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Rigidity Guided Cell Attachment on Inkjet-Printed Patterns

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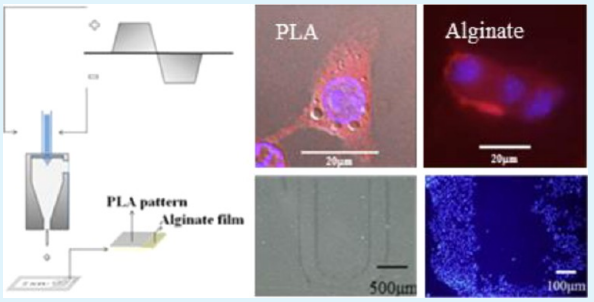
Supporting Information

ABSTRACT: A new approach is presented to control cell attachment behavior on biocompatible substrates. Multiple layers of polylactic acid (PLA) were inkjet-printed on dry alginate films to create composite surfaces with rigidity variation. The printed films were submerged in cell culture medium and fibroblast 3T3-L1 cells were cultured on the printed films. 3T3-L1 cells were found to preferentially adhere on PLA surfaces with higher rigidity. The same approach was also used to create various cell attachment patterns. This study provides a new methodology to fabricate biodegradable matrix for favorable cell adhesion or patterning.



KEYWORDS: *cell attachment patterns, inkjet printing, biodegradable material, alginate, PLA, 3T3-L1 cells*

INTRODUCTION

Cell patterning technique has become an important tool in many fundamental studies, such as tissue engineering[1−3] and drug screening.[4] To arrange cells at specific locations, cell growth factors or inhibitors are regularly printed on biocompactable substrates with various methods, such as soft photolithography,[1,5] laser-directed cell-writing,[6] and dip-pen nanolithography,[7,8] to construct cellular patterns with desired cell morphology. One of the most commonly used methods is soft lithography technique. By applying an elastomeric stamp made of polydimethylsiloxane (PDMS) on planar substrates, one can fabricate biomaterial patterns with feature size down to micrometer scale.[1,5] However, several lithographical steps are required to prepare these elastic stamps, and therefore change of patterns can be cumbersome. In contrast to soft lithography, inkjet printing technology provides a solution-based direct writing approach to deposit liquids accurately on flat or patterned substrates. Because inkjet printing serves as a noncontact direct-writing fabrication technique, one can print any arbitrary patterns accurately without damaging substrates. Thus, it has been widely used to manufacture micropatterns for electronic devices, such as light-emitting diodes (LEDs) and thin film transistors (TFT). Recent study has also shown the feasibility to inkjet-print polymers with cell growth factors for cellular patterning.[9−11]

Although many cell attachment/growth control methods have been developed in the past decade, most previous studies control cell culture patterns by printing cell growth factors, such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), on biocompatible substrates. By adjusting the chemical environment on substrate surfaces, cells adhesion can be controlled locally to generate specific patterns. Besides chemical modification, related studies also suggested that cells can respond to mechanical forces exerted by surrounding environment, i.e., physical properties of substrate surfaces can also affect cell attachment. Following this idea, cell attachment or growth behavior are found to be responsive to the rigidity of substrate surfaces.[12−15] On the basis of this cell attachment preference on substrate rigidity, in this study, we systematically investigate the feasibility of creating cell patterns from rigidity-guided cell attachment. An inkjet printing method is developed to print stiff PLA patterns on relatively soft alginate films to create composite patterns with rigidity variation. 3T3-L1 fibroblast cells, which have been used extensively for cell growth studies and viral transformation,[16] are cultured onto the printed alginate films for cell attachment tests.[13,15] Viable 3T3-L1 cells are allowed to actively explore on the alginate substrates with PLA patterns with a rigidity variation. Our results indicate that 3T3-L1 fibroblasts can indeed detect and respond to substrate stiffness gradient, i.e., 3T3-L1 cells prefer to adhere on patterns of higher rigidity without addition of any cell growth factors or inhibitors.

EXPERIMENTAL SECTION

Materials. Sodium alginate, calcium chloride powder, chloroform, acetonitrile, and tris-buffered saline (TBS, pH 7.4), 4′,6-diamidino-2-phenylindole (DAPI) and were purchased from Sigma Aldrich, USA. Dulbecco's Modified Eagle medium (DMEM) was purchased from Thermo Scientific, USA. Fetal bovine serum (FBS) was purchased from Biolegend, USA. Polylactic acid (PLA Polymer 2002D) particle was purchased from NatureWorks, USA. All the chemicals were of analytical grade and were used as received without any further purification.

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Preparation of Substrates. Sodium alginate powder was first mixed with distilled water under stirring for an hour and then sonicated for about 30 min to remove trapped air bubbles. About 1.5 mL of the resulting clear aqueous solutions was poured into a ($50 \times 30 \times 1$ mm^3) cast, which was placed in a Petri dish. Twenty-five milliliters of calcium chloride solution was then poured into the Petri dish for cross-linking. After 15 min, the cast film was washed with distilled water to removed residual Ca^{2+} cation, and was dried at room temperature under UV lamp sterilization for 1 day. The thickness of the dried alginate films is about 0.1 mm.

Piezoelectric Inkjet Printing of PLA. The PLA ink was prepared by adding 0.2 wt % PLA pallets into an acetonitrile/chloroform mixture of 9:1 weight ratio. The polymer solution was stirred for an hour at room temperature, and was filtered to remove insoluble or airborne particles. A piezo-based inkjet printer (JetLab 4, Microfab, USA) was used to print PLA patterns on dried calcium alginate films. Table 1 lists the printing parameters of the PLA ink. The droplets were ejected at a velocity of 1.31 m/s with a drop volume of 131.4 pL. After printing, the samples were dried for 12 h and rehydrated prior to cell seeding.

Table 1. Printing Parameters for the PLA Ink

rise time (μs)	dwell time (μs)	fall time (μs)	echo time (μs)	rise time 2 (μs)	idle voltage (V)	dwell voltage (V)	echo voltage (V)
10	3	10	3	3	0	20	−25

Scanning Electron Microscopy and Atomic Force Microscopy. Scanning electron microscope (JSM-5310, JEOL) was used to evaluate the cross surface topology of alginate films with PLA coatings. Before the measurement, samples were coated with a thin layer of gold. Atomic force microscopy (EasyScan 2, nanoScience Instruments, with SICON Contact Mode probes from AppNano) was used to measure Young's modulus of alginate films in wet condition. A contact mode probe was used to obtain the force responses upon the indentation for Young's modulus measurement.

Cell Staining and Fluorescence Microscopy. Before cell culture, PLA-patterned calcium alginate substrates were sterilized in 70% (v/v) ethanol solution for approximately 5 min and then washed with DMEM for at least three times to remove unwanted chemicals or dusts. The culture medium was DMEM supplemented with glucose, 10% fetal bovine serum, and 1% Penicillin/Streptomycin/Amphotericin B solution. 3T3-L1 cells were seeded on PLA-patterned calcium alginate substrates at a density of 5×10^4 3T3-L1 cells/cm^2. The cells were allowed to attach to substrates in an incubator at 37 °C under 5% CO_2 for 24 h.

To observe cell nuclei after cell attachment, cultured samples were first washed with 3.7% formaldehyde and 0.2% Triton-X100 solution in TBS at room temperature to clean the substrate surfaces and fix the attached cells. Samples were then incubated with DAPI and fluorescent phallotoxins (Invitrogen, USA) solution simultaneously at room temperature for 30 min to stain cells adhering onto the substrates. After staining, samples were further washed with PBS to rinse off extra staining solution. The rinsed samples were dried overnight in the dark room at 2 °C, and were examined using a fluorescence microscope (Olympus IX71).

RESULTS AND DISCUSSION

Printed PLA Patterns on Alginate. Figure 1 shows various printed PLA patterns on dry alginate films. Inkjet printing method provides a flexible tool for pattern creation simply by connecting printed dots, which are the deposit liquid disks from ejected droplets. These dots are the primary component of all printed patterns. Under the given printing condition in Table 1, with a dot-to-dot distance of 120 μm, one can observe a line of single PLA dots (Figure 1a) of about 90 um in diameter, which is the finest feature size in this study. By tuning the dot-to-dot distance down to 25 μm, one can manufacture continuous lines and solid fill patterns (Figure

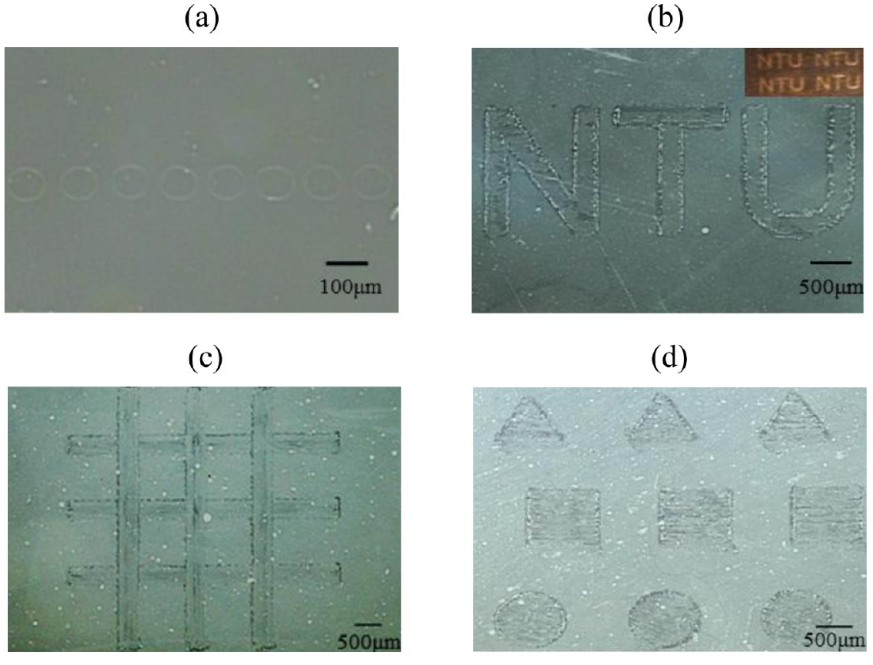

Figure 1. Images of various printed PLA patterns on alginate cast films under optical microscope.

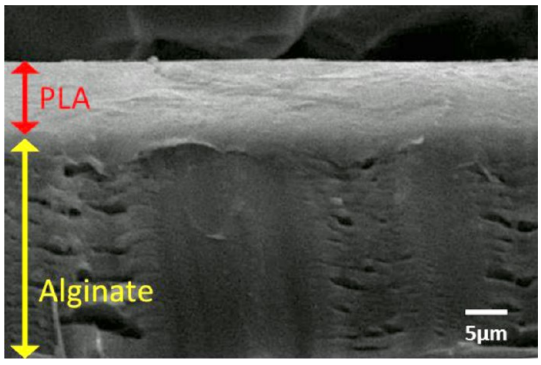

Figure 2. SEM cross-sectional image of a printed PLA film on dry alginate film. To enhance the visual effect, we stacked five repetitive printed layers of PLA on the alginate film.

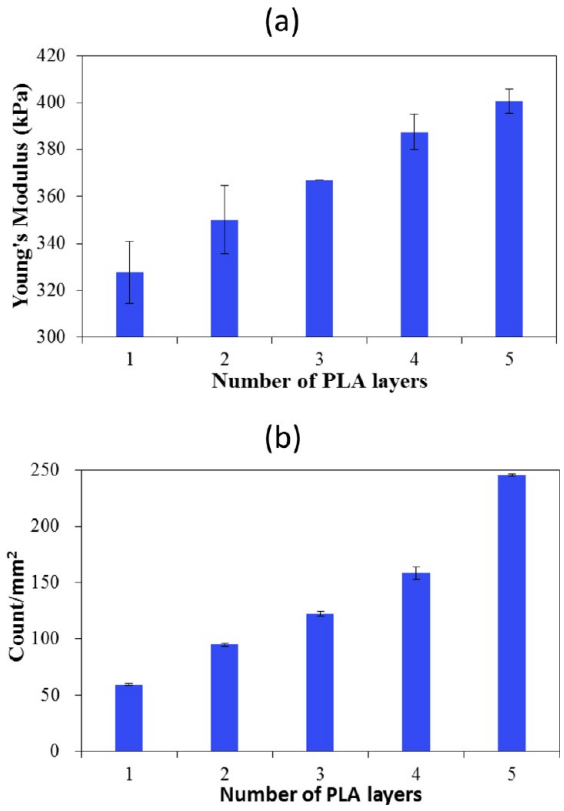

Figure 3. (a) Young's moduli and (b) cell densities on PLA films of various thicknesses. The Young's modulus of alginate film is 192 kPa, and nearly no cell attachment on bare alginate surface after cell culture (see the Supporting Information for more details).

1b–d). Because of the high precision in repetitive printing, one can also print multiple layers to increase the pattern thickness.

The microstructures of printed patterns are examined by scanning electron microscope. Dry alginate films compose of pores of micrometer sizes[17] and absorb liquids quickly. Because of this porous nature, PLA ink can penetrate into alginate films and create dry PLA film anchored on the alginate. Figure 2 shows the cross sectional view of a printed PLA/alginate composite film. The printed PLA film is about 10 μm thick with a 1–2 μm thick transition layer, where PLA and alginate mix together due to liquid imbibition in printing process. This transition layer creates anchors for PLA patterns and helps PLA adhesion on alginate surfaces. Thus, cells on PLA patterns after

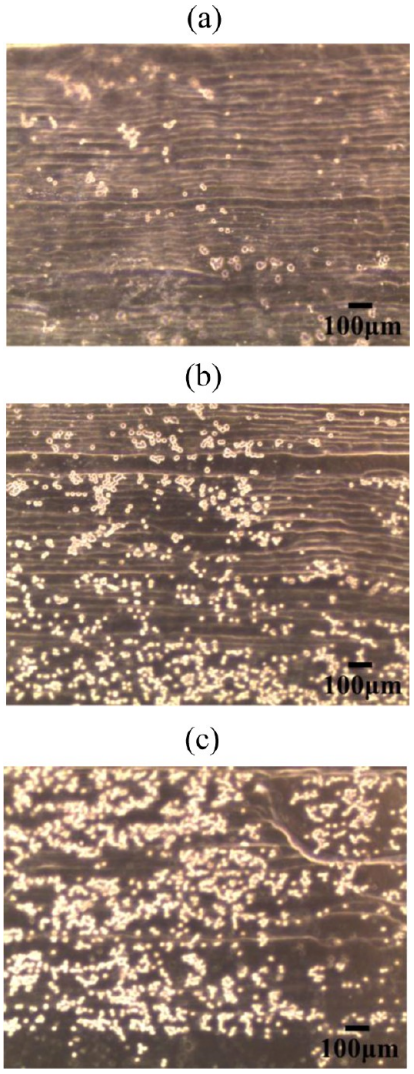

Figure 4. Microscopic images of cell morphology on PLA films of various thicknesses: (a) one, (b) three, and (c) five stacked layers. Bare alginate film has nearly no cell attachment (see the Supporting Information for more details).

Table 2. Roughness of Printed PLA Layers of Various Thickness

no. of PLA layers	1	2	3	4	5
roughness average (nm)	44.4	57.5	50.5	59.3	57.8

culture and/or staining process can be observed clearly on the planar alginate surfaces (Figure 5).

Effect of Rigidity on Cell Attachment. To investigate effects of rigidity variation on cell attachment, we printed multiple layers of PLA on alginate to generate composite substrates with different stiffness. The stiffness measurements of printed PLA films after submerged in culture medium are summarized in Figure 3a. Through AFM indentation, force–distance curves can be used to calculate Young's moduli of composite films from Sneddon model[18]

$$E = \frac{F\pi(1-\nu^2)}{2\tan(\alpha)(S_0 - S)^2} \quad (1)$$

where E is Young's Modulus of sample, F is the loading force (18 nN), $(S_0 - S)$ is the indentation distance, ν is Poison ratio

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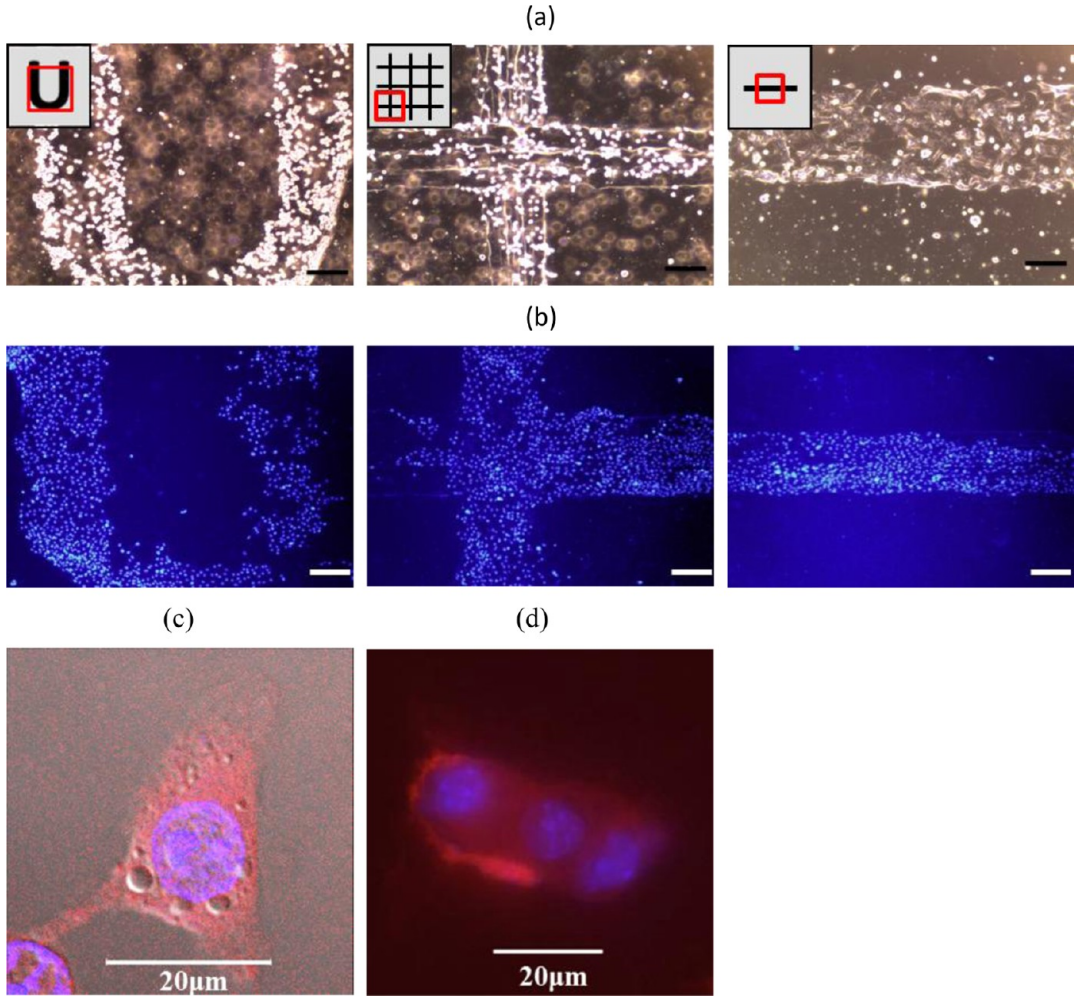

Figure 5. (a) Micrographs of 3T3 cells on PLA-patterned alginate films after 1-day culture. (b) Fluorescent micrographs of 3T3 cells on PLA-patterned alginate substrates. The scale bars represent 200 μm. (c) Fluorescent micrographs of 3T3 cells attached on bare alginate substrate. (d) Fluorescent micrographs of single 3T3 cell spread out nicely on the stiffer PLA surfaces.

(0.36), and α is the half cone angle of AFM probe tip (10°). Because pure PLA has a much larger Young's modulus than wet alginate films (10.625 vs 0.192 MPa from tensile test (SHIMPO FGP-0.5, Japan)), Young's moduli of composite films increases with PLA thickness as expected.

The composite alginate/PLA films were submerged in DMEM for cell culture, and cell attachment after seeding was examined by optical microscopy. Figure 4 shows the microscopic images of printed PLA films after cell culture. From direct observation, it is clear that the attached cell numbers strongly depend on the PLA thickness. Significantly more cells were attached to PLA with more printed PLA layers, or composite films with higher Young's modulus. Cell densities on films of different PLA thicknesses were further calculated by counting cells in the micrographs, such as those in Figure 4, by image process software (Image J), and are summarized in Figure 3b. Cell density increases with number of layers, implying that cells prefer attaching to thicker PLA films. Because PLA surface topography may influence the behavior of cell attachment,[19] surface roughness of printed PLA films was also examined by AFM (see the Supporting Information for detail). The roughness (Table 2) was found to be in the same order and hardly has variation in printed surfaces regardless of numbers of PLA layers applied. Thus, the cell attachment preference is most possibly attributed to stiffness variation generated by multiple PLA layers. These finding are similar to those found in the previous study. Because the results showed better cell attachment with thicker PLA layers, in the following examples, patterns with five stacked PLA layers were printed on dry alginate film to investigate feasibility of the rigidity-guided cell patterning technique.

Cell Culture Patterns. The rigidity-guided cell attachment can be applied to induce cell attachment/growth patterns. Patterns in Figure 1 were used in cell culture to test 3T3-L1 cell attachment preference. Figure 5a shows direct optical microscopic images after 1 day culture. Due to magnification limits of the microscope, only part of the patterns are shown here. 3T3-L1 cells preferentially adhered on the printed PLA patterns, even after slight DI water rinsing. By observing stained cell morphology on alginate film with PLA patterns, as shown in Figure 5b, 3T3-L1 cells indeed attached on PLA patterns only, indicating substrate rigidity influences cell attachment. Micrographs at higher magnification are shown in panels c and d in Figure 5 to understand the mechanism of rigidity-guided cell attachment. On bare alginate films, because of the low rigidity, attached cells are unable to generate adhesion sites. Thus, the cells attached on bare alginate are in round shapes (Figure 5c). On the other hand, cells spread out nicely on the stiffer PLA

surfaces with irregular protrusions (Figure 5d), indicating the formation of great adhesion.[12−15] This evidence shows that 3T3 cells can detect and respond to substrate rigidity difference. Because cell attachment is the initial process of manipulating the tissue engineered microenvironment, the desired morphology of cell candidates can then be designed by manipulating the mechanical properties of printed patterns.

CONCLUSIONS

An inkjet printing method is developed to fabricate PLA micropatterns on alginate cast films. Multilayer PLA square patterns are printed to investigate the relationship between pattern stiffness and cell attachment. The Young's moduli of printed PLA layers on alginate are determined with AFM, and are larger as PLA thickness increases. After one-day culture, more 3T3-L1 fibroblast cells were found to attach on thicker PLA layers, indicating that 3T3-L1 fibroblasts can indeed detect and respond to substrate rigidity gradient. This rigidity-guided attachment approach can be used to create cell culture on various patterns. Through fluorescence microscopy study, 3T3-L1 cells indeed preferentially attach to PLA patterns. Similar inkjet approach can be used to print patterns with rigidity variation for cell separation and controlling cellular behavior.

ASSOCIATED CONTENT

Supporting Information

AFM data for roughness tests and cell culture micrographs are shown separately. This material is available free of charge via the Internet at http://pubs.acs.org/."

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Notes

The authors declare no competing financial interest.

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