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Piezoelectric inkjet printing of polymers: Stem cell patterning on polymer substrates

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ABSTRACT

Generating patterns of cells on surfaces is of great significance not only for fundamental studies in biomedical science but also for the creation of functional customized tissue or organs in regenerative medicine. In this paper, arbitrary, complex stem cell patterns were created using piezoelectric inkjet printing of biocompatible polymers. After a systematic study with different inkjet process variables, various poly(lactic-co-glycolic acid) (PLGA) patterns were fabricated on a polystyrene (PS) substrate. Human adipose-derived stem cells (hASCs) were isolated from subcutaneous adipose tissue and were seeded on the PLGA-patterned PS substrate which consists of areas either favorable (PLGA) or unfavorable (bare PS) to cell adhesion. The hASC stably attached, proliferated within the PLGA patterns, thus, complex and confluent hASC patterns were created. Polymer micro-patterning by inkjet printing could be an effective technique to control cell adhesion geometry, leading to arbitrary cell patterning on surfaces.

1. Introduction

Cell patterning to arrange cells in desired locations has become an increasingly important tool in biotechnology because of its broad range of applications [1], particularly for cell-based bioassay [2], tissue engineering [3,4], cell-based drug screening [5], and fundamental studies of cell biology [6]. Cellular patterns are generally achieved by surface engineering in which the patterns of biomaterials either support or inhibit cell adhesion and growth. For generating patterns of biomaterials and cells, many lithographical techniques have been extensively studied such as photolithography [7–9], dip-pen nanolithography [10,11], scanning probe lithography [12], and microcontact printing [13–16].

In contrast to these lithographical methods, inkjet printing can be described as a solid free-form (SFF) fabrication process, which is a solution-based, direct-writing technique [17–20]. In essence, an inkjet printer is a simple dosing robot and the pattern geometry is defined by the computer-controlled motion of the nozzle along a substrate. Inkjet printing has many attractive advantages as a fabrication technique of micro-patterned structures such as simplicity, arbitrary geometries, low cost of process, low usage of materials, and flexibility. Indeed, inkjet printing is entering a mature state in electronic devices such as light emitting diodes (LEDs) and thin film transistors (TFTs). Recently, inkjet printing has been adapted to various biomedical applications including cell patterning, tissue engineering, DNA micro-arrays, protein micro-arrays, and biosensors because most advanced biomedical devices require micro-patterning of biomaterials [21–25]. Cell patterning can be achieved by the direct inkjet printing of cells or by directing cell behavior through patterning biomolecules such as cell adhesive proteins [20,26].

In this paper, we report the feasibility of inkjet printing of synthetic biocompatible and biodegradable polymers for stem cell patterning. Compared with biomolecules such as collagen, synthetic polymers provide many options in terms of physico-chemical properties of inks and their final patterns. The key advantages of use of synthetic biodegradable polymers include the ability to tailor mechanical properties and degradation kinetics to suit various applications. However, it is not simple to create well-defined polymer micro-patterns by piezoelectric inkjet printing systems. Serious problems such as high viscosity, nozzle clogging, cavitation bubbles, undesired satellite drops, precipitation, and uncontrollable drying patterns are frequently encountered in inkjet printing of synthetic polymers. In this study, poly (lactic-co-glycolic acid) (PLGA), one of the most widely used polymers in medicine, was explored for stem cell patterning because of its excellent biocompatibility, biodegradability, and low toxicity after degradation *in vivo*. The inkjet printability of PLGA inks was systematically investigated under different process conditions. Finally, we prepared various viable stem cell colony arrays on polystyrene (PS) substrates with PLGA patterns.

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2. Experimental section

2.1. Materials

Poly(lactic-co-glycolic acid) (PLGA) (LA/GA 70:30) was provided by the Korea Institute of Industrial Technology (KITECH, Korea). Fluorescein diacetate (FDA) was purchased from Sigma–Aldrich. Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL, USA. All other chemicals were of analytical grade and were used as received.

2.2. PLGA ink preparation and characterization

PLGA inks were prepared by dissolving PLGA in *N*,*N*-dimethylformamide (DMF) at different polymer concentrations (1, 2, 3, 4, and 5 g/dl). The polymer solutions were stirred for 3 h at 35 °C, and were passed through a 0.2 μm syringe filter to eliminate insoluble particles. The viscosity of an ink was measured at 30 °C by varying the shear rate from 20 s^{-1} to 100 s^{-1} in a digital viscometer (Brookfield Engineering Laboratories, USA). A surface tension analyzer (CAHN Instruments Inc., USA) was used to measure its surface tension.

2.3. Piezoelectric inkjet printing of PLGA

Printing was conducted on a piezoelectric inkjet printer (FUJIFILM Dimatix Inc., USA) in a clean bench. The inkjet printing system consisted of an inkjet head with multi-nozzles, a print head driver, an XY stage plate, an XY stage controller, and a host computer with software. Each cartridge in the inkjet head has 16 nozzles linearly spaced at 254 μm. The internal diameter of a nozzle is 21.5 μm. The typical drop volume was about 10 pl. The printing area is approximately 200 mm × 300 mm with an adjustable Z height. The substrate stage is a hotplate controlled by a regulator. A CCD camera equipped with an LED light was installed to monitor drop ejection. Patterns were designed using Photoshop CS.

The condition of PLGA inkjet printing was investigated under different processing conditions. The temperature of the plate was set to 30 °C. The head temperature of a cartridge and the firing voltage varied over a range of 30–40 °C and 15–30 V, respectively. A square (10 × 10 mm) cut from an untreated polystyrene (PS) dish was used as a substrate. After printing, the patterns were dried for 2 h in a drying oven and rehydrated prior to cell seeding.

2.4. PLGA pattern analysis

After printing, the morphology of the pattern and spatial distribution of the resulting polymer deposits after solvent drying were investigated using an optical microscope (Leica, Germany) and a high accuracy non-contact surface profiler (Nano System, Korea).

2.5. Contact angle measurement

A contact angle analyzer (SEO Inc., Korea) was used to measure the static contact angle using the sessile drop method. Contact angles were determined from magnified images of sessile drops about 5 μl deposited on the PLGA-printed PS substrate with a miniature syringe.

2.6. Isolation and characterization of hASCs

With informed consent of the donors, human adipose-derived stem cells (hASCs) were isolated from subcutaneous adipose tissue of healthy females between 20 and 40 years of age who had undergone liposuction at the Kangnam Plastic Surgery Clinic (Seoul, Korea). The human adipose tissue was washed several times with phosphate buffered saline (PBS) to remove red blood cells, and the tissue was digested with 0.1% collagenase type II (Gibco-BRL, USA) for 1 h at 37 °C. The digested tissue was filtered through a 100 μm mesh to remove undigested debris, the filtered cell suspension was centrifuged at 200*g* for 7 min, and the pellet was

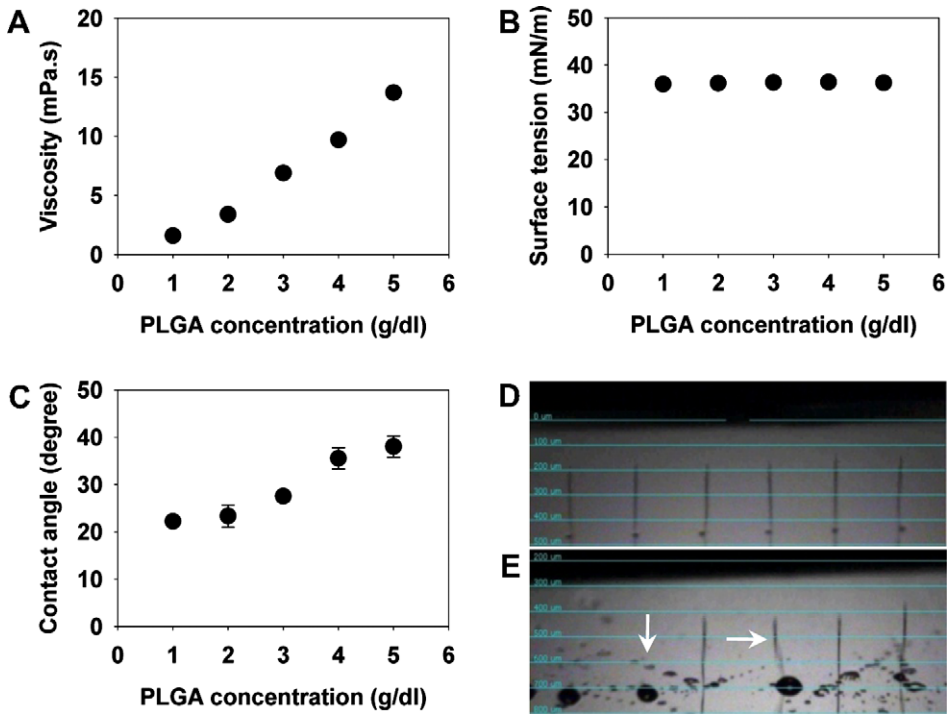

Fig. 1. Basic properties of PLGA inks in DMF at different polymer concentrations. (A) Viscosity, (B) surface tension, (C) contact angle, (D and E) pictures showing droplet ejection, (D) PLGA concentration 1 g/dl, (E) PLGA concentration 2 g/dl.

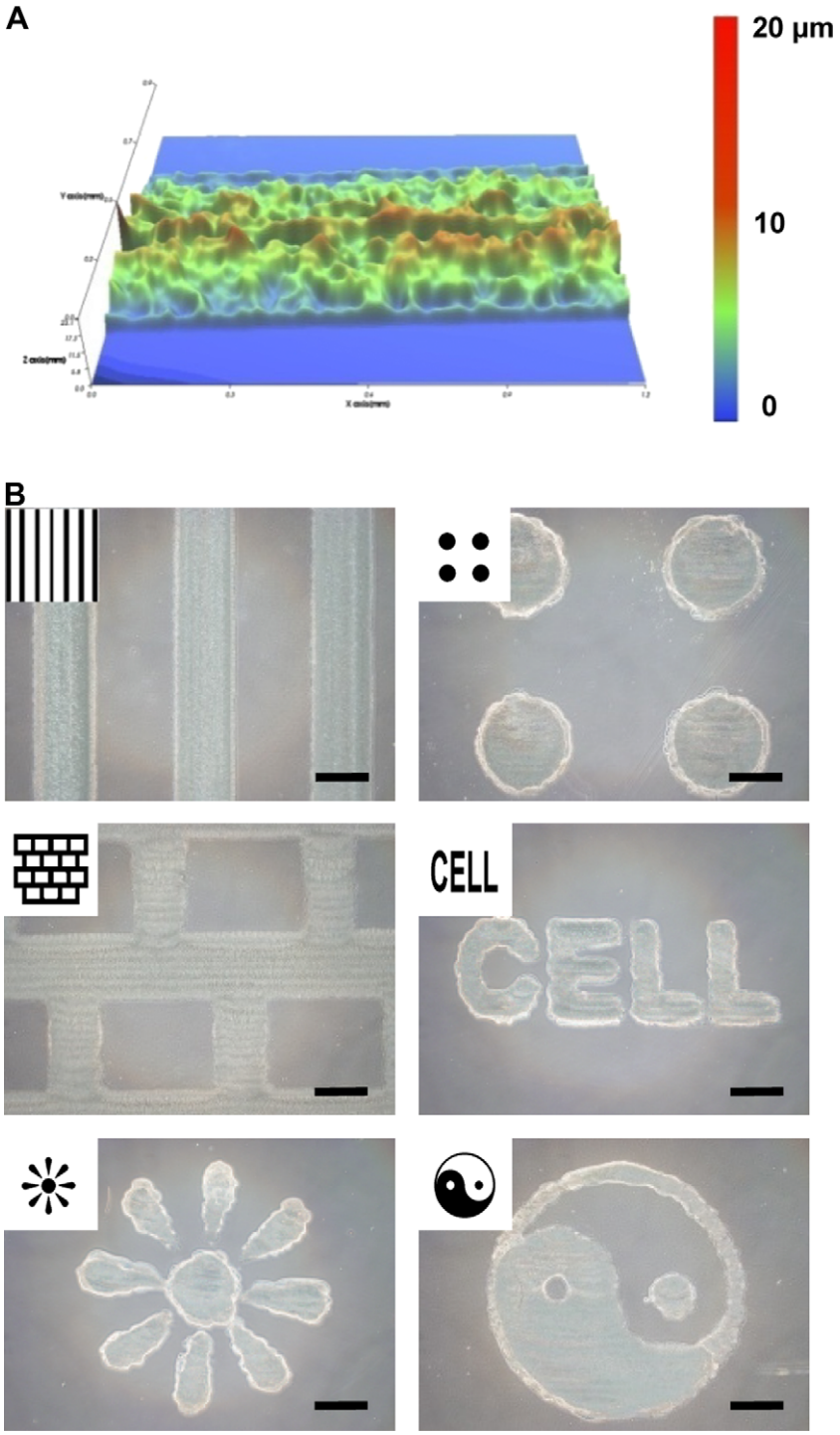

Fig. 2. Optical micrographs of inkjet-printed PLGA patterns. The inserts represent the patterns designed using a Photoshop CS software. (A) Surface profile of line pattern, (B) microscopic images of various patterns. Scale bars represent 500 μm.

washed with PBS. The isolated cells were maintained with DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco-BRL, USA) at 37 °C under 5% CO_2 in a humidified atmosphere.

The phenotype of cultured hASCs was evaluated by measuring cell surface protein expression with flow cytometry using a FACscan argon laser cytometer (BD Biosciences, USA). hASCs were harvested at a concentration of 5×10^5 cells/ml, washed with PBS, and centrifuged at 200*g* for 5 min hASCs were incubated for 30 min at 4 °C with an appropriate amount of fluorescein-conjugated primary antibody to the following CD antigens: CD29, 34, and 44 (Biolegend, USA).

2.7. Cell adhesion and proliferation assays

PLGA-patterned PS substrates were sterilized in 70% (v/v) ethanol solution for approximately 10 min in a clean bench and then washed in serum-free DMEM overnight. Cell adhesion assay was performed using the WST-1-based colorimetric assay (Roche, Switzerland), which relies upon the ability of living cells to reduce a tetrazolium salt into a soluble colored formazan product. hASCs were routinely cultured in DMEM to reach a subconfluent state. Cells were harvested by trypsin-EDTA treatment, resuspened in

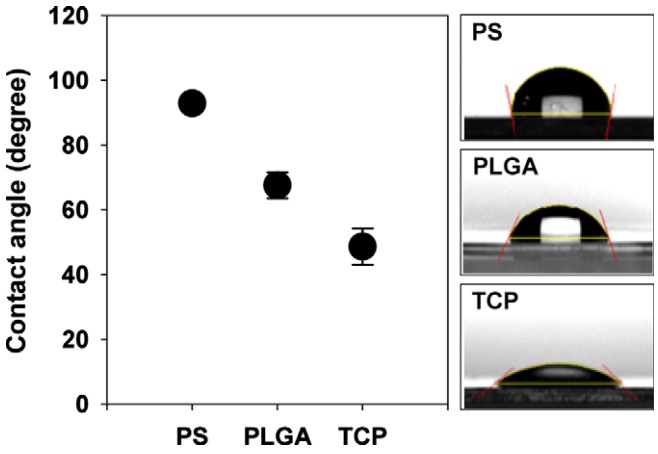

Fig. 3. Water contact angle of PS, PLGA-printed PS, and TCP.

serum-free medium, and seeded on PLGA-printed PS substrates at a density of 5×10^4 cells/cm^2. The cells were allowed to attach to the substrate in an incubator for 24 h. To remove unattached cells, the cell-seeded substrates were rinsed several times with PBS, and were transferred into a new 24 well plate. The WST-1 reagent was added to each well containing the hASC-seeded substrates, and the plates were incubated at 37 °C for 1 h under 5% CO_2. The optical density was then measured at 440 nm using a microplate spectrophotometer (Bio-Tek Instruments, USA). The cell adhesion ratio was expressed as the absorbance of adherent cell density divided by that of cell seeding density as a percentage. For a cell proliferation study, the cells were allowed to proliferate for 72 h on PLGA-printed PS substrate, and the number of attached cells was determined by the same method. The culture medium was refreshed every day.

2.8. Scanning electron microscopy

After removal of the culture medium, the cell-seeded substrates were rinsed twice with PBS and then fixed by using a 2.5% glutaraldehyde solution for 30 min. After cell fixation, the samples were rinsed with PBS, dehydrated through a series of graded ethanol solutions, and lyophilized overnight. The samples were observed by SEM (Hitachi High-Technologies Europe GmbH, Germany) after being coated with platinum by a sputter at an accelerating voltage of 15 kV.

2.9. Fluorescence microscopy

The cell-seeded samples were stained with 100 μl of FDA reagents at 37 °C for 30 min in darkness and then were observed using a fluorescence microscope (Olympus, Japan) equipped with a digital camera. Green fluorescence by FDA reaction indicates live cells.

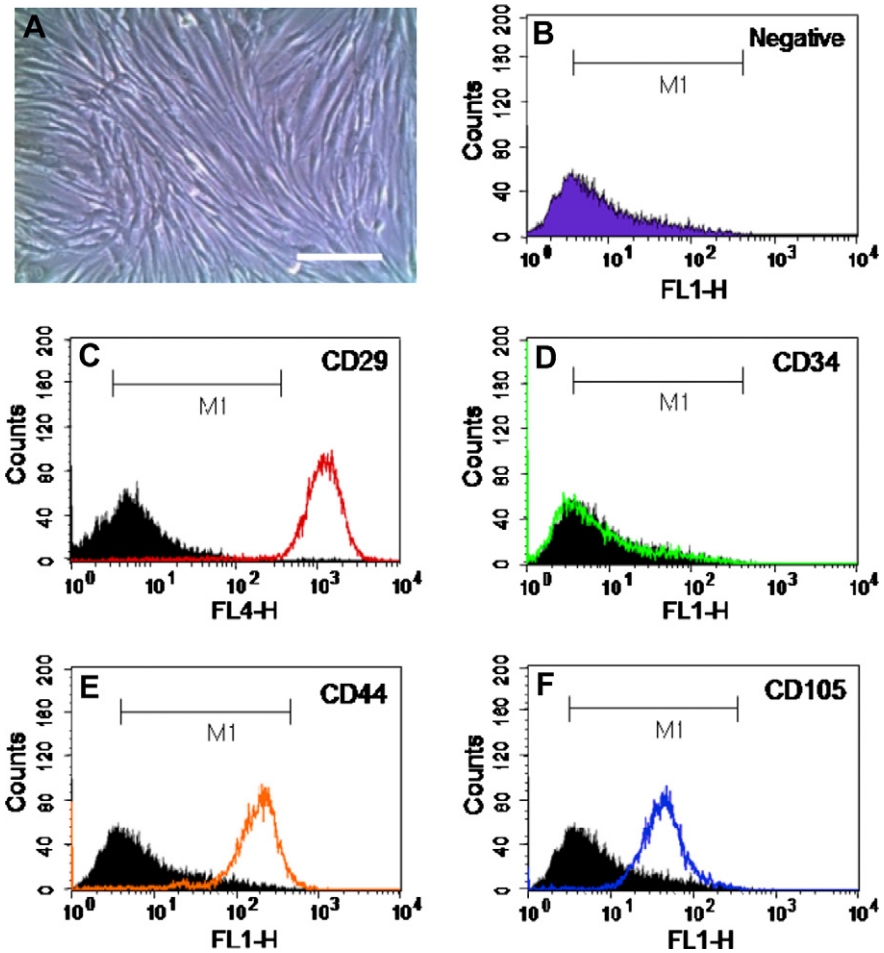

Fig. 4. Characterization of hASCs at passage 9. (A) Morphology of hASCs, (B–F) Immunophenotype of hASCs. Cells were stained with a fluorescein-conjugated primary antibody and analyzed by fluorescence-activated cell sorting. White bar represents 200 μm.

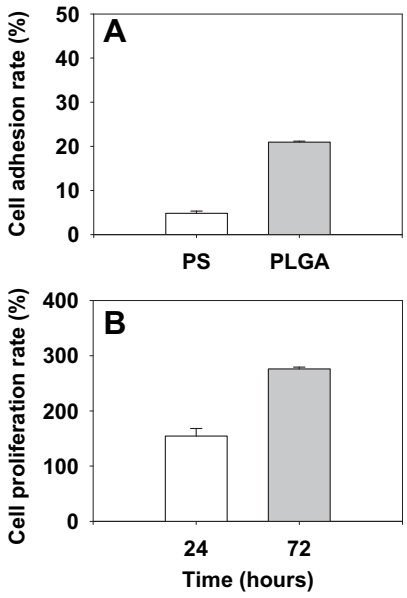

Fig. 5. (A) Cell adhesion on PS and PLGA-printed PS substrates for 24 h and (B) Cell proliferation on PLGA-printed PS substrates for 24 h and 72 h.

3. Results and discussion

3.1. Inkjet printability of PLGA ink

The inkjet printing of polymers offers a great opportunity to develop a powerful mold-free fabrication technology which can create arbitrary and complex polymer micro-patterns. The physicochemical properties of ink significantly affect the inkjet printability, pattern accuracy, and pattern resolution. Even a small amount of polymers in ink greatly changes its rheological properties such as viscosity, and thereby the inkjet printability is strongly influenced [27–32]. The selection of solvent is the first step for polymer ink formulation. The homogeneity of polymer ink is a prerequisite because poor solvents lead to polymer precipitation and eventual clogging of the nozzle. When volatile solvents like chloroform were used as a solvent for PLGA, its evaporation time was incredibly short, within a few seconds, which resulted in frequent PLGA clogging, especially when the nozzle was undisturbed. The use of the non-volatile solvent DMF with a boiling point of 153 °C significantly reduced nozzle clogging [27,33].

Fig. 1 shows basic properties of PLGA inks at different PLGA concentrations. It has been reported that viscosity should be low, ideally below 10 cps, in piezoelectric inkjet printing systems [17,34,35]. At higher viscosities, an inkjet printer may not eject drops from nozzles because the force generated by a piezoelectric inkjet printer is limited [17,28,29]. The presence of PLGA in ink significantly increased its viscosity from 1.5 cps at 1 g/dl to 15 cps at 5 g/dl (Fig. 1A). Surface tension allows spherical droplets to emerge from inkjet nozzles. The surface tension of ink for piezoelectric inkjet printing should be in the range of 30–70 mN/m, high enough to prevent dripping of the ink from the nozzle and low enough to allow spreading over the substrate [34–36]. Fig. 1B shows the surface tensions of PLGA solutions in DMF at different PLGA concentrations. The presence of the polymer in ink did not much affect its surface tension. The values of PLGA inks in DMF were about 35 mN/m, irrespective of PLGA concentrations, which were higher than the bottom limit (30 mN/m). That is, the surface tensions of all PLGA inks in DMF were low but within the range suggested by the literature [34–36]. The contact angle of polymer ink reflects the wettability between ink material and a substrate. The wettability may affect the pattern accuracy and pattern resolution. Fig. 1C shows the contact angles of PLGA inks on PS substrates at different PLGA concentrations. The contact angle gradually increased with PLGA concentration, from 22.2° at 1 g/dl to 38.0° at 5 g/dl.

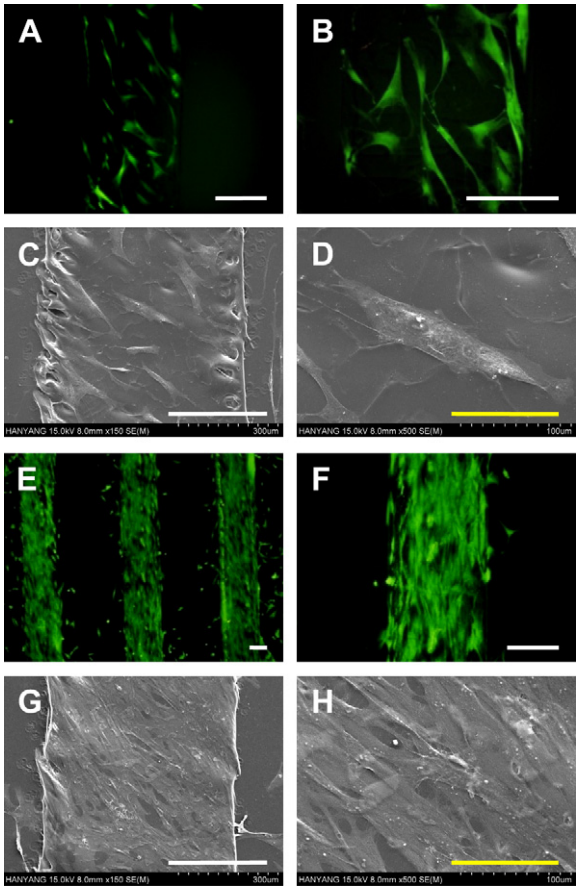

Fig. 6. Fluorescence micrographs and SEM images of hASCs on PLGA-patterned PS substrates. (A–D) after 1 day of culture, (E–H) after 3 days of culture. White bars represent 300 μm and yellow bars represent 100 μm.

Drop ejection was monitored by a CCD camera attached to an inkjet printer, as shown in Fig. 1D and E. At 1 g/dl of PLGA concentration (Fig. 1D), PLGA droplets were continuously and stably ejected from the nozzles. A long tail connecting drop and nozzle was observed, probably due to its high viscosity and low surface tension. At 2 g/dl of PLGA concentration, some nozzles did not stably eject polymer ink droplets. At higher PLGA concentrations (3, 4, and 5 g/dl), most nozzles did not eject polymer ink droplets, probably due to high viscosity. The PLGA ink with a concentration of 1 g/dl was selected for the next steps. The appropriate jetting voltage and firing frequency for printing with 1 g/dl PLGA ink were determined to be 30–35 V and 5 kHz, respectively.

3.2. PLGA patterning on PS substrates

Inkjet printing is a simple method to create controlled polymer architectures on surfaces. If a pattern change is required, a simple design modification can be done quickly on a software interface, without any additional materials. Various PLGA patterns were printed on PS substrates, as shown in Fig. 2. The patterns were analyzed using a high accuracy non-contact surface profiler

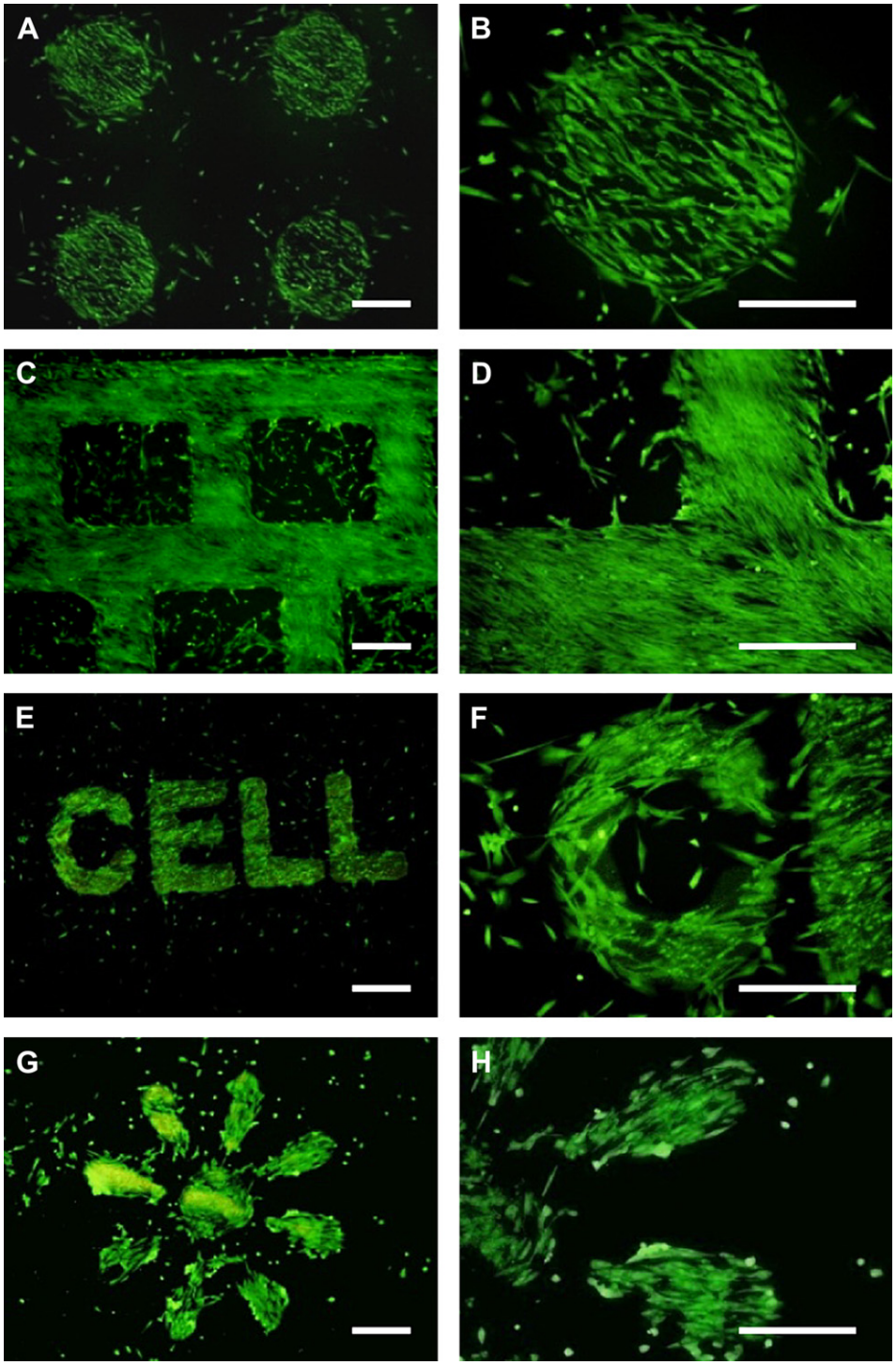

Fig. 7. Fluorescence microscope images of hASCs on PLGA-patterned PS substrates after 5 days of culture. (A and B) Dot pattern, (C and D) brick pattern, (E and F) 'CELL' letter pattern, and (G and H) flower pattern. White bars represent 500 μm.

(Fig. 2A) and an optical microscope (Fig. 2B) after PLGA deposition. The average width and height of line pattern were about 570 μm and 6.4 μm, respectively. The pattern width was approximately 10% larger than that of the designed pattern. The display of color represents the surface roughness of line pattern (Fig. 2A). Surface topography may influence the behaviour of adhering cells. For example, osteoblasts were sensitive to surface roughness and exhibited great attachment rates to rough Ti surfaces [37,38].

3.3. Water contact angle of PLGA-printed PS substrates

The water contact angles of PS and tissue culture plate (TCP) were about 92.8° and 48.6°, respectively (Fig. 3). PS has a long chain hydrocarbon with every other carbon connected to a phenyl group. Hence, bare PS is hydrophobic. TCP is made up of PS treated with a variety of ionic functional groups that support attachment and spreading of cells. PLGA-printed PS had a contact angle of about

67.6°, which is comparable to that of TCP. The surface wettability could be an important contributor to potential cell attachment and proliferation [39]. A few studies showed that cells well spread on substrates with moderate hydrophilicity [40–42].

3.4. Isolation of hASCs

hASCs are multipotent mesenchymal stem cells which have the ability to differentiate along multiple pathways including the adipocyte, chondrocyte, osteoblast, endothelial, epithelial, and neuronal lineages [43,44]. Because adipose tissue is the most prevalent tissue in the human body and can be easily obtained in relatively large quantities, hASCs isolated from adipose tissue have generated intense interest as an alternative source of stem cells [45]. In the present study, hASCs were isolated from the subcutaneous adipose tissue of healthy female donors who had undergone liposuction. The freshly isolated human adipose tissue-derived stromal vascular fraction (SVF) cells were maintained until passage 9, which showed a fibroblast-like morphology [46] as shown in Fig. 4A. Representative flow histograms for the hASCs are shown in Fig. 4B–F. The hASCs expressed stem cell surface markers including CD29, CD44, and CD105, but did not express the hematopoietic marker CD34. The cell surface marker expression confirmed that the isolated hASCs had the capacity of stem cells under controlled conditions *in vitro*.

3.5. Cell adhesion and proliferation

hASCs were seeded into two different substrates (PS and PLGA-printed PS substrates) and cultivated for 24 h. As shown in Fig. 5A, PLGA provided better substrates for cell adhesion than bare PS. Significantly more cells were attached to PLGA. The behavior of cell adhesion on the substrates may be partly due to the surface wettability and roughness [47,48]. As described above, they are important surface properties that could regulate the protein adsorption and cell behavior. The proliferation of hASCs on the PLGA-printed PS substrates is shown in Fig. 5B. hASCs gradually proliferated on PLGA patterns for 72 h. The proliferation rate of hASCs on PLGA was about 154% and 275% after 24 and 72 h, respectively. The PLGA patterns were supportive of the adhesion and proliferation of hASCs.

3.6. Stem cell patterning

A surface-treated PS dish is a commonly used substrate for most cell culture because it is cheap and compatible with most microscopes. Untreated PS prevents cell adhesion, allowing inkjet-printed PLGA to act as an adhesive pattern. One-step inkjet printing of PLGA on untreated PS could produce polymer patterns which consist of areas either supporting or inhibiting cell adhesion. Fig. 6 shows inkjet-printed surfaces which allow the formation of patterned stem cell arrays. At 1 day post-seeding, hASCs attached and stayed on the PLGA patterns. Although the cell patterning was incomplete due to low cell density (Fig. 6A–D), it was clear that the PLGA patterns on PS substrates provided places for cell adhesion. Most cells attached on PLGA patterns seemed to align with the pattern within the boundary. On the contrary, most cells did not attach on non-patterned regions of PS substrates. Therefore, before the first media exchange on day 1, it was common to have many rounded cells floating in the media. A few of the hASCs seemed to spread in non-patterned regions of PS substrates, but these cells were easily rinsed away due to weak cell-substrate interaction. After 3 days of culture, the stem cells attached to PLGA patterns proliferated but were nicely constrained within the PLGA patterns, as shown in Fig. 6E–H. As the cell density increased, the patterns were readily recognized. Specific, complex cellular patterns were also constructed on PLGA-printed PS substrates as shown in Fig. 7. After 5 days the stem cells reached confluence within PLGA patterns. Overall results demonstrate that the PLGA-patterned PS substrate which consists of areas either favorable or unfavorable to cell adhesion controlled the position of cells in a well-defined manner, leading to specific and complex cellular pattern formations.

When compared to lithographic techniques including photolithography and soft lithography, inkjet printing is an extraordinarily simple method to create arbitrary patterns. The lithographical techniques currently show much better pattern accuracy and resolution than inkjet printing. Nonetheless, the simplicity, arbitrary geometries, low cost and flexibility make inkjet printing attractive for application to patterning of cells and polymers. Recently, the technology of inkjet printing has been rapidly developing. The size of inkjet-printed droplets has been steadily decreasing; currently inkjet printers can eject droplets of only a few pico-liters. The corresponding increase in resolution allows inkjet printing to fabricate complex shapes with features in micrometer ranges with the aid of computational topology design (CTD). This study demonstrates the potential of polymer inkjet printing as a system to fabricate distinct patterns of biomaterials and cells. We hope that this approach will generate further interest in stem cell patterning and trigger detailed investigation regarding specific geometrical effects on stem cell functions.

4. Conclusions

The present study describes the creation of stem cell patterns on surfaces using an inkjet printing system. After a systemic study with different inkjet process variables, the inkjet printing system for PLGA micro-patterning was optimized and various PLGA patterns were fabricated on PS substrates. The PLGA patterns were supportive of the adhesion and proliferation of hASCs. The spread of hASC was efficiently constrained by the PLGA patterns. The adaptation of inkjet printing technology to stem cell patterning is a potential strategy for developing customized functional tissues with complex anatomical shapes.

Acknowledgements

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