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Additional Information

CORRELATING SYNTHESIS PARAMETERS WITH PHYSICOCHEMICAL PROPERTIES OF POLY(GLYCEROL SEBACATE)

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Abstract

Poly(glycerol sebacate), PGS, is an elastomeric biodegradable polyester increasingly proposed in a variety of biomedical applications. It is prepared by polycondensation of sebacic acid and glycerol in a first stage in which a prepolymer is obtained, followed by a curing to conveniently crosslink it. In this work, synthesis parameters such as the curing temperature and time, and the molar ratio between reactants, were systematically varied to correlate them with the physicochemical properties of the resulting polymer networks. The efficiency of each manufacturing process was quantified through the relative mass effectively crosslinked and insoluble in tetrahydrofuran. Infrared spectra gave an estimation of the ratio of non-condensed polar terminal groups. These results were correlated with swelling results, which in turn provided the means to calculate the chains density through Flory-Rehner equilibrium swelling equation for lightly crosslinked polymers. The role of the synthesis parameters on the physical state of the resulting polymers, as well as their proneness to hydrolyze, were followed. The results

obtained highlight the relevance of rinsing them following synthesis, to remove non-crosslinked chains that easily diffuse to the surrounding medium. Curing under mild conditions equimolar mixtures of sebacic acid and glycerol proved to lead to poorly crosslinked swellable networks, which hydrolyze easily in bulk mode. Alternative molar ratios yield sticky and difficult to handle materials at higher polyol fractions in the reactive mixture, whilst an excess of acid terminal groups leads to a faster mass loss by hydrolysis in aqueous media together with surface salts deposition, concomitant with a lesser cell viability in *in vitro* culture. PGS synthesized from an equimolar ratio between reactants and cured at 130ºC or higher, for 48 h or longer, show suitable features for their use in tissue engineering applications where hydrophobic surface-degradable rubbers are required, without significant differences among them.

Keywords: poly(glycerol sebacate); biodegradable; elastomer; condensation; curing

1. INTRODUCTION

Functional, partial or total failure of tissues and organs is one of the most serious health concerns and economically costly. Traditional strategies involving the use of artificial prostheses and mechanical devices have improved and saved the lives of millions of patients, but entail a number of inconveniencies, such as long-term mechanical failure following implantation that can trigger an immune response in the host, damaging healthy tissues around the implant. Surgical reconstruction of tissues and organs is feasible in occasions, but pose the risk of surgical and postoperative complications and the development of malignant tumors in the affected areas [1].

Aiming to solve these issues, progress has been made in the tissue engineering field leading to the development of new polymeric biomaterials. Within this group, the most widely evolving in recent decades have been the synthetic biodegradable polymers, for their easier manufacture monitoring in a vast variety of porous structures and more precise tailoring of the degradation process against those of natural origin [2-4]. Furthermore, they are highly versatile in terms of controlling their physicochemical properties and the ease of adaptation to the desired application. Indeed, the mechanical properties and degradation rate of the implant are determined not only by its chemical composition but, to some extent, by the conditions of synthesis of the polymer [5].

Among the many bioresorbable synthetic polymers, poly(glycerol sebacate), PGS, is a tough, flexible and biodegradable elastomer with excellent biocompatibility and physical characteristics similar to biological soft tissues and has subsequently emerged for soft tissue engineering applications [6,7] and as drug carrier [8]. Its synthesis procedure was first reported by Wang *et al.* in 2002 [9] and later enhanced by Gao *et al.* [10]. PGS is able to stay and recover from deformation in dynamic environments and

furthermore displays shape memory, *i.e.* it is able to return after a phase of deformation to its original state thanks to the action of an external stimulus [11]. Another interesting feature is that, unlike other polymers, PGS is degraded mainly by surface erosion, due to its hydrophobic nature, undergoing a linear mass loss over time. This allows the polymer to largely retain its structural integrity and mechanical properties [12], and allows it to be used for controlled drug release throughout its degradation process.

The versatility of PGS synthesis, coupled with its excellent characteristics of biocompatibility and elasticity, make it a polymer of great interest in tissue engineering applications. One of the most important performance areas is the cardiovascular system, where it has been proposed for myocardial regeneration and reconstruction of blood vessels [13] or cardiac valves, where it is used together with other polymers such as collagen [14,15]. Similarly, PGS has also been developed for its use in cartilage tissue engineering, mainly for degenerative arthritis [16], or as regenerative solution to musculoskeletal problems, and the reconstruction of nerve pathways [17].

PGS is obtained by polycondensation of glycerol and sebacic acid [11]. Sebacic acid is a natural intermediary metabolite of ω fatty acid oxidation and long chain glycerol is one of the major components of lipids, both being US Food and Drug Administration approved monomers. The synthesis process comprises two stages: a first prepolymerization phase, resulting in a viscous prepolymer by formation of linear chains between reacting monomers, and a second curing step in which the chains intertwine to form the polymer network. The reaction for the formation of PGS is an esterification between a dicarboxylic acid, sebacic acid, and an alcohol, glycerol. The hydroxyl groups of the polyol act as initiators of the reaction. Thus, initially, a primary hydroxyl group of glycerol attacks a carboxyl group to form a monoester with a free carboxyl group and water as byproduct [18]. This monoester reacts next with another

primary group of glycerol to form another monoester, and so on with all available functional groups.

The prepolymerization is usually carried out at a temperature of about 120ºC-130ºC for 24 h while maintaining an inert atmosphere, usually nitrogen, though a microwave-assisted process that can be carried out in minutes with no need of purge gas has been reported [19]. The latter induces an intensive glycerol evaporation leading to stiffer PGS networks. The synthesis conditions of the curing stage may vary (120ºC/48 h in [9] or 130ºC/1 to 12 days in [12]), resulting in polymers with different mechanical properties and biodegradability [20] to fit with different applications. At this stage, the inert atmosphere is no longer required for crosslinking to occur. However, no rinsing or conditioning process following synthesis has been conducted anywhere, other than vacuum-drying before characterization or sterilization by UV radiation and soaking in growth medium before cultures [9]. Thus, the presence of unreacted monomers and especially non-crosslinked chains has not been yet considered in previous discussions.

The enormous interest that involves the use of this polymer in the tissue engineering field raises thus the question of the relationship between the physicochemical and biological characteristics and the mechanical behavior of PGS with its synthesis conditions, and which would be the optimal preparation protocol according to the targeted application. Herein, the purpose is thus to highlight the relevance of such rinsings following synthesis, and correlate the properties of the networks obtained with their structural parameters after removing the effect of non-crosslinked species. To do this, synthesis parameters such as the curing temperature and time, and the molar ratio between reactants, have been herein systematically varied and the polymeric networks obtained have been characterized after their thorough rinsing to eliminate any unreacted monomers and non-crosslinked chains.

2. MATERIALS AND METHODS

Preparation of PGS films

The synthesis of poly(glycerol sebacate) was carried out in two stages: an initial prepolymerization to allow the polycondensation of the reactants, glycerol (VWR International) and sebacic acid (Sigma-Aldrich) yielding ester bonds and thus branched chains, followed by a curing, when crosslinks eventually result in a three-dimensional network.

The prepolymerization assembly was based on a Dean-Stark assembly [21]. Briefly, an equimolar mixture of glycerol and sebacic acid was placed in a round-bottom three-way flask on a hot plate (Heidolph MR Hei-Standard) as described in [22], with a magnetic stirrer to provide agitation as the reaction takes place. A distillation setup was adapted to condense the water formed as a byproduct of the reactions. The second way was connected to a nitrogen supply, to create the inert atmosphere required by the reaction [23]. The latter was connected through a needle to a nitrogen trap containing water, so that the gas left the flask assembly through the needle by bubbling the water in the trap. This assembly ensured that monomers did not react with external agents and avoided any overpressure inside. The stirring was switched at 100 rpm as soon as the hot plate reached 130°C and these polymerization conditions were kept for 24 h.

The viscous prepolymer was then withdrawn from the flask and poured into Teflon® open square moulds 1 mm in depth, sides 30 mm long. The curing was carried out in a forced ventilation oven (J.P. Selecta Vaciotem) at 130ºC for 48 h, in order to obtain solid films 1 mm-thick.

This procedure was modified so as to correlate materials properties with polymerization conditions: a series of films was cured at different temperatures (110, 120, 130, 140 and 150ºC) for 48 h and another for different times (24, 48, 72 and 96 h) at 130°C; in a third set, half and twice the equimolar sebacic acid:glycerol (SA:G) ratio were compared, all of them cured at 130ºC/48 h (0.5:1, 1:1 and 2:1 SA:G molar mixtures).

Rinsing protocol and quantification of non-condensed reactants

Films were rinsed in tetrahydrofuran (THF; Sigma-Aldrich) to remove non-crosslinked chains and monomer residues, in an orbital shaker for 2 days with renewal each day. This solvent was progressively changed for ethanol (Sigma-Aldrich) the third day and samples were rinsed in pure ethanol another day. Ethanol was changed for deionized water for 2 days more. Samples were allowed to dry overnight at room conditions and next under vacuum for 24 h, and finally cut as required for each assay.

A Mettler AX 205 balance (Mettler-Toledo Inc., Columbus, OH, U.S.A.) with a sensitivity of 0.01 mg, was used to weigh dry samples 5 mm in diameter before and after these rinsing to quantify the mass loss, 3 replicates per sample type.

Fourier-Transform Infrared Spectroscopy scans

Fourier-transform infrared (FTIR) spectra were collected in a Thermo Nicolet Nexus FTIR spectrometer (Thermo Fischer Scientific Inc., Waltham, MA, USA), in the attenuated total reflection mode (ATR), to unveil differences between samples cured differently or prepared from different molar ratios between reactants. The spectra resulted from averages of 128 scans at 16 cm^{-1} resolution, between 600 and 4000 cm^{-1}. Specimens were 20 x 5 x 1 mm^3.

Wettability tests

The water contact angles (WCA) were measured on the surface of dry samples in the sessile drop mode. Samples were 20 x 5 x 1 mm^3. A Dataphysics OCA instrument (DataPhysics Instruments GmbH, Filderstadt, Germany) was used for this purpose. A minimum of ten 3 µl-drops of ultrapure water were analyzed for each sample type. WCA of wet samples (equilibrated in water) were also measured, after carefully removing the water on their surface.

Density tests

The previously mentioned balance, equipped with a Mettler ME 33360 density accessory kit, was used to determine the density of dry samples through Archimedes' principle. Samples, being 5 mm in diameter, were weighed in air and immersed in n-octane (reagent grade 98%, Aldrich, $\rho_{n\text{-}octane}$ = 0.703 gcm^{-3}) at room temperature. Samples swelling in n-octane was previously estimated until equilibration so as to confirm that swelling in it during measurements could be neglected (data not shown). The density, ρ, was determined as the ratio of the weight of the sample in air, $m_{in\ air}$, through the volume of *n*-octane displaced, $V_{displaced}$:

$$\rho = \frac{m_{in\,air}}{V_{displaced}} = \frac{m_{in\ air}}{\left(m_{in\ air} - m_{in\ n\text{-}octane}\right) / \rho_{n\text{-}octane}} \qquad \textbf{(1)}$$

where $m_{in\ n\text{-}octane}$ is the weight of the sample immersed in *n*-octane. Each weighing was done in triplicate for each sample type.

Swelling in water

Swelling in water was quantified by weighing small pieces (5 mm in diameter) of dry samples and at different time points, using the above mentioned balance, until

equilibration to constant weight at 37ºC. Measurements were done in triplicate. The equilibrium water content, EWC, was defined as $EWC = m_{water}/m$, m_{water} and m being the mass of water and dry mass of the sample, respectively.

The molecular mass between crosslinks (M_c) was calculated in each case using the density and elastic modulus of the samples through the equation described by Flory's molecular theory of rubber elasticity [24]:

$$E = 3\,\frac{n_c RT}{V} = 3\,\frac{\rho RT}{M_c} \qquad \textbf{(2)}$$

where n_c/V is the chains density, R is the universal gas constant and T is the temperature.

Flory-Huggins parameter, χ, was estimated for each sample type by Flory-Rehner equation [25], which relates the volume fraction of polymer at equilibrium swelling in water, $\phi = \frac{V}{V_{water}+V} = \frac{1/\rho}{EWC/\rho_{water}+1/\rho}$, and the elastic modulus, E:

$$0 = ln(1-\phi) + \phi + \chi\phi^2 + v_{water}\,\frac{n_c}{V}\,\phi^{1/3} \qquad \textbf{(3)}$$

where v_{water} is the molar volume of water and the 0 on the left hand stems from $\ln \hat{a}_{water}$, being $\hat{a}_{water} = 1$ in pure water. This is why swelling experiments were conducted in water and not in conventional media such as phosphate-buffered saline. On the other hand, it was intended to avoid any deposition of salts that would have affected the swelling results.

Mechanical compression tests

Mechanical compression tests were performed in a Seiko TMA/SS6000 device (Seiko Instruments Inc., Chiba, Japan), from 0.5 to 1500 mN at 100 mNmin^{-1}, at room temperature. Specimens were disk-shaped, 5 mm in diameter and 1 mm-thick, and each set consisted of five replicas. 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 on the surfaces, and until strains of 0.15.

Differential Scanning Calorimetry measurements

Differential Scanning Calorimetry (DSC) measurements were performed in a Perkin Elmer DSC 8000 device to correlate the chains density with the thermal properties of the samples. Each test specimen consisted of approximately 7 mg of material accurately weighed in a standard aluminum pan and sealed. The pans were cooled to -80ºC at 40ºC·min^{-1}, stabilized for 2 min and scanned up to 60ºC at 20ºC·min^{-1}, under nitrogen atmosphere.

Stability of PGS films in aqueous media

To assess the stability of crosslinked PGS films, *in vitro* acellular studies were conducted using water and a basic medium to accelerate degradation. Samples were 5 mm in diameter and 1 mm thick and three replicates per condition and time point were tested. Swelling and degradation experiments in cell growth medium were not conducted to avoid the attachment of organic molecules onto the surfaces of those samples with relatively high crosslinking density, which seem to impede the diffusion of ions into the polymeric network, as described in [20]. Besides, phosphate buffered saline (PBS) was also discarded to avoid any deposition of salts that could disguise an eventual mass loss.

Degradation experiments were carried out by immersing the samples in 2 ml of Milli-Q water or in a 0.01 M NaOH (Scharlau) basic aqueous medium to catalyze hydrolysis reactions, at 37ºC. A set of samples was withdrawn after 14 days and the rest after 28

days, next gently rinsed with Milli-Q water for 1 h to remove any surface residue and vacuum dried. The media were renewed after 14 days. Samples were dry weighed before and after the tests to quantify the mass loss, calculated as follows:

$$mass\ loss\ (\%) = \frac{m_0 - m_t}{m_0} \cdot 100 \qquad \textbf{(4)}$$

where m_0 and m_t are the initial weight and weight at each set time of each sample.

Fibroblasts culture

To condition the samples prior to cell culture, 5 mm-diameter discs of each type were washed for 5 min in an ethanol/water 70/30 mixture, changed for fresh mixture for 2 h more. Samples were then kept in culture medium for 24 h at 37°C. Culture medium was prepared with Dulbecco's Modified Eagle's Medium (DMEM) (Fisher) high glucose supplemented with 10% fetal bovine serum (FBS; Innoprot) and 1% penicillin-streptomycin (P/S; Innoprot).

L929 fibroblasts (from mouse C3H/An connective tissue, Sigma Aldrich) in their 10th passage were seeded on PGS discs as 50 ul droplets, each one containing 10^4 cells. The samples were incubated for 30 min, to allow cells to attach before filling the wells with 400 ul of fresh medium each. The culture environment was maintained at 37ºC and 5% CO_2 up to 7 days, changing the medium every day.

Cell viability assay

The non-destructive AlamarBlue viability assay was performed after 5 h, 4 and 7 days of cell culture, three replicates per sample type. This test has been used in other works to test cell viability on PGS samples [23,26,27]. Briefly, 40 ul of culture medium was withdrawn from each well and substituted by AlamarBlue reactive (Life Technologies). Samples were incubated protected from light for 4h at 37ºC and 5% CO_2. Then, the

supernatants were placed in a new 96-well plate and absorbances at wavelengths of 570 and 600 nm were read. 400 µl of fibroblasts culture medium were added to each well to pursue the culture. A blank of AlamarBlue reactant solution incubated in wells with acellular materials was scanned. A 100% reduced form of Alamar Blue worked as positive control; to prepare it, the AlamarBlue reactive was diluted ten times in culture medium and the solution was autoclaved for 15 minutes before measuring fluorescence.

Fluorescent nuclear staining

Cultured cells were rinsed in 0.1 M DPBS, fixed with 4% paraformaldehyde (Panreac) for 20 min at room temperature, and next washed twice with PBS at 4ºC. Next, the cellular samples were stained with Sudan Black, to avoid PGS autofluorescence, following the procedure described in [22]. To do this, the samples were incubated in Sudan Black (Sigma Aldrich) diluted to 0.3% in 70% ethanol for 15 min at 37ºC, followed by 4 rinses in DPBS.

Cells' membrane was then permeabilized with 200 ul per well of blocking buffer, based on 0.1 M PBS, 10% FBS and 1% Triton X 100 (Sigma Aldrich) for 30 min. Nuclei were stained for 10 min with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich), diluted 1/5000 in PBS. Finally, the samples were rinsed twice in PBS for 5 min and fluorescence images were taken in a Nikon Eclipse 80i microscope at 461 nm.

Cell morphology analysis by SEM

Scanning electron microscopy (SEM) images allowed observing fibroblasts morphology on the different samples. Cells were fixed at each set point with 3.5% glutaraldehyde (Electron Microscopy Science) in 0.1 M PBS for 1 h at 37ºC, washed twice with DPBS and with 0.1 M PB for 10 min, post-fixed with 2% osmium tetroxide (Aname) in 0.2 M

PB for 1 h in the dark and rinsed four times with cold Milli-Q water, 10 min each. Dehydration was performed by immersing the samples in increasing concentrations of ethanol in MilliQ water solutions (30%, 50%, 70%, 96%), 10 min each, and twice more at 96%. The dehydrated cells were critical-point dried by using an Autosambri 814 device (Rockville, MD), and sputter-coated with gold before observation under SEM (JSM-6300, JEOL) at 15 kV and 9 mm of working distance.

Statistical analysis

Results are expressed as mean ± standard deviation from at least three replicates. In all swelling in water, density and Young's modulus experiments, a multiple mean comparison test was used in order to reveal differences between groups of study. A first assumption of normality was made for the data distribution of all samples, and it was assessed through the analysis of the standardized skewness of each group. Due to the reduced sample size, three one-way ANOVA tests were performed (one for each factor of study: curing temperature, curing lapse and reagents ratio), and the honest significant differences, HSD, Tukey's test (confidence degree of 0.95) was selected for the multiple mean comparison, since this is a conservative method in terms of α and β errors. An additional separate-variance Student's t-test was performed independently for the few groups under comparison that presented standard deviations with statistically significant differences (p-value < 0.05). All those further tests led to the same conclusions as in the Tukey's HSD test.

3. RESULTS AND DISCUSSION

Efficiency of the manufacturing process and composition of the resulting films

The prepolymerization following the procedure described above in a Dean Stark assembly allowed obtaining a viscous but manipulable liquid prepolymer, which could be shaped as solid films following different conditions of curing. Figure 1 (a-c) shows PGS films cured at different temperatures (110ºC, 120ºC, 130ºC, 140ºC and 150ºC) for 48h, and for different times at 130ºC (24, 48, 72 and 96 h), as well as those with different SA:G ratios in the reactive mixture and cured at 130ºC/48 h (0.5:1, 1:1, 2:1). Films are transparent, soft and flexible, and get increasingly yellowish and tough as temperature or curing time increases. As for the sebacic acid:glycerol ratio, the films with two-fold the equimolar ratio showed similar characteristics in terms of color and manipulability, whereas those manufactured with a 0.5:1 molar mixture were too sticky and difficult to handle.

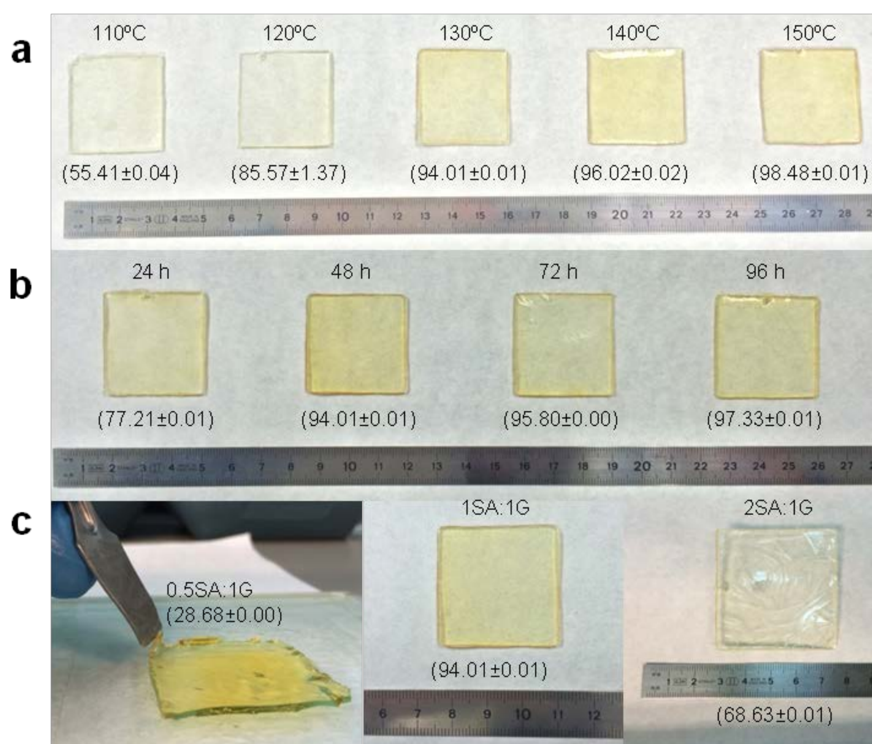


Figure 1. PGS films prepared from equimolar SA:G ratios and cured (a) at increasing temperatures (from left to right: 110ºC, 120ºC, 130ºC, 140ºC and 150ºC) for 48 h, and (b) for different times at 130ºC (24, 48, 72 and 96 h). (c) PGS films prepared from 0.5:1, 1:1 and 2:1 sebacic acid:glycerol ratios and cured at 130ºC/48 h. The values between brackets are mass fractions (%) of efficiently crosslinked chains, *i.e.*, ratios of experimental mass after rinsing per reagents unit mass at different synthesis conditions.

All films swelled significantly when immersed in tetrahydrofuran to remove non-crosslinked chains and monomer residues after manufacture, behaving as fragile gels, and progressively shrinked as the solvent was substituted by ethanol and even more in water to get back to their initial consistency of a rubber xerogel. In no case the films dissolved completely in tetrahydrofuran, which would have meant that the materials prepared were rather a branched prepolymer than a more or less densely connected network. The relative mass loss during such rinses depends clearly on the previous

curing temperature, decreasing strongly from 44.59% at 110ºC to 5.99% at 130ºC and slightly to 1.52% at 150ºC. The same applies for the curing time, with which it can be reduced from 22.79% for 24 h to 5.99 for 48 h and 2.67% for 96 h. The ratios of experimental mass after rinsing per reagents unit mass, Figure 1, provide an idea of the crosslinking efficiency at different synthesis conditions, understood as condensations yielding percolated chains, impeding their solubility in affine solvents such as tetrahydrofuran: longer times or higher temperatures yield denser networks with less non-condensed hydroxyl or carboxyl terminal groups. As shown in the figure, there is no need of curing above 130ºC for longer than 48 h, for which the mass fraction of efficiently crosslinked chains already attains 94%.

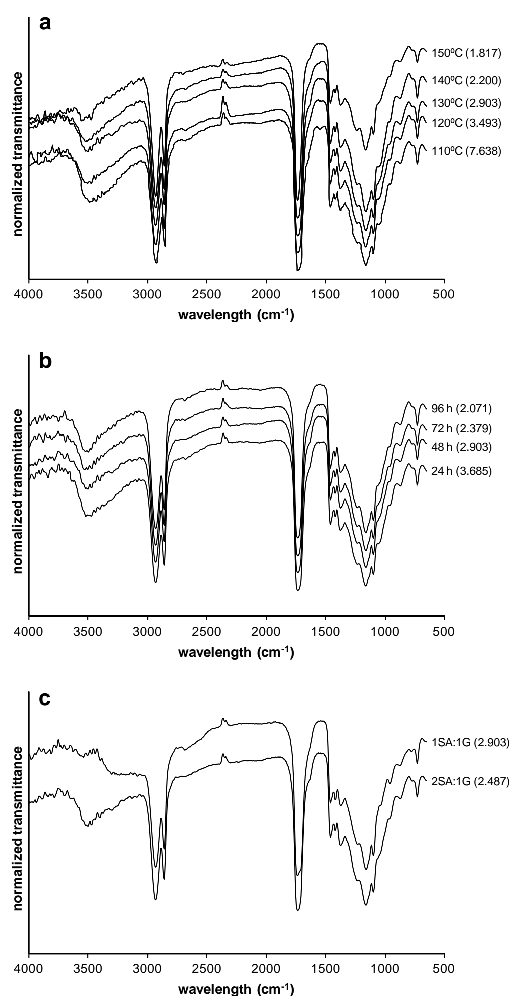
The 0.5:1 sebacic acid:glycerol mixture suffered a mass loss of 31.37%, significantly greater than that of 5.99% for the equimolar one, as expected since the latter is closer to the stoichiometric ratio (2 carboxyl groups instead of 1 are available in for condensation with 3 hydroxyl groups of the polyol). As for the materials resulting from a 2:1 sebacic acid:glycerol molar ratio (4 carboxyl groups instead of 1 are available in for condensation with 3 hydroxyl groups of the polyol), which appeared to promise the lowest mass loss because all hydroxyl groups of the trifunctional crosslinker could eventually be condensed, left though a residue of 71.32%. This result, together with the difficulties in its manipulation, justified discarding such combination.

These results highlight the necessity of purifying the materials after curing, in order to eliminate not only unreacted monomers but also those chains not bound to the main network. Otherwise, these chains can easily diffuse to the surrounding medium as the material swells or is surface hydrolysed, and may provoke an adverse response of the material in a cell culture or after implantation.

Figure 2 (a-c) displays the normalized FTIR spectra of the PGS samples, which reveals the presence of polar carboxyl or hydroxyl non-condensed groups. The broad band between 3700 and 3100 cm^{-1} and the shoulder at 1650 cm^{-1} next to the carbonyl peak, characteristic of OH groups, appeared in all cases, and was found to be inversely related to the curing temperature and time, as well as to increase for samples synthesized with less polyol fraction in the monomeric mixture. The CH_x asymmetric and symmetric stretching peaks appearing at 2962 and 2888 cm^{-1}, respectively, do not show significant differences between samples. The well defined strong peak at 1700 cm^{-1} corresponding to the C=O stretching peak of either carboxyl or condensed ester groups, does not seem to differ, neither. At 1160 cm^{-1}, the intensity of the peak corresponding to C-O bonds slightly decreases for stricter curing conditions.

As stated in [12], as the degree of esterification increases, the intensities of the C=O bond-induced peak increase whereas peaks from O-H bonds decrease, indicating an increased crosslinking density. For a better interpretation of the results, the ratio between transmittances at 3480 and 1720 cm^{-1} was herein calculated for the different samples and is listed in the insets of Figure 2. The drop of such ratio provides greater insight into how the crosslinking density increases.

These results, showing that milder synthesis conditions lead to more open networks with fringes ending in non-condensed polar groups are in agreement with the mass losses quantified above, and will hereinafter be useful in understanding their behaviour under physico-chemical and biological experiments.



SA:G molar ratio	curing T (ºC)	curing t (h)	WCA_{dry} (º)	WCA_{wet} (º)
1:1	110	48	106.26±2.99	91.00±2.80
1:1	120	48	95.98±2.03	89.31±3.60
1:1	**130**	**48**	86.49±1.84	88.69±2.52
1:1	140	48	84.88±2.68	87.13±2.23
1:1	150	48	84.02±3.14	109.93±0.61
1:1	130	24	91.49±1.90	87.28±4.25
1:1	130	72	81.48±1.23	105.23±1.47
1:1	130	96	79.99±0.86	109.18±0.46
2:1	130	48	101.61±3.03	86.48±5.04

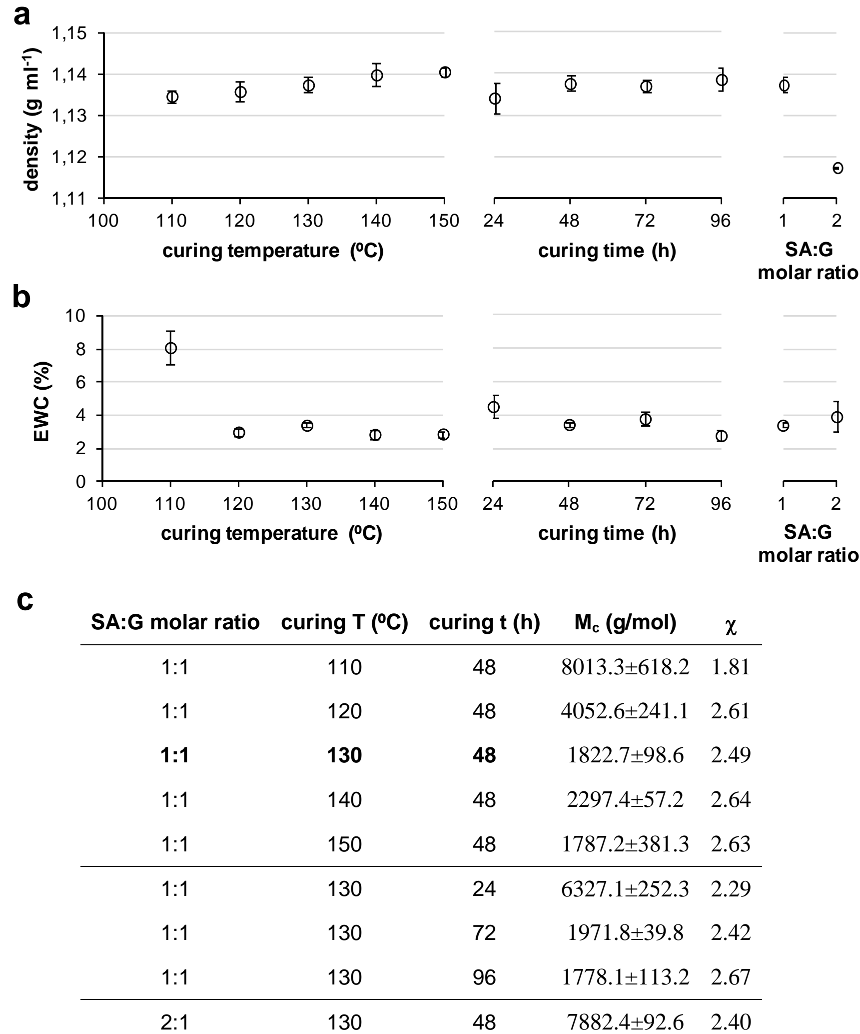
Figure 2. FTIR spectra of PGS films prepared from equimolar SA:G ratios and cured (a) at increasing temperatures for 48 h, and (b) for different times at 130ºC, and (c) PGS films prepared from 1:1 and 2:1 SA:G ratios and cured at 130ºC/48 h. The values between brackets are transmittances ratio between 3480 cm^{-1} and 1720 cm^{-1}. (d) Water contact angles (WCA) on samples prepared following the different procedures, in their dry state and equilibrated in water.

Figure 2(d) presents the average water contact angles of the different samples. As for the curing temperature, at 110ºC water droplets form an angle of 106.26º on the surface, which strongly decreases to 86.49º for 130ºC and slightly hereafter to 84.02º at 150ºC. Such trend is manifested as well for longer curing, the angle for 24 h being 91.49º whereas that for 96 h is 79.99º. The wettability of films fabricated with a 2:1 sebacic acid:glycerol molar ratio is significantly lower (101.61º) than that of samples prepared from an equimolar ratio. These outcomes seem to contradict those described before, but could find though explanation in intra- and inter-molecular hydrogen-bonding interactions between polar terminal groups in the dry state of the samples. This effect has also been observed in dry rubbery poly(hydroxyethyl acrylate), which exposes its hydroxyl groups and thus, behaves as a wettable material only when hydrated [28]. Indeed, the water contact angles of analogous samples equilibrated in water show the opposite trend: those cured at 150ºC/48 h or at 130ºC/ 72-96 h are the least wettable ones.

PGS crosslinking degree and swelling

The dependence of the samples density with the curing temperature and time and the composition of the reagents mixture, Figure 3(a), shows the same trend as the ratio of experimental mass after rinsing per unit mass. The density of PGS synthesized from equimolar mixture and cured at 130ºC/48 h, 1.138 $g \cdot ml^{-1}$, slightly increases at higher curing temperatures and longer times, whereas that of the samples polymerized from a 2:1 sebacic acid:glycerol reactive mixture drops to 1.118 gml^{-1}. These results, in agreement with those found in [20] where the densities of PGS cured at 110ºC and 130ºC/48h/vacuum (but non-purified afterwards) are given, support the hypothesis

previously stated that mild curing conditions (110ºC, 24 h), as well as an excess of acid, lead to defective branched networks, whilst there are no significant differences in terms of these properties beyond 130ºC and 48 h of curing.



SA:G molar ratio	curing T (ºC)	curing t (h)	M_c (g/mol)	χ
1:1	110	48	8013.3±618.2	1.81
1:1	120	48	4052.6±241.1	2.61
1:1	**130**	**48**	1822.7±98.6	2.49
1:1	140	48	2297.4±57.2	2.64
1:1	150	48	1787.2±381.3	2.63
1:1	130	24	6327.1±252.3	2.29
1:1	130	72	1971.8±39.8	2.42
1:1	130	96	1778.1±113.2	2.67
2:1	130	48	7882.4±92.6	2.40

Figure 3. (a) Density, (b) equilibrium water contents of PGS films as a function of curing temperature and time and sebacic acid:glycerol molar ratios. (c) Molecular mass between crosslinks (M_c) of the PGS networks estimated through the relationship between the crosslink density and elastic modulus for rubbers, and Flory-Huggins parameter (χ) estimated through Flory-Rehner equation, which relates the volume fraction of polymer at equilibrium swelling with the crosslink density. In (a), 110ºC *vs* 150ºC and 1:1 *vs* 2:1, $p<0.05$. In (b), 110ºC *vs* others and 24 h *vs* 96 h, $p<0.05$.

Equilibration of the materials in liquid water at 37ºC was used to investigate the influence of the synthesis parameters on the PGS capacity to swell, and allowed estimating its crosslinking density. The elastic moduli of dry samples were required for this purpose. The equilibration of the samples in n-octane was used to confirm that swelling could be neglected and this solvent could be used to perform density measurements. No sample swelled more than 3.5% at equilibrium in n-octane.

Figure 3(b) displays the equilibrium water contents of the PGS networks, referred to their dry mass. In general, water is not as good solvent (herein understood as swelling medium, because continuous networks will not dissolve) for PGS samples as others like THF or even an aqueous basic medium (that used in the accelerated hydrolysis experiment). Samples cured at 130ºC for 48 h conditions swelled 3.43±0.15%. Those cured at 110ºC swelled remarkably more (8.11±1.02%) than those cured at higher temperatures, amongst which differences were not significant. These former conditions were, indeed, those leading to the highest mass loss while rinsing. Nonetheless, swelling in water is not sensitive enough to the curing time or the composition of the reagents mixture to shed significant differences.

The compressive elastic modulus, E, of each sample type was obtained from the stress–strain curves, shown in Figure 4. The values obtained, typical of elastomers, markedly change from around 1 MPa for PGS cured at 110ºC/48 h, or only 24 h/130ºC or prepared with an excess of sebacid acid, up to 4.63±0.24 MPa for materials cured at 130ºC/48 h from an equimolar ratio between reagents. From that point on, following the same trend, differences in terms of mechanical properties are not significant. These harsher curing conditions yield condensed structures that hinder chains mobility when loading. The efficiency of the crosslinking does not seem, though, to improve beyond

that reached at 130ºC/48 h in terms of its impact on the rubbery elastic modulus. Other publications report Young's moduli in the range 0.05-1.2 MPa under tensile tests for PGS cured at 110ºC-130ºC/48 h/vacuum and tested without further purification [19,20].

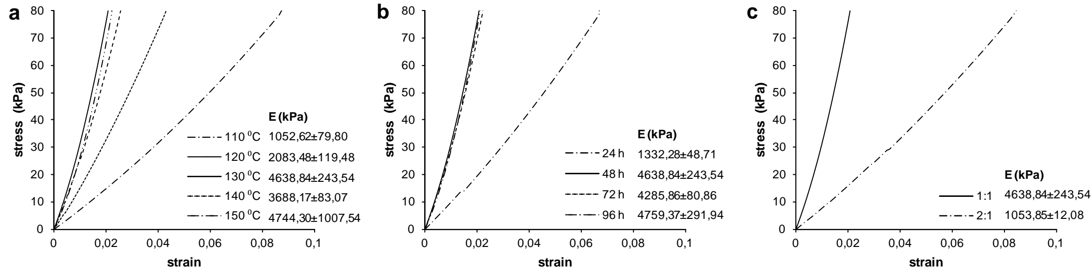


Figure 4. Stress–strain curves obtained from compressive experiments performed on PGS films as a function of (a) curing temperature and (b) time and (c) sebacic acid:glycerol molar ratios. Insets: initial Young modulus, *E*; for samples cured at 110ºC *vs* 130, 140 and 150ºC, those cured at 120ºC *vs* 130, 140, 150ºC, those cured at 24 h *vs* 48, 72, 96 h and those prepared from SA:G 1:1 *vs* 2:1, $p<0.05$.

As for the molecular mass between crosslinks (Figure 3c), no significant differences were observed among samples cured at 130ºC or higher for 48 h or longer, which gave values of the order of 1900 g/mol, whereas those cured under milder conditions, or those prepared with an excess of acid led to molecular masses two or three times greater.

Flory-Huggins parameters, estimated for each sample type by Flory-Rehner equation, proved to depend on the curing conditions and the reactive mixture, increasing from 1.81 for samples cured at 110ºC/48 h to 2.63-2.69 for those cured at 150ºC/48 h or 130ºC/96 h, the latter being the less affine for water.

Thermal properties

Figure 5 displays the specific heat during the heating scan of the DSC measurements, obtained from the heat flow (*dQ*/*dt*) divided through the heating rate (*dT*/*dt*) and scaled by sample mass. The glass transition step lies between -35ºC and -20ºC for samples cured at mild conditions or with an excess of sebacic acid, and shifts up to the -30 to -15ºC range as polycondensations get more extensive and chain movements are more impeded. The glass transition temperature, T_g, was calculated for each sample type as the inflexion point of the step and is listed in the inset. Differences, again, are largest between samples cured at 110ºC or 120ºC, or for 24 h, with those cured at 130ºC for 48 h, but beyond these conditions variations are not so pronounced.

Next, less cured samples show an exothermic crystallization peak followed by the melting peak of those chains able to crystallize. In [6,11] the authors also found that PGS behaved as a semicrystalline polymer when cured at 120ºC/48 h. The maximum of this peak, the melting temperature, is located at 1.50ºC and -4.38ºC for samples cured at 110ºC and 120ºC for 48 h and at -11.57ºC for those cured at 130ºC for 24 h, and tends to get closer to the glass transition and vanish for more strict curings, being hardly discernible for PGS cured at 150ºC/48 h or at 130ºC/72 h, which seem to be completely amorphous. Curiously, an excess of sebacic acid displaced the glass transition of the amorphous phase towards lower temperatures yet the melting peak of the crystalline phase did not appear immediately after or could be discerned. The explanation for this cannot be found in the molecular mass between crosslinks (Figure 3) of these PGS networks, which is not dissimilar to that obtained when curing under mild conditions, but rather could be found, as explained in [29] on the impeded previous crystallization due to interactions among the carboxyl terminal groups of the polymeric chains, which provoke a decrease in the chain mobility.

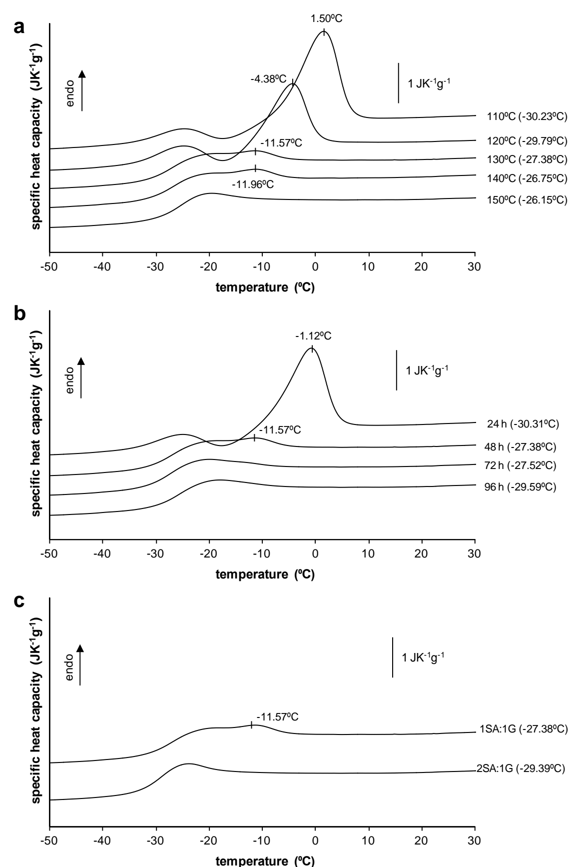


Figure 5. DSC thermograms plotting the specific heat capacity as a function of temperature of PGS films depending on (a) curing temperature and (b) time and (c) sebacic acid:glycerol molar ratios. The values between brackets are the glass transition temperatures, T_g. The melting temperatures, T_m, have been located, when possible, at the maximum of the endothermic melting peak.

Stability of PGS

The immersion in water at 37ºC did not imply a significant hydrolysis in any sample type, the mass loss being in all cases below 3% after 28 days, Figure 6. Previous works have also shown a relatively inert character of PGS in pure water [30], because PGS is hydrophobic and hydrolysis occurs at the surface level [17,30]. Nonetheless, the accelerated degradation assay in 0.01 M NaOH (aq) did allow observing differences

between samples. PGS is more swellable in basic medium and thus degradation proceeds homogeneously in bulk mode. Those samples cured at 110ºC and 120ºC lost 22.22±4.42% and 5.06±0.51% of their weight, respectively, in one month, the latter behaving similarly to those cured for 24 h at 130ºC. As for PGS samples prepared with an acid excess, their mass loss reached 34.78±1.46% and their surface became rough. This assay did not reveal, though, any difference between samples cured at 130ºC or higher, for 48 h or longer. Indeed, swelling in NaOH (aq) of these samples did not surpass 2.7%. In contrast, Nijst *et al.* [31] found that PGS cured at 120ºC/24 h completely degraded in a NaOH 0.1 mM solution (100 times more diluted than the one used here) at 37ºC in few hours; they used the material, though, without further purification, which makes the results unmatched and highlight anew the need of a post-processing rinsing.

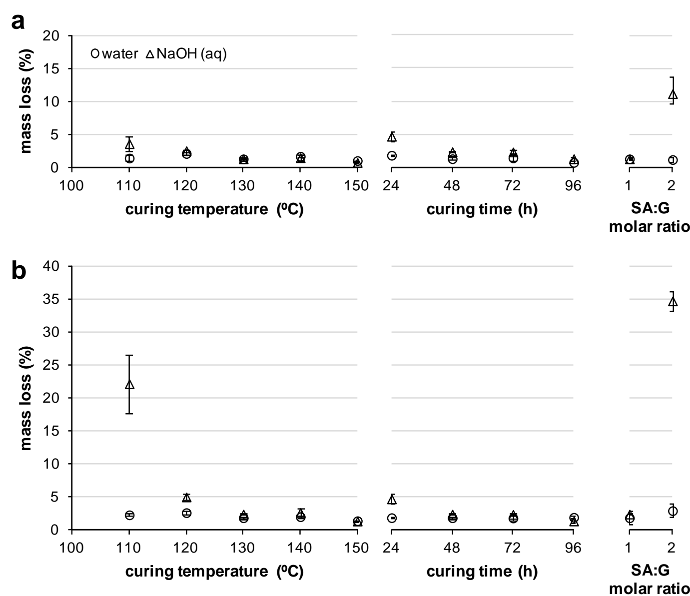


Figure 6. Mass loss of PGS films as a function of curing temperature and time and sebacic acid:glycerol molar ratios after (a) 14 and (b) 28 days in water and 0.01 M NaOH (aq) at 37ºC.

Fibroblasts adhesion and proliferation

PGS samples cured at 130ºC/48 h (starting from equimolar and 2:1 sebacic acid:glycerol ratios), at 150ºC/48 h and 130ºC/96 h were selected for cell culture up to 7 days. Absorbance readings after AlamarBlue stainings did not reveal any significant difference between samples, nor at different time points. Indeed, DAPI nuclear stainings (Figure 7) and SEM images (Figure 8) showed a good cell adhesion after 5 h of culture, which allowed cells to proliferate until largely coat the surface at day 4. Fibroblasts kept their typical morphology of elongated fusiform cells. In contrast, in [23], the authors found cytotoxicity in the extract medium of PGS cured at 130ºC/2 days after a 2 days culture of mouse fibroblasts, though not in extracts of PGS cured at the same temperature for 4 and 7 days. The authors attributed such toxicity to unreacted carboxylic acid groups and/or those produced by aqueous hydrolysis and subsequent acidification of the medium. Those samples were, though, used as obtained, skipping the consecutive rinses in THF and ethanol herein described. After 7 days, cells even formed clusters on those samples cured at 150ºC/48 h or 130ºC/48 h, acquiring then a rounder shape characteristic of senescent cells.

The evidence after this culture is that longer times or higher temperatures of curing do not longer improve the good cell behavior on PGS samples cured at 130ºC/48 h, but those with a higher number of non-condensed polar end groups showed a different behavior *in vitro*: on the one hand, a higher number of cells displayed a senescent rounder morphology. On the other hand, on samples prepared using an excess of acid, typical formations of early crystals can be seen. Such samples probably acquire a negative surface charge when carboxyl groups dissociate and promote the deposition of calcium and phosphate ions, together with others like magnesium, sodium, potassium cations and chloride or carbonate anions, which eventually would give place to

hydroxyapatite cauliflowers. This bioactivity has been observed in previous works with acrylate polymers with carboxy and hydroxy groups, whereby the surface polarity acts synergistically with an appropriate network expansion leading to an exposure of a larger number of apatite nucleating sites and to enhanced intrapolymer diffusion [32]. Of course, confirmation of this hypothesis must wait for further results with *in vitro* acellular experiments in simulated body fluid.

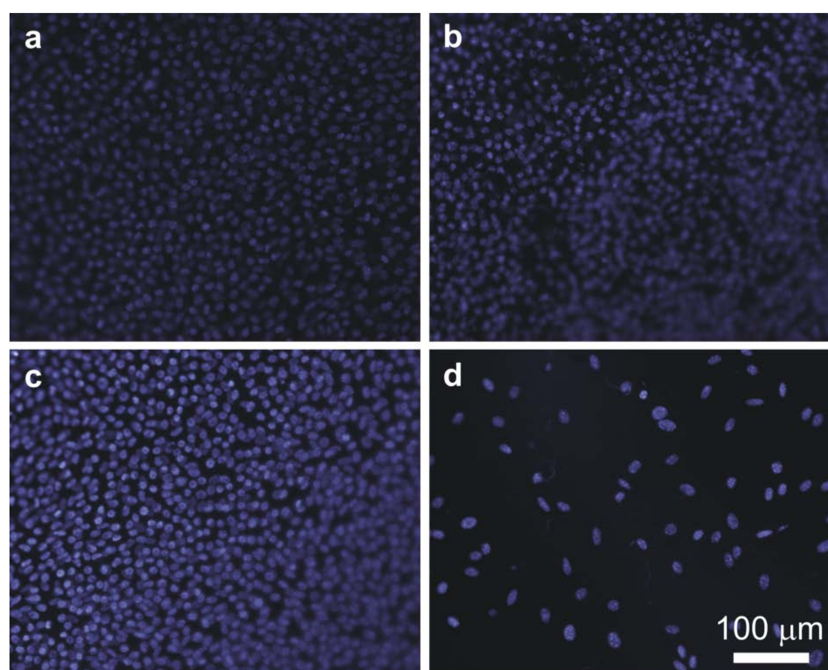


Figure 7. Fluorescence microscopy images of the PGS surfaces cultured with fibroblasts for 7 days. Samples were prepared from equimolar sebacic acid:glycerol mixtures and cured (a) at 130ºC/48 h, (b) at 130ºC/96 h and (c) at 150ºC/48 h and (d) prepared from a 2:1 sebacic acid:glycerol mixture and cured at 130ºC/48 h. Surfaces were pretreated with Sudan Black to avoid autofluorescence and stained with DAPI (cell nuclei) in blue.

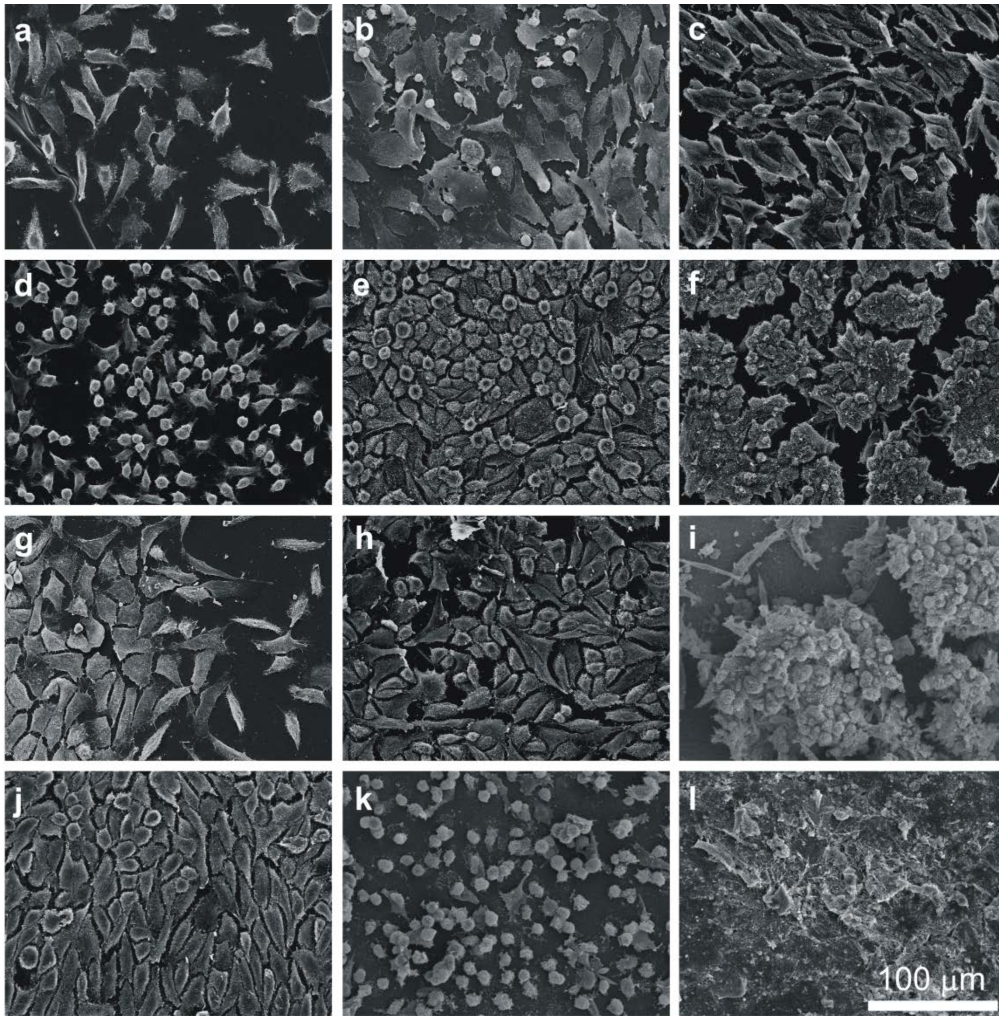


Figure 8. SEM images of the PGS surface cultured with fibroblasts for (a, d, g, j) 5 h, (b, e, h, k) 4 days and (c, f, i, l) 7 days. Samples were prepared from equimolar sebacic acid:glycerol mixtures and cured (a-c) at 130ºC/48 h, (d-f) at 130ºC/96 h and (g-i) at 150ºC/48 h and (j-l) prepared from a 2:1 sebacic acid:glycerol mixture and cured at 130ºC/48 h.

4. CONCLUSIONS

Synthesis parameters such as the curing temperature and time, and the molar ratio between reactants, were systematically varied to correlate them with the physicochemical properties of poly(glycerol sebacate) networks. The efficiency of each manufacturing process was quantified through the mass fraction effectively crosslinked. These results highlight the relevance of rinsing these materials in an affine solvent following synthesis, to remove any non-crosslinked chains that easily diffuse to the surrounding medium, for relative mass losses may range from 6% when PGS is cured at 130ºC/48 h, up to 44.6% when cured at 110ºC.

Infrared spectra gave an estimation of the ratio of non-condensed polar terminal groups, quantified as the relation between transmittances ratio at 3480 cm^{-1} (O-H) and 1720 cm^{-1} (C=O). It ranges from 7.6 to 1.8 under the curing conditions considered herein, being 2.9 for networks cured at 130ºC/48 h. These results were correlated with swelling results, which in turn provided the means to quantify the chains density through Flory-Rehner equilibrium swelling equation. Swelling in water increased from 3.43% for samples cured under standard conditions up to 8.11%, which meant that under mild conditions the molecular mass between crosslinks can be up to 4-fold the former, 1823 g/mol. Variations on their thermal transitions, as well as in their mass loss resulting from hydrolysis were followed. Curing under mild conditions leads to a slight decrease in the glass transition temperature with respect to networks manufactured under standard conditions (-27.38ºC) and allows crystallization and melting thereafter. The networks were found to be stable in water at least for one month, but the hydrolysis proceeded much faster in a basic medium, which facilitates the expansion of the networks, and depended in that case on their crosslinking densities.

All in all, equimolar ratios between sebacic acid and glycerol and curing at 130ºC for 48 h led to dense networks with a number of polar end groups low enough to avoid swelling even in a basic medium and thus, hydrolysable only at a surface level without mass loss implications at least in one month, and with a good biological response in an exploratory cell culture. No significant differences between samples cured for longer times or higher temperatures were observed. Oppositely, curing under milder conditions equimolar mixtures of sebacic acid and glycerol proved to lead to poorly crosslinked swellable networks, which hydrolyze easily in bulk. Alternative molar ratios yield sticky and difficult to handle materials at higher polyol fractions in the reactive mixture, whilst an excess of acid terminal groups led to a fast mass loss by hydrolysis mainly in basic medium, which would be suitable for different applications in the tissue engineering field.

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