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3-D Nanofibrous electrospun multilayered construct is an alternative ECM mimicking scaffold

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Abstract Extra cellular matrix (ECM) is a natural cell environment, possesses complicated nano- and macro-architecture. Mimicking this three-dimensional (3-D) web is a challenge in the modern tissue engineering. This study examined the application of a novel 3-D construct, produced by multilayered organization of electrospun nanofiber membranes, for human bone marrow-derived mesenchymal stem cells (hMSCs) support. The hMSCs were seeded on an electrospun scaffold composed of poly ε-caproloactone (PCL) and collagen (COL) (1:1), and cultured in a dynamic flow bioreactor prior to in vivo implantation. Cell viability after seeding was analyzed by AlamarBlue™ Assay. At the various stages of experiment, cell morphology was examined by histology, scanning electron microscopy (SEM) and confocal microscopy. *Results*: A porous 3-D network of randomly oriented nanofibers appeared to support cell attachment in a way similar to traditionally used tissue culture polysterene plate. The following 6 week culture process of the tested construct in the dynamic flow system led to massive cell proliferation with even distribution inside the scaffold. Subcutaneous implantation of the cultured construct into nude mice demonstrated good integration with the surrounding tissues and neovascularization. *Conclusion*: The combination of electrospinning technology with multilayer technique resulted in the novel 3-D nanofiber multilayered construct, able to contain efficient cell mass necessary for a successful in vivo grafting. The success of this approach with undifferentiated cells implies the possibility of its application as a platform for development of constructs with cells directed into various tissue types.

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Introduction

The shortage of donor organs, the high cost and possible complications of transplant surgery have created the need for an alternate source of mammalian tissue [1]. Absorbable polymeric biomaterials, in the form of tissue-like supports, have recently been recognized for their application in the growing field of tissue engineering research and reconstructive surgery [2]. The role of the scaffold is to allow target cells to attach, multiply and transform from a nonspecific primitive state into cells exhibiting specific functions. Scaffold requirements include biocompatibility and biodegradability [3]. Many issues must be addressed in developing a viable 3-D construct for in vivo implantation. These include the selection of an appropriate polymer with controlled porosity and permeability, scaffold fabrication methodology, choice of cell culture conditions, optimization of cell-seeding technologies and in vivo animal models [4–6]. The recent understanding that extra celluar matrix (ECM) is a natural 3-D cell supporter with com-

plicated ultrastructure [7] determines the direction of scaffold design development.

The technique of electrospinning is a promising direction for producing artificial tissues. This process utilizes an electrostatic field to control the formation and deposition of polymer nanofibers [8–10]. The procedure, which is technically feasible for the fabrication of filaments ranging in the nanometer to micrometer scale with a certain degree of alignment [11, 12], is remarkably efficient, rapid and inexpensive. Synthetic polymers such as polylactic acid (PLA) and poly(lactic-co-glycolic acid) PLGA, and natural macromolecules such as collagen and fibrinogen, have been processed into fibrous non-woven scaffolds for use in tissue engineering research and have been shown to support stem cell growth and differentiation [13–16].

The major challenges in the field of scaffold bioengineering are the operative assimilation and expansion of cells on the scaffold as well as the provision of an optimal supply of nutrients and oxygenation to the entire volume of the 3-D structure. Media perfusion bioreactor systems have been developed to improve mass transport throughout 3-D tissue engineered constructs cultured in vitro [17, 18]. This work examined the potential of the electrospun nanofiber multilayered construct to support hMSC proliferation by the use of a dynamic flow system, followed by in vivo implantation for biocompatibility evaluation. In order to provide a proof of concept our study was performed with undifferentiated cells. Since hMSC were shown to be capable of differentiation to multiple lineages [19] future studies are needed to demonstrate that by changing culture conditions with specific additives, this system may serve as a wide platform for constructing implants with cells differentiated into various cell types (osteoblasts, chndroblasts myoblasts, adipocytes) building target tissues.

Materials and methods

Production of electrospun scaffolds

PCL (~80 kDa) (Aldrich, USA) and bovine collagen type I (Nitta Gelatin, Japan) were dissolved separately in dichloromethane/dimethyl foramide (75:25) to give a 10% solution (w/v). Both solutions were then combined at a ratio of 1:1. The solution was extruded from a 1 mL syringe connected to a hypodermic needle (bore size 0.5 mm; flow rate 0.2–0.5 mL/h). The strength of the electrostatic field was 1 kV/cm. The electrospun nanofibers were deposited onto a grounded collector in the form of a non-woven sheet. The end stage product (100–120 μm thick) was cut into circular shapes (8 mm diameter) to fit into 96-well tissue culture plates, sterilized by oxygen plasma and soaked in DMEM medium (24 h) prior to seeding of the cells.

Cell source and culture conditions

Isolation of human MSCs (hMSCs) was performed as following: human bone marrow cells were obtained by aspiration from the posterior iliac crest of healthy adult donors according to the Declaration of Helsinki, washed twice with PBS, resuspended in Dulbeco modified Eagle medium (DMEM), 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine all from Biological Industries, Israel. Cell number and was assessed by using Turk's solution, following by plating at the density 30 million per 10 cm tissue culture dish. Human bone marrow-derived mesenchymal stem cells were cultured (37 °C, 5% CO_2, 1 week) in the same medium (DMEM, 10% FCS, 2 mM L-glutamine and Pen-Strep (100 U/mL, 100 μg/mL)). At 70–80% confluence the cells were trypsinized, seeded onto electrospun scaffolds (40,000 cells in final volume of 1 mL) and further incubated (24 h) in the same medium prior to transfer to the dynamic flow system.

Cell viability assay

Adhesion studies were performed in 96-well culture plates (1.5×10^4 cells/well). Cell viability after seeding was assessed by the AlamarBlue™ Assay (Serotec, USA) according to the standard protocol. Briefly, cells were incubated (37 °C) with 10% (v/v) AlamarBlue reagent in DMEM for 2 h. Following incubation, data was collected using a FLUOstar Galaxy fluorometer. Fluorescence was recorded at 540 nm excitation and 580 nm emission.

Bioreactor and multilayered electrospun scaffold construct

Thirty individual scaffold mats containing hMSCs (as described above) were impaled onto a sharp rod, made of titanium, forming a 3-D cylindrical shaped construct composed of densely-stacked layers. A sketch of the assembly is presented in Fig. 1. The 3-D scaffold was then transferred to a dynamic flow system utilizing a PluriX™ plug-flow bioreactor (Pluristem Life Systems, Inc., USA). The bioreactor was based on a controlled continuous vertical axial flow system (6.4 mL/min). The system contained a gas reservoir, dissolved O_2 monitor, pH monitor, peristaltic pump with advanced flow control and a medium exchange container. Basic parameters of 37 °C, O_2 and CO_2 were maintained constant throughout the entire 6-week culture period. Medium was exchanged once a week.

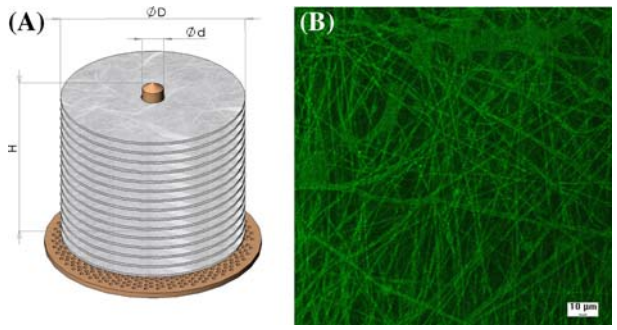

Fig. 1 (**A**) 3-D multilayered electrospun scaffold device. H = 3 mm, D = 8 mm, and d = 0.7 mm. (**B**) Confocal microscopic image of electrospun non-woven mat

Microscopic analysis

Light microscopy histology

For general morphology studies, scaffolds loaded with cells were prefixed in 4% neutral buffered formalin (0.1 M phosphate buffer, pH 7.4) and stained with Hematoxylin and Eosin (H + E), Masson's Trichrome or Toluidine blue stain.

Scanning Electron Microscopy

For SEM studies, samples of scaffolds containing cells 24 h after seeding, 6 week following the dynamic flow culture or after additional 6 week in vivo implantation, were fixed in glutaraldehyde (24 h), 1% OsO_4 (1 h) and 2% tannic acid. They were then dehydrated in graded ethanol solutions, sputter coated with gold palladium and photographed by scanning electron microscope (100 QT operating at 100 volts).

Confocal Microscopy

For imaging and visualization, cells were labeled with CellTrace Far Red DDAO-SE (Molecular Probes (Invitrogen), USA) and Propidium Iodide (PI) (Sigma, USA), according to the manufacturer's instructions. Briefly, scaffold samples (24 h after seeding; 6 week following the dynamic flow culture) were incubated in a solution of DDAO (15 µM, 15 min, 37 °C), washed (PBS), incubated in culture medium (24 h, 37 °C). Subsequent to a 24 h incubation period, the membranes were washed with PBS, stained with PI and immediately examined by confocal microscopy. The scaffold-cell complexes were visualized using a Nikon E600 upright microscope equipped with Bio-Rad Confocal laser scanning system (Radiance 2000). The imaging was performed using an argon laser (488 nm), green HeNe laser (543 nm) and red diode laser (633 nm) for *excitation* and HQ515/30, HQ600/50 and HQ660LP *emission* filters, respectively. Transverse slicing (~50 µm depth) from 25 consecutive sliced images (1.85 µm apart) were used for the cumulative projection image. Each membrane was flipped over and examined from both sides. Using ImageJ 1.345 NIH imaging program coronal reslicing of the projected image was performed.

Animal implant model

Male CD1-***Foxn1^{nu}*** mice (5- weeks-old, Harlan, Israel) weighing 20–25 g were used in the experiment. Animals were provided with food and water ad libitum. The light cycle and the room temperature were automatically controlled. Before the experiments, animals were housed in these conditions for 3–4 days to become acclimatized. Animal care was in accordance with the guidelines of the Committee for the Supervision of Animal Experiments, Technion, Israel Institute of Technology. Following 6 week culture in plug-flow bioreactor cell-scaffold constructs were implanted subcutaneously on the back of the mice (1 construct per animal) under general anesthesia (Ketamine 100 mg/mL, xylazine 1:1). Upon termination of the experiment (additional 6 week) the implants were carefully removed from adjacent tissues and examined by light and scanning microscopy, as described above.

Statistics

Viability data was analyzed by using the *t*-test, and the significant level was set at $p < 0.05$. The results were expressed as mean ± SD, n = 6.

Results

Seeding conditions

Assessment of cell viability 24 h after seeding on the electrospun nanofiber multilayered construct revealed similar metabolic activity of the hMSCs on the tested scaffold and the control polysterene culture plate (Fig. 2). SEM imaging of the scaffolds revealed that the electrospun scaffolds were composed of nonwoven randomly oriented fibers (Fig. 3A). Light microscopy of Toluidine Blue stained samples (Fig. 3B) demonstrated typical elongated cells. While confocal micrographs of the scaffold (Fig. 3 C,D) visualised a 3-D meshed structure containing elongated cells aligned and oriented along the scaffold fibers. The cells (DDAO labeled cytoplasm (blue) and PI labeled nuclei (red)) were attached to and between the green autofluorescent fibers. Visualization of the scaffold layers from both the upper and undersides presented similar results (results not shown).

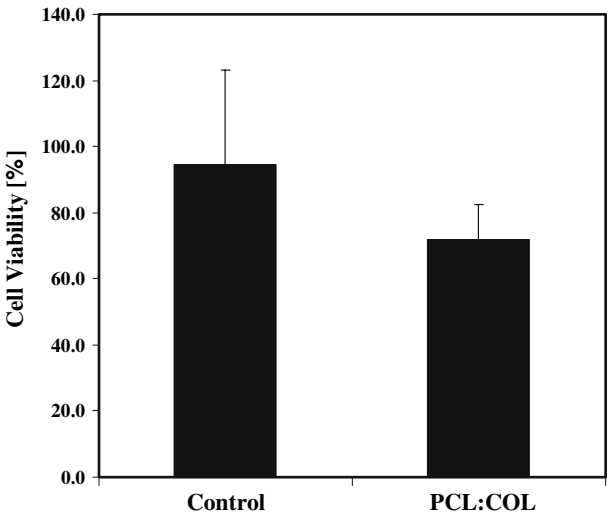

Fig. 2 Viability of hMSC seeded on PCL:COL scaffold compared to standard polysterene culture plate (control) as determined by AlamarBlue™ Assay. No significant change is observed. Data are mean ± SD ($p < 0.05$, *t*-test)

projection analysis by confocal microscope revealed high cell density. (Fig. 4B). Coronal slice (Fig. 4C) performed in the middle of the scaffold demonstrated that cells penetrated into underlying areas (~50 μm depth). Cell nuclei staining with propidium iodide (Fig. 4D) showed their even distribution throughout the scaffold.

In vivo subcutaneous implantation

Multilayered constructs implanted subcutaneously in nude mice revealed no inflammatory response and good integration with the surrounding tissues (Fig. 5C) after six weeks. The membranes were loaded with cells and matrix, and rich vascular infiltration was present between the electrospun fibers (arrow) (Fig 5A, B). An overall high cell and matrix density comparable to the surrounding tissue was observed. SEM imaging revealed a densely packed multilayered construct (arrows) loaded with cells and extracellular matrix (Fig. 5D).

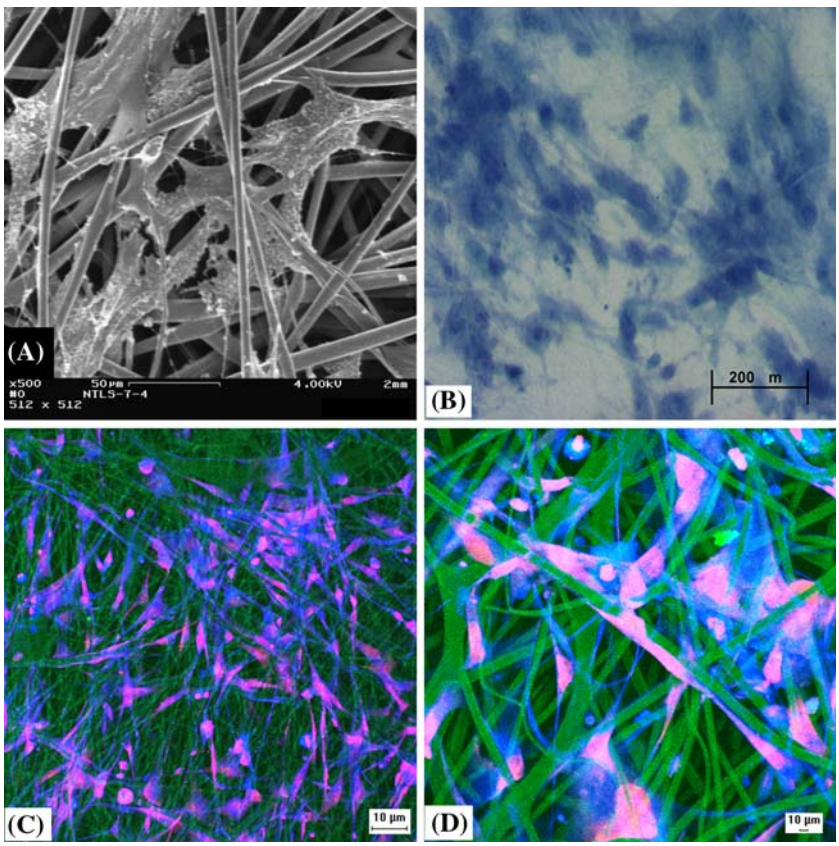

Fig. 3 Light, scanning and confocal microscopy evaluation of hMSCs cultured (24 h) on nanofiber PCL:COL electrospun scaffold. (**A**) SEM micrograph. Elongated cells are aligned along and between fibers. (**B**) Light microscopy, Toluidine blue stain. Typical elongated cells are observed. (**C**) Confocal microscopy image demonstrating numerous elongated cells throughout the scaffold. Note, autoflourescence of electrospun fibers (green), cell cytoplasm (blue) and nuclei (red); ×20; Zoom 1.2. (**D**) Higher magnification of confocal microscopy image demonstrated in C. The cells are between and beneath the nanofibers and are attached and aligned along the nanofibers; ×60; Zoom 1.6

Dynamic culture

SEM imaging of the scaffold layers after 6 weeks of culture in the bioreactor disclosed dense cell arrangements accompanied by extracellular deposits (Fig. 4A). Merged

Discussion

A biomaterial scaffold creates a milieu within which cells are instructed to form a tissue or organ [20]. The ideal scaffold has been already invented by nature in the various

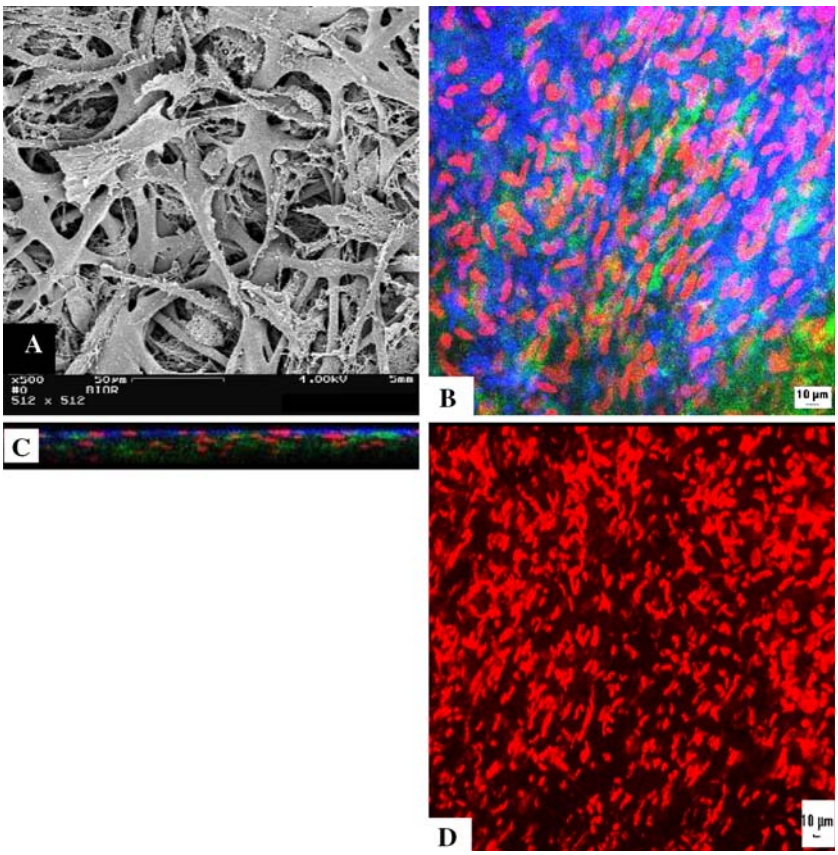

Fig. 4 *SEM and confocal images of hMSCs following culture (6 weeks) in the dynamic flow system.* (**A**) SEM micrograph. Note a distinct 3-D meshed structure of the scaffold containing cells which are aligned along and between the fibers. (**B**) Confocal microscope image of hMSCs cultured on PCL:COL. Numerous elongated cells are seen throughout the scaffold. Note cell cytoplasm (blue) and nuclei (red), ×20; Zoom 2. (**C**) A projection image of coronal section of (**B**), 50 micron depth. (**D**) Confocal microscopy image of hMSCs cultured on the PCL:COL scaffold stained with propidium iodide (PI). The cells are evenly dispersed throughout the scaffold

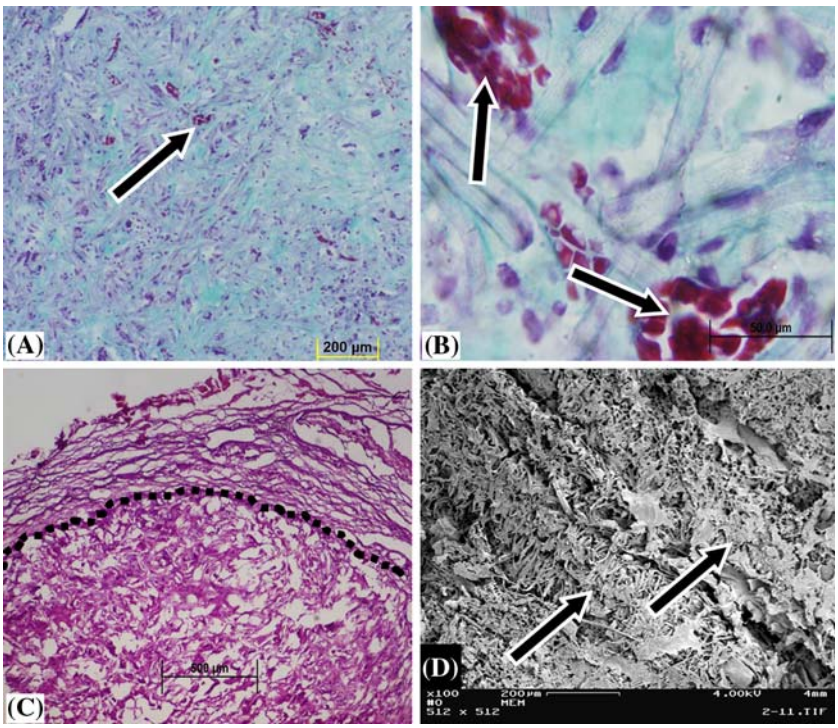

Fig. 5 Electrospun nanofiber multilayered scaffold after 6 weeks of in vivo subcutaneous implanting in nude mouse. (**A**, **B**) Light microscopy images of Masson's Trichrome stained graft samples. Dense cell distribution and matrix containing infiltrated blood vessels (arrow) are observed. (**C**) Light microscopy image of Hematoxylin and Eosin (H&E) staining demonstrates the fine integration of the implant with the surrounding tissues. (**D**) SEM micrograph reveals layered arrangement of the scaffold (arrows) embedded within in the tissue

forms of extra cellular matrix (ECM), satisfying all cells requests on their way to tissue development or rebuilding. The molecular architecture of ECM is very complex and exists at the nano- and macro levels [7, 21, 22]. Traditionally, tissue engineering research is focused on the investigation of macrolevel structures to generate real-size

organ/tissue, though apparently it is clear that adequate tissue renewal won't be possible without its nanostructure reconstruction [7, 20]. There are few recent works, which try to mimic both levels of ECM architecture in the bone [23] and urinary bladder [22]. The novel approach of our study in bioscaffold design was based on the combination of electrospinning technology with multilayer technique, producing a construct with nano- and macro- architecture.

Many polymers are adaptable for an electrospinning process, such as polyethylene oxide, DNA, polycaprolactone, PLA, PGA, polyaniline, etc. [24]. Collagen is a major component of the ECM, composes approximately 30% of total body proteins and has been used as scaffold. However, when used as a scaffold it is rapidly degraded and lacks strength when compared with synthetic materials such as PCL, PLA and PLGA [2]. As a result, collagen based substances alone are unlikely candidates for tissue engineering, specifically for hard tissues such as bone and cartilage. Our study was performed with PCL/collagen mix, based on the results obtained after screening of various compositions and ratios of PCL/PLA, PCL, PCL/PEO, PCL/COL mixtures (data not shown) for their ability to support hMSC adhesion and proliferation. The following combinations of the polymers PCL:PLA 9:1, 3:1, PCL:PEO 9:1, 3:1 and PCL:COL 3:1, 1:1 were tested. The results indicated that hMSCs adhered and proliferated better on the PCL:COL 1:1 electrospun scaffold as compared to all other studied compositions.

This approach combines the benefit of both materials, specifically, the strength and low degradability rate of PCL combined with the good cell attachment and rapid biodegradability of collagen. The demonstrated results (cell viability and cell morphology) indicated that the electrospun PCL/collagen membranes supported growth of the human bone marrow derived mesenchymal stem cells. Electrospinning process provides a nanoscale niche for cell arrangement, being relatively simple technological process, with inexpensive components and straightforward handling of the final product. The major problem of such electrospun scaffold is its low volume capacity, which was overcome in this work by introducing multilayer technique. The stacked system has been used in order to solve the need for 3-D construct with larger volume containing sufficient amount of cells for the vivo implanting. Using this method enabled the design of a construct bearing larger volume and containing sufficient amount of cells for in vivo implanting.

The PCL/COL nanofiber multilayered carrier provided an environment for maximum cell attachment and penetration as viewed by confocal imaging. A series of sliced images were produced by confocal microscopy (Fig. 4C) in order to verify that the cells had infiltrated about 50 μm depth from each side of the membrane reaching the overall depth of the scaffold structure and were present, on the whole, throughout the depth of each scaffold layer. However, to ensure the viability of cells inside 3-D scaffolds bearing large volume, it is necessary to provide better transport of nutrients and constant flow of gases. Media perfusion bioreactor systems have been developed to improve mass transport throughout 3-D tissue engineered constructs cultured in vitro [17]. The micrographs of confocal microscopy reveal the even cell distribution after 6 week culture in dynamic flow system.

The results obtained in this study after in vivo implanting, demonstrated that the multilayered scaffold was biocompatible and was fully integrated with the surrounding tissues with a cell density comparable to the surrounding tissue. In addition the engrafted construct supported neovascularization which is imperative for the survival of any implant and tissue.

Conclusions

Multilayered electrospun meshed scaffolds in concert with a dynamic flow bioreactor may thus provide the solution for long-term cell viability of in vitro cultured cells on synthetic scaffolds which are intended for in vivo application. This combined system provides physiological milieu including a constant supply of essential oxygen and nutrients, thus closely mimicking the in vivo conditions.

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