In-situ cryo-SEM investigation of porous structure formation of chitosan sponges

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A B S T R A C T

Aiming at better understanding of porous structure formation unique in-situ experiments on freeze-drying the chitosan-based solvents were carried out by cryo-SEM method. It allowed us to visualize the lyophilization process and to follow the formation of the porous structure in the chitosan sponge. It was clearly shown that the chitosan solutions just after freezing possess an evident microstructure consisting of the walls of the future pores and of the frozen solvent filling the space between them. The diameter of the pores and their wall thickness are controlled by pre-freezing temperature. An incorporation of nano fillers (chitin nanofibrils and montmorillonite) into the chitosan solution did not affect the pore size and wall thickness. However, they can promote the formation of the layered structure of the pore walls.

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1. Introduction

It is well known that there are some polymers which are able to form sponges with porous structure [1–3]. Chitosan [4] being a partially deacetylated derivative of chitin is one of such polymers. Polymeric materials prepared in a spongy-form can be very useful in tissue engineering as scaffolds which could reinforce, replace and support some organs of a body. Such scaffolds are used for living cell proliferation and, therefore, must possess a number of very important properties like biocompatibility, biodegradability (if necessary), absence of cytotoxicity. Chitosan belongs to very perspective material for these purposes.

Nowadays, different techniques are developed to prepare sponge-like structure of polymeric scaffolds, namely, phase separation, electrospinning, freeze-drying, etc. The microstructure of the obtained scaffolds is very intensively investigated and described in literature [5–7]. It is found that the porosity and pore size distribution of the chitosan-based scaffolds prepared by freeze-drying method are very sensitive to preliminary cooling rate, pre-freezing temperature and solution concentration. The lower the pre-freezing temperature was used, the smaller pores can be obtained [1,5]. However, the opposite behavior was observed for cooling rate and chitosan concentration, i.e. decreasing of these parameters led to increased pore diameters and porosity [8]. It was also revealed that variation of the porous structure parameters effected water absorbance of the sponge [8], compressive strength and strain [7,9], tensile strength [1] and degradation percentage [5].

Nevertheless, the process of the porous structure formation is not still sufficiently studied and described in the literature. Therefore, the aim of the present work was in-situ investigation of the chitosan sponge structure formed during freeze-drying (lyophilization) with help of the cryo-chamber in a scanning electron microscope (cryo-SEM). Such in-situ experimental results have been never described before in the literature. The method of cryo-SEM is successfully used to study very sensitive and/or hydrated samples, which should be saved and investigated in original non-dried state: biological objects [10], food [11,12], different emulsions and solutions [13,14]. This technique allows one to visualize directly the wet microstructures of the specimens and obtain unique and very useful and valuable information.
2. Experimental part

The solutions of 4 wt% chitosan (Fluka Chemie, BioChemika line, MM = 255 kDa, a deacetylation degree of 80%, ash content of 0.5%) in 2% acetic acid were prepared. Additionally, two composite chitosan-based solutions with different nanofillers (chitin nanofibrils (CN) and montmorillonite (MMT)) were prepared as well. The concentrations of MMT and CN were varied in a range of 0.5–20% of chitosan weight.

Small drops of the chitosan-based solutions were very quickly frozen in liquid nitrogen slush at the temperature about –200 °C. Then these specimens were put inside the cryo-chamber PP 2000T (Quorum Technologies, England), broken there, transferred onto cold stage inside SEM (SUPRA 55 VP, Carl Zeiss, Germany) and examined at 2–5 kV and the temperature about –160 °C. Taking into account a high vacuum conditions inside SEM, it was possible to imitate the freeze-drying of the chitosan solution with following sponge structure formation.

Mean pore diameters and pore wall thicknesses were determined by image analysis (Scandium, ©OLYMPUS Soft Imaging Solutions) of SEM micrographs of the fully dried sponges. For this purpose SEM images of several areas perpendicular to the pore direction were captured. At least 50 pores and 70 pore walls were analyzed from different locations of the same sample.

3. Results

The presented here research work was conducted with the goal to follow the porous structure formation process in the chitosan-based solutions and to investigate the obtained sponges.

**Fig. 1** demonstrates the cryo-SEM images of the frozen chitosan solutions. It is clearly seen in **Fig. 1(a) and (c)** that the solutions just after freezing possess a well pronounced microstructure. This microstructure consists of the walls of the future pores (beige color (in the web version)) and of the frozen solvent (blue color (in the web version)) filling the space between them. Thus, even at the stage of cryo-freezing a phase separation of the homogeneous polymer solution occurs. Therefore, the porous structure is generated during phase separation. We suppose that the reason is the strong interaction between the chitosan macromolecules, which are semi-rigid and have a tendency to form the structured network inside the solution. It allows one to assume that the elements of this semi-rigid microstructure are displaced to the periphery of the solvent crystals during freezing. When the solvent removes, the formed polymeric walls surrounding the solvent regions must be strong enough for preventing pore collapse and, hence, for keeping the porous microstructure.

Taking into account that the cryo-SEM experiments are conducted under high-vacuum condition, a sublimation of the frozen solvent inside the cryo-chamber takes place. This implies that such experiments make it possible to simulate and to visualize in-situ a lyophilization process. Therefore, the presented here unique SEM images are very valuable. The arrows between the SEM images in **Fig. 1** enable to trace step-by-step the freeze-drying process and the appearance of pronounced pore structure of the fully dried sponge (**Fig. 1(b and d)**).

During the sublimation of the frozen solvent contained in the chitosan solution, the space originally occupied by the solvent becomes the pores in the obtained sponges. The size distribution of the pores is strongly dependent on the pre-freezing temperature.
and cooling rate. In Fig. 2 the SEM images of the pure chitosan solution pre-frozen in a refrigerator at −4 °C are presented for comparison. The pre-freezing temperatures and the cooling rates in the refrigerator and in liquid nitrogen slush are very different. So, we assumed to get a chitosan sponge microstructure, which would clearly differ from that prepared by pre-freezing in the nitrogen slush. This difference is evidently seen in Fig. 2. The pores become much larger in diameter and their walls are also much thicker. It can be explained by slower cooling inside the refrigerator that leads to appearance of less number of ice crystallizing nuclei and gives them an opportunity to grow up to rather big sizes. This observation is fully in agreement with the literature data [1,5].

Coming back to Fig. 1, one should notice that the lyophilization generates an interconnected porous microstructure and the pores are arranged in the channels running almost parallel to each other. It is a consequence of a heat transfer process during freezing. It was shown in Ref. [15] that the solution of chitosan in acetic acid being under the action of shear stress is capable of self-organization, i.e. the orientation of macromolecules along the force field. We assume that in the presence of a temperature gradient the similar orientation of the chitosan macromolecules occurs, leading to formation of a solution structure anisotropy and, as a result, to a birth of directed pore channels. However, this hypothesis requires further experimental verification, which we plan to hold in the future.

In Figs. 3 and 4 the SEM data obtained on the nanocomposites (chitosan with added chitin nanofibrils (CN) or MMT) are shown. To avoid an overload of the paper with numerous SEM images, we present here the pictures of only one concentration of each nanofiller. It is well seen that the structures of the chitosan+C and chitosan+MMT solutions are very similar to those of pure chitosan (see Fig. 1). It means that these nanofillers do not affect the generating microstructures of the composite sponges. The fillers are pushed out the solvent crystals as well as chitosan. However, the fine nanostructures of the formed pore walls differ in dependence of the nanofiller used. It is worthy to note that, in case of CN filler, the layered nanostructure of the pore walls is revealed (it is better seen in the SEM image of the fully dried sponge in Fig. 3(b)). Calculation of the interaction energy between the macromolecules of chitosan and CN using molecular dynamics simulations showed that the orientation of the chitosan occurs on the surface of chitin nanofibrils promoting the formation of a layered composite structure [15]. Such layered nanostructure is a characteristic feature of chitosan+C nanocomposite and it was observed before in the solution-cast fibers [15].

A mixing of the chitosan solution with MMT causes also similar layering of the sponge pore walls. Moreover, an incorporation of the exfoliated MMT nanoparticles into these walls makes their surface to become rough and relief (Fig. 4).

The analysis of the SEM images given in Figs. 3 and 4 shows that the pore microstructure and/or pore wall nanostructure depends dramatically on the nature of the added nanofillers.

As seen in Fig. 5, the mean pore size (diameter) and the mean wall thickness are significantly controlled by the pre-freezing temperature and cooling rate. This is in a good coincidence with the literature data [1,5]. As discussed above, pre-freezing at the higher temperature (i.e. −4 °C) leads to the formation of a smaller amount of the solvent crystal nuclei, which can grow up to rather big sizes. It is also evident that in the case of growing the big pores, their walls should be much thicker comparing to those frozen at
very low temperatures (i.e. $-200\,^\circ C$ in nitrogen slush).

If one compares the pore diameters and wall thicknesses of the pure chitosan and composite sponges with added CN and MMT, there is not a big difference between them (all values are in a range of statistical data spread).

4. Conclusions

In the present work we have successfully attempted to follow and to visualize in-situ the freeze-drying process and the formation of the chitosan-based sponges by cryo-SEM method. It is shown that the porous structure of the chitosan-based sponges obtained by freeze-drying is formed on the phase separation of the solution. During freezing the formation of the solvent crystallites occurs with simultaneous diffusion of the polymer towards crystal periphery; the following sublimation of the solvent leads to the formation of pores. Pore size is determined by the size of the solvent crystallites and, therefore, depends on the pre-freezing temperature and cooling rate. Introduction of the MMT nanoparticles and chitin nanofibers promotes the formation of the layered structure of the composite, however, does not significantly change the pore size and the wall thickness.

It is evidently demonstrated that cryo-SEM technique can be effectively used for in-situ investigations of sublimation/lyophilization processes.

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References


