AN APPROPRIATE METHOD FOR ASSESSING HYDROGEL PORE SIZES BY CRYO-SEM

[1]Katerina ADAMKOVA, [2]Monika TRUDICOVA, [1]Kamila HRUBANOVA, [2]Petr SEDLACEK, [1]Vladislav KRZYZANEK

[1]The Czech Academy of Sciences, Institute of Scientific Instruments, v. v. i., Brno, Czech Republic, EU, krzyzanek@isibrno.cz

[2]Brno university of technology, FCH, Brno, Czech Republic, EU, sedlacek-p@fch.vut.cz

Abstract

The aim of our work was to examine and describe ultrastructure of the agarose hydrogel and any possible structural concentration dependencies, and to assess the distribution and size of pores of agarose hydrogel in dependence on its concentration. Four concentrations were prepared (0.5 %, 1.0 %, 2.0 % and 4.0 % of dry weight content) and cryo-SEM and turbidimetry methods were executed on wet (original) samples in order to image the ultrastructure and measure the pore sizes within.

Reasonable results were obtained for the wet samples as they were closer to their native state they are usually used for applications in. Cryo-SEM and turbidimetry provided comparable results of pore diameters and allowed to compare pore diameters dependant on the concentrations; moreover, it showed more detailed and realistic structure.

Keywords: Hydrogel, agarose, cryo-SEM, freezing methods, image analysis

1. INTRODUCTION

Hydrogels are ubiquitous materials with three dimensional polymer network in which water is trapped due to its surface tension effect. Recently, there has been an increasing interest in hydrogels, since they can be used in a broad field of applications, e.g., human health and cosmetics (contact lenses, wound healing dressings, and artificial replacement tissues), drug delivery systems, bioengineering etc.

Structure differences between prepared hydrogel concentrations and its respective pore sizes were observed and analysed using freezing, freeze-fracture, freeze-etching, imaging in cryogenic scanning electron microscope (cryo-SEM) and image analysis. Simultaneously, it was necessary to find the best way to prepare hydrogel samples for freezing and the most suitable freezing method. Cryo-SEM imaging was applied because hydrogels, as their name implies, consist a large amount of water, and aqueous samples cannot be observed in high vacuum that is needed to be maintained in the SEM chamber without any preceding stabilization.

2. EXPERIMENTAL PART

2.1. Agarose hydrogel preparation

Agarose hydrogel was prepared and examined. Agarose is a hydrophilic [1] polysaccharide with gelling properties in solution that can be obtained by extraction from marine red algae. The agarose hydrogel is of high elasticity and comprises a thick [2] rigid [1] network of agarose chains with incorporated large water pores [2]. This polymer is similar to living tissue and extracellular matrix fluid, and therefore it is used for biophysical phenomena [1]. The agarose used within this experiment was Sigma Aldrich type II, medium EEO; CAS number 9012 36 6.

To prepare agarose hydrogel, the agarose powder was dissolved in deionized water; subsequently the mixture was stirred and heated for several minutes and left to cool down at the room temperature. There were four concentrations of this hydrogel prepared - 0.5 %, 1.0 %, 2.0 % and 4.0 %. Their density increased proportionally with the increase of the concentration.

2.2. Agarose hydrogel sample preparation and freezing

The agarose hydrogel samples were frozen by means of two freezing techniques that proved the most successful within preliminary testing. The techniques were: plunge freezing (technique based on immersing samples into a liquid cryogen [3, 4]) and high pressure freezing - HPF (hyperbaric freezing in hyperbaric device Leica EM HPM100 - the specimen “sandwiched” between two carriers is jet-freezed with liquid nitrogen under the pressure raised of 2100 bar, thus, ice crystal nucleation is limited [4]). The agarose hydrogel dries out when exposed to the air, which results in concomitant ultrastructure alterations, so there had to be fresh samples prepared prior each freezing process. Moreover, samples have to be frozen in very thin layers and very small volumes, otherwise they would not be frozen evenly - it would take too long for the deeper areas to get frozen and the ultrastructure would be damaged by the growth of ice crystals. The agarose solution density, however, increases rapidly at thin layers and small volumes at the room temperature (this process is called gelatinization), which makes the sample preparation more difficult.

For plunge freezing, small metal tubes filled with each concentration of the hydrogel were held in forceps and swiftly immersed in liquid nitrogen before the gelatinization took place. As for the HPF, the first attempt was to cut off slices using a scalpel and a special puncher. Due to their consistency, hydrogels are not easy to be sliced and the slices were too thick, however. In the end, it was decided to change the strategy completely and work with the mixture before it lost heat and thickened. From several alternative application methods that were tested, dropping a small amount of hot agarose hydrogel directly on the carrier by means of a pipette and covering it with a microscope slide until it thickened was chosen. The rapid density increase in small volumes had to be averted by quick manipulation with the pipette.

After having been frozen, freeze-fracture (scratching the surface superficially by a sharp blade at high vacuum and low temperature [3]) and freeze-etching (letting the surface ice sublime under the vacuum [3]) procedures were performed on all samples.

2.3. Agarose hydrogel imaging

All the frozen samples were imaged in the cryo-SEM FEI Magellan 400L at the temperature of -120 °C, then three steps of freeze-etching succeeded: the first and second steps meant to increase the temperature to -100 °C, and after it stabilized, decrease it back to -120 °C. Within the third step, the temperature was risen to -100 °C and lowered back to -120 °C after 15 minutes. Images were taken before the freeze-etching and after each step respectively.

The plunge frozen samples showed unwanted ice crystal growth effects altering the structure (e.g. enlarging the pores) - they are shown in the **Figure 1**. The most significant alterations are marked with red arrows - a) before and b) after the first step of freeze etching.

The **Figure 2** shows differences between the agarose hydrogel of the same concentration frozen using the plunging technique and the high pressure freezing a) and b) before the freeze etching, c) and d) after the third step of the etching. Again, structure alterations and enlarged perturbed pores can be seen in the plunge frozen samples images. The structure of high pressure frozen samples stays unaltered and undamaged, so the images could undergo further analysis as follows.

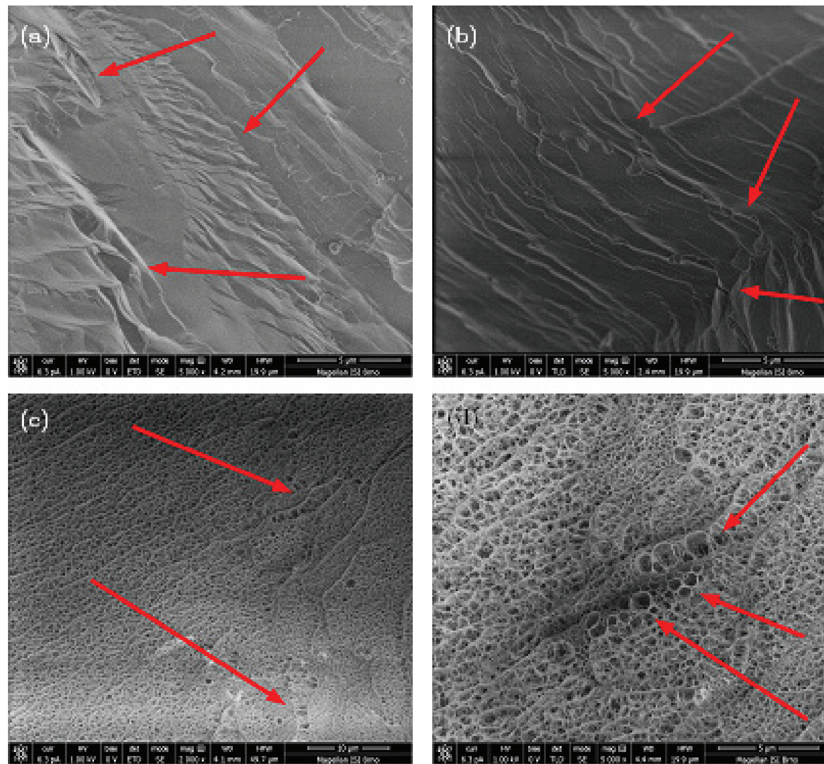

Figure 1 Unwanted structure alterations caused by ice crystal growth prior to the freeze etching (a, b) and after the first step of freeze etching (c, d) marked with red arrows

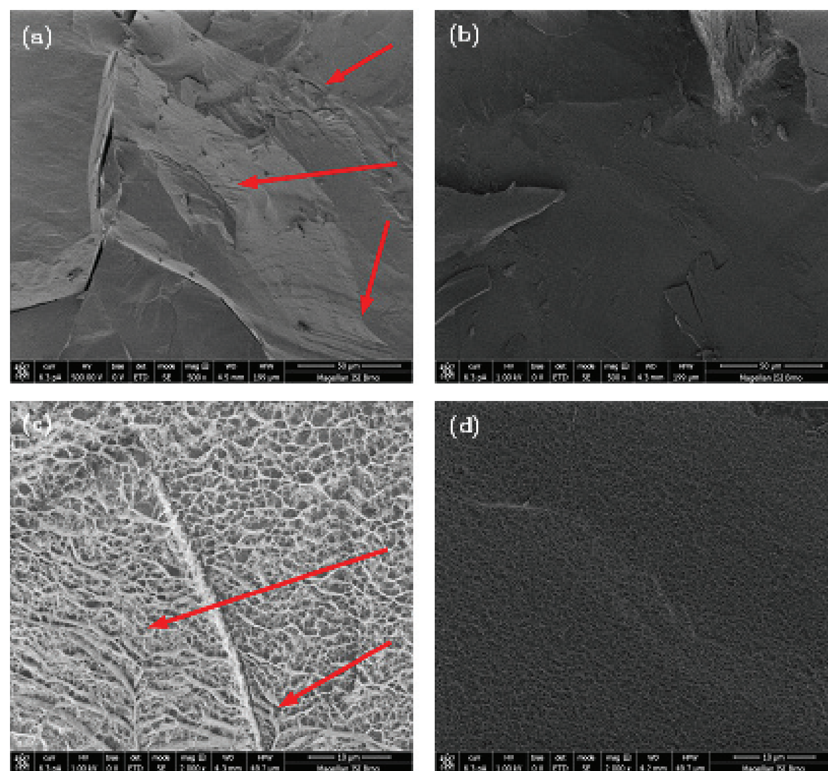

Figure 2 The comparison of agarose hydrogel of the same concentration frozen using the plunging technique (a, c) and the high pressure freezing (b, d), imaged before the freeze etching (a, b) and after the third step of the etching (c, d)

2.4. Image processing and analysis

Basically, the images obtained by the optimized cryo-SEM technique serve as a visual illustration of the qualitative characteristics of internal structure of the studied gel networks. Nevertheless, they can also be further processed in order to gain some quantitative structural parameters. For this purpose, we have applied two approaches of the image processing that are implemented in open-source scientific image processing toolbox ImageJ and that are proposed for the analysis of porous structures. Both analytical approaches were performed on the similar square sections (512×512 pixels) of the images recorded at the same level of magnification (see the first column in **Figure 3**). Before the very analysis, the processed images need to be converted from a grayscale to black & white projections with an effort to display only the uppermost layer of the 3D network structure. For this conversion, the image sections were thresholded using MaxEntropy algorithm. Subsequently, the actual analysis of the internal structure proceeded.

Firstly, the Particle analysis tool (an automatic particle segmentation algorithm implemented in ImageJ) was used to identify individual pores of the gel network. The outlines of the detected pores are shown in the third column in **Figure 3**. As a numerical result of this analysis, every pore which is detected in the structure is described by its area and perimeter. Wherever it is necessary to take care of the pores which are displayed in the binary picture touching one another, the Watershed algorithm can be used prior to the Particle analysis. This algorithm uses a density profile to determine if one object with a peninsula should actually be two objects. If it determines that they should, it will draw a line to separate them. From the Particle analysis, distribution of pore areas and perimeters is obtained and processed into statistical parameters, e.g. average or mean values.

Skeleton analysis represents another image processing technique, applicable in the morphological analysis of porous materials. In this technique, the network structure displayed in the analyzed image is skeletonized, i.e. replaced by the line skeleton using a topology-maintaining medial axis thinning algorithm (skeletonized representations of the analyzed images is shown in the second column in **Figure 3**). Using the Skeleton analysis tool, branches and junctions of such a skeleton are then classified, counted and measured. As an example of a quantitative parameter, useful in description of the porosity of the analyzed structure, average branch length is provided among the results of the Skeleton analysis.

Comparison of the quantitative results of the two image-analysis approaches is shown in **Figure 4**. For both methods, average value of a linear parameter which represents the pore size is provided as a function of concentration of agarose in the gel. In the case of Particle analysis, the average size of the pore was calculated from the average pore area (using the simplified assumption of the circular cross-section of the pores); from the Skeleton analysis, average branch length was used for the same purpose. It can be seen that results of both methods are in good agreement. Nevertheless, we propose the Skeleton analysis as a more suitable method because, unlike the Particle analysis approach, it does not suffer from the improper assumption of the circular pore shape.

Finally, the results of image analysis of cryo-SEM images were compared to the indirect pore size estimation by means of turbidimetry according to Aymard et al. [2]. In this approach, it is assumed that the pore size in agarose gels corresponds well with the correlation length, calculated from the wavelength dependence of the turbidity in spectral region of 800-900 nm. As can be seen in **Figure 4**, the absolute values of the correlation lengths as determined by turbidimetry are significantly lower (although of the same order of magnitude) than the linear parameters resulting from the cryo-SEM image analyses. Nevertheless, the decreasing trends of the correlation length with the agarose concentration is comparable with the concentration dependence if the cryo-SEM results.

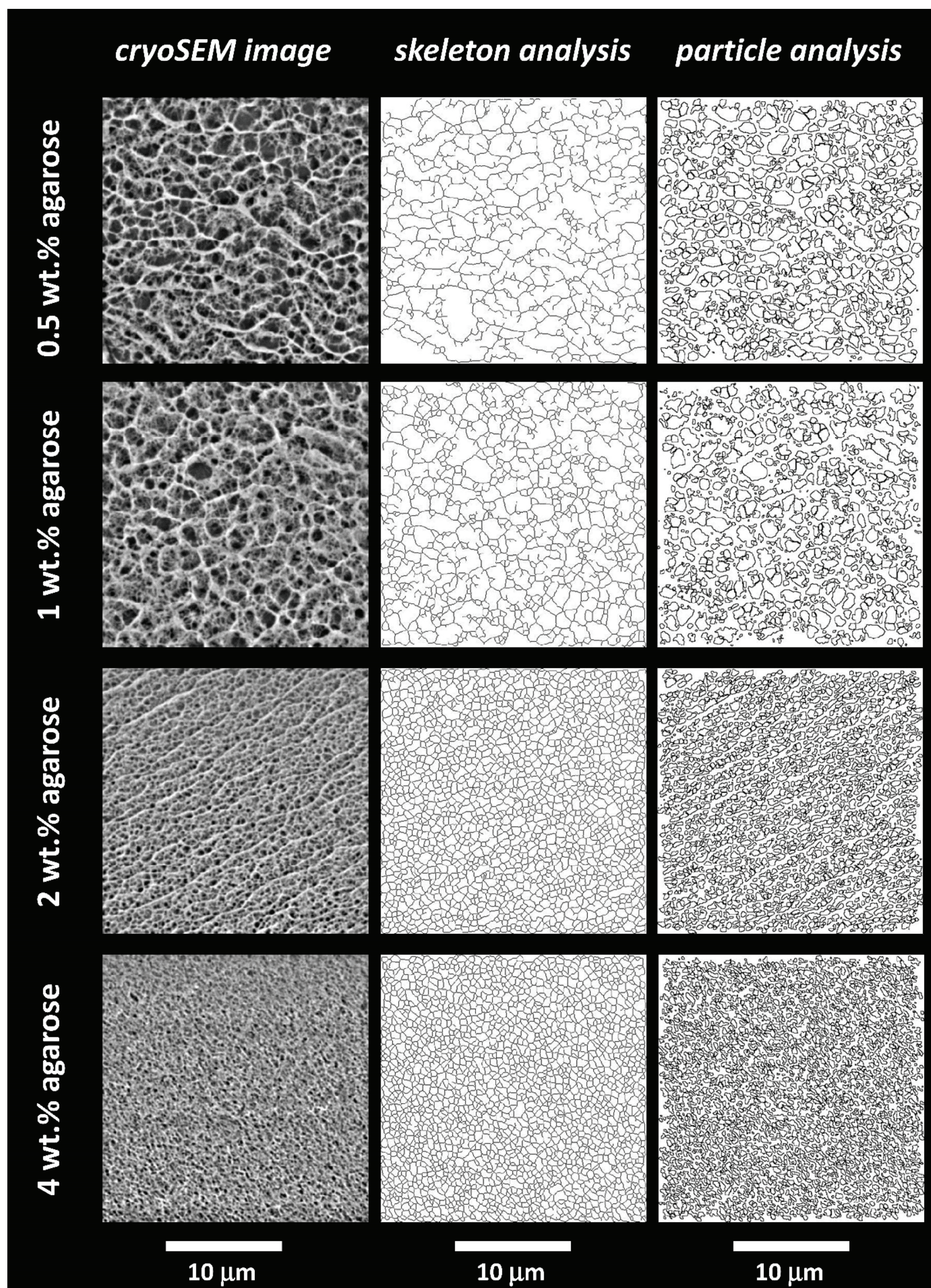

Figure 3 Image processing of the cryo-SEM images of various agarose gels. Sections of the micrographs which were subjected to further processing and analysis (left). Mask representation of the same sections processed for the Skeleton analysis (middle) and Particle analysis (right) in ImageJ.

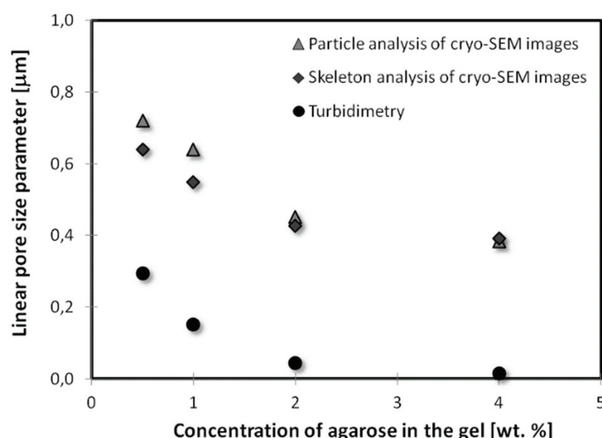

Figure 4 Linear parameters representing the average pore size of the agarose gels

3. CONCLUSION

Four concentrations of agarose hydrogel were prepared in order to observe and examine its structure and any concentration-dependant differences, as well as to perform image analysis. The samples were frozen by means of plunge-freezing and HPF. As for the plunge freezing, the hydrogel was filled into small metal tubes. For the HPF freezing, various sample preparation methods were tested because of rapid thickening of small volumes of the agarose hydrogel at the room temperature. From these preparation methods, only the direct application on the carrier was suitable for all the concentrations. All the frozen samples were then freeze fractured, freeze etched and imaged in the cryo-SEM. The plunge freezing technique proved insufficient, because ice crystal growth caused unwanted structure alterations and damage. HPF frozen samples showed no or minimum contamination, damage or alterations, so the structures of all four concentrations could be examined and compared. The cryo-SEM imaging provided a network like structures revealed by the progressive freeze etching. The agarose hydrogel of the lowest concentration was the most “airy” with the sparsest network and largest pores. The final output of this method, however, does not need to be mere image illustrating the structure - quantitative parameters can also be obtained, which was verified applying two approaches - particle analysis and skeleton analysis.

ACKNOWLEDGEMENTS

The research was supported by CSF (17-15451S), MEYS CR (LD15047 and LO1212), its infrastructure by MEYS CR and EC (CZ.1.05/2.1.00/01.0017).

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