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period. Chondrocyte viability in the scaffolds cultured under dynamic loading was significantly higher ($p<0.05$) than the scaffolds cultured statically. Dynamic cell culture of the scaffolds improved cell viability and decrease the time of in vitro culture when compared to statically cultured constructs. Optimizing culture conditions and scaffold properties could generate optimal tissue/constructs combination for cartilage repair.

Design and Dynamic Culture of 3D Scaffolds for Cartilage Tissue Engineering

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ABSTRACT

Engineered scaffolds for tissue-engineering should be designed to match the stiffness and strength of healthy tissues while maintaining an interconnected pore network and a reasonable porosity. In this work, we have used 3D-ploting technique to produce poly-L-Lactide (PLLA) macroporous scaffolds with two different pore sizes. The ability of these macroporous scaffolds to support chondrocyte attachment and viability were compared under static and dynamic loading *in vitro*. Moreover, the 3D-plotting technique was combined with porogen-leaching, leading to micro/macroporous scaffolds, so as to examine the effect of microporosity on the level of cell attachment and viability under similar loading condition.

Canine chondrocytes cells were seeded onto the scaffolds with different topologies, and the constructs were cultured for up to 2 weeks under static conditions or in a bioreactor under dynamic compressive strain of 10% strain, at a frequency of 1 Hz. The attachment and cell growth of chondrocytes were examined by scanning electron microscopy (SEM) and by MTT assay. A significant difference in cell attachment was observed in macroporous scaffolds with different pore sizes after one, 7, and 14 days. Cell viability in the scaffolds was enhanced with decreasing pore size and increasing microporosity level throughout the culture period. Chondrocyte viability in the scaffolds cultured under dynamic loading was significantly higher ($p<0.05$) than the scaffolds cultured statically. Dynamic cell culture of the scaffolds improved cell viability and decrease the time of *in vitro* culture when compared to statically cultured constructs. Optimizing culture conditions and scaffold properties could generate optimal tissue/constructs combination for cartilage repair.

Keys words: scaffold, solid freeform fabrication, porogen leaching, chondrocytes, cartilage tissue engineering.

INTRODUCTION

Articular cartilage plays an essential role in freely moving joints because it provides a near-frictionless and low-wear bearing surface of the articulating bones and helps to absorb mechanical loads. Articular cartilage has a limited capacity to repair and regenerate under injury or disease, due to its avascularity and relative acellularity [1,2]. Current clinical solutions to repair cartilage include autologous chondrocyte implantation, microfracture and mosaicplasty. However, there is still uncertainty about tissue quality of the repair and its ability to restore long-term function [3]. Therefore, alternative cartilage repair strategies are needed.

Cartilage tissue engineering approach using polymeric scaffolds offers an alternative to conventional repair techniques, with the goal of restoring tissue functionality and favourable clinical outcome [4,5]. It consists of seeding chondrocytes on three-dimensional (3D) highly porous biodegradable scaffolds *in vitro* in order to produce cartilage tissue that will be implanted in the chondral defect [6]. The synthetic materials most frequently used in tissue engineering scaffolds fabrication are poly L- and D- lactic acid (PLLA and PDLA), polyglycolic acid (PGA), and their copolymers poly(lactic-*co*-glycolic acid) (PLGA). These polymers have been used in products for a variety of applications, which have gained FDA approval for human use. These polymers have been shown to exhibit excellent biocompatibility and biodegradation [7].

The design and fabrication of such constructs is critical for cell attachment, survival and matrix production. Pore size, strand diameter and geometry, overall porosity and material used are all critical factors that influence chondrocyte biology. To achieve a construct with constant biological content throughout the scaffold, these parameters must be closely

controlled in the fabrication process. The 3D-plotting technique has shown great potential in making reproducible 3D scaffolds featuring interconnected pores and controlled architectures [8]. Solid freeform fabrication (SFF) techniques can fabricate complex shapes automatically from computer-aided- design (CAD) models and provide excellent control over scaffold external shape and internal geometry, but offer limited micro-scale resolution [9]. Using SFF, the pore morphology can be varied from structure to structure by changing the laydown pattern, which includes changing the angle of deposition, the width of the deposited material and spacing between the strands [10]. Various attempts to develop scaffolds for tissue-engineered cartilage have led to materials with biomechanical properties and biochemical composition far inferior to those of the natural tissue [11]. The topology optimization using SFF seems to be a logical solution to satisfy the conflicting requirements of sufficient porosity for cell migration and nutrient transport combined with mechanical strength comparable to native cartilage [12]. Therefore scaffolds should be designed to match the stiffness and strength of healthy tissues while maintaining an interconnected pore network and a reasonable overall porosity level.

Mechanical loading has been proposed as a stimulatory factor for the *in vitro* development of tissue engineered cartilage constructs. Mechanical stimulation is an important factor regulating chondrogenic differentiation and cartilage matrix formation [13,14]. Thus, application of mechanical loading, using dynamic bioreactor, may be critical to the *in vitro* development of tissue engineered cartilage constructs. Producing a scaffold with biomechanical properties similar to native cartilage has many potential advantages once implanted *in vivo*. Its stiffness would be sufficient to withstand immediate weight bearing. Early force transmission through the repair site could stimulate new cartilage with

biomechanical properties closely matching those of surrounding articular cartilage. Moreover, it would mean faster rehabilitation for patients.

Therefore, the goal of this study is to test the performance of the designed biomimetic 3D-scaffolds in the context of cartilage tissue engineering. The 3D-plotting and porogen-leaching techniques enabled us to analyze; (1) the effect of pore size, (2) effect of microporosity (0% *vs.* 30%), and (3) the effect of mechanical loading on chondrocyte attachment and viability. Furthermore, the performance of all these scaffolds were investigated under dynamic loading *in vitro* using a bioreactor. We believe that 3D-plotting technique could consistently produce scaffolds with mechanical properties similar to native articular cartilage.

MATERIALS AND METHODS

Scaffold fabrication

The plotting material was prepared by dissolving PLLA with a molecular weight of 220 kDa (Biomer, Krailling Germany) in methyl ethyl ketone (Laboratoire MAT, Beauport, Canada). Subsequently, a part of the solution was mixed with salt particles (NaCl; 30% w/w), previously sieved to obtain a desired particle size distribution (30 to 75 µm) in order to generate micro/macroporous scaffolds. The optimal concentration of the polymer in solvent was determined based on the viscosity constraints of the 3D plotter while targeting optimal syringe deposition. It was found that a concentration of 0.4g PLLA/0.6g of solvent provided an adequate viscosity without compromising the smooth deposition of the paste after incorporation of the porogen (NaCl).

3D scaffolds were fabricated using a bioplotter (Envisiontec, Gladbeck, Germany), which is essentially an XYZ plotter as described by Landers *et al*. [15]. Briefly, the plotting material with and without the presence of the porogen was transferred to the plotting cartridge and was dispensed layer by layer, forming a 0°/90° strand structure (Figure 1). A CAD file specifying the geometry of the scaffold was used as input to produce the physical model by the apparatus. Bricks of 20 mm x 20 mm wide and 2.6mm thick were fabricated during this process. Subsequently, each scaffold was punched into discs of 4 mm of diameter and then immersed in water at 50 ^{o}C for up to 6 hours to extract the salt particles, air-dried for 24 hours and vacuum-dried for 48 hours to allow complete evaporation of the solvent/water. Determination of the plotting parameters, rheological characterization of the paste, and porosity measurements are based on previously published work [16].

To evaluate how the scaffold pore size influences the cell attachment and viability within macroporous scaffolds, two different pore sizes were studied (250 and 400 μm). Moreover, micro/macroporous scaffolds were produced at 30% microporosity level (pore size of 250 μm), so as to examine the level of cell attachment and viability in the presence of micropores. Throughout this paper, the three scaffold topologies are denoted as macroporous scaffolds (150/250 and 150/400 μm) and macro/microporous scaffolds (150/250 μm), where the first number indicate the strand diameter, and the second number represents the distance between the strands. Marcoporous scaffolds contained no porogen.

Mechanical characterization

The unconfined compression tests were conducted on the disk-shape scaffolds and on canine cartilage using a displacement-controlled apparatus (Enduratec, ELF3200 series) in a saline bath with 0.009 g/cm^3 salt concentration. Three successive ramp strain of 3% were

applied at a displacement rate of 0.115 μm/s and the samples were allowed to relax for 30 min after each ramp.

Parameter Estimation

The equilibrium modulus (E_z) was estimated based on the biphasic model [17]. The slope of the stress response *vs.* deformation curve, representing the compressive Young's modulus (E), was estimated for all samples. For cartilage, these parameters were estimated using the unconfined compression data on bovine cartilage from the literature, which was characterized under similar testing conditions [18]. Table 1 gives the estimated porosity, the equilibrium modulus, and the compressive Young's modulus for the scaffolds and for the bovine cartilage. The scaffolds after *in vitro* cell culture were not mechanically tested, as our previous studies have shown no effect on mechanical properties up to 5 weeks of cell culture (data not presented).

In vitro Study (Static vs Dynamic)

Canine chondrocytes were obtained from adult male canine (≥ 25 kg) right stifle joint. Animal experiments were conducted in accordance with the Canadian Council on Animal Care recommendations and with the approval of the ethics committee on animal experimentation. Cartilage specimens were rinsed, diced, and the cells were released after cartilage enzymatic digestion. Briefly, cartilage pieces were digested at 37°C in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Burlington, Ontario, (Canada) with 1 $mg.ml^{-1}$ of Pronase (Sigma, Oakville, Ontario, Canada) for 1 hour, followed by 2 $mg.ml^{-1}$ of type IV collagenase (Sigma) for 6 hours, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg ml^{-1} of streptomycin (supplements from Gibco BRL). After cartilage digestion, isolated chondrocytes were

seeded at high density in culture flasks containing 10% FBS/DMEM and maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air until they reached confluence.

Scaffold surface modification and cell seeding

We next verified if our biomimetic scaffolds could support cell attachment and viability. Prior to *in vitro* culture, the PLLA discs were sterilized by soaking in two changes of ethanol (70%) for 30 min. To improve cell attachment, the scaffolds were dipped in a sterilized solution of phosphate Buffered Saline (PBS) (Sigma-Aldrich Chemical, Oakville, Canada) containing gelatin (1%) (J.T Baker, Phillipsburg, USA) for 2 hours. After washing in PBS and drying, and prior to cell seeding, the scaffolds were conditioned inside distinctive wells of 24-well plates in culture medium overnight at 37 ^{0}C.

Then, 10^6 chondrocytes were loaded on the top of each scaffold, and incubated for 2 hours at 37 ^{0}C in an orbital shaker at 5 rpm. After 24 hours, the constructs were transferred to 24-well plates. Half the constructs were dynamically cultured in a bioreactor (Enduratec, Bose, Minnesota, USA) and the remaining scaffolds cultured under static condition for 7 or 14 days. The bioreactor was connected to the biorheometer to apply dynamic sinusoidal compression (strain of 10% for 1 hour follow by 7 hours relaxation, at 1 Hz frequency). Continuous infusion of culture medium into the bioreactor was made possible by using a peristaltic pump (10ml/min) and the culture medium renewed every two days.

MTT assay

Cell viability and number in the scaffolds (n=3) were quantified using MTT assay [19]. The constructs were immersed after 7 or 14 days of culture in MTT solution (500µl) for 2-3 hours. MTT solution was added on scaffolds without the presence of cells as a control. The

supernatant was next aspirated and the crystals were lysed by addition of isopropanol/HCl. The formazan was dissolved, by shaking the plates for 15 min on an orbital shaker, and 2 aliquots of 100 µl of each sample were transferred into a 96-well plate. The formazan absorbance was determined at 570 nm using a universal microplate reader (Bio-Tek Instruments Inc., Winooski, USA). The absorbance of formazan indirectly reflected the level of cell metabolism and this process is taken as a measure of the viability of cells in culture. The results were converted and expressed as cell number.

Histology and scanning electron microscopy

After 2 weeks of cell culture, constructs (n=3) from static and dynamic culture were sectioned in the transverse plan (10 µm thick) and stained with Hematoxylin-Eosin (H&E). The structure was then examined using light microscopy in order to evaluate cellular infiltration and growth. In the SEM study, the scaffolds with and without cells were washed in PBS, and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 for 2 hours. After thorough washing with PBS, the cells were dehydrated through a series of graded ethanol washings followed by critical point drying. The scaffolds were coated using palladium with gold plating and examined by SEM at 10kV.

Statistical analysis

All quantitative data are expressed by mean±standard deviation. A student t test was used to analyze the statistical significance of differences in viability for different samples. Comparaison has been made between each two samples (macroporous 250 *vs.* 400 µm and macroporous 250 µm *vs.* micro/macroporous 250 µm) and between culture conditions (static *vs.* dynamic). P values < 0.05 were considered as significant.

RESULTS

Figure 2 shows the compressive loading followed by stress relaxation for the third ramp strain. The 3D-plotted sample with 85% porosity had a comparable stress response to that of bovine cartilage. We then compared the estimated parameters for the scaffolds (macroporous and micro/macroporous, respectively) with those of bovine cartilage [18]. The estimated parameters indicate that the 3D-plotted constructs at 83% and 85% porosity closely mimic both the equilibrium modulus (E_z) and the compressive Young's modulus (E) of the bovine cartilage. These results indicate that, except in one case (78% porosity), our PLLA scaffolds possess similar mechanical properties compared to articular cartilage.

MTT assay was performed in order to investigate the cell viability on scaffold with different topologies (Figure 3). After one day of static culture macro/microporous scaffolds yielded significantly more cell attachment than macroporous scaffolds. Moreover, increased cell viability was observed on scaffolds with smaller pore size (250 μm) as compared to lager pore size (400 μm). These results suggest that macro/microporous scaffolds have improved surface topography (Figure 4a *vs.* Figure 4c), allowing more cell attachment, which leads to higher initial cell density in the scaffolds and to enhanced cell viability/number at 2 weeks.

Culturing these constructs under dynamic mechanical compression (10% strain for one hour every seven hours) significantly enhanced the number of cells within the scaffolds when compared to unloaded constructs cultured statically ($p<0.05$). Again, the macro/microporous constructs yielded the highest cell number compared to macroporous scaffolds. The lower cell growth rate was observed for the macroporous scaffolds with the

highest pore size (400 μm). Moreover, significantly higher cell growth was obtained ($p<0.05$) with decreasing the pore size (250 μm), probably due to increased cell-cell interaction. Incorporating the porogen into the scaffolds yielded an increase in cell viability compared to macroporous scaffolds. Cell growth was increased from scaffolds cultured under dynamic culture as compared to static culture. Based on MTT test, it is suggested that micro/macroporous scaffolds provide a better environment for cell growth under loading.

Using SEM analyses, cell spreading onto macro/microporous scaffolds was observed at 1 and 2 weeks of static (Figures 4b and 4c) and dynamic culture (Figure 4d and 4e). Compared to unseeded scaffold (Figure 4a), cell density appears to be low and in most cases, located onto the strands in the statically cultured scaffolds. For macroporous scaffolds (150/250 and 150/400 μm), much lower cell density was observed under static culture condition (figures not presented). However, under dynamic condition cell density was enhanced onto and between the strands as early as one week. Increased cell distribution on strand was also observed on 10 μm cross-sections of the constructs after dynamic culture (Figures 4f and 4g). After 14 days of dynamic culture, cells covered the entire scaffold surface and penetrated inside the construct strands (Figures 4e and 4g). At that time point, chondrocytes and matrix had bridged the strands in dynamically cultured scaffolds. SEM micrographs demonstrate that culturing the constructs under mechanical loading leads to a significant improvement in chondrocytes attachment and distribution within the scaffolds.

Tissue sections were next stained with H&E staining to determine the infiltration of the cells within the scaffolds (Figure 5). The histology slides confirmed the results observed

via SEM. At 1 week, H&E staining demonstrated sparse cellularity and uniform distribution of cells with little observable ECM deposition after one day and 7 days of static culture (Figure 5b). In contrast, increased cellularity and ECM production were observed in the macro/microporous construct submitted to dynamic culture for one week (Figure 5c).

DISCUSSION

Chondrocytes and the surrounding extracellular matrix composition are modulated by mechanical loading. In joints, biomechanical properties of cartilage exhibit topographical variation in response to different loading area. One of the difficulties with current cartilage repair techniques is their inability to consistently produce a neocartilage composed of hyaline articular cartilage necessary to assure long-term durability. With the use of a biomimetic scaffold in cartilage tissue engineering, biomechanical properties closely matching adjacent cartilage may be achieved because of early load transmission in the repaired tissue *in vivo*.

This study was conducted to investigate the ability of the designed 3D-plotted biomimetic scaffolds to support chondrocytes adhesion and viability under dynamic loading. In this study, we demonstrated that these scaffolds support cell attachment and growth under static and dynamic culture. We have shown here the ability of this fabrication technique to produce biomimetic scaffolds with constant architecture and desired properties. Moreover, cell attachment and density within scaffolds were greatly influenced by pore size and mechanical stimulation during two weeks of culture. It is already known that cell function and morphology on synthetic polymers is related to physical and chemical properties of the scaffolds. Scaffold's physical structure regulates the diffusion of nutrients and cell-interaction [20] whereas scaffold surface chemistry indirectly affects cell adhesion, morphology and activity [21]. Culturing the cells onto 3D scaffolds with smaller pore size (250 μm) was more suitable for cell growth than scaffolds with larger pore sizes (400 μm).

The poor adhesion observed within the scaffolds of large pore size (400 μm) as compared to small pore size (250 μm) could be due to limited cell attachment to the scaffolds because of inferior surface area and to decreased cell-cell interaction as a consequence of its large pore size. Moreover, cell attachment and viability were enhanced in 3D macro/microporous PLLA scaffolds after the incorporation of porogen (30% salt), which improved surface topography (Figure 4a *vs.* Figure 4c). This observation is in agreement with other studies showing that cartilaginous tissue formation was reported in scaffolds with smaller pore size of 174 μm and 115-335 μm [22,23,24]. Furthermore, a study by Grad *et al.* [25] has shown that a decrease in pore size might help to maintain the appropriate phenotype expression of the seeded chondrocytes and that cells are more likely to dedifferentiate when cultured in a 3D scaffold with large pore sizes (30 times the cell diameter, which is approximately 10-15 μm) [26,27].

Scaffolds provide a 3D structure that is essential for chondrocyte function and synthesis of cartilage-specific matrix proteins such as type II collagen, aggrecan, and sulfated proteoglycans. Liu *et al.* [28] reported that the cells within 3D scaffolds can be grown at larger numbers and for longer time periods. The presence of macropores in 3D scaffolds is important for promoting cell migration [29], however microporosity could promote mass transport, while increasing cell-cell interactions within the construct, leading to significantly improved formation of 3D tissues and *in vivo* performance of tissue-engineered constructs.

It is well known that articular cartilage is subjected to a combination of mechanical compression and fluid shear during *in vivo* loading, and in particular chondrocytes are

exposed to a variety of physical stimuli [30]. Application of mechanical stimulation and fluid shear is considered an effective strategy to influence cellular behavior in load bearing tissues. Mechanical stimulation is important for chondrocyte function and may be necessary for successful cartilage tissue engineering. In the present study, we use a cyclic strain bioreactor system to examine the effect of dynamic mechanical compression (10 % for one hour every seven hours) on chondrocyte viability. In this study, the cell number in scaffolds under compressive strain and perfusion during one-week culture was increased by two folds as compared to unloaded culture. This enhancement of cell number was observed after only two weeks of loading culture. Furthermore, Mauck *et al.*, (2000) have found that sulfated glycosaminoglycan content and hydroxyproline content was greater in dynamically loaded disks (10% strain, at 1Hz for one hour every 7 hours) compared to controls [31]. Although matrix composition was not specifically examined here, dynamic compression on chondrocytes matrix production and composition has been well characterized [32,33,34,35,36]. Subjecting the cartilage to stress induces mechanical, electrical and physicochemical signals that modulate the synthesis and the degradation of the extracellular matrix, so mechanical stresses are essential to improve the generation of extracellular matrix. On the other hand, long-term immobility causes a loss of mechanical properties and structure, thus mechanical stresses are directly linked to cartilage differentiation [37]. Furthermore, our PLLA scaffolds were able to support canine articular chondrocytes adhesion and growth for 2 weeks under dynamic and static culture. Dynamic culture led to increased cell density within the constructs.

Our data showed that the micro/macroporous scaffolds and dynamic culture provide a better environment for cell attachment and growth as compared to macroporous scaffolds

and static culture. Designing of biomimetic 3D scaffolds and dynamic cell culture in a perfused bioreactor improved cell viability and decreased the time of *in vitro* culture when compared to statically cultured constructs.

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FIGURE LEGENDS

Figure 1: Schematics of the scaffold fabrication technique forming a 0°/90° strand structure.

Figure 2: Stress relaxation under ramp compression (3rd ramp) for the scaffolds at different porosity levels compared to that bovine cartilage.

Figure 3: Cell viability in scaffolds at different pore sizes with and without the presence of micropores; macro/microporous (150/250 μm), macroporous (150/250 μm), and macroporous (150/400 μm) after one day, one week, and two weeks of static and dynamic culture. Error bars correspond to standard deviation. *$p<0.05$ as compared to static culture for each topology.

Figure 4: Top surface views of micro/macroporous scaffolds by SEM; (a) before cell seeding (pore size = 400 μm) (100X), (b & c) after one and 2 weeks of static culture (pore size = 150 μm) (150X and 100X), (d & e) after one and two weeks of dynamic culture (pore size = 150 μm) (100X and 95X).

Figure 5: Photographs taken at original magnification after Hematoxylin-Eosin staining of sliced micro/macroporous (250 μm) scaffolds after one day (a), one week of static culture (b), and dynamic culture (c) (scale bars are shown in the images).

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Table 1: Estimated porosity, equilibrium modulus, and the compressive Young's modulus for the scaffolds and for the bovine cartilage

Scaffold topologies	Porosity (%)	E_z [MPa]	E [MPa]
Macro/Microporous (150/250 microns)	83	1.9	1.7
Macroporous (150/250 microns)	78	3.3	6.5
Macroporous (150/400 microns)	85	1.0	1.1
Bovine cartilage	-	1.1	1.4

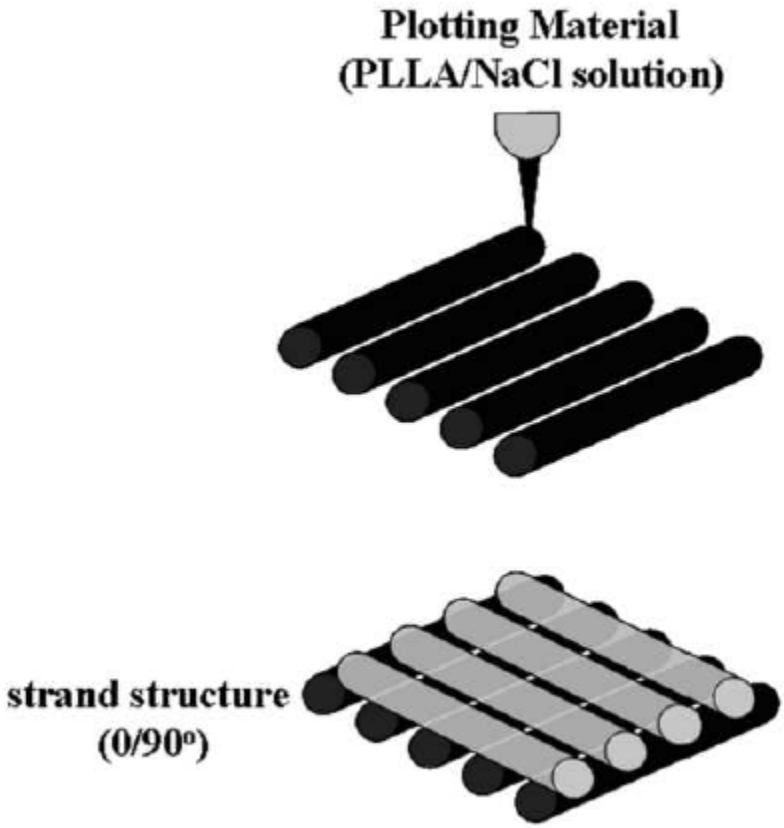

Figure 1, El-Ayoubi

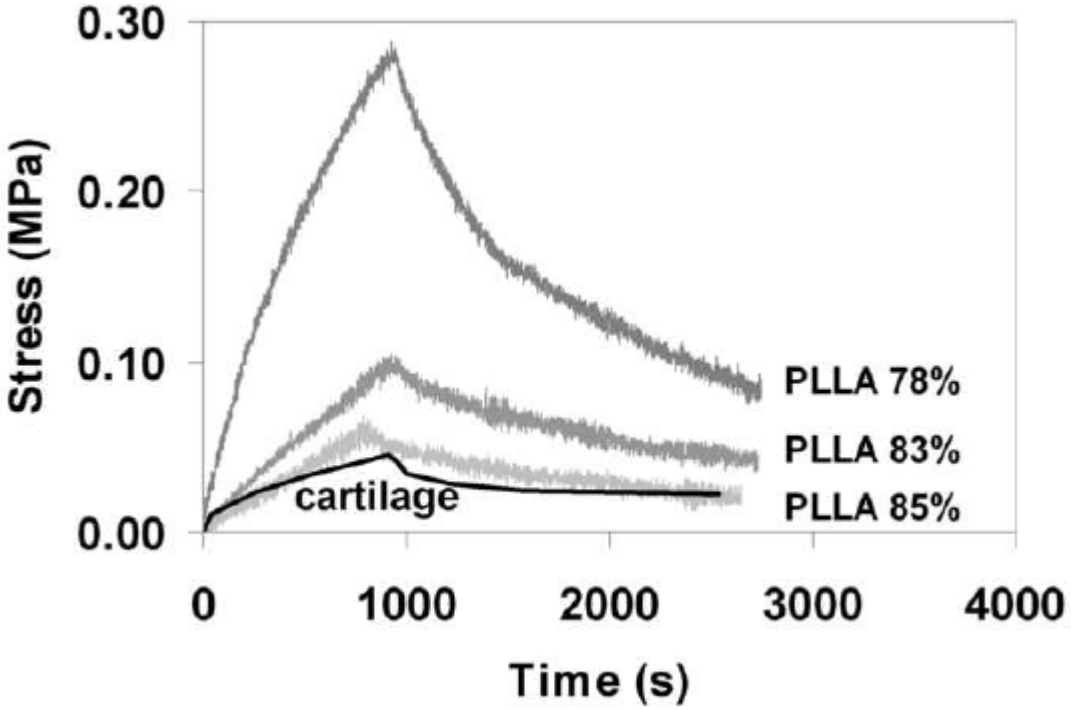

Figure 2, El-Ayoubi

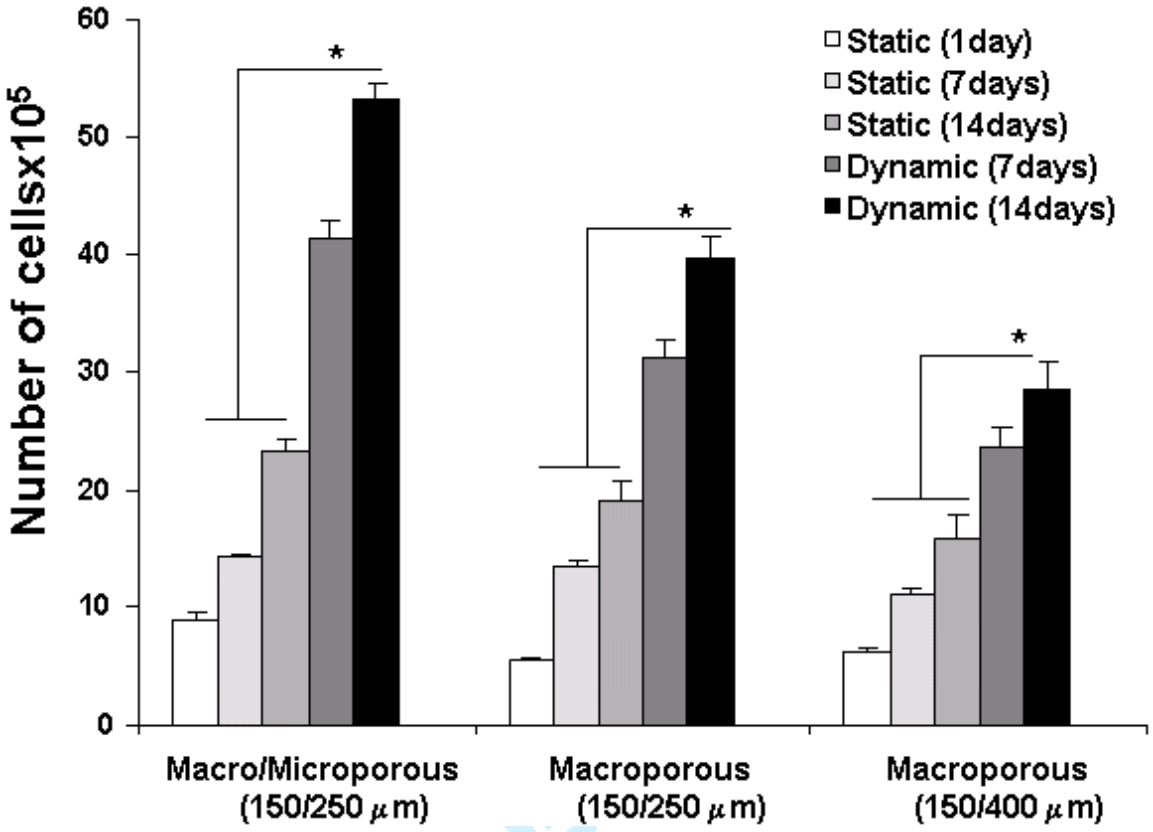

Figure 3, El-Ayoubi

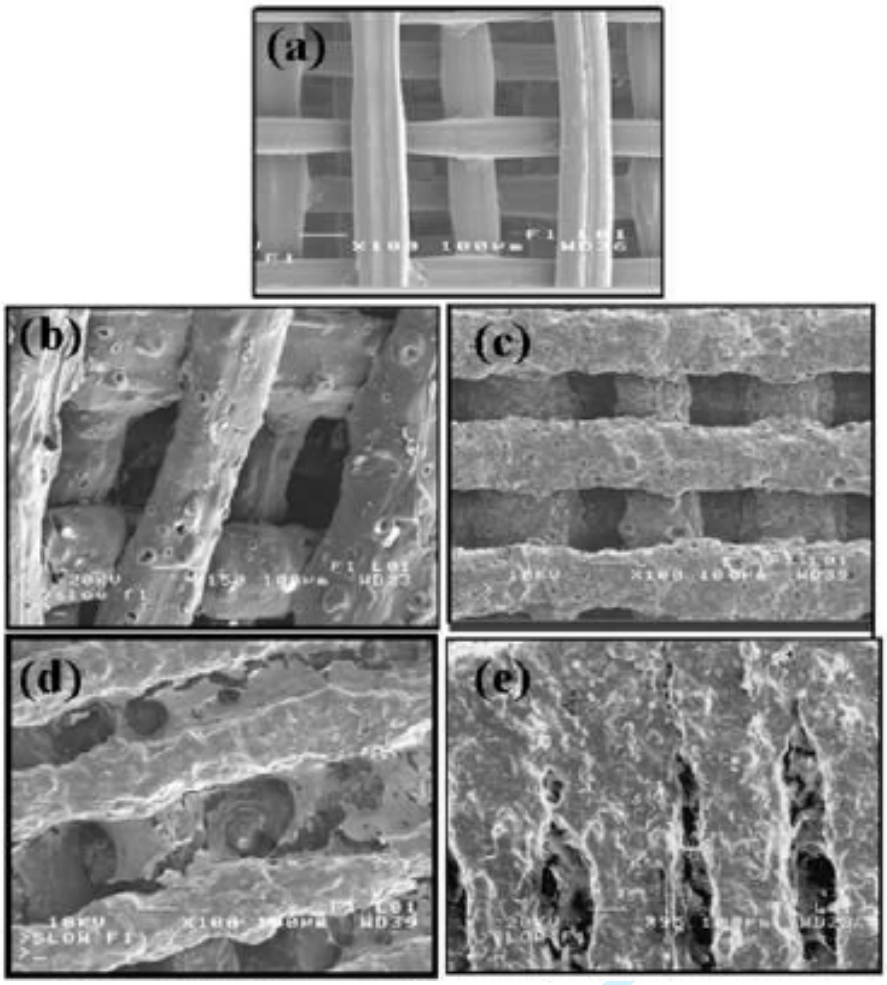

Figure 4, El-Ayoubi

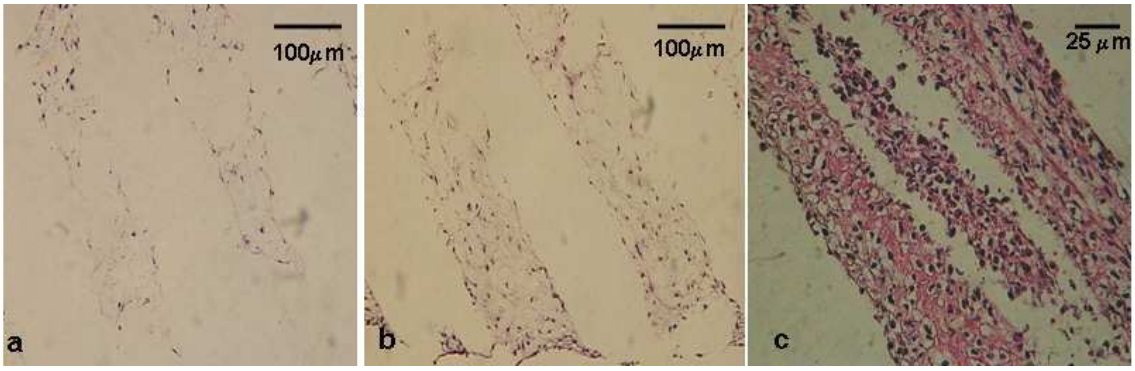

Figure 5, El-Ayoubi