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Fatigue Prediction on Fibrin Poly-ε-caprolactone Macroporous Scaffolds

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Abstract

Tissue engineering applications rely on scaffolds that during its service life, either for *in-vivo* or *in vitro* applications, are under mechanical solicitations. The variation of the mechanical condition of the scaffold is strongly relevant for cell culture and has been scarcely addressed. Fatigue life cycle of poly-ε-caprolactone, PCL, scaffolds with and without fibrin as filler of the pore structure were characterized both dry and immersed in liquid water. It is observed that the there is a strong increase from 100 to 500 in the number of loading cycles before collapse in the samples tested in immersed conditions due to the more uniform stress distributions within the samples, the fibrin loading playing a minor role in the mechanical performance of the scaffolds.

Introduction

Tissue engineering has arisen as a therapy for regenerating damaged or diseased tissues. The most common strategy relies in the use of three-dimensional (3D) scaffolds, in combination with a cell source and signaling factors (Nerem, 2007). The scaffold should provide the architecture to guide new tissue formation, allowing cell-cell and cell-matrix interactions (Chen et al., 2013). In order to achieve this purpose scaffold morphology should have a geometry of interconnected pores to allow cell seeding, proliferation, extracellular matrix (ECM) formation, diffusion of physiological nutrients and removal of metabolic waste products (Mikos et al., 1993).

Scaffold mechanical properties are an important issue concerning tissue and biomedical engineering, as in addition to protect the cells when their extracellular matrix is still not developed, they regulate the biomechanical environment that offers stimulatory cues providing a better integration with the surrounding tissue. In particular in the regeneration of musculoskeletal tissues, like cartilage and bone, supported loads provide cues for the right gene expression and synthesis of ECM (Huang et al., 2010b; Kelly and Jacobs, 2010; McCullen et al., 2010; Riehl et al., 2012).

Polymer materials with a wide range of mechanical stiffness and viscoelastic properties can be prepared and polymer scaffolds can be designed to match the mechanical properties of living musculoskeletal tissues (Puppi et al., 2010). The evolution of mechanical properties of scaffold-cells culture during culture, due to the formation of synthetized ECM is an important parameter characterizing cell growth and differentiation (Khatiwala et al., 2006). The right production of ECM *in vitro* might require to perform culture under dynamic loading conditions. This has been performed by the development of a broad plethora of bioreactors which induce mechanical stimulus in different ways, such as confined or unconfined compression and hydrostatic pressure, among others (Knecht et al., 2006; Schulz and Bader, 2007; Wong M Fau - Carter and Carter, 2003). It was found that cyclic applications of these stimuli generally produce higher differentiation responses due to a cascade of signaling events that has been called mechanotransduction (Chen et al., 2009; De Croos et al., 2006; Huang et al., 2010a; Mahmoudifar and Doran, 2010).

Prediction of the mechanical behavior, ultimate properties and fatigue resistance of a polymer scaffolds implanted in the host tissue is an important issue that has been addressed insufficiently

in the literature. The properties of the dry scaffold are not representative of that of the scaffold with the pores filled by a growing tissue or simply by a fluid. In dry scaffolds, mechanical properties mainly depend on its inner morphology, in particular pore size, geometry and interconnectivity (Spiller et al., 2008). However, in the scaffold immersed in a liquid medium other factors can influence the mechanical properties as well, such as the hydrodynamics and permeability inside the scaffold. Since compressibility of water is low, any factor limiting water permeation through the material is expected to increase apparent scaffold stiffness, in particular under cyclic loading, which means that the influence of liquid media, such as cell culture medium cannot be disregarded. Nevertheless, to our knowledge, only few investigations deal with the description of the mechanical behavior of polymer scaffolds under liquid environment (Blasi et al., 2005; Hutmacher et al., 2001) and none under cyclic loading in aqueous media.

The resistance of the material to mechanical fatigue can be influenced by several factors such as mechanical loading history, environmental conditions, polymer composition and to certain aspects of stress-strain constitutive behavior (Mars, 2004). Several mathematical models firstly developed to predict metallic materials and composites during load-recovery cycles such as Coffin–Manson, Smith-Watson-Topper (SWT) or Morrow models (Ince and Glinka, 2011). Coffin-Manson model is based on the plastic strain range measured by subtracting the elastic strain range from the total strain range from the middle of the mechanical hysterics loop ($\Delta\varepsilon_t$), while SWT model assumes that the fatigue life cycle for any situation of mean stress depends on the product of the maximum stress (σ_{max}) and $\Delta\varepsilon_t$ (Bourago et al., 2011). 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 behavior (Morrow, 1965).

Two failure regimes can be identified in thermoplastic materials: the low regime cycle, where the material fails after a low number of cycles and is the main failure mechanism is heat generate during the load-recovery cycle and is called the *thermally dominated domain.* The second regime occurs at high stress and the high cycle regime at low stress, where the polymer can hold out a large number of cycles before failure. In this regime, the observed materials failure is britle of

nature and only litle energy dissipation is found in the hysteresis loops and is called *mechanically dominated domain* (Janssen et al., 2008).

Glassy polymers such polycarbonate, poly(methyl methacrylate) or poly(vinyl chloride) as well as semicrystalline polymers like poly(tetrafluorethylene), poly(oxymethylene) or high density polypropylene, has been reported that failure is due to the thermally dominated domain (Janssen et al., 2008). Fu et al. (Fu et al., 2013) reported that compressive strength of bioactive scaffolds decreases with the porosity increase present in the samples. Moreover, fatigue performance of bovine bone during cyclic loading results in a reduction of the elastic modulus and accumulation of residual strain leading to a progressive reduction of fatigue life with increasing stress levels (Ganguly et al., 2004).

Scaffold testing in conditions that simulate in some extent the situation during cell culture seems therefore to be an important issue for a correct interpretation of stress transmission to the cells cultured in bioreactors.

In vivo ECM provides generally mechanical resistance by the capability of ECM components of retaining water, articular cartilage being a good example (Schulz and Bader, 2007). This phenomenon will appear as well in cells cultured *in vitro* inside the pores of the scaffold, seeded alone or encapsulated in fibrin, collagen and others (Lee and Mooney, 2001). In the present work, fatigue life cycle of a poly-ε-caprolactone, PCL, scaffold with and without fibrin as filler of the pore structure was characterized both dry and immersed in liquid water; in order to provide some insight in the fatigue behavior of the scaffolds for application in cell cultures under dynamic loading.

Materials and methods

Materials: Poly-ε-caprolactone (PCL, molecular weight of 43-50 kDa) and dioxan were purchased from *Sigma-Aldrich*. Poly(ethyl methacrylate) (PEMA - Elvacite 2043) in the shape of spheres with mean diameter of 200 μm was purchased from *Lucite*. Fibrinogen from human plasma 50-70% protein (≥80% of protein is clottable) and thrombin from human plasma lyophilized powder, ≥2,000 NIH units/mg protein (E1%/280, 18.3) were purchased from *Sigma-Aldrich* and glutaraldehyde (50 % H_2O) was purchased from *Panreac*.

Sample preparation: PCL was dissolved in dioxan (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. Dioxan was extracted from the frozen plates with ethanol at - 20 ^{0}C for three days, changing ethanol every day. Porogen leaching was performed in ethanol at 40 ^{0}C for one day. The porous samples were cut into cylinders with 6 mm diameter and a thickness of approximately of 2 mm. To achieve a complete removal of the porogen, further leaching for each cylinder was performed in ethanol at 40 ºC for nine more days, with daily change of ethanol.

A fibrinogen solution with concentration of 20 μg/ml in saline solution and a thrombin solution of 10 U/ml in TBS with 20 mM of $CaCl_2$ were prepared. Two chromatography syringes with beveled needles were prepared containing 20 μl of fibrinogen or thrombin solutions each. Finally, both solutions were injected in the PCL scaffold and fibrin was allowed to coagulate for 1 h at 37 ^{0}C and subsequently kept in distilled water for no more than 24 hours before the tests.

Characterization: Sample overall porosity, ϕ, was calculated by weighing the scaffolds after complete filling the pores with ethanol. The sample was sealed in a container under high vacuum in which ethanol was injected. Porosity was determined by:

$$\phi = \frac{V_p}{V_p + V_{PCL}}$$

$$V_p = \frac{(m_w - m_d)}{\rho_{EtOH}}$$

$$V_{PCL} = \frac{m_d}{\rho_{PCL}} \qquad (1)$$

where m_d is the dry weight of the sample, m_w the weight with the sample filled in ethanol, V_p the volume of pores, V_{PCL} the volume occupied by the polymer and $\rho_{EtOH} = 0.785$ g/cm^3 and $\rho_{PCL} = 1.135$ g/cm^3 are the densities of ethanol and PCL at room temperature. Porosity values are the average of 5 measurements.

Sample microstructure was assessed by scanning electron microscopy. PCL filled with fibrin was fixed with a 2.5% glutaraldehyde (GA) solution for 1 h at 4 ^{0}C. CryoSEM was performed in a *JEOL JSM-5410* equipment. Water was sublimated at 5 kV, -50 ^{0}C for 20 min, and carbon-sputtered inside SEM chamber before analyses. PCL pristine samples were coated with a thin gold layer using a sputter coating (*Polaron*, model SC502) and their morphology was analyzed using aforementioned equipment.

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 (~25 ºC). In fatigue experiment, samples were submitted at a compressive-strain cycle load up to 1000 cycles (or until material reaches the plastic plateau) at a strain of 15%. Strain deformation was measured by machine cross-head displacement and mechanical stress and strain parameters were obtained on an average of five measurements. The mechanical experiments were performed in dry PCL samples and in PCL and PCL filled with fibrin samples immersed in deionized water. Samples uptake was performed through the injection of water with the help of a chromatography syringe with beveled needles, in a similar process of fibrin filling described above. In order to ensure the maximum uptake, samples were immersed in a water bath and placed in chamber (Vacuum-Temp from Selecta) under vacuum conditions (10^{-2} mmHg) until the sample drops to the bottom of the bath, and only these samples were submitted to the mechanical experiments.

Results and Discussion

The pore architecture of the PCL scaffolds consists in a double porosity: macropores obtained with the porogen spheres with diameters in the order of 200 microns and pores in the order of few microns produced by the freeze extraction technique (Figure 1) that interconnect the bigger ones. This double pore structure allows producing scaffolds with quite high porosity that have been previously proposed for cartilage and bone engineering (Gamboa-Martínez et al., 2011; Izal et al., 2012; Santamaría et al., 2012). Microporosity favors permeability of the scaffold to nutrients and waste products of cell metabolism and can be used to retain different active components (Deplaine et al., 2010; Lebourg et al., 2010a; Lebourg et al., 2010b). Nevertheless,

the apparent stiffness of the scaffold becomes significantly smaller than in similar sponges lacking microporosity, also used in cartilage engineering (Martinez-Diaz et al., 2010; Olmedilla et al., 2012).

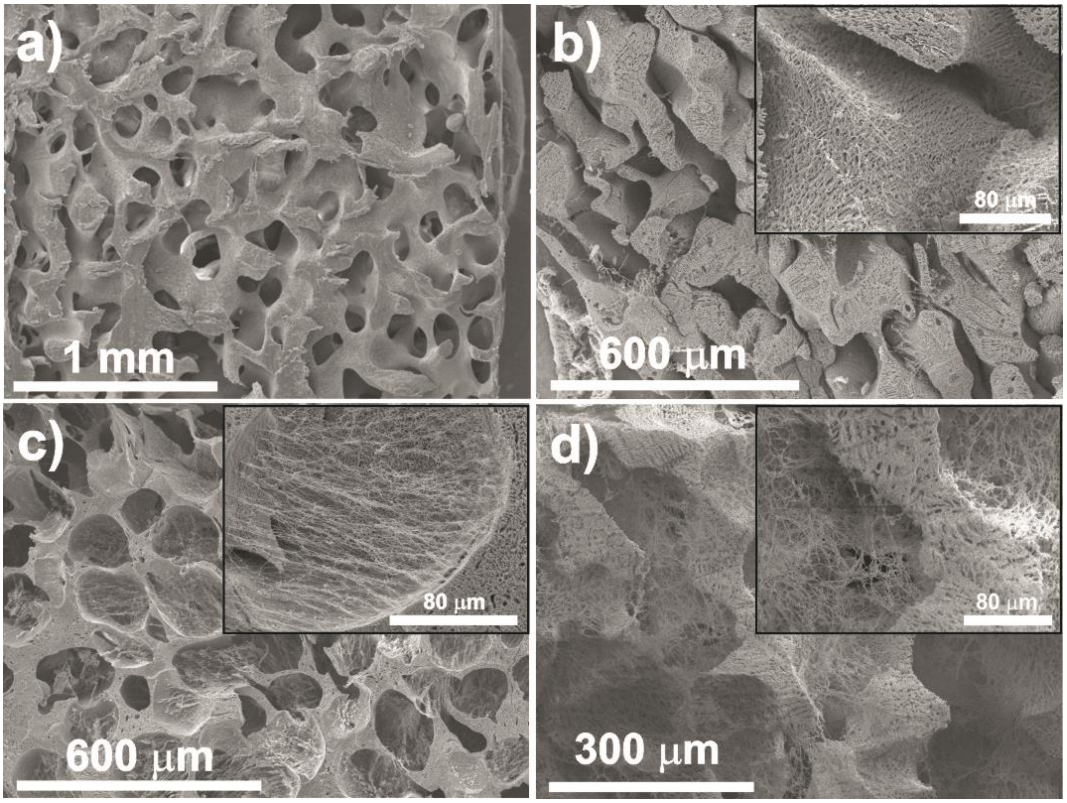

Figure 1 –

These samples with and without fibrin were submitted to cyclic mechanical loading under compressive mode up to a 15% strain, which is a typical deformation in experiments with bioreactors for chondrogenic differentiation (Appelman et al., 2009; Michalopoulos et al., 2012; Nicodemus and Bryant, 2008). Stress-strain curves in 10 consecutive loading-unloading cycles are shown in Figure 2a for the dry sample. The difference between the first and second cycle indicates that some of the PCL trabeculae suffer permanent deformation during the first compression ramp. In successive cycles, the continuous increase of irreversible processes leading to permanent deformation is reflected in a slight decrease of the maximum stress reached in each stress-strain loop (Figure 2c) and in the continuous narrowing of the mechanical hysteresis cycle. It is also worth noticing that dry sample microstructure suffers slightly changes after a few load-

recover cycles, being the porous structure more compact (figure 1a and b) and in the case of the PCL filled with fibrin and measured immersed in water, the fibrin fibrillar structure is destroyed (figure 1 c and d). Macropores partially collapse although an interconnected pore structure still remains, with the open macropores presenting a more oval shape. The micropore structure, on the other hand, do not seems to be affected (see the inset in Figure 2b) in this situation.

PCL is quite hydrophobous with a contact angle of ~ 92º (Little et al., 2009) and filling the pores of the sponge with water requires the application of high vacuum to extract completely air from the micropores before introducing water. Simultaneous injection of fibrinogen and thrombin solutions into the pores and further coagulation allows producing a fibrin gel homogeneously distributed in the whole pore volume. This gel has a nanofibrillar structure, as observed in CryoSEM (Figure 2c) after sublimating the water from the sample. Filling the pores with fibrin facilitates water diffusion through the scaffold.

The mechanical behavior of the samples immersed in water is quite different to the one obtained for dry PCL scaffolds. Dry samples has a maximum stress almost two times higher than immersed ones, this behavior is probably related to the small amount of water absorbed by the PCL polymer (Olmedilla et al., 2012) that consequently act as plasticizer, contributing for material softening (figure 2c).

The shape of the stress-strain plot reveals a larger curvature of the recovery data up to zero strain with respect to the dry sample, indicating a lower permanent deformation and lower contribution of viscoelastic effect (figure 2 a and b). This fact is supported by the SEM images shown in Figure 1d, presenting the macropores nearly unaffected by the mechanical compression experiment. After 10 loading-unloading cycles it was observed that the fibrin starts to lose its characteristic fibrillar structure, suggesting a weak bonding between the fibrin and the PCL scaffold (Figure 1d). The small but continuous decrease of the maximum stress in the successive load-recovery cycles is also apparent in the samples tested in immersion (Figure 2c).

Figure 2 –

Morrow Energy Model: Plastic Strain Energy Density–Life Model

The plastic strain energy density can be physically interpreted as distortion energy associated to the change in shape of a volume element and related to permanent deformation, particularly under conditions of ductile behavior. It can be evaluated as the inner area (W_p) of the stress-strain hysteresis loop for a compressive experiments. Morrow's model describes fatigue behavior in terms of the plastic strain energy density (Kanchanomai and Mutoh, 2004):

$$N_f^m W_p = C \quad (2)$$

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.

Mechanical behavior obtained for dry PCL and immersed PCL with fibrin samples submitted to compressive strain load is presented in figure 3. The plot of Figure 3, shows that Morrow's model can predict with accuracy the decrease of the overall equivalent behavior similar to plastic strain energy of PCL scaffolds at least up to 100 deformation cycles. The experimental results were fitted to equation (2) to characterize material behavior to the mechanical cycle loading before collapse of the samples. The calculated lines are also represented in Figure 3 together with the experimental data and fitting parameters are presented in table 1. It is observed a general decrease of the fatigue exponent and coefficient for the samples immersed in deionized water with and without fibrin with respect to the sample tested under dry conditions (table 1). Moreover, the m value found for the immersed PCL and immersed PCL with fibrin are quite similar, which suggests that the main contribution for samples mechanical performance is given by water. Deviation from the straight line predicted by Morrow's equation is considered the fatigue failure criterion (Kanchanomai and Mutoh, 2004). It was observed that dry PCL stops to follow the mechanical loading cycle after 100 cycles, which means that the sample takes more time to recover to the initial position ($\varepsilon = 0$ %) then the uncompressing loading speed. Finally, samples that were immersed in water (with and without fibrin) seems to deviate from Morrow's straight line after 500 cycles each means that the polymer scaffold reached to the plastic plateau, giving origin to a permanent material deformation. This behavior reflects the relevant role of the

aqueous filling in the of PCL scaffolds mechanical behavior, promoting a homogeneous distribution of the applied mechanical stress and diminishing stress concentration in the PCL trabeculae and work as a plasticizer, hich is the main reason for the premature collapse of the dry scaffold.

Figure 3 –

Table 1:

Based on the experimental data and in the fitting parameters obtained from Morrow's model (table 1), mechanical performance to mechanical cycle behavior was calculated and compared to the experimental results. The calculated number of cycles to reach scaffolds plastic deformation for the different samples are plotted *vs* the experimental load-recovery cycles (figure 4). In this figure, perfect correlation would be represented by data points lying on the solid diagonal line, and when the experimental data deviates more than 10 % from the theoretical line (Morrow's model prediction) (Kanchanomai and Mutoh, 2004). It is clear from figure 4 that Morrow´s mathematical model can successfully predict the load recovery cycles behavior of dry and immersed PCL specimens, with and without fibrin. Further, it is possible to observe that the main contribution for the increase of number of cycles before permanent deformation of PCL scaffolds is due to the presence of water that occupies the scaffolds, promoting a uniform distribution of the applied stress along the sample and not only localized in the trabeculae PCL and work as a plasticizer. When the porous scaffold is filled with air, local distribution of the stress mainly in the sample walls occurs and due to the porous nature of the scaffolds and the different geometry and dimensions of the pore walls (figure 1), a heterogeneous distribution of the applied mechanical stress occurs that leads to the collapse of the structure after 100 cycles, whereas for the sample immersed in water, mechanical stability is strongly increases up to 500 cycles. Is the balance between the homogeneous distribution of the mechanical stress and the plasticizer effect promoted by water that are in the origin of better mechanical performance of PCL scaffolds.

Finally, fibrin is commonly used as an extracellular matrix and in this work, the use of fibrin was to ensure the effect of this material in the mechanical performance of PCL scaffolds. It was observed that fibrin does not have a strong bonding to the scaffold and acts mainly as a plasticizer, especially for first cycles, contributing for sample softening.

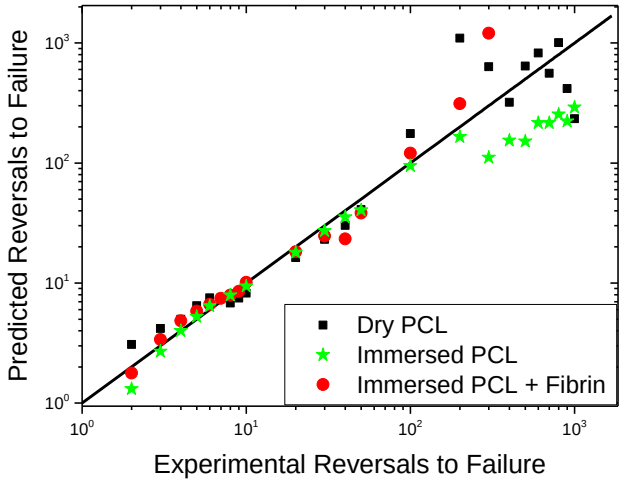

Figure 4 –

Conclusions

The mechanical stability of the scaffold is strongly important for tissue engineering applications and, in particular, in situations in which mechanical solicitations are applied. Fatigue life cycle of poly-ε-caprolactone scaffolds has been studied both with and without fibrin as filler of the pore structure and under dry and immersed in liquid water conditions. It has been proven that collapse of the samples occur after 100 cycles under dry conditions and the number of cycles increase to 500 i in the samples tested in immersed conditions. This fact is due to the more uniform stress distributions within the samples when the samples filled with water. It is also observed that the fibrin loading does not play a relevant role in the mechanical performance of the scaffolds.

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