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EFFECTS OF HIGHLY POROUS ELECTROSPUN HYPERBRANCHED POLYGLYCEROL SCAFFOLDS ON THE ADHESION AND SPREADING FIBROBLASTS IN VITRO

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Abstract. *This work introduces hyperbranched polyglycerols (HPGL's) to produce scaffolds with high porosity and interconnectivity for tissue engineering. Electrospinning, a high-voltage driven spinning technique, was used to produce nonwoven HPGL's fibers for cell culture of normal human fibroblasts. Fibrous scaffolds with increased porosities were obtained after the electrospinning of HPGL's with ethanol:dimethylformamide (1:1) as a spinning solvent. The morphology and microstructure of HPGL's fibers were investigated by scanning electron microscopy (SEM) and mercury porosimetry. HPGL's fibers exhibited a circular cross-section with a smooth surface. To assay the cytocompatibility and cell behavior onto the HPGL's scaffolds, cell attachment and spreading of normal human fibroblasts seeded on the HPGL's scaffolds and interaction between cells and HPGL's fibers were studied by fluorescence microscopy (FM) and SEM. The cells adhered to and easily proliferated on the surface of the HPGL's scaffolds. The FM studies indicated that HPGL's scaffolds not induce apoptosis of adhered fibroblast cells. The results of this study demonstrate the successful fabrication of HPGL's nonwoven fibers by electrospinning technique generating scaffolds with pore sizes suitable for cellular infiltration. Our results indicate that the HPGL's scaffolds may be a good candidate for the biomedical applications, such as wound dressing and scaffolds for tissue engineering.*

Keywords: *Hyperbranched Polyglycerol, Electrospun fibers, Tissue Scaffolds, Electrospinning, Fibroblasts.*

1. INTRODUCTION

Nowadays, the repair of an injured biological tissue through the use of polymeric scaffolds presents a significant medical challenge in regenerative medicine. Polymeric scaffolds have been used to induce tissue repair by undamaged cells at the site of injury promoting cell adhesion, proliferation and extracellular matrix deposition stimulating the migration and differentiation of the stem cells into damaged areas to form a new biological tissue [Griffith et al., 2002; Langer et al., 2004; Langer et al., 1993; Ma, 2004].

Fibroblasts (FBs) are mesenchymal cells that can be readily cultured in *in-vitro* conditions and play a significant role in epithelial-mesenchymal interactions, secreting various growth factors and cytokines influencing the epidermal proliferation, differentiation and formation of extracellular matrix [Junker et al., 2008; Junker et al., 2010; Lorenz et al., 2008; Nolte et al., 2008; Wong et al., 2007]. FBs cells have been incorporated into various commercial scaffolds such as Epicel® (Genzyme Inc.), Permaderm® (Regenicin Inc.), Orcel® (Ortec Inc.), Dermagraft® (Advanced BioHealing Inc.) and Apligraf ®(Novartis Inc.) and used as skin substitutes for wound care including the treatment of burns, chronic venous ulcers and several other clinical applications [Jones, et al., 2002; MacNeil, 2007; Mansbridge, 2006; Raguse et. al, 2005; Naughton, 2002].

Recent progress in micro and nanofabrication techniques has enabled the production of a diverse array of polymeric scaffolds and a large numbers of polymeric scaffolds from different biomaterials are available to provide a friendly micro-environment for 3D FBs scaffold development. These include nanodot arrays, nanoporous structures, nanoparticles and nanofibers [Chandrasekaran et al., 2011; Chung et al., 2013; Idris et al., 2010; Li et al, 2012; Liu et al, 2009; Middelkoop et al, 2002; Ojeh, et al., 2001; Kontonasaki et al., 2007].

The high porosity of nonwoven fibrous scaffolds provides larger surface areas to adsorb proteins and high-density cells with much more biding sites available which contribute to maintain the phenotypic cell morphology [Stevens, et al., 2005]. To produce nonwoven fibrous 3D-scaffolds the electrospinning technique is a common method. Electrospinning is a process that produces a nonwoven fibers by pushing a millimeter diameter liquid jet through a nozzle with an electric field [Hohman et al., 2001]. As a polymer jet is drawn out of the needle orifice, these jets travel downward and become thinner as the solvent evaporates producing nonwoven mesh polymeric fibers at micro/nano-scale in a collecting plate [Reneker et al., 2000].

The use of electrospun fibrous structures using synthetic polymeric hydrogels has drawn increased interest for use in the production of 3D FBs scaffolds due to their similar elastic properties of the biological tissue [Ji et al., 2006; Cukierman et al., 2001; Grinnell et al., 2003; Ji et al; 2006; Schindler et al., 2005]. The three-dimensional interconnected pore networks of electrospun nanofibers generate structures that resemble native extra-cellular matrix (ECM) elements that will be interesting to their uses in would healing processes.

Linear and hyperbranched polyglycerol (HPGL) gels, a branched polyether polymer, have evoked our interest because of their highly hydrophilic and biocompatible properties which can lead to production of scaffolds for cartilage tissue engineering [Kainthan et al., 2006; Du et al., 2014; Mendelson et al., 2013; Weinhart et al., 2011; Khandare et al., 2010].

Motivated by the development of polymeric scaffolds for soft tissue engineering based on electrospun nanofibers, we report the preparation of electrospun HPGL hydrogel fibers. The aim of this work was to study the *in vitro* biological properties of electrospun HPGL fibers obtained through electrospinning technique. The mechanical, water absorption and biological assays were investigated. The biological studies were conducted by *in vitro* conditions to investigate the biocompatibility properties and the potential use of HPGL electrospun fibers for the direct applications to tissue engineering.

2. MATERIALS AND METHODS

Preparation and characterization of HPGL nonwoven electrospun fibers.

Initially, polyglycerol (PGL) with a molecular weight (Mn) of 12 kDa and narrow polydispersity (M_w/M_n= 1.5) was synthesized through the reaction of partially deprotonated 1,1,1-tris(hydroxymethyl)propane with glycerol carbonate [Rokicki et al., 2005, Raether et al., 2012]. After neutralization by filtration over cation-exchange resin, the HPGL was purified by precipitation in acetone, and subsequently dried for 24 h at 80°C under vacuum.

The HPGL was derivatized with methacrylate groups after reaction with glycidyl methacrylate (GMA) for the covalent crosslinking of the HGPL chains and formation of the hydrogel [Oudshoorn et al., 2006; Van Dijk-Wolthuis et al., 1997; Taylor et al., 2011]. HPGL was dissolved in dimethyl sulfoxide (10 mL) and 4-(N,N-dimethylamino)pyridine (DMAP) (2.0 g). GMA (1.0 mmol) was added under gentle stirring to obtain a HPGL with GMA substitution degree of 10% (w/w). The HPGL derivative was re-precipitated three times in diethylether, washed three times with the same solvent and subsequently dried overnight under vacuum at room temperature (25°C).

The HPGL nonwoven fibrous structures were prepared by electrospinning from neat 30 wt% HPGL solution in 50:50 v/v methanol and DMF solution. The HPGL solution was placed in a 10 mL glass syringe fitted to a needle with a tip diameter of 0.9 mm. A high electrical field of 0.2-2.0 $kV.cm^{-1}$ was applied to the needle using a high voltage power supply. The ground collection plate of aluminum foil was located at a fixed distance of 20 cm from the needle tip. A syringe pump was used to feed the polymer solution to the needle tip at a feeding rate of 1.5 mL h-1 under UV radiation (200 W UV lamp, 280 nm) to crosslinking HPGL chains by photopolymerization of GMA groups. The HPGL electrospun fibrous membrane was carefully detached from the collector and dried under vacuum for 48 h at room temperature (25° C) to remove solvent molecules completely. After that, they were soaked in pH 7.4 phosphate-buffered saline (PBS) and then dehydrated by lyophylization. The pore sizes and morphology of electrospun HPGL membranes were determined by scanning electron microscopy (SEM; Phillips XL 30). The porosity of the electrospun HPGL scaffolds were calculated using Eq. (1) and Eq. (2), below [He et al., 2005]. The thickness and diameter of the specimens were measured using a micrometer.

$$D_{Scaff} = \frac{m_{Scaff}}{T_{scaff} x A_{Scaff}} \qquad (1)$$

$$P(\%) = \left(1 - \frac{D_{Scaff}}{D_{HPGL}}\right) \qquad (2)$$

where D_{Scaff} is scaffold density (g/cm^3), D_{HPGL} is hyperbranched polylycerol density (g/cm^3), T_{scaff} is scaffold thichness (mm) and m is the scaffold mass (g).

Mechanical property measurements.

The mechanical properties of the electrospun HPGL fibers were evaluated using a tensile testing instrument (EZ Test-100N, Shimadzu) mounted with a 100 N capacity load cell. The test procedure was based on the ASTM D 882–75 method using flat-faced metal grips with surfaces laminated with sand paper for better hold [ASTM, 1988]. The initial gauge length was set at 40 mm, and the extension speed was 4 mm min-1. The tests were carried out in ambient conditions of 20 ± 2 °C and 55 ± 2% relative humidity (RH). Four mechanical properties, namely tensile strength, percentage elongation at break, elastic modulus and work of failure, were computed from the load–strain profile and the film dimensions, respectively. At least five measurements were taken, and the average was calculated for each film

Swelling measurements.

The swelling capacity of the electrospun HPGL fibers was measured in PBS solution (2.38 g Na_2HPO_4, 0.19 g KH_2PO_4 and 8.00 g NaCl per liter of distilled water adjusted to pH 7.4) at 37° C. Each electrospun HPGL fibers (surface area 2.0 cm^2) was weighed and placed in a previously weighed stainless steel wire mesh with sieve opening of 600 μm. The mesh containing the film sample was then submerged into 10 mL PBS pH 7.4 contained in a plastic container (diameter 5.0 cm, height 2.0 cm). The increase in weight of the electrospun HPGL membranes was determined at pre-set time intervals until a constant weight was observed. Each measurement was repeated four times.

The degree of swelling (M_t/M_∞) was calculated using the Eq. (3).

$$\frac{M_t}{M_\infty} = \frac{(W_t - W_o)}{W_o} \qquad (3)$$

where W_t is the weight of film at time t, and W_o is the initial weight of the film.

Cytotoxicity evaluation.

In this study, Chinese ovary hamster (CHO-K1, ATCC) cells were used to assess the cytotoxicity of the electrospun HPGL membranes following the guidelines of ISO 10993-5 [ISO 10993-5, 1999]. The citotoxicity of electrospun HPGL membranes was assayed by addition of their extracts to a cell culture on a 96-multiwell plate, sterilized by gamma rays from ^{60}Co (25 kGy), individually poured into 15-mL glass flasks with 10 mL DMEM. The sample extracts were diluted from 100 to 6.25%. The microplates were incubated for 24h at 37°C in a humidified 5% CO_2 atmosphere. The cell viability was measured by adding 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)/ phenazine methosulfate (PMS) (20:1) solution. Before adding the solution of MTS/PMS, extracts incubated with the cells were removed from the wells. After this was added 100mL of 80% composed of RPMI culture medium with serum and 20% of MTS/PMS (20:1) solution and incubated for 2h in the CO_2 incubator. The absorbance was measured with a reference wavelength of 490 nm by microplate reader. The results were compared with a negative control of HDPE (High-density polyethylene) and a positive control of phenol 0.3% in PBS (phosphate buffered saline) solution. The Inhibitory Concentration (IC) for 50% of cell viability was taken from graphical interpolation.

Fibroblasts culture.

BALB/3T3 fibroblasts (ATCC) were previously grown in culture dishes and subsequently applied to the electrospun membranes with culture medium Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic/antimycotic .The membranes were stored in duplicate in 12- well plates and sterilized with gamma radiation. After this step, the culture medium was applied (DMEM 10% FBS, 1% L-glutamine, 1% antibiotic/antimycotic) for conditioning the membrane for 20 minutes. The BALB/3T3 fibroblasts (ATCC) were cultured at a concentration of $1x10^4$ on membranes with the aid of a stainless steel ring 1cm in diameter, thus defining the area of application. After 48 hours, the rings were removed and DMEM culture medium (10% FBS, 1% L-glutamine, 1% antibiotic / antimitotic) was changed. After another 48 hours the cells were fixed.

After 96 hours of fibroblasts cultivation on the membranes, cell attachment was promoted as follows. After remotion of the culture medium, the HPGL electropun fibers were washed four times with phosphate buffered saline (PBS). The membranes were immersed in 0.9% saline for 15 minutes and then added glutaraldehyde 2.5% for 10 minutes for fixation. The membranes were washed four times with PBS and dried at room temperature (25°C). The electrospun HPGL membranes were immersed in acridine orange solution 0.01% (v/v): PBS (1:150). After three minutes the membranes were observed on a fluorescence microscope Zeiss Axioskop 40. Sequential illumination at wavelengths of 592nm was used to highlight stained cells. A total of two specimens were examined in this experiment.

3. RESULTS AND DISCUSSION

Morphology of the electrospun HPGL nanofibers.

Typical SEM images of electrospun HPGL fibers with a fifty micron fiduciary marker in the lower right are shown in Fig. 1 (A). The images obtained from scanning electron microscope were studied for the effect of the polymer concentration and electric field on fiber diameter and its distribution. Figure 1(B) shows the effect of the electric field on the measured average fiber diameter of the fibers at three different concentrations of the polymer solution. It is evident from the Fig. 1 that increase the electric field at all concentrations, the average diameter of the electrospun HPGL fibers decreases. The porosity of these fibrous membranes was estimated by the absorption of hexadecane, a low surface tension and low viscosity liquid. The porosities of the membranes were found to be 73 ± 5%.

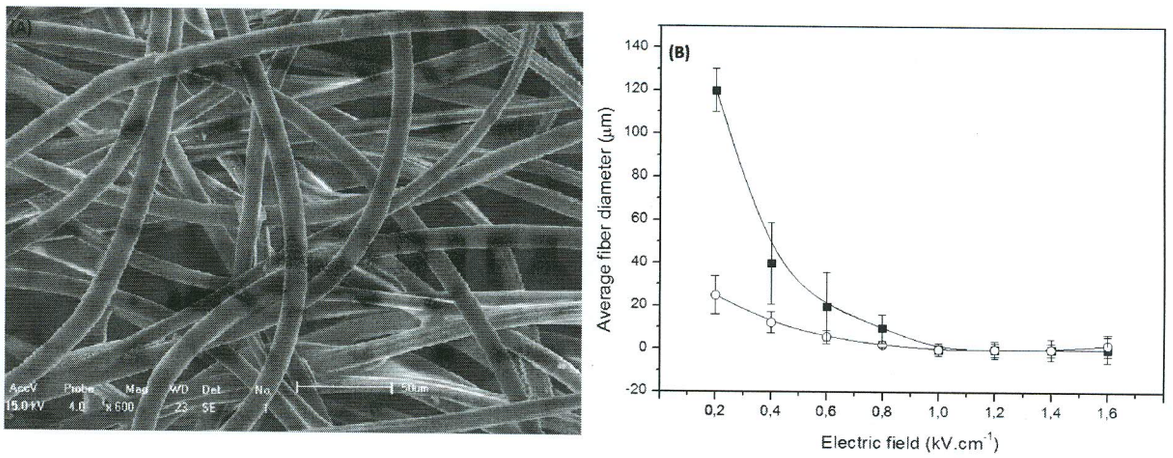

Figure 1. SEM micrographs (A) and the effect of electric field and polymer concentration on the measured average diameter of electrospun HPGL fibers (B). HPGL concentration: 50 wt% (○) and 30 wt% (■) in 50:50 v/v methanol and DMF solution.

Porosity may be considered an important descriptor when selecting the fibrous scaffold for tissue engineering. The porous scaffolds can afford not only cell attachment, proliferation, and differentiation, but also sufficient transport for nutrients and waste removal. The apparent density and porosity of electrospun HPGL membranes sheet were calculated by using Equation (2). The calculated apparent density and porosity of HPGL nonwoven fibers membrane fabricated with the electrospinning technique were found to be 0.368 g/cm^3 and porosity of 68±3% respectively. This highly porous scaffold appears to be convenient for adhesion and proliferation of biological cells. The high surface area to volume ratio and high porosity of electrospun HPGL membranes will be efficient for the nutrient delivery, fluid absorption and excretion of the metabolic wastes [Venugopal et al., 2008].

Swelling tests.

Fluid uptake is an important parameter, which influences the physical characteristics of the scaffolds after and prior to cell seeding. Figure 2 displays the water uptake curve of electrospun HPGL amembranes at 37 °C in the form of M_t/ M_∞ vs. immersion time. The maximum water uptake at equilibrium was 90% (in reference to the original specimen weight). The diffusion coefficients were calculated from the initial linear region of this curve. The diffusion coefficients determined at 37 °C was 2.35x10^{-13} m^2/s. The amount of water

absorbed by electrospun HPGL membranes was related to the porosity and the amount of available liquid water at the surface of the material.

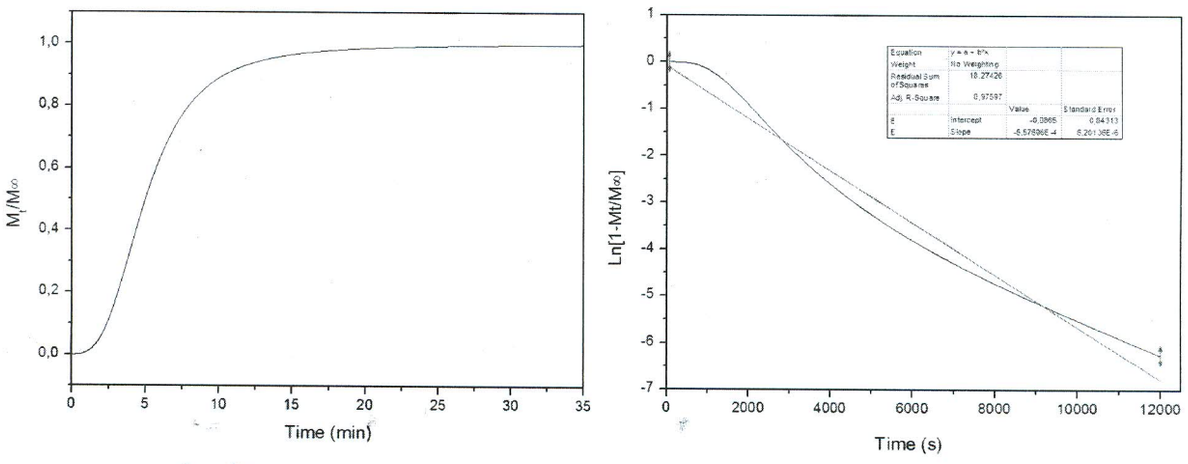

Figure 2. Swelling measurements of electrospun HPGL membranes in PBS solution pH 7.4 at 37 °C.

Mechanical properties.

An ideal material for use in soft tissue engineering should be elastic and flexible but sufficiently strong for clinical handling. The tensile testing provides an indication of the strength and elasticity of the membrane, which can be reflected by the elastic modulus. The Young's modulus of electrospun HPGL membranes is 5.3 $\pm$ 0.7 MPa and is similar to soft tissues and therefore will provide a supportive matrix for chondrocyte activity and cartilage ECM secretion [Mow et al., 2005].

Cytotoxicity evaluation.

The *in vitro* results cytotoxicity of electrospun HPGL fibers are shown in Fig. 4. The biocompatibility evaluation of the electrospun HPGL fibrous membranes provides encouraging indications for long-term safety. In fact, in the cytotoxicity study, the material extracts did not induces toxic effects on the CHO cells, showing high cell viability. Such kind of biocompatible and highly porous scaffolds with high surface area can be applied might be promising as scaffold material for soft tissue engineering application.

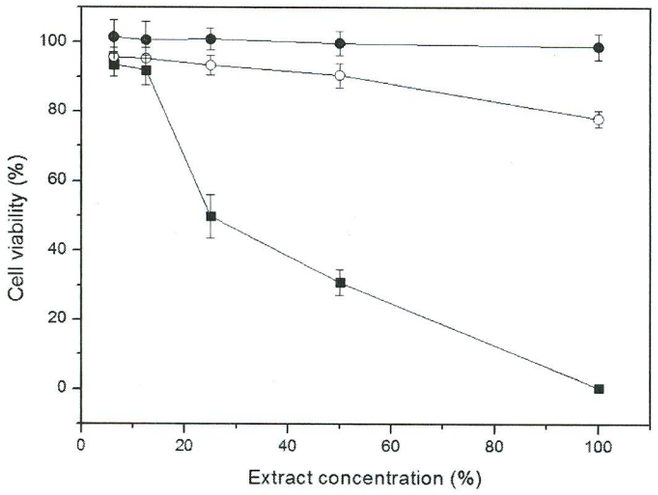

Figure 4. Cytotoxicity profile of electrospun HPGL membranes extracts (○) against CHO cells. Ultra-height molecular weight polyethylene (UHWMPE) (●) was used as negative control and phenol was used as positive control (■).

Cell Adhesion and Proliferation.

In construction of scaffolds for tissue engineering the main strategy for modulating the cell-material interactions will be the creation of the mimetic biological surface that will be favorable to the adhesion and proliferation of cells. In this study, fibroblasts were seemed to electrospun HPGL membranes which have the advantage of the elasticity and water uptake needed for the BALB/3T3 cells. The basic studies of fibroblasts adhesion and proliferation in electrospun HPGL membranes were presented in Fig. 5.

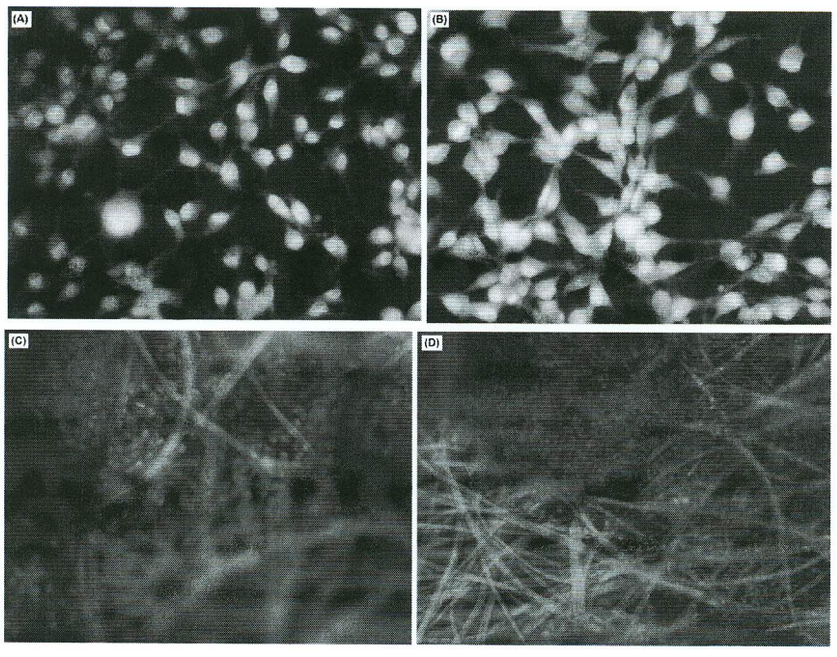

Figure 5. Acridine orange on the first day (24h) after BALB/3T3 seeding onto control plates (A,B) and electrospun HPGL membranes (C,D) (x40).

Accordingly, the fibroblasts responded favorably to electrosupun HPGL membranes. As expected the highly porous scaffold did not affect the cell proliferation, which showed a significant increase over time. It was observed (Fig. 5) that fibroblast cells tightly attached to the surface of the scaffold, and then proliferated and clustered on the electrospun HPGL membrane. Provided that the results of this in vitro study can be directly extrapolated to the clinical situation, one may expect that electrospun HPGL membranes to be a safe scaffold for soft tissue engineering.

4. CONCLUSIONS

In the present study, electrospun HPGL membranes were prepared by electrospinning and its properties including morphological, mechanical and biological assay, were investigated. The electrospun HPGL fibrous sheets showed highly randomly fibrous morphology with nearly uniform diameter around 10 μm range and porosity reaches to 73%. The Young's modulus of electrospun HPGL membranes was 5.3 $\pm$ 0.7 MPa, similar to soft tissues and providing a supportive matrix for chondrocyte activity and cartilage ECM secretion. The cytotoxicity studies indicates that electrospun HPGL membranes did not induces toxic effects on the CHO cells, showing high cell viability. The BALB/3T3 fibroblasts responded favorably to electrosupun HPGL membranes. As expected the highly porous scaffold did not affect significantly the cell proliferation, which showed a significant increase over time. From the results, we can conclude that the electrospun HPGL scaffold had adequate biocompatibility and mechanical properties, which is an important principle property of scaffolds and appears to be considered ideal for soft tissue engineering. Because of their size, high surface area and porosity electrospun HPGL fibers have the potential to be a good replacement for a natural extra-cellular matrix (ECM), opening the door for many tissue engineering applications.

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