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Polymeric scaffolds used in regenerative therapies are implanted in the damaged tissue and submitted to repeated loading cycles. In the case of articular cartilage engineering, an implanted scaffold is typically subjected to long-term dynamic compression. The evolution of the mechanical properties of the scaffold during bioresorption has been deeply studied in the past, but the possibility of failure due to mechanical fatigue has not been properly addressed. Nevertheless, the macroporous scaffold is susceptible to failure after repeated loading-unloading cycles. In this work fatigue studies of polycaprolactone scaffolds were carried by subjecting the scaffold to repeated compression cycles in conditions simulating the scaffold implanted in the articular cartilage. The behavior of the polycaprolactone sponge with the pores filled with a poly(vinyl alcohol) gel simulating the new formed tissue within the pores was compared with that of the material immersed in water. Results were analyzed with Morrow's criteria for failure and accurate fittings are obtained just up to 200 loading cycles. It is also shown that the presence of poly(vinyl alcohol) increases the elastic modulus of the scaffolds, the effect being more pronounced with increasing the number of freeze/thawing cycles.

Keywords

pore, filling, vinyl, alcohol, gel, mechanical, poly, vitro, caprolactone, macroporous, scaffolds, cartilage, fatigue, tissue, behavior, engineering, influence

Disciplines

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***In vitro* mechanical fatigue behaviour of poly- ϵ -caprolactone macroporous scaffolds for cartilage tissue engineering. Influence of pore filling by a poly(vinyl alcohol) gel.**

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Abstract

Polymeric scaffolds used in regenerative therapies are implanted in the damaged tissue and submitted to repeated loading cycles. In the case of articular cartilage engineering, an implanted scaffold is typically subjected to long term dynamic compression. The evolution of the mechanical properties of the scaffold during bioresorption has been deeply studied in the past, but the possibility of failure due to mechanical fatigue has not been properly addressed. Nevertheless, the macroporous scaffold is susceptible to failure after repeated loading-unloading cycles. In this work fatigue studies of polycaprolactone scaffolds were carried by subjecting the scaffold to repeated compression cycles in conditions simulating the scaffold implanted in the articular cartilage. The behaviour of the polycaprolactone sponge with the pores filled with a poly(vinyl alcohol) gel simulating the new formed tissue within the pores was compared with that of the material immersed in water. Results were analyzed with Morrow's criteria for failure

and accurate fittings are obtained just up to 200 loading cycles. It is also shown that the presence of poly(vinyl alcohol) increases the elastic modulus of the scaffolds, the effect being more pronounced with increasing the number of freeze/thawing cycles.

Introduction

Macroporous biodegradable polyesters are interesting scaffolds for cartilage tissue engineering applications, since they are able to sustain the mechanical loading produced in the joint during motion, which in turn produce suitable signals for the development of the extracellular matrix (ECM). The interconnected pores may provide a structure suitable for cell attachment, proliferation and fluid flow for nutrients and waste transport^{1,2}. The overall mechanical properties of the scaffold are determined by material mechanical properties, permeability and pore structure. These properties regulate load transfer and evolution of the ECM in a feedback process where the growing ECM matrix changes with time together with the aforementioned properties. Thus, prediction of the scaffolds mechanical behavior, in particular when a cyclic load is applied, is necessary for the proper understanding of the mechanical effect on cell response.

The retrieval of scaffolds after implantation in animals for measuring mechanical properties is hard, expensive and raises ethical issues. On the other hand, the measurement of the mechanical performance of scaffolds during *in vitro* experiments involves the use of growth factors and bioreactors to stimulate ECM synthesis, needing also large experimental time to obtain information concerning material mechanical performance. Therefore, finding a model capable to predict material behavior during *in vitro* and *in vivo* experiments is an important challenge for tissue and biomedical engineering.

Mechanical fatigue behavior of polymer scaffolds have been barely assessed in the literature^{3,4}, despite their large importance in actual applications. The mechanical properties of dry scaffold are not representative of the behavior of the material when immersed in cell culture media or during *in vivo* experiments, as the porous will be filled with aqueous media and/or growing tissue that will strongly contribute to the scaffold mechanical response. For dry scaffolds, the mechanical properties will depend mainly on the inner morphology, in particular, pore size and polymer wall thickness and interconnectivity⁵. On the other hand, when the scaffold is immersed

in an aqueous media, there are other factors that contribute for scaffold mechanical behavior as well, such as hydrodynamics and permeability inside the porous structure^{5,6}. Water is known for its low compressibility and any factor limiting water permeation through the material will increase apparent scaffold stiffness and therefore the influence of liquid media cannot be disregarded⁶. However, only few investigations mention the mechanical behavior of polymer scaffolds under liquid environment^{7,8} and none of them under cyclic loading in aqueous media.

In this work a micro and macro porous poly(ϵ -caprolactone) (PCL) scaffold has been employed, it is a semicrystalline hydrophobic polymer with an “*in vivo*” degradation time of 2-4 years, raising interest in cartilage tissue engineering^{9,10}. By combining a freeze extraction process and the use of porogen microparticles a scaffold structure that is characterized by large interconnected spherical pores with microporous walls can be processed.¹¹⁻¹³

Fatigue behavior of materials can be influenced by several factors such as thermal and mechanical loading history, environmental conditions, polymer composition and other aspects of stress-strain constitutive behavior¹⁴. Several mathematical models have been developed mainly to predict fatigue life cycle of metallic materials, including the Coffin–Manson, Smith-Watson-Topper (SWT) and Morrow models^{15,16}. The Coffin-Manson model is based on the plastic strain amplitude and number of elapsed cycles, while in the SWT model¹⁷ the product of maximum stress (σ_{max}) and $\Delta\epsilon_t$ is assumed to control the fatigue life cycle for any given situation¹⁸. Moreover, Morrow developed a model to predict fatigue life cycle of metals based on the plastic strain energy density that can be physically interpreted as the distortion energy associated to the change in shape of a volume element and can be related to failure, in particular under conditions of ductile behavior¹⁹.

It has been shown⁶ that fatigue life cycle of poly- ϵ -caprolactone (PCL) scaffolds, with and without fibrin within the porous structure strongly depends on the presence of water, the fatigue life cycle increasing from 100 up to 500 loading cycles in samples tested under dry and immersed conditions, respectively. This effect was attributed to a more homogeneous stress distribution promoted by water within the samples. On the other hand, the presence of fibrin

inside the macroporous structure plays just a minor role in the mechanical performance of the scaffolds.

Poly(vinyl alcohol) (PVA) gels are often applied as cartilage substitutes as they may mimic the elastic modulus of cartilage^{20,21}, as they offer the possibility of formation of physical cross-links during freezing/thawing cycles without the need of toxic monomers used typically in chemically cross-linked gels²² such as gelatin²³ or chitosan²⁴. During exposure to cold temperatures promoting water freezing, phase separation leads to regions of high PVA concentration, hydrogen bonding and crystallite formation due to the higher packing of the PVA chains²⁵. These interactions remain intact before thawing and create a non-degradable three-dimensional hydrogel network. Increasing the number of freezing/thawing cycles, the degree of polymer phase separation, hydrogen bonding and crystallite formation can be increased leading to tailored mechanical properties by controlling the freeze/thaw process and the number of cycles²⁶.

In a previous study a macro and micro porous PCL scaffold was filled with an aqueous solution of PVA and exposed to up to 6 cycles of freezing and thawing. The mechanical properties of the PCL and PVA construct reached values of natural articular cartilage and meniscus. The PCL and PVA construct is then considered suitable as an in vitro model for simulating the growing cartilage inside of the scaffold of the PCL scaffold²⁷ In the present work the same protocol was applied to obtain PCL – PVA constructs, whose fatigue mechanical properties were measured. These properties are critical for the use of these scaffolds in cell cultures under dynamic loading.

Materials and methods

Materials: Poly- ϵ -caprolactone (PCL, 43-50 kDa) and 1,4-dioxan were purchased from *Sigma-Aldrich*. Poly(ethyl methacrylate) (PEMA - Elvacite 2043) spheres (mean diameter of 200 μm) were purchased from *Lucite*. Poly (vinyl alcohol) with Mw 13 kDa and >99% hydrolyzed was obtained from *Sigma-Aldrich*.

Sample preparation: PCL scaffolds were prepared as previously described elsewhere ⁶. Briefly, PCL was dissolved in dioxane (25% w/v) and this solution was mixed with PEMA microspheres (1:1 w/w). Then, the mixture was placed in Teflon Petri dishes and submerged in liquid nitrogen for a minute. Dioxane was extracted from the frozen plates with ethanol at -20 °C for three days, changing ethanol every day. Porogen leaching was performed in ethanol at 40 °C for one day. The porous samples were cut into cylinders with 6 mm diameter and a thickness of approximately of 2 mm. Further leaching for each cylinder was performed in ethanol at 40 °C for nine days, changing ethanol daily.

In order to ensure the maximum water uptake for measurements in immersion, after complete porogen leaching, all samples were immersed in a distilled water bath and placed in a chamber (Vacuum-Temp from Selecta) under 10^{-2} mmHg conditions until the samples dropped to the bottom of the bath.

PVA was dissolved in water (10% w/v) at 90 °C for 1 hour. After the solution was obtained it was allowed to cool to room temperature. The viscous solution was introduced into PCL scaffolds by syringe vacuum. All PCL scaffolds under immersion were placed inside a syringe with 10 ml of PVA solution 10%, at room temperature. The syringe barrel was closed with a syringe cap and vacuum was made by moving the piston alternately at maximum (50 ml) and minimum to displace and substitute the air inside the pores by PVA. The operation was repeated 30 times. The remaining PVA in the external surface of the scaffolds was removed. Hydrogels of different stiffness were obtained by freezing PCL-PVA constructs at -20 °C for 16 hours and then thawed back to room temperature for 8 hours in humid environment. Three series of samples were prepared with 1, 3 or 6 freeze-thawing cycles respectively.

Sample characterization:

Sample morphology and gel construct was assessed by scanning electron microscopy using a JEOL JSM-5410 apparatus equipped with a cryogenic device. Images were taken at an accelerating voltage of 10 kV. Samples were previously immersed in water during 24 h and then frozen at -80 °C. Then, the samples were cryo-fractured and water was sublimated during 40 min before coating with a gold thin layer.

Mechanical experiments were performed on cylindrical samples with 6 mm diameter and a height of ~2 mm in a *Shimadzu AG-IS* universal testing machine in compression mode at a test velocity of 1 mm.min⁻¹ and at room temperature. In fatigue experiment, samples were submitted to a compressive-strain cycle load at a strain of 15% and up to 1000 cycles, which is typically the strain range of interest considered to be the maximum magnitude physiological deformation suffered for articular cartilage^{28,29}. Strain deformation was measured by machine cross-head displacement. Mechanical stress and strain parameters were obtained after an average of five sample replicas for each set of constructs (PCL with PVA after 1,3 and 6 freeze-thawing cycles). All mechanical experiments were performed after sample immersion in deionized water.

Results

Morphology, morphology variation and mechanical response

It was observed that PCL porous architecture consists on a double porosity: macropores ranging from 120 up to 200 µm obtained from the leaching of the porogen spheres and micropores resulting from dioxane crystals formed during the freeze extraction process that interconnect the bigger ones (examples indicated with arrows in figure 1a and b). This double porous structure results in an overall high porosity (83.4±2.6%⁶) and has been previously proposed for cartilage and bone replacement³⁰⁻³². Such structure favors scaffold permeability to nutrients and waste products of cell metabolism and can be used to retain active components^{11,33,34}. However, apparent scaffold stiffness becomes smaller than in similar sponges lacking microporosity^{13,35}. Further, when PVA solution is introduced in PCL scaffolds in which the air has been extracted from the pores, it is able to fill both the macropores and the micropores. On crosslinking by freeze-thawing cycles, PVA forms a continuous polymer network filling the pore structure. The continuity of the PVA phase between the micropores and macropores even made it difficult to identify the interphases with PCL in SEM pictures. Only small discontinuities can barely be found in particular points of the SEM pictures (area in figure 1c) Due to the viscosity of PVA solution some micropores can remain empty, an example can be observed in the inset of figure 1c. Small detachments at the interphase between PVA and PCL appear in some points in but they could be produced during cryofracture in sample preparation (inset of figure 1c). Scaffolds observed after cyclic mechanical loading for 1000 cycles show that the PCL macropores collapse

and their shape changed from circular to a more ellipsoid one (figure 1 a and b). In the case of the samples filled with PVA after fatigue testing (figure 1d) cryogenic fracture shows increased roughness with respect to the smooth surface observed before fatigue (figure 1c) and the shape of macropores is not clear.

Figure 1

Mechanical analysis

Mechanical performance of PCL and PCL – PVA samples obtained from the evaluation of the elastic modulus for the first cycle determined at $\varepsilon = 5\%$ show that PVA contributes for an increase of the elastic modulus, especially for the samples submitted to higher freezing/thawing cycles (figure 2a). Further, the incorporation of PVA inside of the PCL porous matrix leads to an increase of the maximum stress when compared to the pristine polymer (figure 2b). Such behavior is ascribed to the increase in the degree of crystallinity present in the PVA hydrogel that promotes a homogenous distribution of the stress among the sample crystalline regions^{25,26}.

Figure 2 –

Moreover, the maximum stress decreases after each cycle, suggesting that the material undergoes permanent deformation with increasing the number of loading cycles during the first ~40 cycles, stabilizing after that (figure 3). Initial maximum stress is higher while PVA freezing/thawing cycles are increased.

Figure 3

Morrow energy model: plastic strain energy density-life model

Hysteresis loops behave differently when PVA is added, especially after 6 freeze/thawing cycles (fig.4). Cyclic loading has a major impact for immerse PCL without PVA. These differences may be observed in detail with fatigue analysis.

Figure 4

Under cyclic loading, the plastic strain energy per cycle is considered a measure of the amount of fatigue damage per cycle. The amount of plastic strain by the material and the hysteresis energy absorbed during cyclic loading has been postulated as a basis for failure analysis. According to Morrow's model¹⁹, the relation between plastic strain energy density and the fatigue life can be expressed as:

$$N_f^m W_p = C \quad (1)$$

where W_p is the overall equivalent behavior similar to plastic strain energy density; N_f is the fatigue life and m and C are the fatigue exponent and coefficient, respectively.

Experimental data were fitted with equation 1 to evaluate the material behavior in response to cyclic mechanical loading before ample collapse with $R > 0.98$. The fitting results are represented in figure 5 as solid lines, together with the experimental data points, and the fitting parameters are presented in table 1. A general decrease of the fatigue exponent (slope in Figure 5) and coefficient (y-intercept) for all samples was observed, with respect to the pristine porous PCL sample, which means a lower decrease in dissipated and energy suggests that the PVA is attached to the porous PCL walls and some mechanical loading is supported by PVA gel. According to ANOVA with Tukey test, the slopes are significantly different between PCL samples without PVA and after 6 freezing-thawing cycles.

Figure 5

Table 1

It has been reported³⁶ that freezing/thawing promotes a decrease in the number of hydroxyl groups available for hydrogen bonding caused by an increase of entanglement which hinders

other hydrogen bonding formation from weakening of physical network. Further, decreasing of the glass transition also occurs with increasing number of freezing/thawing cycles.

For higher freezing/thawing cycles, the volume of the PVA increases as well as the degree of crystallinity^{22,25}, and the material can support partially the mechanical load that is transferred from water and from PCL matrix, leading to more stable distribution of the applied mechanical loading and consequently to higher fatigue life cycle behavior.

Based on the fitting parameters (table 1), mechanical life cycle behavior was calculated and compared to the experimental data. The calculated number of cycles to reach scaffolds plastic deformation for PCL and the different PCL – PVA samples are plotted *vs* the experimental load-recovery cycles (figure 6). Good correlation is represented by experimental data points lying on the solid diagonal line with deviations lower than 10 % from the theoretical line³⁷. Figure 6 shows thus that Morrow's model can successfully predict the load recovery cycles behavior for the PCL and PCL-PVA samples, at least until 200 cycles, when intrinsic properties change and the constructs does not respond equally to cyclic loads^{16,37}.

Figure 6

Discussion

PCL porous scaffolds with double porosity and an overall porosity of $83.4 \pm 2.6\%$ ⁶ with and without PVA inside the porous structure has been submitted to cyclic mechanical compression until a 15% deformation. When PVA solution is introduced in PCL scaffolds and after crosslinking by freeze-thawing cycles, a continuous polymer network filling the matrix pore structure was observed (figure 1). It has been reported³⁶ that freezing/thawing promotes a decrease in the number of hydroxyl groups available for hydrogen bonding caused by an increase of entanglement which hinders other hydrogen bonding formation from weakening of physical network. Further, decreasing of the glass transition also occurs with increasing number of freezing/thawing cycles.

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from water and from PCL matrix, leading to more stable distribution of the applied mechanical loading and consequently to higher fatigue life cycle behavior. Similar behavior was observed for PCL scaffolds immersed in water⁶.

Finally, Morrow's fitting parameters presented in table 1 allowed to compare experimental and predicted PCL scaffolds fatigue life cycle and a truthful correlation was obtained up to 200 loading-unloading cycles and for larger number of cycles deviation occurs both for dry and pristine PCL⁶ samples.

Conclusions

The mechanical stability of polymer scaffolds is a key issue for tissue and biomedical engineering applications, particularly, when the materials are immersed in aqueous media and submitted to cycle mechanical loading in conditions resembling their application environment. In this way, PVA gel has been introduced within the PCL macroporous scaffolds aiming to simulate the growing tissue. It is demonstrated that the presence of PVA increases the elastic modulus of the scaffolds, this effect being more pronounced with increasing the number of freeze/thawing cycles. Increasing the number of freeze/thawing for PVA improves the resistance to fatigue, being only noticeably for higher freeze/thawing cycles, which better resemble the conditions of a growing extracellular matrix. However, accurate fitting to the Morrow's energy model is just possible up to 200 loading-unloading cycles. The deviation for larger number of cycles occurs both for dry and pristine PCL⁶ samples. This fact, should be related to complex changes in the porous structure and local interactions among the different phases (PCL, PVA and water). In future works these results will be compared with the effect of cartilage-like ECM generated *in vitro* inside the macropores, in order to further validate the model and to delve in the mechanical integration of ECM and scaffolds, and its effects in fatigue resistance of the overall construct.

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Figure Captions

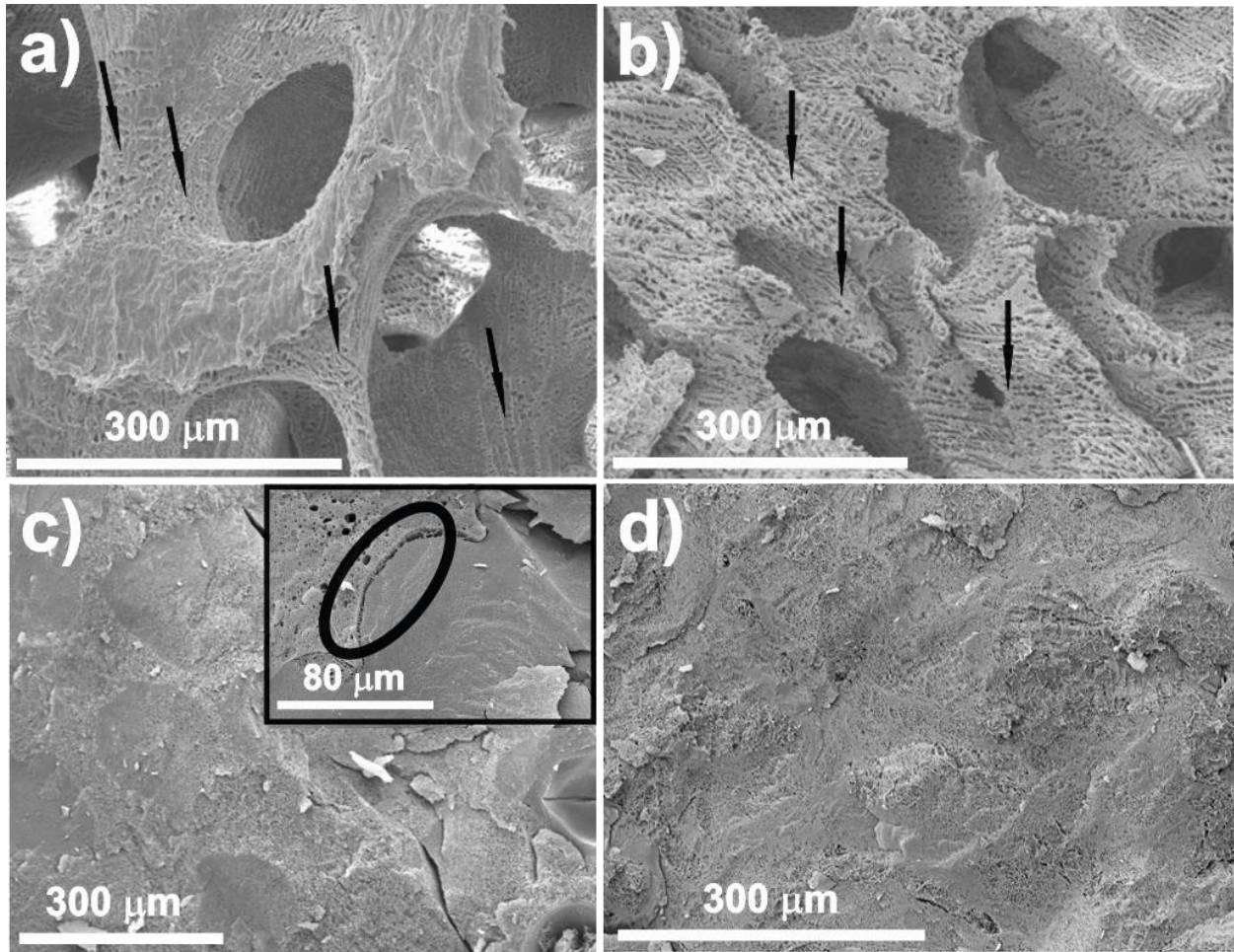


Figure 1 – PCL microstructure: a) pristine scaffold, b) after applied cyclic mechanical loading for 1000 cycles, c) PCL – PVA sample after 6 freeze/thawing cycles before and d) after applied cyclic mechanical loading for 1000 cycles.

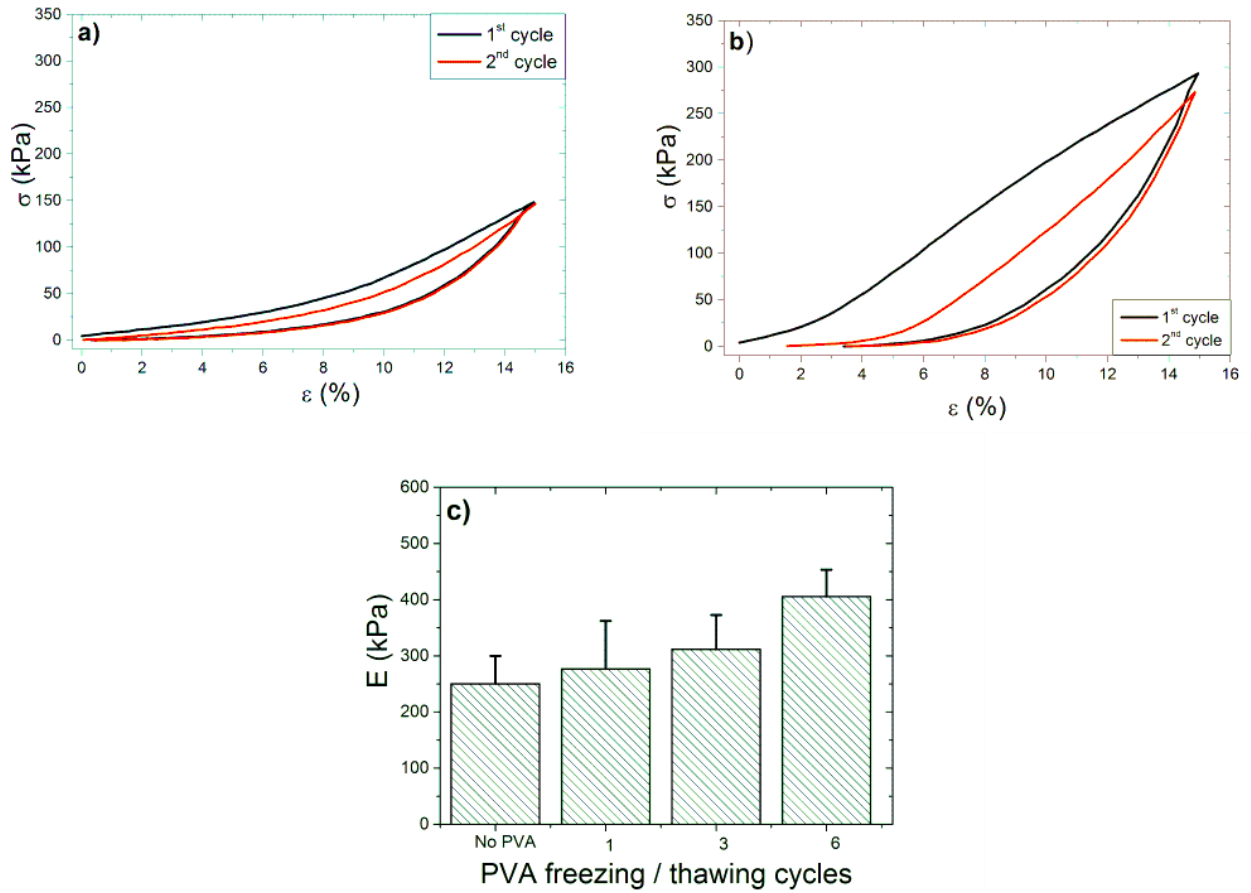


Figure 2 – Hysteresis of first and second loop for a) pristine immerse PCL and b) immerse PCL-PVA after 6 freezing/thawing samples c) Elastic moduli obtained for first cycle in PCL and PCL-PVA samples (1, 3 and 6 freezing/thawing cycles), calculated from first 5% of strain.

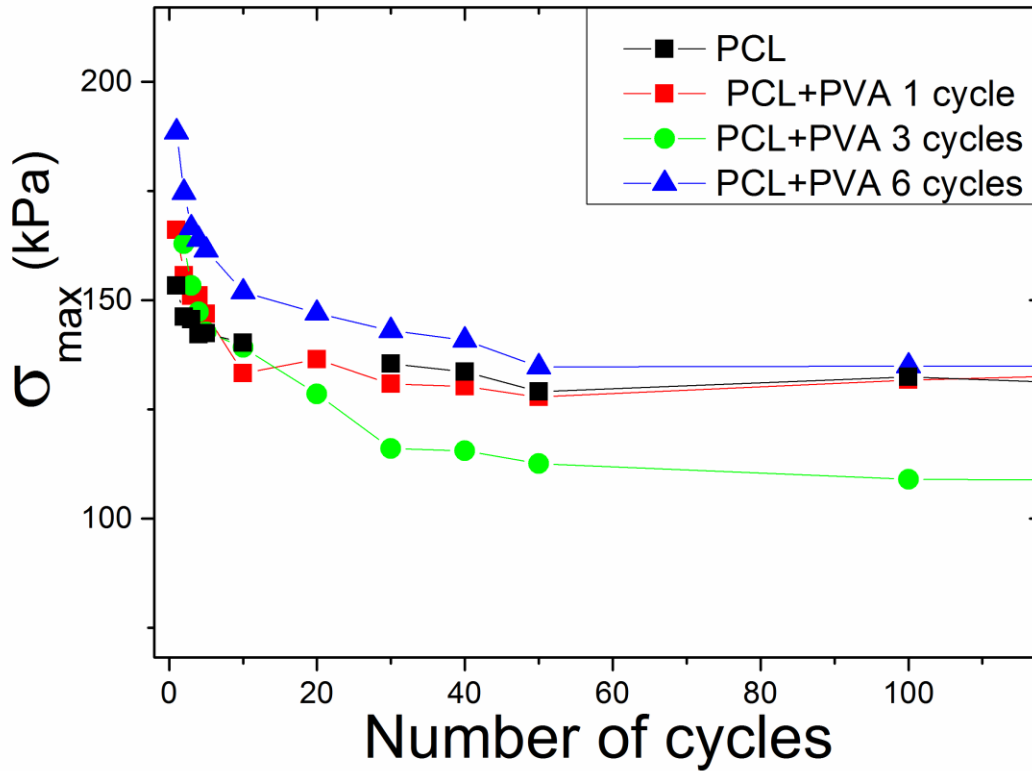


Figure 3. Average maximum mechanical compression stress as a function of the mechanical load cycle number for PCL and PCL - PVA samples after different freeze/thawing cycles.

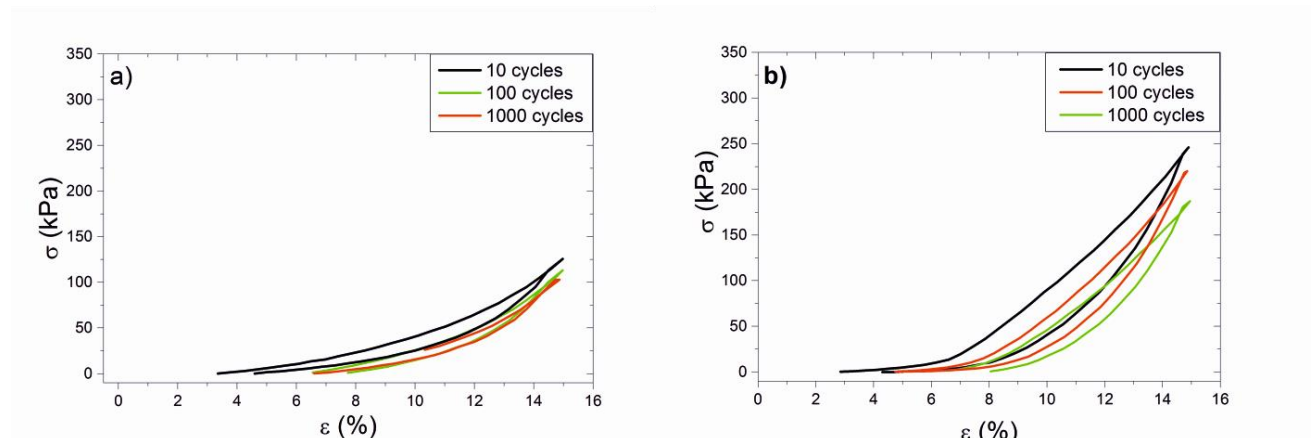


Figure 4 –Hysteresis loops after 10, 100 and 1000 loading cycles, for a) immerse pristine PCL and b) immerse PCL with PVA after 6 freeze/thawing cycles.

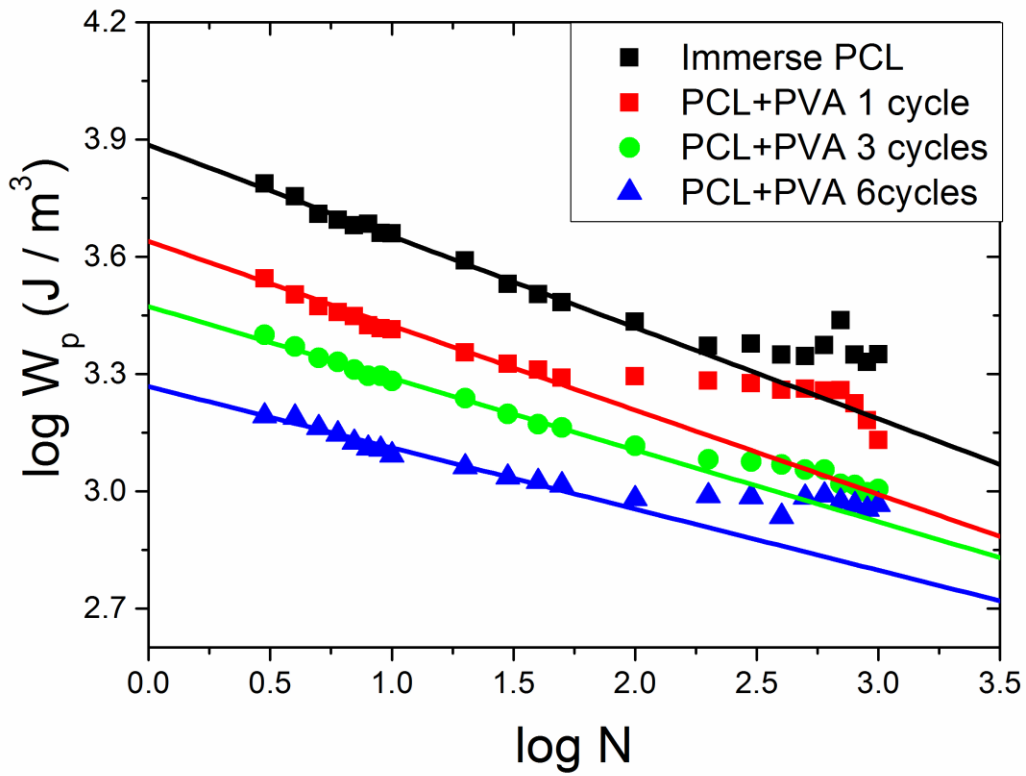


Figure 5 – Relationship between the overall equivalent behavior similar to plastic strain energy density and the number of load-recovery cycles of PCL and PCL-PVA samples.

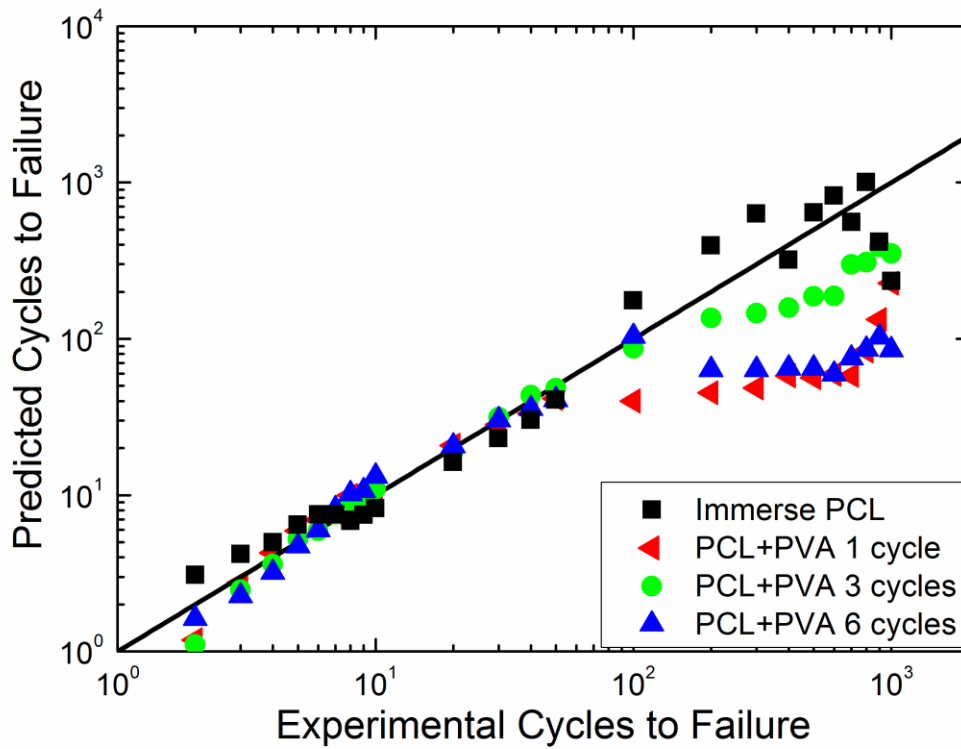


Figure 6 – Comparison of experimental and theoretically predicted fatigue behavior, according to Morrow’s model for PCL and PCL-PVA samples.