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Replica moulded poly(dimethylsiloxane) microwell arrays induce localized endothelial cell immobilization for coculture with pancreatic islets

Anouck L. S. Burzava,1,a) Aurelien Forget,2,3,a) Frances J. Harding,1,4,5 Michaelia P. Cockshell,6 Daniella Penko,7 Camille Rouzaud,1 Vincent Ahmad1, Paula F. Marina,1,2 Darling Rojas-Canales,7 Claudine S. Bonder,6,8 P. Toby H. Coates,7 Michaela Waibel,9 Helen E. Thomas,9 Thomas W. Kay,8 Thomas Loudovaris,9 Anton Blencowe,1,2,b) and Nicolas H. Voelcker5,10,11,12,c) 

1Future Industries Institute, University of South Australia, Mawson Lakes, South Australia 5095, Australia  
2School of Pharmacy and Medical Science, University of South Australia, Adelaide, South Australia 5000, Australia  
3Institute for Macromolecular Chemistry, University of Freiburg, 79104 Freiburg, Germany  
4Cell Therapies Pty Ltd, Peter MacCallum Cancer Centre, Melbourne, Victoria 3000, Australia  
5Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia  
6Centre for Cancer Biology, University of South Australia and SA Pathology, Adelaide, South Australia 5000, Australia  
7Royal Adelaide Hospital, Centre for Clinical and Experimental Transplantation, Frome Road, Adelaide, South Australia 5000, Australia  
8Adelaide Medical School, Faculty of Health Sciences, University of Adelaide, Adelaide 5000, Australia  
9St Vincent Institute, 9 Princes Street, Fitzroy, Victoria 3065, Australia and The University of Melbourne, Department of Medicine, St. Vincent’s Hospital, 44 Victoria Parade, Fitzroy, Victoria, 3065, Australia  
10Melbourne Centre for Nanofabrication, Victorian Node of the Australian National Fabrication Facility, Clayton, Victoria 3168, Australia  
11Commonwealth Scientific and Industrial Research Organization (CSIRO), Clayton, Victoria 3168, Australia  
12Monash Institute of Medical Engineering, Monash University, Clayton, Victoria 3800, Australia  

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PolyJet three-dimensional (3D) printing allows for the rapid manufacturing of 3D moulds for the fabrication of cross-linked poly(dimethylsiloxane) microwell arrays (PMAs). As this 3D printing technique has a resolution on the micrometer scale, the moulds exhibit a distinct surface roughness. In this study, the authors demonstrate by optical profilometry that the topography of the 3D printed moulds can be transferred to the PMAs and that this roughness induced cell adhesive properties to the material. In particular, the topography facilitated immobilization of endothelial cells on the internal walls of the microwells. The authors also demonstrate that upon immobilization of endothelial cells to the microwells, a second population of cells, namely, pancreatic islets could be introduced, thus producing a 3D coculture platform. Published by the AVS.

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I. INTRODUCTION

Spherical cellular aggregates are of great interest for applications in tissue regeneration12 and drug testing.3 Conventional spheroid culture formats, including hanging drop,5 rotating vessel,5 or microwell platforms,5 force the cell populations to aggregate into spheroids composed of one or several cell types.7 In microwell systems, the cell aggregation is typically driven by the antifouling or nonadhesive properties of the microwell material.8 Several materials have been utilized for the fabrication of microwell arrays including collagen,9 poly(ethylene glycol),10 electrospun poly(ethylene oxide terephthalate)-poly(butylene terephthalate),11 and poly(dimethylsiloxane) (PDMS).12 Once associated into spheroids, the cell aggregates have been employed for drug testing,13 tumor models,14 and cartilage tissue regeneration.15 To enhance the range of applications of cell spheroids, and to understand their interactions and behavior in contact with other cell populations, experiments have been conducted with spheroids cultured in direct contact with cell monolayers,16 or in the same vessel separated via transwell inserts.17 However, these systems do not maintain the spherical geometry of the spheroids as they expose the spheres to a planar geometry. Therefore, a technique that would enable the manufacture of coculture systems with nonplanar geometry would have the potential to maintain the shape of spheroids or cell aggregates. As an example, a particular cell population immobilized in curved microwells could allow for coculture systems whereby the immobilized cell population can interact with cell aggregates under favorable conditions for maintaining their spherical geometry.

For the fabrication of microwell arrays, replica moulding has been widely used to pattern surfaces. Silicone derivatives have been established as exceptional materials for the replica mould manufacturing technique as they are liquids that can follow finely detailed moulds and later cross-linked to form transparent and flexible solid replicas that can easily be
removed from the moulds. In particular, PDMS with its good gas permeation, low cytotoxicity, has been intensively utilized for microfluidic applications and customized cell culture vessels. The fabrication of PDMS microfluidic devices or microwells has been performed with different manufacturing techniques including lithography, frozen droplets, and micromachining. However, all of these techniques produce smooth PDMS surfaces that are generally nonadherent for cells. While this property is highly valuable for some microfluidic applications, for other applications that require immobilization of cells, further chemical treatment is needed. One approach is to chemically functionalize the PDMS surface with cell adhesive peptide sequences. Alternatively, the surface topography can be tuned via this approach exhibit a surface topography that is particular functionalization for cell immobilization. The surface topography was directly measured using optical profilometry and compared to the replica moulded PDMS microwell arrays (PMAs), which were also characterized via Fourier-transform infrared (FTIR) spectroscopy and contact angle measurements.

II. EXPERIMENT

A. Fabrication of PDMS microwells

The design of the microwell devices was created in Solidworks (Dassault Systems, France), a computer-aided design (CAD) software, and then exported as an STEP file for 3D printing of the casting moulds. The moulds were printed from temperature resistant RGD525 plastic using a PolyJet printer (Objective 3D, Australia). Before use, the moulds were washed thoroughly with deionized water and dried. For PDMS replica moulding, Sylgard 184 (Corning, USA) was prepared according to the supplier’s specifications and degassed under vacuum (500 mbar) until a clear solution was obtained. The Sylgard solution was then poured into the moulds and cured at 60 °C for 16 h. The cured replica PMAs were removed from the mould, sonicated in acetone (100 ml) for 20 min followed by ethanol (100 ml) for 20 min, and then dried for 1 h at 100 °C.

B. Fourier-transform infrared spectrophotometry

FTIR spectroscopy was performed using a Hyperion 1000 FTIR Microscope from Bruker (Ettlingen, Germany). An attenuated total reflectance (ATR) accessory with a germanium crystal and a liquid nitrogen-cooled mercury cadmium telluride detector was used to record the spectra. All spectra were collected as an average of 64 scans, with a resolution of 4 cm⁻¹, and over the range of 650–4000 cm⁻¹. A background of air was subtracted from the raw spectra. Spectra were analyzed using the Opus software from Bruker (Ettlingen, Germany).

C. Contact angle measurements

A custom-built sessile drop apparatus with an Olympus SZ-PT microscope and lens system mated to a Sony CCD camera was employed to measure the wettability of the surfaces. A 10 μl syringe (Hamilton, Reno, USA) was used to disperse droplets of Milli-Q water (1 μl) on the sample. A minimum of three contact angle measurements were taken from each surface. Angle analysis of captured droplets was performed with ImageJ software v1.50 with the DropSnake plugin.

D. Optical profilometry

PDMS replica surface topography was directly measured on an NT1100 optical profilometer (Veeco, USA). 3D printed moulds were coated with a 21 nm gold layer to allow a good reflection of the sample surface for its characterization by means of an optical profilometer. For all samples, the topography was measured in vertical scanning interferometry mode with a 40x magnification.

E. Atomic force microscopy

Atomic force microscopy (AFM) images were collected utilizing PeakForce Tapping in air on a MultiMode 8 AFM (Bruker, US) equipped with a Nanoscope V controller (Bruker, US) and a scanner E. The AFM scans were collected with a ScanAsyst AIR probe (Bruker, US) of a nominal spring constant of 0.2 N m⁻¹ and a nominal resonant frequency of 70 kHz. A 0.99 Hz or lower scan rate was employed as the PDMS surfaces were slightly sticky. The WSXM 5.0 DEVELOP 9.0 Edition software was used to process (to remove scanner tilt/bow) and analyze (to perform the cross-sectional analysis) the AFM scans.

F. Scanning electron microscopy

Samples were sputter coated with a gold layer using a Polaron SC7640 sputter coater operating at 3 kV and 20 mA,
at a pressure of 4 × 10^{-2} mbar for 120 s. The samples were imaged using an INCA-X Act (Oxford Instrument) environmental scanning electron microscope.

G. Late blood OEC cell culture

Human studies were given ethical clearance from the Royal Adelaide Hospital Human Research Ethics Committee (HREC), Adelaide, SA, Australia with informed written consent obtained in accordance with the “Declaration of Helsinki.” Human peripheral blood samples were taken in Lithium Heparin blood collection tubes from healthy subjects. Red blood cells were lysed, and bloods were processed with Lymphoprep™ (Stemcell Technologies, VIC, Australia) to isolate the mononuclear cells. Primary human outgrowth endothelial cells (OECs) were isolated based on an established protocol. These were cultured on 48-well plates prepared with Collagen (Sigma) coating in endothelial selective media, EGM-2 (Lonza) with 20% fetal calf serum (FCS) (Hyclone). After 4 weeks, colonies of OECs appear and were expanded. Characterization of OECs was via flow cytometry using a previously published panel including CD133, CD117, CD34, CD31, CD144, VEGFR2, CD146, CD90, CD45, CD14, and CD38. Following addition of the OECs to the PMAs, cell media was changed every 2 days to provide fresh nutrients.

H. Murine endothelial progenitor cell isolation and cell culture

All animal protocols were approved by the University of Adelaide Animal Ethics Committees and undertaken as per the guidelines established by the “Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.” Murine endothelial progenitor cells (mEPCs) were derived from male C57B6 mice (Laboratory Animal Services, The University of Adelaide, Adelaide, Australia) as previously described. Bone marrow cells were obtained by flushing femurs and tibiae of 6- to 12-week-old mice using M199 medium (Sigma). Cells were cultured on fibronectin (50 μg/ml; Roche, Switzerland) in 20% FCS (Gibco/Invitrogen). The medium was supplemented with endothelial cell growth (BD Biosciences), a tissue extract containing multiple growth factors including fibroblast growth factor, vascular endothelial growth factor, endothelial cell growth factors α and β, and heparin (all at 15 μg/ml). Cells were harvested with 0.1% trypsin-ethylenediaminetetraacetic acid (Sigma Aldrich) for ~3 min at 37 °C within 7 days of initial seeding. EPC-conditioned media was obtained from 90% confluent, day 7 mEPC cultures, centrifuged (800 g, 5 min), sterile-filtered (0.4 μm), and used immediately.

I. Human islet isolation

Human pancreases were obtained with informed consent from next of kin, from heart-beating, brain-dead donors, with research approval from the HREC at St Vincent’s Hospital, Melbourne. Human islets were purified by intraductal perfusion and digestion of the pancreases with collagenase followed by purification using Ficoll density gradients. Purified islets were cultured in Connaught Medical Research Laboratories (CMRL) 1066 medium (Invitrogen) supplemented with 10% human serum albumin, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (complete CMRL), in a 37 °C, 5% CO₂ humidified incubator.

J. Coculture of endothelial cells with human islets and cell staining

PMAs were sterilized in a 0.4 mg/ml penicillin/streptomycin solution (Thermo Fisher Scientific) and left in a 37 °C, 5% CO₂ humidified incubator the day before seeding the endothelial cells. The cytoskeleton of OECs and MEPCs was stained using CellTracker™ Red CMTPX (Life Technologies). Lyophilized dye was dissolved in DMSO to afford a 10 mM solution that was diluted in serum-free media to a final concentration of 20 μM. Cell culture media was first removed, and cells were incubated for 30 min with the CellTracker solution. Cells were then rinsed five times with phosphate-buffered saline (PBS), before being trypsinized, resuspended in fresh complete cell media and seeded at a density of 10⁵ cells/ml onto the PMAs. The cell nucleus was stained immediately before imaging using Hoechst 33342 dye. Cells were stained with a 2 μg/ml Hoechst solution at 37 °C for 30 min. Human islets were first washed with PBS, then stained with 1 mM Newport Green™ DCF diacetate (Thermo Fisher Scientific) for 1 h in a 37 °C, 5% CO₂ humidified incubator, and washed with PBS. The stained human islets were then deposited on top of the OEC coated PMA and incubated for 4 days.

K. Fluorescence microscopy

Cells were imaged on an Eclipse TiS inverted fluorescence microscope from Nikon (Tokyo, Japan). Confocal images were obtained from a Nikon AIR resonant scanning multispectral confocal microscope (Nikon Eclipse Ti, Tokyo, Japan).

III. RESULTS AND DISCUSSION

Important design criterion for the utilization of PDMS moulded cell culture vessels is their compatibility with commonly used labware consumables and laboratory instruments such as fluorescence microscopes. Therefore, the 3D printed moulds were designed specifically to provide replica PMA inserts that fit securely into 12-well culture plates. This enabled manipulation of the PMAs in sterile containers and for a series of arrays to be used under the same culture conditions. To assure a continuous waterproof junction between the PMAs and the well of the well-plate, the array was designed with a wall that confined the cell culture media on top of the array without leaking under the PMA insert. Initially, a CAD was prepared for a mould that would provide replica PMA inserts with an inner diameter of 18 mm, a height of 15 mm, a wall thickness of 2 mm, and an array of 425 microwells in a circular pattern [Fig. 1(a)]. The diameter and depth of the individual microwells were each 500 μm. The CAD was then 3D printed from RGD525 photopolymer, to afford a mould that could sustain the PDMS curing process.
temperature without deformation [Fig. 1(b)]. The PDMS precursor solution was then poured into the mould and allowed to cure at 60 °C for 16 h to afford the PMA inserts [Fig. 1(c)]. The size and quality of the microwell array were assessed via light microscopy [Fig. 1(d)], which revealed that the replica reproduced the features of the mould without any defects.

Moulds produced via PolyJet 3D printing can potentially contain monomers that were not cross-linked during the manufacturing process, which could leach out of the object and be transferred onto the PDMS surface during replica moulding which could impact the physical properties of the PMA surface. Therefore, the surface chemistry of a flat surface of the replica moulded PMAs and of the 3D printed mould was compared to flat PDMS controls cured on tissue culture polystyrene (TCPS) Petri dishes via ATR FTIR. On the PMAs spectra, none of the characteristic peaks observed on the spectra of the 3D mould were observed, and the PMA spectra were identical to the PDMS moulded on tissue culture polystyrene [Fig. 2(a)]. These results suggest that the composition of the PMA is identical to PDMS, indicating that there was no contamination from the mould.

Surface wettability is a major factor in cell immobilization on polymer materials. Therefore, the static water contact angle was measured on the flat surface of the replica moulded PMAs (91.3°) and compared to the flat PDMS control (100.3°) [Fig. 2(b)]. The small difference in contact angle is unlikely to affect protein adsorption on the surface or cell immobilization. Indeed, it has been reported that a contact angle of 20°–40° is optimal for fibroblast cell immobilization, and PDMS treated with oxygen plasma (contact angle = 10°) was only able to marginally improve epithelial colorectal adenocarcinoma (Caco-2) cell immobilization compared to untreated PDMS. Therefore, the observed change in surface wettability is unlikely to contribute toward cell immobilization on the PMAs.

Additive manufacturing techniques such as the one used in this study to prepare the microwell array moulds produce objects that have a distinctive surface topography. This surface topography originates from the addition of successive layers in the z-plane and spatial resolution in the x- and y-planes. To obtain a quantitative analysis of the fine features, we used optical profilometry (Fig. 3), and the topography of the convex hemispherical mould [Fig. 3(c)] was compared to that of the replica moulded microwell forming a concave hemispherical feature [Fig. 3(d)]. For the mould and replica, the surface root mean square roughness ($Rq$) was determined to be of $2.83 \pm 0.95 \mu m$ and $2.81 \pm 0.78 \mu m$, respectively [Fig. 3(f)]. This was measured across four samples, with a $p = 0.0907$ as determined by an unpaired $t$-test, indicating that the surface roughness of the 3D printed mould is transferred to the PDMS replica during the moulding and curing process. In comparison, the surface roughness of the flat PDMS control cast from TCPS Petri dishes [Fig. 3(e)] was $0.04 \pm 0.01 \mu m$, 2 orders of magnitude lower than the inner surface of the PDMS microwells. Thus, the observed surface roughness of the PMAs indeed originates from the 3D printed mould.

The results obtained from optical profilometry were also complimented with microscopy techniques. Many instruments allow for the characterization of surface topography. For example, AFM is often used to analyze surface roughness, although this technique generally requires a planar surface and is better suited to the analysis of nanoscale features. Nevertheless, AFM scans were acquired for a representative area of the hemispherical convex and concave features of the 3D printed mould [Fig. 4(a)] and PMA replica [Fig. 4(b)], respectively. These scans indicated the transfer of nanoscale surface features from the 3D printed mould onto the PMA, and the cross-section analysis revealed a linear periodicity on the mould which is believed to originate from the 3D printing manufacturing process in the z-direction.

![Fig. 1. Fabrication of PDMS microwell arrays (PMAs): (a) Starting from a computer-aided design, (b) microwell array moulds were 3D printed, and then (c) the PMA was obtained by replica moulding. (d) Brightfield microscopy image of a PMA.](image1)

![Fig. 2. (a) ATR FTIR surface characterization of the PDMS microwells compared to the 3D printed mould and (b) sessile-drop water contact angle measurements of the replica PDMS microwell compared to PDMS cured in TCPS Petri dishes.](image2)
[Fig. 4(c)], and this was transferred to the PDMS replica [Fig. 4(d)]. To further confirm the profilometer analysis, scanning electron microscopy (SEM) was used to image the concave hemispheres of the PDMS replica [Fig. 4(e)] and convex hemispheres of the mould [Fig. 4(f)], which revealed concentric circular patterns similar to those observed for the profilometer results.

Late blood OECs are circulating cells that are highly proliferative and have the potential to differentiate into vascular endothelial cells. They are therefore thought to play a significant role in vascular repair.\(^{42,43}\) It has been reported that OECs have the potential to improve the survival and integration of cell transplants such as pancreatic islets.\(^{44-47}\) Furthermore, OECs are thought to play a critical role in supporting the function of pancreatic islets.\(^{48}\) Whilst the formation of heterotypic cell spheroids composed of dissociated islets and OECs have been proposed,\(^{49}\) in vitro coculture systems are likely to play an important role in understanding the interaction between OECs and cell spheroid transplants. In this study, we aimed to demonstrate that the PMAs could be used as a platform for spheroid coculture systems by initially immobilizing OECs in the microwells, followed by the addition of pancreatic islets in the microwells.

Initial experiments to investigate cell immobilization onto the replica moulded PMAs and flat PDMS control were performed using murine bone marrow derived endothelial progenitor cells (mEPCs).\(^{50}\) Generally, cross-linked PDMS surfaces are characterized by low cell immobilization.\(^{23}\) To validate this characteristic with mEPCs, the cells were cultured on the flat PDMS control and TCPS well plates. After being stained with CellTracker to visualize the cytoskeleton, mEPCs were grown for 48 h on both surfaces and finally stained with Hoechst to visualize the nuclei (Fig. 5). As expected, the mEPCs were found to adhere and spread on the TCPS surfaces to form a near confluent monolayer [Fig. 5(a)] with typical elongated cell morphology. In contrast, the few remaining cells on the flat PDMS control had an atypical spherical morphology [Fig. 5(b)], indicating that the mEPCs are not immobilized to the smooth PDMS surface.

Gulati et al. previously reported that the surface topography of 3D printed anodized titanium implants was responsible for improvements in the adhesion of osteoclasts.\(^{51}\) It was also
demonstrated that surface nanotopography can direct the osteo-
genic differentiation of dental pulp derived stem cells.\textsuperscript{52}
Therefore, we hypothesized that the surface roughness $(R_q \pm 0.78 \mu m)$ within the wells of the PMAs could be sufficient
to immobilize the mEPCs even though they cannot be immo-
ibilized on to flat PDMS surfaces. Therefore, the CellTracker
labeled mEPCs were cultured on the replica PMAs and then
stained with Hoechst before fluorescence microscopy
imaging, which revealed that the cells predominately resided
within the wells and not on the flat areas between the wells
[Figs. 6(a) and 6(b)]. High-resolution confocal imaging of
the microwells revealed that the cells were immobilized to the
internal wall of the microwells and adopted a typical elon-
gated shape [Fig. 6(c)]. Furthermore, the 3D reconstruction of
the confocal images confirmed that the cells are deposited not
only on the bottom of the microwells, which could be
attributed to gravity, but also on the sides of the microwells as
they contour to the microwell shape [Fig. 6(d)].
Finally, to demonstrate that the PMAs can be utilized as
a coculture platform, human OECs were immobilized in the
microwells and then human pancreatic islets were added. CellTracker labeled human OECs were initially cultured in the
PMAs for 3 days to allow immobilization within the micro-
wells, and this was followed by incubation with human pancre-
atic islets for an additional 4 days. Fluorescence microscopy
images (Fig. 7) of the PMAs revealed that the OECs [Fig. 7(a)]
and islets [Fig. 7(b)] resided solely within the microwells. The
islets were stained with Newport Green DCF indicator to visu-
alize viable insulin-producing $\beta$-cells via binding to intracellu-
lar zinc. Interestingly, the OECs remained immobilized on
the microwell surface and did not migrate onto the islets, and islet
spheroids remained intact and did not dissociate. These results

\begin{figure*}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{AFM height images of a representative area of the hemispherical convex and concave features of the (a) 3D printed mould and (b) PDMS replica. The cross sections along the dotted lines in the AFM height images are also provided for the (c) mold and (d) replica. SEM images of a (e) concave hemisphere of the replica and (f) convex hemisphere of the mould; arrows point at concentric patterns and insets show a 3D rendering of the concave replica and convex mould.}
\end{figure*}
suggest a strong immobilization of the OECs on the microwell surface and that this manufacturing technique could be used to easily design coculture vessels of various shapes by leveraging on the inherent topography of 3D printed parts.

IV. SUMMARY AND CONCLUSIONS

The results presented in this study demonstrate that the intrinsic surface roughness of 3D printed hemispherical microwell array moulds can be transferred to replica moulded PDMS microwell arrays. In turn, this particular topography can be utilized to immobilize late outgrowth endothelial cells evenly and precisely within the microwells, without immobilization to the flat areas between the microwells. Finally, the immobilization of one cell population within the hemispherical shape of the microwells can be used to create a spherical coculture system whereby cell spheroid aggregates, such as pancreatic islets, can interact with the immobilized cells while maintaining their shape and integrity. As the immobilized cell population remains on the microwell surface, it is envisaged that this manufacturing technique could be utilized to design coculture platform of various shapes without the recourse of surface functionalization for the immobilization of cells.
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