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Research Paper

Multilayer cell culture system supported by thread

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ABSTRACT

Cell culture systems have validated to be able to mimic living organisms for various biological applications. In the current study, we demonstrated a method of fabricating three-dimensional (3D) cell culture system by stacking multiple layers of polydimethylsiloxane (PDMS) embedded with functionalized hydroxypropyl cellulose methacrylate (HPC-MA) porous scaffolds, which were connected through thread as a readymade hydrophilic channel. Thread could wick and distribute micro volumes of liquid samples from single inlet point into arrays of detection and working zones without the requirement of either pump or external pressure. Thread with an average thickness of 300 μm provided sufficient rate to continuously supply the media into the system in less than 250 s and remove waste from the cell culture system. The number of COS-7 cells present in the multilayer culture system was comparatively identical to the cells grown in the tissue culture dish and scaffold in a microwell plate. This multilayer cell culture system can be applied to implantable biodegradable poly(lactic-co-glycolic acid) (PLGA) suture as well as initiating cell differentiation in multilayers.

1. Introduction

Cell culture systems that can mimic the *in vivo* condition are vital to tissue engineering, cell biology, drug discovery and development, biochemistry and pharmacokinetic studies [1,2]. The majority of studies on cell and tissue regulation have relied upon the analysis of cells grown on two-dimensional (2D) surfaces, such as tissue culture plates, microwell plates and petri dishes. Although this 2D *in vitro* models are inexpensive, convenient to use, conducive to systematic and also possess high cell viability [1,3], they have distinct disadvantages as well. For example, cells cultured in 2D *in vitro* models typically do not maintain their differentiated functions and are morphologically different compared to the cells grown in a three-dimensional (3D) environment [4]. Therefore, 2D cell culture systems do not reflect the *in vivo* situation where cells grow within a complex 3D microenvironment. To address these disadvantages, 3D cell culture systems were developed in which cells are grown within microfabricated devices containing 3D structures that facilitate the accurate replication of living organisms, where vascular perfusion continuously supplies and removes metabolites and catabolites, respectively.

There are many different platforms that can be used for 3D cell culture and they can be generally categorized as paper- [5], glass/silicon- [6,7] and polymer-based systems [8]. Paper-based systems have been extensively studied for the fabrication of microfluidic device [9–12] and 3D cell culture because microfabrication of polymer-based and silicon/glass-based platform often requires specialized engineering instruments and fabrication methods. In addition, paper is biocompatible with various biological samples and can be modified by a wide range of functional groups that can be covalently bonded with proteins, small molecules and nucleic acids [13]. 3D paper cell culture device can be fabricated by stacking paper sheets with impregnated with cell suspension in matrix hydrogel. The stacked paper-based system can be de-stacked for layer-by-layer molecular analysis after 3D cell culture [5]. Compared to paper-based and polymer-based platforms, the use of glass/silicon-based as 3D cell culturing system involved higher processing cost, longer fabrication time as well as require facilities and equipment in a clean room for fabrication. However, glass/silicon-based platform is still widely used due to its strength of anodic bonding that allows an excellent resistance to high pressure and offers better optical properties that are useful in high-resolution fluorescence microscopy [14]. For example, Webster and co-authors fabricated a microfluidic system for tissue biopsy culture using glass where the response of normal col-

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orectal tissue and neoplastic biopsies to hypoxia was assayed by determining the release of vascular endothelial growth factor [15]. Glass/silicon-based cell culture systems were also integrated with concentration gradient generators in order to test the cytotoxicity and passaging the cells [16].

Polydimethylsiloxane (PDMS) is a common polymeric material for fabricating 3D cell culture systems due to its low cost, high permeability to oxygen and ease of soft lithography technique. Other polymers such as polycarbonate (PC) [17], poly (methyl methacrylate) (PMMA) [18], collagen [19] and agarose [20] have also been utilized for fabricating microfluidic cell culture systems with different structural features, such as micropillars [21,22], microchannels [23,24] and micro-wells [25,26]. Some of the polymers can also provide an *in vivo*-like environment resembling living tissues. In the recent studies, PDMS has been often used with other material platforms to fabricate 3D cell culture devices.

In the current study, we reported the fabrication of a 3D cell culture system by using PDMS in combination with cotton thread and functionalized hydroxypropyl cellulose methacrylate (HPC-MA) scaffold. Recently, textile has been presented as a low-cost [27–30] and low-volume substrate [31] for the fabrication of analytical diagnostic systems. Thread is a readily available and durable material having a porous structure. This porous nature of thread facilitates continuous transportation of aqueous solutions from wet to dry regions over a period of time by capillary action regardless the nature of the sample. Herein, we present a 3D cell culture system that employs thread as a cost-effective transportation channel to overcome diffusion limitation by continuously supplying nutrients and removing waste and catabolites. Cotton thread has been used as a media diffusion channel for the fabrication of multilayer cell culture device by sewing the thread through multiple layers of PDMS embedded with cellulosic scaffolds.

2. Materials and methods

2.1. Fabrication of multilayer cell culture system

The multilayer cell culture system was constructed using PDMS as the support stand, functionalized hydroxypropyl cellulose methacrylate (HPC-MA) scaffold as the cell adhesion structure, and cotton thread as the liquid transporting system. The desired pattern on the PDMS layer, with $22 \times 22 \times 5\ mm^3$ dimensions, was designed using AutoCAD software (Autodesk Inc., San Rafael, CA), from which the master mold was printed using a 3D printer(Objet Eden 3D, Stratasys Inc., Eden Prairie, MN) and UV-curable polymeric material (ObjetFullCure720TM). The fabricated molds were immersed in 10% (v/v) sodium hydroxide (NaOH) solution for 2 h after printing in order to remove all the remaining printing support materials before air-drying for overnight. The freshly prepared PDMS prepolymer was then cast onto the master mold, cured at 70 °C for 1 h in an oven and the PDMS layer was subsequently removed from the master mold. The first layer of the PDMS mold consisted of one inlet reservoir for cell culture media. The second layer consisted of one inlet reservoir, one main chamber for embedding the functionalized scaffold and one channel connecting the inlet reservoir to the main channel. The third and fourth layers consisted of one channel connecting to the chamber. Natural cotton thread with different thicknesses was obtained from the School of Fashion and Textiles, RMIT University, Melbourne, Australia. Natural cotton thread is hydrophobic and requires treatment to allow the wicking of the liquids. Hydrophobic cotton can be treated by plasma oxidation [27], mercerization [29] or washing with sodium carbonate [32].

In order to obtain hydrophilic cotton thread, the thread was treated with lab-scale vacuum plasma reactor (K1050X plasma asher, Quorum Emitech, UK) for 1 min at power of 50 W [27]. The rate of supplying liquid solution in the system was studied by sewing 10 cm of cotton thread (with the thicknesses of 200, 300 and 600 μm) into 3 pieces of cellulosic paper (0.5×0.5 mm) before setting up it on a ruler (Fig. S1). A total of 500 μL of food dye solution was applied on one end of the thread. The time required for the papers to be saturated with the dye solution was then measured, and therefore thread with an average thickness of 300 μm was selected to employ in the cell culture system.

The functionalized HPC-MA scaffold was prepared according to the protocols described in our previous works with minor modifications [33,34]. After HPC-MA was synthesized, it was dissolved in 20% deionized water (w/v), to which a photoinitiator 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone was added to a concentration of 4% (w/w) of the polymer weight, equivalent to a final concentration of 0.8%(w/v) for HPC-MA-20%. The solution was first warmed to 40–45 °C to allow the formation of phase emulsion before cross-linking. A total volume of 0.5 mL of the solution was then added to the chambers on the PDMS mold and cross-linked with UV light (320–405 nm, 200 mW cm^{-2}) at a distance of approximately 100 mm using a UV cross-linker (HonleUV Technology, UV-F 400, Germany) for approximately 6 min. The cross-linked gels were then washed with deionized water to remove any uncross-linked HPC-MA and the photoinitiator and later frozen at −20 °C, followed by lyophilization under vacuum for 48 h. The lyophilized HPC-MA scaffolds were functionalized by activating in 40 nM 1,1′-carbonyldiimidazole (CDI) in acetone at room temperature for3 h. The activated scaffolds were then washed in acetone for 3–4 times to remove any unreacted traces of CDI. After washing, the activated scaffolds were treated with gelatin (1.16 mg mL^{-1}) in a sodium bicarbonate ($NaHCO_3$) buffer (50 mM, pH 9) at room temperature for 24 h. The conjugated scaffolds were then washed twice with sterile phosphate buffered saline (PBS) before lyophilization.

The scaffolds have dimensions of $5 \times 5 \times 2.5\ mm^3$ that needs to be inserted into the chambers in PDMS layers. The device was finally assembled by pinning three layers of the patterned PDMS with correct registration. The thread was sewn through the three layers using a household sewing needle and crossing the scaffolds, which were positioned into the channels of the PDMS to wick the media from resource to the growing cells into the scaffold. Finally, the entire device was compressed by tightening the thread and pinned after seeding the cells onto the scaffolds.

2.2. Cell culture and addition of channels to the system

All PDMS layers, functionalized hydroxypropyl cellulose methacrylate (HPC-MA) scaffolds, and the cotton thread were decontaminated by soaking in 80% (v/v) ethanol overnight, followed by washing with sterile PBS and air-dried overnight. African green monkey kidney cells (COS-7 cells) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 ml-glutamine, and 50 units/ml penicillin-streptomycin (P/S) at 37 °C in a 5% CO_2. The cells were then seeded onto the scaffold and allowed to penetrate prior to stacking up the PDMS layer. Cell seeding was conducted at a density of 2×10^4 per scaffold and each layer of the PDMS was subsequently stacked up. The PDMS device was placed inside a covered glass container, within the container there are two open reservoirs containing medium as showed in Fig. 3, where the medium reservoir provide a humidified environment and we observed negligible loss of water in the 3D device.

In order to transport more culture media to the cells and remove waste from each layer, some threads as readily made hydrophilic channels were added into the system. The addition of channels into the multilayer cell culture system was achieved by carefully

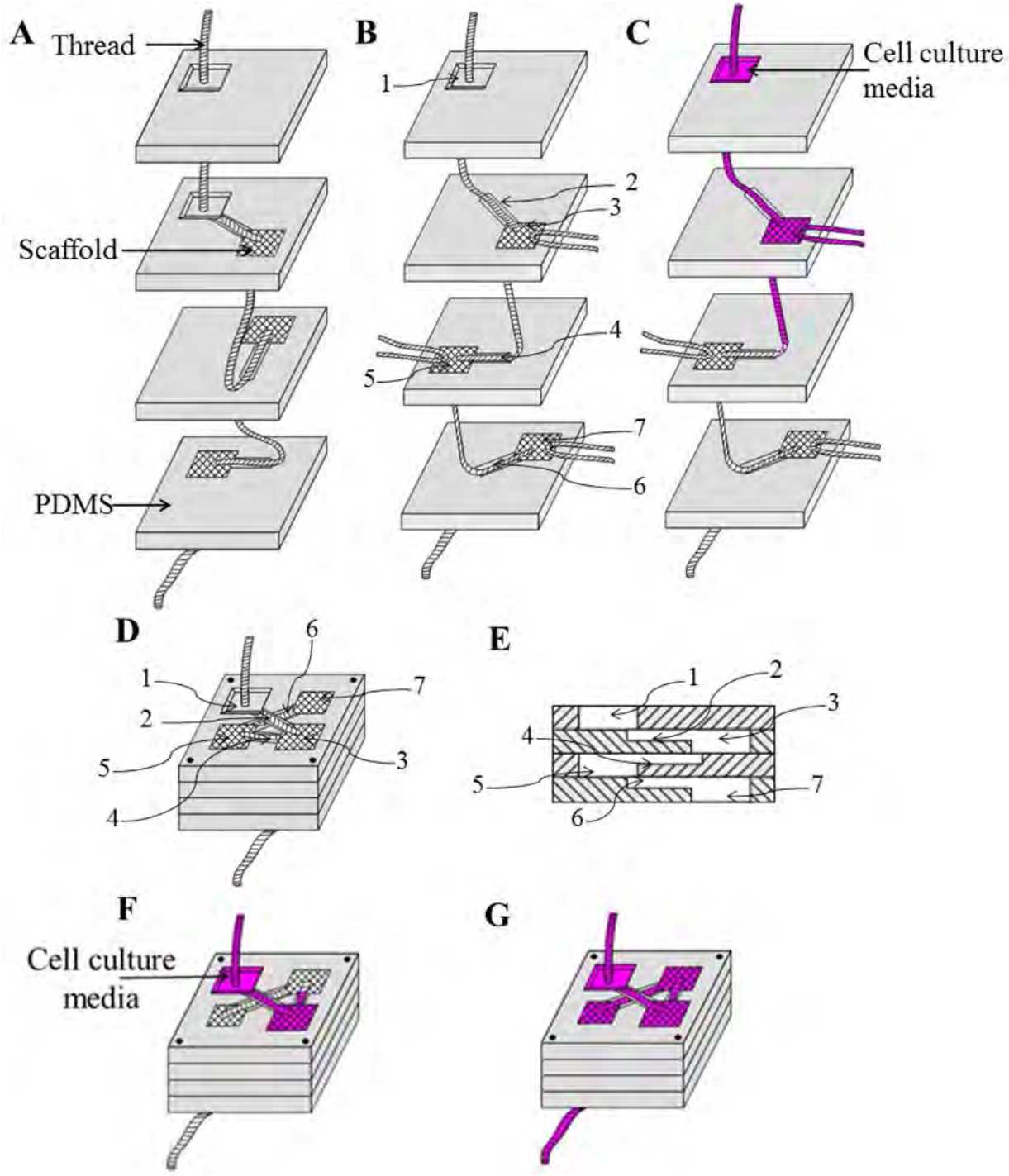

Fig. 1. Schematic illustration of multilayer cell culture system supported by thread. The multilayer device is fabricated from PDMS before inserting the scaffolds into the chambers. A piece of thread sewn into the device (A) before placing additional thread to each layer (B). Then, cell culture media can transport from the chamber in the first layer to the second, third and forth layers through the thread (C). The device is pinned together after cell seeding (D). Cross-sectional view of the pined device (E). Addition of cell culture medium into the system after 40 s showing the cell culture media transported from the first layer and filled up the scaffold in the second layer (F). After 5 min, the media completely filled up the scaffolds in different layers (G).

inserting the decontaminated cotton thread into the middle of the scaffold that was embedded in different layers of the cell cultur devices.

2.3. Measurement of cell culture media flow rate in scaffolds

The scaffolds in the 3D device were sewn together using hydrophilic cotton thread before the cell culture media in the first layer was added. The media distributed to the layers of scaffolds from wet to dry regions. The images of scaffolds were then captured in color (Apple iPhone 5 Camera) over a period of time and the images were transferred into Adobe Photoshop for the measurement of mean intensity in CMYK color mode. The average color intensity of each layer was transferred to Excel to provide a model for scaffold penetration rate. The flow rates of the culture media using single and multiple threads were determined by capillary action of the multilayer cell culture device to transport 5 mL of culture media over a period of time from the external culture media

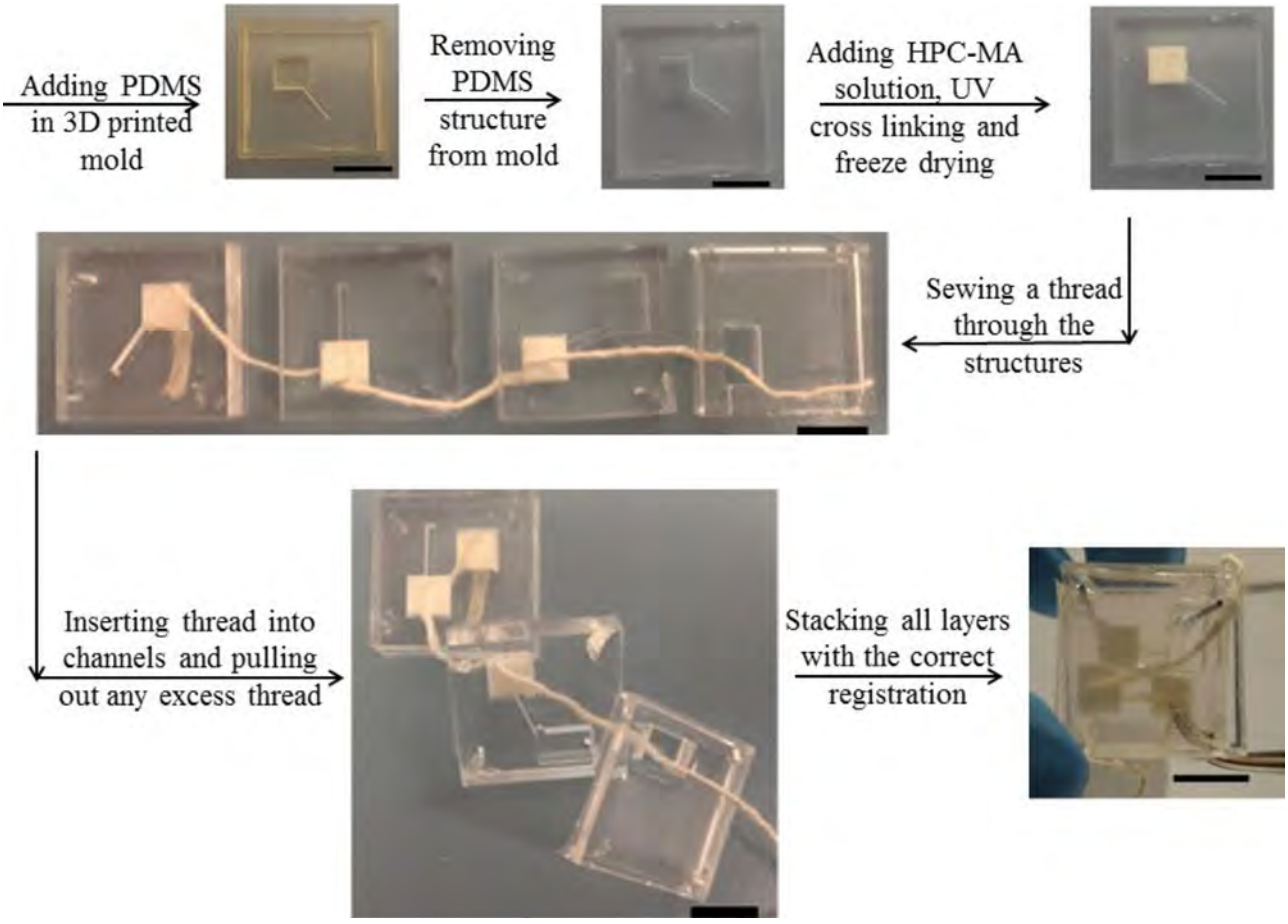

Fig. 2. Preparation of single layer structure, stacking multiple layers to form 3D cell culture system and adding extra threads to continuously remove the waste from the system. Scale bar is 1 cm.

reservoir to the outlet of the last layer. The flow rate of the single and multiple threads was measured three times and the average value of the measurement was calculated.

2.4. Cell proliferation assay

Media and thread-free scaffold cell culture system were used as a control system. The viability of cells after immobilization in the hydrogel fibres was examined using a live/dead viability assay kit. Prior to staining, scaffolds were removed from their respective layers. COS-7 cells in the scaffolds were stained by first staining the scaffolds with 5 μM calceinacetoxymethyl ester solution (Life Technologies, Australia) and later 10 μM propidium iodide and incubated for half an hour and 5 min, respectively, at 37 °C in dark. Visualization of the stained cells was executed using an inverted confocal fluorescence microscope (Nikon A1 Rsi MP, Australia).

The number of cells proliferating on the hydrogel was determined by quantifying the DNA content using PicoGreen assay kit (Quanti-iTPicoGreen dsDNA Reagent, Life Technologies, Australia) according to the manufacturer's protocol. At different time points, the cells in the scaffold were trypsinized twice and centrifuged for 5 min at 1200 rpm to ensure that all the sizes were collected. The cell pellet was rinsed by using cold PBS twice, each followed by centrifugation for 5 min at 1200 rpm. The resultant cell pellet was then collected. The cells were lysed using 100 μL of NP40 cell lysis buffer (Life Technologies, Australia) for 30 min on ice and vortexed at 10 min intervals. The extract was transferred to microcentrifuge tubes and centrifuged at 13,000 rpm for 10 min at 4 °C. The clear lysate was aliquoted to clean microcentrifuge tube where 100 μL of PicoGreen reagent was added and allowed to incubate for 5 min at room temperature in dark. The final solution was transferred into a 96-well plate, and the fluorescence was read using a microplate reader at excitation wavelength 480 nm and emission wavelength 520 nm. The number of cells in the sample was determined by correlating the DNA content with a DNA standard curve. The DNA standard curve was determined using cell lysates with a known number of cells.

2.5. Statistical analysis

All experiments were performed in at least three replicates. Results were reported as average value ± standard deviation. One-way analysis of variance (ANOVA) was used to compare multiple groups of data statically and *P* values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Measurement of the flow of cell culture media

The multilayer cell culture system was fabricated by stacking four layers of PDMS molds with embedded functionalized scaffold and a cotton thread running through all of the four layers as shown in Fig. 1. Thread as a hydrophilic channel distributed the fluidics within and between scaffolds from top to bottom layers separated by PDMS. The durability and wicking property of the thread makes

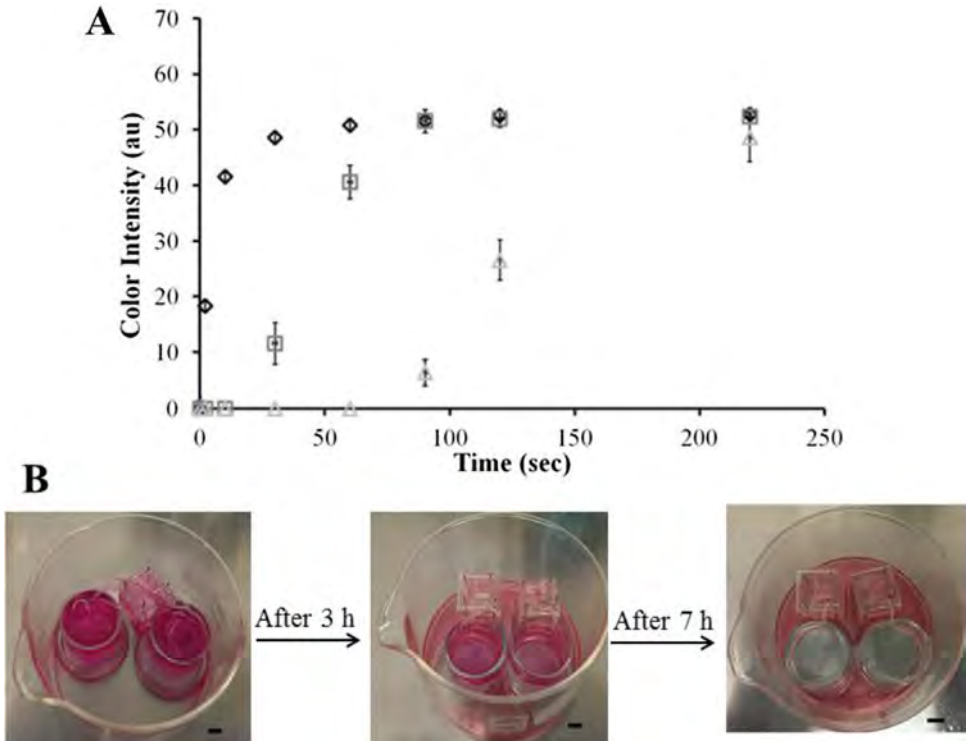

Fig. 3. Penetration rate of media into scaffold using cotton thread at different layers, second (◇), third (□) and fourth layers (△), ($p < 0.05$, n = 6). The data are shown as mean values with standard deviation as error bars in the form of mean value ± standard deviation. Diffusion of cell culture media over a period of time (A). Diffusion of cell culture media with multiple thread system over a period of time (B). Scale bar is 1 cm.

it possible to employ it as a ready microfluidic channel that can wick aqueous solution from liquid resources to dry regions (Fig. 2).

The thread can distribute media into scaffolds in different layers and remove waste from the system, which make it possible to measure the saturation levels of media in each layer of scaffolds. The real samples shown in Fig. 3 indicated that 5 mL of cell culture media could be transferred to scaffolds within 7 h. In addition, a graph of the average color intensity of red (measured as the mean intensity of magenta in CMYK format in Adobe Photoshop) in scaffolds in the four different layers *versus* time is shown in Fig. 3. Thread possessing an average thickness of 300 μm was capable of transporting the media into scaffolds within 250 s till scaffolds were completely saturated (Fig. S1). The trend line of the measurements indicated that the scaffold in the second layer was fully saturated before the next layers starting to absorb media due to the fact that scaffolds is a highly absorptive substrate and can absorb the media from thread till they are completely saturated.

3.2. Cell proliferation in multilayer cell culture system and additional channels

COS-7 cells were used in the current study to assess the appropriate functionality of the multilayer cell culture system as these cells possess rapid division and growth characteristics [35], which allowed testing to be fast and efficient. COS-7 cells were seeded onto the scaffold in different layers in the single thread system, and allowed to grow for 6 days. On Day 3 and Day 6, the cells were observed under the confocal microscope. As shown in Figs. 4 and S2 , the cells were able to proliferate over a period on Day 3, however; these cells were unable to survive until Day 6 due to the lack of the delivery of nutrients and removal of waste from the scaffold construct (Fig. S3). Hence, additional cotton threads were placed to each layer of the cell culture after Day 3 to provide sufficient nutrients to the growing number of cells present in the scaffold. The flow rate of the single thread and multiple thread systems was determined to be 12 ± 4 μL/min and 21 ± 8 μL/min, respectively. The increase in flow rate indicated that more nutrients and waste were delivered and removed through the system. The fluorescent live/dead stained COS-7 cells were cultured in both multilayer cell culture system and control multilayer cell culture system for 3 and 6 days, respectively. The cells in the multilayer culture system with the diffusion of culture medium and waste removal through both cotton thread and additional cotton thread exhibited sufficient viability and healthy morphology with cell proliferation along with time increase (Fig. 5). These results were further confirmed by PicoGreen assay. The number of the cells present in both the cell culture system and control was individually quantified by measuring DNA content. The proliferation of cells in multilayer cell culture system over a period of 6 days is shown in Fig. 6. The number of the cells on the scaffold in the microwell plate, tissue culture dish, second, third and fourth layers of scaffold increased gradually over a period of 6 days ($P < 0.05$, n = 6) and decreased gradually over a period of 6 days ($P < 0.05$, n = 6) for the control scaffold. In addition, the number of the cells present in the multilayer culture system was comparatively identical to the cells grown on the tissue culture dish and scaffold in a microwell plate. The increased number of cells on Day 3 and 6 also suggested that the cells have an adequate amount of nutrients with the addition of thread in the third and fourth layers.

4. Conclusion

A multilayer cell culture device supported by thread was developed in the current study by stacking layers of PDMS embedded with the functionalized scaffold. The thread was sewn into four different layers using a household sewing needle and crossing the

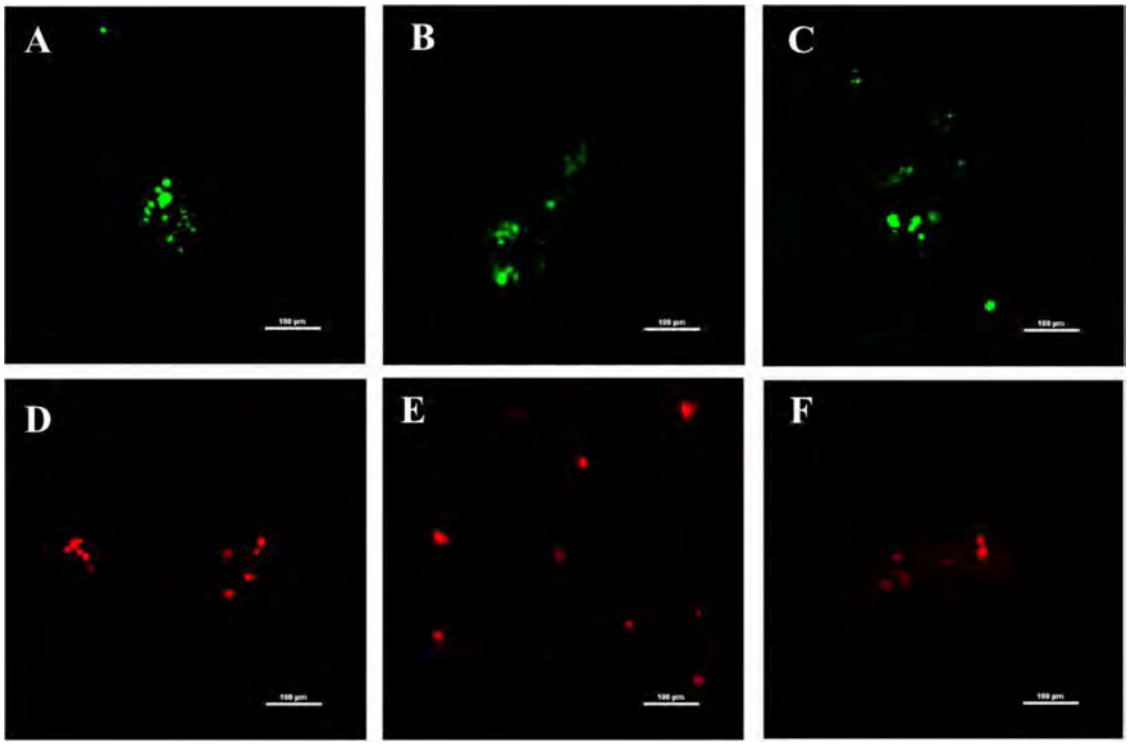

Fig. 4. Fluorescent images of COS 7 cells growth on Day 3 on second (A), third (B), and forth layers (C) of scaffolds. Fluorescent images of dead COS 7 cells on second (D), third (E) and fourth layers (F) of scaffolds in the single thread system. Scale bars are 100 μm.

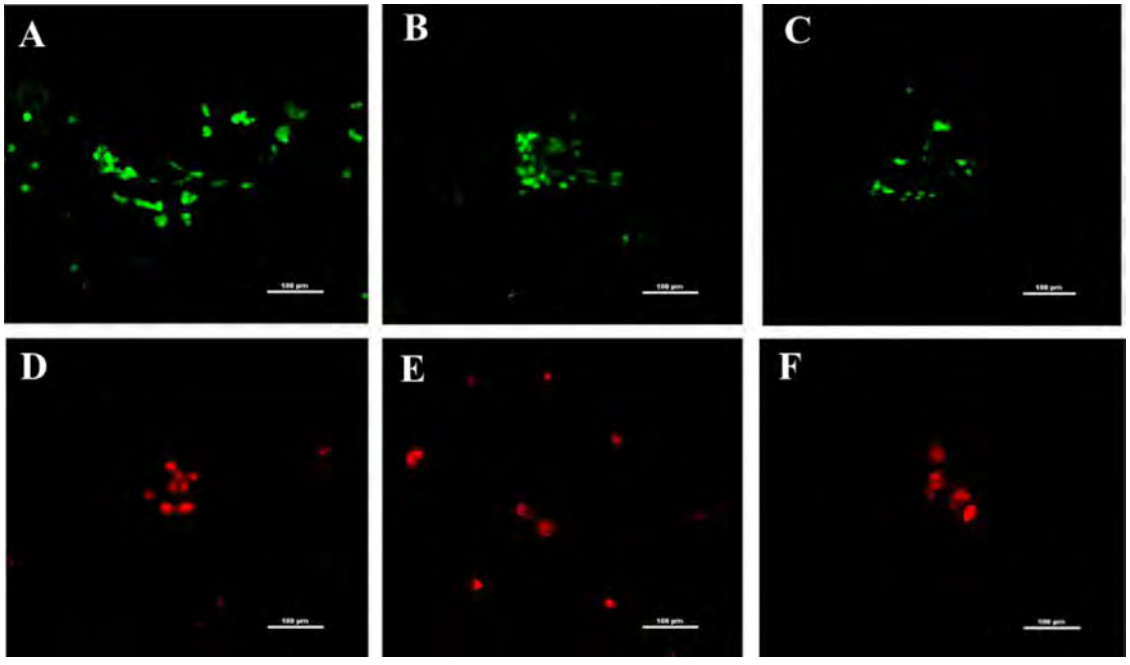

Fig. 5. Fluorescent images of COS 7 cells growth on Day 6 on second (A), third (B), and forth layers of scaffolds (C). Fluorescent images of dead COS 7 cells on second (D), third (E) and fourth layers (F) of scaffolds after inserting additional thread to each layer. Scale bars are 100 μm.

scaffolds, which were positioned into PDMS channels for the transportation of media to the cells in scaffold and waste removal from the scaffold construct. The cells were able to proliferate over a period of 3–6 days by adding some additional threads to the cell culture system after 3 days. For future design, cotton thread can be replaced by biodegradable PLGA suture for implantation to allow the connection of different organs in the body. Further, different types of media (*e.g.*, differentiation media) can be added to different layers in order to initiate cell differentiation. The developed multilayer cell culture system is low-cost, simple, biocompatible, flexible in design and easy to construct. It can potentially be used with different combinations of scaffold materials or thread (made of different materials) to tailor the flow rates and support multilayer cell culture system.

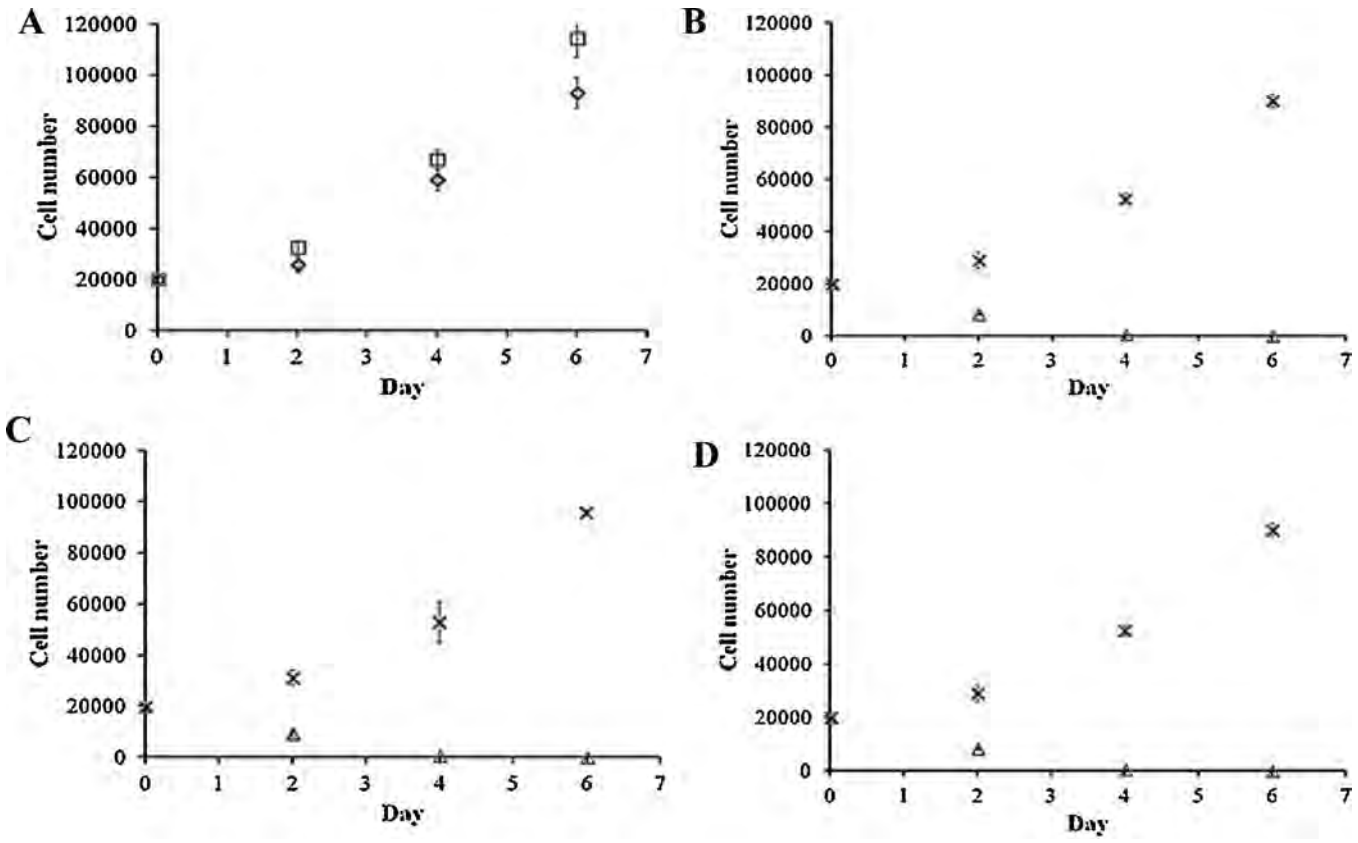

Fig. 6. Proliferation of COS-7 cells on scaffold in the micro well plate (◆) and on culture dish (□) (A); on the second layer of the device (x) and control scaffold (▲) (B); on the second layer of the device (x) and control scaffold (▲) (C); and on the third layer of the device (x) and control scaffold (▲) (D). The number of cells on the scaffold in well, culture dish, second, third and fourth layers of the devices increased gradually over a period of 6 days ($p < 0.05$, $n = 6$) and decreased gradually over a period of 6 days ($p < 0.05$, $n = 6$) for the control scaffold. The data shown as mean values with standard deviation and error bars in the form of mean value ± standard deviation.

Conflict of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.snb.2017.10.186.

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