THE EFFECT OF SALT FUSION PROCESSING VARIABLES ON STRUCTURAL, PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES OF POLY(GLYCEROL SEBACATE) SCAFFOLDS

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Abstract

Poly(glycerol sebacate), PGS, is a biodegradable elastomer recently proposed in the form of scaffolds for cardiac, vascular, cartilage or neural applications. In the present work, several processing variables for the fabrication of PGS scaffolds by the salt fusion method were systematically studied, namely the pre-polymer/porogen ratio, the salt particles average size, use of tetrahydrofuran to dissolve the pre-polymer for its injection in the porogen template, and the curing pressure. The effect of these variables on their structural, mechanical and biological properties was assessed to select those leading to optimal ones in terms of their potential performance in tissue engineering applications.

Keywords: poly(glycerol sebacate); salt templates; scaffold; mechanical modulus; cell culture

1. INTRODUCTION

Since tissues and organs which Young's moduli range 0.01 – 5 MPa (i.e. *soft* tissues) are considerably more abundant than *hard* tissues like bone or enamel (E ~ 5 - 20 GPa) [1], most biomaterials for soft tissue engineering are based in natural or synthetic polymers, either used as substrates or as combined strategies (3D scaffolds, composites, hydrogels…). Besides suitable mechanical properties, other features like lightweight, high versatility and fair biocompatibility have led to an intensive research on and use of elastomers, especially polyesters like polylactic acid [2,3], polycaprolactone [4,5] polyethylene terephthalate [6] or polyhydroxyalkanoates [7,8].

In pursuit of developing cheaper and more versatile polyesters, Wang and collaborators developed in 2002 a polymer from the mild crosslinking of glycerol and sebacic acid, which biodegradation and biocompatibility could be tailored via minor changes in the synthesis parameters [9]. Although the synthesis and enzymatic decomposition of the so-called poly(glycerol sebacate), or PGS, had previously been described [10], its potential as a polyvalent material for tissue engineering applications unleashed a considerable interest about its use as myocardium patches [11-13], heart valve leaflets [14,15], synthetic blood vessels [16], adipose tissue substitute [17], nerve conduits [18] and more recently for immature bone regeneration [19-21].

It is remarkable that most of the aforementioned studies used PGS-based porous scaffolds or membranes, with a wide variety of three-dimensional cavity structures. Most strategies are based in the salt (NaCl) fusion method, while other authors opted for inverse hydroxyapatite moulds [22,23], ceramic templates [14] or electrospun membranes [14,24]. Despite the extensive use of partially-fused NaCl particles as porogen, the technical parameters for

obtaining the salt templates and curing the PGS within them have been overlooked or frequently considered a minor issue. This is probably due to the fact that most of the contemporary studies are based on the first mention of the procedure, where the NaCl particles were described as "*of appropriate size*" [9]. This led to a wide spectrum of particle size ranges, from 75-150 μm [21,25] to 350-500 μm [19], when indicated. Considering the remarkable differences in the bulk polymer properties depending on the curing temperature [12,26,27], the glycerol:sebacic acid ratio [23,27] or the method of curing [28], it can be inferred that some aspects of the PGS crosslinking within the NaCl moulds will probably have an effect on the corresponding scaffold and, thereby, on the behaviour of the lodged cells. Actually, Gao *et al.* [29] developed porous PGS scaffolds based on fused and non-fused salt moulds, revealing that certain factors could significantly impact the Young's modulus, porosity and growth rate of 3T3 cells cultured within them.

Hence, here we analysed systematically a set of factors like the size of NaCl particles, the pressure applied during sintering, the PGS-to-NaCl ratio and the use of an organic solvent to pour PGS into the moulds on the density, porosity, structural morphology, Young's modulus and biocompatibility of the scaffolds produced that way. Thus, the significant parameters during the fabrication of PGS sponges through salt fusion have been discriminated, and the optimal conditions unveiled.

2. MATERIALS AND METHODS

Preparation of PGS scaffolds

Poly(glycerol sebacate) scaffolds were prepared following a salt leaching procedure based on that reported by Gao *et al.* in 2006 [29]. Briefly, a viscous PGS pre-polymer was dispensed and allowed to cure in sintered salt templates so that micropores are origined from the salt crystals as the pre-polymer crosslinks, whereas smaller pores are likely due to evaporation of glycerol during PGS curing.

PGS pre-polymer (hereinafter referred to as pre-PGS) was previously obtained following the procedure described by Wang *et al.* in 2002 [9], which was later on implemented and refined by the authors [27]. An equimolar mixture of glycerol (VWR International) and sebacic acid (Sigma Aldrich) was stirred in a round-bottom three-way flask at 130ºC on a hot plate under an inert nitrogen atmosphere for 24 h. This assembly ensured that monomers did not react with external agents and avoided any overpressure inside, since the gas left the flask assembly through a needle after bubbling through water in a cold trap. Water resulting as by-product of the polycondensation of the reactants condensed in the glass connection. This pre-polymerization yields ester bonds preferably leading to linear and branched chains.

In parallel, cylindrical sintered salt templates were produced, so that they could be afterwards cut to yield three replicates per cylinder. Briefly, 0.5 g of ground and sieved sodium chloride particles (NaCl) of diameters either of 150-212 μm or 212-250 μm were placed in the holes (7 mm in diameter and 10 mm high) of a Teflon® mould. Salt particles were next sintered in two steps: firstly using glass rods of the same diameter half introduced in the holes and a metal plate of 1400 g on top. Templates were compacted in this way for 10 min at room

temperature, and next the Teflon moulds were introduced (without the metal plate, to ensure the wetting of the salt particles) in a chamber previously pre-conditioned at a relative humidity of 97% by means of a potassium sulphate (K_2SO_4; Scharlau) saturated solution kept at 37ºC for 4 h. The porogen templates were next compacted for a second time in the humid chamber for 10 min. Finally, the sintered templates were dried in an oven at 130ºC up to 24 h, before further processing.

The quantity of pre-PGS that could be obtained in the pre-polymerization assembly was found to be very limited due to the heat flow provided by the hot plate below. Since a considerable quantity of pre-polymer was needed for the curing under different conditions to get the scaffolds, an oil (Thermo Haake SYNTH260, Sigma-Aldrich) bath pre-conditioned at 130ºC was used to lodge the round bottom flask with the equimolar mixture inside and overcome this inconvenient. The reaction could thus be carried out isothermally. A magnetic stirrer was placed in each container to ensure the stirring of the reactants mixture.

Two sets of pre-PGS, either pure or dissolved in tetrahydrofuran (THF; Sigma-Aldrich) at a 70/30 wt. ratio were injected in the moulds to wet the templates completely. The choice of the solvent was based on its high affinity with pre-PGS compared to other solvents proposed in the literature. The pre-polymer/porogen weight ratio was 1/27, 1/12, 1/9, 1/3 or 1/1, to extend the reach of the study by Gao *et* al. [29]. In the second set, the THF was allowed to evaporate for 15 min in a desiccator under continuous extraction and connected to a cold trap where it could condense, following injection. Next, the Teflon moulds were placed in a vacuum oven to allow the pre-PGS to cure at 130ºC for 48 h. The materials were then allowed to cool down, were withdrawn from the moulds, and cut into 1 mm-thick samples having 7 mm in diameter.

Thus, the processing variables under study were: the porogen size (150-212 μm or 212-250 μm), the templates compaction pressure (0.63, 1.25, 1.54 and 3.56 bar), the use or not of a solvent to inject the pre-polymer in the templates (pre-PGS pure or dissolved at a 70/30 wt. ratio in THF) and the pre-polymer/porogen weight ratio (1/27, 1/12, 1/9, 1/3 and 1/1).

Preparation of PGS films

Pre-PGS was poured in Teflon® open square moulds 1 mm in depth and sides 3 cm long. The curing was carried out in a vacuum oven at 130ºC for 48 h, in order to obtain solid 1 mm-thick films. Films were finally casted in 7 mm circular discs to be used as controls during the characterisation assays.

Rinsing protocol of PGS scaffolds and films

Cured PGS/salt cylinders were first rinsed in 50 mL of deionized (Mili-Q) water each to remove the salt porogen. The rinsing protocol was established by following the electric conductivity of the rinsing water with time: 2 days with two renewals/day under continuous orbital shaking were enough to ensure the removal of the salt crystals (resistivity > 18 MΩ·cm) in all tested samples at room temperature. Next, PGS scaffolds (and films) were rinsed, as described in [27], for two days in 10 ml tetrahydrofuran each with renewal the second day. This solvent was progressively changed to ethanol (Sigma-Aldrich) on the third day and to deionized water on the fourth day. Samples were finally allowed to dry under vacuum for 24 h (films) or 48 h (scaffolds).

Scaffold morphology analysis by SEM

Scanning electron microscopy (SEM) images of the scaffolds were obtained in a JSM-6300 JEOL device to estimate their porosity and interconnectivity. Materials were previously gold-coated and images of their sections were taken at 10 kV.

Porosity and density tests

The density of the PGS networks in scaffolds and films was quantified through Archimedes' principle with a Mettler Toledo AE-240 balance, equipped with a Mettler ME 33360 density accessory. For this purpose, samples were weighed in air and immersed in n-octane (reagent grade 98%, Sigma-Aldrich, $\rho_{n-octane}$=0.703 g·cm^{-3}) at room temperature. The density ρ was determined as the ratio of the weight of the sample in air, m, through the volume of n-octane displaced, $V_{displaced}$:

$$\rho = \frac{m}{V_{displaced}} = \frac{m}{\left(m - m_{imm}\right) \Big/ \rho_{n-octane}} \quad (1)$$

where m_{imm} is the weight of the sample immersed in n-octane.

As for the scaffolds, samples were weighed in air before (m) and after n-octane perfusion within their pores (m_{perf}) and next immersed in n-octane (m_{imm}). Perfusion of n-octane was carried out by immersing the scaffolds in a vial containing the solvent, and placing it in a desiccator working at continuous gas extraction for 3 min. To quantify the total porosity, the following equation was used, which relates the volume of pores (V_{pores}) with the total volume (V_{scff}+V_{pores}). Three replicates of each scaffold typology were used to calculate the mean porosity and density.

$$\pi = \frac{V_{pores}}{V_{scff} + V_{pores}} = \frac{m_{perf} - m}{m_{perf} - m_{imm}} \quad (2)$$

Mechanical compression tests

Mechanical compression tests were performed on cylindrical porous scaffolds and solid discs obtained from films, from 0 to 1500 mN at 100 $mNmin^{-1}$, at room temperature.. Three replicates per condition were measured. A Seiko TMA/SS6000 device (Seiko TMA, SS6000) was used for this purpose. The compressive elastic moduli, *E*, were obtained from the initial slope of the stress-strain curves, after disregarding the initial convex zone due to lack of parallelism of the surfaces, in order to ensure that the load was homogeneously distributed.

Cell culture in PGS scaffolds

L929 mouse fibroblast cells (C34/An connective tissue; Sigma Aldrich) were used for the cell viability study. Six replicates per synthesis condition and culture time (1, 2 and 4 days) were used. To sanitize the samples, they were soaked for 1 h in vials with 70% ethanol, and next transferred to culture plates under sterile conditions to be progressively soaked in 70, 50, 30% ethanol and pure deionized water (10 min in each). The samples were then kept in culture medium in an incubator (Thermo Fisher scientific, 3111 model) at 37ºC, 5% CO_2 until cell seeding. Culture medium was prepared with Dulbecco's Modified Eagle's Medium high glucose (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Innoprot).

L929 fibroblast in their 11^{th} passage were seeded on all materials as 5 µl droplets, each one containing $2x10^4$ cells. Next, materials were incubated for 45 min to facilitate cells attachment, and subsequently wells were filled with 1 ml of fresh medium each. The culture

environment was kept at 37ºC and 5% of CO_2 up to 4 days, changing the medium every day. After withdrawal, samples were transferred to new culture plates to be frozen at-80º C to stop cellular activity until further experiments.

DNA quantification assay

A DNA quantification assay was carried out to quantify the total amount of DNA in each scaffold sample and control film. Samples were incubated for 16 h at 60ºC in 250 µl of Proteinase K (Roche) solution at 5 %vol. in DPBS (Dulbecco's Phosphate Buffered Saline). Next, PicoGreen reactant (Invitrogen) was prepared and the assay was carried out following the manufacturer's instructions. Fluorescence was read at a wavelength of 520 nm in a Victor 3 fluorescence multiplate reader (Perkin Elmer).

Statistical analysis

In order to find the correlation between the synthesis parameters and different outputs, a two-way ANOVA test was applied to data resulting from the density, porosity and cell proliferation tests, with further multiple mean comparisons (Fisher's LSD test) when necessary. Because several parameters were under study at the same time, special attention was paid to interaction effects. Normality of data was assessed through the analysis of residuals for all samples.

3. RESULTS AND DISCUSSION

In this work, several sets of poly(glycerol) scaffolds were successfully obtained by varying the following synthesis conditions: the salt size, the salt templates compaction pressure, the use of THF as solvent for the pre-polymer injection in the templates and the pre-polymer/porogen weight ratio.

It is important to highlight that, to the best of our knowledge, this is the first time that an oil reactor is used to overcome the inconvenience of temperature homogeneity of the PGS reactants mixture in order to obtain larger batches of the pre-polymer, and also the first time the measurement of water conductivity is used to establish the rinsing procedure to ensure the total elimination of the porogen.

Morphological parameters obtained from SEM images and porosity tests, density values and elastic moduli, and DNA quantities after a fibroblasts culture were obtained to establish a correlation with the synthesis conditions. Shown below is the effect of each synthesis variable on the PGS scaffolds' properties. In each section, all synthesis conditions were fixed except the one under evaluation.

Variables related to the salt porogen templates

Porogen size

Salt diameters were of 150-212 μm and 212-250 μm, and PGS/salt weight ratios were 1/12, 1/9, and 1/3, based on the hypothesis that all could plausibly lead to pores of appropriate dimensions to host cells. The rest of the synthesis conditions were fixed: the salt templates were sintered with a weight of 3.56 bar and pre-PGS was used pure for the injection. As SEM images in Figure 1 show, it does not seem that the tested porogen sizes have a significant

effect on the scaffold morphology in terms of porosity and interconnectivity. Pores of around 200 μm in diameter are in general connected to others through 50-100 μm-sized throats, in occasions leading to greater pores and open structures (see c and e as example). Salt particles of 212-250 μm were preferred, though, to prepare the rest of the scaffolds since the fraction of porogen particles of 150-212 μm was scarce as obtained by grounding and sieving table salt.

Pressure applied in the compaction of the salt templates

Four different weights were used to sinter the salt templates: 0.63, 1.25, 1.54 and 3.56 bar. The rest of the synthesis conditions were fixed: the pre-PGS/porogen weight ratio was 1/3 and the PGS pre-polymer was used pure in the injection. SEM images are shown in Figure 2. SEM images did not show significant differences in terms of interconnectivity of the pores, so the pressure applied in the salt sintering was discarded as a variable for the synthesis procedure, at least in the range of pressures tested herein. Thus, the pressure applied in the following sections was set up at 3.56 bar.

Variables related to the injection of pre-PGS in the salt templates

Pre-PGS/salt ratio

The following pre-PGS/salt weight ratios were tested: 1/1, 1/3, 1/9, 1/12 and 1/27. The pre-polymer was dissolved in THF for the injection in all cases. SEM images in Figure 3 show the morphologies of the scaffolds obtained. Qualitatively, porosity increases as the pre-PGS/salt ratio decreases (further evidence can be found below in this manuscript). Indeed, very few pores were obtained with the 1/1 ratio, and consequently this ratio was discarded for further analyses. It cannot ruled out, though, that other drying processes such as

lyophilisation could lead to a more open and porous structure. However, on the other end, the 1/27 ratio was also discarded since one-piece scaffolds could only be obtained if the pre-polymer was dissolved in THF prior to injection, but not when used pure.

Dissolution of the pre-polymer in THF

Figure 4 shows the SEM images of scaffolds obtained by injecting either the viscous pure pre-PGS or its solution in THF. 1/12, 1/9 and 1/3 pre-PGS/salt wt. ratios were compared to evaluate the effect of this condition. According to these images, dissolution of the pre-polymer in THF did not seem to have a significant effect on the morphology of the scaffolds (but did have a strong effect when the pre-PGS/salt decreased to 1/27). However, it was decided to dissolve always the pre-polymer in further analyses because of the much easier manipulability of the pre-polymer during injection.

Porosity, density and mechanical compression measurements as well as a DNA quantification assay were next performed to correlate quantitatively the effect of these last two processing variables on the scaffolds properties.

Porosity and density

To estimate quantitatively the influence of the pre-PGS/salt ratio and that of the dissolution of the pre-polymer in THF on the morphology of the scaffolds, their porosity and density were measured, as shown in Table 1.

According to these results, the density of PGS networks is independent of the synthesis conditions, and is identical in all cases to the value of casted films. However, porosity does depend on them. The pre-PGS/salt ratio resulted to play a significant effect on the porosity (p-value=0.001), while the effect of the concentration of the pre-polymer in THF was not

significant. Thus, the values for each pre-PGS/salt ratio were averaged considering both sets of scaffolds, those obtained with and without THF (see last column in Table 1). The statistical analysis of these values shows that 1/12 and 1/9 pre-PGS/salt ratios yielded scaffolds of similar porosity (around 75%), higher than those obtained when a 1/3 ratio was used.

Elastic properties

Figure 5a shows the average stress-strain profiles obtained for each set of PGS scaffolds. These curves are characteristic of polymeric scaffolds, showing two elastic (linear) deformation regions, at low and high strain, the second one with a steeper slope, and a typical *plateau* between them, when the progressive collapse of the pores occurs. The elastic modulus, E, was calculated as the slope of the linear regression at low strain, Figure 5(b), in each set of samples. As can be observed, a pre-PGS/salt ratio of 1/3 leads to stiffer scaffolds regardless of dissolving the pre-polymer in THF or not. This is certainly due to their lower porosity in comparison to the others.

Previously reported elastic modulus of PGS-based porous constructs fabricated under similar conditions do match data obtained for these scaffolds [21,23]. Furthermore, the moduli does coincide with the predicted so-called reduced tensile modulus, E_r, which is calculated according to [30] by normalization taking into account its previously measured porosity:

$$E_r = E_{bulk}\left(1-\pi\right)^2 \quad \textbf{(3)}$$

Here, E_{bulk} is the modulus obtained for the bulk polymer, for PGS films in this case, which value was estimated around 250 kPa [31,32]. Interestingly, such values are found only for solid films or discs obtained for short-term (< 25 h) or vacuum-free curing processes, where a noticeable amount of glycerol is still present in the bulk. For longer curing periods [26],

increased initial sebacic acid:glycerol ratios [31] or when unreacted reagents and free oligomers are thoroughly removed [27], the Young's moduli are one order of magnitude higher. Thus, the unreacted glycerol content in crosslinked PGS constructs must be considered as a main factor influencing key physicochemical parameters. We strongly encourage washing material structures based on cured PGS in an affine solvent prior to its application, even when a high degree of conversion is reached in the curing reaction.

Biological performance

The PicoGreen assay allowed to quantify the total amount of DNA present in each set of scaffolds and on the disc-shaped films used as controls, Figure 6. Mean values are represented against culture time, for scaffolds prepared from (a) dissolved and (b) pure pre-PGS, injected in salt templates at different ratios.

The interaction effect between both variables (the pre-PGS/porogen ratio and dissolving or not pre-PGS in THF), was found to be statistically significant (p-value < 0.05) regarding cell proliferation. The relationship between each variable and culture time was significant too (p-value < 0.001), so that the effect of them cannot be assessed individually. According to Figure 6, it seems that cells preferably grow in scaffolds prepared from pre-PGS/salt ratios of 1/3 (no matter if dissolved or not in THF, in this case) or 1/9 (with the pre-PGS this time dissolved in THF). The rest of the scaffolds did not show any outstanding performance. In scaffolds obtained from 1/12 pre-PGS/salt ratio, the amount of DNA registered was lower, probably because their more porous structure yields slightly less PGS surface available for cell attachment or permits cell migration. Flat structures (films) showed the lowest amount of DNA and a characteristic short-term (1 to 4 d) stagnation in cell viability [27,33], clearly highlighting the role of a three-dimensional environment to upregulate cell growth.

4. CONCLUSIONS

The main processing variables associated with the fabrication of PGS scaffolds by curing the corresponding pre-polymer in a porogen template made of sintered salt particles were systematically followed, to evaluate their individual effect on the structural, physicochemical and biological properties of these scaffolds. In particular, the role of those parameters related to the salt porogen templates (the salt particles average size and sintering pressure) and with the injection of the pre-polymer in the porogen template (the pre-PGS/porogen ratio and the pre-PGS dissolution in tetrahydrofuran) was investigated.

In relation to the range of diameters of the salt particles used, no differences were observed in the morphology of the scaffolds and those with diameters between 212 and 250 μm were chosen because of its simpler gathering by sieving. As for the pressure applied in the sintering of the porogen template, it proved to be non-significant in terms of microstructure.

Regarding the pre-PGS/porogen ratio, qualitative differences were observed in the morphology of the scaffolds for the ratios used. The 1/3 wt. ratio resulted in less porous scaffolds compared to those from 1/9 and 1/12 proportions. Cells cultured in PGS scaffolds seem to colonize preferably less porous materials (1/3 wt. ratio, regardless of whether the pre-polymer is dissolved in tetrahydrofuran or not), likely because of a better balance of PGS surface per scaffold volume unit. The use of tetrahydrofuran for the injection of the pre-polymer in the template improves the manipulation of the mixture, resulting in more porous and homogeneous scaffolds, and subsequent higher cell colonization.

Thus, in view of the morphological parameters and physicochemical properties, and the ease of manipulation and biological behaviour, it can be stated that PGS scaffolds obtained from salt particles sized 212-250 μm and sintered at 3.56 bar, with its pre-PGS dissolved in THF

and injected in the template at a pre-PGS/salt wt. ratio of 1/3 for its curing at 130ºC for 48 h, seem to be the most promising for their application in tissue engineering strategies where PGS could be of interest for its elastomeric, hydrophobic, biodegradable and biocompatible features. It cannot be, though, ruled out that other combinations can be more beneficial for other applications.

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Figures

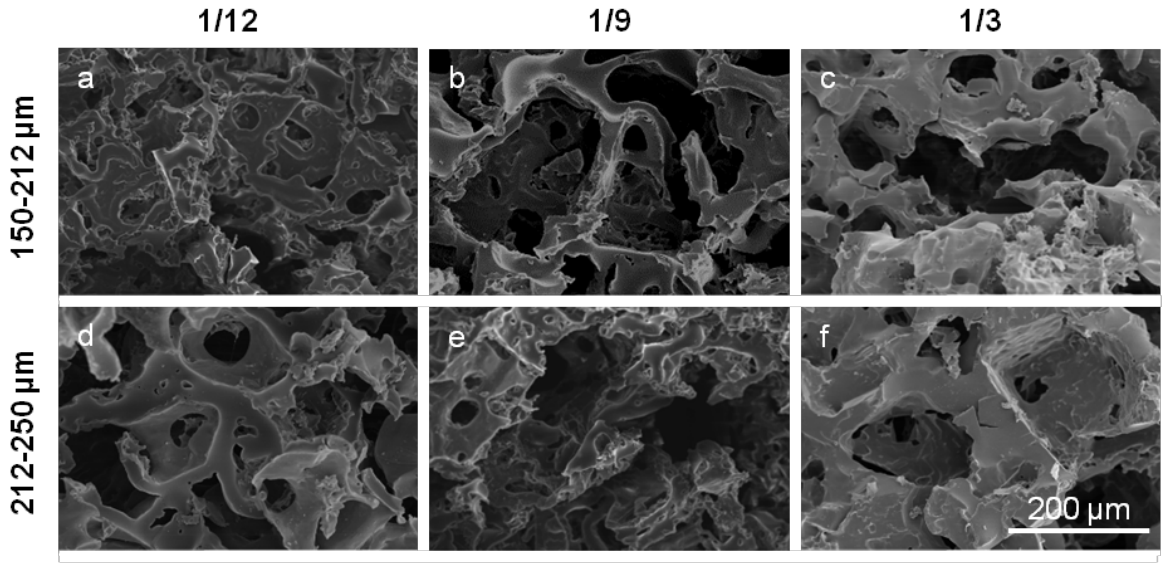


Figure 1. SEM images showing the influence of the particles size: (a-c) 150-212 µm and (d-f) 212-250 µm, and the pre-PGS/salt weight ratio on the scaffolds morphology: (a,d) 1/12, (b,e) 1/9 and (c,f) 1/3. The salt compaction pressure was 3.56 bar and pre-PGS was used pure for the injection in the templates. Scale bar: 200 µm.

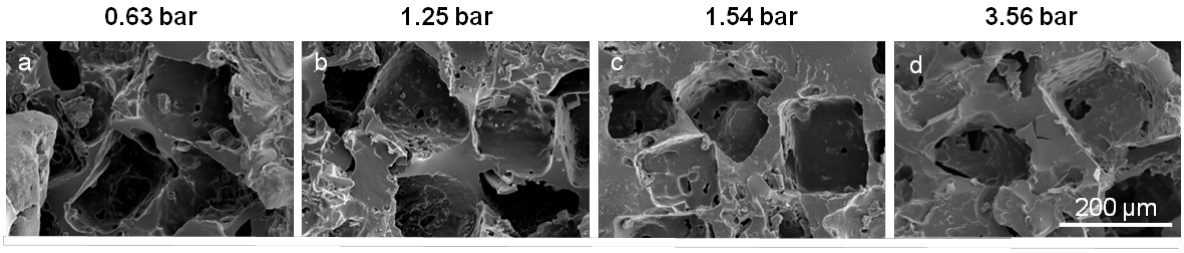


Figure 2. SEM images showing the influence of the pressure applied to prepare the porogen templates: (a) 0.63 bar, (b) 1.25 bar, (c) 1.54 bar and (d) 3.56 bar. The pre-PGS/porogen wt. ratio was 1/3 and pre-PGS was used pure in the injection. Scale bar: 200 µm.

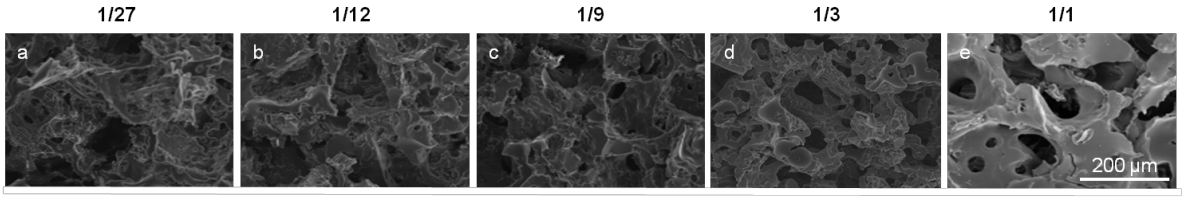


Figure 3. SEM images showing the influence of the pre-PGS/salt weight ratio on the morphology of the scaffolds: (a) 1/27, (b) 1/12, (c) 1/9, (d) 1/3 and (e) 1/1. The salt compaction pressure was 3.56 bar and pre-PGS was dissolved in THF for the injection in the templates. Scale bar: 200 µm.

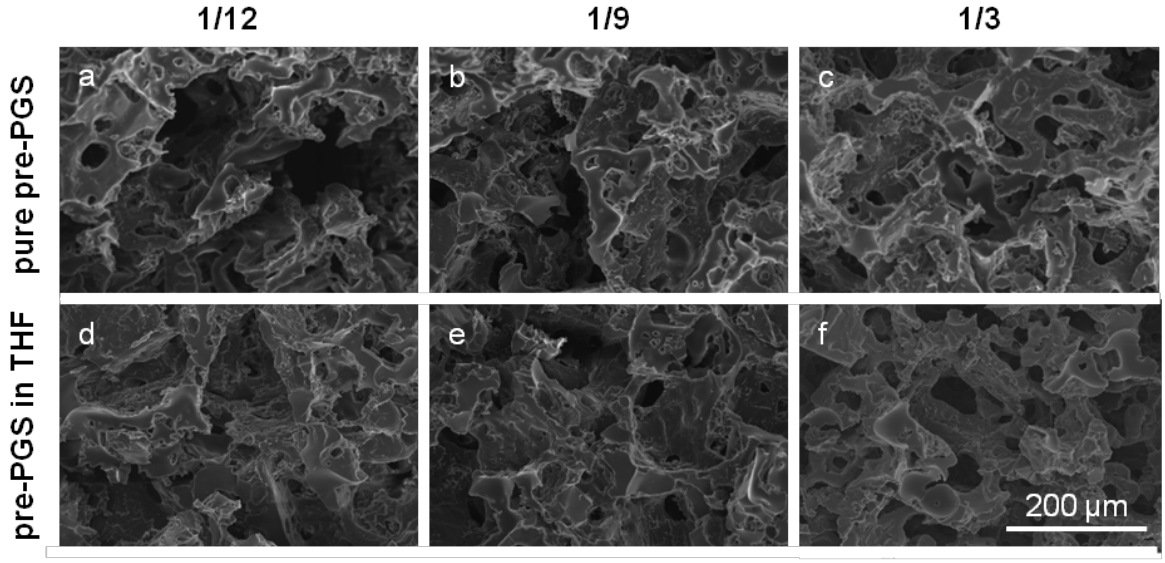
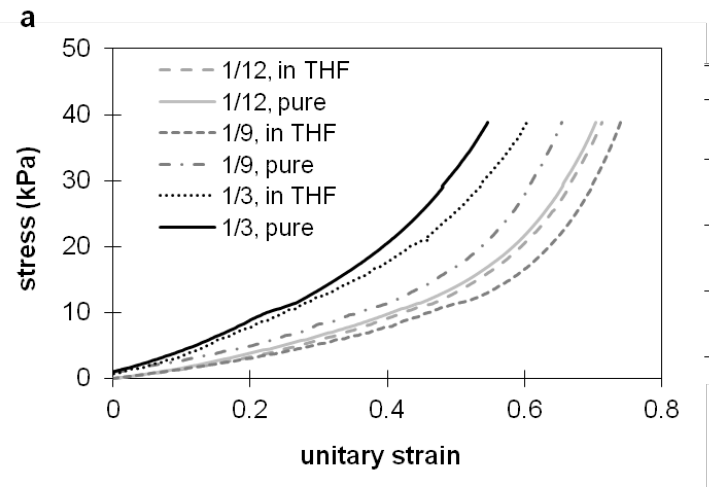


Figure 4. SEM images showing the structural effect of using pre-PGS in its pure form (a-c) or dissolving it in THF before injection (d-f), and injected at pre-PGS/salt ratios of (a,d) 1/12, (b,e) 1/9 and (c,f) 1/3. Scale bar: 200 µm.



b

samples		*E* (kPa)
pre-PGS/salt ratio	concentration of pre-PGS in THF	
1/12	70/30 wt	19.74 ± 0.19
	100%	16.38 ± 0.14
1/9	70/30 wt	19.56 ± 0.08
	100%	15.36 ± 0.09
1/3	70/30 wt	36.68 ± 0.32
	100%	36.22 ± 0.33

Figure 5. (a) Stress-strain curves for each set of scaffolds. (b) Elastic moduli, *E*, calculated as the slope of the initial linear fit, together with their standard error.

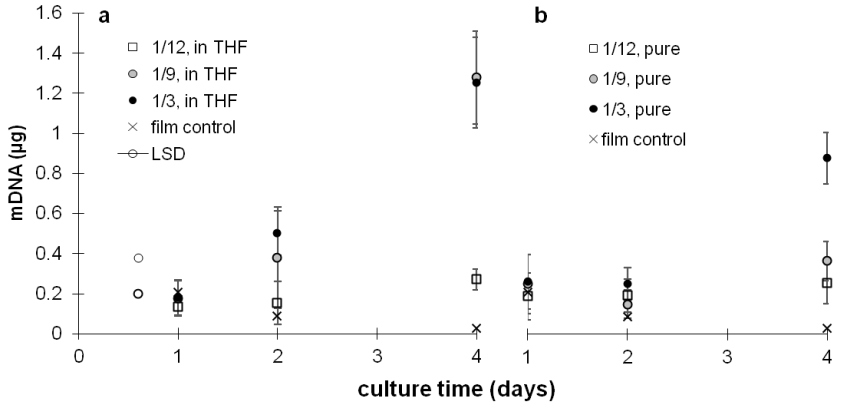


Figure 6. DNA mass quantification for each cultured set of scaffolds ± standard deviation *vs*. culture time: (a) scaffolds obtained from dissolved pre-PGS, and (b) from pure pre-PGS, injected in the templates at different pre-PGS/salt ratios. Films were used as control. LSD segment shows the distance below which two DNA quantity means cannot be considered significantly different (for both *a* and *b*).