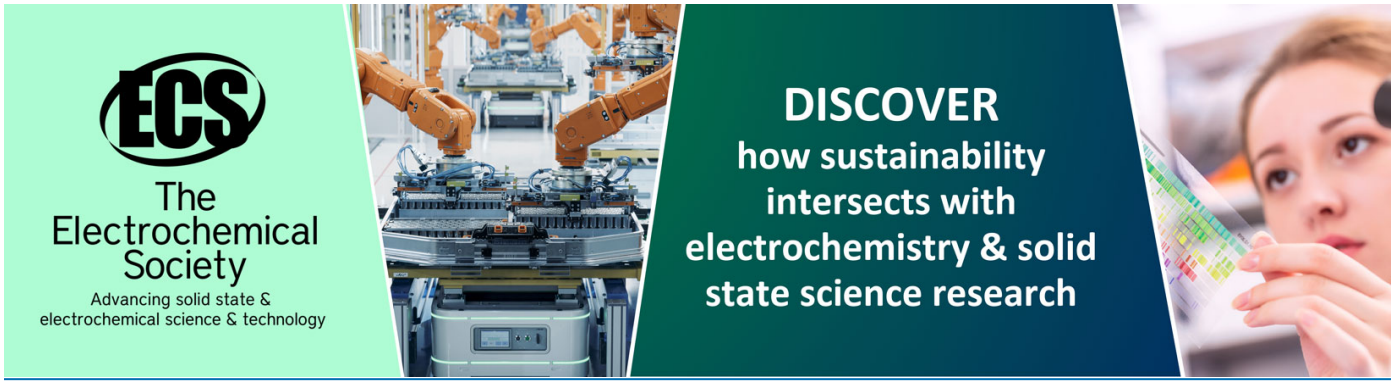
Dynamical ‘in situ’ observation of biological samples using variable pressure scanning electron microscope



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Dynamical "in situ" observation of biological samples using variable pressure scanning electron microscope

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Abstract. Possibilities of "in-situ" observation of non-conductive biological samples free of charging artefacts in dynamically changed surrounding conditions are the topic of this work. The observed biological sample, the tongue of a rat, was placed on a cooled Peltier stage. We studied the visibility of topographical structure depending on transition between liquid and gas state of water in the specimen chamber of VP SEM.

1. Introduction

The variable pressure scanning electron microscopy allows observation and investigation of specimens that are difficult or impossible to image in a conventional high vacuum scanning electron microscope (SEM). The basic difference between the variable pressure microscope (VP SEM) and SEM is that the specimen chamber of VP SEM can contain a certain amount of gas, mostly water vapour [1]. The collisions with gas molecules influence both primary electrons between the small holes in the pressure-limiting apertures and the sample, as well as signal electrons emitted from the sample. The high pressure of gases in the specimen chamber of VP SEM makes completely different conditions for SE detection than in a conventional SEM. The construction of detectors must be based on different physical principles. The most efficient detector of secondary electrons (SE) in the high pressure conditions in the specimen chamber of VP SEM uses the principle of gas ionisation that proceeds as a cascade between a grounded specimen holder and a detector signal electrode supplied with a positive voltage, placed under the pole piece of the objective lens [2, 3].

The modern VP SEM allows specimens to be observed in two high-pressure modes as well as in the high vacuum mode. In the "low vacuum", also natural mode, the pressure ranges from 13 – 330 Pa (0.1 – 2.5Torr) and in the "environmental" mode it is over 330 Pa (2.5Torr). The "environmental" mode enables very wet non-conductive samples to be observed without covering the surface with a conductive coating and their natural fully hydrated surface structure is preserved [4].

The pressure of water vapour in the specimen chamber of the VP SEM together with specimen temperature play a crucial role in obtaining and maintaining the state of thermodynamic equilibrium between the environment of the specimen chamber and the sample itself, as demonstrated by Cameron and Donald [5]. In thermodynamic equilibrium of 100% relative humidity we must follow the dependence of saturated water vapour pressure on the specimen temperature. A small change of temperature from the stable pressure of the water vapour can be used for the study of transition between the hydration and dehydration phenomena but also to a more limited extent for the study of reactions [6]. These dynamical "in-situ" experiments are the aim of this work.

2. Material and methods

The rat tongue has a good stability to maintain natural hydration inside the sample and the change of structure of siliform papillae on its surface can indicate the decrease of relative humidity; for this reason it is a suitable sample for dynamical "in-situ" experiment. The sample was not treated with any preparation technique and its surface was not covered by any conductive layer. The rat tongue was placed on a cooled specimen holder (Peltier stage) and the temperature of the specimen holder was adjusted and kept at 2°C. For this temperature the saturated water vapour pressure is 708 Pa.

Our experimental VP SEM AQUASEM-II was designed in the Institute of Scientific Instruments (ISI) of the Academy of Sciences of the Czech Republic as a non-commercial apparatus for research on VP SEM detection systems and VP SEM techniques [7]. The electron optical column with a tungsten hairpin filament and electronics was delivered by Tescan Ltd. The special specimen chamber, the differentially pumped chamber, the Peltier-cooled specimen holder and the hydration system [8], allowing precise control of water vapour flow into the chamber, were designed at ISI Brno. The pressure measurement is provided by a system of capacity gauges made by the Pfeiffer company. The microscope works also in high and low vacuum modes. The single crystal YAG:Ce^{3+} (yttrium aluminium garnet activated with trivalent cerium) has a hole in the centre, so that it simultaneously acts as a pressure-limiting aperture which restricts the gas flow between the specimen chamber and the differentially pumped chamber [9]. It is used for the detection of backscattered electrons in the low and high vacuum modes. In the low vacuum and environmental mode the secondary electron signal was detected by an electrode with inner diameter 4 mm deposited on the input surface of the single crystal detector. With a positive bias of 370V with respect to the sample it acts similar to the environmental secondary detector [2,3].

Sufficient and stable humidity in the specimen chamber during the first pumping stage must be preserved. At the beginning of the pumping the pressure is equal to the atmospheric pressure in all parts of the microscope. A small cup with distilled water is inserted into the specimen chamber and temperature of sample is reduced to 2°C. The microscope is pumped using rotary and turbo-molecular pumps and the specimen chamber is pumped only trough the pressure limiting aperture. Then the pressure in the chamber stabilizes at about 2000 Pa, this value is given by the thermodynamic balance corresponding to the pressure of saturated water vapour in the cup. When the water from the cup evaporates, the pressure in the specimen chamber is decreasing and water vapour is supplied from a hydration system [8] to establish the required pressure in the chamber.

All experiments were carried out under constant operating conditions of VP SEM (beam accelerating voltage 20 kV, probe current 200 pA, working distance 2.5 mm, positive bias of the electrode system 370V) and in the water vapour environment with high relative humidity.

3. Results and discussion

This experiment was focused on be study the transition from the hydration to dehydration state and to study the topographical changes depending on dehydration using the VP SEM. The first image (Figure 1A) of the sample was recorded after the pressure in the specimen chamber was decreased from 2000 Pa to 730 Pa at the stage temperature 2°C and after 10 minutes of pumping the specimen chamber. The specimen is fully hydrated and its surface is covered with water, so that no topographical details are visible on the surface. Figure 1B shows a sample at 715 Pa and 2°C which are conditions slightly above the dependence of saturated water vapour on the sample temperature (liquid phase region). After that the pressure was decreased to 708 Pa. Slow local temperature increase of the observed area due to the influence of primary electron beam and low temperature conductivity of the biological sample violate the thermodynamic balance and cause a very slow evaporation of water from the sample surface dependent on the observation time [9]. It can be seen in Figure 1A-E, recorded in 5 min intervals. With the decreasing water layer the fine topographical structure starts to be visible.

Evaporation of water from the sample is not even and it depends on temperature, pressure, topography and local material properties in the given place. The thermal effects of the beam depend on the beam

current and voltage due to the change of interaction volume, which in biological sample can be relatively large.

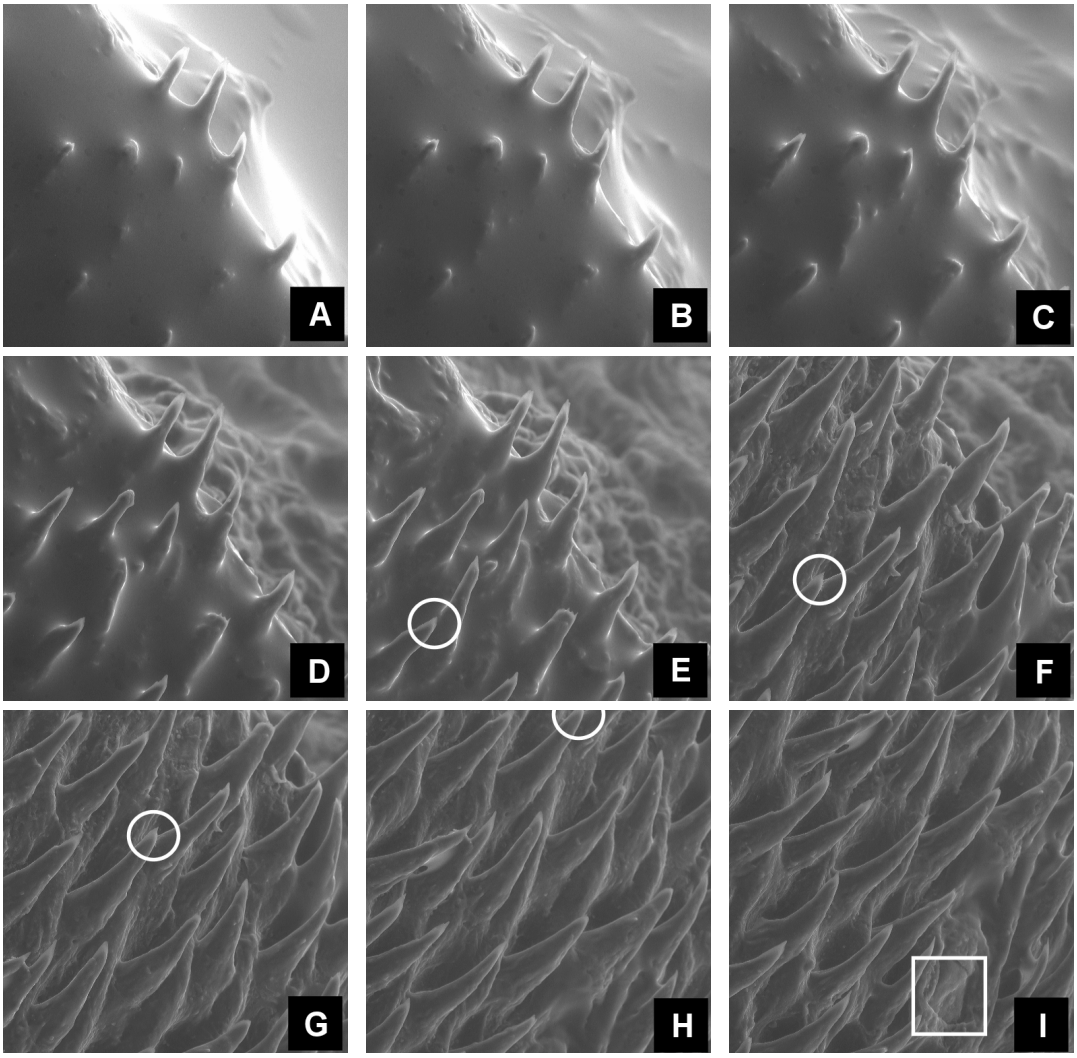

Figure 1. Siliform papillae on the rat tongue held at 2°C observed at the different water vapour pressure. A) 730 Pa, B) 715 Pa C-I) 708 Pa. Figures A-E were recorded in 5 minute intervals F-I) were recorded in 10 minute intervals. (Field of view 250μm, beam voltage 20 kV, probe current 100 pA, working distance 2.5 mm, bias of the electrode system 370V).

A very thin layer of water on the surface of siliform papillae and preservation of their natural undistorted shape demonstrate optimum conditions for the observation of the sample in Figure 1E. Further increase of the local sample temperature leads to evaporation of water from inside the sample. It is accompanied by slow sample shifts, which imply the dehydration of its volume and its stronger damage. These shifts are demonstrated by changing the position of the object marked with white circles in Figure 1E-I that were recorded in 10 min intervals. Figure 1I shows that the sample has shifted due to dehydration so much that the object marked with the white ring before is no longer visible; the shape of a taste-cell marked by a white square in this final figure and large inclination of papillae demonstrate that the damage of the sample is already significant. Undamaged, fully hydrated natural state of a taste-cell is characterized by a hemispherical shape without cracks.

4. Conclusion

Dynamical "in-situ" experiment focused on transition from hydration to dehydration state in the observation of biological sample by VP SEM underline the significance of thermodynamic stability close to the sample, considering thermal influence of the beam, temperature conductivity of specimen, temperature stability of cooled specimen holder etc. This experiment also shows physical conditions for evaporation of thin water layer from the sample surface and topographical changes of the sample influenced by dehydration.

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