IN VIVO BONE TISSUE ENGINEERING: CHARACTERIZATION AND OSTEOINDUCTIVE ASSESSMENT OF BIOLOGICAL SCAFFOLDS WITH ENDOGENOUS STEM CELLS

C. Katrilaka[1,6], A. N. Tzavellas[2], N. Karipidou[1,6], S. Moirotsos[1], M. Sachinidis[1], N. Michailidis[3,6], A. Cheva[4], E. Tsiridis[2], T. Choli-Papadopoulou[5] & A. Aggeli[1,6,*]

[1]Department of Chemical Engineering, Aristotle University of Thessaloniki, Thessaloniki, Greece

[2]3rd Department of Orthopedics, Aristotle University of Thessaloniki, Thessaloniki, Greece

[3]Department of Mechanical Engineering, Aristotle University of Thessaloniki, Thessaloniki, Greece

[4]Department of Pathology, Aristotle University of Thessaloniki, Thessaloniki, Greece

[5]School of Chemistry, Aristotle University of Thessaloniki, Thessaloniki, Greece

[6]Centre for Research & Development of Advanced Materials (CERDAM), Center for Interdisciplinary Research and Innovation, Balkan Center, Thessaloniki, Greece

*(*aggeli@auth.gr)*

ABSTRACT

Biomedical engineering is an emerging discipline that is defined by the simultaneous application of engineering, biological and medical principles, processes and technologies for the design and fabrication of medical devices, in order to address real-world biological and medical challenges. One of these challenges is tissue regeneration, and specifically for this study, bone tissue regeneration. These medical devices that are intended to be used for bone tissue regeneration in vivo, known as scaffolds, must provide a sufficient three-dimensional micro-environment for cell migration, attachment, proliferation and viability, while they maintain the structure of the regenerated tissue. The fabrication of these devices is still challenging. Freeze drying is a widely used process for the development of medical devices in the form of collagen-based, sponge-like implants, that fulfil/match all the above-mentioned properties, but they face major drawbacks. First, thorough characterization of these types of scaffolds is not common and second, scaffolds alone cannot induce tissue regeneration, therefore, functional molecules, like growth factors, should be co-delivered. The aim of this interdisciplinary study is firstly to fully characterize the novel collagen-based carrier, which is fabricated in our lab, and then to compare its osteoinductive abilities with those of commercially available products in vivo. The fabricated scaffolds undergo microscopic analysis in order to obtain pore size and architecture. Furthermore, they are subject to water retention, degradation and thermogravimetric analysis tests, in order to assess their physicochemical properties, as all of the above are decisive factors for cell adhesion and proliferation. Their good osteoinductive abilities in conjunction with their physicochemical properties, make these scaffolds interesting both as model systems and for commercial applications in bone tissue regeneration.

KEYWORDS: in vivo tissue engineering, bone regeneration, collagen-based scaffold, stem cells

INTRODUCTION

Biomedical engineering combines engineering, biology, and medicine, collaborating with healthcare professionals to develop innovative solutions for various medical challenge [1]. Research in biomedical engineering focuses on medical devices, such as scaffolds, which can be used for tissue regeneration. Especially, hard tissue engineering can be applied to bone defects caused by operations, fractures, and bone diseases. Scaffolds are required to be capable of osteoconduction, osteoinduction, and osteogenesis in vivo [2]. Moreover, in order to confirm these requirements are met, it is necessary to evaluate the structure of the scaffolds, as well as their physicochemical

properties [3],[5]. In this study freeze dried collagen scaffolds undergo microscopic analysis in order to obtain pore size and architecture. Furthermore, they are subject to water retention, degradation and thermogravimetric analysis tests, in order to assess their physicochemical properties, as all of the above are decisive factors for cell adhesion and proliferation.

Firstly, water retention influences the ability of the scaffold to maintain a hydrated environment, which is crucial for proper cell function and nutrient transport. Secondly, the degradation rate of the scaffold refers to the rate at which it decomposes over time. If the scaffold degrades too quickly, it may not provide sufficient structural support for cell attachment and proliferation. On the other hand, if it degrades too slowly, it might impede the natural remodelling of the tissue.

Additionally, tissue development is influenced by the inner structure of the scaffold, like pore size, curvature, shape, and porosity. According to bibliography, cells have been shown to prefer concave surfaces of high curvature, while convex surfaces are less favourable, and in addition to that scaffolds with pore sizes equal or greater than 300μm are preferred, especially for in vivo applications [4]. Optical microscopy is a practical, effective, and low-cost method to study the geometry of micro-scale scaffolds.

Finally, by using the technique of Thermogravimetric Analysis (TGA) the thermal stability and composition of materials as a function of temperature are examined. In other words, this method provides insights into the thermal stability, degradation profiles, and composition of the scaffolds, and therefore contributes to a better understanding of their behaviour under various temperature conditions.

METHODOLOGY

Water retention

A piece of dry scaffold was weighed and recorded as W_{dry}. The sample is placed in a vial and submerged in a regulatory solution to simulate conditions within the blood and left to soak. Subsequently, the scaffold is removed from the liquid and excess of fluid is removed. Finally, the wet scaffold is weighed and recorded as W_{wet}. The water retention is calculated according to the equation [6].

$$W_r=(W_{wet}-W_{dry})/W_{dry} *100\ \% \quad (1)$$

Degradation

The degradation rate of the scaffold was evaluated by measuring the change in sample weight over time under conditions simulating physiological conditions. The initial (dry) weight of the samples is defined as W_0, and the samples were immersed in appropriate solution. The samples were removed regularly from the degradation medium, dried, weighed and recorded as W_t. The degradation rate, D, is calculated according to the equation [6]

$$D=(W_0-W_t)/W_t *100\% \quad (2)$$

Microscopy

The scaffolds were imaged using optical microscopy under transmitted illumination to quantify pore size distribution. The scaffolds are cut into thin, flat slices using a surgical blade. The reason why this process is needed is because the depth of field of an optical instrument becomes very narrow in high magnifications. Image analysis was then carried out and the data that was extracted contained: surface area, circularity, axes of the best fitting ellipse, and roundness, among others. Pore diameter was calculated using surface area, by assuming circular shape, according to the equation (3)

$$r = \sqrt{A/\pi} \quad (3)$$

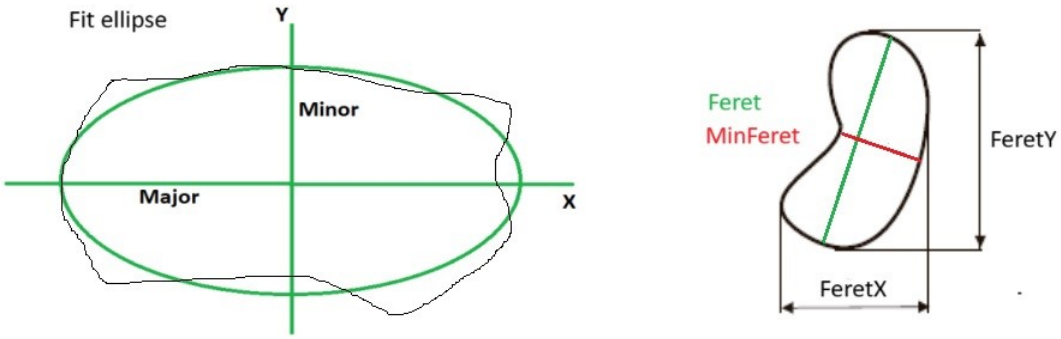

FIgure 1. *Geometrical features measured by image analysis. Circularity gives a measure of the shape's likeness to a perfect circle and it is measured as:* $Circ. = \frac{4 \cdot \pi \cdot Area}{Perimeter^2}$. *Major axis, minor axis,* $AR = \frac{Major}{Minor}$ *and* $Roundness = \frac{1}{AR}$ *apply for the best fitting ellipse that is calculated. FeretX and FeretY represent an object's diameter along the axes X and Y, while Feret is the the longest distance between two points, and MinFeret is the minimum caliper diameter.*

Thermogravimetric Analysis

Thermogravimetric analysis (TGA) was performed using a Discovery SDT 650 thermogravimetric analyzer of *TA Instruments | Waters* from 25 to 275 °C in a nitrogen environment. Samples weighing between 5 and 10 mg were examined in an alumina pan with a heating rate of 10 °C/min [7].

RESULTS AND DISCUSSION

Water retention

In the context of this study, the variation of water retention was studied in relation to the percentage of dry scaffold mass of the sponge, as well as the immersion time of the sponge in solution. Following the experimental procedure mentioned above, the following diagrams are obtained.

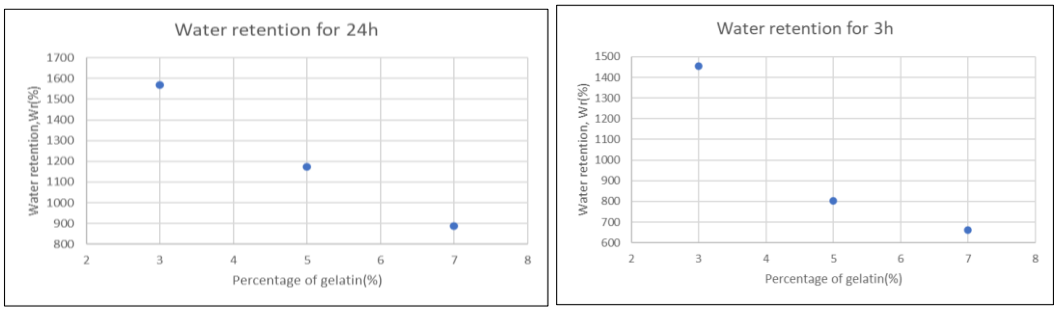

Figure 2. *Effect of the concentration of the scaffold on water retention ratio*

In the first diagram, the correlation between water retention and the percentage of gelatine is shown over 24 hours immersion, while in the second diagram, it is shown for 3 hours. In the first few hours of immersion, the sponge might quickly absorb water, saturating its outer layers. After a certain point, the sponge may reach a saturation level where further absorption slows down and water absorption now depends on diffusion through the sponge material to reach deeper layers over time, for this reason, there is a difference in the value of water retention between 3 and 24 hours. From both diagrams, it appears that as the percentage of gelatine increases, water retention

decreases. This occurs because the less gelatine the sponge is made of, the more "empty" space there is available for water absorption.
Furthermore, the alteration in water retention over immersion time (6,12,24,48h) is studied for a constant gelatine concentration and the following diagram is obtained:

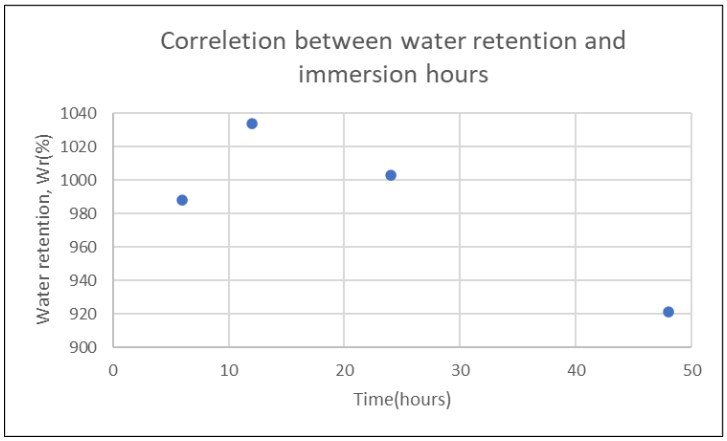

Figure 3. *Impact of immersion time on water retention ratio*

Within the first 24 hours, there is a small range of variation in water retention. The presence of the peak in 12 hours may be attributed to experimental error or by the swelling of the sponge absorbing water. This swelling can change the sponge's structure, making it more difficult for water to penetrate deeper layers, explaining the decrease of water. Also, in 48 hours degradation has already started, which is likely why water retention has decreased.

Degradation

In the following diagram the average degradation rate of the scaffold over time is displayed.

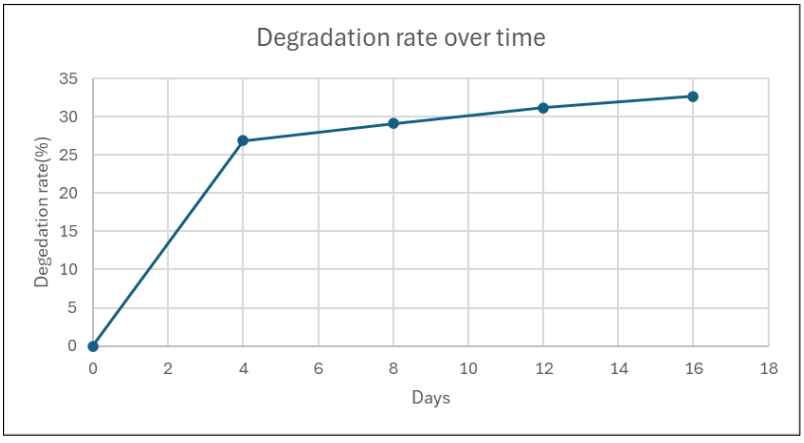

Figure 4. *Degradation of scaffold over time*

In the initial 4-day period, there is a fast degradation, which subsequently slows down. One possible explanation to the above-mentioned observation is that when the sponge is immersed in solution, due to its hydrophilic nature it absorbs water and swells This swelling can lead to increased mobility of polymer chains within the gel matrix, facilitating the diffusion of water molecules and degradation into the gel structure. According to [8, 9], swelling happens the first hours and after that the sponge remains in a steady state, where it can't be enlarged any more, and hence the degradation rate decreases.

Microscopy

In this section, three different types of sponges were tested under the microscope, one was the

control, and two other which were further stabilised. For every sample, 2-4 slices were obtained. Pore sizes were quantified by assuming a circular pore shape. Representative photos of the samples are given below (Figure 5).

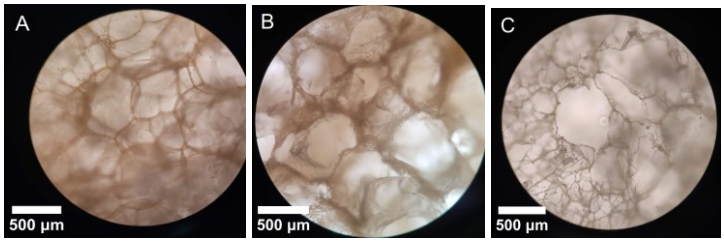

Figure 5. *Microscope photos of a scaffold section (A) Control, (B) with a Higher crosslinker concentration and (C) with Higher crosslinking time*

Pore size distributions for every scaffold group is represented in the following chart in Figure 6:

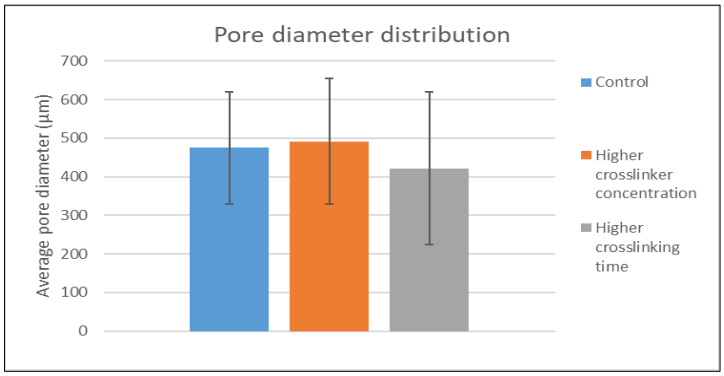

Figure 6. *Pore diameters for every scaffold group. Column heights represent average pore diameters, and error bars represent standard deviations*

These preliminary results suggest that the pore size does not seem to be greatly affected by the stabilisation method. This is an expected result since the scaffold concentration remains constant here, and this is the main parameter that is known to affect pore size distribution. In addition other geometrical features, like porosity and pore curvature are also under investigation.

Thermogravimetric Analysis

Two of the above-mentioned types of sponges were analyzed in the Discovery SDT 650 thermogravimetric analyzer. The results are presented in the following diagram in figure 7.

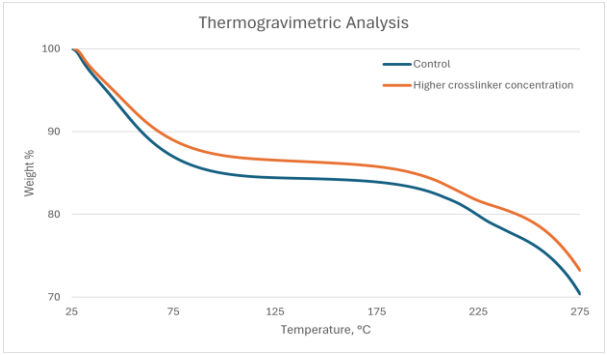

Figure 7. *Graphic representation of Thermogravimetric Analysis. % of Weight loss over Temperature (°C) increase*.

As it is expected, for a temperature rise from 25°C to 100°C there is a drop of about 10-15% of sponges initial weight, as the remaining free water of each sponge evaporates. The slight difference between the two types of sponges is probably due to the fact that the higher crosslinker concentration results in higher and stronger bonds between gelatin strains, and therefore results also to a slower release of the free water. Lastly, gelatin's degradation seems to start at a temperature of approximately 250°C, where the sponge undergoes endothermic reactions of hydrolysis and oxidation.

Their good osteoinductive abilities in conjunction with their physicochemical properties, make these scaffolds interesting both as model systems and for commercial applications in bone tissue regeneration.

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