ASSESSMENT OF THE MICROSTRUCTURE AND MECHANICAL PROPERTIES OF POROUS GELATIN SCAFFOLDS

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Abstract

Gelatin scaffolds are in the interest of tissue engineering and drug release. The scaffold porosity and microarchitecture are of great importance in proper tissue regeneration. In this work, the freeze-drying method was used to produce the scaffolds. The effect of concentration of the initial gelatin solution and pre-freezing temperature on the scaffold's microstructure and microarchitecture (porosity, pores size, shape, and distribution) was evaluated. The mechanical tests of samples were performed. Moreover, the influence of the gentamicin sulphate addition on the gelatin scaffolds microstructure and mechanical properties was also studied.

The linear relationship of porosity to the concentration of the initial solution was observed. Therefore, it is possible to obtain a scaffold with a planned porosity. Pores were interconnected with an aspect ratio between 1.5-1.8. For porosity 74 ± 9% the average pore size was 0.7 ± 0.6 mm, with most pores in the range 0.2-0.4 mm. For the samples with porosity 57 ± 14%, the average pore size was 0.2 ± 0.2 mm, with most pores in the range 0.05-0.2 mm. The process of pre-freezing the solution in liquid nitrogen caused the highest porosity of the sample, the smaller pores size and the lower pores size distribution in comparison to the sample pre-frozen in -20°C. The mechanical parameters for all the samples are sufficient for filling bone defects. The addition of a drug to gelatin caused only slight changes in the pore architecture. This material could be applied as a scaffold in the bone loss correlated to bacterial infection.

Keywords: *scaffold, microstructure, tissue engineering, gelatin, freeze-drying*

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Introduction

Porous scaffolds used in tissue engineering are fraught with many challenges. Designing biomaterials that meet the scaffolds requirements is very difficult. Choosing a proper method of scaffold production and designing its parameters is one of the key aspects affecting the final product [1-3]. The most popular techniques to fabricate gelatin scaffolds are electrospinning, phase separation, porogen leaching and self-assembly. Depending on the production method, properties, such as microstructure and mechanical strength may be modified. The lowest mechanical parameters are characteristic for woven biomaterials obtained in the electrospinning process. An extremely promising method of manufacturing porous substrates is the freeze-drying [4-6]. It avoids problems related to a solvent residue in the biomaterial and the use of high temperatures. Moreover, this method allows for introducing drugs or biological active agents to the scaffold [7].

The scaffold porosity and microarchitecture (pore size and shape, pore size distribution, pore connectivity) is of great importance in the cells adhesion and proliferation and in the proper tissue regeneration [8-10]. The trabecular bone porosity is about 60%. The largest frequency of pore size is in the range from 200 μm to 300 μm. The most optimal scaffold microarchitecture for trabecular bone regeneration is the porosity of about 60% and the pore size from 300 μm to 500 μm [11,12]. The choice of the method of scaffold manufacturing and also the selection of process parameters, such as the type and concentration of the solvent, the type and share of additives, temperature, allows to shape the microstructure for specific tissues. The porosity and the pores size have a big impact on the mechanical properties of scaffolds [12,13]. The high porosity and the presence of large pores negatively affect mechanical strength. Therefore, finding a compromise between the required porosity and the strength of scaffold plays a huge role in the design of porous substrates used in tissue engineering [8].

Gelatin is widely used in tissue engineering of bone, skin, cartilage and kidney [1,10,14,15]. It is a natural biopolymer derived from collagen hydrolysis. Gelatin scaffolds are biocompatible and polyampholyte, therefore they can induce the regeneration of tissue and organs. Moreover, this biopolymer may be modified by calcium phosphate ceramics or blended with synthetic polymers to improve mechanical properties and osteointegration [16]. Gelatin is also useful for controlled drug delivery. Most often, gelatin crosslinking is applied to slower drug release from samples [17]. Bacterial infections of bone tissue are a common problem after surgical operations and the inflammations result in bone defects. The most common drug used for bone healing is gentamicin sulphate [18]. Therefore, this study focuses on the possibility of gentamicin sulphate incorporation to the gelatin scaffold.

The aim of this work was to evaluate how the concentration of the initial gelatin solution influenced the microstructure of scaffolds obtained via a freeze-drying method. In this study, the freeze-drying method was selected due to the low temperature which allows for incorporation of biological substances to the scaffold (protein, drug).

Moreover, such a method does not require any additional substances such as porogen or solvent. The effect of pre-freezing temperature was also assessed. The scaffolds porosity, pores size, shape and distribution were analysed. The influence of the samples microstructure on their mechanical properties was also investigated. It was tested how gentamicin sulphate added to the gelatin solution affected the microstructure and pore microarchitecture. The gentamicin sulphate addition to the non-cross-linked gelatin structure was aimed to fasten the drug activity in the cases of bone losses often correlated to bacterial infection.

Materials and Methods

Sample preparation

Porous gelatin scaffolds were obtained by the freeze-drying method. Gelatin powder (bovine skin, type B, Sigma-Aldrich, bulk density 0.58 g/cm^3) was dissolved in distilled water in 3 different concentrations: **Sample 1** Cp = 11 wt%; **Sample 2** Cp = 14 wt%; **Sample 3** Cp = 20 wt%. The solutions were well mixed and then poured into a cell culture plate. 4 samples were obtained from each solution with the appropriate concentration. In the next step, they were placed in a freezer at about -20°C for 20 min. For comparison, one group of samples were frozen in liquid nitrogen (**Sample 1",** Cp = 11 wt%). Next, they were freeze-dried (Labconco freeze dryer). The process temperature was -60°C, the pressure was -0.09 mBar. The obtained samples were shaped as cylinders with a diameter of 12 mm and a length of 10 mm (FIG. 1). In the analogous way **Sample 4** with gentamicin sulphate (GS) was obtained. GS distributed in a small amount of water was added to the gelatin solution to obtain the final proportion of gelatin to water Cp = 11 wt%, and GS to gelatin 1 wt%.

Microstructure

The microstructure of scaffolds was assessed using a ZEISS stereoscopic microscope (StereoMicroscope, Zeiss). Porosity was determined using the point method as a probability of hit to the analyzed phase of point thrown randomly to the surface. The tests were performed using a 100-point grid (10 x 10) put to the surface 22 times. The average pore size D was calculated in a formula (1) where D1 is a longer diameter and D2 is a shorter diameter of pores. The aspect ratio K was calculated in the formula (2).

$$D = (D1+D2)/2 \quad (1)$$

$$K = D1/D2 \quad (2)$$

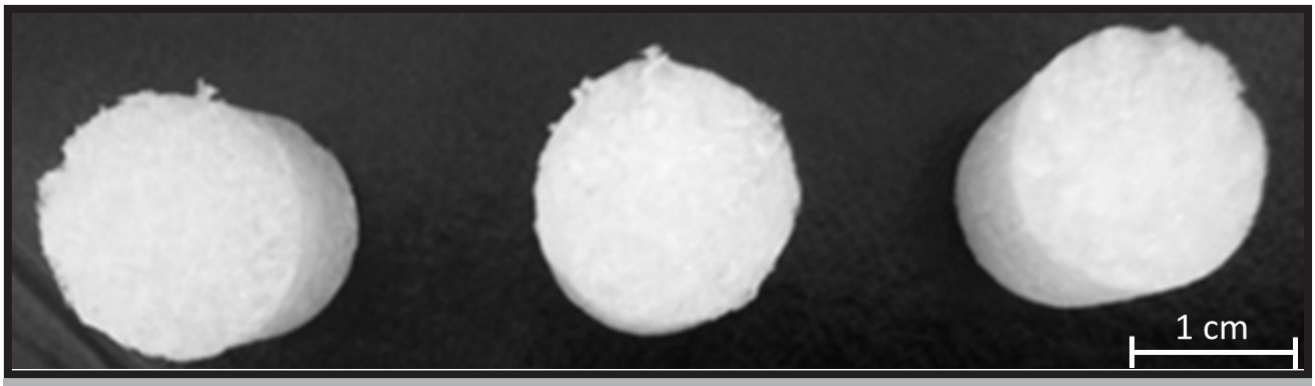

FIG. 1. Shape of the samples.

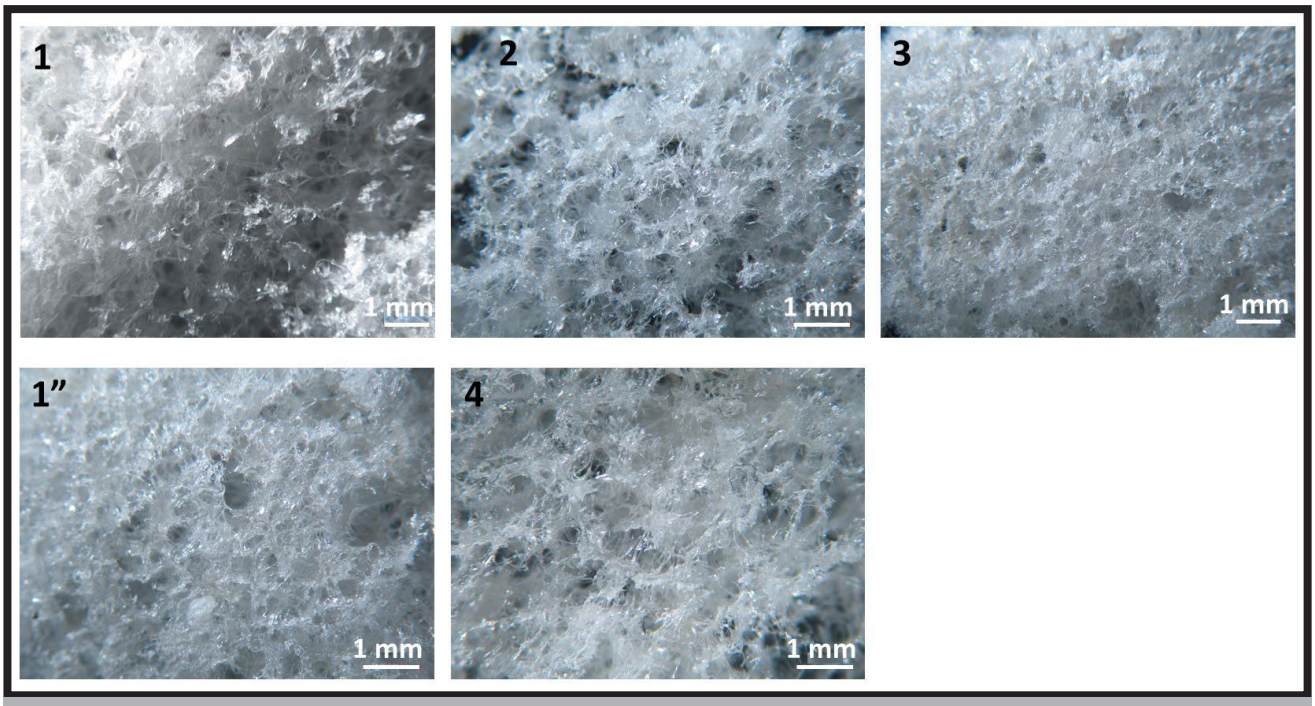

FIG. 2. SEM micrograph of the Samples 1 - 4 and 1".

Mechanical tests

Mechanical properties were measured in a compressive test by using a universal testing machine (Zwick 1435). The compression speed was 2 mm/min. The test was finished when the displacement reached 5 mm.

Statistical analysis

The statistical analysis was performed with Student's t-test (with confidence level 0.95). The data were expressed as the mean ± standard deviation.

Results and Discussion

FIG. 2 shows the microstructure of the obtained scaffolds. All the samples have irregular, connected pores. It is an important feature regarding tissue engineering as it allows for cells migration, and for the transport of the biological active agents. The pores sizes and aspect ratio of pores for all the samples are presented in TABLE 1. The measured values of the longer and the shorter diameter of pores confirm their irregular, elongated shape. This is also indicated by the aspect ratio, which is appr. between 1.6-1.8. It is also clearly visible that the average pores size is strongly influenced by the concentration of initial solutions. The largest average pore size is observed for Sample 1 (0.747 mm), for Sample 2 the pore size is equal to about 0.318 mm, and for Sample 3 the smallest pore size is measured (0.229 mm). The histograms show the larger dispersion of average pore size for Sample 1 (FIG. 3). The most pores are in the range of 0.2-0.4 mm, but there are also pores above 1 mm in size. Sample 2 contains less varied pores. The most pores are in the range of 0.2-0.3 mm, however, the large frequency of pore size is also in the range from 0.075-0.5 mm. For Sample 3 the small dispersion of pore size is visible, and the most pores are in the size range of 0.05-0.2 mm. The largest pores are present only individually. The characteristic of obtained scaffolds is similar to the microstructure of natural tissue, especially to bone tissue [8].

The analysis of the microstructure of obtained scaffolds shows that porosity is highly dependent on polymer concentration. The real and theoretical porosity of scaffolds are presented in TABLE 2. The theoretical porosity was calculated from the volume ratio of water used for preparing the gelatin solution. The measured porosity or Samples 1, 2 and 3 is appropriately: 74, 68, 57%. As assumed, the lowest concentration of polymer (Sample 1) has the highest porosity, and the highest concentration of polymer (Sample 3) has the lowest porosity. What is important, there are no statistically significant differences between the theoretical and the measured porosity. A large standard deviation of porosity is visible. It results from the high variation in pore size and the uneven distribution of pores in the structure of the scaffolds. The linear relationship of porosity to the concentration of the initial solution allows to determine what concentration should be used to obtain a scaffold with the planned porosity (FIG. 4).

TABLE 1. Pore size and shape of the samples.

Pore size and shape	Longer diameter D1 [mm]	Shorter diameter D2 [mm]	Average pore size D [mm]	Aspect ratio K
Sample 1	0.940 ± 0.778	0.554 ± 0.428	0.747 ± 0.595	1.744 ± 0.404
Sample 2	0.381 ± 0.251	0.254 ± 0.152	0.318 ± 0.198	1.493 ± 0.308
Sample 3	0.293 ± 0.277	0.179 ± 0.144	0.229 ± 0.204	1.661 ± 0.542
Sample 1"	0.342 ± 0.259	0.228 ± 0.148	0.293 ± 0.158	1.552 ± 0.249
Sample 4	0.872 ± 0.522	0.487 ± 0.334	0.652 ± 0.455	1.79 ± 0.401

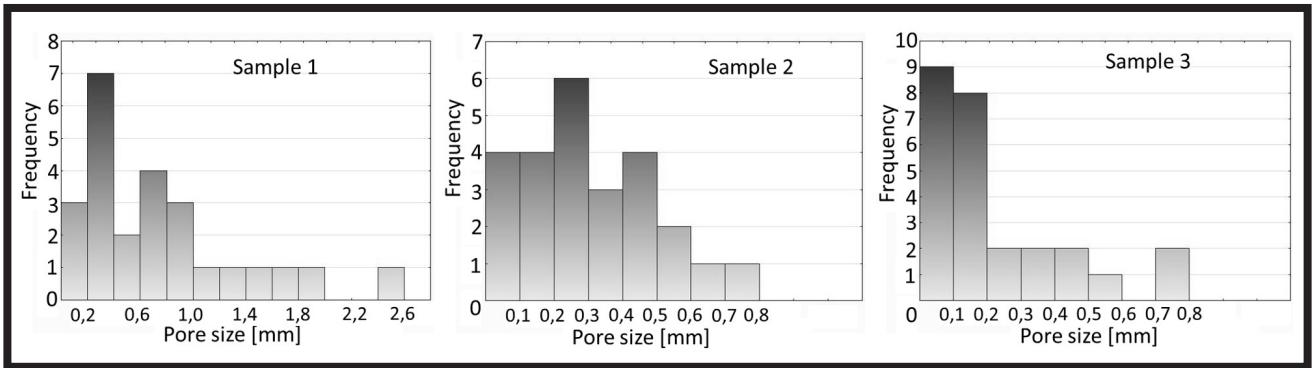

FIG. 3. Frequency of the pore size in a particular range for samples 1, 2 and 3.

TABLE 2. Porosity of the obtained samples in comparison with theoretical values.

	Sample 1	Sample 2	Sample 3	Sample 1"	Sample 4
Measured porosity [%]	74 ± 9	68 ± 11	57 ± 14	78 ± 5	73 ± 10
Theoretical porosity [%]	82	78	70	82	82

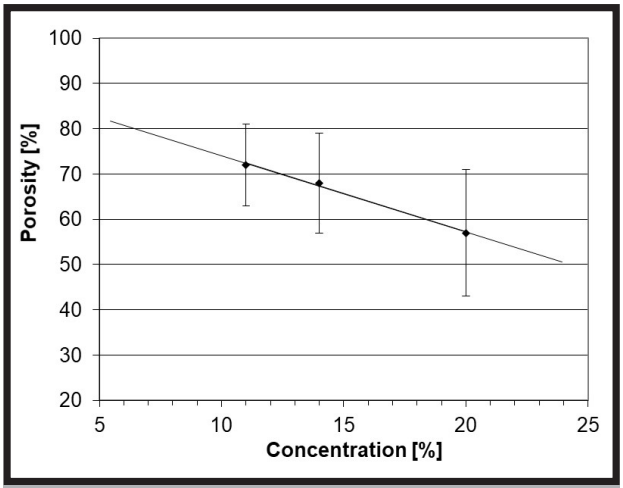

FIG. 4. The relation of porosity from the concentration of initial gelatin solution.

For comparison, the results for Sample 1" frozen in liquid nitrogen before freeze-drying are presented. In this case the pores are significantly smaller than for Sample 1 (average pore size for Sample 1" is 0.293 ± 0.158 mm and for sample 1 is 0.747 ± 0.595 mm). Moreover, the standard deviation is also lower, and it indicates that the pores size is less diverse. The porosity of Sample 1" is more adequate to theoretical value than for Sample 1. The largest frequency of pore size is in the range from 0.1 mm to 0.3 mm (FIG. 5). This manner of sample preparation i.e. freezing in liquid nitrogen can be used when the scaffold should be applied for smaller cells than bone cells.

The addition of the drug to gelatin scaffold caused a slight reduction in the size of pores, however, the changes are in the error range (TABLE 1). The histogram of Sample 4 is also different when compared to Sample 1 (FIG. 5). Dispersion of results is lower, the most pores are in the range 0.2-0.3 mm, however the high frequency of pore size is also in the range 0.1-0.7 mm. The total porosity of Sample 4 is not changed (TABLE 2).

The mechanical parameters of the obtained samples were investigated in the compression test (TABLE 3). This study shows a close dependence of mechanical parameters on the porosity of the samples. The lowest Young's modulus is observed for Sample 1 with the highest porosity. Moreover, in this case, the lowest force is required (103 N) to cause a 10% deformation. For comparison, such a deformation of Sample 3 requires a force of 156 N. The compression stress at 50% strain was also evaluated, and the values for all the samples equalled 3.01-3.4 MPa. The highest value of this parameter (3.4 MPa) was observed for Sample 3 characterized by the lowest porosity. The measured forces are sufficient for the resulting substrates to act as a scaffold for bone tissue regeneration [19-21]. Scaffolds obtained from natural polymers are usually characterized by poor mechanical properties, and the compression strength very often does not exceed 1 MPa [22]. Apart from total porosity, the shape and orientation of pores also affect the mechanical properties of scaffolds. Arora et al. reported maximum mechanical properties for aligned pores [23]. Vetrik et al. observed the greater compressive strength for more complex morphological architecture [24]. Roosa et al. described that the matching the pore size to the cell dimension provided better mechanical strength after implantation due to the initial pores filling with cells [25].

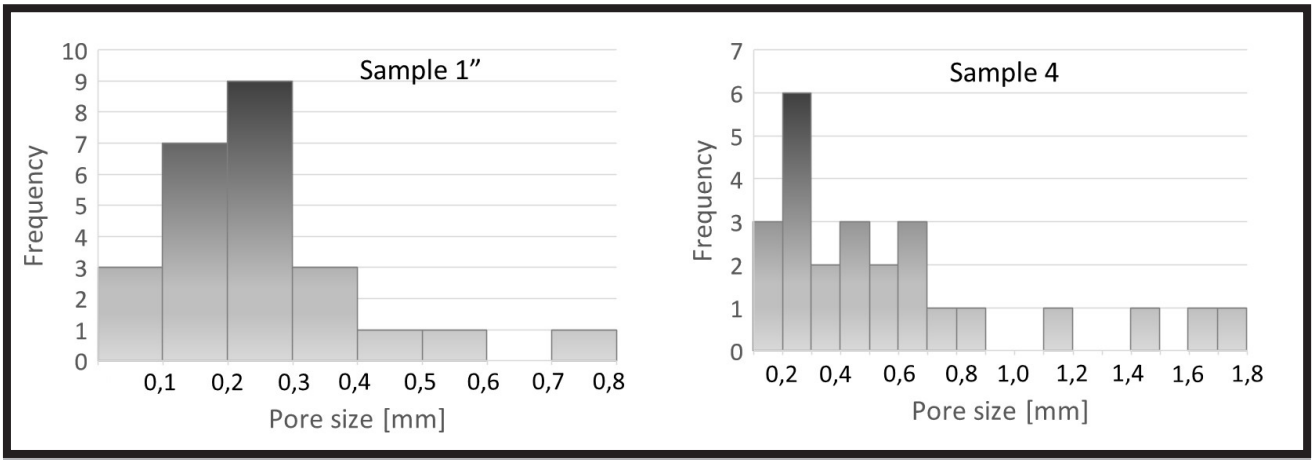

FIG. 5. Frequency of pore size in particular range for Samples 1" and 4.

TABLE 3. Mechanical properties of scaffolds.

	Sample 1	Sample 2	Sample 3	Sample 1"	Sample 4
Young's modulus E [GPa]	0.05 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.08 ± 0.01
Compression strength $\sigma_{(\varepsilon = 50\%)}$ [MPa]	3.01 ± 0.15	3.16 ± 0.42	3.40 ± 0.39	3.13 ± 0.17	3.09 ± 0.20
Force at 10% strain [N]	103 ± 4	123 ± 10	156 ± 6	121 ± 5	115 ± 7

Sample 1" which was obtained by pre-freezing in liquid nitride is characterized by higher mechanical properties when compared with Sample 1. It can be related with the presence of smaller pores in the microstructure and their more homogenous distribution. Moreover, the addition of gentamicin sulphate also improves the mechanical properties. In this case, the force causing 10% deformation is higher as compared to Sample 1, moreover elasticity of Sample 4 is lower. This is important during the initial stage after implantation. However, as gelatin is no crosslinked, the rapid drug release and fast gelatin degradation can be expected, and in consequence the fast mechanical parameters decrease. In this stage of the study, a rapid antibacterial action of the scaffold was planned.

Conclusions

Freeze-drying is a very useful method for manufacturing scaffolds with additives (e.g. drug) for tissue regeneration. Changing the initial polymer solution and pre-freezing of the samples makes it possible to create the desired microstructure and to precisely control the microstructure parameters. The linear relationship of porosity to the concentration of the initial solution is observed. Such a dependence allows to determine what concentration should be used to obtain a scaffold with planned porosity. For all the samples, the pores are interconnected, with longitudinal shape, aspect ratio between 1.5-1.8.

The lower initial gelatin solution concentration caused higher porosity (74 ± 9%) and the bigger pores size (average pore size 0.7 ± 0.6 mm, most pores in the range 0.2-0.4 mm). For the samples with a higher solution concentration porosity was 57 ± 14%, with an average pore size 0.2 ± 0.2 mm and most pores in the range 0.05-0.2 mm.

The pre-freezing of the solution in liquid nitrogen caused the highest porosity of sample, the smaller pores size and the smaller pores size distribution in comparison to the sample pre-frozen in -20°C.

The obtained samples microstructure (especially for Sample 1) is adequate for bone tissue regeneration. The mechanical parameters for all the samples are sufficient for this application.

The addition of drug to gelatin caused only slight changes in the pore architecture, and the observed changes were within the error margin. However, the addition of gentamicin sulphate improved the mechanical properties of the scaffolds.

The next step of the studies will be investigating a drug release profile and assessing the influence of gelatin crosslinking on the drug release speed.

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