

Artificial Cells, Nanomedicine, and Biotechnology

An International Journal

ISSN: 2169-1401 (Print) 2169-141X (Online) Journal homepage: informahealthcare.com/journals/ianb20

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To cite this article: Anuradha Elamparithi, Alan M. Punnoose, Sarah Kuruvilla, Maddaly Ravi, Suresh Rao & Solomon F. D. Paul (2016) Electrospun polycaprolactone matrices with tensile properties suitable for soft tissue engineering, *Artificial Cells, Nanomedicine, and Biotechnology*, 44:3, 878-884, DOI: [10.3109/21691401.2014.998825](https://doi.org/10.3109/21691401.2014.998825)

To link to this article: <https://doi.org/10.3109/21691401.2014.998825>



Published online: 24 Jan 2015.



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Electrospun polycaprolactone matrices with tensile properties suitable for soft tissue engineering

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Abstract

The extracellular environment is a complex network of functional and structural components that impart chemical and mechanical stimuli that affect cellular function and fate. Cell differentiation on three dimensional scaffolds is also determined by the modulus of the substrate. Electrospun PCL nanofibers, which mimic the extra cellular matrix, have been developed with a wide variety of solvents and their combinations. The various studies have revealed that the solvents used influence the physical and mechanical properties, resulting in scaffolds with Young's modulus in the range of 1.8–15.4 MPa, more suitable for engineering of hard tissue like bone. The current study describes the use of benign binary solvent-generated fibrous scaffolds with a Young's modulus of 36.05 ± 13.08 kPa, which is almost 50 times lower than that of scaffolds derived from the commonly used solvents, characterized with myoblast, which can be further explored for applications in muscle and soft tissue engineering.

Keywords: electrospinning, matrix modulus, polycaprolactone, 3D scaffolds

Introduction

Three dimensional (3D) cell cultures are proving invaluable for several applications, and this approach has given hope for situations that were hitherto impossible, such as for the repair or replacement of irreversibly damaged, fully differentiated, specialized tissue. Several factors are important for 3D cell cultures, which can extend their utility for tissue engineering purposes. Two major parameters are the matrices and the choice of cells that are used. Of these, the matrix is of primary importance, as a suitable and flexible matrix can support a wide variety of cell and tissue lineages. Thus, the biocompatible nature, the physical and mechanical properties, and the ease with which a material

can be molded into a 3D cell culture matrix, hold the key for further advancements in this new area of experimental science, with far reaching health care implications. Recent research emphasizes matrix elasticity towards cell differentiation of organ-specific lineage. The behavior of cells on 3D scaffolds is not only dependant on extracellular matrix (ECM) adhesion but also on the stiffness and elasticity of the materials. Engler et al. (2006), confirmed the sensitivity of matrix elasticity to the specificity of stem cell lineage. They observed that when the matrix elasticity mimics the brain, with a modulus of 0.1–1.0 kPa, it is suitable for directing a stem cell population towards neuronal lineage. When the stiffness measures between 8 and 17 kPa, the cells take up a myogenic lineage, while an osteogenic lineage is induced when the modulus measures 25–40 kPa (Engler et al. 2006).

Several other studies also have shown that the modulus of the matrices is also a determining factor in cell migration, proliferation, organization, and differentiation. Fibroblasts displayed significant differences in migration patterns with changes in density and stiffness of the matrices (Zaman et al. 2006). Neonatal ventricular cardiomyocytes cultured on substrates of ~10 kPa developed aligned sarcomeres and produced maximal force, whereas those on stiffer scaffolds had more stress fibres and unaligned sarcomeres (Jacot et al. 2008). The morphology and function of neural stem cells is also affected by changes in the elastic modulus of the scaffolds (Saha et al. 2008). Human bone marrow stromal cells cultured on matrices with varying moduli underwent osteogenic differentiation in the absence of osteogenic differentiation supplements, and the effect was more pronounced on stiffer substrates (Parekh et al. 2011).

Electrospinning, a technique patented in 1934 by Formhals, is commonly used to create fibrous scaffolds from nanofibers that are synthetic, natural or a combination of both, with a nanotopography that mimics the native

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(Received 8 November 2014; accepted 11 December 2014)

environment and promotes cell spreading and proliferation. Nonwoven matrices have been well-studied as promising materials, and have been developed for a wide range of medical applications, such as wound dressing, artificial skin, and heart valves (Pham et al. 2006). Polycaprolactone (PCL) is a synthetic polymer that has been approved by the FDA; its properties of solubility, elasticity, inertness, and slow rate of degradation into non toxic products make it favorable for use in regenerative medicine (Sun et al. 2006, Ciardelli et al. 2005). In general, electrospun PCL matrices tend to have increased stiffness and more rigidity (Gunatillake and Adhikari 2003), and are more favorable for hard tissue engineering applications (Middleton and Tipton 2000). The choice of solvent is essential for nanofiber generation, as the solvent influences the physical and mechanical properties of the electrospun PCL (Mondal 2014, Asran et al. 2010). Some commonly used solvents for electrospinning PCL are acetone, dichloromethane, methanol, chloroform, dimethylformamide, and acetic acid (Johnson et al. 2009). Fluoroalcohols, such as Hexafluoroisopropanol (HFIP) and Trifluoroethanol (TFE), have also been used to generate nanofiber scaffolds with natural polymers (Thakkar et al. 2013, Zhang et al. 2005). Min Sup Kim et al. have also reported electrospinning of PCL composites using HFIP and Trifluoroacetic acid (TFA) (Kim et al. 2012a). Both HFIP and TFE are expensive solvents, and along with TFA, are also corrosive and not environment-friendly. There is also evidence to suggest that the integrity of the natural polymer might be affected during the electrospinning process when using fluoroalcohols as solvents (Zeugolis et al. 2008). The blending of natural polymers with PCL is primarily to provide bioactive surfaces for improved adhesion. However, the cost, source, batch to batch variability, and shelf life of the biopolymers are also factors to be considered.

PCL scaffolds generated using the above mentioned solvents were used in experimental approaches for applications in tissue engineering, such as hyaline cartilage, autologous chondrocytes, and bone replacement (Kim et al. 2012b). Mesenchymal stem cells seeded on electrospun PCL

scaffolds and cultured in a rotating bioreactor displayed matrix mineralization and deposition of Type I collagen after 4 weeks (Yoshimoto et al. 2003). Analysis of similar constructs *in vivo* in rats revealed osteoblast-like cells in multiple layers, with a woven bone-like structure coupled with the presence of osteocyte-like cells within the mineralized matrix (Shin et al. 2004b). Bovine fetal chondrocytes were able to preserve their chondrocytic morphology on PCL nanofibers during 3 weeks in culture, and also showed an upregulated expression of Collagen type II B, a marker for mature chondrocytes (Li et al. 2003). PCL nanofiber scaffolds seeded with bone marrow-derived mesenchymal stem cells differentiated into chondrocytes in the presence of transforming growth factor beta. The constructs also displayed a typical zonal morphology with a cartilaginous matrix of type II collagen, aggrecan, and cartilage proteoglycan link protein (Li et al. 2005). The various studies elucidate that nanofiber scaffolds of PCL have been widely investigated for engineering of tissues where the native elastic modulus is above 1 MPa, such as bone, cartilage, ligament, tendon, trileaflet valve (Gaudio et al. 2008), and vascular tissues.

The current study describes the process used to generate electrospun matrices of PCL with a Young's modulus of elasticity 50 times lower than that of scaffolds derived from the commonly used solvents (Table I). The solvent used was a binary solvent comprising acetic acid and dimethyl sulfoxide. Scanning electron microscopy (SEM) of the electrospun matrices revealed an intricate network of random fibers with fiber diameters ranging from 200 to 600 nm. Myoblasts seeded on the PCL nanofibers proliferated well, as ascertained by the viability assay, and were found to maintain their phenotype for longer time points. Fluorescence microscopy analysis of immuno-stained sections revealed that the scaffolds were strongly positive for muscle-specific actin and desmin. The lower modulus of elasticity of the PCL nanofiber scaffolds generated make them interesting for potential applications in muscle, vascular, and soft tissue engineering applications.

Table I. Tensile property of PCL with different solvents.

S.no	Polymer	Solvent	Tensile strength in Mega Pascals	Tissue studies/constructs	References
1	10% Polycaprolactone	TFE	2.7	Bone tissue engineering (bone marrow stromal cells)	Zhang et al. (2005)
2	14% Polycaprolactone	Tetrahydrofuran/ dimethylformamide (1:1)	2.1 ± 0.4	Bone tissue engineering (Human mesenchymal stromal cells - fibrochondrocytes)	Li et al. (2007)
3	Polycaprolactone	Acetone	1.3 ± 0.04	ND	Johnson et al. (2009)
4	15% Polycaprolactone	Dichloromethane/methanol	3.5 ± 1.1	Neural tissue engineering	Hackett et al. (2010)
5	12.5% PCL/collagen (separately electrospun)	HFP	5.0 ± 1.7	Neural tissue engineering (stem cells-neural cells)	
6	PCL/collagen (1/1)	Acetic acid	1.2 ± 0.4	ND	Yogeshwar et al. (2011)
7	Polycaprolactone	Acetic acid	1.88 ± 1.3	ND	
8	10% Polycaprolactone	TFE	1.74 ± 0.18	Ocular surface engineering (Limbal epithelial cells)	Sharma et al. (2011)
9	5% polycaprolactone	HFP/TFA (9/1)	15.4 ± 2.3	Skin tissue engineering (human dermal fibroblast)	Kim et al. (2012a)
10	5% PCL/chitin (1/1)	HFP/TFA (9/1)	34.1 ± 6.8	Skin tissue engineering (human dermal fibroblast)	

Depicts the review of solvents used to electrospin polycaprolactone and their resultant higher modulus, in MPa.

Table II. Porosity measurement of binary images with 3 different thresholds.

Sample	Magnification	Top layer (P1)	Middle layer (P2)	Lower layer (P3)	Average pore size
1	2000	75.9	56.7	19.2	50.6
2	2000	80.2	56.9	33.7	56.9
3	2000	81.9	54.1	26.3	54.1
4	2000	73.2	56.1	18.0	49.7
				MEAN	52.8
				STDEV	2.36

Represents the porosity measurements of 4 SEM images with 2000X magnifications using ImageJ software. Porosity percentages of 3 threshold layers of the nanofiber mats were measured as P1, P2 and P3. Total porosity percentage was the average of the three, and the overall percentage of porosity was estimated by calculating the mean and standard deviation.

Materials and methods

Electrospinning of polycaprolactone

Polycaprolactone (PCL) (Mw-80,000) was procured from Sigma Aldrich, and the solvents acetic acid and dimethylsulfoxide (DMSO) were of analytical grade and were obtained from SRL, Mumbai and Merck, USA. PCL was dissolved in a binary solvent of acetic acid and DMSO (93:7), and homogeneous solutions of 10, 12 and 15% (weight/volume) were prepared. The electrospinning apparatus was a custom set up consisting of a stabilized high voltage supply, Premier Combines, Chennai, India; a programmable peristaltic pump, Ravels Tech, Chennai, India; silicone tubing, a 26-G needle and a grounded metal plate as a stationary collector. The PCL solutions were maintained at a flow rate of 0.2 ml/min, and nanofibers were generated at 16 KV and collected onto a stationary collector. The distance between the needle and the collector plate was 23 cm. Prepared scaffolds were cut into 1 cm squares, sterilized by ethanol treatment and stored in sterile PBS.

Characterisation of electrospun polycaprolactone membranes

Scanning electron microscopy and measurements. Electrospun PCL scaffolds of 3 different concentrations – 10, 12 and 15%, were morphologically evaluated by SEM (S-3400N SEM). The matrices were fixed and dehydrated by serial dilutions of ethanol. They were sputter-coated with gold before being analyzed. Digital images of 3 different scaffolds were captured, and the average fiber diameters were measured using ImageJ Software, from 30 measurements chosen at random across each image set. The porosity of the scaffolds was analyzed by examining the SEM images, using ImageJ software as previously described. [24] The SEM images with a gray scale level of 256 were converted to binary images by calculating the threshold, and the porosity of the scaffolds was measured using the formula $P = (1 - n/N) \times 100$, where n represents the number of white pixels, N is the total number of pixels in the binary image, and P is the percentage of porosity of the binary image. The 3 thresholds that aid in classifying various layers of the nanofiber mat were selected as described by (Mobarakkeh et al. 2007), and their porosity percentages were measured as P_1 , P_2 and P_3 . Four SEM images of magnifications of 2000 and 5000 were analyzed for P_1 , P_2 and P_3 , and the overall percentage of porosity was estimated by calculating the mean and standard deviation (Table II).

Tensile property

PCL nanofibers were collected for 4 h and prepared into strips with dimensions of 30 mm in length and 15 mm in width, and stored in PBS. The mechanical properties were measured using a universal tensile tester (INSTRON 3365) with a 10 N load cell, and measured at a speed of 1 mm/min (Zorlutuna et al. 2009) (Table III). A total of 4 samples were tested and the mean and standard deviation were calculated.

MTT assay

PCL scaffolds were cut into 1 cm squares, washed in three washes of sterile PBS, followed by sterilization with serial dilutions of ethanol. Scaffolds were washed well with sterile PBS and stored in PBS supplemented with antibiotics, at 4°C. Rat skeletal L6 myoblasts were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum and antibiotics. Bovine collagen (0.2%) was coated on PCL scaffolds prior to cell seeding for 1 h. L6 cells were seeded onto scaffolds in non-adherent culture dishes at a seeding density of 2.5×10^4 cells/scaffold and incubated at 37°C in an atmosphere containing 5% carbon dioxide. The culture medium was refreshed every 2 days. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) test assesses the cytotoxicity by measuring the cell viability and proliferation (Mi et al. 2000). After culturing for 2, 4 and 6 days, the scaffolds were transferred to fresh wells of a 12-well plate and incubated for 4 h with 1 ml of medium and 100 µl of MTT reagent at 5 mg/ml. After 4 h, the medium was discarded and 500 µL of DMSO was added, incubated for a further hour, and the resulting mixture was gently shaken for 10 min. Then, 200 µL of the solutions were removed and measured at 595 nm using a spectrophotometer.

Table III. Tensile property of the nanofibrous matrices collected for 4 h.

Nanofibrous membrane	Tensile stress (kPa)	Elongation (%)	Modulus (elasticity) (kPa)
Sample 1	33.47	174.56	31.41
Sample 2	28.68	137.29	27.05
Sample 3	33.18	122.22	34.26
Sample 4	33.92	82.73	49.23
Sample 5	18.03	163.15	19.66
Sample 6	43.55	95.77	54.67
Mean	31.80	129.29	36.05
SD	8.32	36.33	13.38

The tabular column represents the mean and standard deviation of the tensile strength, elongation, and Young's modulus of 6 different samples of polycaprolactone nanofibrous matrices.

Immunofluorescence staining with muscle-specific actin

Seeded scaffolds were cryopreserved for a 6-day time point and cryosectioned. The sections were fixed with 3.7% formaldehyde for 10 min, rinsed twice with PBS, and permeabilized with 0.5% triton X 100 in PBS for 10 min. After blocking for 1 h, monoclonal anti muscle-specific actin antibody (Biogenex, USA) was added at (1:50) and incubated overnight. Sections were washed well and stained with Goat anti Mouse FITC (Merck), counter stained with DAPI to highlight the nuclei, and analyzed on a Zeiss LSM 510 Laser Scanning Confocal Microscope. The slides were rinsed in PBS and followed by nuclear staining with DAPI at 5 $\mu\text{g}/\mu\text{l}$ added for 30 min. The slides were then observed on a Nikon TE Eclipse inverted fluorescence microscope, and images were analyzed using Image Pro Software.

Reverse transcription polymerase chain reaction (RT-PCR)

Using RNA Plus (MP Biomedicals), total RNA was isolated and converted to cDNA using the Verso cDNA synthesis kit (ABgene) according to manufacturer's protocol, from PCL matrices seeded with myoblasts for 6 days. Polymerase chain amplification (PCR) was performed as follows: 94°C for 30 s for denaturation, 60°C and 63°C for 30 s for annealing of GAPDH and desmin respectively, and 72°C for 30 s for extension. PCR was carried out with 35 cycles, which were analyzed using agarose gel electrophoresis. GAPDH primers 5'-ACC ACA GTC CAT GCC ATC-3' (forward) and 5'-TCC ACC ACC CTG TTG CTG-3' (reverse) with 556bp product size; desmin primers 5'-CAA CCT TCC GAT CCA GAC CT-3' (forward) and 5'-GAG TGG AAA AGG CTG GCT TC-3' (reverse) with 224 bp product size.

Statistical analysis

All numerical data were expressed as mean $\pm$ standard deviation (SD).

Results

Characterization of PCL scaffolds

PCL fibers were generated in all three concentrations that were tested. However, the scaffolds varied in optimal characteristics. Beaded fibers were observed at the concentration of 10% and were not present at higher concentrations. The fiber diameters were increased at higher concentrations. 10% PCL matrices showed fiber diameter ranging from 150 to 210 nm, 12% PCL matrices showed fiber diameter ranging from 200 to 350 nm, and 15% PCL matrices showed fiber diameter ranging from 250 to 595 nm (Figure 1). The average

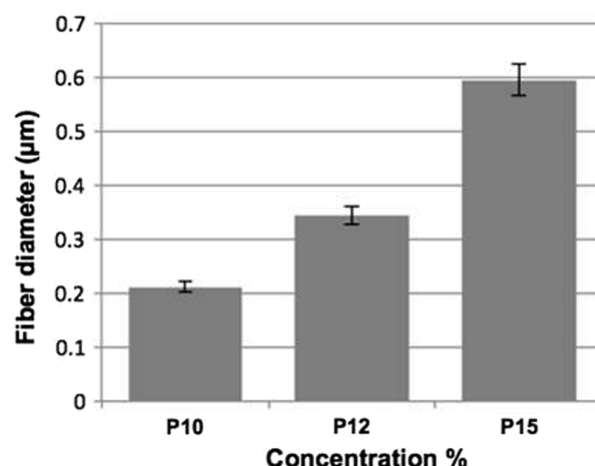


Figure 2. Depicts the graphical representation of the measurement of fiber diameter of electrospun matrices with different concentrations of 10% wt, 12% wt, and 15% wt, using ImageJ software. The fiber diameter increases with an increase in concentration.

fiber diameter of the three different concentrations, plotted as a graph, showed the increase in fiber diameter (Figure 2). Scaffolds generated with 12% polymer concentration were chosen for further tests. Porosity measurements carried out using the methods of Mobarakeh et al. revealed an average porosity percentage of 52.8 ± 2.36 (Table II), which is optimal for tissue engineering applications. Tensile measurements of PCL scaffolds revealed a remarkably low modulus of elasticity with a mean value of 36.05 ± 13.38 kPa (Table III). The mechanical properties of the PCL scaffolds indicate that they are ideal for soft tissue engineering applications.

Biocompatibility assays

Rat skeletal myoblasts adhered onto PCL scaffolds and proliferated well, as examined by the MTT assay. The average was 3 samples per scaffold, at 2, 4, and 6-day time points (Figure 3). The seeded scaffolds were compatible and the 3D environment was optimal for the proliferation of muscle cells, as analyzed over defined time points of 2, 4, and 6 days, with no reduction in colorimetric signal. Cryosections of the seeded scaffolds on day-6 revealed the presence of muscle-specific actin and desmin, indicating that the matrices were conducive for maintaining the native architecture of myoblasts (Figures 4 and 5). This was also confirmed by gene expression of desmin on 3D PCL matrices using Reverse Transcription Polymerase Chain Reaction (RT-PCR) (Figure 6). Briefly, 250 ng of cDNA of day-6 of matrices with

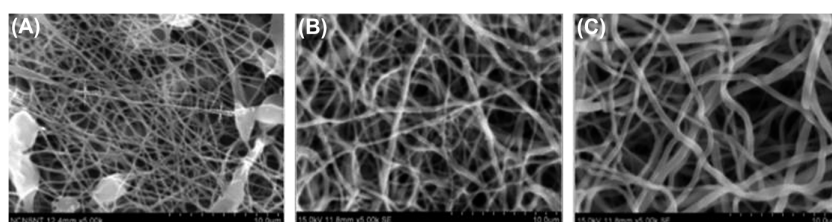


Figure 1. (A), (B), and (C). SEM of electrospun membranes of polycaprolactone, with the concentrations of 10%, 12%, and 15% (Magnification $\sim 5000\times$).

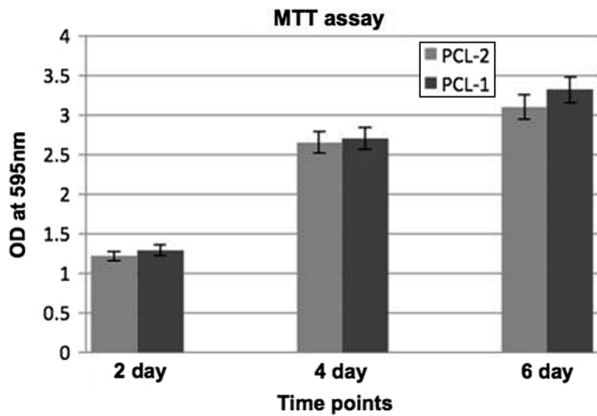


Figure 3. Biocompatibility of the polycaprolactone membrane, evaluated by the MTT assay using L6 rat skeletal myoblast cell growth tested at 3 different time points (2, 4, and 6, days in culture).

myoblasts were harvested and compared with 2D cultures of the same time point, for expression levels of both desmin and GAPDH, as described. We observed that there was moderate increase in expression levels of desmin in 3D PCL matrices when compared to the 2D culture, showing active expression of factors important for the function of muscle. Our study confirms that the electrospinning of polycaprolactone using acetic acid and DMSO can generate favorable nanofiber matrices with suitable modulus for application in muscle tissue engineering applications.

Discussion

The physical and mechanical properties of the scaffolds are dependent on the choice of the solvent. We have shown in our work that it is possible to generate nanofibers of PCL using an economical and benign solvent combination, which also results in matrices with a reduced modulus of elasticity, thus making it favorable to use the FDA-approved biomaterial PCL in soft tissue engineering applications. Kanani and Bahrami 2011, studied the different solvents and their combinations, such as glacial acetic acid, 90% acetic acid, methylene chloride/dimethyl formamide, and formic acid/acetone, and compared them with the morphology of the PCL nanofibrous matrices; they concluded that the usage of glacial acetic acid creates a non uniform fiber diameter (Kanani and Bahrami 2011). Schueren et al. (2011), also evaluated the morphology with solvents such as chloroform, methanol, ethanol, and formic acid/acetic acid, and concluded that the optimal solvent combination

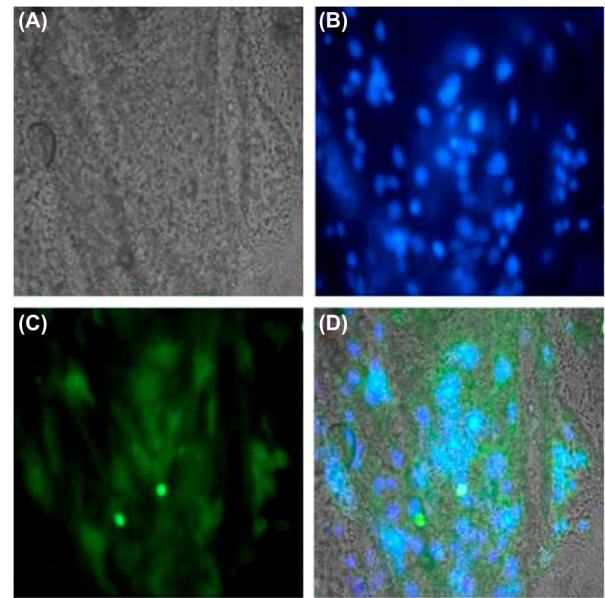


Figure 5. Cryosectioned matrices seeded with rat skeletal myoblasts were stained with (A) Phase contrast, (B) nuclear staining with DAPI, (C) desmin with FITC, and (D) Merged image of all.

is a mixture of formic acid and acetic acid. A review of the literature shows that ideal solvents for electrospinning PCL are dichloromethane, tetrahydrofuran, trifluoroethanol and hexafluoro isopropanol, which are generally considered as toxic and not environmentally friendly solvents (Schueren et al. 2011). Juliana Dias et al. (2013), conducted a study with acetic acid and triethylamine, and also emphasized the accuracy of the polymer concentration. The benign binary solvent combination, acetic acid and dimethyl sulfoxide, used to electrospin PCL, resulted in beaded fibers at lower concentrations and smooth nanofibers at higher concentrations of the polymer. The matrices prepared from a 12%wt concentration had smooth fibers without beads between 200 and 350 nm, with a modulus 50 times lower when compared with those generated by other groups with commonly used solvents, as reported in the literature (Table I). The novelty in the study is the preparation of PCL substrates of lower modulus, without blending with natural polymers, which can be beneficial in soft tissue engineering.

Desmin is an intermediate protein of the contractile filament which is expressed in all stages of the development of muscle, seen in both proliferating as well as in differentiated muscle. It connects different components in cytoplasm, to

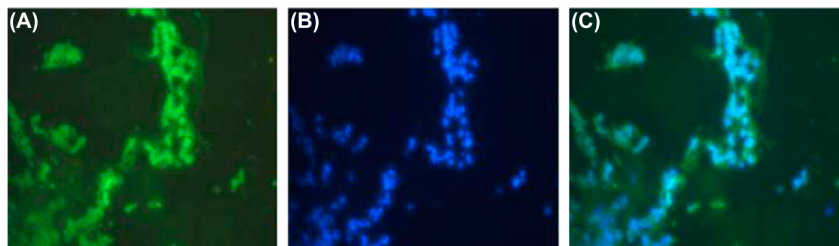


Figure 4. Cryosectioned polycaprolactone electrospun matrices seeded with rat skeletal myoblasts were stained for muscle-specific actin with FITC (A), DAPI for nuclear staining (B), and merged (C).

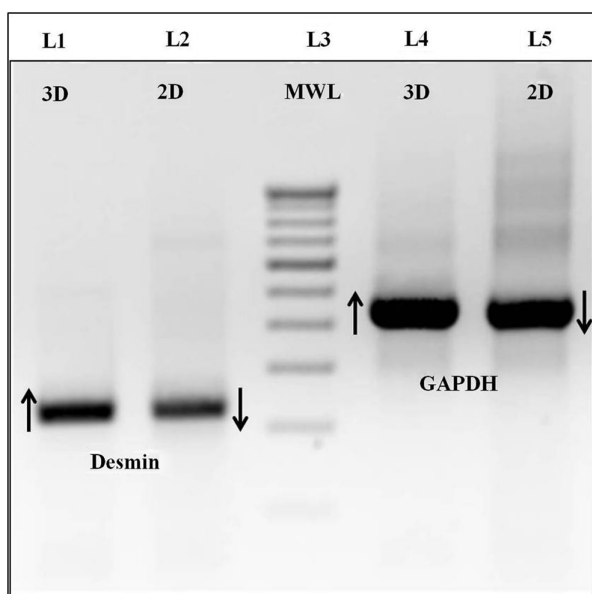


Figure 6. Agarose electrophoresis image of RT-PCR analysis of desmin and GAPDH. (Lanes 1 and 2) – Desmin 3D and 2D. (Lanes 4 and 5) – GAPDH 3D and 2D.

maintain the cell architecture and structure with the contractile apparatus. In the current study, myoblast-seeded electrospun PCL nanofibrous matrices showed an appreciable increase in proliferation upto 6 days in growth medium, in comparison with the 2D culture. Boontheekul et al., showed C2C12 cell adherence, spread, and proliferation on hydrogel matrices, with a stiffness between 1 and 45 kPa, in contrast to the primary myoblast which showed adherence and proliferation only on matrix modulus between 12 and 45 kPa and not in the 1–10 kPa range (Boontheekul et al. 2007). The current study establishes electrospun PCL matrices by using acetic acid and dimethyl sulfoxide with a modulus of elasticity of 36.05 ± 13.38 kPa, which is compliant for myoblast cultures, and also in the range for primary myoblast culture. Generally, such a low modulus is achieved with hydrogels, but we were able to establish such a low modulus in nanofibrous scaffolds, which can be effectively used as a tissue patch. Seeded matrices actively express muscle actin and desmin after a week in culture, and RT-PCR results revealed a healthy expression of desmin on day-6. This correlates well with the study conducted by Boontheekul et al., and the elasticity of the matrices was found suitable for 3D muscle culture. Shin et al. have shown contracting cardiomyocytes on PCL nanofibrous membranes, with the thickness of 10 μm , and concluded that the stacking of cell sheets would potentially lead to a cardiac graft (Shin et al. 2004a, Ishii et al. 2005). Our PCL substrates generated with the benign solvent combination thereby open the possibility for their use in soft tissue and organ-specific tissue engineering applications.

Conclusion

Our study concludes that the synthetic polymer PCL can be fabricated into scaffolds with tensile properties suitable for muscle and soft tissue engineering applications without

blending with natural polymers. The benign solvent combination is non toxic and economical, and can electrospin matrices of low modulus of elasticity, similar to hydrogels, and can be potentially used as an ideal matrix for stem cell differentiation for organ-specific tissue engineering. These synthetic matrices have the potential to be further improved as functionally bioactive scaffolds with the inclusion of cytokines, cell adhesive peptides, and growth factors, to deliver therapeutic drugs and enhance their integration with native tissue in regenerative therapies using stem cell technology.

Acknowledgements

We are grateful to the Department of Biotechnology, Government of India, for funding this work (BT/PR11228/BRB/10/677/2008). We thank the members of the Biotechnology wing of Sree Chitra Thirunal Institute of Medical Science and Technology for their help with tensile testing.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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