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**Design and Fabrication of 3D Porous Scaffolds to Promote
Cell-Based Gene Therapy**

**Rouwayda El-Ayoubi¹, Nicoletta Eliopoulos², Robert DiRaddo¹, Jacques Galipeau²,
Azizeh-Mitra Yousefi¹**

1) Industrial Materials Institute, National Research Council of Canada, Boucherville,
Qc, J4B 6Y4, Canada

2) Lady Davis Institute for Medical Research, Jewish General Hospital, McGill
University, Montreal, QC, H3T 1E2, Canada

Corresponding author

Azizeh-Mitra Yousefi

Industrial Materials Institute, 75, de Mortagne Blvd, Boucherville,

QC, J4B 6Y4 Canada

Phone: 450-641-5386

Fax: 450-641-5106

Email: Azizeh.Yousefi@imi.cnrc-nrc.gc.ca

Abstract

Biomaterials capable of efficient gene product delivery by embedded cells provide a fundamental tool for the treatment of acquired or hereditary diseases. A major obstacle is maintaining adequate nutrient and oxygen diffusion to cells within the biomaterial. In this study, we combined the solid free-form fabrication (SFF) and porogen leaching techniques to design 3D scaffolds, with bimodal pore size distribution, for cell-based gene delivery. The objective of this study was to optimize the design of scaffolds, in order to improve cell viability and simultaneously drug delivery. Genetically engineered murine bone marrow-derived mesenchymal stromal cells (MSCs) were seeded onto Poly(L-Lactide)(PLLA) scaffolds with different microporosity, in order to deliver erythropoietin (Epo) as a vehicle protein. Over a period of 2 weeks in culture, an increase in cell proliferation and metabolic activity was observed with increasing microporosity of the scaffold. The concentration of Epo detected in supernatants increased with increasing microporosity level in scaffolds. Our study shows that the highly porous 3D matrix can promote cell viability and the release of their therapeutic protein. This study clearly demonstrates the capacity of the designed constructs for the dual role of scaffold for tissue regeneration and as a delivery system for soluble gene products.

Keys words: 3D scaffold, solid free-form fabrication, porogen leaching technique, gene therapy, erythropoietin, mesenchymal stromal cells.

1. Introduction

The combination of gene therapy and tissue engineering scaffolds represents a promising approach. Cell-based gene therapy uses genetically engineered cells to deliver a therapeutic gene product for the treatment of acquired or hereditary diseases [1]. The success of this strategy is dependent on mass transport to promote cell survival and protein delivery. Among important variables, the transport of oxygen and nutrients is typically the limiting factor for the survival of transplanted cells [2–3].

Cell-based gene therapy involves *in vitro* genetic manipulation and expansion of cells and their subsequent implantation *in vivo*. Cells that express the transgene serve as bioreactors for localized protein production. In this study, we used genetically engineered bone marrow-derived mesenchymal stromal cells (MSCs) for *in vivo* delivery of murine erythropoietin (Epo), as a vehicle protein. Epo is a haematopoietic growth factor stimulating the proliferation and differentiation of erythroid progenitor cells and inhibiting their apoptosis [4], and is used for the treatment of anemia. An alternative to the use of recombinant proteins, such as human erythropoietin (rhEpo) for instance, is to instead use autologous somatic cells after they have been gene-modified to secrete the plasma soluble gene product. MSCs are optimal as such autologous cells since they are plentiful and accessible in humans of all ages, can be obtained with minimal morbidity and discomfort, can be easily handled, expanded, and genetically engineered *in vitro*, can be clonally selected for high protein expressors, and can be reimplanted in the donor without a conditioning regimen [5–8].

To couple scaffold-based tissue engineering and gene therapy, we have previously embedded Epo gene-modified MSCs within the mouse-compatible material Matrigel™

[9], as well as within the FDA-approved bovine collagen-based matrix Contigen™ [10]. Furthermore, we tested this latter neo-organoid strategy in a mouse model of anemia induced by experimental chronic renal failure, and consequently noted that Epo-secreting MSCs embedded in Contigen™, when implanted subcutaneously in these anemic mice, led to a rise in plasma Epo concentration, an increase in hematocrit that was cell dose dependent, as well as an enhancement in exercise capacity [11]. However, a greater cell density was observed in the collagen implant periphery, while a sparse and more necrotic cell population was seen in the implant core. There was little neovascularization in the Contigen. This failure to vascularize most likely contributed to the death (coagulation necrosis) of implanted MSCs localized in the implant core and to the fragmentation of the organoid material [10]. Likewise, studies from Mooney's group [12] found that after transplanting hepatocytes on PLGA scaffolds (3 mm thick), the majority of these cells in the central area of the scaffold died within a short time. The death of cells was due to the limitation of nutrient transport and the greatest distance from blood vessels in the host tissue. Recently, Hurtado et al. [13] seeded genetically modified Schwann cells onto macroporous scaffolds to promote axonal regeneration in the injured spinal cord; however, they observed poor cell viability in the scaffolds following implantation.

To overcome this kind of problem, the architecture of the scaffold needs to be optimized in order to provide a nutrient rich environment and to prevent cell death. Ideally, a scaffold should possess the following characteristics to bring about the desired biological response [14]: 1) three-dimensional and highly porous with an interconnected pore network for cell growth and flow transport of nutrients, oxygen and metabolic waste, (2) biodegradable or bioresorbable with a controllable degradation and resorption rate to

match cell growth in vitro and/or in vivo, (3) suitable surface chemistry to allow cell attachment, proliferation and differentiation, (4) mechanical properties matching those of tissues at the site of implantation, and (5) controlled release of drug/gene product.

The synthetic materials most frequently used in tissue engineering scaffolds are polylactic acid (PLA) and polyglycolic acid (PGA). These biodegradable polymers have gained FDA approval for human use in a variety of applications [15], and have been extensively investigated as drug carriers in controlled drug delivery, due to their excellent biocompatibility, biodegradability, and controlled drug release. The design and fabrication of porous constructs based on these materials to act as scaffolds for cell attachment and growth is critical for the success of the tissue engineering field. The scaffold fabrication technique should be flexible to generate alternative scaffold architectures in order to allow optimal designs. Therefore, the emerging solid free-form fabrication (SFF) techniques are becoming the method of choice for tissue engineering applications. Among these processes, the 3D plotting technique has shown great potential in making reproducible 3D scaffolds featuring interconnected pores and controlled architectures [16].

This study aims to fabricate 3D scaffolds with controlled architectures to promote cell viability and functionality. We have introduced a new scaffold fabrication technique by combining the solid free-form fabrication and the porogen leaching techniques, in order to create a bimodal pore size distribution. The produced scaffold architecture allows cell migration through the 3D-plotted macropores, while promoting the oxygen and nutrient transport via the micropores created by the porogen.

2. Material and methods

2.1. Scaffold material

The plotting material was prepared by dissolving the PLLA with a molecular weight of 220 kDa (Biomer, Germany) in methyl ethyl ketone (MEK). The dissolution process was on average one to three days for grinded PLLA granules, and three to five days for whole polymer granules. The mixture was stirred thoroughly at least once a day to speed up the dissolution process. Subsequently, the solution was mixed with 10% to 50% w/w of salt particles (NaCl), previously sieved to obtain a desired particle size distribution (30 to 75 μm). 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).

2.2. Scaffold fabrication

3D scaffolds were fabricated using a bioplotter from Envisiontec, which is essentially an XYZ plotter as described by Landers et al [16]. Figure 1 shows the schematics of the proposed scaffold fabrication technique leading to a bimodal pore size distribution. Briefly, the plotting material was transferred to the cartridges with 250 μm dispensing needle tips and was dispensed layer by layer, forming a 0°/90° strand structure (Figs. 1a to 1d). 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 1.13 mm thick were fabricated during this process (Fig. 1e). At the end of the fabrication process, each scaffold was punched into discs of 6 mm of diameter and 1.13 mm thick (Fig. 1e) and

then immersed in water at 50 °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.

A variety of plotting patterns can be created if the strands within each layer are laid without or with offset with respect to the previous layers in order to form non-staggered or staggered patterns as presented in Fig. 2a and 2b, respectively. Both patterns were considered in this study so as to investigate the possible effect of the scaffold pattern on cell growth and Epo release.

2.3. Determination of the plotting parameters

Since the plotting parameters affect the physico-mechanical properties of the scaffolds, a series of experiments were initially conducted to determine the influence of the plotting parameters on scaffold porosity and mechanical properties [17]. The primary parameters were the internal diameter of the dispensing needle, layer thickness (h), strand diameter (D), and the distance between the strands (L), representing the pore size of the constructs (see Fig. 1c). Needles with larger tip diameters ($> 250\ \mu\text{m}$) generally produced scaffolds with thicker strands, which had lower porosities and higher Young's Modulus [17]. Therefore, needles with a tip diameter of $250\ \mu\text{m}$ were used in this study. On the other hand, increasing the layer thickness compromised the adhesion of the strands between two successive layers, and as a consequence the integrity of the scaffold. It was found that a layer thickness below $140\ \mu\text{m}$ provided adequate adhesion between the layers for a needle tip diameter of $250\ \mu\text{m}$.

The optimal distance between the strands was determined targeting the highest scaffold overall surface available for cell attachment (effective surface), while respecting the

channel interconnectivity by avoiding excessive overlap between the successive bioplotted strands (staggered pattern in Fig. 2b). To this end, for the strand diameter of 150 μm used in this study, the distance between the strands was also set to 150 μm . The effective surface of the 3D plotted constructs was subsequently determined as a function of scaffold plotting parameters (D, L, and h in Fig. 1c) and scaffold geometry (P, W, and t in Fig. 1e). Taking $h = L$ and $W = P$, and knowing that the number of strands in planar direction (n_p) and in thickness direction (n_t) are given by:

$$n_p = \frac{P + L}{D + L} \quad (1)$$

$$n_t = \frac{t}{D} \quad (2)$$

the effective surface of the brick-shaped scaffolds, without micropores, was approximated by:

$$S_e = \left(\frac{P + L}{D + L} \right) \left[\pi P t - \left(\frac{P + L}{D + L} \right) \left(\frac{\pi D}{4} \right)^2 \left(\frac{t}{D} - 1 \right) \right] \quad (3)$$

where the subtracted term in the right hand side accounts for the contact surface between the successive layers (S_c), assuming a square contact area ($S_c = n_p^2 (\pi D / 4)^2 (n_t - 1)$). In Table (1), the effective surface of the brick-shaped scaffolds, estimated for different distances between the strands (L), is compared with that of a non-porous brick ($S = 2P^2 + 4Pt$). It can be seen that once the distance between the strands is reduced to 150 μm , the effective surface available for cell attachment is increased up to 5 times that of nonporous brick.

2.4. Porosity measurements

The true volume of each scaffold was calculated based on the polymer density ($\rho_{PLLA}=1.25 \text{ g/cm}^3$) and scaffold mass (m) using $V_t = m/\rho$. The scaffold porosity (in vol%) was calculated using the following equation:

$$\phi = \frac{(V_a - V_t)}{V_a} \times 100 \quad (4)$$

where V_a is the apparent volume of the scaffold (in cm^3) estimated based on the geometry of each disk (thickness and diameter). Table (2) gives the overall porosity of the scaffolds at different microporosity levels.

2.5. Microcomputed tomography analysis

To verify the presence of salt particles after porogen leaching, the microcomputed tomography (micro-CT) technique was used. The specimens were scanned using an MS-130 high-resolution Micro-CT scanner at the University of Michigan (GE Medical Systems, London, ON, Canada) to visualize and quantify the amount of salt before and after leaching. Samples with different porogen concentrations were scanned, and each constituent (polymer and salt) was identified. Three-dimensional isosurface renderings were made with MicroView Version 1.1.1 (GE Medical Systems).

2.6. Mechanical characterization

The unconfined compression tests were conducted on the disk-shape scaffolds using a displacement-controlled apparatus (Enduratec, ELF series) in a saline bath (0.009g/cm^3 salt concentration). A ramp strain of 3% was applied at a displacement rate of $0.115 \text{ } \mu\text{m/s}$. Three samples were tested at each porosity level (0%, 20%, and 30% of salt) for the non-staggered pattern (without offset). The slope of the stress response vs.

deformation curve, representing the compressive Young's modulus, was estimated for all samples. The Young's modulus for these scaffolds is given in Table 2.

2.7. Isolation and genetic engineering of mesenchymal stromal cells

Murine bone marrow-derived mesenchymal stromal cells (MSCs) were isolated as we have previously reported [9–11], from female 15-20g C57Bl/6 mice (Charles River, Laprairie Co). Briefly, mice were sacrificed and whole bone marrow, collected by flushing the femurs and tibias with complete media (DMEM supplemented with 10% FBS and 50U/ml Pen/Strep), was plated in tissue culture dishes, and incubated at 37°C with 5% CO₂. Five days later, the non-adherent hematopoietic cells were discarded and the adherent MSCs were maintained in culture for 10-15 passages in complete media. These MSCs were then genetically engineered by several rounds of retroviral transduction with filtered (0.45µm) retroviral particles previously generated to express the cDNA for murine Epo via transfection of our Epo containing retroviral construct into ecotropic retrovirus packaging cells GP+E86, as we have published [10]. The Epo MSCs utilized in the present study were a monoclonal population isolated after plating the polyclonal population at limiting dilution. The secretion of Epo *in vitro* by these gene-modified cells was quantified by enzyme-linked immunosorbent assay (ELISA) (Roche Diagnostics, Indianapolis, IN) performed on conditioned media, and revealed that Epo MSCs secrete 3 Units of Epo per million cells per 24 hours [10].

2.8. Scaffold surface modification and cell seeding

Prior to *in vitro* culture, the PLLA disks were sterilized by autoclave at 200°C for 10 min. In order to improve cell attachment, the scaffold surface was modified by coating the sample with gelatin (Sigma Chemical, Co). The scaffolds were dipped in a sterilized

solution of PBS containing gelatin (1%) 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°C.

A total of 1×10^6 cells/ml was loaded directly on the top of the scaffold, and incubated for 2 hours at 37°C to allow the cells to build adhesion plaques on the scaffold. Afterwards, 1 ml of complete medium was added to each well. Cell-scaffold constructs were then cultured for 2 weeks. The media was changed every 2-3 days for each time point. The seeded scaffolds (n=4) were removed at specific time points after incubation for evaluation of adhesion, proliferation and viability. To confirm that murine Epo-transduced MSCs do secrete murine Epo *in vitro*, and to quantify the amount, we utilized supernatant (200 µl) collected from these MSCs in an ELISA for human Epo (which also can be used to detect murine Epo). As control, scaffolds without cell enrichment (n=4) were also tested at the same time intervals and conditions.

2.9. Cell attachment and growth

The attachment and growth of MSCs on the scaffolds were examined as a function of culture time, surface modification (coating) and scaffold architecture (level of micropores). Cell attachment and growth were monitored by histological study and scanning electron microscopy (SEM), and the cell activity by MTT assay.

2.9.1 Histological study and scanning electron microscopy

At the end of each time point, seeded scaffolds were fixed in glutaraldehyde and prepared for histological examination. Tissue blocks were sectioned at 10 µm in the transverse plan and stained with Hematoxylin-Eosin (H&E). The slides were prepared in triplicates,

the composite structure was then observed using light microscopy in order to evaluate cellular infiltration and growth.

For electron microscopy 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 scanning electron microscope.

2.9.2. MTT assay

Cell activity in the scaffolds (n=4) was quantified using MTT assay. The yellow tetrazolium salt (MTT) is reduced in metabolically active cells to form insoluble purple formazan crystals. The reduction process requires functional mitochondria. This assay provides information on metabolic activity as well as the cell number when used with an accurate standard curve. The scaffolds seeded with MSCs were removed after each testing time, and incubated at 37°C in MTT solution (200µl) for 2-3 hours. The supernatant was 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 optical density (O.D.) was determined at 570 nm using a universal microplate reader (Bio-Tek Instruments Inc.). MTT assay was also performed on direct counts of MSCs consisting of the following numbers: 2.5×10^5 , 5×10^5 , 10^6 , 2×10^6 , 4×10^6 . The absorbance values were plotted against the counted cell numbers, and thus a standard calibration curve was established. Viable cells grown on scaffolds were determined based on their MTT results.

2.10. Statistical analysis

All quantitative data are expressed by mean $\pm$ standard deviation. Student t tests were used to compare the proliferation and viability for different topologies. P values < 0.05 were considered as significant.

3. Results

Micro-CT conducted on scaffolds before and after extraction of salt showed the presence of salt particles (dark spot) in scaffolds before salt extraction (3a) and proved that all the salt was removed after extraction (3b). In terms of scaffold mechanical properties, the results of Young's modulus in Table (2) indicate a decrease in compressive stiffness of the constructs as the microporosity increases. The mechanical testing results at the highest microporosity (50% salt) are not shown in this work because of poor reproducibility for these constructs, mainly due to the low sample thickness (1.13 mm).

The pore distribution and the surface topography of the 3D scaffolds were analyzed by scanning electron microscopy (Fig. 4). SEM analysis revealed a very good consistency between the machine settings (bioplotter) and the measured parameters (pore size and strand diameter). SEM analysis of the scaffold with and without micropores showed variation in surface morphology (Fig. 4). The surface of the scaffolds without porogen appeared very smooth with cubic shape of macropores (Fig. 4a). Before salt extraction, the presence of salt particles (30 to 75 μ m) was observed inside the scaffold, leading to a rough surface (Fig. 4b). After salt extraction, SEM photomicrographs revealed high microporosity throughout the scaffold, with micropores ranging from 5 to 75 μ m. The larger micropores (30 to 75 μ m) were created by leaching out the salt particles (Figs. 4c

and 4d), while the smaller pores (5-25 μm) resulted from solvent evaporation. SEM images following 2 weeks static seeding showed cell attachment onto (Fig. 4e), between the strands and inside the micropores (Fig. 4f), indicating the attachment of the cells on, between and into the porous surface of scaffolds.

Hematoxylin-eosin staining of whole scaffolds (Fig. 5a) and cross sections (Fig. 5b) (10 μm thickness) at day 14 revealed that the highly porous scaffolds (30% and 50% salt) appeared to have a greater number of cells distributed across the sections. The majority of the cells had intact nuclei, indicating the cell viability inside the 3D constructs.

The cells-seeded scaffolds were cultured for 1 and 2 weeks and were tested to determine whether they maintained sufficient metabolic activity by MTT assay. MTT assay was used as a measure of relative cell viability. MTT results indicate that all cellular scaffolds exhibited measurable metabolic activity as compared to acellular scaffolds.

Fig. 6 displays the cell viability of MSCs seeded on scaffolds without and with different density of micropores. The growth of cells was poor on untreated PLLA scaffold due to the absence of micropores. The metabolic activity and the number of cells seemed to increase time dependently and with the increase of microporosity in scaffolds. The scaffolds with low level of microporosity (10%) demonstrated low cell numbers after 2 weeks of seeding, as compared to the 30% and 50% microporosity (Fig. 6). The increase of micropores led to an improvement in cell growth. The enhancement of metabolic activity and cell number in scaffolds (30 and 50% microporosity) was likely caused by more efficient transport of oxygen and nutrients to the cells loaded in the scaffolds.

Fig. 7 shows the effect of the plotting pattern (with or without offset) on the cell viability, as a function of in vitro incubation time. It can be seen that the offset pattern improves cell attachment, resulting in higher cell viability, possibly because the cells can be retained inside the scaffold more easily during and after seeding.

The functional capacity of MSCs to secrete Epo is demonstrated in Fig. 8, where the release of Epo, from MSCs after one week of culture increased with increasing microporosity level. The concentration of Epo ($\text{mU}/10^6 \text{ cells}/24\text{h}$) from scaffolds (50% salt) significantly increased (2 fold) as compared to scaffolds without microporosity.

4. Discussion

Our present study reports a method to produce 3D porous scaffolds with a well-defined architecture and high porosity. We combined solid free-form fabrication and porogen leaching techniques, in order to create a bimodal pore size distribution within the 3D plotted scaffolds. These scaffolds could be implanted in vivo for drug delivery and for cell and gene therapy applications.

A variety of techniques is currently being employed for fabrication of porous polymeric scaffolds, including phase separation [18–19], emulsion freeze-drying [20], gas foaming [21], fiber bonding [22] and porogen leaching [23]. The conventional scaffold fabrication techniques can create highly porous scaffolds with smaller pores, but they have a very limited control on scaffold architecture and pore interconnectivity. On the other hand, the emerging solid free-form fabrication techniques could create complex scaffold architectures with interconnected pores; however, the smallest attainable feature size is usually limited to > 100 microns [24]. Among these, the 3D plotting technique permits to

design and fabricate scaffolds with controlled external and internal architecture (shape, geometry, porosity, and orientation) to match implant site geometry and to yield biomimetic structures [25]. Our present investigation introduces a new scaffold fabrication technique using a combination of the SFF (3D-plotting) and porogen leaching techniques [26] that could resolve the shortcoming of each technique, when used individually.

We have here demonstrated that the combination of the two techniques may be adopted to create 3D biomimetic scaffolds that support cell proliferation and function. In the present in vitro study, murine MSCs genetically engineered to secrete murine Epo were seeded onto these scaffolds for 2 weeks, in order to investigate cell viability and protein release. We demonstrated that the uniform distribution of the seeded cells within the scaffolds depended on scaffold properties, such as shape, surface, and porosity.

Several studies have shown that the 3D geometry can provide greater available surface area for cell attachment, spreading, proliferation, and transmission of biochemical substances than 2D [27]. In addition, Liu et al, (2006) have reported that compared to 2D tissue culture plates, cells differentiated on porous 3D scaffolds possess significantly higher expression levels of extracellular matrix (ECM)-related genes, as well as genes that regulate cell growth, proliferation and differentiation [28]. Cells in 3D scaffolds can be grown at larger number and for longer time periods [29]. We have also observed in this study that cell adhesion and proliferation within scaffolds were greatly enhanced during two weeks of culture.

Attachment of cells to substrate is a major factor controlling their structure, function and cell cycle [30]. Generally, since PLLA is hydrophobic, it can reduce cell attachment and

proliferation. Therefore, introducing bioactive molecules on the surface of scaffolds would have an immediate effect on cell behavior. In this study, the scaffolds were coated with gelatin to improve cell adhesion to the scaffold. Our preliminary study showed a poor cell adhesion for non-coated scaffolds (data not shown). Therefore, this investigation was performed using gelatin-coated scaffolds. This is consistent with other studies using PLGA [31] and PLLA scaffolds, where coating led to enhanced cell growth [32–33].

We observed an increase in cell proliferation with increasing microporosity level of the scaffolds, and consequently a difference in the cellular response. The increased surface area, resulting from the micropores, led to a higher amount of cells attached to the scaffolds. Moreover, improved nutrient and oxygen diffusion, also due to the micropores, promoted cell growth and function [34–36]. On the other hand, the presence of macropores is important for promoting cell migration.

Cell proliferation was not only dependent on porosity, but also on fiber deposition and orientation. An increase of cell proliferation in the off set structure was noticed. The effect of structure changing profile could be due to the facility of cell-cell communication during and after seeding, since pore size and shape influence cell attachment and in growth.

Epo release from MSCs embedded in the scaffolds was measured after one week of culture, and revealed that the level of Epo release increased with the increasing microporosity density within the scaffolds. We hypothesize that the use of these scaffolds in clinical studies will allow elevated bioavailability of the soluble protein gene product, as well as prolonged viability of the MSCs engineered to deliver this “drug”.

Numerous studies have shown the capacity of synthetic polymers to serve as delivery systems. Newman and McBurney among others, have demonstrated the capacity of PLGA microspheres to serve a dual role as both delivery systems of bioactive factors and as scaffolds for pluripotent stem cells for tissue engineering and regeneration [37] and [38]. For gene therapy applications, several criteria are associated with obstacles, such as cell population to target and the extent of transgene expression for a therapeutic effect. However, these limitations could be circumvented through the use of cell and gene therapy, or more precisely through the transplantation of autologous somatic cells, such as MSCs, genetically engineered to deliver a beneficial soluble gene product. Genetically engineered cells have been employed in numerous models; such for bone [39–40] and nerve regeneration [41]. MSCs, other than their use as cellular delivery vehicles for therapeutic gene products, have also been exploited for regenerative medicine due to their progenitor cell properties and innate abilities to differentiate into a wide range of cells which include chondrocytes, osteocytes, cardiomyocytes, adipocytes, astrocytes [42–45].

5. Conclusions

In this study, we have optimized the design of 3D scaffolds to maintain cell viability and delivery of therapeutic protein, through precise manipulation of scaffold shape, porosity, and internal pore architecture. By combining tissue engineering and gene therapy techniques we could generate a neo-organoid device that could be used to treat a wide variety of medical problems, including cancers. This technology could circumvent the need for protein purification and would be particularly useful for complex molecules requiring potential delivery.

The results of this study can be easily adapted to many cell types and may be employed for different cell and protein therapies. Further studies could involve optimization of the scaffold architecture to control the release of the protein. Optimizing the design of these delivery systems will potentially have a great impact on the ability of clinicians to treat several diseases, including cancer, trauma, diabetes, and cardiovascular illnesses.

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Figure legends

Figure 1: Schematics of the proposed scaffold fabrication technique leading to a construct with bimodal pore size distribution; (a) Preparation of initial paste, (b) & (c) 3D plotting, (d) porous scaffold after salt leaching, (e) final disk-shaped scaffolds after punching.

Figure 2: SEM images of the side view showing the plotting patterns; (a) without off set (non-staggered) (60X), (b) with off set (staggered) (80X) .

Figure 3: Micro-CT images of the scaffolds fabricated by combination of 3D-plotting and porogen leaching techniques; (2a) before salt leaching, (2b) after salt leaching. The salt and polymer are represented by pink and cyan colors, respectively.

Figure 4: Top surface views of scaffolds by SEM; (a) before salt incorporation, (b) before salt leaching (100X), (c& d) after salt leaching (100X and 300X), (e,f) after cell seeding on the scaffolds (100X and 200X).

Figure 5: Photographs taken at original magnification (40X); (a) after hematoxylin staining of the whole scaffolds, (b) cross section of scaffolds at different microporosity levels (10 %, 30 %, and 50 %).

Figure 6: Cellular metabolic activity and number of cells in scaffolds at different microporosity levels after one and two weeks of culture. Error bars correspond to standard deviation.

Figure 7: (a) Cell growth in the scaffold with highest level of microporosity (50%), at different culture times, (b) photographs taken after hematoxylin staining of cross sections. The results are presented for both plotting pattern.

Figure 8: Release of Epo by embedded genetically engineered marrow-derived mesenchymal stromal cells (MSCs) within scaffolds (different microporosity) after one week of culture. * $P < 0.05$ scaffolds (20% and 50%) as compared to scaffolds without microporosity (0%).

Table (1): Effective surface of the brick-shaped scaffolds as a function of the distance between strands (L); D = 150 μ m, P = 20 mm, t = 1.13 mm.

L (μm)	Non-porous Brick (S in mm²)	Porous Brick (S_e in mm²)	S_e/S
300	890	2226	2.5
200	890	3293	3.7
150	890	4360	4.9

Table (2): Overall porosity and Young's modulus for the 3D-plotted scaffolds at different levels of microporosity (% of salt).

% of salt	Porosity (%)	Modulus (MPa)
0	56 $\pm$ 0.5	3.64 $\pm$ 0.17
10	58 $\pm$ 0.7	-
20	62 $\pm$ 1.5	1.58 $\pm$ 0.20
30	64 $\pm$ 1.1	0.51 $\pm$ 0.05
50	69 $\pm$ 2.7	-

Fig. 1. El-Ayoubi, R.

Fig. 2. El-Ayoubi, R.

Fig. 3. El-Ayoubi, R.

Fig. 4. El-Ayoubi, R.

Fig. 5. El-Ayoubi, R.

Fig. 6. El-Ayoubi, R.

Fig. 7. El-Ayoubi, R.

Fig. 8. El-Ayoubi, R.