

Moreover, there are some important specific contributions to the field of clinical and environmental microbiology:

- A methodology for the preparation and visualization of clinically significant and biofilm positive microbial strains using scanning electron microscopy was developed with the aim of visualizing under high magnification the ultrastructure of the extracellular matrix produced during cultivation and the relationships among the microbial cells in the biofilm as well as to study areas of the biofilm where the microbial cells adhere to the substrate/surface.
- A methodology for the preparation and visualization of microbial cultures connected with an environmental/biotechnological potential using scanning electron microscopy was improved with the intention to study polymer production as well as polymer biodeterioration.

The educational contribution of this doctoral thesis is represented by supervising one bachelor thesis which was successfully defended in 2014 and two master theses where the author acted as a consultant. Several students' measurements resulted in co-authoring conference papers. Furthermore, authors educational activities were connected with the preparation of two international workshops "*Recent trends in cryo-SEM applied to biology and chemistry, 2016*" and "*Correlative microscopy of beam sensitive samples, 2018*" organized by the research group Microscopy for biomedicine (BioEM) at Institute of Scientific Instruments of the CAS, v. v. i. in Brno. Both events were aimed at students and professional public and drew nearly one hundred participants. Last but not least, participating in the establishment of a new BioEM lab can be considered to be an indirect contribution by this work's author.

1 SEM OF SENSITIVE SAMPLES

It is easy to forget that practically all of the organelles and cellular inclusions were actually discovered or described in detail thanks to the electron microscope (EM). Its description laid the foundations for the design of experiments which led to the revelation of cellular functions and processes that contributed to the understanding of cell structures in natural and experimental environment. Electron microscopy in biology is not only used for imaging of preparations, but also for analysis of chemical composition or physical properties. The use of high-vacuum scanning electron microscope (SEM) in biological applications where the studied samples are composed mainly of elements with low atomic number and have a high water content, brings with it many difficulties. As well as the development of microscopes themselves, detection systems, equipment and software for image processing is important, the development of specialized techniques for sample preparation and handling is also important. This area will be discussed in more detail in the following text.

1.1 MODERN SCANNING ELECTRON MICROSCOPES IN BIOLOGY

In the 1950s the scanning system was incorporated in the electron microscope thanks to a scientific group from Cambridge, led by Oatley [1]. His group invented an improved detector for secondary electrons (SE) and backscatter electrons (BSE) [2] and also focused on the development of electronics needed for screening with the electron beam and for recording the detected signal as an image. Today's modern SEM microscopes owe most of their important functions to the knowledge and work done by this very famous group as well.

1.1.1 The function of electrons in SEM

In comparison with light or X-rays, electrons are almost ideal excitation source for SEM imaging. Electrons in SEM include four important functions:

- They are an easily accessible and easily sustainable monoenergetic source with a high specific brightness value.
- The wavelength can be very short and microscope lenses can focus the electron beam into a Gaussian spot size with a diameter about 0.5-2 nm depending on the accelerating voltage.
- Due to the existence of the electron charge, it is possible to use electromagnetic field for fast and accurate scanning of a sample surface.
- Energetic electrons can, after they hit the sample, excite various detectable signals, such as secondary electrons, Auger electrons, backscatter electrons, also characteristic and continuous X-rays, electron-hole pairs, and cathodo-luminescence. Because these signals are produced only in the place and time of the interaction between the sample and the electron beam, they are immediately detected and processed in order to create an image or signal maps/curves.

The use of electrons in the SEM has, particularly for biological materials, limitations which are generally known and accepted, because benefits of the electron microscopy cannot be denied [1; 3]. The most significant sources of their limitations are defects of electromagnetic lenses; existence of the electron charge; radiation damage; vacuum in SEM; contamination of the sample surface and low chemical contrast. On the other hand, modern SEMs enable working with low energies and high resolution, and if the appropriate parametrs setting and the optimal scanning mode are selected, many of these phenomena can be suppressed. For example, if image integration is used, a dose the sample is subjected to during SEM imaging can be minimized, and thus the effect of charging and contamination can be eliminated and the resulting image with needed contrast and resolution obtained.

1.2 BIOLOGICAL SAMPLE PREPARATION FOR SEM

Biological specimens, such as individual cells or tissues, usually contain a significant amount of water. For example, in the case of microbial biofilms which are, to a large extent, also a research subject within this thesis, the water amounts up to 97 % of their weight [4]. Therefore, it is no exaggeration to consider the preparation methodology of biological and other hydrated sensitive samples to be very important and extensive scientific and experimental area where a number of disciplines, such as physics, chemistry, biology, instrumental development and construction meet. A proof for this statement is a number of publications dealing with procedures and instructions for individual groups of samples, where various methods and techniques that can be used are discussed [5-7]. Biological samples usually contain water which must be removed from them prior to the imaging. The method depends on the specimen type and the information we want to obtain about it.

The conventional chemical preparation of the hydrated object for room temperature SEM includes the following steps [1; 7] and this whole process takes a few hours, sometimes even days:

- Fixating the preparation by immersing it in a fixative agent that binds to organic molecules such as lipids and proteins. The fixative agents (e.g. glutaraldehyde (GA) and osmium tetroxide (OsO_4)) [6; 7] are usually dissolved in a buffer with suitable pH and molarity. Sometimes multiple fixation steps can be applied, individual protocols are adapted to the sample type.
- Washing out the fixative solutions using a buffer which is repeated several times.
- The water content is removed from the sample using a dehydration series, where in several steps the water is replaced by solutions with increasing concentrations of ethanol or acetone. This process is known as dehydration.
- Drying of the sample (e.g. critical point drying, chemicals with low surface tension miscible with products of the dehydration series).
- The final step is to attach the sample to a special holder, alternatively it is possible to increase its surface conductivity by a thin layer of metal or carbon.

The physical preparation of a sample is represented by cryogenic methods and subsequent SEM imaging at low temperature (cryo-SEM), or at room temperature for completely dried samples. The advantage of this approach is mainly its swiftness, thanks to which we avoid a chemical preparation lasting several hours as well as possible artefacts associated with exposure to chemicals. This preparation usually includes the following steps [5; 7]:

- The main requirement is to fix the sample by freezing so that no water crystals that would damage it irreparably are created inside.
- After the cryo-fixation, the sublimation process follows, which can only take a few seconds or minutes to sublime just a thin layer of ice off the sample surface [8; 9], or it can be a multistage process for complet freeze-drying.
- Optionally coating the sample.

The whole physical preparation lasts from a few minutes to several hours. Its main advantages include the possibility of fixating the sample swiftly so it is close to its natural state at the given moment, also the preparation can be studied in its fracture, so besides the surface, internal structures can be imaged as well. On the other hand, it is a process physically demanding to maintain the necessary rates and other parameters in individual steps so that we can avoid all artefacts.

1.3 CRYO-SEM

1.3.1 Introduction to cryo-SEM

SEM became commercially available 30 years after the first TEM and the first low-temperature images were published [10]. One of the reasons for the advance development in cryo-SEM is the benefit of large specimen chamber – it is easier to construct cold stages. Cryogenic electron microscopy can be recognized as the only way to visualize the natural structure of materials such as lung and leaf tissue, foams and foods, and soils. The conventional preparative techniques remove the liquid phases and distort the solid phases, and even freeze-drying produces a sample in which the gaseous and liquid phases are indistinguishable. Another attribute is that it is a relatively fast technique for obtaining medium high resolution information about a wide range of samples. With the appropriate equipment, hydrated samples can be frozen, fractured, coated, and imaged in shorter time in contrary with conventional approach. Therefore, it is possible to visualize dynamic processes occurring in the aqueous phase in high-resolution. In addition, the necessary ancillary preparative equipment associated with cryo-SEM allows a single sample to be sequentially analyzed by a process of repeated fracturing further into or across the sample.

1.3.2 The properties of water in liquid and solid state

Although low-temperature microscopy is not only associated with hydrated systems, most studies are concerned with systems where water, either as ice or amorphous water, is an important structural component. More about the subject can be found in several studies [11-13].

Water is in many aspects a substance with some unexpected properties. At 0 °C (Fig. 1) the solid, liquid, and vapour phases coexist [14]. The presence of hydrogen bonds affect the internal chemical energy of the water molecule. The relatively low density is caused by continually breaking and reforming of hydrogen bonds. Because energy can be stored in hydrogen bonds, water has a high specific heat and can lose or store large amounts of thermal energy. The large heat of vaporization provides the basis for an effective cooling mechanism and it has a large influence on the kinetics of crystallization [12]. Depending on the temperature and pressure, solid water can exist currently in 17 experimentally-confirmed polymorphs of ice. Hexagonal ice is the polymorph of ice that forms when water is cooled quite rapidly. Cubic ice is metastable form of ice which may be formed in the laboratory when water vapour is condensed at below –30 °C. At the pressure of approximately 20 bar and the temperature in the range between –20 °C and –90 °C it should be possible to undercool water without the formation of the two polymorphs of ice. If heat is removed from water at these conditions, the liquid can crystallize to form of high-density, high-pressure polymorphs of ice. Amorphous ice is a monocrystalline form ice and in cryo-SEM it is considered as an ideal state of water. The term of "*vitreous ice*" is also used to describe this state [15].

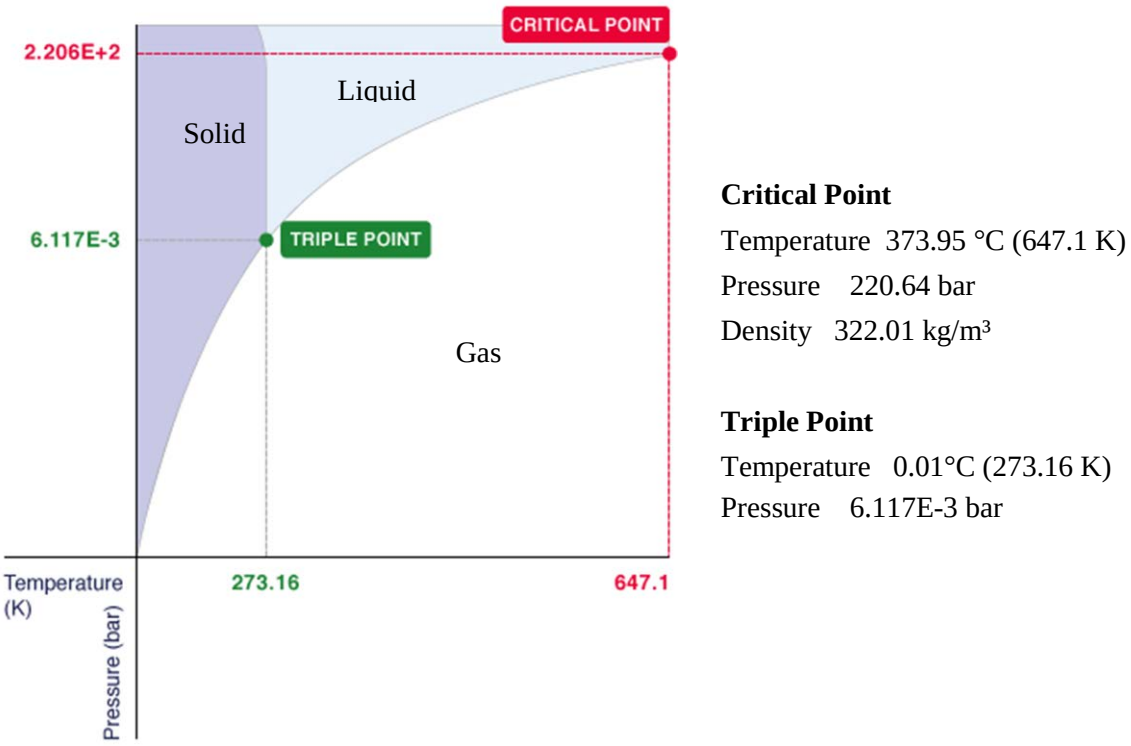


Figure 1: Schematic drawing of H_2O phase diagram. Adapted from [14].

1.3.3 Cryo-fixation

Cryo-fixation by vitrification is an excellent method for preserving biological material. It makes it possible to observe samples without going through the major transformations of chemical fixation, dehydration and drying. Vitrification is the process by which the viscosity of the sample is increased to such a high value that

molecular movements become negligible before ice crystal formation has time to start. There are two main advantages to be gained by vitrification: the provision of a solid matrix suitable for manipulation and microscopy, and an effective immobilization of dynamic processes [12]. In principle, the method is simple. It should now be clear that if a purpose is to convert the water in a sample to a vitreous or even a microcrystalline state by lowering the temperature, then the sample must be cooled as rapidly as possible. The low thermal conductivity of water prevents high cooling rates in the centre of a thicker specimen and the gaining of undercooled water in whole bulk of specimen is impossible. Undercooled water is important in the preparation of samples for cryo-EM. The water in small droplets and thin layers when is rapidly cooled will first undercool and in the absence of any nucleating agents can be transformed to a noncrystalline, glasslike state. The existence of this phenomenon has received some support from the studies of Sceats and Rice [16].

The cooling speeds calculated in study of Studer et al in 1995 [17] cannot be significantly increased because the limiting factor is the thermal conductivity of water. There are, however, two other parameters that can favour vitrification: the addition of a cryoprotectant and high pressure. The former acts by reducing the capability of water molecules to participate in crystal formation. For example, in a saturated sugar solution, water molecules are so involved in their interaction with the sugar that they cannot crystallize. Most soluble substances act as a cryoprotectant. The material inside a cell, the cytoplasm, which typically corresponds to 15-30 % of dry weight, is a cryoprotectant that nature provides freely [18].

There are described eight principal ways of sample freezing for EM in one of major literature sources obtained sample preparation protocols for EM [18]:

- *Combination of chemical preparation and freezing* into a liquefied organic gas such as propane at a temperature of from –160 °C to –190 °C.
- *Plunge-freeze method* involves dipping the specimen into a liquid cryogen. This method is the simplest, widely used procedure especially for cryo-TEM.
- *Cold metal block freeze method* involves bringing rapidly exposed, thin and flat specimens into contact with a polished metal surface which has been uniformly cooled to –190 °C with liquid nitrogen or to –254 °C with liquid helium.
- *Cryogen jet freeze method* involves spraying liquid propane, cooled by liquid nitrogen up to a temperature of –190 °C, onto the specimen at high speed.
- *Spray-freeze method* is based on freezing in a microcrystalline state that is achieved when an efficient cryogen acts specimens of very small size.
- *Punch-freeze method* can be used that the specimen is punched out of the living organ by means of a syringe chilled with liquid propane or liquid nitrogen.
- *High-pressure freezing (HPF) method* becomes to cutting edge freezing methods and for this thesis means a crucial sample preparation technique.

This section summarizes several studies [5; 19-21] and contains a discussion about the improvements in the technology and the methodology of **high-pressure freezing (HPF)** nowadays. In the current state of HPF instrumentation and preparation methods, the technique has not still reached its full theoretical potential. Cryo sample

preparation takes several requirements such as to achieve optimal freezing quality of the sample without distorting the organization of the tissue in short time.

The effect of high pressure on the critical freezing rate of aqueous samples is related to the principle of Le Chatelier – when water freezes, its volume increases. High pressure inhibits this expansion and thereby the crystallization. These changes result in less heat being produced by crystallization, thereby reducing the amount of heat that has to be extracted per unit time by cooling. The cooling rate can be reduced. The pressure zone for affecting the freezing behaviour of water is about 2 050 bar. At this pressure the melting point of water is depressed to approx. –20 °C, and the zone of possible supercooling is reduced to –90°C. These changes result in the lowering of the critical cooling rate of pure water and biological specimens as well. With any method, the actual depth of good freezing will vary with the chemistry of the particular cell type. In general, cells with less free water will freeze better than those that have more aqueous cytoplasm. Most cells also have natural cryoprotectants, such as high sugar and/or protein contents. Despite the fact, it was experimentally recognized a preserving fine ultrastructural details of bulky samples up to 200 μm without any visible deformation caused by ice crystallization. These results proved that HPF has not yet reached its full theoretical potential.

Nowadays, there are currently three commercially available HPF instruments on the market: the Wohlwend Compact HPF03 from Wohlwend Engineering, Sennwald, Switzerland; the Leica EM ICE from Leica Microsystems and the ABRA HPM010 from Fluid AG. The question of which high-pressure freezer to choose depends primarily on several factors such as the size and shape of the sample which needs to be frozen and for special application required light and electrical stimulation there exists only one option. Another important criteria are number of samples, which are able to be frozen in one hour, as well as a purchase price. The Leica microsystems archived product HPF EM PACT2 was described in details by Studer et al. [22] and Vanhecke and Studer [23]. In particular, the standard specimen cups were smaller and less versatile. The specimen well was 1.5 mm in diameter and depths are limited to 100 or 200 μm. Because of the small specimen size this machine has been especially used in applications where frozen samples have been processed for TEM, typically after their freeze-substitution. The HPF EM PACT2 was used in experimental part of this thesis where its usability is shown in microbiological experiments and its unique combination with cryo-SEM observation; see Results and published papers [24; 25].

Freeze-fracturing means a separation of a specimen along a line of least resistance parallel to the applied force. It may be achieved either by applying tensile stress by the knife edge. The process of fracturing is described in several studies [6; 21; 24; 26]. Completely brittle material will fracture without deformation, whereas completely ductile material does not fracture but will exhibit plastic deformation. The main aim of fracturing at low temperature is to obtain fractures with a minimum of plastic deformation. In an ideal brittle fracture, deformation is limited only to the molecules that are actually pulled apart. The applied force of the fracturing tool causes stress to build up in localized regions in the sample. This stress overcomes the cohesive

properties of the specimen and then suddenly spreads rapidly through the sample, causing it to fracture. The way the sample fractures will depend on its brittleness, which is influenced not only by the properties of the sample but by decrease in temperature, rate of stress application, and the presence of discontinuities such as minute cracks within the sample (Fig. 2). In order to utilize the process effectively it is necessary to integrate the fracturing process into a system which allows the fracture faces to be processed further before being examined and analyzed in a deep frozen state in the SEM (e.g. ACE600, ACE900 and BAF060 (Leica Microsystems) [27], the ALTO series (Gatan) [28] or a cryo-system from Quorum Technology).

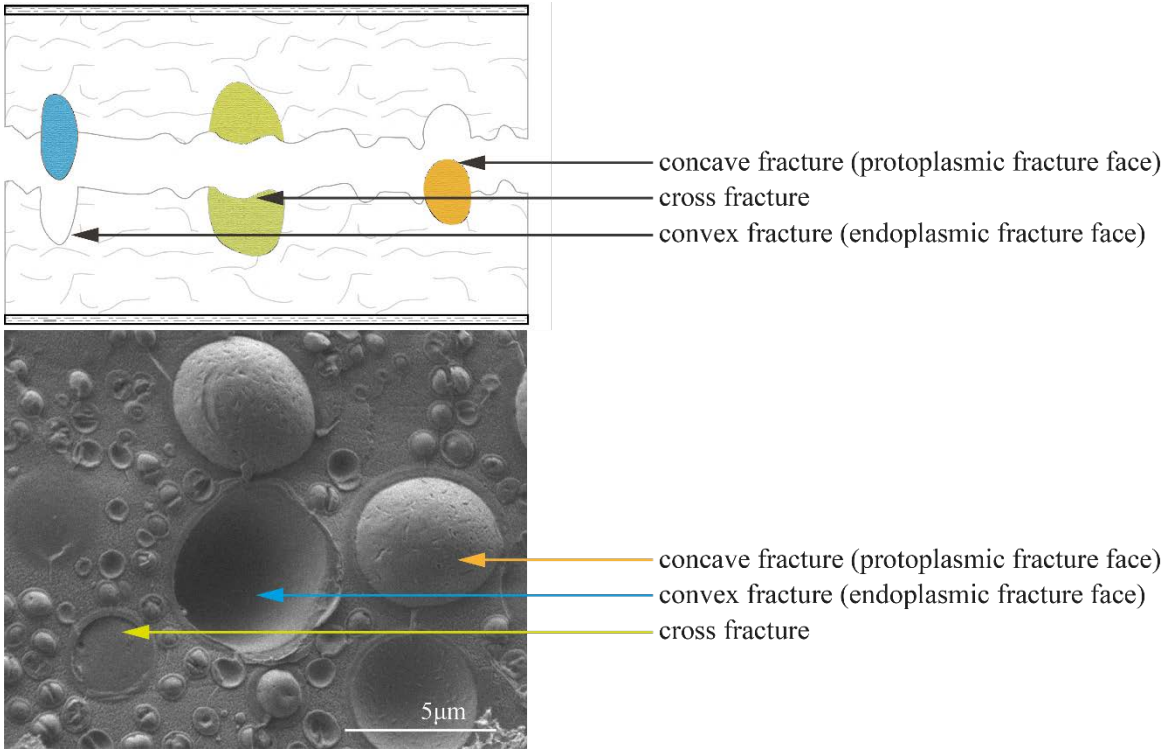


Figure 2: A principle of freeze-fracturing method.

Because water plays such an important part in both the properties and structure of molecules in hydrated systems, its removal must be done as gently as possible in order to minimize structural damage and distortion. Following terminology is used: **Sublimation** is defined as a process of the transition of a substance directly from the solid to the gas phase, without passing through the intermediate liquid phase. Sublimation occurs at temperatures and pressures below a substance's triple point in its phase diagram. The reverse process of sublimation is deposition or desublimation, in which a substance passes directly from a gas to a solid phase. The sublimation rate is proportional to the difference of P_{sat} - P_{part} and high vacuum in cryo-preparation chamber as well as in SEM [12]. **Freeze-drying** is one of a number of dehydration processes, which effectively removes water from hydrated systems and minimizes structural collapse. The term **freeze-etching** is defined as removal of ice from the surface of the fractured specimen by vacuum sublimation, before making imaging by cryo-SEM or preparing the replica. Frozen specimens with a temperature of –120 °C have a saturation pressure of about 10^{-7} mbar (Fig. 3) [8]. If this pressure is established

in the chamber, condensation and evaporation are in equilibrium. At a higher pressure the condensation rate is higher than the sublimation rate [8]. This has to be avoided by all means. A colder (than the specimen) plate placed above the specimen reduces the local pressure and works as a condensation trap (also cooled as an anticontaminator). Water molecules driven up from the specimen preferentially attach to the colder surface of the anticontaminator and therefore it is advantage to have the area as large as possible. At a lower pressure than the saturation pressure more molecules sublimate than condensate and freeze-etching takes place.

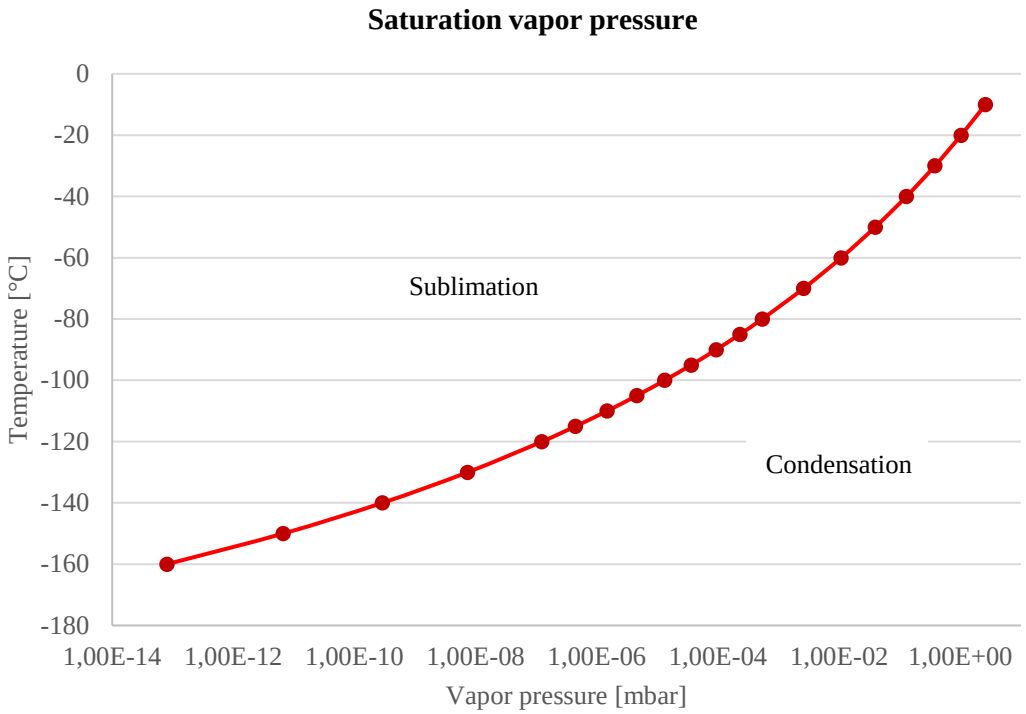


Figure 3: Curve of saturation vapour pressure of water. Redrawn from [8].

1.4 SAMPLE HOLDER DEVELOPMENT IN CRYO-SEM

Cryo-SEM was originally developed by biologists or biophysicists. Among the pioneers in that area was Patrick Echlin, who developed an early methodology of cryo-SEM [29]. While cryo-TEM is now a well-established methodology that can be applied in the study of a wide range of biological/chemical applications; note that for the often used cryo electron tomography a Nobel prize in chemistry was awarded end of 2017 [30], quite often research question needs to be completed by cryo-SEM examination. Nowadays, SEM cryo-holders are available from a number of manufacturers. Nevertheless, some researchers have produced their own home-made sample holders which enable specific holding of the sample and its investigation by EM. Group around Paul Walther developed a new type of high-pressure freezing cryo-holder [31] and Jacob Bastacky and his colleagues developed innovative sample

holders [32] as well. The combination of HPF fixation and freeze-fracturing together with imaging by cryo-SEM was presented in the work from Yong Wu et al [33] on *Staphylococcus aureus* biofilm. In HPF fixed biofilms grown on sapphire disks (6 mm in diameter), artifacts in extracellular matrix (ECM) structure were presented.

The main objectives solved in this thesis is focused on instrumental developments and improvements related with low temperature sample preparation. Within this thesis, also temperature measurement during freeze-etching process was performed in the cryo preparation chamber ACE600. Behind the original temperature sensor which is an original part of the chamber at the cryo stage that is cooled down by metal belts coupled with Dewar containing liquid nitrogen, two additional temperature Pt100 sensors were directly mounted on – the first one on the sample holder to the place of the sample (Fig. 4, the blue line) and the second one on the cryo stage near the original temperature sensor (Fig. 4, the red line). The grey line in Figure 4 shows the temperature measured by the original Leica sensor. The set freeze-etching temperature is corresponding with this grey line. In the presented experiment the freeze-etching parameters were set as following: starting temperature at –140 °C, increasing the temperature to –90 °C with a speed of 5 °C/min, subsequent staying at –90 °C for 10 min and finally lowering down to initial temperature of –140 °C. The pressure in cryo preparation chamber was 2.7×10^{-7} mbar. It is not surprising that measured red and blue curves have time shift (time needed for cooling down the sample holder). On the other hand, as an unexpected result a temperature shift of original sensor has been recognized which is decisive in freeze-etching procedure. This temperature shift was verified by measurement of liquid nitrogen temperature. It can be summarized that real sublimation has been running at different conditions set and the sublimation speed is lower than would be expected.

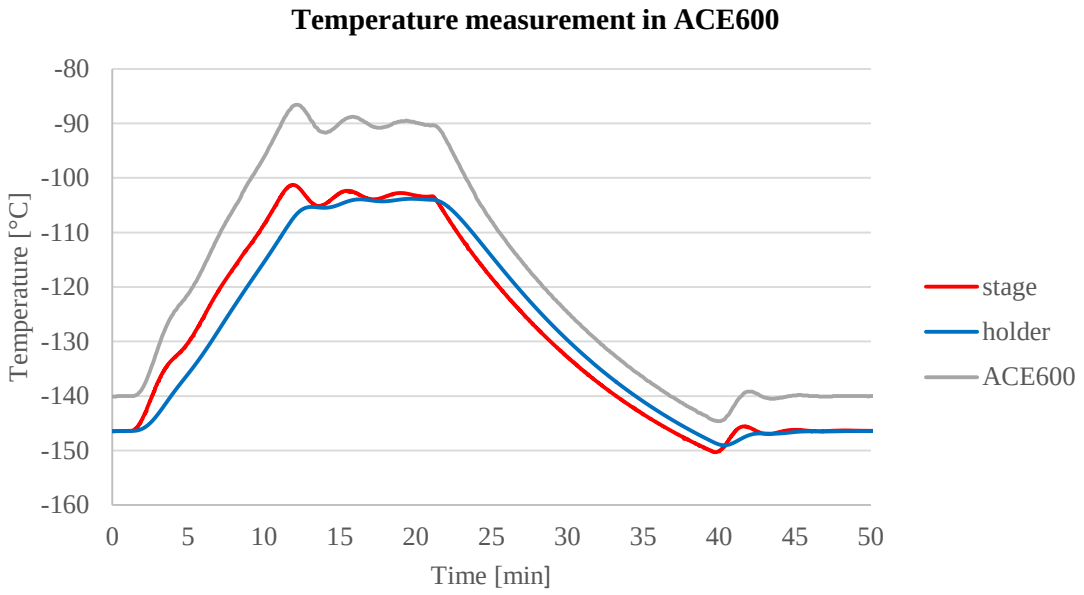


Figure 4: Temperature measurement in cryo preparation chamber ACE600. Lines show the temperature: red on the cryo-stage, blue on the sample holder, and grey line represents temperature measured by original sensor on the cryo-stage.

1.5 THE ROLE OF SEM IN MICROBIOLOGY

Yeast and bacteria are microorganisms that can live as planktonic cells or in an organized formation called biofilm [34; 35]. During their adherence to surfaces or interfaces, the cells are embeding themselves into an amorphous extracellular matrix (ECM) [35-37]. The presence of ECM is often considered an important characteristic of a biofilm [34] and water content can sometimes be as high as 90 % of the total biofilm mass. Biofilms are important in many aspects of health, biotechnology, etc. [37; 38]. However, the detailed architecture of the interior of the ECM has not yet been fully elucidated.

The yeast *Candida parapsilosis* and bacterium *Staphylococcus epidermidis* are found among the normal human microbiota [38; 39]. However, in a medical context they can cause difficult-to-treat infections [40; 41]. Understanding their biofilm structure can contribute to develop a more efficient treatment strategy for biofilm infections. *Bacillus subtilis* which is a nonpathogenic, Gram-positive soil bacterium are also capable of forming a biofilm. Although biofilm formation on polymer surfaces contributes to changes in surface conditions and in this way influences the biodegradation rate. A research [42] carried out to study the effect of various nutrition conditions on the biofilm formation and related PCL film biodeterioration by submerged cultivation of *B. subtilis CCM* 1999.

In the work [43] the cryoprotective effect of PHB-3-hydroxybutyrate (3HB) for lipase was tested as a model enzyme as well as for selected eukaryotic (*Saccharomyces cerevisiae*) and prokaryotic (*Cupriavids necator*) microorganisms. In addition, using cryo-SEM, the role of intracellular PHB granules with respect to the survival of bacterial cells during freezing and thawing was studied.

Single microorganisms cells as well as microbial biofilms are usually investigated by various microscopic techniques including confocal laser scanning microscopy (CLSM) and conventional SEM [42; 44], transmission electron microscopy (TEM) [45], Focused Ion Beam (FIB)-SEM and by special SEM techniques, such as cryo-SEM or environmental SEM (ESEM) [24; 46]. The main limitation of the light microscopy techniques is the restricted magnification. This can be resolved by the use of SEM, which provides high-magnification images, which is important for understanding the morphology and physiology of biofilms [9]. However, a conventional SEM is limited due to the chemical preparation [5]. Fixation with aldehydes and osmium tetroxide preserve cell morphology and enhance contrast, dehydration with ethanol or acetone series is used for the gradual replacement of the water inside the sample. However, this phase of preparation also causes some artefacts, such as cell membrane discontinuities. In the case of cryo-fixation, the biofilm is not dehydrated but kept frozen to obtain high-resolution images closer to the native state of the sample. It has been proven that in cryo-fixed biofilms, the bacterial ultrastructure preservation and the biofilm organization improved significantly [33]. This can be performed by the HPF technique [20; 21; 47; 48]. Microscopic results reveal that cryo preparation of microbiological samples seems to be meaningful approach.

2 MOTIVATION AND RESEARCH STRATEGY

As mentioned in the thesis assignment: "*The aim of the doctoral thesis is an instrumental and a methodological development in the field of scanning electron microscopy and its application in biomedicine and sensitive nanomaterials. An integral part of the work is the specific preparation of samples, both chemical and physical (e.g. cryofixation, immunostaining)*"; the motivation was clearly specified. A suitably selected sample preparation methodology and appropriate instrumental equipment are key for obtaining meaningful and publishable results in microscopic research, especially in biological applications.

There were several important topics and tasks related to the thesis. All the results may be divided into successive tasks depending on their contribution to the thesis:

- *Instrumental & Methodological developments in cryo-SEM*. The motivation in this part was to design and build innovative tools, especially various sample holders, and to optimize the related methodology for the preparation of fully hydrated samples which should be applicable using available laboratory equipment. Specific tasks were the building of sample holders allowing perpendicular freeze-fracturing of small sapphire disks as well as gripping of multiple coper tubes. The author's contribution to the development of a new assembly for temperature control of the sample came from a need to have a defined temperature in the area of the examined sample that is considered to be an important parameter in most applications.
- *The merit of SEM in microbiological research.* A demanding challenge in the field of microbiological research was to develop an optimal methodology for preparation of biofilm samples, with the aim of visualizing the internal structure of a biofilm layer at a high resolution reached by means of a scanning electron microscope. Another microbiological project required developments in sample preparation and freeze-fracturing methodology which were useful in studies of environmentally significant microbial strains.

The research strategy in this thesis started with setting out the scientific problems and identifying the research question and objectives. This part included literature studies and numerous discussions with experts in the field of microbiological/biotechnological and microscopic research. Subsequently, it could seek optimal solutions. In this work, methods and instrumental designs are proposed which were realized and tested on real samples. The new instrumentation was developed with the aim of enabling sample investigation at set conditions and of determining the answers to scientific questions. The results were published in several journals and thus recognized as useful for the scientific community.

3 RESULTS

3.1 INSTRUMENTAL & METHODOLOGICAL DEVELOPMENTS IN CRYO-SEM

Author's scientific outputs related to the Chapter 3 Results [AI – AVII] are cited in the Section: *OUTPUTS INCLUDED IN ANNEXES OF THE THESIS* (page No. 31).

3.1.1 Sample holder I. – perpendicular freeze-fracturing of sapphire disc

Author's contribution to the publications [AI; AII]:

Design of the sample holder construction, sample processing, design of the experiments with microbial biofilm, biofilm incubation**,** imaging in cryo-SEM, results interpretation, preparation of manuscripts as the first author.

Results description:

In this study an innovative method for the preparation of fully hydrated samples of microbial biofilms of cultures *Staphylococcus epidermidis*, *Candida parapsilosis* and *Candida albicans* was presented. Cryogenic scanning electron microscopy (cryo-SEM) and high-pressure freezing (HPF) rank among cutting-edge techniques in the electron microscopy of hydrated samples such as biofilms. However, the combination of these techniques is not always easily applicable. Therefore, a method of combining high-pressure freezing using EM PACT2 (Leica Microsystems) was presented, which fixed hydrated samples on small sapphire discs, with a high resolution SEM equipped with the widely used cryo-preparation system ALTO 2500 (Gatan). Using a holder developed in house, a freeze-fracturing technique was applied to image and investigate microbial cultures cultivated on the sapphire discs. Our experiments were focused on the ultrastructure of the extracellular matrix produced during cultivation and the relationships among microbial cells in the biofilm. The main goal of these investigations was a detailed visualization of areas of the biofilm where the microbial cells adhere to the substrate/surface. The feasibility of this technique was shown and clearly demonstrated in experiments with various freeze-etching times.

A new sample holder for sapphire discs (Fig. 5) with a diameter of 1.4 mm for the HPF instrument EM PACT2 (Leica microsystems) was designed and built. Due to small dimensions of the sapphire discs (diameter 1.4 mm, thickness 50 μm) several requirements for holder construction were specified such as easy fixing of the sapphire disc in liquid nitrogen, perpendicular fracturing of the sapphire disc with the sample in the preparation chamber ALTO 2500 using the original scalpel-manipulator, and the ability to observe both sides of the sapphire disc in the cryo-SEM without removing it from the ALTO 2500. The dimensions and material of the holder main body were analogous to the original dimensions and material, so that the disc fit in the ALTO 2500 instrument. Note that these experiments (Fig. 6) were performed in cooperation with the Biology centre of the Czech academy of sciences, Ceske Budejovice; the holder developed at ISI Brno is still being used to tackle other projects at the Biology Centre.

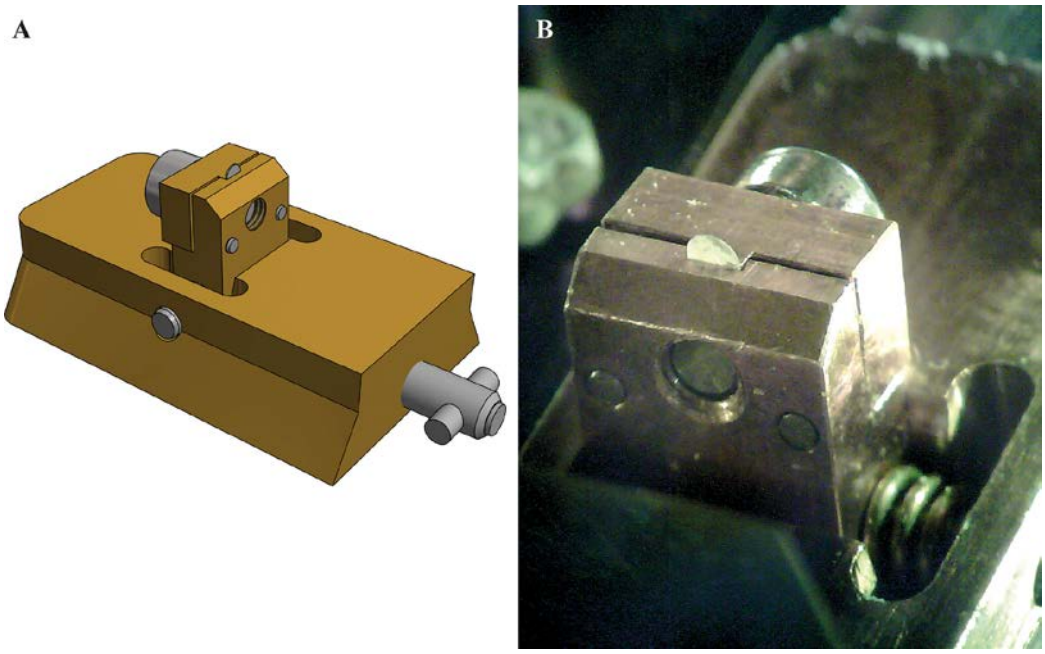


Figure 5: A holder allowing gripping and perpendicular cross freeze-fracturing of the 1.4mm sapphire disc compatible with the ALTO 2500 cryo-preparation system. (A) a schematic drawing and (B) actual picture taken during the experiments in the cryo-preparation chamber.

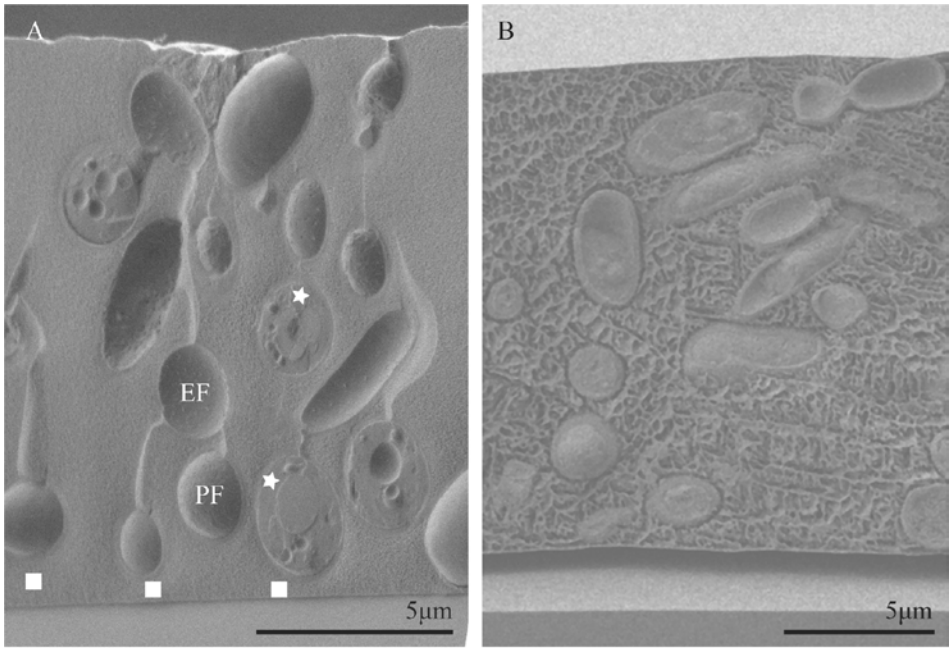


Figure 6: Freeze-fracture of *Candida parapsilosis*. (A) *Candida parapsilosis* grown on a sapphire disc and fixed using HPF. (B) *Candida parapsilosis* grown on a glass substrate and fixed by plunging in nitrogen slush. Endoplasmic (EF) and protoplasmic (PF) fracture faces exhibit typical invaginations and corresponding ridges, broken cells show a preserved ultrastructure with visible cell organelles (stars) and quite large distances between cells and an adhesion surface/substrate (square).

3.1.2 Sample holder II. – freeze-fracturing of samples in Cu tubes

Author's contribution to the publication [AIII]:

Contribution to the construction of the sample holder, sample processing, design of microbial sample preparation for cryo-SEM, imaging in cryo-SEM, cryo-SEM results interpretation, preparation of a SEM part of the manuscript.

Results description

A new sample holder for the cryo-SEM technique was designed and built (Fig. 7). It enables investigation of frozen samples covered in small copper tubes (Cu tubes) with various diameters ranging from 0.8 mm to 1.2 mm. The main body of the new holder fits onto the transfer rod and the rail system of the VCT100 cryo high-vacuum transfer system and the ACE600 cryo preparation chamber (both Leica Microsystems). Its upper part allows one to attach up to eight samples at a time. Therefore, it is possible to observe eight samples in cryo-SEM under the same conditions. Cryogenic fixation of the samples in the Cu tubes by plunging into liquid cryogen can be applied. Although that is not the ideal freezing methodology, it could be beneficial in some experiments such as in the following study.

Accumulation of polyhydroxybutyrate (PHB) seems to be a common metabolic strategy adopted by many bacteria to cope with cold environments. This work aimed at evaluating and understanding the cryoprotective effect of PHB. At first a monomer of PHB, 3-hydroxybutyrate, was identified as a potent cryoprotectant capable of protecting model enzyme (lipase), yeast (*Saccharomyces cerevisiae*) and bacterial cells (*Cupriavidus necator*) against the adverse effects of freezing-thawing cycles. Further, the viability of the frozen/thawed PHB-accumulating strain of *C. necator* was compared to that of the PHB non-accumulating mutant. The presence of PHB granules in cells was revealed to be a significant advantage during freezing. This might be attributed to the higher intracellular level of 3-hydroxybutyrate in PHB-accumulating cells (due to the action of parallel PHB synthesis and degradation, the so-called PHB cycle), but the cryoprotective effect of PHB granules seems to be more complex. Since intracellular PHB granules retain highly flexible properties even at extremely low temperatures (observed by cryo-SEM), it can be expected that PHB granules protect cells against injury from extracellular ice. Finally, thermal analysis indicates that PHB-containing cells exhibit a higher rate of transmembrane water transport, which protects cells against the formation of intracellular ice, something that usually has fatal consequences.

Figure 8 shows cryo-SEM microphotographs of PHB non-containing (*C. necator* PHB 4) and containing (*C. necator H16*) bacterial cells. In PHB-containing cells, needle-like plastic deformations were observed, while these structures were absent in cells without the polymer, which indicates that these deformations can be clearly attributed to PHB granules. Despite the fact that the mechanism of the genesis of these deformations during freeze-fraction has not yet been explained, we can state that

frozen PHB granules exhibit completely different mechanical and physico-chemical properties than any other components of bacterial cytoplasm and that their flexibility, even in deeply-frozen states, is significantly higher than that of PHB isolated from bacterial cells. In cryo-SEM microphotographs we observed elongation corresponding to a value of more than 100%.

Figure 7: A holder is able to grip up to 8 copper tubes in order to freeze-fracture and image the sample inside.

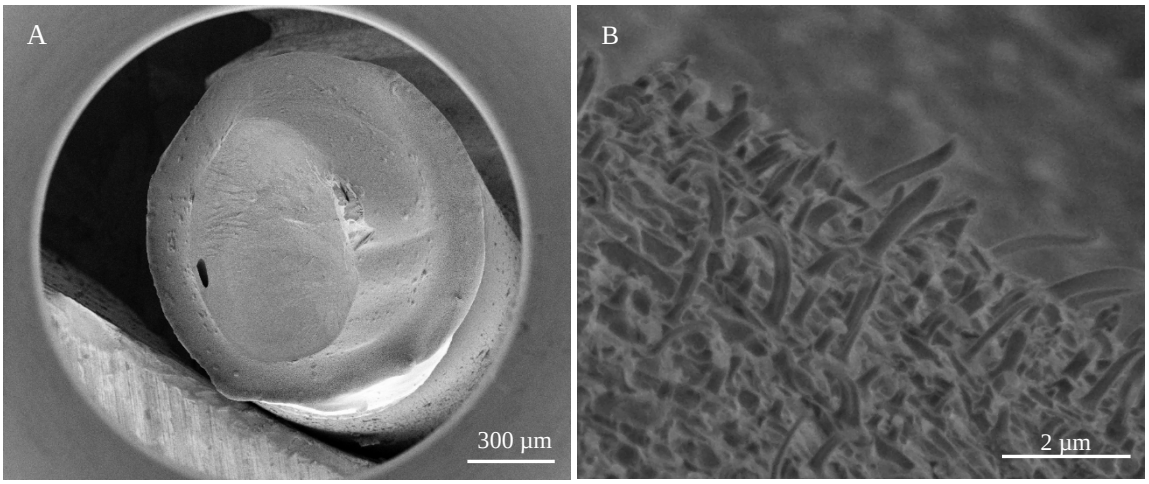


Figure 8: Cryo-SEM images of freeze-fracture of *Cupriavidus necator* sample frozen in copper tube: (A) an overview magnification and (B) a fracture face of bacterial cells containing intracellular PHB granules.

3.1.3 A sample temperature control assembly

Author's contribution to the utility model [AIV]:

Contribution to the design of the shape of an assembly for sample temperature control, sample processing/installation, preparation of the technical documentation.

Results description

Cryogenic electron microscopy is recognized as an important technique in the biological as well as material sciences. It is particularly useful for analysing biological samples or polymers with a high water content. The precise control of a defined temperature in the area of the examined sample is important for most samples, but it seems to be essential in some applications like investigation of hydrogels that are very sensitive to sublimation. Currently, commercial cryo-attachments have a temperature control located in the area of the stage where the sample holder with a specimen is placed on. The main disadvantage of this solution is that the temperature is measured quite far away from the specimen, therefore the measured temperature means the value of the complete system including table + holder + sample. The rate of temperature control is burdened by the whole entire system including a large mass of the material consisted on. Moreover, during sublimation the sample holder as well as the stage is heated and cooled, therefore this standard solution leads to a delay in temperature response. Moreover, it does not allow the analysis of a frozen sample in the transmission mode, i.e. using the STEM detector, with the temperature control. Our main innovations in the presented sample temperature control assembly are new designs for a cryo-stage and the sample holder where a heating element and a temperature sensor are a new part thereof. Both elements are electrically interconnected with the cryo-stage as well as with a temperature controller using a set of connectors. The cryo-stage is connected to the Dewar containing liquid nitrogen via the cooling bands. The main body of the sample holder is based on a commercial solution in order to maintain compatibility with the VCT100 high-vacuum transfer system and other cryo-equipment produced by Leica Microsystems. The construction of the sample holder's upper part is completely innovative (Fig. 9; the Utility Model No. 32258). The assembly is made of a gold-plated copper alloy.

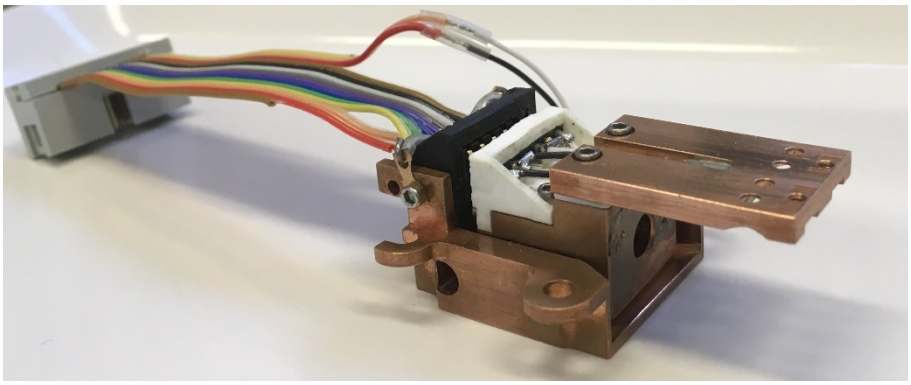


Figure 9: The new sample temperature control assembly.

3.2 THE MERIT OF SEM IN MICROBIOLOGICAL RESEARCH

3.2.1 Biological application I – Medical significant biofilm positive microbial strains

Author's contribution to the publication [AV]:

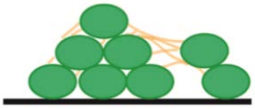
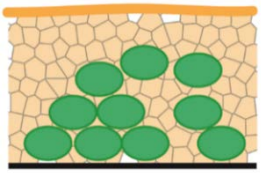
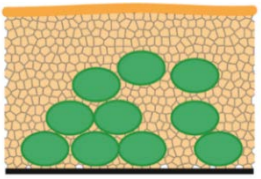
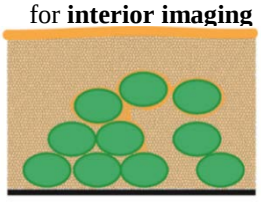
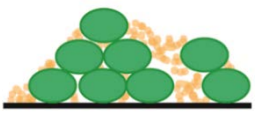
Design of the experimental concept, biofilm cultivation, sample preparation for SEM and cryo-SEM, imaging using SEM and cryo-SEM, SEM and cryo-SEM results interpretation, preparation of the manuscript as the first author.

Results description

The biofilm-forming microbial species *Candida parapsilosis* and *Staphylococcus epidermidis* have been recently linked to serious infections associated with implanted medical devices. In this work, microbial biofilms were studied using high-resolution SEM. Observation using SEM allowed us to visualize the biofilm structure, including the distribution of cells inside the extracellular matrix and the areas of surface adhesion. The work evaluated the merits of a classical SEM involving chemically fixed samples with cryo-SEM, including physical sample preparation based on plunging the sample into various liquid cryogens as well as high-pressure freezing (HPF). For imaging the biofilm interior, the freeze-fracture technique was applied. In this study, it was shown that the different means of sample preparation have a fundamental influence on the observed biofilm structure. The SEM was employed to study the ECM content and distribution in the biofilm, and the way it translates into a Raman spectral characteristics. The SEM images helped us to estimate the relative proportion of the ECM, which in most cases ranges between 20 % and 50 % of the total biofilm volume. This means that in the Raman spectra the signal was observed both from the bacterial cells and from the ECM, the proportion of which depends on the growth stage of the biofilm. The proportion of the ECM increased with the age of the biofilm.

All the samples were imaged by SEM, both at room temperature and at low temperature. Author describes all the sample preparation protocols for SEM and the limitations of these six different preparation techniques on bacterial and yeast biofilms (M1–M6 in the Table 1). The benefits and influence on the biofilm structure are summarized in the Table 1. A comparison of the applied techniques for microbial biofilm studies was presented and it showed that a combination of Raman spectroscopy with selected SEM techniques can provide a deeper insight into the chemistry and composition of biofilms.

Table 1: Comparison of benefits and limitations of sample preparation protocols for SEM (Methods M1-M6) and their influence on biofilm structure as shown in schematic drawings. Our best candidates for sample preparation techniques are labelled green.

M	Advantage	Disadvantage	Schema
M1 – air-drying	Speed of sample preparation Simplicity Repeatability of measurement in SEM at room temperature Suitable for **surface imaging**	The loss of the 3D structure Deformation of microbial biofilm Deformation of ECM The possibility of imaging only the sample surface (not interior)	Deformation of biofilm 
M2 - conventional chemical preparation	Repeatability of measurement in SEM at room temperature The 3D structure is preserved. Suitable for **surface imaging**	Long-term procedure Damage of soft biofilm sample due to multi-steps washing Artefacts with chemicals treatment (the change of gel-like ECM into fiber structures) The sample surface imaging	Biofilm is washed out 
M3 – plunging LN_2; cryo-SEM	Speed of sample preparation The 3D structure of microbial cells is preserved Possibility of biofilm **interior imaging** (used freeze-fracturing technique also suitable for M3-M5)	Artefacts with freezing procedure Freezing is sufficient for very thin samples Limitation for surface imaging because of water content in biofilm samples	"Large"ice crystals 
M4– plung. Ethane; cryo-SEM	Speed of sample preparation The 3D structure of microbial cells is preserved Possibility of biofilm **interior imaging**	Artifacts with freezing procedure (smaller ice crystals inside biofilm than by M3) Freezing of thin samples Limitation in surface imaging because of water content in biofilm samples	"Small" ice crystals 
M5 – HPF freezing and cryo-SEM	Speed of sample preparation 3D structure of microbial cells / ECM is nicely preserved The best freezing technique for samples with thickness up to 200 µm (exp. tested) Biofilm **interior imaging**	Limitation in surface imaging (water content in biofilm) Limitations connected with HPF machine – cultivation substrate (sapphire discs for freeze-fracturing; Al or Cu-gold discs)	**Optimal** prep. of biofilm for **interior imaging** 
M6 - Combined preparation	Speed of sample preparation 3D structure of biofilm Biofilm **surface imaging** Repeatability of measurement in SEM at room temperature after freeze-drying Less washed out biofilm	Artifacts from chemical fixation (the change of gel-like ECM) The imaging of sample surface	**Optimal** prep. of chemically fixed biofilm for **surface imaging.** 

3.2.2 Biological application II – Environmentally significant microbial strains

Author's contribution to the publication [AVI]:

Microbial samples preparation for SEM, sample processing, imaging using SEM, SEM results interpretation, preparation of SEM part of the manuscript.

Results description

Widespread studies on the biodegradation of plastics have been carried out in order to confront the environmental problems associated with synthetic plastic waste. From this point of view aliphatic polyesters represent a class of biodegradable materials with various potential applications. Research interest has been also paid to enhancement of biodegradation of resistant plastics by incorporation of aliphatic ester linkages to aromatic ones or modification of polyolefins with aliphatic polyesters. Polycaprolactone (PCL) as a representative of aliphatic polyesters undergoes biodegradation by the action of enzymes and microorganisms and hence it is a suitable candidate for the study of biodeterioration and biodegradation.

The biodeterioration activity of a *Bacillus* strain, naturally occurring in the soil environment, on the surface of polycaprolactone films in mineral salt and mineral salt-yeast extract enriched media at 30 °C after 21 days was studied. Polyester square specimens (10×10 mm) were sectioned from film prepared by melt pressing. Control abiotic tests were carried out by immersion of the studied samples into the media in the absence of microorganisms. As described in [145, 187] the surface erosion pattern which developed is ascribed to the formation of degradation products ended with carboxylic groups in dissociated forms as the first step. Subsequently, the accumulation of more hydrophilic species supports both the acceleration of chain degradation through autocatalysis and the diffusion of water. Changes in the film surface were evaluated by means of SEM using a Magellan 400/L (Thermo Fisher Scientific) at a primary energy of 2 keV. For characterization of the material surface, here the presented samples lacked any chemical treatment and were air-dried, and they were coated with 4 nm of platinum. The microcracks which developed on the surface of biotically-aged films in mineral salt and mineral salt with yeast extract media were randomly distributed. The formation of irregular holes on the surface may be associated with higher enzymatic activity in a mineral salt medium enriched with yeast extract. The polycaprolactone sample, due to its semicrystalline and hydrophobic character, displayed only a low water uptake in abiotic control media and consequently no weight loss along with no surface erosion proceeded.

3.2.3 Biological application III – Environmentally significant red yeast strains

Author's contribution to the publication [AVII]:

Microbial sample preparation for SEM and cryo-SEM, sample processing, imaging using SEM and cryo-SEM, SEM and cryo-SEM results interpretation, preparation of the abstract and poster.

Results description

Several investigations of the influence of different cultivation conditions on lipids production by red yeasts, using SEM and Raman spectroscopy techniques, have been reported. Here, SEM uses an electron beam to gain information about the morphology of cells which reflects the cells' response to the applied stress. Consequently, Raman spectroscopy was used for the determination of carotenoids and lipids present in the biomass. Thus, this study presented some factors which could lead to efficient industrial production of carotenoids and lipids in selected biotechnological production. Applied stress can induce changes in cell composition, metabolism and physiology. These investigations were focused on red yeasts which also could produce a high amount of lipid compounds. Carotenoids are membrane-bound lipid-soluble pigments, which can act as effective antioxidants and scavenge singlet oxygen. In red yeast, controlled physiological and nutrition stress can be used for enhanced pigment and also oil production. Namely, in the presented experiments osmotic stress was induced by incubation in two different media which led to production of lipids or carotenoids. Note that also the morphology of cells can be changed. Both media contain the same: salts, glucose and ammonium sulphate with different C/N ratios. The first growth medium (medium 1) indicated positive results for carotenoid production and the second medium (medium 2) with a high C/N ratio led to an increased lipid production.

For SEM imaging the samples of *Cystofilobasidium infirmominiatum* were prepared in the following way: (a) the samples were cultivated aerobically at 25 °C in the two different media for 144 hours; (b) cell suspensions were fixed in 2.5% glutaraldehyde in PBS for 2 hours and 30 min in 1% OsO_4, dehydrated by ethanol series and dried using HDMS on the glass slides. Both images of prepared samples were scanned without any metal coating at an electron beam energy of 1 keV and a beam current of 6.3 pA in a SEM Magellan (FEI). The samples of *Sporobolomyces shibatanus* were cultivated as mentioned above and observed by cryo-SEM. The sample was quickly frozen in liquid nitrogen, moved into a vacuum chamber (ACE600, Leica Microsystems) where it was freeze-fractured and sublimated at –95 °C for 5 minutes. In the next step, the sample was moved under high vacuum using a shuttle (VCT100, Leica Microsystems) into the SEM (Magellan, FEI) equipped with a cold stage and the fractured structure was observed with a 1 keV electron beam at –120 °C and a beam current of 6.3 pA without any metal coating. Our results showed a combination of SEM and Raman spectroscopy approaches could be applied for.

4 CONCLUSIONS

Electron microscopy in biology can provide high-resolution images and other specific information originating from electron-sample interactions that are useful for a deeper understanding of the structural and functional quintessence of complex biological systems. Consequently, specimen preparation protocols related to this powerful technique and imaging procedures should form the basis for the correct interpretation of the hydrated sample images and analyzed data.

In the introductory part, the author describes sample immobilization techniques such as the preparation protocols of aquagenous materials that are necessary for their transition to a solid state in which a specimen can resist physical conditions during imaging and analysis. These techniques are divided into two main groups depending on the means of sample fixation and on the observation temperature under a scanning electron microscope (SEM). For conventional SEM, i.e. under room temperature, a chemical fixation to preserve soft structures, subsequent dehydration and metal coating is required. All of these steps have their own limits and often create a lot of artifacts which have been identified in several studies which are cited in the thesis. Many of the problems associated with conventional preparation techniques of hydrated electron beam sensitive materials can be avoided by using cryogenic SEM (cryo-SEM), which allows observation of an aquagenous specimen close to its native state. However, to understand the imaged structures properly, it is important to determine the main processes that occur during the cryo-preparation, because potential pitfalls in cryo-SEM can be also recognized. In addition, this theoretical section of the thesis also includes an overview of already extant instruments for SEM sample preparation, such as critical point dryers and cryo-related equipment which are designed with the aim of dealing with the water content in biological and other liquid-based specimens.

This dissertation thesis contains innovations and improvements in the field of specimen preparation and cryo-SEM technique that were produced at the Institute of Scientific Instruments of the CAS in Brno. The most important results, including the developed tools and their verification in real experiments, are described in the Chapter *3 Results*.

In particular, the new constructions of two sample holders are presented. The functionality of the first sample holder for perpendicular freeze-fracturing of small sapphire discs, which are usually used for high pressure freezing (HPF), is presented in experiments with microbial samples. Among the investigated microbiological strains are the biofilm positive bacteria *Staphylococcus epidermidis* and yeasts such as *Candida albicans* and *Candida parapsilosis*, which are considered to be clinically significant because they are often involved in serious infections. The main aim of this study is to suggest that cryo-SEM in combination with HPF and perpendicular cross freeze-fracturing through a sapphire disc is an excellent technique for imaging highly hydrated samples. Details of biofilm formation can be recognized and further studied

in the natural hydrated state, thus allowing for detailed investigations of the ultrafine structure and morphology in near-life-like conditions.

These strains were used also in the methodological study where positives and limits of a room temperature SEM and cryo-SEM technique are evaluated on the quality of biofilm structure. We compared the merit of various sample preparation protocols including chemical fixation as well as physical fixation by plunging into various liquid cryogens and freezing by HPF. It was shown that the different means of sample preparation have a fundamental influence on the observed biofilm structure. SEM was employed to study the ECM content and distribution in the biofilm, and the way it translates into Raman spectral characteristics.

The second presented sample holder was designed with the aim of improving and increasing the reproducibility of experiments which required the same temperature/sublimation conditions. It was proved successful in the field of microbiological samples with biotechnological potential. Its upper part allows one to attach up to eight samples covered in cooper tubes at a time. Therefore, it is possible to observe eight samples in cryo-SEM under the same conditions. Its usefulness is shown in the evaluation of the cryoprotective effect of polyhydroxybutyrate (PHB) inside bacterial cells of *Cupriavidus necator*. In this case, the cryogenic fixation in Cu tubes by plunging into liquid cryogen can be applied. Even though it is not the ideal freezing methodology, it is beneficial in these experiments.

A sample temperature control assembly presents an innovation in the area of increasing temperature control during sublimation as well as imaging by cryo-SEM. This valid utility model couples a new design of the cryo-stage and a sample holder which is enhanced by means of a temperature sensor and heating element.

The merits of the methodological improvements are proven also in other experimental applications such as the investigation of the influence of different cultivation conditions on lipid production by red yeasts such as *Cystofilobasidium infirmominiatum* and *Sporobolomyces shibatanus*, using a combination of SEM and Raman spectroscopy. Furthermore, the effect of the biofilm formation of *Bacillus subtilis* on biodeterioration and biodegradation of poly-ε-caprolactone films was studied.

The author would like to note that the carefully selected sample preparation methodology and appropriate instrumental equipment described within this thesis are key in obtaining meaningful and publishable results in microscopic research, especially in biological applications. Besides the author’s results, which have been published and therefore recognized as useful for the scientific community, the presented thesis can be considered to represent a review of the current state in the area of high-vacuum SEM in the life sciences as well.

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AUTHOR'S SCIENTIFIC OUTPUTS

The following information were available in Scopus database and ASEP database.

Date: 26. 06. 2019

Impacted Journal Article: 8

Journal Article: 1

Conference Papers: 25

Valid utility model: 1

Functional sample: 2

OUTPUTS INCLUDED IN ANNEXES OF THE THESIS

[AI] Hrubanová, K., Nebesářová, J., Růžička, F., Krzyžánek, V. The innovation of cryo-SEM freeze-fracturing methodology demonstrated on high pressure frozen biofilm. *Micron*. 2018, 110(JUL), 28-35. ISSN 0968-4328, doi: 10.1016/j.micron.2018.04.006.

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CURRICULUM VITAE

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Education:

2012 – 2019 Doctoral study at Physical Engineering, Brno University of Technology (BUT). Advisors: Ing. Vladislav Krzyžánek, Ph.D., Topic: Scanning electron microscopy and its applications for sensitive samples, Research work in the area of electron microscopy especially at low temperature for microbiological studies.

2009 – 2011 Master study Masaryk University in Brno (MU). Advisor: Mgr. Karel Kubíček, PhD, Topic: Study of physical properties of biomembranes

Professional experience:

2011 – Research assistant, ISI of the CAS, Brno, Czech Republic, Department of electron microscopy, Group: Electron microscopy for biomedicine

Parental leave 2014 and 2017

Teaching activities:

- BUT 2012/2013 2F, 3F; 2013 3F
- Supervisor of bachelor's thesis (2014) and consultant of two master's theses (2014 and 2018)

Participations in scientific projects:

- the Czech Science Foundation (projects 19-20697S;17-15451S;16-12477S;15-20645S;14-20012S;1929651L)
- the Ministry of Industry and Trade of the Czech Republic (project TRIO FV30271)
- the Technology Agency of the Czech Republic (projects TN01000008; TE01020118)
- the Ministry of Education, Youth and Sports of the Czech Republic (projects LO1212; CZ.1.07/2.3.00/20.0103).

Author's scientific project: Thermo Fisher Scientific/CSMS scholarship (2015-2017) and TACR TN01000008 (DP21; 2019-2020).

Language skills:

Czech, English, German

Scientific activities:

SEM, cryo-SEM, biological sample preparation, workshop's organization for scientific community

ABSTRACT

The presented dissertation thesis titled "*Scanning electron microscopy and its applications for sensitive samples*" describes an instrumental and methodological development in the field of scanning electron microscopy leading to an innovative solution that could be particularly applicable in microbiological research. A summary of the history and current state of electron microscopy (EM) as a scientific imaging and analytical technique is provided in the introductory chapters. The undeniable contribution of EM in the biological and medical sciences is evidenced by many cited scientific publications. This dissertation thesis contains innovations and improvements in specimen preparation and cryogenic scanning electron microscopy (cryo-SEM) produced at the Institute of Scientific Instruments of the CAS in Brno. In particular, the new constructions of special sample holders together with methodological development in the field of microbiological sample preparation resulted in finding optimal parameters for individual processes. In the experimental part there is showed a verification of methodological procedures in the study of hydrated and electron beam sensitive specimens. Subsequent comparison of different methodological approaches on a defined microbiological system contributes to extending the interpretation of the hitherto known results. Among the microbiological strains investigated were the biofilm positive bacteria *Staphylococcus epidermidis* and yeasts such as *Candida albicans* and *Candida parapsilosis*, which are considered to be clinically significant because they are often involved in serious infections and especially threaten immunocompromised patients. Furthermore, the effect of the biofilm formation of *Bacillus subtilis* on the biodeterioration and biodegradation of poly-ε-caprolactone films was studied. The new development in low temperature cryo-SEM was employed in the research of microbes with biotechnological potential such as *Cupriavidus necator* and *Sporobolomyces shibatanus*.

ABSTRAKT

Předložená dizertační práce s názvem „*Rastrovací elektronová mikroskopie a její aplikace pro senzitivní vzorky*“ pojednává o problematice rastrovací elektronové mikroskopie v kontextu instrumentálního a metodologického vývoje vedoucího k inovativnímu řešení, které je dobře aplikovatelné zejména v mikrobiologickém výzkumu. Součástí práce je rozprava o historii a současném stavu elektronové mikroskopie (EM) jakožto vědecké zobrazovací a analytické techniky, tato část se nachází v úvodních kapitolách. Nepopiratelný přínos EM v biologických a lékařských oborech je dokazován mnoha citovanými vědeckými publikacemi. Předložená dizertační práce přináší novinky z oblasti přípravy preparátů a kryogenní rastrovací elektronové mikroskopie (cryo-SEM) vyvinuté na pracovišti Ústavu přístrojové techniky AV ČR, v.v.i. v Brně. Jedná se především o návrhy a výrobu speciálních držáků vzorků a vývoj nových metodik v oblasti přípravy mikrobiologických preparátů vedoucích k nalezení optimálních parametrů jednotlivých procesů. V experimentální části se nachází ověření metodologických postupů při studiu hydratovaných a na elektronový svazek senzitivních preparátů. Následné srovnání různých přístupů na definovaném biologickém systému z oblasti mikrobiologie přispívá k rozšíření interpretace doposud známých výsledků. Mezi zkoumanými mikrobiologickými kmeny byly biofilm-pozitivní bakterie *Staphylococcus epidermidis* a kvasinky jako *Candida albicans* a *Candida parapsilosis,* jež jsou považovány za klinicky významné, protože se podílejí na vzniku závažných infekcí zejména u imunokompromitovaných pacientů. Dále byl studován vliv růstu biofilmu bakterie *Bacillus subtilis* na biodeteriorizaci a biodegradaci poly-ε-kaprolaktonových fólií. Vývoj v oblasti cryo-SEM byl aplikován ve výzkumu mikrobů s biotechnologickým potenciálem, jako jsou např. *Cupriavidus necator* a *Sporobolomyces shibatanus.*