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Rapid isothermal substrate microfabrication of a biocompatible thermoplastic elastomer for cellular contact guidance

Maxime D. Guillemette [a,b,c,1], Emmanuel Roy [a,*], François A. Auger [b,c], Teodor Veres [a]

[a] Industrial Materials Institute, National Research Council, Boucherville, QC, Canada J4B 6Y4
[b] Department of Surgery, Faculty of Medicine, Laval University, Quebec City, QC, Canada G1V 0A6
[c] LOEX, Centre de Recherche FRSQ, CHA Universitaire de Quebec, Quebec City, QC, Canada G1S 4L8

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ABSTRACT

The use of microstructured substrates to study and influence cell orientation, which plays an important role in tissue functionality, has been of great interest lately. Silicon and poly(dimethylsiloxane) substrates have typically been used, but long processing times and exogenous protein surface coating, required to enhance cell viability, limit their use as large-scale platforms. There is thus a need for a non-biodegradable biocompatible substrate that allows rapid and low cost microfabrication. In this paper a styrene–(ethylene/butylene)–styrene block co-polymer (SEBS) microstructured by a rapid replication technique using low pressure an isothermal hot embossing approach has been demonstrated. SEBS substrates were treated with oxygen plasma to enhance cell adhesion and sterilized using ethylene oxide gas. While cell adhesion to and proliferation on these substrates was as good as on tissue culture polystyrene, cellular alignment on microstructured SEBS was also very high (97.7 ± 0.5%) when calculated within a 10° angle variation from the longitudinal axis. Furthermore, tissue sheets on microstructured SEBS have been produced and exhibited cellular alignment within the engineered tissue. In addition, these results were obtained without coating the material with exogenous proteins. Such substrates should be helpful in the culture of tissue engineered substitutes with an intrinsic orientation and to elucidate questions in cell biology.

1. Introduction

The production of living tissue engineered substitutes [1] requires adequate cell proliferation. Until now the most widely used cell culture surface has been flat polystyrene (PS). In order to mimic the organization of physiological tissue in tissue engineering cells have to grow in an oriented manner. This can be achieved via contact guidance, a principle by which cells align following physical cues, such as the silk fibers of spider webs [2] or surface topography, as used more commonly nowadays [3–5]. Tissue functionality is intimately related to tissue orientation, with such spatial three-dimensional organization giving the cornea its strength and transparence [6–8], tendons their mechanical properties [9,10] and smooth muscle cell tissues from different organs their elasticity and compliance [11,12]. For many tissue engineering and cell culture applications contact guidance provides an important stimulus to the cells and consequently dictates their physiological orientation [13–15].

The use of poly(dimethylsiloxane) (PDMS) in microfabrication has frequently been explored [16,17], as its optical, mechanical and biocompatible properties allow imaging, stretching and, to some extent, cell culture. Unfortunately, PDMS has a long processing time and limited biocompatibility. In order to promote cell adhesion for tissue engineering purposes it is necessary to use exogenous protein, like collagen or fibronectin, which then create regulatory obstacles for clinical applications [18]. Hard thermoplastic polymers like polystyrene (PS) [19] and poly(methyl methacrylate) (PMMA) [20] have also been used as contact guidance substrates. Usually produced by hot embossing using silicon master molds fabricated by standard lithographic techniques and reactive ion etching (RIE), hard thermoplastic substrates are less time consuming to produce compared with PDMS elastomer substrates. However, thermoforming of such PS and PMMA materials, among others, requires solving some specific issues related to mold fabrication and demolding due to the overall thermal and mechanical constraint upon the molding process, thus major attention is needed to establish the stability and integrity of the master mold over embossing runs [21,22]. The use of metallic molds for hard plastics can be considered, but their production is cost-effective

* Corresponding author. Tel.: +1 450 641 5394; fax: +1 450 641 5105.
E-mail addresses: emmanuel.roy@imi.cnrc-nrc.gc.ca, Emmanuel.roy@cnrc-nrc.gc.ca (E. Roy).
[1] Present address: Harvard–MIT Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

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only for production runs and requires fabrication processes and equipments that are not commonly found in laboratory set-ups, especially in tissue engineering and biology laboratories. Thus there is a need for new materials and fabrication techniques that could provide rapid and low cost microstructuration, while providing biocompatibility and long-term viability. In this study the possibility of using a robust and high throughput thermoforming method in order to microstructure a low cost thermoplastic elastomer (TPE) has been demonstrated. By using a low pressure (1.6 bars) and rapid isothermal (3 min) hot embossing approach a large number of styrene–(ethylene/butylene)–styrene (SEBS) substrates have been fabricated. Using a low cost and highly stable SU-8 master mold prepared by standard lithography we have replicated more than 50 substrates of 6 inch^2 area having gratings of 8 μm period and 1.5 μm depth.

Prior to use the SEBS substrates were treated with oxygen plasma and sterilized with ethylene oxide gas. By using SEBS substrates without any exogenous protein coating we were able to grow smooth muscle cells (SMC) and show cell proliferation comparable with commercially available tissue culture PS. Contact guidance, providing cellular orientation, was evaluated and more than 95% of cells were within a 10° angle shift from the longitudinal axis of the substrate grating. Tissue sheets were also produced using SMC cultured on microstructured SEBS substrates and cellular alignment persisted throughout the experiment.

2. Materials and methods

2.1. Substrate fabrication

A Versaflex TPE SEBS block co-polymer was purchased from GLS Corp. (McHenry, IL). Versaflex was used in the form of sheets (3.0 mm thickness, 160 mm width and >10 m length) prepared by extrusion using a Killion KL100 single screw extruder (Killion Laboratories Inc., Houston, TX).

The master mold for TPE thermoforming was fabricated by standard SU-8 photolithography. SU-8 GM1040 photo-resist (Gersteltec, Pully, Switzerland) was spin-coated on a 6 inch silicon wafer (Silicon Quest International Inc., Santa Clara, CA) to achieve a layer thickness of 1.5 μm, followed by pre-bake steps at 65 and 95 °C for 5 and 15 min, respectively. The resist layer was exposed to UV light (Hg i-line) through a laser-written high definition sodalime photomask (HTA Photomask, San José, CA) using an EVG 6200 mask aligner (EV Group, Schärding, Austria). For the post-exposure bake the same conditions as for the pre-bake were used. The resist was developed in propylene glycol monomethyl ether acetate (Sigma–Aldrich Corp., St. Louis, MO) and then hard-baked at 160 °C overnight. The master was finally treated with trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Sigma–Aldrich) using vapor phase deposition to generate an anti-adhesive layer on its surface.

Hot embossing was carried out using an EVG 520 HE system (EV Group). Embossing experiments were carried out by an isothermal embossing molding approach (Fig. 1). The TPE sheet was first placed on the microstructured Si/SU-8 mold during which an almost perfect contact is achieved due to the viscoelastic properties of the polymer. Additionally, a Si counter wafer treated with an anti-adhesive layer was positioned on top of the TPE foil. The stack was then introduced into the pre-heated chamber of the system (170 °C) and a pumping step was applied in order to reach a primary vacuum of 1 mbar. In order to ensure a compelled filling of micrometric cavities a pressure of 1.6 bars was then applied for 3 min. Once the force was released the stack was removed from the system for room temperature cooling. De-embossing was performed manually by peeling off the polymer layer from the mold, as with the PDMS material for soft lithography.

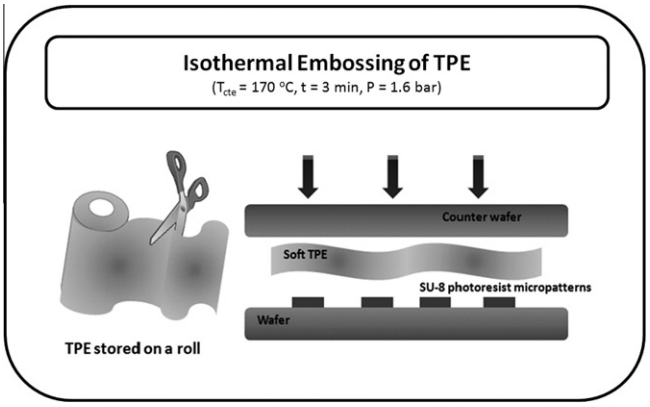

Fig. 1. Schematic illustration of the rapid isothermal microfabrication of the TPE substrate. The TPE material was extruded on a roll for storage and used on demand. A piece of TPE was diced from the roll and placed on a Si/SU-8 master mold for micrograting isothermal molding. Once a primary vacuum of 1 mbar was reached in the pre-heated chamber a pressure of 1.6 bar was applied to the counter wafer plate for 3 min, and then the stack was removed from the embossing chamber for rapid external cooling.

2.2. Surface treatment and characterization

All substrates were treated using plasma oxidation (Plasmalab80, Oxford Instruments, Bristol, UK) for 4 min at a power of 70 W and a pressure of 50 mTorr. Contact angles were measured with a goniometer from Kerenco Instruments Inc. (El Paso, TX) using deionized water as the probe liquid. Roughness and contrast phase imaging of TPE microstructured surfaces were carried out using a multi-mode Nanoscope IV atomic force microscope (Veeco Metrology Group, Santa Barbara, CA), operated under ambient conditions in both contact and tapping mode using silicon nitride high aspect ratio cantilevers (AR-10-NCHR-10, Veeco) with a spring constant of 42 N m^{-1}. Scanning electron microscopy (SEM) images were taken using an S-4800 scanning electron microscope (Hitachi, Mississauga, ON) operated at an acceleration voltage of 1.0–1.5 keV.

2.3. Cell and tissue culture

The study was approved by the Saint-Sacrement Hospital Ethics Committee and all tissues were obtained after informed consent was given. SMC isolation was carried out as described previously [23]. Briefly, umbilical cords were obtained from healthy newborns and then veins were cannulated and washed with thermolysin solution (Sigma, Oakville, Canada) for endothelial cell separation. Collagenase H solution (Roche Diagnostics, Laval, Canada) was then perfused for 30 min at 37 °C. Cells were centrifuged and resuspended in a 3:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 modified medium (Invitrogen, Burlington, Canada) supplemented with 10% fetal calf serum (HyClone, Logan, UT), 100 U ml^{-1} penicillin (Sigma), and 25 μg ml^{-1} gentamicin (Schering, Pointe-Claire, Canada). SMC identity was confirmed by specific α-SM-actin immunostaining. For proliferation and orientation assays SMC were seeded on regular 60 mm tissue culture polystyrene Petri dishes (BD Falcon, Mississauga, Canada) or on SEBS (flat and microgrooved) at a concentration of 12,000 cells cm^{-2}. SMC sheets were obtained following 13 days culture in the presence of 50 μg ml^{-1} ascorbate (Sigma). To remove the tissue sheet from the substrate we peeled the sheets apart using tweezers. The use of enzymes is not necessary, since the cohesive forces created in the tissue sheet, bonding cells and extracellular matrix are together greater than the forces bonding the cells to the substrate

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