Development, Fabrication and Evaluation of a Novel Biomimetic Human Breast Tissue Derived Breast Implant Surface

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Abstract: Breast implant use has tripled in the last decade with over 320,000 breast implant based reconstructions and augmentations performed in the US per annum. Unfortunately a considerable number of women will experience capsular contracture, the irrepressible and disfiguring, tightening and hardening of the fibrous capsule that envelops the implant. Functionalising implant surfaces with biocompatible tissue-specific textures may improve in-vivo performance. A novel biomimetic breast implant is presented here with anti-inflammatory in-vitro abilities.

Topographical assessment of native breast tissue facilitated the development of a statistical model of adipose tissue. 3D grayscale photolithography and ion etching were combined to successfully replicate a surface modelled upon the statistics of breast tissue.

Pro-inflammatory genes IL β 1, TNF α , and IL6 were downregulated ($p < 0.001$) and anti-inflammatory gene IL-10 were upregulated on the novel surface. Pro-inflammatory cytokines Gro-Alpha, TNF α and neutrophil chemoattractant IL8 were produced in lower quantities and anti-inflammatory IL-10 in higher quantities in culture with the novel surface ($p < 0.01$). Immunocytochemistry and SEM demonstrated favourable fibroblast and macrophage responses to these novel surfaces.

This study describes the first biomimetic breast tissue derived breast implant surface. Our findings attest to its potential translational ability to reduce the inflammatory phase of the implant driven foreign body reaction.

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15th August 2016

To,
Professor W.R. Wagner
Editor-in-chief
Acta Biomaterialia

Dear Professor Wagner,

I am writing to you with regard to submitting an original manuscript for consideration for publication in Acta Biomaterialia. In this novel translational study, we describe a combined materials and biological approach to addressing a common longstanding clinical problem, referred to as breast capsular contracture which occurs following surgical breast implantation. Women experience (rates of up to 17% in 450 000 per annum in the US alone) capsular contractures (a painful and disfiguring fibrotic reaction to the silicone implant surface) following breast augmentation for cosmetic or oncological reasons. Capsular contractures have long been associated and thought to arise due to the body's reaction to the non-biomimetic, non-biocompatible, roughened surfaces of the commercially available silicone breast implants.

This original work describes a unique approach taken in designing, producing and testing a biomimetic implant surface derived directly from the intricate micro-anatomical features of human breast tissue. In addition, we demonstrate the *in-vitro* ability of this surface to successfully reduce the inflammatory phenotype of human fibroblasts and macrophages. We believe this unique approach will prove to be an inimitable resource and likely to be cited first in future literature describing the next generation of breast implant surface development and manufacture.

Importantly, of note, lately there has been a concerted effort in order to reduce breast implant associated capsular contracture, so as to prevent and reduce the unwanted development of anaplastic lymphomas associated with some of the existing implant surfaces. We believe this emerging work is the next intuitive step in realising a truly biocompatible and biomimetic implant surface designed for the surface of the breast tissue.^[1]

I look forward to hearing from you.

Yours Sincerely,

A handwritten signature in purple ink, appearing to read 'Bayat', with a stylized flourish at the end.

Dr. Ardeshir Bayat
Associate Professor
Plastic and Reconstructive Surgery Research

Development, Fabrication and Evaluation of a Novel Biomimetic Human Breast Tissue Derived Breast Implant Surface

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20th October 2016

Dear Professor W.R. Wagner

Re: Response to Reviewers

Thank you for requesting reviews of our paper and for your additional comments received on the 16/11/16. We have made amendments to our manuscript to reflect these recommendations. Below is an itemised list of our alterations and responses to the reviewers.

We look forward to hearing from you.

Yours Sincerely,

Dr Ardeshir Bayat

Associate Professor

Plastic and Reconstructive Surgery Research

Reviewer #1: *This interesting pre-clinical paper looks at a breast implant surface texture that mimics the topography of adipose tissue removed from patients undergoing breast reduction surgery. The methods are well described and show how technological advances in surface topography can be transferred onto medical grade silicone. When this surface was mixed with macrophages and fibroblasts in vitro, it showed down-regulation of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines. Imaging demonstrated good adhesion of cells to the surface.*

This is very early in the development of biologically derived implant surfaces and I encourage the authors to proceed with further development and in vivo trials of their newly derived implant surface.

It is emerging, however, that biofilm infection onto implant surfaces (both breast and other implants) play a major role in causing re-operation and implant failure. The combination of a more bio-compatible surface with anti-infectives should be the ultimate goal for all biomaterials to ensure that the body has time to incorporate the new surface free of infection. The authors should make reference to the growing body of work linking biofilm to contracture.

Response: Thank you for your suggestion to include a statement on the link between biofilm and capsular contracture. We recognise the effect that implant bacterial colonisation has on the development of capsular contracture and have re-structured paragraph 3 of the introduction to reflect this.

Reviewer #2: *The authors deal with the problem of breast implants; their use is wide even if capsular contracture is a very frequent complication. The onset of periprosthetic capsule is strictly linked to inflammation and often associated to ALCL. In this scenario, the possibility to improve the quality of breast implant surface and consequently their features deserves attention.*

I think that the manuscript is well written, the methodological approach is detailed and complete with the use of different and updated techniques.

I have just some minor concerns:

-the introduction is too long, especially if compared with the discussion. Please, make shorter the introduction and develop more the discussion

Response: Thank you for your comments. We have shortened the introduction section and developed the discussion section.

-better clarify the time of cell culture in material and method section

Response: We have added a second paragraph to section 3.15 of the methods section to clarify the periods of cell culture.

-add "a" and "b" to graph 1

Response: We have added "A" and "B" to graph 1

-make attention to some typing errors

Response: We have proof read our paper and corrected any typing errors identified

Reviewer #5: *The authors present a generated, produced and analysed breast implant texture derived from human breast tissue. Topographical assessment of native breast tissue facilitated the development of a statistical model of adipose tissue. Photolithography and ion etching were employed to successfully replicate the surface model. Immunocytochemistry and scanning electron microscopy images indicate favourable fibroblast and macrophage responses to these novel surfaces with anti-inflammatory in-vitro abilities.*

The overall work has been conducted with care and, in my opinion, is of a significant advance in the field. The experimental fabrication results are interesting.

I have the following suggestion/comments for the authors:

1. In Figure 4, it will be nice to put the graphs A2 and B2 in the same graph, to label with units the scale (horizontal and vertical). If the authors decide to keep the 3D images represented in the Figure A3 and B3, then also the scale bars should be clear represented with units.

Response: Thank you for your suggestion. We have updated Figure 4 and the manuscript and figure captions to reflect these changes.

Additional Changes:

Any further changes to the manuscript have been underlined and highlighted in yellow. The reference numbers have been changed to reflect any changes.

Editor:

1. Please list all author names in the references as per the Guide for Authors

Response

This has been corrected in the manuscript

2. Please include scale bars in Figure 2 as well as define all scale bar lengths for Figures 1, 2 (once added), 3, 5 in their respective figure legends

Response

This has been corrected in figure 2 and in the legends for figures 1,2,3 and 5.

TISSUE COLLECTION
AND PROCESSING

LASER CONFOCAL
IMAGING OF
TISSUE

3D PHOTOLITHOGRAPHY
OF ADIPOSE SURFACE

TRANSFER OF
ADIPOSE SURFACE
TO SILICONE

NOVEL ADIPOSE SURFACE

PCR & CYTOKINE EXPRESSION
DEMONSTRATES REDUCTION
IN MACROPHAGE INFLAMMATION
BY NOVEL SURFACE

STATEMENT OF SIGNIFICANCE

Breast implants are still manufactured using outdated techniques and have changed little since their inception in the 1960's. Breast implants can cause a medical condition, capsular contracture which often results in disfigurement, pain, implant removal and further surgery. This condition is due to the body's reaction to these breast implants. This article describes the successful manufacture and testing of a novel breast implant surface inspired by the native shapes present in breast tissue. Results show that this novel implant surface is capable of reducing the negative reaction of human cells to these surfaces to help reduce capsular contracture. This work represents the first steps in producing a more successful breast implant.

1 ABSTRACT

Breast implant use has tripled in the last decade with over 320,000 breast implant based reconstructions and augmentations performed in the US per annum. Unfortunately a considerable number of women will experience capsular contracture, the irrepressible and disfiguring, tightening and hardening of the fibrous capsule that envelops the implant. Functionalising implant surfaces with biocompatible tissue-specific textures may improve *in-vivo* performance. A novel biomimetic breast implant is presented here with anti-inflammatory *in-vitro* abilities.

Topographical assessment of native breast tissue facilitated the development of a statistical model of adipose tissue. 3D grayscale photolithography and ion etching were combined to successfully replicate a surface modelled upon the statistics of breast tissue.

Pro-inflammatory genes IL β 1, TNF α , and IL6 were downregulated ($p < 0.001$) and anti-inflammatory gene IL-10 were upregulated on the novel surface. Pro-inflammatory cytokines Gro-Alpha, TNF α and neutrophil chemoattractant IL8 were produced in lower quantities and anti-inflammatory IL-10 in higher quantities in culture with the novel surface ($p < 0.01$). Immunocytochemistry and SEM demonstrated favourable fibroblast and macrophage responses to these novel surfaces.

This study describes the first biomimetic breast tissue derived breast implant surface. Our findings attest to its potential translational ability to reduce the inflammatory phase of the implant driven foreign body reaction.

2 INTRODUCTION

Breast Implant based surgery is performed in a variety of settings, from reconstruction for congenital anomalies and post-mastectomy defects, to augmentations for cosmetic purposes. 1,773,584 breast augmentations for aesthetic reasons were declared worldwide to the International Society of Aesthetic Plastic Surgery in 2014.(1) Breast implants are not without their inherent complications, with the pathological contracture of the capsule being the most frequent and predominant cause for patient dissatisfaction post-implantation.(2, 3) Contracture rates occur in a not insignificant 17.5% of implant based procedures.(4)

Capsular contracture is the pathological fibrous amplification of the foreign body response to the breast implant. Typical progression of the foreign body reaction results in a functional capsule, which helps to maintain implant position.(5) In contracted capsules this response is exaggerated and the capsule becomes thickened, fibrotic and less pliable, which can manifest as mastalgia, breast firmness and a poor aesthetic result ~~and which~~ often culminates in re-operation.(6)

Capsular contracture is a ~~condition with~~ multifactorial ~~origins condition~~. Filler material, sub-muscular placement of the implant, adjuvant radiotherapy, ~~and~~ bacterial colonisation ~~and implant surface texture~~ have been implicated in its development.(7) Recently a growing body of evidence has linked biofilm formation with capsular contracture.(8) Sterile precautions have proved important in reducing capsular contracture rates.(9) Capsular contracture has also been linked to implant surface texture, the focus of this paper.

Current breast implants, with an elastomer shell and saline or silicone gel filler are derived from a 1960's design, which evolved into a textured, polyurethane coated implant in the late 1960's.(10, 11) Concerns that polyurethane was pro-cancerous led to a moratorium on

this surface in the US market. Recognising the ability of polyurethane to alleviate contracture rates and due to a conceived perception that this was a consequence of implant texture, several silicone implant surface textures which attempted to mimic the polyurethane surface were developed.(12, 13) ~~This basic shell and filler construction has endured.~~ Implant textures which are currently available are manufactured by either imprinting salt or polyurethane foam into the uncured implant shell or by moulding the implant surface from a pre-textured, often sandblasted mold.(14) Whilst the manufacturing techniques employed are crude, their development is constrained by a litigious legacy and the textures produced remain poorly characterised. Recent systematic review and a meta-analysis, have both demonstrated the protective effect of implant texture on capsular contracture. (15-17) No study has managed to standardise the confounding variables of capsular contracture and demonstrate that one particular implant surface is most efficient at reducing this condition. ~~, however recent reports have found a link between one specific type of implant surface texture and anaplastic large cell lymphoma.~~

Biological inspiration has been utilised in many branches of engineering to elucidate new approaches to technical problems; biomimicry is the extension of this principal.(18)
Biological surfaces have evolved over millions of years to produce materials and topographies that are highly organised from the macro- to the nano-scale. Micro and Nano surface topographies have been shown to influence cell proliferation, attachment, adhesion, migration and morphology.(19) Many of the morphological topographies, which exist *in-vivo* that interact with cells are those from the extra cellular matrix (ECM). The ECM of different tissue types promote the production of distinct tissue morphologies from where they are derived.(20, 21) ~~Disruption of the planar arrangement of the capsule which surrounds the implant and the promotion of breast tissue ingrowth by textured surfaces have been~~

postulated to be the factors that help reduce contracture rates. The most aggressive implant textures, the “macro textures” with deep surface interstices, have also been shown to shed particulate silicone, increase inflammation within implant capsules and potentiate the development of double capsules and late seromas.

Biological inspiration has been utilised in many branches of engineering to elucidate new approaches to technical problems; biomimicry is the extension of this principal. Biological surfaces have evolved over millions of years to produce materials and topographies that are highly organised from the macro to the nano scale. Each of these topographical hierarchies plays an important role in the function of these natural surfaces. Surfaces that mimic the physiological ECM have been theorised to decrease the foreign body reaction and modulate the immune response, improving the bodies reaction to these surfaces.(22)

Breast implants are frequently inserted into the sub-glandular tissue plane, between the adipose tissue of the breast anteriorly and the pectoralis muscle fascia posteriorly. In this study adipose tissue was isolated from this tissue interface to generate a novel surface, the “original adipose” surface. This surface represents a tissue specific extension of a recent publication by another group member, Kyle et al. who generated an acellular dermal matrix inspired surface.(23)

In the process of achieving this novel adipose surface, it was recognised that breast tissue has an inherent, measurable surface structure that was modelled and generated to produce a secondary “modelled adipose” surface. Nano-technology has largely unaffected the development of breast implants and thus the aim of this study was to integrate nano-technological approaches with biological inspiration. (24) The rationale behind this approach was that these textures could promote responses from cells important in dictating

the foreign body response to the implant at a nano and micro scale, whilst also providing a surface which was comprised of textures with larger tissue dimensions capable of integrating with breast tissue.

To assess the ability of these surfaces to mediate the foreign body and inflammatory response in an *in-vitro* environment macrophages, the cell type shown to have the most powerful effect on the processes of tissue repair, remodelling and biocompatibility were utilised.(25) THP-1 human macrophages were cultured on the novel surfaces developed and their morphology, gene expression and cytokine secretions quantified to ascertain the effects of these surfaces.(26)

The ultimate aim of this study therefore was to produce a site-specific, hierarchically textured implant topography with provenance from the breast.

3 MATERIALS AND METHODS

This article describes the characterisation and fabrication of two novel polydimethylsiloxane (PDMS) implant surfaces derived from native breast tissue topography; the original adipose and the modelled adipose surface. Tissue samples used in this study were obtained through the Plastics and Reconstructive Surgery Research (PRSR) Skin and Tissue Bank ethics (North West Research Ethics Committee Ethics Code – 11/NW/0683 Date: 2/11/2011). Informed consent was obtained from patients for the use of their tissue in this study. All breast tissue processing was done at a Human Tissue Authority licensed laboratory.

3.1 COLLECTION OF BREAST TISSUE AND SAMPLE FIXATION

Breast tissue from three patients was collected from elective cosmetic breast reduction operations and transferred to the laboratory in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, UK) supplemented with 1% penicillin and streptomycin (PAA laboratories, Pasching, Austria), 1% L-glutamine (PAA) and 10% Fetal Bovine Serum (PAA). Patients had no past medical history of any malignancy or fibrotic conditions, none were obese and none smoked. Patient demographics are included in Supplementary Table 1.

Breast tissue was washed thoroughly in warmed phosphate buffered saline (PAA) supplemented with 1% Penicillin and Streptomycin (PAA) before the lobules of breast adipose tissue were dissected. Breast adipose tissue was fixed in paraformaldehyde 2% (Sigma-Aldrich), glutaraldehyde 2.5% (Sigma-Aldrich) and 0.1M hepes buffer (Formedium, UK) for 7 days at 4°C.

Adipose tissue was washed four times in distilled water for 15 minutes each and then post fixed in osmium tetroxide 1% (Agar Scientific, UK) in 0.1M hepes (Formedium, UK) for 1 hour. Following two further wash steps in distilled water of 15 minutes each, the tissue was

dehydrated using graded acetone steps of 25%, 50%, 75%, 90%, and 100%, for 15 minutes at each step. Three further washes in 100% acetone were then performed before the tissue was critical point dried (Quorum Technologies Ltd., UK).

3.2 IMAGING, SAMPLE MEASUREMENT AND GENERATION OF ORIGINAL ADIPOSE SURFACE

Fixed adipose tissue was mounted on a scanning electron microscopy (SEM) stub and scanned using an X-100/X-200 series 3D laser confocal microscope with a 50x objective (Keyence, Japan). SEM mounted samples were sputter coated with gold and palladium for 120 seconds using a SC7620 sputter coater (Quorum Technologies Ltd, UK) and imaged using an Quanta 250 FEG SEM (FEI, Oregon, USA). Images from the laser confocal microscope were then exported as an .asc point group data file. This .asc file was opened in Gwyddion, Image background subtraction using a polynomial fit function was used before the data was converted into an 8bit grayscale bitmap, containing the height data in this file as 256 grayscales. (27) This created the original adipose surface.

3.3 MEASUREMENT OF THE ADIPOSE SURFACE DEFINES THE MODELLED ADIPOSE SURFACE

The original adipose surface was further analysed to define its statistical characteristics. Matlab code was engineered to recognise hemisphere boundaries within grayscale images using watershed segmentation. Maximal height of each of these segments was then established by the code to recognise the centroid position of each hemisphere within these segments, before a 3D sphere fit function established the closest fit of a sphere to each of these segments. Code also generated data for the nearest neighbour distance (the distance between one centroid and the next) and hemisphere diameter. Combining the hemi-sphere diameter and relation of each hemi-sphere to its nearest neighbour a new modelled adipose

surface was generated with the same statistical attributes as the native tissue and exported as an 8bit grayscale bitmap.

3.4 MEASUREMENT OF THE SECONDARY ADIPOSE TEXTURE

As micro and nano textures have been demonstrated to influence cell characteristics, the micro and nano surface texture on the surface of the adipocytes was characterised. Fixed adipose tissue was scanned using a Dimension Icon® Atomic Force Microscope (AFM) (Bruker, UK). Samples were scanned using TAP-150A (Bruker) tips (nominal $k = 5\text{N/m}$). Areas on the surface of the adipocytes measuring $20 \times 20\mu\text{m}$ were imaged in PeakForce Tapping™ mode. Surface scans were exported to Gwyddion and flattened using the polynomial fit function. Height data was exported at this point and plotted as a histogram to establish the height profiles of the surface. To measure the fibres on the surface of the adipocytes a laplacian of Gaussian edge technique was used to highlight fibres within the scan and exported as an 8bit bitmap. These images were then opened in Fiji, a binary threshold established, before fibres were scanned using the BoneJ plugin to establish fibre widths, spacing and fractal dimension.(28) Angles of fibres were also measured to an arbitrary 0 degrees, to establish fibre direction.

3.5 3D PHOTOLITHOGRAPHY

Following the generation of a grayscale bitmap of both the original adipose and modelled adipose surfaces this data was transferred into PDMS using maskless grayscale lithography of a SU-8 photoresist mould.

3.6 GENERATION OF THE BASE LAYER

In a class 100 clean room a $4 \times 4\text{ cm}$ plain silicon wafer was sonicated for 10 minutes in acetone, isopropyl alcohol (IPA) and distilled water, dried with nitrogen gas and baked on a

hot plate for 10 minutes at 150°C. Hexamethyldisilazane (Microchem, USA) was then spun onto the wafer at 3000rpm for 45 seconds before it was returned to the hotplate for a further 10 minutes. As an adhesion layer, SU8-2000.5 (Microchem, USA) was spun onto the wafer at 3000rpm and ramp baked from 25°C to 95°C before being held at 95°C for 5 minutes. The SU-8 coated wafer was flood exposed to 454nm wavelength light for 20 seconds before being returned to the hotplate for a further 5 minutes. The SU-8 coated wafer was developed in EC solvent (Chestech Ltd, UK) for 1 minute before being rinsed in IPA and baked at 150°C for 10 minutes.

3.7 THE PRIMARY TEXTURE

A secondary, thicker layer, of SU-8 2025 (Microchem, USA) was then spun onto the surface of the base layer coated wafer at 4000rpm, corresponding to a thickness of 30µm. This was followed by a ramped pre-exposure bake from 25°C to 95°C before being held at 95°C for 10 minutes.

3.8 EXPOSURE

The wafer was exposed to either the original adipose or modelled adipose pattern using a laser writer (LW405, Microchem, Italy). Bitmap images were inverted prior to use using imageJ.(29) Inverted bitmap images were loaded into the laserwriter software and pixel size was registered to 0.5µm in X and Y. A 40x objective was used to expose the photoresist and laser power dose was dictated by the corresponding grayscale level (0 being no dose and 256 being maximum dose).

3.9 DEVELOPMENT

Once the pattern had been transferred, the wafer was subject to a post exposure bake for 10 minutes at 95°C before development of the pattern in EC solvent for 10 minutes with gentle agitation. Substrates were then rinsed in IPA and dried.

3.10 SECONDARY TEXTURE

In the case of the modelled adipose surface, to impart a roughness onto the surface which had statistically similar properties to that of native adipose tissue, an oxygen etch recipe was used to texture the surface of the SU-8 master. A Plasmalab 100 System (Oxford instruments, England) was used to etch the SU-8 surface for 6.5 minutes at 5mTorr of pressure, with an RF power of 5, ICP pressure of 300 and an O₂ flow rate of 45Sccm.

3.11 TRIMETHYLCHLOROSILANE VAPOUR ACTS AS A RELEASE LAYER BETWEEN SILICONE AND MASTER MOLD

Trimethylchlorosilane (TMCS) (Sigma, UK) was used as a releasing layer to passivize the surface of the SU-8 mould and to reduce silicone bonding to this master template. The SU-8 master and 0.5mls of TMCS were placed under vacuum in a dessicator. The vacuum was held for 1 hour in order to silanize the wafer.

3.12 MEDICAL GRADE SILICONE CREATES ADIPOSE SURFACES IN SILICONE

To mold subsequent silicone from each master wafer, MED 6215 silicone (Nusil California, US), a medical grade silicone with permission for use in humans was used. Silicone was spun onto the wafer at 200rpm and cured overnight in an oven at 65°C before being peeled from the surface of the SU8 to produce both the original adipose and modelled adipose surfaces.

3.13 SUBSTRATE CHARACTERISATION

Fabricated implant surfaces were characterised using AFM (Dimension Icon® Atomic Force Microscope, Bruker, UK) a laser confocal microscope (X-100/X-200 series 3D laser confocal microscope Keyence, Japan) and a Scanning Electron Microscope (Quanta 250 FEG SEM, FEI, Oregon, USA).

3.14 PREPARATION OF CULTURE SURFACES

Manufactured surfaces were cut into 15mm disks using a punch cutter, before being adhered to the bottom of a 24 well plate (Corning Incorporated, New York, USA) using a drop of uncured MED 6215 silicone, which was cured overnight at 65°C. Tissue culture plastic (TCP) and a smooth MED6215 silicone surface, manufactured by curing PDMS on a plain silicon wafer were used as controls. Prior to cell seeding manufactured surfaces were washed twice with PBS and sterilised using 70% ethanol for 1 hour. Manufactured implants were air dried for half an hour and washed twice with PBS.

3.15 GROWTH OF THP-1 MACROPHAGES

Human THP-1 monocyte cells were cultured in RPMI 1640 medium, “normal media” (Sigma Aldrich, UK) supplemented with 1% penicillin and streptomycin (PAA), 1% L-glutamine (PAA) and 10% Fetal Bovine Serum (FBS) (PAA) in T75 TCP flasks (Corning Incorporated, USA). Monocytes were incubated at 37°C in 5% CO₂ and media was changed weekly. THP-1 monocytes were treated with 25nM phorbol myristate acetate (PMA) (Sigma Aldrich, UK) for 24 hours to facilitate differentiation into macrophages. After differentiation, macrophages were washed twice with normal media and rested for a further 24 hrs in normal media. Prior to seeding, macrophages were serum starved for 24hrs in 0.5% FBS

media to synchronise these cells. Cells were seeded at 250,000 cells per well of a 24 well culture plate (Corning Incorporated, USA) and each experiment was performed in triplicate.

Gene expression and cytokine analysis was performed at 12 and 24 hour time points, whilst immunocytochemistry and SEM was performed at 24 hours.

3.16 RNA EXTRACTION, CDNA SYNTHESIS AND QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION

Cells were washed once with PBS, before being lysed in buffer RLT (Qiagen, UK) and the lysate collected. RNA was extracted using the Qiagen RNA Mini kit as per manufacturer's instructions. RNA purity and quantity was assessed using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) before RNA was transcribed to cDNA using a qScripts cDNA synthesis kit (Quanta Biosciences, USA). Quantitative real time polymerase chain reaction (RT-PCR) was performed on a LightCycler 480 machine (Roche Diagnostics, Germany) as described previously.⁽³⁰⁾ The gene expression of Tumour Necrosis Factor Alpha (TNF alpha), Interleukin Beta1 (ILB1), Interleukin 6 (IL6), Interleukin 10 (IL10) and Mannose Receptor (CD206) were analysed. Primers and probes were designed using the Universal Probe Library and purchased from Sigma Aldrich, UK.

3.17 INFLAMMATORY MARKER CYTOKINE ARRAY

Cell culture media was aspirated from the cell culture well at each time point and stored at -80°C until further use. Luminex analysis was performed by ProcartPlex™ Multiplex Immunoassay (eBioscience, Austria) for human IL-1RA, IL-1beta, IL-6, IL-8, IL-10, IL-12, TNF alpha, IFN gamma and GRO alpha as per manufacturer's instructions.

3.18 IMMUNOCYTOCHEMISTRY

Immunocytochemistry was performed on breast-derived fibroblasts for vinculin, F-Actin and DAPI. Immunocytochemistry was performed on macrophages using Integrin α -v, F-Actin and DAPI. Disks of the manufactured implant surfaces were cut with a 6mm punch biopsy, adhered to 8mm circular cover-slips using 3 μ l of mixed MED-6215 silicone and cured at 65°C overnight, sterilising and seeding with 10,000 macrophages or 5,000 fibroblasts.

After 24 hours of cell growth, cells were fixed in 10% neutral buffered formalin (Sigma-Aldrich, UK) for 1 hr, washed in a Tris Buffered Saline (TBS) (Sigma-Aldrich, UK) and unreacted formalin was quenched by incubating in 1% glycine for 30 minutes. Fixed cells were permeabilised with 1% Triton-X 100 (Sigma-Aldrich, UK) for 30 minutes. Cells were then washed twice before blocking in 10% Bovine Serum Albumin (BSA, Sigma-Aldrich, UK) for 1hr. After washing, fibroblasts were incubated in Anti-Vinculin antibody at a dilution of 1:200 in 10% BSA (V9131, Sigma-Aldrich, UK) for 1 hr at room temperature (RT). After washing macrophages were incubated in Anti-Integrin α -v at a dilution of 1:750 in 10% BSA (ab124968, Abcam, Cambridge, UK) for 1 hr at RT. Cells were washed in TBS-Tween (TBST, 0.1% Tween in TBS) and incubated in the secondary antibody, anti-mouse (anti-rabbit) Alexa-Fluor-488 dye (Invitrogen, UK) at a 1:200 concentration for 1hr at RT in the dark. Cells were washed with TBST, incubated with Rhodamine Phalloidin (Sigma-Aldrich, UK) at a concentration of 1:1000 for 45 minutes, with 4', 6-diamidino-2-phenylindole (DAPI, 1:500 in TBST, Invitrogen, UK) for 15 minutes before they were washed twice and placed in PBS at 4°C until imaging.

3.19 CONFOCAL MICROSCOPY

Images were acquired using a Leica SP5 (Leica, Wetzlar, Germany) inverted laser-scanning confocal microscope with an x40 immersion lens. Samples were imaged in PBS and ImageJ was used to compile z-stack slices using the ImageJ maximum projection tool.

3.20 SCANNING ELECTRON MICROSCOPY

Growth media was removed and cells were washed twice with 0.1M hepes buffer (Formedium, UK). Cells were fixed in 2.5% glutaraldehyde (Sigma-Aldrich, UK) and 0.1M hepes buffer (Formedium, UK) for 1hr at RT. Following two wash steps in distilled water of 15 minutes each, the tissue was dehydrated through a graded series of ethanol, 25%, 50%, 75%, 90%, and 100%, for 15 minutes at each step. Three further washes in 100% ethanol were then performed before the cells and implant were critical point dried (Quorum Technologies Ltd., UK).

3.21 STATISTICAL METHODS

Experiments were performed three times in triplicate. To determine the difference in gene expression between the surfaces created, Δ CT values were calculated by subtracting CT values from the averaged reference gene Beta Actin. Relative gene expressions to TCP were calculated using the $\Delta\Delta$ CT method. Data was analysed in Graphpad Prism 6 and analysed using an ordinary one way Anova. If the *P* value was significant ($P < 0.05$) then a Tukey multiple comparisons test was used to compare values. Standard deviation was calculated

4 RESULTS

4.1 ADIPOCYTES AND ADIPOSE TEXTURE RETAIN STRUCTURE AFTER TISSUE FIXATION

S.E.M. illustrates that the breast adipose locule surface is composed of close packed spheres, with their outermost cap projecting and a web of ECM fibres running over their surface (Figure 1A).

4.2 LASER CONFOCAL MICROSCOPY DEFINES THE ORIGINAL ADIPOSE TEXTURE

Laser confocal measurements (Figure 1C) show that the adipocytes, the individual building blocks of this surface, retained their spherical nature and had not collapsed during fixation.

A bitmap of the surface measurement data, where height was represented as 256 greyscales was extracted from the laser confocal images (Figure 1B). This defined the original adipose surface.

4.3 EXTRACTION OF STATISTICAL DATA FROM THE ADIPOSE SURFACE

Code was written to extract statistical data of nearest neighbour distances and sphere diameters (Figure 2). Sphere diameters, or diameters of the adipocytes on the surface of breast adipose tissue, ranged from 15.5 μm to 102 μm (mean 42.82 μm , median 40.5 μm , S.D. 12.19 μm) (Graph [1A 1B](#)), whilst nearest neighbour distances (from one sphere or adipocyte centre to its closest neighbour's centre) ranged from 33.8 μm to 152 μm (mean 68.98 μm , median 66.7 μm , S.D. 16.7 μm) (Graph [1B 1A](#))

4.4 MATLAB CODE DEFINES THE BASE STRUCTURE OF THE "ADIPOSE SURFACE"

Based upon the recognised spheres on the surface of a sample (Figure 2B), Matlab code generated the base texture of the new grayscale modelled adipose surface. Figure 2C shows that the modelled adipocyte surface represents the surface seen in Figure 1B and the

hemispheres on its surface are aligned with those in Figure 1B's texture. Figure 1D also shows that when projected as a height map there is very little space between each hemisphere and the close packing nature of the surface is retained in the modelled surface.

4.5 AFM AND SEM ILLUSTRATE THE SURFACE TEXTURE ON THE SURFACE OF THE ADIPOCYTES

Further analysis revealed that the adipose surface was covered in a meshwork of intersecting extracellular matrix fibres, which lie across the surface of the adipocytes. Figure 3A and B illustrate a 20 x20 μm SEM and AFM representative scan of the surface of an adipocyte. By thresholding these AFM images in Gwyddion(31) and the BoneJ plugin in FIJI(32), data pertaining to the fibrous surface of the adipocyte can be established (figure 3C and D). Fibres were shown to have an average width of 107nm with an average spacing of 238nm. Fibres are also shown to be randomly aligned and have a fractal dimension of 1.956 when assessed using the box counting method. (See table 1)

4.6 AFM STATISTICALLY QUANTIFIES THE ADIPOSE SURFACE

To statistically replicate the secondary texture on the surface of the adipose surface height profiles on the surface of the adipocytes were measured. The height profiles varied between each adipocyte, but the information gained allowed the optimisation of the etch recipe used to texture the surface of the modelled adipose surface. AFM scans of the adipocyte surface gave the detail of the height profiles of these adipocytes as is illustrated in Graph 2A. The SU-8 mould was etched using a 6.5 minute oxygen etch recipe which produced a texture on the surface of the mould with statistics and a surface height which lay within the range of that measured in native breast adipocytes (Graph 2B).

4.7 LASER CONFOCAL MEASUREMENT DEMONSTRATES SUCCESSFUL TRANSFER OF THE MODELLED ADIPOSE SURFACE INTO SILICONE

Height data generated by the Matlab code and the actual PDMS surface created from this computer generated template are represented in Figure 4. [4:1A 4B](#) and [4:2A 4B1](#) and their corresponding profiles illustrate the successful transfer of this surface into PDMS.

4.8 GROWTH OF HUMAN DERIVED FIBROBLASTS AND MACROPHAGES SHOWS RECOGNITION OF SURFACE MACRO-TEXTURE

Fibroblasts and macrophages were grown in culture on the surface of the original adipose, modelled adipose and smooth control surfaces and imaged using immunocytochemistry and SEM (See figure 5)

Fibroblasts adhered to the original adipose, modelled adipose and smooth surface with a classical spindle shaped morphology illustrated by vinculin staining in Figure 5 A,E and I. Topographical cues on the surface of the original adipose surface can be seen to have prompted these fibroblasts to align to the valleys between the hemispherical shapes on its surface. This was not true of the modelled adipose surface, which may indicate that the secondary texture on the surface of the modelled adipose surface masked the primary hemispherical nature of the underlying topography. This may also indicate that the modelled adipose secondary texture allowed the fibroblasts to climb the side of the secondary topography and stretch over the primary texture beneath.

Macrophage adhesion to the surfaces included in this study showed that this cell type was not constrained by the underlying primary topography (See Figure 5 D and H). Macrophages were present in contact with the whole surface and the primary topography did not cause contact guidance of these cells. Macrophages have been shown to develop a more

classically activated phenotype when well spread on the surface of biomaterials. Macrophages cultured on the surface of the modelled adipose surface were less spread than those on the original adipose and smooth surfaces (Figure 5D, H and L).

4.9 MODELLED ADIPOSE SURFACE INDUCES A POSITIVE ALTERATION IN GENE AND CYTOKINE PROFILES CULTURED ON ITS SURFACE WHEN COMPARED TO SMOOTH AND ORIGINAL ADIPOSE SURFACES

Macrophages are classically considered to exist along a spectrum of M1 to M2 phenotype where M1, or classically activated macrophages are associated with inflammation and M2, or alternatively activated macrophages are considered wound healing.(33) Macrophages with a higher M2/M1 phenotype have been shown to reduce the fibrotic reaction to implants.

Genes and cytokines were chosen to assess the influence that these surfaces had on influencing the cellular phenotype. TNF-alpha, IL-6, IL-8 and IL-1 β are pro-inflammatory genes and associated with an M1 phenotype, whilst IL-10, CD206 and CCL2 are associated with the M2 macrophage.(33) Results demonstrate that the modelled adipose surface provoked a pro-M2 polarization in THP-1 macrophages when compared to the original adipose surface and smooth surfaces (see Figure 6).

At 12 hours macrophage genetic expression and cytokine production of the pro-inflammatory gene and cytokine TNF alpha was lowest on the modelled adipose surface (expression of TNF-Alpha vs original adipose $P= 0.0008$, vs smooth $P= 0.0008$. Cytokine production of TNF-alpha vs original adipose $P<0.0001$ and vs smooth $P= 0.0186$). At 12 hours the anti-inflammatory cytokine IL-10 was produced in highest quantities on the modelled adipose surface (production of IL-10 vs original adipose $P=0.0032$ and vs smooth

$P= 0.0186$) and had the highest expression on the modelled adipose surface at 24 hours (expression of IL-10 vs original adipose $P= 0.0007$, vs smooth $P= 0.0006$).

At 12 hours the pro-inflammatory genetic expression of IL-6 (vs smooth $P=0.0006$) and IL-1 β (vs original adipose $P=0.0007$ and vs. smooth $P=0.0006$) were lowest on the modelled adipose surface. At both 12 and 24 hours, the modelled adipose surface provoked the lowest expression of the neutrophil chemoattractant IL-8 (at 12 hours vs original adipose $p=0.0035$ and vs smooth $p= 0.0134$, at 24 hours vs original adipose $p=0.0098$ and vs smooth $p= 0.0477$). CD206, which is a marker of M2a phenotype had a higher relative expression in the modelled Adipose surface when compared to the smooth surface at 12 hours ($P=0.0001$) and a higher than the original Adipose surface ($P<0.0001$).

5 DISCUSSION

We have generated, produced and analysed the first breast implant texture derived directly from human breast tissue. This modelled surface texture has been shown to reduce the inflammatory response of macrophages and induce an alternatively activated macrophage phenotype in the initial biomaterial *in-vitro* response.

Breast implant textures are at the crucial interface between biomaterial and body tissue and have been shown to have demonstrable effects on the rates of capsular contracture. Macro-textured implants, with large coarse features have been theorised to promote the ingrowth of breast tissue and reduce the planar arrangement of fibroblasts on their surface, an effect which has been postulated to increase capsular contracture.(34) These textures have also been associated with the development of late seromas and double capsules.(35)

Macro-textures are often larger than the size of the cells that dictate the foreign body reaction, but are relatively smooth on the micro and nano-scale, the level of cellular recognition. (36) Identifying the importance of both of these levels of texture, this study describes the design of a biomimetic implant with a primary and a secondary texture engineered to positively influence the reaction to this implant at a cellular and tissue level.

Human derived breast adipose tissue has for the first time been examined and fixed. A custom built surface assessment algorithm has permitted the texture on the surface of native breast adipose tissue to be mapped. Defining the shapes and features within breast adipose tissue has allowed the statistical replication of these features in silicone which will ultimately facilitate the production of this surface at a scale required for commercialisation. Photolithographic techniques and oxygen plasma etching, techniques usually reserved for the microelectronics industry, have been employed to create a complex texture in the surface of medical grade silicone which mimics that found within the breast.

This novel surface is comprised of a plurality of close packed convex hemispheres. This close packing truncates the top of these hemispheres producing a network of interconnecting valleys which will permit the tessellation of native breast adipocytes and encourage the implants integration with breast tissue.

To examine the *in-vitro* effect of implant texture we used macrophages, a novel cell type when assessing the *in-vitro* reaction to breast implant surfaces and the cell type with an undisputed role in the regulation of the foreign body reaction.(37) The initial effect of the biomaterial *in-vivo* is dictated by the inflammatory response to the material and macrophages are pivotal to this process.

We compared our novel surface to three controls; TCP and two surfaces manufactured using the same medical grade silicone and curing schedules at the modelled surface, but with a smooth surface on one of these controls and a texture derived directly from the shape of adipose tissue itself; the original adipose surface. Macrophages arrive at the biomaterial wound interface within the first few hours, remain for several days and dictate the downstream foreign body reaction.(38) Pro-inflammatory cytokines IL-1 β ,(39), IL-6(40), TNF alpha(41) and IL-8(42) involved in macrophage activation and IL-10(43) a potent anti-inflammatory cytokine, all have important roles in the reaction of macrophages to biomaterial surfaces and in dictating the remainder of the foreign body reaction.(43) IL8 and TNF-Alpha have also been found to be upregulated in contracted fibrotic capsules.(44) Each of these genes or cytokines were favourably modulated by the texture on the surface of our novel modelled implant surface.

Traditionally, fibroblasts have been used to assess the *in-vitro* reaction to breast implant surfaces as they generate extra cellular matrix, the main component of the capsule and differentiate into myofibroblasts, the contractile smooth muscle cells that are thought to contribute to contracture. We chose to include fibroblasts in our assessment of the surfaces of the implants as an indicator of cell alignment, as this, with the smooth surface implants, has been theorised to increase contracture rates.(45) Fibroblasts are recruited from approximately 24-48 hours after the wound is created at the end of the inflammatory phase and at the beginning of the proliferative phase and are activated by the chemo-attractants and cytokines produced by macrophages.(46) Fibroblasts recognised the primary texture of the original adipose surface and tracked along the valleys between each hemisphere on the surface in a range of different directions, thus breaking the alignment seen in the smooth surface implants. Our results reinforce the evidence that two cell types are influenced by

surface topography and that the modulation of this response is possible by altering the topography of biomaterial surfaces independent of implant chemistry.(47)

The favourable response that appears to be provoked by these novel surfaces on the *in-vivo* environment is difficult to quantify; as are the *in-vitro* results presented by this study. The true effect on these implant surfaces and those already on the market, will be measured by *in-vivo* and animal studies.

6 CONCLUSION

We have identified a surface native to the human breast tissue, statistically analysed this surface, replicated it in medical grade silicone and assessed its *in-vitro* reaction using two cell types crucial in the development of the foreign body reaction. PCR and cytokine results demonstrate the favourable reaction of these cells to this novel surface and act as a foundation for further *in-vivo* work. Capsular contracture is a complex multifactorial condition, but these results demonstrate an important new direction in improving the implant texture of breast implant surfaces to potentially mitigate against the inflammatory response to biomaterial implant surfaces.

ACKNOWLEDGEMENTS

We would like to acknowledge our clinical colleagues and collaborators who have contributed and supported this work.

DISCLOSURE

This work was funded by Establishment Labs, Costa Rica. Establishment Labs did not take part in the study design, collection or analysis of the data; in writing the journal article or the decision to submit for publication.

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Figure 1

Figure 2 Revised

Figure 3

Figure 4 revised

Figure 5

Figure 6
[Click here to download high resolution image](#)

RT-PCR RESULTS

CYTOKINE RESULTS

A

B

Table 1

Width of fibres	107nm (S.D. 41nm)
Average spacing of fibres	238nm (S.D. 312nm)
Angle of fibres	92.361 degrees (range 0-179.7 degrees)
Fractal dimension	1.956 ($R^2= 0.999$)

Table 1: Properties of Nano Fibres Identified on the Surface of Adipocytes

Figure 1: Characterisation of the Adipose Surface. [\(scale bars = 500µm\)](#). A: SEM image of fixed native adipose tissue. B Laser Confocal Height map of the Original Adipose surface. C 3D projection of image B cross sectional profile.

Figure 2: Matlab Code Generated modelled Adipose Surface Work Flow [\(scale bars = 300µm\)](#). A: Watershed diagram recognising the valley boundaries between each hemisphere on the original adipose surface ([Figure 1Figure 1B](#)). B: Sphere centre position within each of the boundaries marked by a cross on the upper cap of each adipocyte recognised in A. C: New modelled adipose projection based on the statistical definition of the original adipose surface. D: 3D Projection of the modelled adipose surface.

Figure 3: Assessment of the Secondary Texture of the Adipose Surface [\(scale bars = 5µm\)](#). A & B: SEM and AFM images of an adipocyte illustrating the fibrous surface texture of this surface. C: image of spacing between fibres identified in B by “BoneJ”. D: Fibres identified in B.

Figure 4: [Laser Confocal Imaging Confirms Transfer of Adipose Surface in to Silicone. A Height map of modelled adipose surface. A1 Profile of height map of modelled adipose surface. B Laser confocal scan of actual PDMS adipose surface. B1 Profile of height map of actual PDMS adipose surface. C Graph of profiles of modelled and actual adipose surfaces.](#)

Figure 5: Immunocytochemistry And Scanning Electron Microscopy (SEM) Images Reveal Fibroblast and Macrophage Response to Original, modelled and Smooth Implant Surfaces [\(scale bars = 100µm\)](#). Surfaces are shown in columns and cell types or surface preparations in rows. Immunocytochemistry of vinculin (green) and F-actin (red) in figures A,E and I show that there were few notable differences in focal adhesion formation between each surface and that fibroblasts were well spread and spindle shaped. Fibroblasts morphology was affected by original adipose implant primary topography. B shows that fibroblasts aligned along the valleys of the surface, whilst fibroblasts on the modelled surface (F) were spread evenly across the surface and didn't appear to recognise underlying primary topography. Macrophages, stained for Integrin αV and F-actin,

showed that they were did not align to the underlying primary topography and could be found to adhere to the uppermost surfaces of the original and modelled adipose surfaces (D,H). Macrophages were more spread on the surface of the smooth and original adipose surfaces (D,L) when compared to the modelled adipose surface (H) which has been shown to be associated with an increased inflammatory response.

Figure 6: Relative genetic expression and cytokine production of the manufactured surfaces when compared to TCP. (* $P < 0.05$). Results show a positive influence of the modelled surface to provoke an M2 macrophage phenotype.

Graph 1: A: Sphere diameter and B: nearest neighbour distances for the three patients included in the study.

Graph 2: Height Data Allows Generation of a Statistically Matched Secondary modelled Adipose Texture. A: Histogram of surface heights of 12 adipocytes. B: Histogram of etched modelled surface illustrates that 6.5 mins of etching produces a surface wit with comparable height statistics to native tissue.

Supplementary Table 1
[Click here to download Supplementary Material: Supplementary Table 1.docx](#)

Figure 2

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Dear Professor W.R. Wagner

Re: Response to Reviewers

Thank you for requesting reviews of our paper. We have made amendments to our manuscript to reflect their comments. Below is an itemised list of our alterations and responses to the reviewers.

We look forward to hearing from you.

Yours Sincerely,

Dr Ardeshir Bayat

Associate Professor

Plastic and Reconstructive Surgery Research

Reviewer #1: *This interesting pre-clinical paper looks at a breast implant surface texture that mimics the topography of adipose tissue removed from patients undergoing breast reduction surgery. The methods are well described and show how technological advances in surface topography can be transferred onto medical grade silicone. When this surface was mixed with macrophages and fibroblasts in vitro, it showed down-regulation of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines. Imaging demonstrated good adhesion of cells to the surface.*

This is very early in the development of biologically derived implant surfaces and I encourage the authors to proceed with further development and in vivo trials of their newly derived implant surface.

It is emerging, however, that biofilm infection onto implant surfaces (both breast and other implants) play a major role in causing re-operation and implant failure. The combination of a more bio-compatible surface with anti-infectives should be the ultimate goal for all biomaterials to ensure that the body has time to incorporate the new surface free of infection. The authors should make reference to the growing body of work linking biofilm to contracture.

Response: Thank you for your suggestion to include a statement on the link between biofilm and capsular contracture. We recognise the effect that implant bacterial colonisation has on the development of capsular contracture and have re-structured paragraph 3 of the introduction to reflect this.

Reviewer #2: *The authors deal with the problem of breast implants; their use is wide even if capsular contracture is a very frequent complication. The onset of periprosthetic capsule is strictly linked to inflammation and often associated to ALCL. In this scenario, the possibility to improve the quality of breast implant surface and consequently their features deserves attention.*

I think that the manuscript is well written, the methodological approach is detailed and complete with the use of different and updated techniques.

I have just some minor concerns:

-the introduction is too long, especially if compared with the discussion. Please, make shorter the introduction and develop more the discussion

Response: Thank you for your comments. We have shortened the introduction section and developed the discussion section.

-better clarify the time of cell culture in material and method section

Response: We have added a second paragraph to section 3.15 of the methods section to clarify the periods of cell culture.

-add "a" and "b" to graph 1

Response: We have added "A" and "B" to graph 1

-make attention to some typing errors

Response: We have proof read our paper and corrected any typing errors identified

Reviewer #5: *The authors present a generated, produced and analysed breast implant texture derived from human breast tissue. Topographical assessment of native breast tissue facilitated the development of a statistical model of adipose tissue. Photolithography and ion etching were employed to successfully replicate the surface model. Immunocytochemistry and scanning electron microscopy images indicate favourable fibroblast and macrophage responses to these novel surfaces with anti-inflammatory in-vitro abilities.*

The overall work has been conducted with care and, in my opinion, is of a significant advance in the field. The experimental fabrication results are interesting.

I have the following suggestion/comments for the authors:

1. In Figure 4, it will be nice to put the graphs A2 and B2 in the same graph, to label with units the scale (horizontal and vertical). If the authors decide to keep the 3D images represented in the Figure A3 and B3, then also the scale bars should be clear represented with units.

Response: Thank you for your suggestion. We have updated Figure 4 and the manuscript and figure captions to reflect these changes.

Additional Changes:

Any further changes to the manuscript have been underlined and highlighted in yellow. The reference numbers have been changed to reflect any changes.