Physico-chemical characteristics of coated silicone textured versus smooth breast implants differentially influence breast-derived fibroblast morphology and behaviour

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\textbf{Abstract}

Capsule formation is an inevitable consequence of silicone breast implantation. Clinically challenging dense fibrocollagenous capsular contractures occur at different rates between smooth compared to textured surfaces. Host response is influenced by several factors including implant surface texture, chemistry and interactions between cells and the extracellular matrix (ECM). Specific coatings can modify the physico-chemical properties of implant surfaces eliciting specific cellular reactions. Therefore, we evaluated the physico-chemical characteristics of coated smooth versus textured silicone breast implants on breast-derived fibroblast morphology and behaviour using (a) confocal laser microscopy, (b) Raman spectroscopy and (c) the effect of four unique protein and glycosaminoglycan (GAG) coatings (aggrecan, collagen I, fibronectin and hyaluronic acid) on breast-derived fibroblast attachment, proliferation, morphology, spreading, cytotoxicity and gene expression. Collagen I, fibronectin and hyaluronic acid coatings exhibited satisfactory fibroblast adhesion ($p<0.001$) in comparison to uncoated surfaces. Cell adhesion was less on smooth surfaces compared to textured surfaces ($p<0.001$). Fibroblasts cultured on collagen I, fibronectin and hyaluronic acid coated implants demonstrated improved cell proliferation than uncoated surfaces ($p<0.001$). LDH assay showed that coating surfaces with collagen I, fibronectin and hyaluronic acid did not induce cytotoxicity. Alpha-actinin expression and fibroblast adhesion to the substrate were upregulated ($p<0.001$), in textured versus smooth surfaces. FAK, vinculin and paxillin expression were upregulated ($p<0.001$), in all surfaces coated with fibronectin and collagen I.

In conclusion, we present original data for expression of adhesion-related genes, cell
1. Introduction

Capsule formation is an inevitable consequence of implant insertion into a body cavity. Breast capsules are thus typically formed after silicone breast implant insertion into the breast cavity; however, some capsules can undergo contracture formation. A fibrous capsule usually forms around silicone breast implants. This is a relatively hypocellular membrane of rather uniform thickness which is rich in collagen. There may be a thin discontinuous layer of activated epithelioid myofibroblasts next to where the implant was situated and a thin acellular protein film between the implant and capsule. Both within and directly below this membrane, there are usually foam cells and lymphocytes, often in large numbers (Van Diest et al., 1998). However, whilst aetiology remains unknown, a variety of associations have been proposed that may predispose implants to capsular contracture formation including the filler material, implant placement technique, surface texture, presence of foreign bodies (such as glove talcum powder), subclinical infections near the area of implantation, hematoma and seroma (Berry et al., 2010). Breast capsular contracture is a clinical challenge for both the patient and the clinician in view of the degree of physical severity and availability of limited options for management. There are two types of surfaces for the most commonly used silicone breast implants today (Fig. 1). Silicone breast implant surface texture is considered to influence the rate of breast capsular contracture formation (Barr and Bayat, 2011). An implant surface is thought to interact directly with the breast tissue once inserted. A number of prospective studies have shown evidence of the benefit of textured compared to smooth implants in the first year post-implantation, although this benefit is maintained at 5 and 10 years (Poepl et al., 2007, Barnsley et al., 2006, Hakelius and Ohlsen, 1997, Malata et al., 1997, Coleman et al., 1991, Ersek, 1991, Ma and Gao, 2008). Meta-analyses calculated the occurrence of breast capsular contracture on textured surfaces to be about fivefold less in comparison to smooth surfaces, which was maintained for 3 years (Wong et al., 2006, Barnsley et al., 2006). However, one previous study showed no statistically significant difference between saline-filled smooth and textured breast implants (Fagrell et al., 2001). Moreover, studies carried out in animal models are conflicting, as two studies found an increase in the rate of capsular contracture in smooth surface implants (Brohim et al., 1993, Clugston et al., 1994), while other studies found thicker and tighter capsules around textured surfaces (Barone et al., 1992, Bucky et al., 1994, Bern et al., 1992). Rationale behind the efficacy of reducing capsular contracture with textured breast implants is based on the fact that cells grow into and around the interstices of the surface resulting in an environment where contractile forces tend to cancel each other out, resulting in thinner capsule formation by contact inhibition (Harvey et al., 2013). Smooth surfaces elicit a fibrous reaction where collagen fibrils align cumulatively in a connective-tissue capsule adjacent to the implant.

Third-generation biomaterials are designed to stimulate cell behaviour in a specific manner at molecular level (Hench and Polak, 2002). Molecular modifications on the surface of the implants induce specific interactions with cell receptors such as integrins directing cell proliferation, differentiation and ECM production and organisation. Coating surfaces is an alternative route to influence the implant surface topography by creating cues for cellular adhesion and the subsequent induction of tissue integration (Harvey et al., 2013, Hauser et al., 2009). Different techniques of coating have been performed on breast implants with the aim of reducing the rate of capsular contracture. Polyurethane covered breast implants consists of silicone shell covered with fine-cell urethane and filled with silicone gel. The polyurethane coating is 1 mm thick and the septum is built into the prosthesis featuring a Y-shape thin-walled that allows the implant fixes within the chest wall (Ashley, 1970). This capsule surrounding the polyurethane consisted of five layers: a single layer of macrophages, foreign body giant cells, and epithelioid cells, a layer of subacute inflammatory tissue, a plasmaacytic infiltrate, a thick layer of connective tissue and a layer of lax connective tissue along the breast parenchyma (Vazquez, 1999). The polyurethane coating induces a vascular foreign body reaction that prevents fibroblasts from producing collagen in a continuous plane so the contracture of the capsule is minimum and only 10% of patients have shown capsular contracture at 4 years; however, 25% of the patients showed capsular contracture at 10 years, and this may be due to the disintegration of the polyurethane coating (Berry and Davies, 2010). In the 1990s, polyurethane-covered Même breast implants were withdrawn from the market due to the risk of chemical breakdown of the polyurethane foam to carcinogen 2-toluenediamine (Collis et al., 2000). Currently, polyurethane is joined at the base to the implant, instead of being glued to the implant which was the case before it was withdrawn. Therefore, polyurethane does not become detached and a capsule is formed only around the polyurethane and not between the foam and the implant as it was previously (Vazquez, 1999, Vazquez and Pellon, 2007). Roca studied autologous fat grafting with textured silicone gel implants in porcine models showing softer capsules around the implants (Roca et al., 2014). Park covalently coated silicone implants with a biomembrane-mimicking polymer (PMPC) and showed a significant decrease in capsular thickness compared to non-coated implants in rat models (Park et al., 2014). Zeplin coated silicone implants with recombinant spider silk proteins and showed reduced post-operative inflammation and fibrosis in rat models only (Zeplin et al., 2014).
The extracellular matrix (ECM) in the breast tissue is comprised of specific proteins and proteoglycans (Muschler and Streuli, 2010). Fibroblasts use surface receptors to interact physically with their immediate environment (Bershadsky et al., 2003). ECM receptors, mainly integrins, provide attachment to the surrounding stroma. The associations of the heterodimeric receptors formed by integrins define the influence to different ECM components. To successfully elicit...
specific cellular responses and direct new tissue formation, biological cues are created through the design of biomimetic scaffolds that modify biomaterials with ECM molecules (Shin et al., 2003). The interaction of ECM proteins with cells via cell-surface integrin family receptors results on focal contacts; they provide support to cellular processes and maintain the tissue architecture. Cell spreading, adhesion, proliferation and migration are influenced by the signal transduction cascades initiated by the binding of an ECM molecule and integrin.

Protein, proteoglycan (PG) and glycosaminoglycan (GAG)-based coatings have been used to promote fibroblast activity modification (Steward et al., 2011, Li et al., 2012a, Hauser et al., 2009). Aggrecan is a proteoglycan found in the ECM of cartilage, which has a molecular mass of >2,500 kDa (Roughley et al., 2006). Another constituent element of ECM is collagen, which enhances cell attachment and proliferation and can be found in the form of filaments, sheets and fibrils (Li et al., 2012b), providing tensile strength to the tissue scaffolding. Collagen type I was chosen to coat the surfaces because it is a major constituent of breast tissue and supporting structures. In addition, collagen type I fibres are recognised by integrin cell surface receptors and thus appropriate as an ideal interface for the cell-surface interaction (Mwenifumbo and Stevens, 2007). An essential component of ECM related to cell adhesion is fibronectin, a 450 kDa dimeric glycoprotein that can bind specifically to two widely expressed cell surface receptors, integrins α5β1 and αvβ3 (Elter et al., 2012). Fibronectin plays an important role in the adhesion of cells to material surfaces; it can even strengthen the cell-surface adhesion (Elter et al., 2012). Hyaluronic acid transports the metabolites and nutrients, provides tissue resistance to compressive forces and controls cell migration and proliferation (Cohen et al., 2003). Hyaluronic acid provides the perfect scaffold for cells to proliferate and migrate (Collins and Birkinshaw, 2013, Korurer et al., 2014).

The above four candidates were chosen from a whole genome microarray study (Kyle et al., 2013) performed in cDNA of 23 breast capsules: 12 capsules of Baker grades I and II and 11 capsules of Baker grades III and IV; 122 genes were found to be upregulated and 22 downregulated (Baker grades I and II refer to normal capsules compared to grades III and IV which refer to severely contracted capsules) (Baker, 1975). The candidate genes were selected from the microarray data by grouping genes according to three categories. The first category consisted of all the downregulated genes, the second category comprised of the genes related to cell adhesion and ECM and the third category was formed based on the smallest statistical p-value. Genes that matched the three categories were selected, and a literature review into gene and protein expression of fibrosis and breast capsules was also conducted in order to corroborate the microarray data.

Therefore, the aim of the study was to compare the efficacy of different types of ECM coatings and known smooth versus textured silicone breast implant surface topographies. To this end, the effect of four specific coatings on initial cell attachment, viability, proliferation and gene expression on these surfaces was investigated. We examined the hypothesis that micrometre-sized surface textures affect adhesion-related breast tissue fibroblast function to a different degree depending on the scale of micro-topography and the properties of the coatings by assessing cytotoxicity, cell morphology, adhesion, proliferation and the expression of α-actinin, vinculin, paxillin and FAK. By focusing on integrin-mediated focal adhesion structure and intracellular signalling molecules, we examined the effect of topography and coating on cell function, morphology and behaviour.

2. Materials and methods

The study was conducted following two stages: the first stage consists of characterising the surface of silicone breast implants by measuring the arithmetic surface roughness and analysing the chemical composition and the second stage consisted of studying the in vitro adhesive interactions of breast fibroblasts with implants surfaces in order to determine how implant surface textures and surface coatings affect specific functions of the cell directly involved in cellsurface adhesion. These stages are described in Fig. 2.

2.1. Sample preparation

The silicone breast implants studied were chosen from some of the commonly available implants in clinical practice: (1) Textured Surface-1 (TS1) (Mentor Siltex® (Mentor Worldwide LLC, Skyway Circle North Irving, Texas)); (2) Smooth Surface (SS) (Mentor® Smooth (Mentor Worldwide LLC, Skyway Circle North Irving, Texas)); (3) Textured Surface-2 (TS2) (Allergan Biocell® (Allergan, Inc, Santa Barbara, California)). Sample preparation was performed following the methodology of Valencia (Valencia-Lazcano et al., 2013). Surfaces were coated with either 5 μg/ml aggrecan (Sigma-Aldrich, USA), 5 μg/ml collagen type I (BD Biosciences, USA), 5 μg/ml fibronectin (Sigma-Aldrich, USA) or 10 μg/ml hyaluronic acid (Sigma-Aldrich, USA) solution and incubated for 1 h at 37 °C and washed twice with PBS (PAA laboratories, Austria).

2.2. Characterisation

Physical properties of smooth and textured breast implants were evaluated by looking at their topographical features using confocal laser microscopy following the procedure described previously by Valencia (Valencia-Lazcano et al., 2013). The procedure enables highly accurate 3D images of implant surfaces to be produced from which surface roughness parameter values can be obtained. Chemical characterisation was carried out recording Raman images and spectra of the implants using confocal Raman spectrometer equipped at an excitation of 532 nm and a magnification of 50x air objective (N.A.=0.7) (Horiba, USA).

2.2.1. Adsorption

The adsorption of aggrecan, collagen, fibronectin and hyaluronic acid onto the breast implant surfaces was determined by first measuring the thickness of the coating with the confocal laser microscope following the procedure described previously by Valencia (Valencia-Lazcano et al., 2013). From the measured thickness (d), the surface mass density of the
coating was determined by the Feijters formula:

\[
\text{Adsorbance} = \frac{dn_{\text{protein}} - dn_{\text{buffer}}}{dn/dc}
\]

where \(n_{\text{protein}}\) and \(n_{\text{buffer}}\) are the refractive index of the protein and buffer, respectively, and \(dn/dc\) is the refractive index increment for a given concentration.

### 2.3. Cell culture

Cultured human breast tissue fibroblasts were established from tissue biopsies taken from healthy female patients \((n=3)\) undergoing routine elective surgery. All patients had given full written and verbal consent for the use of discarded tissue for the experimental purposes of this ethically approved study. All cultures were passage 2 and were grown to confluence in 125 ml culture flasks in Dulbecco's Culture Medium (DMEM) (Sigma-Aldrich, UK) substituted with 10% foetal bovine serum (Sigma-Aldrich, USA), 1% l-glutamine (PAA laboratories, Austria), 1% non-essential amino acid solution (Sigma-Aldrich, USA) and 1% penicillin/streptomycin (PAA laboratories, Austria) at 37°C in a 5% CO2 atmosphere. Prior to seeding the fibroblasts on the surfaces, fibroblasts were arrested to take them to the G0/G1 phase.

### 2.4. Cytotoxicity

Cytotoxicity was tested after 24 h incubation; media from each well was aspirated and centrifuged at 1600 rpm for 4 min. The media was transferred to a 96-well plate at a volume of 100 μL per well by triplicate, and 100 μL of LDH (Roche, USA) was added to each well. The plate was protected from light and incubated at room temperature for 30 min. Absorbance was measured spectrophotometrically (Molecular Devices, USA) at a wavelength of 485 nm. The background absorbance of the multi-well plates was measured at 690 nm and this value was subtracted from the primary wavelength measurement. The number of replicates was 3 per surface; the control group corresponds to the uncoated surfaces.

### 2.5. Fibroblast adhesion to surfaces

Adhesion assay was tested after 2 h of seeding the fibroblasts onto the surfaces. Dissociated cell suspensions were washed twice with PBS (PAA laboratories, Austria) to be later re-suspended in media without serum. Five microlitre of calcein AM solution (Molecular probes, USA) was added to a cell suspension of \(5 \times 10^6\) cells/ml and incubated at 37 °C for 30 min. The cells were washed twice with media and re-suspended in fresh media. The cells were seeded on breast implant surfaces in a 96-well plate in a final volume of 100 μL/well of the calcein-labelled cell suspension (\(5 \times 10^4\) cells) culture medium (Sigma-Aldrich, UK) and incubated for 2 h in a humidified atmosphere of 37 °C, 5% CO2. Non-adherent calcein-labelled cells were removed by washing four times with warm media (Sigma-Aldrich, UK); finally 200 μL of PBS (PAA laboratories, Austria) was added. Fluorescence was measured using a micro-plate reader (Molecular
37 rpm. After 4 h incubation in a humidified atmosphere of 37 °C, 5% CO2, media was removed and poured at 100 μL of Trizol reagent (Invitrogen Ltd, UK) (5 min). An equal amount of ethanol 80% (Fisher Scientific, USA) was added. Centrifugation was performed for 15 s at 14500 rpm for 15 min. Three layers were formed and the upper layer was aspirated and transferred into an eppendorf tube; an equal amount of ethanol 80% (Fisher Scientific, USA) was added. Trizol was aspirated and transferred to an RNeasy mini spin column. After 15 s of centrifugation, supernatant was poured away and 350 μL of chloroform (Fisher Scientific, USA). Surfaces were washed three times with 0.1% Tween 20/PBS (5 min each). Surfaces were labelled with secondary antibody Alexa Fluor® 488 dye (abcam, UK) 1:500 dilution for 100 min at room temperature. Surfaces were washed three times with 0.1% Tween 20/PBS (Fisher Scientific, USA) 5 min each. The surfaces were labelled with 4',6-diamidino-2-phenylindole (DAPI) (Molecular probes, USA) for 15 min and then washed with 0.1% Tween 20/PBS (Fisher Scientific, USA) for 5 min.

The surfaces were labelled with rhodamine-phalloidin 1:1000 (Sigma-Aldrich, USA) for 40 min at room temperature and then washed once with PBS (PAA laboratories, Austria), twice with 0.1% Tween 20/PBS (Fisher Scientific, USA) 5 min each and finally twice with PBS (PAA laboratories, Austria). Surfaces were mounted with ProLong® Gold antifade reagent (Invitrogen, USA) and covered with aluminium paper and stored at −4 °C. Immunofluorescence microscopy was carried out using a DeltaVision deconvolution system softWoRx v3.4.5 (Applied precision, USA) which consists of an Olympus IX-70 inverted microscope with an epi-illumination of 100x. Images were analysed using softWoRx v3.4.5 and Image-J software (NIH, USA).

### 2.7. Immunofluorescence

Fluorescence staining of the actin (TRITC) (cytoskeleton), vinculin (FITC) (focal adhesion protein) and cell nucleus (DAPI) was performed using triple staining. Throughout the staining procedure, after 24 h of culture, media was removed from the well plate and the surfaces washed with PBS (PAA laboratories, Austria). Surfaces were covered with 10% formalin (Sigma-Aldrich, USA) overnight and washed with PBS (PAA laboratories, Austria). Surfaces were covered with 0.1% Triton X-100 (Sigma-Aldrich, USA) for 10 min. Each sample was washed twice with PBS (PAA laboratories, Austria) (5 min each), then covered with blocking buffer (LI-COR Biosciences, USA) for 30 min and washed twice with PBS (PAA laboratories, Austria) (5 min each). The surfaces were labelled with first antibody ab2264 rabbit polyclonal to paxillin (abcam, UK) at a concentration of 1:100 overnight at 4 °C. Surfaces were washed three times with 0.1% Tween 20/PBS (5 min each). Surfaces were labelled with secondary antibody Alexa Fluor® 488 dye (abcam, UK) 1:500 dilution for 100 min at room temperature. Surfaces were washed three times with 0.1% Tween 20/PBS (Fisher Scientific, USA) (5 min each). The surfaces were labelled with 4',6-diamidino-2-phenylindole (DAPI) (Molecular probes, USA) for 15 min and then washed with 0.1% Tween 20/PBS (Fisher Scientific, USA) for 5 min.

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### 2.8. Quantification of gene adhesion expression

#### 2.8.1. RNA extraction

After 24 h of culture, total RNA was extracted from each specimen by adding 500 μL of Trizol reagent (Invitrogen Ltd, UK) for 2 min. Trizol was aspirated and transferred to an eppendorf tube and 350 μL of chloroform (Fisher Scientific, USA) was added; the tube was shaken and centrifuged at 14500 rpm for 15 min. Three layers were formed and the upper layer was aspirated and transferred into an eppendorf tube; an equal amount of ethanol 80% (Fisher Scientific, USA) was added and mixed by pipetting it and poured into an RNeasy mini spin column. After 15 s of centrifugation, supernatant was poured away and 600 μL of RWT buffer (Qiagen, Netherlands) was added. Centrifugation was performed for a
was less than 0.05, the difference was regarded as statistically significant.

an eppendorf tube, RNease-free water (Qiagen, Netherlands) was poured into the column and 500 μL of RNease-free water (Ambion, USA) was added; this was then centrifuged for 15 s. Supernatant was poured away and the column was centrifuged for 1 min at 15000 rpm. The column was placed into an eppendorf tube and 10 μL of RPE buffer (Qiagen, Netherlands) was added to the top layer and left for 1 min at room temperature before centrifugation at 15000 rpm for 1 min. RNA concentration and purity were determined using a UV–vis spectrophotometer (NanoDrop, USA) by absorbance measurements.

2.8.2. cDNA synthesis
The quantity of nuclease-free water was calculated based on RNAs (100 ng) concentration; to make a 20 μL solution, 4 μL of cDNA super mix (Quanta Biosciences, USA) was poured into an eppendorf tube, RNease-free water (Qiagen, Netherlands) was added and finally mRNA was added. The tube was centrifuged for 30 s and incubated at 25 °C for 5 min. Synthesis conditions comprised an initial cycle at 42 °C for 1 h and one cycle at 85 °C for 5 min to inactivate the enzyme, and finally the solution was diluted in 180 μL of nuclease-free water (Ambion, USA).

2.8.3. Quantitative real-time polymerase chain reaction
The primers used to amplify the selected genes using real time qPCR were designed using Universal ProbeLibrary (Roche, USA). Reactions were set up in a total volume of 10 μL using 4 μL of cDNA, 5 μL probes master (Roche, Germany), 0.7 μL RNease-free water (Qiagen, Netherlands) and 0.1 μL each of gene-specific primer (Table 2) and performed in a LightCycler® 480 Real-Time PCR System (Roche, Germany). The cycling conditions were 95 °C for 10 min; 45 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 1 s with a single fluorescence measurement; cooling was set at 40 °C for 30 s. Specificity of the PCR products was confirmed by analysis of the dissociation curve.

2.9. Statistical analysis
Statistical analysis was performed using the Prism v.5.0 software package for Windows (GraphPad Software, Inc., USA), applying the two-way ANOVA test; when the p-value was less than 0.05, the difference was regarded as statistically significant.

Table 2 – Sequences of primers used for adhesion-related genes using quantitative real-time polymerase chain reaction.

<table>
<thead>
<tr>
<th>Gene/Primer</th>
<th>Gene ID</th>
<th>Sequence 5’ to 3’</th>
<th>Primer Position</th>
<th>Amplicon Size (bp)</th>
</tr>
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<tbody>
<tr>
<td>(ACTN1) Alpha-actinin L</td>
<td>nm_001102.3</td>
<td>cttgccagcatctcctcat</td>
<td>834–853</td>
<td>70</td>
</tr>
<tr>
<td>(ACTN3) Alpha-actinin R</td>
<td>nm_001102.3</td>
<td>tcagtagctgtgtaggtggt</td>
<td>882–903</td>
<td>70</td>
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<tr>
<td>(VCL) Vinculin (VCL) L</td>
<td>nm_014000.2</td>
<td>gggaggtattacacgcacaaat</td>
<td>2767–2787</td>
<td>88</td>
</tr>
<tr>
<td>(VCL) Vinculin (VCL) R</td>
<td>nm_014000.2</td>
<td>aatgtagctattgccttgcc</td>
<td>2835–2854</td>
<td>88</td>
</tr>
<tr>
<td>(PXN) Paxillin L</td>
<td>nm_002859.3</td>
<td>cccagttgaggagctcttg</td>
<td>785–805</td>
<td>81</td>
</tr>
<tr>
<td>(PXN) Paxillin R</td>
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<td>ctagcgtgctgtgacatg</td>
<td>848–865</td>
<td>81</td>
</tr>
<tr>
<td>(ptk2) Protein tyrosine kinase L</td>
<td>nm_153831.2</td>
<td>gtcgtcttcgcttccacg</td>
<td>73–90</td>
<td>77</td>
</tr>
<tr>
<td>(ptk2) Protein tyrosine kinase R</td>
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<td>126–149</td>
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3. Results

3.1. Physico-chemical characterisation of smooth versus textured implants
Raman spectra showed TS1, TS2 and SS implants have the typical Raman spectrum of pure PDMS at the 490 cm⁻¹ the Si-O stretch and 713 cm⁻¹ the Si-C stretch modes. Implants from the same manufacturer, TS1 and SS possess a similar profile (Fig. 3) in spite of their surface roughness. Confocal laser microscopy showed different surface roughness between these two implants: TS1 is 8.88 μm rough while SS is 0.06 μm. TS2, whose surface roughness was 18.83 μm, showed a different profile and lower intensity levels in comparison to TS1 and SS (Fig. 3).

Surface roughness was modified by coating the surfaces with specific protein, PG and GAG coatings. In this case, the same coating procedure was performed on all surfaces; thus the changes in roughness were likely due to the interactions between implant topography and the coating structure. The surface features of the different breast implants after coating with aggrecan, collagen I, fibronectin and hyaluronic acid were assessed using confocal laser scanning microscopy. 3D topological models were obtained using colour-height information from planar images of the implant sample surfaces (Valencia-Lazcano et al., 2013). Fig. 4 (a)–(c) show the 3-D topography of the uncoated silicone breast implants (a) TS1, (b) SS, and (c) TS2, were used as the control. The arithmetic surface roughness measurements for both the coated and uncoated implant surfaces are shown in (Fig. 5a), where upon inspection it can be seen that surface roughness was significantly different (p<0.001) among the TS1, SS and TS2. Coated surfaces of TS1 were found to be significantly rougher (p<0.001) than the uncoated ones (Fig. 5b). SS surfaces coated with collagen I, fibronectin and hyaluronic acid were significantly rougher (p<0.01) compared to the uncoated surfaces; however, no significant difference in surface roughness was observed between the aggrecan-coated and uncoated SS (Fig. 5c). TS2 coated with fibronectin and hyaluronic acid were found to be significantly rougher (p<0.05) compared to the uncoated surfaces. No significant differences were found in the roughness values of uncoated, aggrecan and collagen I coated TS2 (Fig. 5d).

3.1.1. Adsorption
The adsorption values of aggrecan, collagen I, fibronectin and hyaluronic acid onto TS1, SS and TS2 are presented in
Table 1. The highest adsorption at the peaks occurred on TS2 coated with hyaluronic acid (1.97 mg/cm²) and fibronectin (0.93 mg/cm²), while SS coated with aggrecan showed the lowest adsorption (0.004 mg/cm²) among all surfaces. The highest adsorption at the valleys occurred on TS1 (1.11 mg/cm²) and TS2 (0.98 mg/cm²) coated with fibronectin, while SS coated with aggrecan showed the lowest adsorption (0.004 mg/cm²) among all surfaces.

Fig. 3 – Raman spectra of silicone breast implants. Spectra of the textured and smooth silicone breast implants showed the key Raman features of PDMS in all implants: Si-O stretch (490 cm⁻¹) and the Si-C stretch (713 cm⁻¹).
3.2. Cytotoxic effect of specific coatings on breast fibroblasts

After 24 h of seeding breast fibroblasts onto uncoated TS1, SS and TS2 and surfaces coated with aggrecan, collagen I, fibronectin and hyaluronic acid, lactate dehydrogenase (LDH) activity in the culture media was used as an indicator of cell membrane integrity and thus a measurement of cytotoxicity (Fig. 6). The results presented in Fig. 6 reveal that SS, TS1 and TS2 coated with aggrecan showed a significantly higher \( p < 0.001 \) cytotoxicity compared with the corresponding uncoated surfaces. A decrease in cytotoxicity was found on TS1 coated with collagen I \( p < 0.01 \), fibronectin \( p < 0.01 \) and hyaluronic acid \( p < 0.001 \) when compared with uncoated TS1. No significant difference was found between the cytotoxicity values of SS and TS2 coated with collagen I and fibronectin compared with the uncoated SS and TS2; however, the cytotoxicity of the hyaluronic acid coated surfaces was significantly lower \( p < 0.001 \) than the uncoated surfaces.

3.3. Effect of specific coatings on breast fibroblast attachment

A calcein AM cell adhesion assay was used to measure the adhesion of calcein-labelled breast fibroblasts to uncoated TS1, SS and TS2 and surfaces coated with aggrecan, collagen I, fibronectin and hyaluronic acid (Fig. 7). Cell adhesion was found to be lower for fibroblasts on the smooth surfaces compared to the textured ones \( p < 0.001 \). Cell adhesion was significantly higher on collagen I, fibronectin and hyaluronic acid coated implants compared to the corresponding uncoated surfaces \( p < 0.001 \). Cell adhesion was highest (89%) for collagen I coated TS1 and TS2 and fibronectin coated TS2 (87%). No significant difference was found between fibroblast adhesion to aggrecan coated implants compared to the corresponding uncoated surfaces \( p > 0.05 \).

3.4. Specific coatings induce a variable proliferation rate in breast fibroblasts

Proliferation of breast fibroblasts seeded for 24 h on TS1, SS and TS2 coated with aggrecan, collagen I, fibronectin and hyaluronic acid was quantified using a water-soluble tetrazolium salt-1 (WST-1) cell proliferation assay and compared with control cultures grown on uncoated TS1, uncoated SS and uncoated TS2 (Fig. 8). On smooth surfaces, fibroblasts proliferation rates were lower compared to the textured surfaces for both the coated and uncoated surfaces \( p < 0.001 \). Cell proliferation was significantly higher for fibroblasts seeded on collagen I, fibronectin and hyaluronic acid coated implants compared to the corresponding uncoated surfaces \( p < 0.001 \). The highest cell proliferation rate (0.8) was found in collagen coated TS1, followed by 0.71 for fibronectin coated TS1. Lower proliferation rates were measured on uncoated and aggrecan coated surfaces \( p < 0.001 \).

3.5. Effect of coatings on cytoskeleton organisation

Rhodamine-phalloidin staining of breast fibroblasts cultured on uncoated and aggrecan coated TS1 and SS, and uncoated TS2 revealed cells that exhibited fine stress fibres all around the cell periphery (Fig. 9). Fibroblasts seeded on aggrecan coated SS and TS1, and uncoated TS2 were not able to form detectable cell-material adhesion complexes as well as actin cytoskeleton, and thus remained rounded, non-spread. Fibroblasts seeded on uncoated SS were poorly spread and showed...
Fig. 5 – Changes in surface roughness of the modified coated surfaces examined by confocal laser microscopy. (a) Comparison of measured arithmetic surface roughness among the three implants showed statistical significant differences between them ($p<0.001$). Comparison of measured arithmetic surface roughness among uncoated and coated (b) Textured Surface 1, (c) Smooth Surface and (d) Textured Surface 2. Statistical analysis was performed applying the two-way ANOVA test; when the $p$-value was less than 0.05, the difference was regarded as statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Fig. 6 – Cytotoxicity was evaluated by the quantification of plasma membrane damage by measuring the LDH activity in the cultured supernatant of breast-derived fibroblasts. Bar graphs show the toxicity effects of the coating proteins on breast-derived fibroblasts after 24 h of seeding them onto Textured Surface 1, Smooth Surface and Textured Surface 2 coated with aggrecan, collagen I, fibronectin and hyaluronic acid. Data shows mean ± standard deviation. Statistical analysis was performed applying the two-way ANOVA test; when the $p$-value was less than 0.05, the difference was regarded as statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Fig. 7 – Effect of specific coatings on fibroblast attachment. Textured Surface 1, Smooth Surface and Textured Surface 2 were coated with aggrecan, collagen I, fibronectin and hyaluronic acid. Calcein AM cell adhesion assay was used to compare breast fibroblasts attachment after 2 h compared to control cultures grown onto uncoated Textured Surface 1, Smooth Surface and Textured Surface 2. Data shows mean ± standard deviation and the attachment is represented as a percentage of the total number of cells seeded. Statistical analysis was performed applying the two-way ANOVA test; when the $p$-value was less than 0.05, the difference was regarded as statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).
a more random actin network than on uncoated TS1. Well spread cells in collagen I, fibronectin and hyaluronic acid coated TS1, SS and TS2 exhibited thick fibres throughout the entire cell. In fibronectin coated TS2, collagen I coated SS and hyaluronic acid coated TS1 cells showed a well-organised actin cytoskeleton. Fibroblasts seeded on fibronectin coated TS1 and on hyaluronic acid coated TS2 exhibited an elongated cellular phenotype. However, actin fibres were dense in all the cells in hyaluronic acid coated TS2 in comparison to fibroblasts in fibronectin coated TS1 which showed an abundance of fine fibres throughout the entire cell.

### 3.6. Effect of aggrecan, collagen I, fibronectin and hyaluronic acid coatings on adhesion expression in breast fibroblasts

After 24 h of incubation, total RNA was extracted from breast fibroblasts (n = 3) (passage 2). The expression of alpha-actinin, FAK, paxillin, vinculin mRNA was determined by quantitative reverse-transcriptase polymerase chain reaction to compare the effect of the molecular coatings with the uncoated implants in breast fibroblasts (Fig. 10).

When the expression of the cytoskeleton components was examined, the level of α-actinin was found to be reduced in uncoated and aggrecan coated surfaces, but the α-actinin expression was higher in collagen I and fibronectin coated TS1 (p < 0.01) and hyaluronic acid coated TS1 (p < 0.001), and in collagen I, fibronectin and hyaluronic acid coated TS2 (p < 0.001). The FAK level expression by breast fibroblasts was upregulated in collagen I, fibronectin and hyaluronic acid coated surfaces (p < 0.001), while the FAK expression was lower in both uncoated and aggrecan coated TS1, SS and TS2. The gene expression of paxillin was upregulated in fibroblasts seeded on collagen I and fibronectin coated implants (p < 0.001) and in the hyaluronic acid coated TS1 (p < 0.01), SS (p < 0.05) and TS2 (p < 0.001). Regarding vinculin expression, a higher level was detected in the surfaces coated with collagen I and fibronectin (p < 0.001) compared to the uncoated ones.

### 4. Discussion

In this study, for the first time, we evaluated the physico-chemical characteristics of coated silicone breast implants on breast-derived fibroblast morphology and behaviour. Chemical properties were examined by Raman spectroscopy; topographical features were studied by confocal laser microscopy, and the effect of four unique protein and glycosaminoglycan (GAG) coatings (aggrecan, collagen I, fibronectin and hyaluronic acid) on breast-derived fibroblast cytotoxicity, attachment, proliferation, morphology and gene expression were examined.

The choice of the specific coatings evaluated in this study was based on an in-house whole genome microarray study undertaken in order to determine genes whose expression would correlate with breast capsular contracture formation and on previously published gene and protein research of relevance to breast capsular fibrosis (Kyle et al., 2013). Our results showed that specific coatings can modify the physico-chemical properties of implant surfaces eliciting specific cellular reactions. Moreover, we showed that the coated surfaces excluding aggregan, promoted cell-surface adhesion, proliferation, morphology and the upregulation of adhesion-related genes without any cytotoxic effect. These findings provide valuable information of characteristic expression of adhesion-related genes, cell morphology and proliferation in breast fibroblasts following the application of specific coatings on smooth compared to textured breast implant surfaces.

Foreign body reaction is elicited by the implant placement in the body; this initiates an initial inflammatory phase where the prosthesis is encapsulated or eliminated by the host. Surface characteristics such as roughness, texture, surface free energy, surface charge and chemical composition all play key roles in cell adhesion and growth, and the nature of a biomaterial surface governs the phenotypic response of interacting cells (Prasad et al., 2010). Previous studies (Bacakova et al., 2011) have demonstrated that fibroblast activity can be modulated by specific coatings. Coating the surface of implants with these specific coatings may thus provide enhanced support and anchorage for cells and favourably regulate cell morphology, adhesion and proliferation (Yamamoto et al., 2006, Franz et al., 2011). In this study, specific coatings were employed on the surfaces of smooth versus textured breast implant surfaces in order to study fibroblast behaviour on these different topographies. It has been suggested that ECM proteins can be used to optimise fibroblast reaction to implants (Li et al., 2012a, Li et al., 2012b, Ungaro et al., 2006). Surface roughness and chemistry of the substrate have been shown to modulate cell-surface interaction. Furthermore, cell attachment and proliferation can be improved by utilising coatings and altering the micro-topography of these surfaces.
Fig. 9 – Morphology of breast fibroblasts seeded onto coated and uncoated breast implant surfaces. Immunofluorescence staining of the actin (red) (cytoskeleton), vinculin (green) (focal adhesion protein) and cell nucleus (blue) (DAPI) was performed on breast-derived fibroblasts on day 1 after seeding onto (D–F) aggrecan, (G–I) collagen I, (J–L) fibronectin and (M–O) hyaluronic acid. Coated and uncoated (A, D, G, J, M) Surface Texture 1, (B, E, H, K, N) Smooth Surface and (C, F, I, L, O) Surface Texture 2. Pictures were taken using DeltaVision deconvolution system at 100x magnification.
The study demonstrated that cell adhesion and spreading were sensitive to both the physical and chemical properties of the substrate. Cell proliferation and cytotoxicity were also studied. It was found that surfaces coated with collagen I, fibronectin and hyaluronic acid did not elicit a cytotoxic effect on breast tissue–derived fibroblasts in comparison to the uncoated surfaces. Lower proliferation rates were measured on uncoated and aggrecan coated surfaces. Fibroblast proliferation rate differed significantly among the surfaces investigated. An investigation into cell morphology showed that surface coatings promoted cell morphological modifications and the organisation of actin fibres in all surfaces. Gene expression of adhesion-related proteins was shown to be modified by specific coatings and topography amongst the variety of surfaces tested.

Silicone breast implants investigated in this study are made with polydimethylsiloxane (PDMS); Raman spectra revealed a nearly matching profile for implants from the same company; however, implants from different company showed a dissimilar profile. These results are consistent with FTIR/ATR spectroscopy studies comparing breast implants from two different companies. Breast implants differed in chemical composition, and this could be related to the manufacturing process. In smooth implants, the silicone rubber shell is made with a shiny polished mandrel, and to flatten this outer surface, it is steeped in a solvent, while in textured implants, the silicone rubber shell is made with a negative-contact imprint from a polyurethane foam or by pushing the silicone-coated mandrel into granular salt.

Scanning electron microscopy and light microscopy have been used to analyse the topographic features. Changes in surface roughness after coating the surface were consistent with the adsorption of coatings on the valleys of the surfaces. Fibronectin coated surfaces showed the highest increment of surface roughness up to 277% in SS, 100% in TS1 and 16% in TS2 which correlates well with the highest rates of adsorption (per surface) of fibronectin on SS (0.014 mg/cm²), TS1 (1.118 mg/cm²) and TS2 (0.986 mg/cm²). It was also found that cell adhesion was improved by surface roughness. Cell adhesion on rougher surfaces was higher. This means that the greater area due to texturing available for cell spreading allows increased anchoring ability by fibroblasts. In contrast, the same level of cell adhesion was not achieved on smooth surfaces, where low percentages of cell adhesion were recorded in comparison to the textured surfaces. After evaluating surface roughness, it was determined that, for the surfaces investigated in this study,
fibroblast adhesion required a substrate with a surface roughness of at least 14 μm, at which point the degree of adhesion increased with surface roughness until a maximum adhesion was achieved at 21.94 μm. Furthermore, cell spreading demonstrated an incremental response to increases in surface roughness between 17.46 μm and 21.94 μm. The organisation of the actin cytoskeleton was induced by the microtopography in fibroblasts seeded on textured surfaces alone compared to smooth surfaces.

Increased cell adhesion was found on all implant surfaces coated with fibronectin and collagen I in comparison to the uncoated surfaces, which increased cell spreading. By contrast, implants coated with aggrecan demonstrated reduced cell-surface adhesion which therefore reduced cellular proliferation. These results suggest that collagen I and fibronectin act as potent regulators of cytoskeletal organisation and cell spreading. This interaction is important for cell migration in wound healing (Franz et al., 2007). Cells that are forced to spread over large surface areas, as in the case with textured surfaces, survive better and proliferate faster than cells that do not spread out (Lowery et al., 2010). The stimulatory effect of cell spreading potentially encourages tissues to regenerate after injury. If cells are lost from an epithelial layer, for example, the spreading of the remaining cells into the vacated space will stimulate them to proliferate until they fill the gap (Alberts et al., 2002). It is still uncertain, however, how a cell senses the extent of its own spreading and how it adjusts its behaviour accordingly. Cell behaviour is likely to be affected by the chemical and physical structure of the coating. Fluorescent staining images revealed that surfaces coated with collagen I and fibronectin induced organisation of actin stress fibres and that collagen I affected the cytoskeletal arrangement and cell spreading, factors commonly regarded to be of significance with cell migration and wound healing.

In cell migration, a protrusion is sent in the direction of the movement; the extracellular substrate bound by integrin receptors induces integrin clustering and formation of adhesion complexes (Biname et al., 2010). This leads to the eliciting of adaptor proteins, which connect the adhesion points to the actin cytoskeleton and triggers intracellular signalling. This signal is defined by the proteins created and the type of integrin engaged in the interaction with the specific extracellular substrate. Small temporary adhesion complexes located in the leading edge of protrusions (focal complexes) mature under tension into larger structures known as focal contacts. Focal adhesions are more stable structures connected to actin stress fibres (Berrier and Yamada, 2007). Focal adhesion complexes are well established as a major adhesive and signal transducing component between the internal actin cytoskeleton and the external ECM (Petit and Thiery, 2000). Focal adhesion plays a key role in sensing surface topography in the extracellular environment. Focal adhesion kinase and the adaptor protein paxillin are focal adhesion proteins that can bind to and activate integrin B1 subunit cytoplasmic domains (Sequeira et al., 2012), along with vinculin, which connects integrins to actin filaments.

The adapter proteins that link stress fibres to integrins include alpha-actinin. An increase in alpha-actinin content was observed in the cells that had spread most with netlike actin filaments seeded on hyaluronic acid coated TS1, collagen I coated SS and fibronectin coated TS2. When SS and TS2 were coated with collagen I, and TS1 and TS2 were coated with fibronectin and hyaluronic acid, the alpha-actinin expression was upregulated (p < 0.001), which resulted in firmly attached fibroblasts to the substrate (p < 0.001). FAK has a role in modulation of the assembly of focal adhesions in response to tension exerted by the cytoskeleton on attachments to the extracellular substrate via integrins (Parsons, 2003). We observed that the expression of FAK was reduced in fibroblasts seeded onto the uncoated and aggrecan coated implant surfaces. In contrast, the FAK expression was upregulated in the surfaces coated with fibronectin and collagen I which resulted in increased cell-substrate adhesion; p < 0.001. Paxillin, a focal adhesion-associated adaptor protein is involved in modulating cell adhesion and spreading (Wang et al., 2009). We observed that the expression of paxillin was reduced in fibroblasts cultured on the uncoated and aggrecan coated implant surfaces. However, paxillin expression was upregulated (p < 0.001), which resulted in well-spread fibroblasts, in the surfaces coated with fibronectin and collagen I. Vinculin, a membrane-cytoskeletal protein in focal adhesions that is involved in linkage of integrin adhesion molecules to the actin cytoskeleton (Maheshwari et al., 2000), was found in all surfaces. A low content of vinculin was observed in fibroblasts seeded onto uncoated and aggrecan coated surfaces (p < 0.001). This resulted in weakening of adhesion (p < 0.001), conversely a higher content of vinculin resulted in bigger focal adhesions and greater abundance of focal adhesion points in fibroblasts seeded onto collagen I and fibronectin surfaces. There is a consistent pattern in enhanced cell-surface adhesion when the surfaces were coated with collagen I. Cell proliferation highlights a significant benefit on the presence of collagen I to the surface of TS1, while hyaluronic acid did to SS, and fibronectin to TS2. TS1 represented the optimal substrate to promote spreading when coated with hyaluronic acid, while SS and TS2 did with collagen I. Surprisingly, no significant difference was identified between fibroblast adhesion and proliferation to aggrecan coated implants compared to the corresponding uncoated surfaces (p > 0.05).

Breast capsular contracture aetiology remains uncertain, but it is characterised by dense fibrocollagenous connective tissue with local inflammatory response. Furthermore, breast capsule consists of myofibroblasts, which are implicated in contracting the breast implants (Hwang et al., 2010). Thus we decided to limit our samples to breast-derived fibroblasts. However, future investigation, involving different types of cells found in breast capsules, will be of value. Another potential limitation of our study was the total number of samples used for breast tissue (n = 3); a larger number of samples that includes different ethnicities would be beneficial. Our research represents a study focused on enhancing cell-surface adhesion, spreading and proliferation on fibroblasts seeded on breast implants. Coating deposition has been used successfully to improve cell behaviour of medical devices (Filova et al., 2014), but often results in coatings that are too thick, non-uniform and unstable. However, based on previous reports, we can speculate that coating deposition...
can be improved by functionalising the substrate in order to covalently attach the coatings. Chemically grafting coatings to substrates can improve coating adhesion (Kandel et al., 2014) to the substrate resulting in a thin and more stable layer as described previously for other biomedical research techniques.

The study demonstrated that cell adhesion and spreading were sensitive to smooth and textured surfaces. Cell proliferation and cytotoxicity were also studied. It was found that surfaces coated with collagen I, fibronectin and hyaluronic acid did not elicit a cytotoxic effect on breast tissue derived fibroblasts in comparison to the uncoated surfaces. Fibroblast proliferation rate differed significantly among the surfaces investigated. An investigation into cell morphology showed that surface coatings promoted cell morphology modifications and the organisation of actin fibres in all surfaces. Gene expression of adhesion-related proteins was shown to be modified by specific coatings and topography amongst the variety of surfaces tested.

5. Conclusion

In summary, this study has demonstrated the extent and strength of cell adhesion, and subsequent cell proliferation and differentiation based on the physical interactions between cells and the extracellular environment in the form of topography and on the chemical interactions mediated by specific coatings. We have shown that the surface coating of the silicone breast implants induced over-expression of specific adhesion-related genes in breast-derived fibroblasts. Our findings demonstrate that the most promising candidates in the regulation of adhesion and proliferation in the coated textured implant are fibronectin and collagen I. A good adhesion ensures that the implant holds in place; thus preventing micromotion at the host prosthesis interface, the fibroblasts will not over-produce collagen in response to this host-prosthesis shearing motion. Consequently, capsules around these coated surfaces may be thinner and less contracted in comparison to the capsules surrounding the uncoated textured surface implants. The addition of appropriate coating to the surface of silicone breast implants may reduce the risk of capsular contracture formation in the near future. Further studies are required to provide a better understanding of cell-surface interaction in breast fibroblasts and silicone implants.

References

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