Development and functional evaluation of biomimetic silicone surfaces with hierarchical micro/nano-topographical features demonstrates favourable in vitro foreign body response of breast-derived fibroblasts

Daniel J.T. Kyle\textsuperscript{a, b}, Antonios Oikonomou\textsuperscript{b}, Ernie Hill\textsuperscript{b}, Ardeshir Bayat\textsuperscript{a, *}

\textsuperscript{a} Plastic and Reconstructive Surgery Research, Manchester Institute of Biotechnology, University of Manchester, Manchester, UK
\textsuperscript{b} School of Computer Science, Centre for Mesoscience and Nanotechnology, The University of Manchester, Manchester, UK

\begin{abstract}
Reproducing extracellular matrix topographical cues, such as those present within acellular dermal matrix (ADM), in synthetic implant surfaces, may augment cellular responses, independent of surface chemistry. This could lead to enhanced implant integration and performance while reducing complications. In this work, the hierarchical micro and nanoscale features of ADM were accurately and reproducibly replicated in polydimethylsiloxane (PDMS), using an innovative maskless 3D grayscale fabrication process not previously reported. Human breast derived fibroblasts (n = 5) were cultured on PDMS surfaces and compared to commercially available smooth and textured silicone implant surfaces, for up to one week. Cell attachment, proliferation and cytotoxicity, in addition to immuno-fluorescence staining, SEM imaging, qRT-PCR and cytokine array were performed. ADM PDMS surfaces promoted cell adhesion, proliferation and survival (p < 0.05), in addition to increased focal contact formation and spread fibroblast morphology when compared to commercially available implant surfaces. PCNA, vinculin and collagen 1 were up-regulated in fibroblasts on biomimetic surfaces while IL8, TNF\textalpha, TGF\textbeta1 and HSP60 were down-regulated (p < 0.05). A reduced inflammatory cytokine response was also observed (p < 0.05). This study represents a novel approach to the development of functionalised biomimetic prosthetic implant surfaces which were demonstrated to significantly attenuate the acute in vitro foreign body reaction to silicone.
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1. Introduction

Surgical implants provide a diverse variety of site-specific tissue replacements for a number of functions, which are available to the practising surgeon. Common examples in use today include breast implants, dental implants, nerve conduits, vascular implants and orthopaedic implants [1]. The increasing demand for synthetically engineered body implants is a result of an ageing population and the associated tissue degeneration and malignancy [2]. This trend will continue until tissue regeneration techniques utilising autologous mesenchymal stem cells to engineer tissue-specific replacements becomes perfected and available to routine clinical practice. The biomaterials industry is expected to be worth $58.1 billion in 2014 [3] as medical devices such as breast implants are being increasingly required, with 385,813 breast augmentations/reconstructions (72% silicone implants) performed in the United States alone in 2013, up 2% from 2012 to 32% from 2000 [4].

However, current commercially available silicone mammary implants are not without their complications. For instance, silicone mammary implant surfaces suffer from significant limitation due to the formation of a constrictive fibrotic capsule post-implantation, known as capsular contracture, which results in firmness, deformity and pain in addition to device failure [5]. Capsular contracture formation remains the most common complication associated with silicone mammary implants, with rates ranging between 14.8 and 20.5% [6].

The exact pathoetiology of breast implant surface-related capsular contracture formation remains unclear, however, there
are a number of known risk factors, such as peri-prosthetic bacterial infection and radiotherapy pre or post-implantation, while submuscular placement and importantly, textured as opposed to smooth implants, can reduce contracture risk [7]. In addition to currently practised methods of limiting rates of capsular contracture formation through minimising bacterial infection around the implant (exogenous hypothesis), an alternative approach to this clinically significant complication is necessary to reduce the over-exaggerated foreign body reaction to the implant (endogenous hypothesis) [8].

Current commercially available silicone breast implants were fundamentally designed in the 1960's and the elastomeric implant shell (the site of the tissue/implant interface) is described as being either 'smooth' or 'textured' [9,10]. Textured implants are macroscopically rough surfaces formed either of nodular features or cuboid shaped pits, which are hundreds of microns large [11]. However, current silicone breast implants are intrinsically limited in their performance as the elastomeric shells (surfaces) were not designed with consideration of the mechanisms which promote favourable host response being a primary objective of the implants' function and rather they evolved through "trial and error optimization" [12], resulting in minimal advancement in implant performance. Conversely, due to the availability of advanced fabrication techniques and increased understanding of the variables which influence host-response at the cell-implant surface interface, medical devices manufactured today should aim to be non-toxic and non-immunogenic, whilst performing an active role in host response [13,14].

Micrometric and nanometric surface topographies influence cell attachment, proliferation, migration and differentiation in numerous cell types and on various substrates, both in vitro and in vivo [15–20]. It has been suggested that initial implant cell attachment and subsequent cytokine release may dictate the extent and impact of the foreign body reaction and clinical outcome through cell mechanotransduction and signal transduction mechanisms, which mediate cytokine/chemokine release and extracellular matrix (ECM) deposition [21–23]. This is the rationale behind the current tendency to use acellular dermal matrix (ADM) in implant-based breast augmentation/reconstruction. It is thought that ADM, in addition to providing inflammatory support to the implant, provides peri-implant cells and tissues with a familiar biological microenvironment, containing complex nano and microtopographical cues which delivers stimulus for specific and directed cell response [24–26]. The result is diminished foreign body reaction to the implant and reduced capsular contracture formation [27].

Our intuitive aim here was to design and fabricate a novel biomimetic silicone surface, acellular dermal matrix polydimethylsiloxane fabricated (ADM PDMS F), through reproduction of the complex hierarchical, nano and micro-scale topography of ADM using an innovative maskless 3D grayscale lithography fabrication process, not reported to date. This creative approach to implant surface design combines our understanding of the potential positive effects of micro and nanoscale topographies on cell function with the unique and inherent benefits of biomimetic structures. A silicone surface prepared using a direct casting (soft lithography) technique, acellular dermal matrix polydimethylsiloxane cast (ADM PDMS C), was also created to use as further validation.

Therefore, it is proposed that the biomimetic topographical cues of ADM silicone may mitigate the acute in vitro foreign body response of breast-derived fibroblasts (BFs) in comparison to commercially available smooth and textured silicone implant surfaces. For validation, a comprehensive in vitro biological evaluation of BDF cell function on respective surfaces through analysis of cell attachment, proliferation, morphology, gene expression and cytokine secretion was performed, to elucidate whether early BDF response to silicone surfaces could be positively altered through biomimetic topography.

2. Materials and methods

Tissue samples used in this study were obtained by the Plastics and Reconstructive Surgery Research (PRSR) Skin and Tissue Bank (North West Research Ethics Committee, Ethics code — 11/NW/0083). Patients were recruited and samples obtained following informed consent prior to elective breast reduction (to obtain skin and breast tissue). Patient demographics are found in Supplementary Table S1. Samples were anonymised and coded prior to use. In this work, two polydimethylsiloxane (PDMS) surfaces were fabricated, characterised and biologically evaluated; acellular dermal matrix polydimethylsiloxane fabricated surface (ADM PDMS F) and acellular dermal matrix polydimethylsiloxane cast surface (ADM PDMS C).

2.1. Collection of skin samples and tissue decellularisation protocol

Normal skin (n = 3) was collected from patients undergoing breast reduction surgery and stored in growth media containing Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) (PAA, Austria), penicillin (100 units/ml), streptomycin (100 units/ml) and l-glutamine (2 mM) (PAA Austria) while transported to laboratory. The skin was washed thoroughly in sterile phosphate buffered saline (PBS) supplemented with penicillin (100 units/ml) and streptomycin (100 units/ml), to remove any remaining blood, before hair and subcutaneous fat was removed. The decellularisation protocol followed is outlined in Hogg (2013) [28] and an exhaustive list of reagents used and exact protocol can be found in Supplementary Table S2 and S3, in addition to Hogg (2013) [28]. Briefly, the protocol involved moving the tissue through a succession of reagents, beginning with removal of the epidermis, followed by tissue dissection in peracetic acid, digestion of cellular components in a hypotonic buffer, a detergent wash to remove cellular debris and removal of nuclear contents [28].

2.2. Immunohistochemistry and haematoxylin and eosin staining of ADM

Immunoperoxidase staining was performed for collagen type IV, collagen type VII and laminin V to confirm the presence of an intact basement membrane (BM), using Novacastria® Novolink™ Polymer Detection System (Leica Biosystems, UK), following manufacturer’s instructions. Briefly, formalin fixed, paraffin embedded tissue samples were serially sectioned into 5 μm thickness, mounted onto charged slides (Thermo Scientific, USA) and left to dry overnight. Sections were then deparaffinised, rehydrated and antigen retrieved in sodium citrate buffer (Ph 6 and 60 °C for 1 h). Endogenous peroxidase and non-specific protein binding was blocked and the section treated with primary antibodies overnight at 4 °C (Supplementary Table S4). The sections were then further incubated with Post Primary Block and then with Novalink Polymer before proteins were finally detected using DAB chromogen substrate buffer for peroxidase detection and counterstained with haematoxylin. In addition, sections were stained with haematoxylin and eosin (H&E), to ensure morphology of tissue was not altered by decellularisation protocol, using standard laboratory protocol as previously described [29]. All sections were imaged on an upright Olympus Microscope (BX51, Olympus, UK).

2.3. Fabrication of ADM PDMS F

2.3.1. Characterising ADM using atomic force microscopy (AFM)

ADM samples were placed basement membrane (BM) side up onto microscope slides and allowed to slowly air dry at 4 °C for 24 h. ADM was imaged using a Bruker Dimension Icon™ Atomic Force Microscope (AFM) (Bruker, UK). Samples were imaged using ScanAsyst™ Air probes (Bruker, UK)/silicon nitride, nominal k = 0.4 N/m and in conductive ScanAsyst™ mode. Peak Force Tapping™ (PPT) amplitude was 150–100 nm, and PPT frequency was 1 kHz. Scan rate was 0.5 Hz. A large, intact area of ADM (500 µm) was imaged through obtaining numerous 90 × 90 µm² AFM scans using offsets in the X and Y direction (Supplementary Fig. S1A). Scans were performed in at least 3 different areas of the ADM sample and on 3 different patient soft tissue samples.

2.3.2. Creating a grayscale ADM pattern for exposure

An ADM montage was created using the stitching feature within Mountain Maps™ 7 imaging software (Digital Surf®, France) to stitch together numerous, adjacent raw 90 × 90 µm² ADM images of ADM. The montage was produced using X and Y offsets and similarities in height at the image edge to correctly align images, thereby forming a large intact area of ADM without leaving stitch lines (Supplementary Fig. S1C–D). The ADM montage was converted to an 8-bit grayscale image, consisting of 256 grayscale levels (Supplementary Fig. S1B–D), using the open source scanning probe analysis software Gwyddion (http://gwyddion.net) which could then be read by a laser lithography system.
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2.3.3. Maskless grayscale 3D photolithography

The following fabrication protocol was optimised for the particular equipment in our facility and further details can be found in the patent application [30]. In a class 100 clean room, a 2 cm² plain silicon wafer was sonicated for 5 min each in acetone, distilled water and isopropyl alcohol (IPA), dried with nitrogen gas and dehydrated on a hot plate set at 200 °C for 10 min. Resist adhesion was promoted using hexamethyldisilazane (HMDS) (Sigma-Aldrich, UK) which was spun onto the wafer at 4000 RPM for 60 s. This was immediately followed by spin coating photoresist S1813® (Microsprinter 6180 Series, Shipley, UK) onto the silicon wafer at 3000 RPM for 60 s, producing a thickness of 1.3 μm, followed by a soft bake at 72 °C for 1 min 30 s on a hot plate. Maskless grayscale photolithography was performed using a laser writer (Microtech Laserwriter LW4005, Italy). The prepared grayscale bitmap image was loaded into the laser writer and the pixel size was set at 0.5 μm in X and Y. A 40x objective and 0.65 NA was used. A baseline exposure dose of 0 J/cm² was assigned to black pixels (pixel 0, no exposure) and a dose of 0.31 J/cm² was assigned to white pixels (pixel 256, maximum exposure), with corresponding doses within this dose range assigned to each pixel according to the grayscale pattern. Exposing the photoresist to a beam of 405 nm wavelength leads to modifi- cation of the molecular structure of the photoresist resulting in solubilisation of the exposed areas (for a positive resist) in appropriate developer solution. Essentially, grayscale level corresponds with exposure dose which corresponds with feature depth after development. Optimisation was performed on a grayscale wedge design until linearity of the exposed resist was achieved. The exposed resist was developed in MF® 319 developer solution (Microsprint®, Shipley, UK) for 30 s with gentle agitation followed by 1 min in distilled water to stop the development and then dried with a gentle stream of nitrogen gas.

2.3.4. Fabrication of master template using modified deep reactive ion etching

An Oxford Plasmalab System 100 ICP® (Oxford Instruments, UK) deep reactive ion etcher (DRIE) running a modified Bosch process recipe was used to permanently transfer the exposed ADM pattern from the photoresist into the silicon wafer, which subsequently acted as a template to produce PDMS stamps through soft lithography. The Bosch process is a two-step time-multiplexed etching system which allows for the transfer of deep, highly anisotropic features, through alternating deposition (C₄F₈) and etching (SF₆) steps. Increased isotropy and lower etch selectivity were achieved.

2.3.5. Soft lithography used to create biomimetic silicone surfaces from master template

The PDMS used to create ADM PDMS surfaces for biological evaluation was kindly donated by Mentor Corporation (Mentor Corporation, Texas, USA) and was the same medical grade mixed dimethyl silicone elastomer dispersion used to manufacture the company’s own commercially available implant surfaces. The silicon master was vapour treated with a silanizing agent, Trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane (FTDS) (Sigma-Aldrich, UK) for 10 min in a desiccator under vacuum, reducing surface free energy in order to ease the release of PDMS from the silicon master. The PDMS was spun onto the silicon wafer (100 RPM) and de-gassed in a desiccator for 1 h to remove any bubbles and to aid the transfer of features within the silicon template into the PDMS. To cure and crosslink the PDMS it was baked overnight in an oven at 80 °C. A 1.5 × 1.5 cm² PDMS stamp containing the fabricated surface topography of ADM was then cut out using a scalpel which could then be used for biological evaluation.

2.4. Fabrication of ADM PDMS C

The ADM PDMS C surface was fabricated through a standard soft lithography technique. After drying at 4 °C, ADM was directly silanized with FTDS as described above. PDMS was poured directly onto native ADM and cured for 48 h at room temperature (17 °C). This was then peeled off the ADM surface to create an inverted ADM surface in PDMS. The inverted mold was again silanized, coated with PDMS, cured and removed to reveal a PDMS cast which was a replica of the native ADM surface.

2.5. Substrate characterisation

Commercially available smooth and textured implant surfaces, native ADM, ADM PDMS F and ADM PDMS C surfaces were characterised using AFM, scanning electron microscopy (SEM), optical 3D profiling and 3D laser scanning. Roughness measurements and quantitative topographical data were gathered using AFM. AFM was performed using a Bruker Dimension Icon® Atomic Force Microscope (AFM); Samples were imaged using ScanAsyst® Air probes (silicon nitride, nominal k = 0.4 N/m) and conducted in ScanAsyst™ mode. Peak Force Tapping™ (PFT) amplitude was 150–100 nm, and PFT frequency was 1 kHz. Scan rate was 0.5 Hz. Images were analysed using NanoScope Analysis® software (Bruker, UK) and ISO 25178 (Surface texture: areal, Sa) roughness analysis was performed. Prior to SEM imaging, samples were coated with 2 nm chromium (Cr), acting as an emission layer and 12 nm of gold (Au). Imaging was performed on a Carl Zeiss ULTRA PLUS® system using a working distance of 2.7 mm and EHT voltage level of 7–10 kV.

Textured implant surfaces were also measured with an optical 3D profiler (Bruker Contour CT-G1, UK) and 3D laser scanner as they were too rough for measurement with AFM.

Table 1
Deep reactive ion etching (DRIE) recipe.

| Grayscale DRIE recipe: Etch selectivity: Optimised at 10:1. |
|----------------------------------------------------------|
| **Step** | **Step Time (seconds)** | **Pressure (mTorr)** | **RF power (Watt)** | **ICP power (Watt)** | **C₄F₈ (SCcm)** | **O₂ (SCcm)** |
|----------------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Etch | 3 | 10 | 5 | 300 | 100 | 5 | 0 |
| O₂ etch | 3 | 10 | 5 | 300 | 0 | 0 | 30 |
| Deposition | 4 | 10 | 5 | 300 | 5 | 100 | 0 |
| Repeats | | | | | 80–100 |

Grayscale deep reactive ion etch (DRIE) recipe. SF₆ = Sulfur hexafluoride. C₄F₈ = Octafluorocyclobutane. SCCM = Standard cubic centimetres per minute.

| Table 2 |
|---------------------------------|
| Characterisation of native acellular dermal matrix (ADM), acellular dermal matrix polydimethylsiloxane fabricated (ADM PDMS F) surface, acellular dermal matrix polydimethylsiloxane casted (ADM PDMS C) surface, smooth implant surface and textured implant surface. |
|---------------------------------|
| **Surface** | **Sa (nm)** | **Sq (nm)** | **Sz (nm)** | **Sv (nm)** | **Sp (nm)** | **Ssk** | **Excess Sku** | **Fractal dimension** |
|---------------------------------|
| ADM | 480.4 | 177 | 607.6 | 221.9 | 4020.7 | 1352.3 | -2009 | 827.4 | 2010.9 | 644.2 | 0.01 | 0.32 | 0.03 | 0.49 | 2.33 | 0.05 |
| Native ADM | 484 | 187.9 | 550 | 231.6 | 3366 | 1119.4 | -1692 | 648.2 | 1674 | 472.6 | -0.07 | 0.06 | -0.18 | 0.23 | 2.29 | 0.07 |
| ADM PDMS F | 522.7 | 155 | 664 | 191.3 | 4501.1 | 1205 | -2235 | 883.6 | 2266 | 407.1 | 0.12 | 0.26 | 0.19 | 0.22 | 2.30 | 0.01 |
| ADM PDMS C | 522.7 | 155 | 664 | 191.3 | 4501.1 | 1205 | -2235 | 883.6 | 2266 | 407.1 | 0.12 | 0.26 | 0.19 | 0.22 | 2.30 | 0.01 |
| Smooth | 22.2 | 9.6 | 36.1 | 22.3 | 483 | 258.4 | -144 | 114.7 | 339.4 | 155 | 1.95 | 0.77 | 12.62 | 6.22 | 2.06 | 0.09 |
| Textured | 8240 | 4760 | 10,000 | 5800 | 40,002 | 20,900 | -20,060 | 11,088 | 19,420 | 9640 | -0.02 | 0.46 | -3.21 | 0.60 | 2.05 | 0.17 |

Both ADM PDMS F and ADM PDMS C surfaces were accurately fabricated and possessed similar topographical features as existing within native ADM. However, the ADM PDMS C surface contained more of the features of native ADM, and was a more reliable fabrication method; hence Ss, Ss, and Sp values were more analogous to native ADM than to the ADM PDMS F surface. The native ADM, ADM PDMS F and ADM PDMS C surfaces all had a mean Ssk and excess Sku value of approximately 0 and a fractal dimension of 2.3. Smooth silicone implant surfaces contained nano-scale peak surface irregularities, which significantly deviated from the mean, hence an excess Sku value of 12.62. Textured implants are macroscopically nodular, surfaces containing Ss values in excess of 40 μm. The surface features gradually deviated from the mean and were predictable. Fractal dimension of both smooth and textured implants was approximately 2. Data is displayed as the mean of at least six 90° m² areas of each substrate surface and ± indicates standard deviation. (Sa = arithmetical mean height; Sq = root mean square height; Sz = maximum peak to valley distance; Sv = maximum valley depth; Sp = maximum peak height; Ssk = skewness; excess Sku = kurtosis – 3, FD = Fractal dimension).

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2.6. Isolation of breast derived fibroblasts (BDF) from breast tissue, primary cell culture and cell cycle synchronisation

Breast derived fibroblasts (BDF) were used for all studies, as they are the cells which would encounter the implant surface if they were inserted into breasts in vivo. As fibroblasts from different body sites have distinct genotypic and cytokine profiles, it was important for us to use breast specific BDFs to appreciate how the effect of implant surface topography on cells may be realised in vivo, if used clinically [31,32]. BDFs were enzymatically obtained from breast gland and connective tissue after retrieval from patients undergoing breast reduction; using standard methods previously described in our laboratory [33]. Briefly, breast tissue was washed thoroughly in sterile PBS, before being minced finely with a scalpel and digested in 5% collagenase A (Roche, Mannheim, Germany) for 4 h at 37°C. After digestion, tissue was centrifuged at 1500 RPM for 10 min, supernatant removed and resuspended in fibroblast growth media (outlined above) and incubated in T75 tissue culture plastic (TCP) flasks (Corning Incorporated, USA) at 37°C in humidified 5% CO2 air. Growth media was changed every 48 h and cells passaged at 70–80% confluence. All BDFs used in the following experiments are of passage 3 or lower in an effort to retain the cells innate genotypic and phenotypic characteristics before they’re lost due to excessive passaging [34,35].

Prior to the following experiments, cell cycle synchronisation was performed. To synchronise the cell cycle, fibroblasts are arrested at G0/G1 stage through culture in 0.2% FBS DMEM for 24 h before time point 0.

For all experiments, cells were seeded at a density of 1 x 10⁴ per well, in a 24-well cell culture plate (Corning Incorporated, USA) and performed in triplicate. Cells were seeded on onto ADM FBMS F, ADM FBMS C, implant surfaces and tissue after retrieval from patients undergoing breast reduction; using standard methods previously described in our laboratory [33]. Brieﬂy, breast tissue was washed thoroughly in sterile PBS, before being minced finely with a scalpel and digested in 5% collagenase A (Roche, Mannheim, Germany) for 4 h at 37°C. After digestion, tissue was centrifuged at 1500 RPM for 10 min, supernatant removed and resuspended in fibroblast growth media (outlined above) and incubated in T75 tissue culture plastic (TCP) flasks (Corning Incorporated, USA) at 37°C in humidified 5% CO2 air. Growth media was changed every 48 h and cells passaged at 70–80% confluence. All BDFs used in the following experiments are of passage 3 or lower in an effort to retain the cells innate genotypic and phenotypic characteristics before they’re lost due to excessive passaging [34,35].

2.7. Cell attachment and proliferation

Cell attachment (1–6 h) and proliferation (24 h–1 week) experiments were performed using an MTT cell viability assay (Cell proliferation Kit 1 (MTT), Roche, Germany), as per manufacturer’s instructions and measured using a micro-plate reader at a wavelength of between 570 and 650 nm.

2.8. Cell apoptosis

Cell apoptosis was assessed using the lactose dehydrogenase (LDH) enzyme assay, as per manufacturer instructions (Cytotoxicity Detection Kit, Roche, Germany) and measured on a micro-plate reader at a wavelength of between 490 and 600 nm.

2.9. RNA extraction, cDNA synthesis and quantitative reverse transcriptase chain reaction (qRT-PCR)

Cells were collected in TRIzol buffer (Invitrogen, UK). RNA extraction, cDNA synthesis and qRT-PCR were carried out to manufactures instructions, using standard protocol in our laboratory and as described previously [33,36]. RNA concentration and purity were analysed on a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). RNA concentration was normalised prior to cDNA synthesis and cDNA synthesis was carried out using the QScript cDNA synthesis kit (Quanta Biosciences, USA). qRT-PCR was performed on a LightCycler 480 machine (Roche Diagnostics, Germany), as described previously [33,37]. The gene expression of proliferating cell nuclear antigen (PCNA), vinculin (VCL), collagen type 1 (COL1A1), tumour necrosis factor alpha (TNFα), interleukin 8 (IL8), heat shock protein 60 (HSPO60) and transforming growth factor beta 1 (TGFβ1) were measured. All primers from Sigma-Aldrich, UK. Primers and probes used for qRT-PCR are shown in Supplementary Table S5. CT values were calculated by subtracting averaged RPL32 (reference gene) CT values from averaged CT values of target gene. Relative gene expression levels were calculated by using 2-ΔΔCT method.

2.10. Inflammatory marker Multi-Analyte ELISA

At each time point, cell culture media was aspirated from the cell culture well, centrifuged for 10 min at 2500RPM to remove any particulates and frozen at -20°C until needed. A custom made Human Inflammatory Marker 96-well Multi-Analyte ELISA assay kit (Qiagen, SABiosciences, UK), which quantifies 12 cytokines per plate, was performed following manufactures instructions. The expression of interleukin 1 alpha (IL1A), interleukin 1 beta (IL1B), transforming growth factor beta 1 (TGFβ1), interleukin 4 (IL4), interleukin 6 (IL6), interleukin 8 (IL8), interleukin 10 (IL10), interleukin 12 (IL12), interleukin 17A (IL17A), interferon gamma (IFNγ), tumour necrosis factor alpha (TNFα) and granulocyte macrophage colony stimulating factor (GM-CSF) were detected. Briefly, samples (50 μl) are incubated for 2 h at room temperature (RT) with assay buffer (50 μl) and serial dilutions of the antigen standards (50 μl), followed by washing three times over 5 min with wash buffer. Detection antibody solution (100 μl) was then added and incubated for 1 h, followed again by three washes. Avidin-HRP (100 μl) was then added to each well and incubated for 30 min and again washed, this time four times. This was followed by adding Development solution (100 μl) and incubating for 15 min, followed by adding Stop solution (100 μl) and read on a micro-plate reader at a wavelength of 450 nm. After media was collected for cytokine profiling, cells on implant surfaces were detached from cell substrates using 0.05% trypsin (Sigma-Aldrich, UK) and 0.2% ethylenediaminetetraacetic acid (Sigma-Aldrich, UK) in PBS, aspirated from cell culture well and counted using a haemocytometer so that cytokine secretion levels could be normalised by cell number.

2.11. Immunocytochemistry

Immunocytochemistry was performed on BDFs for vinculin, F-Actin and DAPI. Cells were fixed in 10% Neutral buffered formalin (NBF) (Sigma-Aldrich, UK) for 1 h, washed in PBS and permeabilised in 0.5% Triton-X 100 (Sigma-Aldrich, UK) for 25 min. Cells were then washed and blocked in 1X BSA at pH 7.4 for 1 h at RT, wrapped in foil. After washing in PBS, cells were incubated with rhodamine phalloidin stain (1:200 in PBS) (Sigma-Aldrich, UK) for 45 min at RT. Cells were again washed in PBS before being incubated with 4’,6-diamidino-2-phenylindole (DAPI, 1:500 in PBS, Invitrogen, UK) for 15 min at RT. Surfaces were washed with PBS, mounted with Prolong gold (Invitrogen, UK) and stored in a cold room, wrapped in foil. Surfaces were visualised on an upright immunofluorescence microscope and images recorded. (BX51, Olympus UK Ltd).

2.12. Scanning electron microscopy

Growth media was removed and pre-warmed (37°C) 2% glutaraldehyde (Sigma-Aldrich, UK) in 0.1 M sodium cacodylate buffer (Sigma-Aldrich, UK) was used and cells were fixed for 2 x 30 min. The fixative was then removed, cells washed in 0.1M sodium cacodylate buffer three times over 5 min, before adding 2% osmium (Sigma-Aldrich, UK) in distilled water for 20 min. Cells were washed again with buffer before being dehydrated through a graded series of alcohols (30%, 50%, 70%, 90%, and 100% x 3; 10 min at each concentration), then gradually transferred through a graded series of HMDS (Sigma-Aldrich, UK) (i.e. 100% ethanol, 30%, HMDS, 50% HMDS, 70% HMDS and 100% HMDS; 10 min at each stage). The samples are then left to dry in a fume hood overnight before Cr/Ag (~15 nm) was deposited by E-beam evaporation and the sample was visualised.

2.13. Statistical analysis

All experiments were performed three times, in triplicates. All statistical tests were performed using GraphPad Prism 6 software. Relative optical density (OD) values of the colourimetric MTT/LDH assays were used for cell attachment, cell proliferation and cell survival comparisons. To determine the difference in gene expression between BDFs on different surfaces the relative threshold cycle (Ct) was used, obtained from PCR. Relative gene expression was calculated using the 2-ΔΔCT method and used for comparison. Normalised optical density values of the colourimetric ELISA cytokine array were used for cytokine level comparisons. Two-way ANOVA followed by Turkey post-hoc multi-comparison analysis was performed for all experiments and a p value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Presence of intact basement membrane on ADM surface after decellularisation

After removal of the epidermis and decellularisation of the dermis, immunoperoxidase staining for collagen type IV, collagen type VII and laminin V revealed that the dermal basement membrane was intact (Supplementary Fig. S2 C–E). Furthermore, H&E revealed morphology of the dermis was preserved throughout the decellularisation process (Supplementary Fig. S2A–B).

3.2. Substrate characterisation

Areal 3D surface analysis revealed comprehensive differences in topography and roughness values between ADM and commercially available implant surfaces, as summarised in Table 2. Textured implant surfaces (Sa = 0.24 μm; Sz = 0.48 μm) were ~17 times rougher than smoother implant surfaces (Sa = 0.022 μm; Sz = 0.48 μm). ADM was found to be a gradually undulating, self-similar surface, containing macro, micro and nano-scale features
A skewness and excess kurtosis value of approximately 0 indicates a surface which contains a Gaussian distribution of peaks and troughs, which do not significantly deviate from the mean. Smooth silicone implant surfaces are predominantly flat surfaces with positive skewness and excess kurtosis values, indicating a surface with many random small nanoscale peaks on the surface which significantly deviate from the mean (Fig. 1B i–v). Textured silicone implant surfaces were found to be extremely rough, nodular surfaces with features several hundreds of microns large, and SEM revealed minimal texturing at the micro and nanoscale (Fig. 1C i–v). A positive skewness and negative excess kurtosis indicates a predominance of peaks, which vary gradually and predictably from the mean.

3.3. Characterisation of ADM PDMS F and ADM PDMS C surfaces

ADM PDMS F and ADM PDMS C surfaces were also characterised and compared with native ADM, to evaluate the accuracy with which each fabrication technique reproduced ADM features and is also summarised in Table 2. Both fabrication techniques were able to reproduce ADM topography and roughness in PDMS accurately (Fig. 2A–C) but the direct casting technique of native ADM most reliably reproduced features and is considered the gold standard technique to create biomimetic topographies (Fig. 2C i–iv). Both techniques were able to mimic features down to 10’s of nanometres in scale. It can be seen in the SEM images in Fig. 2A (iv) and 2B (iv) that although the maskless grayscale lithography technique was able to accurately reproduce features at the micro and nanoscale, the undulating features of native ADM at the macro scale were missed, as these were out of the Z range of the AFM scanner and were therefore not included in the grayscale montage which was used to create the ADM PDMS F surface.

3.4. ADM PDMS topography promoted increased cell attachment after 6 h

As seen in Fig. 3A, attachment of BDFs to ADM PDMS F was significantly greater after 4 h than to smooth (p = 0.0051) and textured (p = 0.0005) implant surfaces. After 6 h, there was a significantly increased BDF attachment to both ADM PDMS F and C surfaces.
surfaces in comparison to both smooth (ADM PDMS F \( p = 0.0008 \); ADM PDMS C \( p = 0.037 \)) and textured (ADM PDMS F \( p < 0.0001 \); ADM PDMS C \( p = 0.0012 \)) implant surfaces. Furthermore, there was no significant difference between cell attachment on both smooth and textured implant surfaces after 6 h, and no significant difference between ADM PDMS C and ADM PDMS F surfaces.

3.5. **Cell proliferation was promoted on ADM PDMS surfaces up to 1 week**

As seen in Fig. 3B, after 24 h, significantly increased cell proliferation was observed on ADM PDMS F surfaces in comparison to smooth (\( p = 0.046 \)) and textured implant surfaces (\( p = 0.032 \)). By 48 h there was a noticeable increase in proliferation of BDFs on ADM PDMS F and C surfaces in comparison to smooth and textured implant surfaces which became most significant after 1 week (after 1 week, ADM PDMS F vs. smooth \( p = 0.03 \); vs. textured \( p < 0.0001 \); ADM PDMS C vs. smooth \( p = 0.0001 \); vs. textured \( p < 0.0001 \)). Moreover, after 1 week, there was a significantly increased BDF proliferation on smooth implant surfaces in comparison to ADM PDMS F surface (\( p = 0.042 \)) and significantly increased proliferation of BDFs on ADM PDMS C surface in comparison to the ADM PDMS F surface (\( p = 0.048 \)).

3.6. **ADM PDMS topography promoted increased cell survival at every time point**

As seen in Fig. 3C, LDH assay revealed improved BDF survival on ADM PDMS surfaces at every time point, in comparison to smooth and textured silicone implant surfaces. This was most significant after 96 h and continued up to 1 week (at 1 week, ADM PDMS F vs. smooth \( p < 0.0001 \); vs. textured \( p < 0.0001 \); ADM PDMS C vs. smooth \( p = 0.0001 \); vs. textured \( p < 0.0001 \)). In addition, increased cell death was observed in BDFs cultured on textured implant surfaces in comparison to smooth implant surfaces after 1 week (\( p = 0.0011 \)). Furthermore, at 1 week there was significantly increased cell survival of BDFs on ADM PDMS C surface in comparison to ADM PDMS F surface (\( p = 0.028 \)).

3.7. **ADM PDMS induced a positive alteration in gene expression associated with attachment, proliferation, ECM synthesis and inflammation**

As seen in Fig. 4A, after 24 h, PCNA was significantly up-regulated in BDFs on ADM PDMS F surfaces and by 96 h PCNA was significantly up-regulated in BDFs on both ADM PDMS F and C surfaces in comparison to smooth and textured implant surfaces (ADM PDMS F vs. smooth \( p = 0.036 \); vs. textured \( p = 0.0010 \); ADM PDMS C vs. smooth \( p = 0.0013 \); vs. textured \( p < 0.0001 \)). There was no significant difference in PCNA mRNA expression between BDF on ADM PDMS F or ADM PDMS C surfaces (\( p > 0.05 \)). Vinculin was significantly up-regulated on ADM PDMS surfaces after 24 h which continued up to 1 week, as seen in Fig. 4B (At 1 week, ADM PDMS F vs. smooth \( p < 0.0001 \); vs. textured \( p = 0.008 \); ADM PDMS C vs. smooth \( p < 0.0001 \); vs. textured \( p = 0.0001 \)). Furthermore, there was no significant difference between vinculin gene expression in BDFs cultured on ADM PDMS F, in comparison to ADM PDMS C (1 week, \( p = 0.55 \)), or between smooth and textured implant surfaces.
Fig. 4. Acellular dermal matrix (ADM) polydimethylsiloxane (PDMS) topography induced alteration in genes associated with attachment, proliferation, ECM synthesis and inflammation. Relative gene expression of PCNA (A), vinculin (B), IL8 (C), TNF-alpha (D), TGF-beta 1 (E), Collagen 1 (F) and HSP60 (G) of breast-derived fibroblasts (BDFs) on silicone surfaces from 24 h up to 1 week. Acellular dermal matrix polydimethylsiloxane fabricated and casted topographies (ADM PDMS F and C) induced up-regulation of PCNA, vinculin and collagen 1 in BDFs while IL8, TNF-α, TGF-β and HSP60 were down-regulated, in comparison to BDFs on smooth and textured silicone implant surfaces. Tissue culture plastic (TCP) and collagen were used as controls. mRNA expression was normalised by reference gene RPL32. Data is expressed as mean ± SD of three independent experiments, performed in triplicate and analysed using two-way ANOVA (* p < 0.05).
The expression of IL8 was significantly down-regulated in BDFs after 24 h on ADM PDMS surfaces in comparison to smooth and textured implant surfaces, as seen in Fig. 4C (ADM PDMS F vs. smooth p < 0.0001; vs. textured p < 0.0011; ADM PDMS C vs. smooth p < 0.0001; vs. textured p = 0.0002), which continued up to 48 h for BDFs on ADM PDMS C surfaces in comparison to smooth implant surfaces (ADM PDMS C vs. smooth p = 0.026). Moreover, there was a significant down-regulation of IL8 in BDFs cultured on textured implant surfaces in comparison to BDFs on smooth implant surfaces after 24 h (p = 0.0074). As seen in Fig. 4D, TNFα was significantly down-regulated in BDFs on ADM PDMS surfaces in comparison to smooth implant surfaces only after 24 h (p = <0.05). However, after 48 h TNFα was significantly down-regulated in BDFs on ADM PDMS surfaces in comparison to both smooth and textured silicone implant surfaces (After 48 h, ADM PDMS F vs. smooth p < 0.0001; vs. textured p < 0.0001; ADM PDMS C vs. smooth p < 0.0001; vs. textured p < 0.0001). TGFβ1 was significantly down-regulated in BDFs on ADM PDMS surfaces after 96 h in comparison to smooth implant surfaces and in comparison to both smooth and textured implant surfaces by one week, as seen in Fig. 4E (At one week, ADM PDMS F vs. smooth p < 0.0001; vs. textured p = 0.031; ADM PDMS C vs. smooth p < 0.0001; vs. textured p < 0.0001). Furthermore, at one week, TGFβ1 was significantly up-regulated in BDFs on smooth implant surfaces in comparison to textured implant surfaces (p = 0.0002). As seen in Fig. 4F, collagen type 1 was up-regulated in BDFs on ADM PDMS surfaces in comparison to smooth implant surfaces after 48 h (ADM PDMS F vs. smooth p = 0.0036; vs. textured p < 0.001; ADM PDMS C vs. smooth p < 0.002; vs. textured p < 0.005). By 96 h, collagen type 1 was only up-regulated in BDFs on ADM PDMS C surfaces in comparison to textured surfaces (p = 0.009). By one week there was no difference in expression of collagen 1 in BDFs on any of the silicone surfaces. HSP60 was down-regulated in BDFs on ADM PDMS surfaces in comparison to smooth

Fig. 5. Acellular dermal matrix (ADM) polydimethylsiloxane (PDMS) topography reduced breast derived fibroblast (BDF) pro-inflammatory and pro-fibrotic cytokine secretion. Inflammatory cytokine profile of breast-derived fibroblasts (BDFs) on respective silicone surfaces at 24 h (A), 48 h (B), 96 h (C) and 1 week (D). Tissue culture plastic (TCP) was a control. The secretion of a number of pro-inflammatory cytokines (in particular IL6, IL8, and TNF-alpha) were decreased by BDFs on acellular dermal matrix polydimethylsiloxane fabricated and casted surfaces (ADM PDMS F and C) in comparison to smooth and textured silicone implant surfaces, at each time point. At 96 h, there was a transition from a pro-inflammatory cytokine profile to a pro-fibrotic cytokine profile in BDFs on smooth and textured silicone implant surfaces which was attenuated by BDFs on ADM PDMS F and C surfaces. Conversely, at 96 h, an increase in the secretion of IL10 by BDFs on ADM PDMS F and C surfaces may indicate a switch from a pro-inflammatory to pro-wound healing state. Data is expressed as mean ± SD of three independent experiments, performed in triplicate and analysed using two-way ANOVA (* = p < 0.05 in comparison to smooth silicone implant surface; # = p < 0.05 in comparison to textured silicone implant surface).
implant surfaces only after 48 h and then in comparison to both smooth and textured implant surfaces by 96 h, which continued up to one week, as seen in Fig. 4G (At one week, ADM PDMS F vs. smooth \( p = 0.0037 \); vs. textured \( p < 0.0001 \); ADM PDMS C vs. smooth \( p < 0.0001 \); vs. textured \( p < 0.0001 \)). Furthermore, HSP60 was down-regulated in BDFs on smooth implant surfaces in comparison to textured implant surfaces \( (p = 0.0001) \).

3.8. ADM PDMS topography reduced breast derived fibroblast pro-inflammatory and pro-fibrotic cytokine secretion

The secretion of a number of pro-inflammatory cytokines by BDFs on ADM PDMS F and ADM PDMS C surfaces was reduced in comparison to both smooth and textured implant surfaces, indicating that ADM topography was able to attenuate the acute in vitro foreign body response of BDFs to the silicone surface, up to a 1 week time point. At 24 and 48 h, the secretion of IL6, IL8, and TNFα by BDFs on ADM PDMS surfaces were reduced in comparison to smooth and textured implant surfaces while TGFβ1 was reduced in comparison to smooth implant surfaces only (Fig. 5A). In addition, by 48 h, IL1A, IL17A and GM-CSF secretion by BDFs on ADM PDMS C were also reduced and secretion of IL10 was increased, in comparison to BDFs on textured implant surfaces only. Furthermore, secretion of IFNγ was reduced by BDFs on both ADM PDMS C and F surfaces in comparison to smooth and textured implant surfaces (Fig. 5B). At 96 h, the observed mitigated pro-inflammatory response of BDFs to ADM PDMS surfaces was most prominent and a reduced secretion of IL1A, IL6, IL8, IL12, IL17A, IFNγ, TNFα, TGFβ and GM-CSF by BDFs on ADM PDMS C were observed in comparison to BDFs on smooth and textured silicone implant surfaces, which continued up to one week (Fig. 5C). Moreover, at 96 h, a transition from a pro-inflammatory weighted cytokine profile to a pro-fibrotic weighted cytokine profile of BDFs on smooth and textured implant surfaces may have been taking place, highlighted through an increase in TGFβ1 secretion by BDFs on smooth and textured implant surfaces, which peaked at one week (Fig. 5D). Conversely, increased secretion of the anti-inflammatory and pro-wound healing cytokine IL10 by BDFs on ADM PDMS surfaces was observed at 1 week in comparison to BDFs on smooth and textured surfaces, in addition to the reduced secretion of the aforementioned pro-inflammatory and pro-fibrotic cytokines (Fig. 5D). Together these results indicate that a topographically induced reduction in pro-inflammatory cytokine secretion and enhanced pro-wound healing cytokine secretion by BDFs on ADM PDMS surfaces was observed, which was most significant after 96 h. After this time, a switch from a pro-inflammatory cytokine profile to a pro-fibrotic cytokine profile in BDFs on smooth and textured implant surfaces and to an anti-inflammatory/pro-wound healing profile in BDFs on ADM PDMS F and C surfaces was detected.

3.9. Immunocytochemistry and SEM imaging revealed increased focal contact formation and spindle shaped BDF morphology of breast derived fibroblasts on ADM PDMS surfaces

As seen in Fig. 6A–D, immunofluorescence images revealed specific focal staining of vinculin in BDFs on ADM PDMS surfaces. The formation of focal contacts at the tips of F-actin filaments is characteristic of focal adhesions indicating that BDFs on ADM PDMS surfaces can form stable attachment to the underlying biomimetic topography, and subsequently spread to develop typical fibroblast “spindle like” morphology. SEM images (Fig. 7A–D) demonstrated attachment of BDFs to specific features on the ADM PDMS surfaces indicating that the ADM topography provided topographical cues which the cells were able to sense and interact with. As seen in Fig. 6E–F, immunofluorescence staining of BDFs on smooth implant surfaces revealed round cell morphology, containing diffuse and non-specific vinculin staining with no focal contact formation. BDFs have aggregated on the surface of the smooth implant and are often seen preferentially binding to each other instead of forming focal contacts with the underlying implant surface topography. SEM imaging (Fig. 7E–F) confirmed the poor attachment and spherical shape of BDFs on smooth implant surfaces. BDFs on textured implant surfaces also revealed mostly diffuse and non-specific staining of vinculin and minimal focal contact formation, with a significant number of cells appearing to be trapped in deep troughs between nodules (Fig. 6G–H). However, some focal adhesions were clearly observed but were difficult to demarcate. The cells were able to spread; however, as shown in Fig. 7G–H, it appears that the cells are wedged within the valleys between the steep nodules on the textured implant surface. Taken together, the immunofluorescence and SEM images suggest that BDFs on textured implant surfaces were unable to spread or migrate effectively due to physical restriction by the steep nodule side walls present within the textured surface and also displayed rounded morphology due to lack of micro and nanoscale topographies.

4. Discussion

This study, for the first time, demonstrates that ADM surface topography, accurately replicated in silicone using a novel fabrication technique (not previously reported), induces improved breast fibroblast cell attachment, proliferation, survival and diminished acute in vitro foreign body response to silicone, compared to breast fibroblasts seeded onto commercially available smooth and textured silicone implant surfaces. Extensive characterisation of native ADM in addition to smooth and textured implant surfaces revealed a number of significant topographical and roughness differences between surfaces which may explain the enhancement in cell response to ADM PDMS surfaces. Textured implant surfaces were found to be extremely rough, nodular surfaces at the macro scale (features >200 µm) but, similarly to smooth implant surfaces, are relatively uniform at the microscale and nanoscale. Therefore, although textured implant surfaces undoubtedly influence tissue response in vivo, they have little impact on cellular response, as the textured features are too large to be recognised by a cell’s filopodia and lamellipodia. This is in contrast to the fractal, self-similar, nano and microscale topography of ADM, which contains a range of hierarchical features, all within the size range of features that a cell is able to sense, interact with and respond to. Clinically, a silicone surface containing ADM topographical cues, may facilitate cell adaption but would not be too rough (macroscopically) or porous, as to encourage excessive tissue in growth which may aggravate the local micro-environment and lead to seroma formation, which has been associated with textured implant surfaces [38].

Therefore, the rationale behind this work was to reproduce ADM’s range of complex features in silicone, in an attempt to enhance silicone biocompatibility through biomimetic topography. Thus, the first important outcome was the optimisation of a fabrication technique which enabled reproducible, accurate replication of biomimetic topographies in silicone. We were able to achieve this through an innovative technique combining the use of AFM, 3D grayscale lithography and modified DRIE. As shown in Table 2, comprehensive areal (S) roughness analysis of fabricated surfaces revealed that the optimised fabrication technique was able to accurately reproduce nano and micro-scale features of ADM in silicone to within 10 nm (Native ADM Sa = 480 nm; ADM PDMS F Sa = 484 nm). Furthermore, ADM PDMS F peak to valley measurements were within 0.6 µm of native ADM (Native ADM Sz = 4 µm; ADM PDMS F Sz = 3.4 µm) while skewness, kurtosis and...
Fractal dimension values were all within 0.2 μm of native ADM. 3D AFM rendering, section profiles and SEM images revealed precise reproduction of ADM topography, in addition to roughness. For corroboration of the topographical effects of ADM PDMS F on cell response, a second silicone surface (ADM PDMS C) was prepared through a standard soft lithography (replica moulding) technique, which has been demonstrated to reproduce features accurately at a nanoscale [39]. The ADM PDMS C surface was found to have reproduced the features of native ADM at the macro, micro and nanoscale. Together, the two novel ADM PDMS surfaces permitted the robust investigation of BDFs to ADM topography in silicone.

ADM PDMS surfaces promoted increased BDF attachment, proliferation and cell survival in comparison to smooth and textured silicone implant surfaces, at every time point up to one week. Cell attachment of BDFs was increased on ADM PDMS surfaces in the MTT cell viability assay, through measurement of mRNA transcription levels of vinculin and immunofluorescence staining of vinculin protein within focal adhesions. Vinculin is a membrane-cytoskeletal protein and an essential component of focal adhesions formed between cells and their environment [40]. Its function is important for cell attachment, spreading, migration, proliferation and differentiation [41]. For adherent cells, such as fibroblasts, optimum cell

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**Fig. 6.** Immunocytochemistry revealed increased focal contact formation of breast derived fibroblasts (BDFs) on acellular dermal matrix (ADM) polydimethylsiloxane (PDMS) surfaces. Immunofluorescence staining of breast-derived fibroblasts (BDFs) on acellular dermal matrix polydimethylsiloxane casted (ADM PDMS C) (C–D), smooth silicone implant surfaces (E–F) and textured silicone implant surfaces (G–H). The images show staining of F-Actin (red), vinculin (green) and DAPI (blue). Insets in B and D show focal contact formation of BDFs on ADM PDMS surfaces (White scale bar – 40 magnification). White arrows are pointing to focal contacts. Cells on ADM PDMS F and C surfaces (A–D) were able to attach, form visible focal contacts and spread, revealing typical ‘spindle-like’ fibroblast morphology. This is in stark contrast to BDFs on smooth silicone implant surfaces (E–F) which attached poorly and demonstrated preferential cell–cell attachment than cell-substrate attachment, which can be indicative of stressed/frustrated cell behaviour. Similarly, BDFs on textured implant surfaces (G–H) also demonstrated poor cell attachment but some focal contact formation was observed. Interestingly, BDFs on textured silicone implant surfaces appeared to be ‘trapped’ within the deep troughs on the implant surface and adhered to the steep nodule side walls.
function is dependent upon the formation of stable focal contacts with surrounding ECM (or prosthesis topography) [42]. This is because focal contacts are critical to mechanotransduction pathways (the mechanism through which cells convert mechanical cues within their extracellular environment into alteration of gene and protein expression) [43]. The formation of tissue or substrate specific focal contacts results in appropriately activated mechanotransduction pathways (ErK, JNK, FAK) which alter cell function and control cell fate [44,45]. Hence, the following alterations in gene expression and cytokine secretion begins with cell attachment to substrata via focal contacts and therefore the ability of ADM PDMS surfaces to promote enhanced cell adhesion is fundamental to the success of the surface as a biomaterial. Equally, BDF proliferation was found to be increased on ADM PDMS surfaces through MTT cell viability assay and qRT-PCR for the PCNA gene. PCNA becomes highly expressed during DNA synthesis and repair [46]. Proliferating cells are metabolically active and constantly synthesising new DNA prior to mitosis and therefore PCNA is an indication of these processes [47]. As mRNA transcription does not always result in corresponding protein translation, the MTT cell viability assay confirmed that an increase in cell proliferation (protein level) was indeed promoted by ADM PDMS topographies and validated the

Fig. 7. Scanning electron microscopy (SEM) images revealed specific attachment of breast derived fibroblasts (BDFs) to topographical features on acellular dermal matrix (ADM) polydimethylsiloxane (PDMS) surfaces and spindle shaped BDF morphology. Scanning electron microscopy (SEM) images of breast-derived fibroblasts (BDFs) on acellular dermal matrix polydimethylsiloxane fabricated (ADM PDMS F) (A–B), acellular dermal matrix polydimethylsiloxane casted (ADM PDMS C) (C–D), smooth implant surfaces (E–F) and textured implant surfaces (G–H). Insets show focal contact formation of BDFs to specific topographical features on ADM PDMS surfaces (A and D); while cells are round and poorly spread on smooth and textured surfaces (F and H). (Black scale bars in A, C, E, G = ×400 magnification, in B, D, F, H = ×1000 magnification and insets = ×4000 magnification in (B), ×6000 magnification in (D) and ×2000 magnification in (F) and (H). The borders of BDFs on ADM PDMS surfaces are highlighted with a yellow dashed line to clearly demarcate cells. Black arrows point to cells attached to silicone surface. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
up-regulation in PCNA transcription. The cell apoptosis LDH assay is a measurement of cell survival as LDH is released into cell culture media by cells that have undergone apoptosis. Improved cell survival of BDFs on ADM PDMS surfaces was observed via reduced LDH levels in cell culture media and is consistent with the cell proliferation and immunofluorescence data.

Our results are consistent with the paradigm that cellular functions such as attachment, proliferation and survival can be influenced by underlying substrate topographies, independent of surface chemistry, as it has been shown previously that cells are able to respond to numerous silicone geometries of various dimensions, down to 10 nm in height [48]. However, there have been few in vitro studies which have attempted to mimic basement membrane or ECM topography in silicone and the majority of previous studies were performed on arbitrary, periodic, non-variant topographies such as grooves, pits or pillars, with minimal consideration for mimicking a cell’s in-vivo niche microenvi-

ronment [49]. Nonetheless, there is a body of evidence to which this work adds to, such as the finding that biomimetic intestinal crypt-like topography in PDMS was found to enhance Caco-2 cell metabolic activity [50] and bioengineered limbal crypts in collagen hydrogels regulated limbal epithelial stem cell differentiation [51]. Furthermore, engineered hydrogel skeletal muscle architecture enabled highly differentiated myofiber formation [52]. Recently, an engineered “off the shelf” heart valve was manufactured, which has yet to be biologically evaluated beyond cell viability assays, but may significantly improve long-term valve performance, as a major limitation of currently available valve replacement’s is leaflet fibrosis [53]. In this work we found that at both mRNA transcription and protein translation levels, the secretion of pro-inflammatory/pro-fibrotic cytokines IL8, TNFα and TGFβ1 were reduced by BDFs on ADM PDMS surfaces in comparison to smooth and textured implant surfaces. IL8 and TNFα are acute phase inflammatory chemokines and cytokines, respectively, and have been associated with inflammatory conditions such as rheumatoid arthritis, psoriasis, cystic fibrosis and pulmonary fibrosis [54,55]. TGFβ1 is a pro-fibrotic cytokine which has been implicated in the formation of fibrotic disorders such as hypertrophic scarring, keloid scarring and Dupuytrens contracture/disease via transformation of fibroblasts into myofibroblasts and their persistence within tissue beyond normal physiological time scales [56]. Significantly, IL8, TNFα and TGFβ have all been found to be up-regulated in contracted fibrotic breast capsules around commercially available silicone implants [57–59]. Therefore, it is particularly relevant that ADM PDMS topographies were found to down-regulate these pro-inflammatory/pro fibrotic mediators at both mRNA transcription and protein levels, in comparison to commercially available smooth and textured implant surfaces. HSP60 is a gene which is up-regulated in stressed cells [60]. It has been identified within the peri-implant protein milieu, in association with contracture formation [61]. It was found to be down-regulated in BDFs on ADM PDMS surfaces, which may indicate that cells are less stressed on ADM PDMS surfaces in comparison to their response to commercially available silicone implant surfaces.

At 48 h, collagen type 1 was found to be up-regulated in BDFs on ADM PDMS surfaces in comparison to smooth and textured implant surfaces, which returned to baseline levels by one week. In agreement with our work, nanoscale topographies on low-adhesion

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Fig. 8. A schematic illustration is proposed as a hypothetical mechanism for enhanced cellular response of breast derived fibroblast (BDFs) on acellular dermal matrix polydimethylsiloxane (ADM PDMS) topography in comparison to BDFs on smooth and textured silicone implant surfaces. As ascertainment at mRNA transcription and protein translation levels, BDFs on ADM PDMS surfaces displayed improved cellular attachment to underlying ADM PDMS topography which was clearly visualised through vinculin staining within focal contacts in immunofluorescence stained images (1). The formation of stable focal contacts is critical to mechanotransduction pathways, which are potent mediators of cell function. Therefore, the formation of specific, conformational, focal contacts, directed by ADM topographical cues, enables BDFs to convey messages of their surrounding environment to the nucleus via various mechanotransduction pathways (2). We evidenced that this ‘activation’ of mechanotransduction pathways resulted in the alteration of genes controlling cell attachment, proliferation, inflammation and ECM deposition (3). Furthermore, we found that this alteration in mRNA transcription levels were realised at a protein level through attenuation of the secretion of a number of pro-inflammatory and pro-fibrotic cytokines, in addition to increased secretion of pro-wound healing cytokines (4). Taken together, this in vitro study found ADM PDMS F and C surfaces were able to promote cell attachment, proliferation and survival of BDFs, in addition to mitigating the acute in vitro foreign body reaction of BDFs to silicone surfaces in comparison to smooth and textured implant surfaces. Schematic inspired by Vogel and Sheetz (2006). Local force and geometry sensing regulate cell functions. Nat Rev Mol Cell Biol. 2006; 7:265–75. Adapted from: Vogel V, Sheetz M. Local force and geometry sensing regulate cell functions. Nat Rev Mol Cell Biol. 2006; 7:265–75.
substrates (as PDMS inherently is) have been found to up-regulate collagen 1 expression as a result of improved cell attachment [62]. It follows that if cells are able to form stable focal adhesions with underlying substrate, which promotes metabolic activity, fibroblasts will proliferate and begin to synthesise, degrade and remodel collagens at the surface—cell interface, as they would within native ECM. However, although early up-regulation of collagen 1 was induced in BDFs on ADM PDMS surfaces, to understand how this may relate to peri-implant capsule formation, it is imperative in future work to measure deposition of collagen and other ECM proteins on the substrate surface, in addition to analysis of the levels of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and collagenases. As secretion of TGFβ1 was not increased by BDFs on ADM PDMS surfaces, we hypothesise that the up-regulation of collagen type 1 mRNA transcription was not due to interaction with the TGFβ1 receptor and activation of SMAD signalling pathways but may be due activation of mechanotransduction pathways via integrins [63].

Normalised cytokine profiling revealed the attenuation of a number of inflammatory cytokines by BDFs on ADM PDMS surfaces in comparison to smooth and textured implant surfaces. Although the pathogenesis of capsular contracture appears to be multifactorial, an acute inflammatory response against the implant surface seems central to its formation and contracture rates have been found to correlate positively with levels of inflammation [64]. Moreover, Wolfram (2012) found that dysregulated intracapsular regulatory T cells and a TH1/TH17 weighted immune response may lead to excessive fibrosis and contracture formation. The authors detected increased levels of the pro-inflammatory cytokines IL6, IL8, IL17, IFNγ and TGFβ1 in the media of isolated intracapsular T cells, which may have been contributing to capsular contracture progression [65]. As the secretion of these same cytokines were reduced by BDFs on ADM PDMS surfaces, ADM topography in silicone may potentially avert the in vivo progression from capsule formation to contracture through mitigation of the inflammatory response towards the silicone implant surface.

Taken together, the evidence demonstrated in this work confirms that ADM PDMS topographical cues induce alteration of mRNA transcription and protein translation levels of a number of key mediators of the acute foreign body response to silicone (Fig. 8). This is in agreement with Chen et al. (2010) that found nano and micron scale topography could affect foreign body response of macrophages in comparison to planar controls [66]. In addition, our work corresponds with the results in Yanez-Soto et al. (2013) that demonstrated corneal epithelial wound healing was enhanced on biomimetic basement membrane topography in comparison to flat controls [67]. However, to ascertain whether the positive, in vitro effects of ADM topography have the potential to alter the long term in vivo foreign body response to silicone was beyond the scope of this study. Despite our promising findings, it is acknowledged that these results are purely the outcome of in vitro studies and therefore it is crucial that long term clinical studies in animals or humans are performed to uncover the true performance of this biomimetic surface and analysis of the in vivo response would be highly recommended.

5. Conclusion

Biomimetic silicone topographies were successfully engineered through a novel 3D grayscale fabrication technique, which represents a useful strategy to generate surfaces capable of significantly enhancing silicone implant surface performance. Silicone surfaces functionalised with ADM topography induced specific responses in breast-derived fibroblasts, which resulted in attenuation of the acute in vitro foreign body reaction to surfaces for up to one week. This data indicates that ADM topography may induce activation of mechanotransduction pathways through the formation of specific focal contacts with biomimetic surface features, which leads to down-regulation of pro-inflammatory/pro-fibrotic genes in addition to the reduced secretion of pro-inflammatory/pro-fibrotic cytokines. Biomimetic topographies require considerable consideration in the pursuit to enhance biocompatibility of current implant surfaces. In particular, engineered silicone mammary implant surfaces containing biomimetic ADM topology may potentially lead to reduced rates of capsular contracture formation and further work should seek to evaluate functional performance in an in vivo model.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.02.003.

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