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Technical Note

Direct patterning of mammalian cells onto porous tissue engineering substrates using agarose stamps

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Abstract

This paper describes simple, inexpensive, and potentially generic methodology for generating patterns of mammalian cells on porous scaffolds for tissue engineering using replica printing. Circular patterns (diameter: 200, 700, and 1000 μm) of human osteoblasts were transferred directly from topographically patterned agarose stamps onto porous hydroxyapatite scaffolds or onto fibronectin-coated glass slides. The use of hydrogel stamps provided a "wet", biocompatible surface and maintained the viability of cells adsorbed on stamps during the patterning process. Stamps inked once with suspensions of cells allowed the repeated patterning of substrates. Direct stamping of human osteoblasts (and, potentially other mammalian cells) can be used to control the size, spacing, and geometry of patterns of cells printed on porous tissue engineering substrates. This approach may find use in controlling the spatial invasion of scaffolds, promoting the hierarchical organization of cells, and in controlling cell–cell interactions as a step in preservation of phenotypes of cells.

Keywords: Hydroxyapatite; Cell printing; Osteoblast

1. Introduction

Tissue function is influenced by micron-scale spatial organization of cells. Attempts to engineer physiologically functional tissue in vitro frequently rely on the use of artificial scaffolds to promote the generation of neotissue [1,2]. Such scaffolds, whether composed of synthetic or natural polymers, typically possess a porous macro-architecture to allow the ingress of cells, and to promote their subsequent population of the scaffold. The use of printing technologies may offer a strategy for controlling the invasion of cells *into* the scaffold spatially, and for facilitating the generation of organized tissues. The scaffolds most commonly employed are porous, often heterogeneous, and hydrophilic. These characteristics limit the use of established patterning techniques, including soft lithography with poly(dimethylsiloxane) (PDMS) stamps and microfluidics [3–19].

Here, we demonstrate a new methodology for generating patterns of cells with circular shapes and diameters of 200, 700 or 1000 μm, on the surface of hydroxyapatite scaffolds and glass slides, using replica printing [20]. The surface of hydroxyapatite required no prior chemical modification; cells transferred directly from a topographically patterned agarose stamp onto the surface [21]. The use of a hydrogel for the stamp provided a "wet" surface that kept cells hydrated and maintained cell viability throughout the printing process.

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2. Experimental section

2.1. Cell culture

Normal human osteoblasts (NHOst, CC-2538, Cambrex BioScience Walkersville, MD) were cultured in osteoblast basal medium (OBM, CC-3208, Cambrex BioScience Walkersville, MD) until confluent. Cells were treated with trypsin (0.25%) and ethylenediaminetetraacetic acid (EDTA, sodium salt, concentration $2.5\,g\,L^{-1}$) for 2–3 min, subjected to centrifugation at 800 rpm for 5 min, and resuspended in OBM at a concentration of ~2 million cells mL^{-1}. This suspension of cells was used to ink the posts of the stamps directly as described in Section 2.3.

2.2. Preparation of stamps

Stamps were fabricated using a method that has been described in detail by Mayer et al. [21]. Briefly, we prepared agarose stamps by casting hot solutions of 2–3% agarose (OmniPure, EM Sciences, EMD Chemicals Inc., Gibbstown, NJ; the gel strength of a 1.5% gel is $>3200\,g\,cm^{-2}$) against PDMS (Dow Corning Sylgard 184, Corning, NY) molds to a height of ~3 cm. Once the agarose solution had gelled at room temperature and ambient pressure (~2 h), the agarose stamp was peeled away from the PDMS master, and used to pattern cells.

Molds were fabricated as outlined in Fig. 1: (i) a PDMS replica was prepared by casting a PDMS prepolymer against a 1536-well polystyrene plate (wells had a diameter of 1 mm, a depth of 1 mm, and a pitch of 2.5 mm) (Corning Incorporated, Corning, NY); (ii) the polymer was cured at ~60 °C, rendered hydrophilic with an air plasma, and silanized with a vapor of (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Bristol, PA) under vacuum (~60 mm Hg); and (iii) the prepolymer was cast against the PDMS replica and cured thermally to produce a PDMS mold (Fig. 1). For feature sizes smaller than 1 mm, we used photolithography to prepare masters containing arrays of microfabricated posts in bas-relief in SU8 photoresist (MicroChem Corp., Newton, MA) on silicon wafers (Silicon Sense Inc., Nashua, NH), with the following dimensions: (i) 200 μm diameter posts, 130 μm height, 400 μm pitch; and (ii) 700 μm diameter, 500 μm height, 900 μm pitch. The masters were silanized using a vapor of (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane. Replicas of these masters were fabricated in PDMS and served as molds for preparing patterned agarose stamps.

2.3. Patterning hydroxyapatite with osteoblasts

Hydroxyapatite scaffolds (dimensions 1 cm × 1 cm, thickness 2 mm) (ProOsteon™ 200, Interpore Cross International, Irvine, CA) were imaged using an optical microscope (Leica MZ12) equipped with a digital camera (Nikon DXM1200) and by scanning electron microscopy (FEI Quanta 200 ESEM) at 5 kV and 1 Torr (see Fig. 2). We did not coat the hydroxyapatite substrate with metal before imaging with SEM. The inking and stamping procedure described below was performed in a laminar flow hood. We patterned cells on sterile

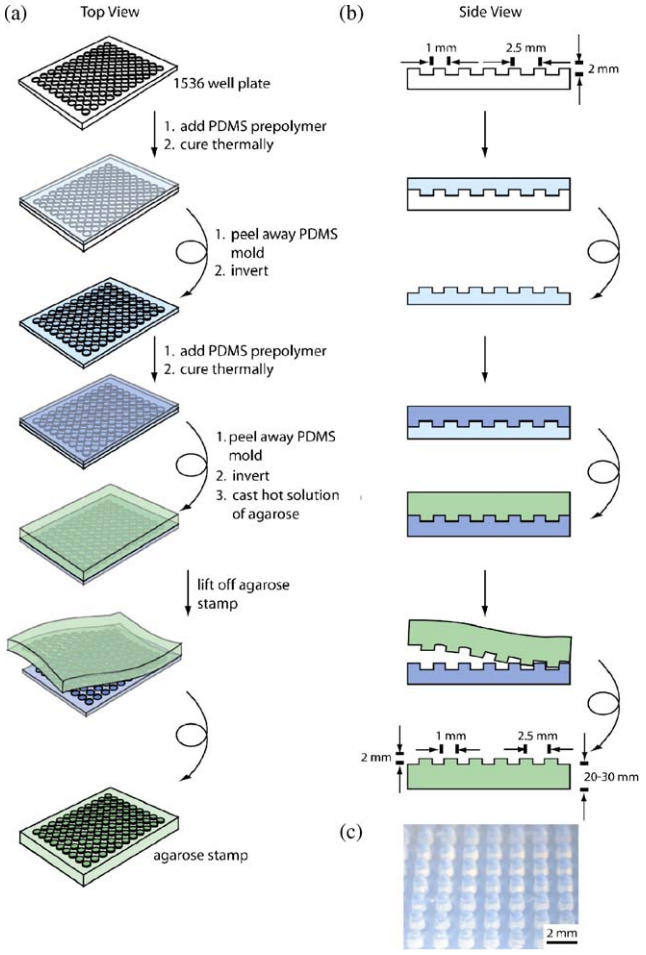

Fig. 1. Schematic depicting the fabrication of micropatterned agarose stamps from a 1536-well plate: top view (a) and side view (b). Image of an agarose stamp (c).

hydroxyapatite scaffolds (used as received) that had been presoaked in OBM or were untreated. Agarose stamps with 700 or 1000 μm diameter posts were "inked" by pipetting ~0.2 μL of cell suspension onto individual posts using a micropipette (repeated 2–5 times). This technique required only minute volumes of suspensions of cells (~1 μL per post, a value corresponding to ~2000 cells). For stamps with 200 μm diameter features, we applied a suspension of cells (100 μL of a suspension containing 2 million cells mL^{-1}) to the entire face of the agarose stamp textured with the posts. The agarose gel absorbed excess liquid within 10 min, leaving cells deposited on the surface of the posts. The stamp thus "inked" was used to pattern cells on hydroxyapatite scaffolds directly. We brought freshly inked stamps into contact with the surface of the hydroxyapatite scaffolds for 15 min, and then peeled away the stamp. After stamping, substrates were immediately immersed in media (OBM, 37 °C) and transferred to an incubator (Model 3110, Forma Scientific, 5% CO_2 at 37 °C).

2.4. Analysis of surface patterns

To ensure that cell attachment had occurred, we imaged scaffolds 24 h after patterning with an inverted microscope

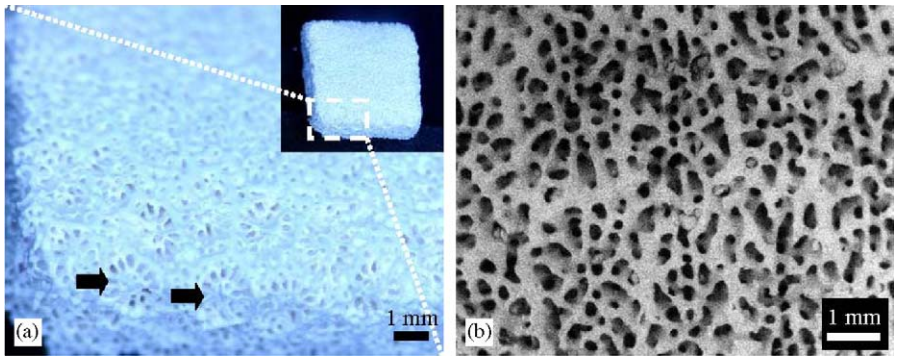

Fig. 2. (a) Optical microscopy images (whole scaffold and detail) and (b) scanning electron micrograph of the hydroxyapatite scaffolds. The arrows in image (a) indicate rosette-shaped arrangements of interconnected pores ~200 μm in diameter.

equipped for epifluorescence microscopy. The scaffolds were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 8 min. Two further PBS washes were performed, and the cells were permeabilized with a solution of Triton X-100 in PBS (0.2%) (Invitrogen, Carlsbad, CA) for 30 min. Cells were stained for actin and DNA using Alexa Fluor® 488 phalloidin and Hoescht 33342 (Molecular Probes, Eugene, OR), respectively, for 20 min using the procedure provided by the vendor. Cells were washed with PBS three times and then imaged using a Zeiss Axiovert 200 inverted microscope equipped with a light source for fluorescence microscopy (HBO 103 mercury vapor short-arc lamp, 100W). For fluorescence microscopy of Hoescht 33342, we used a filter cube with an excitation filter at 360–400 nm and an emission filter at 460–500 nm (Chroma set 31000); for Alexa Fluor® 488 phalloidin we used a filter cube with an excitation filter at 450–490 nm and an emission filter at 515–565 nm (Zeiss Schott set 48-80-10).

3. Results and discussion

The ability to control patterns of cells on surfaces has important applications for biosensor technology [22,23], fundamental studies of cell biology [3,24,25], and tissue engineering. In tissue engineering, the goal of a hierarchical organization of cells to promote the in vitro development of functional tissue may benefit from the spatially controlled placement of cells in specific locations on a substrate.

Soft lithography—a method that uses stamps or channels fabricated in an elastomeric material to transfer patterns of molecules onto surfaces—has been widely used to pattern mammalian cells [3,6,26,27]. Recently, soft lithography has been extended by the use of hydrogel stamps [21,28–30,36,37]. Mayer et al. and Weibel et al. fabricated topographically patterned agarose stamps and used them to print proteins [21] and bacteria [7]. The present work demonstrates that patterned agarose gels can serve as biocompatible, water-based stamps for patterning osteoblasts on porous tissue engineering scaffolds.

We patterned agarose stamps using a procedure illustrated in Fig. 1. Micropatterned agarose stamps were easy to fabricate and mechanically robust. Stamps containing features 700 or 1000 μm in diameter were inked manually by applying suspensions of cells with a micropipette (in the case of 200 μm posts, we applied a suspension of cells to the entire face of the agarose stamp containing the posts). The hydrogel absorbed excess liquid within 10 min, leaving cells absorbed on the surface of the posts; the "wet" hydrogel surface kept cells hydrated.

Hydroxyapatite scaffolds have been widely used in bone tissue engineering [31–33]. In this work, we patterned "ProOsteon^{TM} 200" hydroxyapatite scaffolds with human osteoblasts. As is the case with most scaffolds used for in vitro tissue engineering, the hydroxyapatite scaffold has a macroporous structure, with pores ~200 μm in diameter (see Fig. 2). Fig. 3 illustrates spots of osteoblasts patterned on the surface of hydroxyapatite. These patterns were printed with an agarose stamp having 1 mm diameter posts and a pitch of 2.5 mm. The technique transferred material to the surface of the substrate in parallel, making it possible to pattern multiple spots of cells simultaneously. Figs. 3(a)–(c) display three spots of cells patterned at the same time on the same scaffold, and illustrate the reproducibility of the pattern transfer. We confirmed the viability of cells patterned using this technique by imaging the adhesion of cells and spreading the actin cytoskeleton on the surface (Fig. 3(d)).

The conditions used to ink stamps were important in achieving a good spatial resolution of the patterns of cells. Fig. 4 compares sub-optimal printing conditions (Figs. 4(b) and (c)) with an "optimized" method of printing (Fig. 4(a)). In the optimized method, we avoided leaving cells on the posts longer than required for the stamp to absorb *most* of the excess liquid to maximize survival of cells during the inking procedure. Stamping from posts with cells covered in a thin film of residual liquid yielded spots of viable cells, and good replication of the feature size of the posts

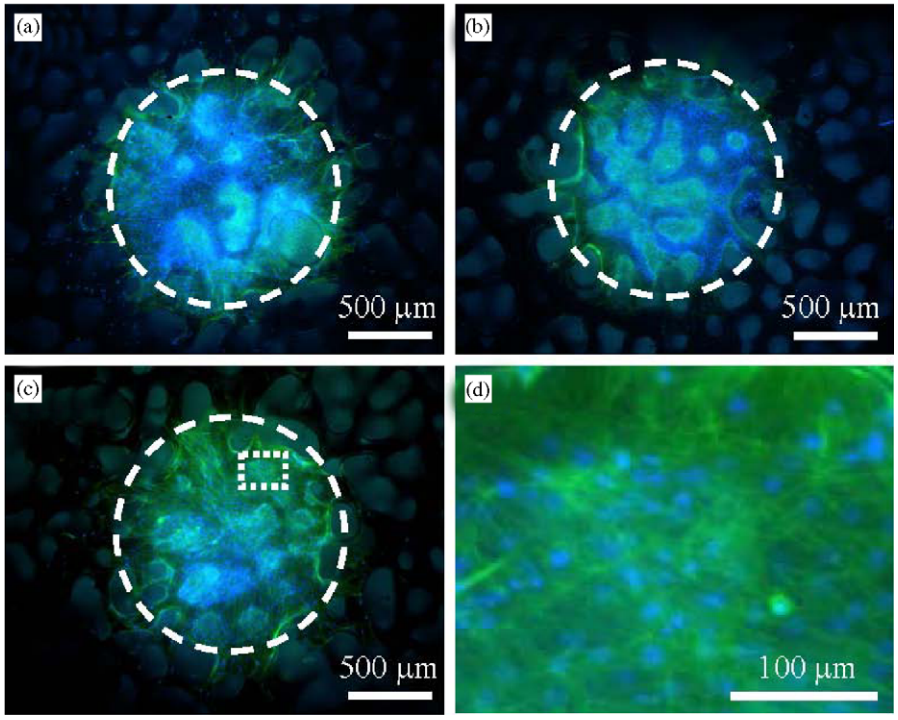

Fig. 3. Different regions of a hydroxyapatite scaffold patterned with osteoblasts using a single agarose stamp with 1000 μm diameter circular features. Images (a)–(c) show an area on the top surface of the same scaffold that was patterned during the same stamping event. Dashed white lines indicate areas patterned with cells. (d) Higher magnification of the area within the white box in (c). Actin was stained bright green with phalloidin and DNA stained bright blue with Hoescht 33342. The dark blue/grey features in the unpatterned background of the images in (a), (b), and (c) are artifacts of fluorescence microscopy, resulting from light reflected from the white hydroxyapatite scaffolds. Images were acquired 24 h after patterning.

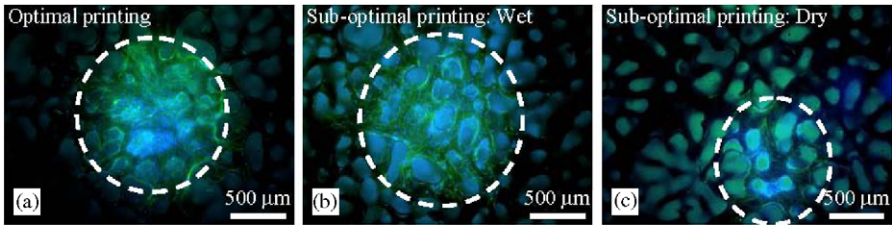

Fig. 4. Comparison of the pattern of cells generated using: (a) a scaffold pre-wetted with medium (OBM); (b) stamping immediately after inking with excess liquid on the posts; and (c) a dry scaffold. Actin is stained green with phalloidin and DNA stained blue with Hoescht 33342. The white dashed circles denote the area patterned with cells. The grey/green/dark blue features in the unpatterned background of the images in (a), (b), and (c) are artifacts of fluorescence microscopy, resulting from light reflected from the hydroxyapatite scaffolds (see Supplementary Fig. 1 for an image of an unpatterned scaffold). Image (c) shows the rosette-shaped pattern of the porous hydroxyapatite scaffold as shown in Fig. 2 (a). Images were acquired 24 h after patterning.

(the diameter of the spots was consistently 20–30% bigger than the diameter of the posts that were used for stamping), as attributed to small amounts of residual liquid and cell spreading over the 24 h period (Fig. 3 and Fig. 4(a)). When the liquid applied to the stamp during inking was not given adequate time to absorb (<1 min) prior to printing, the resolution of the resulting pattern was low (cells spread over an area ~30–50% larger than the dimensions of the posts), and the number of cells transferred was reduced (Fig. 4(b)).

Characteristics of the scaffold were also important for the stamping procedure. When the hydroxyapatite scaffold was not "pre-soaked" with cell culture media prior to printing cells, the liquid from the hydrogel rapidly transferred to the substrate, and cell ingress occurred within the scaffold (Fig. 4(c)). Although not intended in this work, the ingress of cells might be desirable for certain applications.

Stamps containing 700 μm diameter posts also generated patterns of cells with a reproducibly good spatial resolution (Fig. 5(a)). The 200 μm diameter posts on

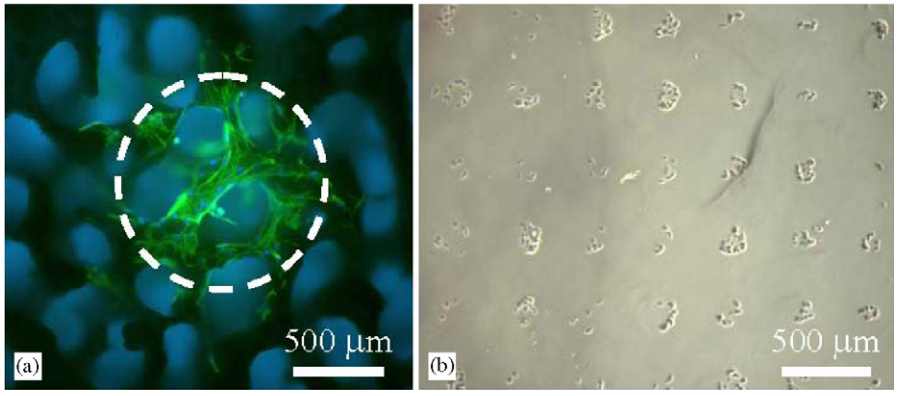

Fig. 5. (a) Scaffold patterned with osteoblasts using an agarose stamp with 700 μm diameter circular features. Actin is stained green with phalloidin and DNA stained blue with Hoescht 33342. The white dashed circle denotes the area patterned with cells. The blue features in the background are artifacts of fluorescence microscopy, resulting from light reflected from the white hydroxyapatite scaffolds. (b) Fibronectin-coated glass slide patterned with cells using an agarose stamp with 200 μm features.

agarose stamps could not be inked individually without the use of robotics; instead we spread a suspension of cells over the posts. Within 10 min, most of the excess liquid had been absorbed by the stamps. When these stamps were used to pattern hydroxyapatite surfaces, we were unable to discern patterns of cells on the scaffolds after three days of incubation. We attribute this result to the transfer of cells from 200 μm diameter posts to the *pores* in the scaffold; the pores are ~200 μm in diameter (see Fig. 2). In contrast, when we patterned flat, fibronectin-coated glass slides with agarose stamps having 200 μm diameter posts, we observed patterns of cells on the slides indicating that cells were transferred. We infer that the difference in the results for the two substrates is due to the absence of pores in the glass substrate, and the affinity of the cells to the exposed RGD groups of fibronectin (Fig. 5(b)).

Once inked with cells, an agarose stamp with 700 or 1000 μm diameter posts could be used to make patterns without re-inking. This observation suggests that the transfer of cells from the stamp to the substrate is incomplete: it may be possible to culture residual cells directly on the posts of the hydrogel stamp, thereby creating a "living" stamp that regenerates its own "ink". This concept has been demonstrated recently with bacteria [7].

An important consideration when employing stamping procedures is the possible transfer of the stamp material to the substrate. Stamping experiments with PDMS or hydrogel stamps demonstrate that typically small amounts of the PDMS or hydrogel transfer from the stamp to the substrate [29,30,34]. In this study, we were able to perform multiple stamping events with the same agarose stamp without losing the fidelity of the patterns of cells, suggesting little transfer of agarose to the substrate. Although we did not attempt to quantify the amount of agarose transferred, it was apparent that the transfer of a biocompatible material such as agarose did not compromise the quality of the patterns or the growth of the cells on the scaffolds.

The method we have described is simple and inexpensive; it may provide a generic technology for patterning many cell types or combinations of cell types on the surface of porous substrates used for tissue engineering. This technique has the primary advantage that it produces patterns of osteoblasts on hydroxyapatite with control over the geometry, size, and spacing between patterns of cells. A disadvantage is that the timing of the inking and stamping procedure and ambient humidity must be tightly controlled to maximize the survival of the cells.

We believe that the technique presented here might be applicable to mammalian cells other than osteoblasts and porous tissue engineering scaffolds other than hydroxyapatite. Stamping mammalian cells directly onto tissue engineering scaffolds may find use in controlling the spatial invasion of scaffolds, promoting the hierarchical organization of cells, and in controlling cell–cell interactions as an essential step in preservation of phenotypes of cells [35].

Acknowledgments

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Appendix A. Supplementary data

The online version of this article contains additional supplementary data. Please visit doi:10.1016/j.biomaterials.2005.05.001.

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