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Additional Information

An “in vitro” experimental model to predict the mechanical behaviour of macroporous scaffolds implanted in articular cartilage.

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Abstract

A model is proposed to assess mechanical behaviour of tissue engineering scaffolds and predict their performance “in vivo” during tissue regeneration. To simulate the growth of tissue inside the pores of the scaffold, the scaffold is swollen with a Poly (Vinyl alcohol) solution and subjected to repeated freezing and thawing cycles. In this way the Poly (Vinyl alcohol) becomes a gel whose stiffness increases with the number of freezing and thawing cycles. Mechanical properties of the construct immersed in water are shown to be determined, in large extent, by the water mobility constraints imposed by the gel filling the pores. This is similar to the way that water mobility determines mechanical properties of highly hydrated tissues, such as articular cartilage. As a consequence, the apparent elastic modulus of the scaffold in compression tests is much higher than those of the empty scaffold or the gel. Thus this experimental model allows assessing fatigue behaviour of the scaffolds under long-term dynamic loading in a realistic way, without recourse to animal experimentation.

Key words: Polycaprolactone, Poly (Vinyl Alcohol), Freezing and thawing, Mechanical properties

1. Introduction

Langer and Vacanti started the emerging field of tissue engineering, applying biology and engineering principles to provide functional solutions to repair damaged tissue[1].Tissue engineering techniques aims to regenerate damaged tissues or organs by transplanting previously seeded cells in a bioresorbable polymeric material. Current tissue engineering strategies uses three-dimensional porous scaffolds or gels that can be of natural or synthetic origin. Depending of the type of tissue to regenerate the scaffold structure and properties very greatly, so also the cell source. The scaffold should be designed to offer an adequate biomechanical environment for cells and newly formed tissue. One of the main objectives when designing a scaffold is to obtain a structure that diminishes the differences in its stress-strain response with respect to the neighboring tissue at the site of the defect[2]. At the same time, the scaffold must transmit appropriate mechanical signals to the cells, which then translate mechanical stimuli to stimulate extra-cellular matrix production[3]. It has been demonstrated that static compression of cartilage explants decreases extra-cellular matrix production while dynamic compression of scaffolds, where mature chondrocytes are seeded, increases the amount of glycosaminoglycans (GAGs) produced. This effect depends of many parameters, such as the strain or frequency of the load applied[4-6]. It seems that various mechanisms are triggered and many factors play important roles in regulating the cartilage metabolism during compression of the tissue. One of the factors that could be of great importance in regulating the chondrocyte metabolism is the fluid flow and hence, the permeability of the scaffold, which would have a large influence in both a 3D culture and “in vivo”[7]. The permeability of the scaffold is important because nutrients and waste products must be transported with the fluid through the scaffold, and cells must be able to migrate through the pores. Accordingly, it is the scaffold’s 3D structure and interconnectivity that determine the permeability of the construct. Porosity, permeability and mechanical strength are closely connected and important considerations in the design and fabrication of the scaffold. In this work we focus on the mechanical behaviour of the scaffold. However, the scaffold does not sustain all the stress alone; “in vivo” the pore structure is being filled

progressively with a tissue that contributes to the mechanical performance of the implant. It is difficult to predict the behaviour of the scaffold “in vivo”. It is also difficult to measure the mechanical properties after the sacrifice in animal models. The mechanical assays performed in the laboratory to characterize the empty scaffold are not representative of the situation “in vivo”. The aim of this work is to develop a model that is able to predict the mechanical performance throughout the lifetime of a macroporous scaffold implanted in a cartilage defect. The model will thus minimize the recourse to animal models in the development of new scaffolding materials for cartilage engineering. This experimental model simulates the growing cartilaginous tissue by a Poly (Vinyl alcohol) gel, and its stiffness is being adjusted by successive freezing and thawing cycles.

Polycaprolactone (PCL) is a hydrophobic semicrystalline polymer with a total degradation time of 2-4 years “in vivo”. This group has previously proposed a procedure to produce PCL or Polylactide scaffolds that combines freeze extraction and the use of porogen microparticles[8-12]. These scaffolds will have a pore architecture characterized by large interconnected spherical pores with microporous pore walls. The porosity of the scaffolds can thus be above 80% and the amount of degradation products that must be metabolized during the regeneration process is quite small. The mechanical strength of these scaffolds depends mainly on the volume fraction of macro and micropores.

Poly (Vinyl alcohol) (PVA) hydrogels can be physically cross-linked by exposing a PVA aqueous solution to cycles of freezing and thawing. By freezing the water, the polymer chains are expelled and form regions of high PVA concentration. The polymer chains come into close contact with each other, crystallize and form hydrogen bonds. During thawing these structures remain and create a non-degradable three-dimensional polymer-network. The stiffness of the gel increases with the number of cycles, molecular weight, duration of each freezing and thawing cycle and the concentration of polymer[13].

Many authors refer to PVA gels as cartilage scaffolds or substitutes[14, 15]. The permeation and solute diffusion coefficient of PVA gels, which have undergone successive freezing and thawing cycles, is determined by studying the transport of ophylline and FITC-dextran.It was shown that the crystalline PVA fraction and the mesh size of the network influence the

solute transport in addition to the gels mechanical properties. For both molecules, the diffusion coefficient was higher for low crystalline PVA fraction than for high crystalline fraction. A lightly cross-linked PVA had greater mesh size and molecular weight between cross-links than a denser cross-linked PVA gel[16].

In this study the mechanical behaviour of a composite consisting of a polymer scaffold made of PCL, whose pores are filled with PVA, has been evaluated. The compliance of the gel inside the pores is adjusted from a viscous liquid to a hard gel by subjecting it to a number of freezing and thawing cycles.

2. Materials and Methods

PCL with molecular weight 70 – 90 000 Da was purchased from Sigma Aldrich. Microspheres of Elvacite 2043 (a mixture of low weight Poly (Ethyl Methacrylate) (PEMA) and Poly (Methyl Methacrylate) (PMMA)) with diameters ranging from 120 to 200 µm were purchased from Lucite International. 1,4 Dioxane from Sigma Aldrich was used as solvent for PCL. Ethanol (99% pure) from Scharlab was used to dissolve the dioxane in the freeze extraction process. Poly (Vinyl alcohol) with Mw 130000 Da and 99+ % hydrolyzed was obtained from Sigma Aldrich. All the chemicals were used as received.

2.1 Scaffold preparation

A PCL scaffold with macro- and micro porosity was fabricated by mixing a 15 wt. % PCL in 1,4 Dioxane solution with PEMA spheres at a weight ratio 1:1.25. The mixture was immediately frozen with liquid nitrogen. After approximately one minute, it was immersed in precooled ethanol and kept at -20ºC with three changes of cold ethanol. Then the scaffolds were washed in ethanol at 38ºC for 8 days changing ethanol every day until the ethanol did not show any traces of PEMA. Probes of the ethanol used in the extraction process were evaporated in a Petri dish to detect any traces of PEMA. After elimination of the porogen, the scaffolds were dried at room temperature under vacuum until reaching constant weight.

2.2 Hydrogel preparation

A 10% aqueous solution of PVA was prepared by stirring at 80°C for 2 hours. After the solution was obtained, it was allowed to cool to room temperature. The viscous solution was then poured into a 48 multiwell cell culture plate. Hydrogels were obtained by freezing the solution at -20°C for 12 hours and then thawed back to room temperature for 8 hours.

2.3 Scaffold / hydrogel construct

The solution was introduced into PCL scaffolds by vacuum injection. The scaffolds were subjected to a number of successive freezing and thawing cycles as described for PVA gels.

2.4 Scanning Electron Microscopy

The morphology of the scaffold and gel construct was observed by Scanning Electron Microscope (JEOL JSM-5410) equipped with a cryogenic device. Images were taken at an acceleration voltage of 10 kV. The samples were immersed in water for 24 hours, then frozen to -80°C. The sample was then cryofractured and water was sublimated for 40 minutes before it was coated with a gold layer. The cross-section was observed. Both the PVA hydrogels and the PCL/PVA construct were observed.

2.5 Mechanical testing

To evaluate the effect of the PVA filling of the scaffolds unconfined compression tests were performed. After every cycle of thawing, for up to 6 cycles, the PVA hydrogels and the scaffold/gel construct were examined. After the last cycle of thawing, the samples were put in distilled water overnight. Mechanical characterization of the PCL scaffolds, PVA hydrogels and the scaffold/gel construct was performed using a Microtest Universal testing machine with a 15 N load cell.

Cylindrical samples of 5 mm diameter and 2 mm height were cut with circular stamps and surgical scalpels in the case of PCL scaffolds. The pure PVA hydrogels were produced in cylindrical form in the culture wells with a diameter of 6 mm and were cut in 4 mm height samples. The samples were exposed to compression cycles in a homemade device that allows the samples to be immersed in water during the assay. A stress-strain representation of the results was used to calculate the maximum stress for 15% deformation and the modulus of the porous sample was calculated as the slope of the curve in the elastic region up to 2% deformation.

3. Results and Discussion

3.1 Morphology

The PCL scaffold shows a double interconnected porosity with macropores ranging from 120 µm to 200 µm approximately. The macropores are produced by the porogen leaching process. The micropores are approximately 10 µm and result from the dissolution of dioxane crystals formed in the freeze extraction process (Figure 1a). The microstructure is similar to that obtained in previous works[8-12]. Figure 1b shows the PCL scaffold filled with the PVA gel after 6 cycles of freezing and thawing. It is clear that the gel enters both the macro and micro pores. Cryogenic-SEM was used to assess the microstructure of the PVA gels to conserve the structural swelling. In conventional SEM, the samples must be dried and the pores of the gel collapsed, producing gel contraction and possibly detachment from the scaffold surfaces. Figure 1c shows the porous structure of the PVA gel that cannot be observed inside the macropores of the scaffold at lower magnifications.

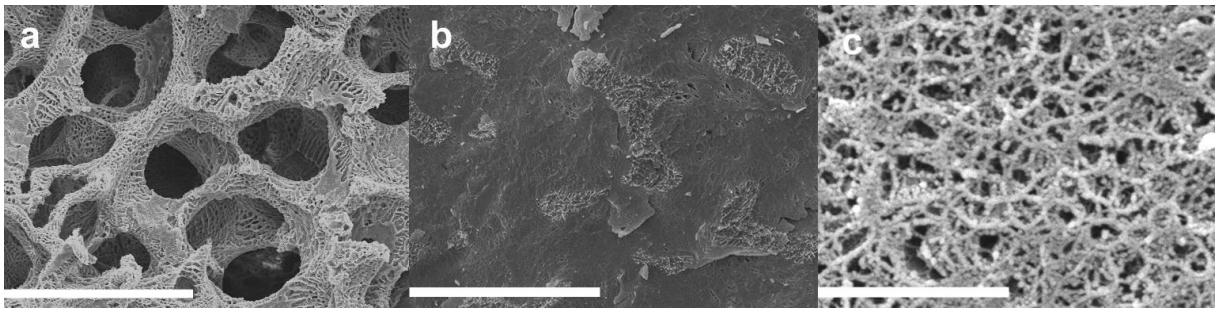

Figure 1. Figure (a) shows the SEM image of the cross section of PCL scaffold, in (b) the cryo-SEM image of the cross-section of a PCL scaffold filled with PVA gel and subjected to 6 freezing and thawing cycles is presented (scale bar in (a) and (b) 300 µm). Figure (c) shows the cryoSEM image of bare PVA gel at high magnification (scale bar 4 µm) in which the nanoporous structure of the gel can be observed after water sublimation.

Previous studies from other authors show that a structural change of PVA hydrogels occurs with increasing number of freezing and thawing cycles[17]. A macroscopic restructuring of the polymer-rich phase results in a progressive increase of polymer-rich zones throughout the first 6 cycles. A progressive increase in porosity was noted through 6 cycles varying from 4.5% after the first freezing and thawing cycle to 41.9% after 6 cycles[17]. Other authors

studied the evolution in cristallinity, swelling capacity and crosslinking density after up to 45 cycles of freezing and thawing. The results showed an increase in cristallinity and cross-link density and an decrease in the swelling capacity for increasing number of cycles of freezing and thawing, indicating that the characteristics of the gels do not stabilize in a short number of cycles[18].

3.2 Mechanical properties

Natural articular cartilage can be modelled as a biphasic structure, with a solid porous permeable phase, the tissue, and a fluid phase. Cartilage tissue consists of extra-cellular matrix produced by chondrocytes, who organize and maintain the structure of the cartilage by continued reorganization activity. Cartilage is mainly formed by collagen type II fibres and glycosaminoglycans (GAG), which are responsible for high water absorption capacity. The particular mechanical properties of articular cartilage arise mainly from the interactions of water, electrolytes and the collagen and proteoglycans polymeric matrix. When cartilage is deformed, water flows through the tissue allowing limited deformation and giving a viscoelastic response. Therefore it is the biphasic structure of cartilage that determines its load-bearing capacity. Pressure makes fluid flow through the permeable solid phase[19-22].

This study intends to obtain an experimental model that simulates the mechanical behaviour of the scaffold when cartilaginous tissue grows inside the pores. The mechanical response of a scaffold immersed in water with pore architecture such as that shown in Figure 1a, is expected to be quite different from that of healthy cartilage because the dynamics of the water flow through the pores is different. Nevertheless, it is expected that the scaffold will be progressively filled with the extra-cellular matrix that is produced by the cells, that are previously seeded or invading the scaffold “in vivo”. This would show that the mechanical properties of the scaffold/newly formed tissue construct vary in time[23-25]. In our model, PVA gel filling the pores of the scaffold simulates the growing extra-cellular matrix. Progressive freezing and thawing cycles can alter not only the stiffness but also water permeation of the PVA gel[17, 26].

The effect of water diffusion on the stiffness of the scaffold under compression is clearly demonstrated by the dependence of the stress-strain curve on the strain rate. Figure 2 shows the stress-strain plots of PCL /PVA constructs measured at different deformation rates from 0.02 to 1 mm/min. At high compression rates, the water occupying the macropores behaves as an incompressible fluid that resists the compression force, which increases the apparent elastic modulus of the construct. At low compression rates, water is able to escape from the sponge making the sample more compliant and thus the measured apparent elastic modulus decreases. We have chosen for the rest of the work the highest rate available in our experiments, 1mm/min. With the thickness of our samples that would correspond to a frequency of 0,03Hz, which is closest to the range normally considered for physiological loading frequencies in articular cartilage, between 0,1 and 2 Hz.

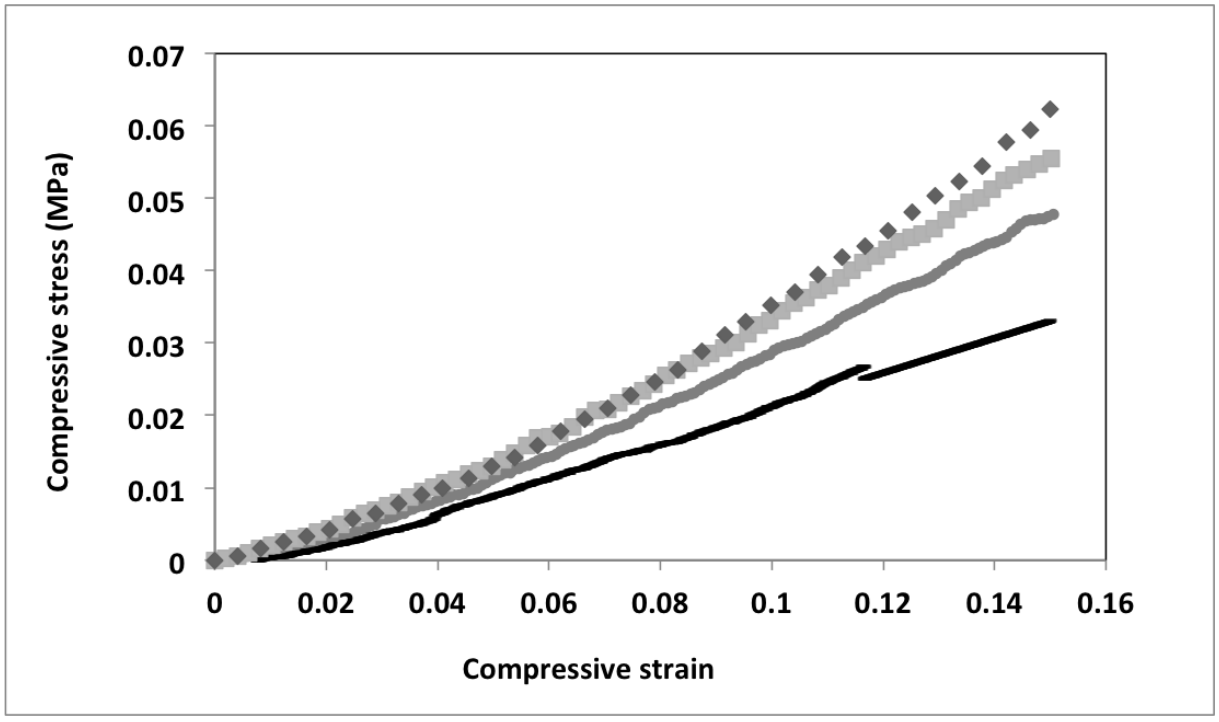

Figure 2.Stress-strain curves obtained at varying compression rates in an unconfined compression test of a sample of PCL scaffold filled with PVA gel and subjected to 6 freezing and thawing cycles. The rates used were 1mm/min (◆), 0.5 mm/min (■), 0.2 mm/min (•) and 0.002 mm/min (–).

Figure 3 shows the compression tests performed at a deformation rate of 1 mm/min in the water-immersed PCL scaffold. Three characteristic regions of scaffold materials can be observed[27]: (I) the elastic region corresponding to low strains, (II) the plateau region in which the polymer struts suffer plastic deformation or fracture and pore structure collapses with large deformations at nearly constant stress, and (III) the high slope stress-strain region corresponding to the behavior of the compacted material. The characteristic regions are labeled in figure 3.

The effect of the PVA gel filling the pores is significant in all regions. The plateau is substituted by a continuous increase of stress that shows that collapse of the PCL struts is partly prevented when the pores of the scaffold are filled by the gel. Interestingly enough, the increase in compressive stress in the PCL scaffold /PVA constructs cannot be explained by the stiffness of the gel that fills the pores. As shown in Figure 3, the elastic modulus of the gel is not very different from that of the PCL scaffold in region II. The clear synergic effect in the construct has to be attributed to the role of the PVA gel to constrain water permeation through the construct after a number of freezing and thawing cycles. Again, incompressibility of liquid water explains the high increase in the compression stress in the construct.

In cartilage application the strain range of interest is limited to 15%, which is considered to be the order of magnitude of physiological deformations suffered by articular cartilage[28, 29]. Values of the apparent elastic modulus were determined in the initial 2% deformation but due to the significant curvature of the stress-strain plot, the stress at 15% strain is also presented.

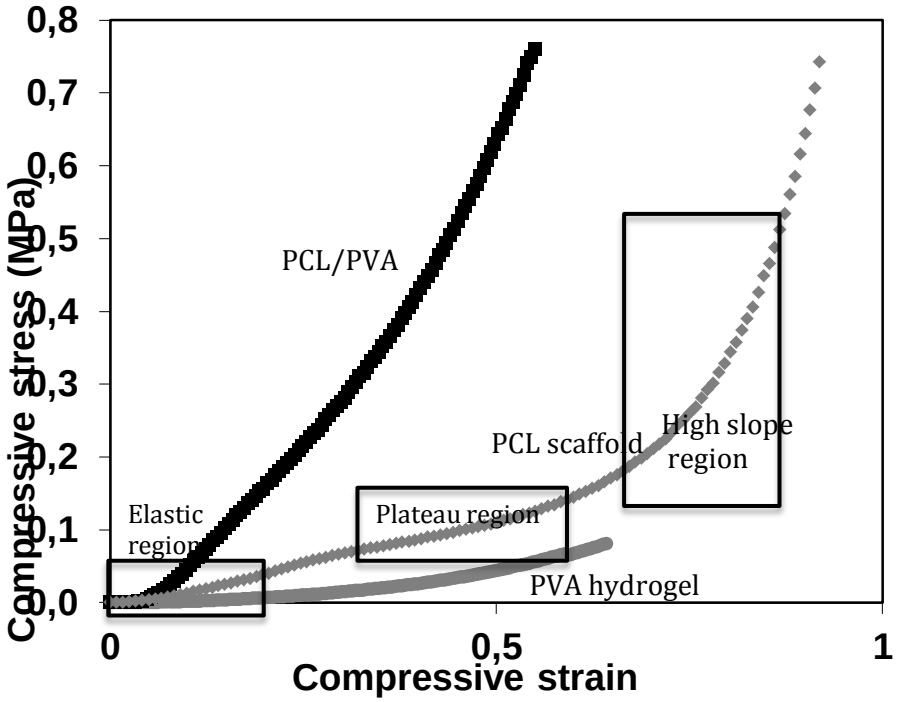

Figure 3. Unconfined compression test of the PCL scaffold immersed in liquid water (◆), PVA gel after 6 freezing and thawing cycles (•) and the PCL scaffold /PVA construct after 6 freezing and thawing cycles (■).

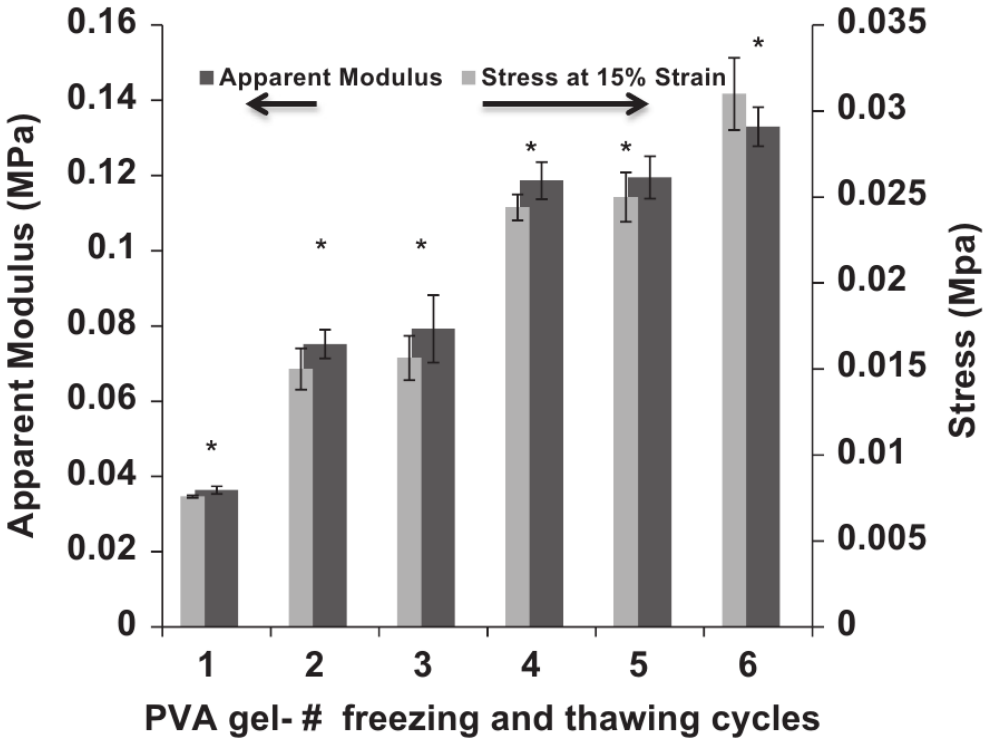

Figure 4. Apparent elastic modulus measured in unconfined compression tests of the PVA gel subjected to different number of successive cycles of freezing and thawing. The apparent elastic modulus was calculated for strains below 2%. The stress at 15% strain is represented in the secondary axis.

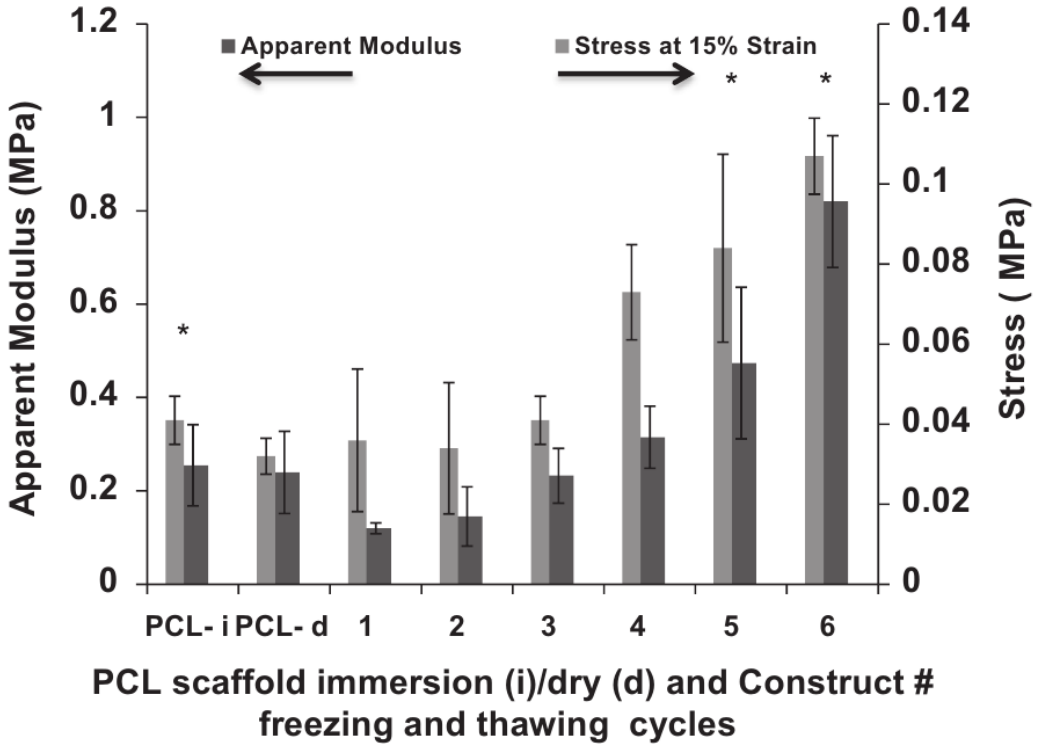

Figure 5.Apparent elastic modulus and stress at 15% strain measured in unconfined compression tests in PCL scaffolds (measured in immersion in water-i or dry-d) and the PCL scaffolds/PVA constructs subjected to a different number of successive cycles of freezing and thawing. The asterisk indicates significant differences with respect to dry PCL scaffold according to ANNOVA tests with $p< 0.05$. The Stress at 15% deformation is represented on the secondary axis.

	Apparent Elastic Modulus(MPa)	**Stress at 15% strain (MPa)**
PVA 6 cycles f/t	0.13 ±0.005	0.032±0.002
PCL empty scaffold	0.25 ±0,088	0.040 ±0.006
Construct 6 cycles f/t	0.82 ± 0.14	0.11 ±0.009
Articular cartilage (rabbit model)	0.26-0.57	0.08-0.3

Table1. The values of the apparent modulus and the maximum stress for the PCL scaffold, the PVA gel and the scaffold / gel construct. Values for articular cartilage, previously reported, are shown for comparison[30]26].

The results obtained in the compression tests of the PVA gel show very low values of one freezing and thawing cycle, around 0.036 MPa, but the apparent elastic modulus of the gel increases for every additional cycle. The stiffness increases with the number of freezing and thawing cycles due to the increasing number of cross-links that will distribute the stress on a higher amount of crystalline regions. This feature has been explained by the increased hydrogen bonding between amorphous and or crystalline regions and the densification of regions of amorphous PVA chains[17]. The modulus for the highest cross-linked gel reaches 0.133 MPa. The trend of the stress at 15% strain shows the same behavior with a linear dependence between the stress and the number of freezing and thawing cycles. It is worth noting that the results in Figure 4 do not show any indication of reaching stable values for the stiffness of the gel. As a consequence, we expect that subjecting the gel to further freezing and thawing cycles would lead to an increase of its modulus.

The compression test of the PCL scaffold/PVA construct shows similar behavior (Figure 5) as the PVA hydrogels. For one freezing and thawing cycle the modulus of the construct is about 0.12 MPa and for every additional cycle up to 6 cycles the stiffness increases until reaching 0.82 MPa. Interestingly enough, the mean value of the modulus do not depend linearly on the number of freezing and thawing cycles, instead there is an increasing effect on the stiffness with each successive cycle. Furthermore, the mean value of the compression modulus for constructs subjected to 1 to 3 freezing and thawing cycles is even lower than for the PCL scaffolds measured dry or in immersion without PVA. The statistical analyses show that the differences are not significant between the dry PCL scaffold and the PCL scaffold neither in immersion nor with the construct for up to 3 cycles of freezing and thawing, giving an interesting result. This feature is more explicit in the results of the stress at 15% strain, shown in Figure 5 at the secondary axis. Therefore, filling the hydrophobic scaffold with a soft gel does not change the stiffness of the construct for the first three cycles of freezing and thawing. Not until a densely cross-linked gel is introduced into the scaffold the stiffness increase. As explained above for higher deformation regions, it seems like the stiffness of the filler itself plays a minor role in the mechanical behavior of the construct, which is mostly determined by the permeability of water in the material. Water diffusion in a very soft gel facilitates the expulsion of water from the scaffold. This would explain that the three first

freezing and thawing cycles do not produce a significant increase in stiffness in the construct. Note that the water content in the gel filling the scaffold pores is on the order of 90%. According to the literature, PVA gel subjected to an increasing number of freezing and thawing cycles develops a nanoporous structure, in addition to increasing crystallinity[17]. For a higher number of freezing and thawing cycles, in spite of increasing nanoporosity, permeability studies suggest a complex relationship between crystallinity, pore interconnectivity, permeability and pore tortuosity that results in a decrease in permeability with the number of cycles[31].

This singularity explains that in the low deformation regime, the stiffness of the PCL scaffold /PVA construct increases for every cycle of freezing and thawing much more than the stiffness of the PVA gel itself. This behaviour is also relevant for the application of macroporous scaffolds in cartilage engineering strategies. The development of newly formed tissue inside the pores could allow the implanted scaffold to reach mechanical properties close to that of the healthy articular cartilage, even if the regenerated tissue is softer than the healthy tissue. This is important for tissue regeneration since it implies that the biomechanical environment of cells hosted in the scaffold pores could be adequate in shorter times than those required for the organization of extra-cellular matrix with the structure of hyaline cartilage. In fact, it is possible that the extra-cellular matrix produced within the macropores of a scaffold do not organize like hyaline cartilage, despite of the fact that chondrocytic markers are expressed.

To emphasize the synergic effect of the scaffold and the hydrogel filling the pores, Table 1 shows the values of the apparent elastic modulus in the elastic regime, which can be compared with those of articular cartilage in human or animal models. In previous work authors have conducted indentation studies of the articular cartilage of rabbits[30, 32]. Values of the modulus ranging between 0.26 and 0.59 MPa were measured and the stress for 15% strain was between 0.08 and 0.3 MPa. This shows that the required modulus can be reached with a quite soft hydrogel filling the pores of the scaffold. This is due to the low water permeability of the gel.

The results obtained with the experimental model proposed in this work show that testing a scaffold to predict its performance during tissue regeneration requires a precise simulation

of the contribution of newly formed tissue and of the behavior of the scaffold/tissue construct. This experimental model could be used to obtain a realistic prediction of the performance of a given macroporous scaffold after a number of deformation-recovery cycles as is experienced “in vivo” during tissue regeneration.

The biphasic theory models natural articular cartilage with two different constituents: a fluid phase and a solid phase. The amount of the fluid phase and its permeability is determined in large extent by the GAG contents of the tissue. Although the chain structure of PVA and GAGs is quite different, its role in the mechanical behaviour of the filled scaffold might be similar. ESEM images show that both cartilage and PVA hydrogels are porous structures with small holes [31]. Water is retained within the polymer network due to the hydrophilic group of the polymer chains. Water content is in the order of 70 to 80% by weight in cartilage and 90% in PVA gels. Water motion inside the cartilage tissue and in PVA is controlled by polymer-water interaction and pore tortuosity. These similarities make PVA a good filling material to simulate the effect of the growing tissue inside the pores of the implanted scaffold in its mechanical performance.

Long-term fatigue testing of unfilled scaffolds could significantly underestimate the performance of the material. Mechanical performance of the scaffold while it is resorbed “in vivo” could be adequately analyzed as well using hydrolytic or enzymatic media during fatigue testing.

4. Conclusions:

An experimental model has been proposed to test the mechanical behaviour of tissue engineering scaffolds. Newly formed tissue inside the scaffold during the regeneration process is simulated by a PVA hydrogel subjected to a series of consecutive cycles of freezing and thawing. The elastic modulus of the PVA gel increases with the number of freezing and thawing cycles. A PCL scaffold with a pore architecture consisting of spherical interconnected macropores with micropore walls could be efficiently filled with PVA. The stiffness of the scaffold/hydrogel construct increases when subjected to repeated freezing and thawing cycles. Interesting enough after three cycles of freezing and thawing the elastic modulus of the construct is much higher than that of either of its components. This result is attributed to

the effect of the PVA gel hindering water permeation through the scaffold when it is subjected to compression loading. This mechanical behaviour is quite similar to that of articular cartilage and could be used to predict the performance of scaffolds during degradation or during long-term fatigue testing.

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