Improving skin integration around long-term percutaneous devices using fibrous scaffolds in a reconstructed human skin equivalent model

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Abstract

The interface between synthetic percutaneous devices and skin is a common area for bacterial infection which may ultimately result in failure of the device. Better integration of percutaneous devices with skin may help reduce infection rates due to the creation of a dermal seal. However, the mis-match in material and chemical properties of devices and skin presents a challenge for closing the dermal gap at the skin-device interface. Here we have used a tissue engineering approach to tissue integration by creating a highly fibrous poly(ε-caprolactone) scaffold using melt electrowriting and seeding this with dermal fibroblasts, followed by maturation and insertion into a full-thickness defect made in an ex vivo skin model. The integration of seeded scaffolds was compared with controls including a non-seeded scaffold and a polymer tube with a smooth surface. Dermal fibroblast inclusion in the scaffold and epidermal upgrowth verses downgrowth/marsupialization around the device were used as measures of integration. Based on these measures, almost all pre-seeded scaffolds performed better than both the non-seeded scaffolds and smooth tubes. The hypothesis is that the fibroblasts act as a barrier to epithelial downwards migration, and provide healthy tissue for nascent epidermal development.

Keywords: dermis, melt electrowriting, percutaneous device, polycaprolactone

Running Head

Skin integration around percutaneous devices in an in vitro skin model
Introduction

Percutaneous devices are widely employed in the medical field to connect internal organs to external devices for the transfer of fluids, forces or electrical currents.\textsuperscript{1} Devices include catheters, bone prosthesis, fixators, and drivelines for mechanical circulatory support.\textsuperscript{1} Large, long-term percutaneous devices that breach the skin create a chronic wound and can act as an entry point for pathogenic organisms, which can lead to infections.\textsuperscript{1-3} The incidence of infection varies from 2-33\% for central venous, peritoneal or urinary catheters and is 5\% for fracture fixation devices over the life of the device.\textsuperscript{4} The mis-match in the physical properties between the artificial percutaneous device and the surrounding skin leads to poor integration around the device and exacerbates the chances of infection.\textsuperscript{5,6}

Many different materials and porous surfaces have been investigated in an attempt to overcome the problem of poor skin integration around long-term percutaneous devices. These include sphere templated porous poly(2-hydroxyethyl methacrylate) implants with interconnected pores, fine trabecularised carbon implants consisting of a pyrolytic carbon core with a tightly woven pyrolytic carbon surface, and hydroxyapatite–silicone composites, whereby the sintered hydroxyapatite micro particles are covalently bound to a silicone elastomer.\textsuperscript{7-9} Other strategies that have been used to reduce infection rates with percutaneous devices include coating the surface with antimicrobials, e.g. chlorhexidine, silver sulfadiazine and triclosan,\textsuperscript{10} or combinations of proteins and antibiotics, e.g. a collagen and gentamicin coating\textsuperscript{3} to indirectly promote skin integration.\textsuperscript{3,11} Physical methods to anchor percutaneous devices to prevent micro-pistoning, that is, the relative movement between tissue and the device\textsuperscript{12} and other forms of movement leading to trauma of the device-skin interface have also been investigated. These include the use of a pedestal, which is anchored to the skull,\textsuperscript{13} or using an anchor with a porous flange to achieve tight integration in the dermis and a non-porous ‘chimney’ to diffuse stress at the skin intersection.\textsuperscript{13,14}
An alternative approach to reduce infections with percutaneous devices is the use of a porous anchor, which is pre-seeded with autologous fibroblasts and placed subcutaneously in the abdominal region. This work was pioneered by Kantrowitz and co-workers in the 1980s, based on the hypothesis that the fibroblasts will form a dense collagen network, hindering epithelial downgrowth,\textsuperscript{15} i.e. epidermal migration parallel to the percutaneous device into the dermis, in an attempt to restore epithelial continuity.\textsuperscript{1} The resulting percutaneous access device technology consists of a polycarbonate conical neck and silicone disk anchor covered in a Dacron fabric with heterogeneous porosity and has successfully been implanted in patients as part of a clinical trial for the Kantrowitz CardioVAD cardiac assist device reported in 2014.\textsuperscript{16} The observed episodes of infections in two patients were not linked to the device-tissue interface. Nevertheless, despite these efforts infections remain a major complication with percutaneous devices.

To address this we have extended on the approach of Kantrowitz and coworkers by designing a conduit with highly regular porosity to act as a tissue engineered interface between the percutaneous device and the skin. Cell-seeded porous scaffolds are already known to improve dermal healing (e.g. Dermagraft ®, OrCel ® or Hyalomatrix ®\textsuperscript{17–20}) and so it is logical to expect that a similar approach may improve integration of a percutaneous device.

To create the porous structure we have used the emerging additive manufacturing technique of melt electrowriting (MEW). The advantage of MEW is the capacity to print fibres in the nano- to micrometer range with controlled fibre deposition.\textsuperscript{21} MEW scaffolds have shown potential as dermal substrates, e.g. fibroblast infiltration throughout an interconnected scaffold within 14 days of \textit{in vitro} culture including expression of key dermal extra cellular matrix proteins, namely fibronectin and collagen I.\textsuperscript{22} Similarly, fibroblasts and keratinocytes grown on heparin and fibroblast growth factor-2 coated MEW scaffolds have been shown to form structures similar to native human skin, with a stratified epidermal and a dermal layer.\textsuperscript{23}
Here, we have used MEW to modify percutaneous device surfaces, pre-seeded the modified surface with human derived primary fibroblasts and studied skin integration in a reconstructed human skin equivalent model (HSE).
Methods

Scaffold manufacture

The scaffolds were manufactured from medical grade poly(ε-caprolactone) (PCL, Corbion Purac, The Netherlands) via MEW using an in-house custom built machine described previously. The molten PCL was extruded through a 23 gauge needle at a temperature of 84 °C. A voltage of 6.7 kV was applied to the needle and the jet was collected on a grounded and motorised x-y collector plate in a lattice with layers at 90° and 45° to each other. The needle-collector distance was set to 6 mm. A laser-cutting machine (ILS12.75, Universal Laser Systems, Inc. USA) was utilised to cut the scaffolds into 10 mm × 10 mm squares.

Solid PCL tubes were produced by dip coating a 4 mm stainless steel mandrel into a 10% w/v solution of PCL in chloroform. The mandrel was attached to a rotor at an angle of 60.5° and immersed into the PCL solution 3 times in intervals of 2 minutes while being rotated at 2.5 Hz. Following annealing in a 70 °C water bath for 15 minutes the mandrel was placed in a vacuum oven at 70 °C and vacuum of 7 mbar to remove air bubbles trapped in the PCL before the MEW scaffolds were wrapped around the heated, PCL coated mandrel. This resulted in scaffold-tube constructs with an outer diameter of 4.5 mm. The PCL-tube constructs were dried and immersed in ethanol to aid in the removal from the mandrels.

Scaffold Characterisation

Scaffolds were characterised using scanning electron microscopy (SEM). Un-seeded scaffolds were gold sputter coated at 30 mA for 75 seconds using a Leica EM-SCD005 Sputter Coater (Leica, Germany). Images were taken on a Zeiss Field Emission scanning electron microscope (SEM) (Carl Zeiss Microscopy, Germany) at an accelerating voltage of 5 kV. To determine the mean pore size and fiber size scaffolds were scanned using micro-computed tomography (μCT, SCANCO Medical AG, Brütisellen, Switzerland, μCT), at 6
µm at isotropic voxel size, 45 kVp energy and 1200 ms integration time, to determine the mean pore size and filament size for each scaffold (approximately 1500 µCT slices per scaffold).

**Pre-seeding the scaffolds**

In total, 18 tubular scaffolds were seeded with human derived dermal fibroblasts. Prior to seeding, the scaffolds were sterilised in 80% ethanol for 30 minutes, dried in a laminar flow hood and exposed to UV light for 20 minutes on either side. The sterile scaffolds were placed in fibroblasts growth medium (Invitrogen, Australia) and centrifuged at 212 x g for 10 minutes to ensure the scaffolds were immersed in the media then incubated at 37 °C overnight to allow for gas equilibration.

Human dermal fibroblasts were isolated from split thickness biopsies from consenting patients undergoing elective surgery according to previously published protocols. Ethical approval was obtained from the Queensland University of Technology Research Ethics Committee (1300000063) and Uniting Healthcare / St Andrew’s Hospital Ethics Committee (0346). Following enzyme digestion in 0.125% trypsin (Life Technologies, Australia) at 4 °C overnight to allow separation of the dermis and epidermis, the dermis was comminuted and incubated in 0.05% collagenase-A (Invitrogen) solution in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen, high glucose) at 37 °C and 5% CO₂ for 48 hours to allow harvesting of the fibroblasts. The resulting solution was centrifuged at 212 x g for 10 minutes prior to resuspension and subsequent culture of the cells in fibroblasts growth medium, consisting of DMEM, supplemented with 10% foetal calf serum, 50 U/mL penicillin, 50 µg/mL of streptomycin and 2 mM L-glutamine (all Invitrogen), up to passage 4.

Pre-incubated tubular scaffolds were placed horizontally in 12 well plates prior to application of 30 µL fibroblasts growth medium containing $2 \times 10^5$ cells onto the scaffolds and
incubation for 30 minutes at 5% CO₂ and 37 °C to allow for initial cell attachment. Scaffolds were then inverted and another 30 μL of growth medium containing 2 × 10^5 cells was placed on the scaffolds. Following a further 30 minute incubation the scaffolds were submerged in 2 mL growth medium, which was replaced every 48 hours. This protocol was repeated with fibroblasts from three unique donors. Un-seeded tubular scaffolds and solid PCL tubes served as controls and were submerged in fibroblasts growth medium and placed in the incubator simultaneously with the pre-seeded samples.

Cellular behaviour on the scaffolds

SEM was performed on two scaffolds per biological replicate. Following 4 days of culture, the scaffolds were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer for a minimum of 2 hours, washed in buffer and post fixed in 1% osmium tetroxide in cacodylate buffer for 30 minutes, before dehydration in a graded series of ethanol (50%, 70%, 90% for 2 × 10 minutes and 100% for 2 × 15 minutes). Finally, the samples were chemically dried in hexamethyldisilazane (Sigma Aldrich, Australia) twice for 30 minutes, gold sputter coated and imaged by SEM.

Imaging on a confocal laser scanning microscope (CLSM) (Leica microsystems TCS SP5, Germany) was performed on 3 scaffolds from 3 biological replicates. The cell – scaffold constructs were stained for cell nuclei and actin filaments after 4 days of culture. For CLSM of the pre-seeded scaffolds, the samples were rinsed in phosphate buffered saline (PBS, Oxoid Phosphate Buffered Saline tablets, ThermoFisher) supplemented with magnesium and calcium and fixed in 4% paraformaldehyde (PFA) for 20 minutes. They were then permeabilised with 0.2% (v/v) Triton X-100 (Sigma) in PBS for 10 minutes, prior to incubation for 10 minutes in 1% BSA/PBS (w/v) blocking solution. Samples were fluorescently labelled by placing them in a working solution containing blocking solution and
0.8 U/mL FITC–conjugated phalloidin (Alexa Fluor 488 Phalloidin, Invitrogen, 200 U/mL) and 5 µg/mL 4′6- diamidino–2–phenylindole (DAPI, Life Technologies, USA). Samples were washed 3 times in PBS between each step.

Cell viability of the fibroblasts 4 days after seeding on the tubular scaffolds was assessed in duplicates by live/dead staining, whereby the samples were incubated in a working solution of PBS with 0.67 µg/mL of fluorescein diacetate (Invitrogen) and 5 µg/mL of propidium iodide (Invitrogen) for 20 minutes. Samples were rinsed in PBS after incubation and two fields of views per sample were imaged by CLSM. To ensure a representative count cell viability was manually calculated from two randomly selected fields of view from each sample.

Cell proliferation was determined at 4 and 7 days post seeding using CLSM micrographs of DAPI stained cell – scaffold constructs. Images of those constructs were captured on a Nikon A1R CLSM (Nikon Co. Ltd.). For each sample, 3 z-stacks were acquired.

**Human Skin Equivalent**

The reconstructed human skin equivalent (HSE) models were established based on split thickness biopsies from patients undergoing elective surgery according to previously published protocols\(^{27,28}\) with modifications. Following enzyme digestion in 0.125% trypsin (Invitrogen) at 4°C overnight, the epidermis and dermis were separated and keratinocytes were harvested from the epidermis underside and papillary face of the dermis. Keratinocytes were cultured on a layer of irradiated 3T3s in keratinocyte growth medium (KGM) prepared as previously described.\(^{27-29}\) The fibroblasts were harvested using the same protocol as the pre-seeded tubular scaffolds as described above.

The keratinocytes and fibroblasts were seeded onto de-cellularised and de-epidermised dermis (DED) originating from skin samples and processed to remove endogenous cells.\(^{27,28}\)
Sterile stainless steel rings (Aix Scientific, Germany) with an internal diameter of 9 mm were placed onto the papillary side of each DED and KGM containing non-patient matched $3.6 \times 10^4$ keratinocytes and $1.8 \times 10^4$ fibroblasts were transferred into the centre of the stainless steel ring. The HSEs were lifted to the air-liquid interface after 48 hours of culture in KGM which was replaced every 48 hours.

**Percutaneous model**

After 10 days post-seeding of fibroblasts and keratinocytes, 4 mm holes were created in the HSE models using a biopsy punch (Stiefel) through which the pre-seeded, un-seeded and PCL-only tubular scaffolds were inserted from the dermal side and carefully pulled through using forceps. Each group consisted of 18 samples in total, 2 technical replicates per time-point and 3 biological replicates. On day 3, 7 and 12 the constructs were fixed in 4% PFA for 20 minutes following a PBS wash and sucrose infiltrated with sucrose-OCT (optimal cutting temperature, Tissue Tek, Finland) solutions to preserve structural integrity of the samples. For the sucrose infiltration a stock solution of 50% sucrose in PBS (w/v) was prepared. This stock solution was then used to prepare the sucrose – OCT solutions with different ratios, further detailed in the following. Following 3 PBS washes the samples were submerged in the stock sucrose solution for 2 hours. The samples were then transferred into a solution containing the sucrose stock solution and OCT in a 2:1 ratio for 2 hours, prior to transferring them into a solution consisting of sucrose stock solution and OCT in a 1:1 ratio for a further 2 hours. Next, the samples were transferred to a solution containing the sucrose stock solution and OCT in a 2:1 ratio, prior to placing the samples in OCT only for another 2 hours. Samples were then snap frozen in liquid nitrogen and stored at -80 °C until required for further processing.

**Histology**
The snap frozen samples were sectioned at 20 µm thickness on a cryostat (Leica Biosystems) prior to rehydration in a series of graded ethanol and water. Cell nuclei were stained by placing the samples in Harris haematoxylin (HD Scientific, Australia), differentiated in 1% acid alcohol and blued in Scott’s water, prior to counterstaining in eosin (HD Scientific). Cover-slips were applied using prolong gold mounting medium (ThermoFisher) and slides were imaged using a Zeiss Axio Imager M2 microscope (Zeiss, Germany).

**Mechanical pull-out testing**

Pull-out testing was performed 12 days following insertion of the implants into the HSE using an Instron MicroTester (Model 5848; Instron, USA) equipped with a 5 N load cell. The HSEs constructed from the tissue from each donor (n=3) were placed on a custom printed plate with a hole to accommodate the implants. A custom printed ring was placed on top of the HSE and secured under compression. The exposed end of the tubes were gripped with a hydraulic clamp connected to the tensile tester. Pull-out was measured at a rate of 1 mm/min until failure. Pull-out testing was repeated with HSEs constructed from the tissue of 3 different donors and a minimum of 3 technical replicates per donor and experimental group. Statistically significant differences in the required pull-out force was determined by One Way Analysis of Variance (ANOVA) with Greenhouse-Geisser correction, followed by Tukey’s Test and was accepted where p<0.05.
Results

Melt electrowriting of tubular scaffolds

The scaffold design was based on a 45° wood-pile pattern resulting in scaffolds with regular triangular pores (Figure 1A). The fibres comprising completed scaffolds had a smooth appearance with no observed defects. Mean porosity of the scaffold was 89±1.9%, with an average pore size and fiber thickness of 113.5±7.3 µm and 13.2±0.5 µm, respectively.

To fuse the PCL scaffolds to the solid PCL tubes the core tube was pre-heated then wrapped with the scaffold resulting in fusing at the interface. Importantly this heat-bonding method did not substantially alter the pore geometry (Figure 1B) and resulted in the bottom layers of the PCL fibres being embedded into the PCL sleeve (Figure 1C). Laser cutting of the scaffolds did not influence the fibre morphology or the structural integrity of the scaffolds (Figure 1D).

Cell morphology

To examine the ability of the scaffolds to support growth of fibroblasts, cells were seeded onto the scaffolds and the cell-scaffold constructs cultured and analysed using CLSM and SEM to determine cell morphology and fibre-cell interactions. The triangular pore design resulted in scaffolds with a mean porosity of 89±1.9%, with an average pore size and fiber thickness of 113.5±7.3 µm and 13.2±0.5 µm, respectively and points of cell attachment were observed not just in single planes but also on different layers of the fibres throughout the depth of the scaffold. Moreover, the elongated spindle-like cells were found to bridge between adjacent fibres with inter-fibre distances ranging from 13 to 85 µm (Figure 2A-C). Cell nuclei and actin filaments were present in the voids, indicative of cellular infiltration into the pores (Figure 2A, B). Evidence of cell sheets within the pores after 4 days of culture was
obtained by SEM (Figure 2C, D). The cell sheets formed within pores at different depth of the scaffold.

**Cell viability and proliferation on the tubular scaffolds**

Cell viability on the PCL scaffolds on the day of insertion into the HSE was assessed by live/dead assay. Some cell death was observed in the cells derived from one patient (Figure 3B) yet viability was still greater than 90% for this and all other samples for the 3 biological replicates (Figure 3).

To assess cell proliferation, cells were cultured on the tubular scaffolds for 4 and 7 days and the cell nuclei labelled with DAPI prior to image capture. The fluorescently labelled cell nuclei were visualised and counted using Fiji software (Figure 4). To ensure a representative cell count 3 different field of views per sample were captured. The average cell count after 4 days of cell culture in all samples was approximately 50-75 per field of view and increased to 100 and 140 for 2 of the 3 biological repeats after 7 days.

**Skin–percutaneous device interface**

The skin–percutaneous device interface model used was a reconstructed HSE cultured for 10 days before being punctured with a 4 mm defect through which either the solid PCL tubes, tube/scaffolds or tube/cell-scaffolds were inserted. The model was then grown for 3, 7 and 12 days before histological analysis. The final time point at 12 days was chosen as previous studies have shown that larger 6 mm defects (compared with a 4 mm percutaneous device for this study) can fully heal within this time.26

H&E stained cross-sections of the HSE-device samples allowed macroscopic evaluation of the integration of the device into the skin (Figure 5). Epidermal growth patterns around the PCL samples varied, with some samples showing epidermal upgrowth and some downgrowth (Figure 5).
Unlike the solid PCL tubes, the un-seeded scaffolds exhibited epithelial cells that were integrated with the implant (Figure 5, middle row) particularly after 12 days, yet the epithelial layer still grew downwards along the tube.

The H&E stained sections of the day 4 pre-seeded MEW scaffold–skin interface exhibited a thin layer of fibroblasts covering the lateral surface of the scaffolds, identified by their spindle-like morphology (Figure 6 C and D). Extra cellular matrix (ECM) deposited by the fibroblasts can be observed in the voids (Figure 6 D).

A summary of all samples and time points and epithelial downgrowth (marsupialization) based on microscopic observations of the H&E stained samples is shown in Figure 7. The cells from the epidermis around the PCL tubes did not seem to follow a particular trend towards epidermal downgrowth or upgrowth throughout the selected time course. At day 3, 2 out of 4 samples exhibited epidermal downgrowth. At day 7, 3 out of 4 samples exhibited epidermal downgrowth and at day 12, 4 out of 6 samples exhibited epidermal downgrowth. Whilst all un-seeded samples exhibited epithelial downgrowth at all time points, the majority of the pre-seeded samples exhibited epithelial upgrowth. At day 3, 3 out of 5 samples exhibited signs of epidermal upgrowth, 6 out of 6 at day 7 and 3 out of 5 at day 12.

**Mechanical integration**

Another measure of integration is the pull-out test where the force required to remove the implant from the skin is measured. The 3 types of implants, smooth PCL, un-seeded and pre-seeded scaffolds were maintained in the HSE for 12 days prior to performance of pull-out testing. The pull-out tests were conducted such that the distance the upper clamp travelled was enough to completely remove the tubes/scaffolds from the skin samples, tearing the samples in the process at the implant/dermis interface. Pre-seeded and un-seeded implants exhibited similar mean maximum pull-out forces of 0.498 N +/- 0.067 N and 0.538 N +/-
0.055 N respectively, whereas the smooth PCL samples had a considerably lower mean maximum pull-out force of 0.076 N +/- 0.032 N (p<0.05) (Figure 8).

**Discussion**

In this study the ability of PCL scaffolds manufactured using MEW to increase skin integration around long term percutaneous devices was investigated. Scaffolds manufactured using MEW have previously shown potential as dermal substrates.\(^{22,23}\) MEW is a relatively new technique for producing porous, fibrous scaffolds.\(^{21}\) This solvent-free method involves extrusion of a polymer melt through a charged needle and collection onto a grounded or oppositely charged moving plate. The computer controlled movement of the collector plate dictates the pattern of the deposited fibres. In this study polycaprolactone (PCL) was chosen as an inert polymer substrate since it is highly amenable to MEW, available as medical-grade and is known to exhibit low-immunogenicity.\(^ {31}\) The 45° wood pile pattern for the scaffolds was selected to achieve pore infiltration by the fibroblasts in a short period of time. Cells seeded onto scaffolds have previously been shown to attach to fibre junctions, from where they then migrate outwards.\(^ {22,32,33}\) The chosen pattern offers increased junctions compared to square pores and consequently it can be expected that this pattern increases initial cell attachment. In agreement with previous studies,\(^ {22,34,35}\) the organisation of the cells was dictated by the scaffold design with the cells attaching to, and elongating along the PCL fibres. Nuclei presence, visualised using CLSM, within the voids of the scaffold indicates that the cells migrated throughout the entire thickness of the scaffold. These results suggest that a 4 day incubation period is sufficient for the cells to attach to the fibres and infiltrate the pores. Moreover, this indicates that the selected pore size is suitable to achieve a regular infiltration by the fibroblasts and that cell numbers were adequate for the scaffold size.
Cell viability was assessed using a live/dead assay and showed slight differences across the biological replicates. It is possible that the small proportion of additional cell death exhibited by replicate 2 is explained by assay variability or donor-to-donor variability. Donor-to-donor variability in outcomes using primary cells may be expected due to genetic variability in the population and intrinsic biological factors. As the fibroblasts utilised for these experiments were harvested from full thickness skin explants, the cell population would consist of reticular, as well as papillary fibroblasts. These two fibroblast populations have been shown to exhibit differences in proliferative rates. Depending on the predominant fibroblast cell type on the sample, this may explain the slightly greater cell density and prevalence of cell sheets exhibited by replicate 3 (Figure 3C). Moreover, donor-to-donor variability in human primary dermal fibroblasts has been reported in regards to mechanical strength, cell growth and cell sheet formation. Overall, however, these results confirm that no cytotoxic solvents remained in the PCL tubes after dipcoating. Consequently, the material and approach has the potential to be used 

The increasing amount of cells on the samples, as determined by cell counts based on CLSM, indicates that the cells proliferate on the tubular scaffolds, although there was some significant patient-to-patient variability after 7 days of culture. Similarly, to the differences observed in the live/dead assay the differences between biological repeats could be explained by inter donor variability.

The abrupt chemical and physical boundary between synthetic percutaneous devices and collagenous skin is a likely source of failure. One of the failure modes is epithelial downgrowth, or marsupialization, describing the formation of an inward folded epidermis, forming a sulcus between the dermis and the device, as the skin tries extruding the device from the implant site by attempting to join the skin below the implant. We hypothesised that using MEW scaffolds in a tissue engineering approach could create a gradient boundary
between the synthetic device and skin thereby promoting lateral skin integration by encouraging uniform cell infiltration of the pores.

Epidermal growth patterns around the PCL samples varied, with some samples showing epidermal upgrowth and some downgrowth (Figure 5 top row).

Despite cellular integration with the un-seeded PCL scaffolds, the epidermis tended to grow downwards along the implant. This was similar to observations by Squier and Collins who, when studying epithelial behaviour around porous filters implanted in porcine skin, found epithelial downgrowth in all implants, with more pronounced migration with smaller pores.\textsuperscript{40} Likewise, limited epithelial migration into porous surfaces was also shown by Fukano et al., who report the absence of a contiguous epidermal layer around porous poly(hydroxyethyl methacrylate) implants.\textsuperscript{7}

It is curious that all the un-seeded scaffolds (n=15) had the epidermis growing downwards, whereas some of the PCL tubes had upwards growth (Figure 7). This surprising result may be an anomaly due to variations in dermis samples or defects in the scaffolds, however, further study would be required to confirm the cause of the variation for the PCL tubes.

The fibroblasts secreted ECM into the pores, as demonstrated by H&E staining, these secreted components have been reported to encourage epithelisation.\textsuperscript{41,42} Coulomb et al. used collagen matrices and human primary fibroblasts and keratinocyte to study the effects of ECM and dermal fibroblasts on epidermal growth. Using this model they were able to show that the fibroblasts secrete diffusible factors which facilitate epidermalisation, with the largest epidermal outgrowth exhibited by epidermis growing on collagen reorganised by living fibroblasts.\textsuperscript{43} They reported good integration between the fibroblasts and the dermis of the HSE. Interestingly, the epidermal tongue tended to grow upwards in parallel to the scaffold
surface on some of the pre-seeded scaffolds, appearing to epithelialize the layer of fibroblast in the scaffold (Figure 6). Winter et al. and Heaney et al. have previously hypothesised that epidermal downgrowth could be halted by healthy collagen bundles. Interestingly the results presented herein seem to support this hypothesis.\textsuperscript{44,45} The absence of epithelial downgrowth in many of the implanted pre-seeded samples is an indication that this approach could overcome the marsupialization failure mode for percutaneous devices. These results with the pre-seeded scaffolds in the HSE compare favourably with other attempts at preventing epithelial downgrowth. For instance, Knabe et al. studied continuous ambulatory peritoneal dialysis catheters made from smooth silicone explanted from humans and found epithelial downgrowth along the catheter surface.\textsuperscript{46} Similar failure of smooth polyethylene percutaneous implants in mice characterised by significant extrusion of the device has also been observed.\textsuperscript{45} The inclusion of porosity alone does not prevent downgrowth. Shin and Akao\textsuperscript{47} measured epithelial downgrowth around porous hydroxyapatite implants in a canine model and observed downgrowth, yet for a non-porous version of the same material they observed good integration. Conversely, in the same study non-porous glassy carbon and $\beta$-tricalcium implants exhibited downgrowth.\textsuperscript{47} These observations suggest that epithelial downgrowth is not dictated by the presence or absence of pores alone, other factors that may contribute to the divergence of these observations may be the different materials used, the size of the pores and the overall implant, the duration of the study and the host species in which the implants were evaluated.

Additionally to H&E staining of the skin-device interface, we measured integration using mechanical pull out testing. The similar forces between seeded and un-seeded scaffolds suggests that the scaffold itself plays a greater role in the pull-out force than the cells. It should be noted that the pull-out force is independent of up- or down-growth (Figure 7),
rather the pull-out force is a measure of the adhesion of the dermis and the cell/scaffold construct.

The pull-out forces measured here are similar to results reported by Furuzono et al.\textsuperscript{9} who observed forces of 0.03 N required to remove smooth silicone and 0.49 N required to remove silicone modified with HA from rat skin. Despite the significant improvement in pull-out force with the scaffold-wrapped tubes, the forces are still relatively small and would not prevent rupture in case of trauma to the device exit site.

The scaffolds could, however, provide sufficient stability to reduce micro-pistoning. It is thought that tissue adhesion to implants can only take place if relative movement can be prevented. Reduction of micro-pistoning is a key factor in achieving better skin integration and to allow the skin to heal around the percutaneous device.\textsuperscript{48}

The absence of cellular attachment to the solid PCL tubes was reflected in the pull-out testing, with a maximum force of 0.076 N +/- 0.032 N required to rupture the skin-implant interface and during the histological processing of the samples, with the tissue separating from the implant. Separation between the tissue and implant during histological processing was not observed with the pre-seeded or un-seeded implants. Together with the observations from the H&E micrographs this indicates that the scaffolds provide better cues for cell adhesion, for both, the fibroblasts and the keratinocytes. Fibroblast, as well as keratinocyte adhesion to fibres has previously been reported by Sun et al. who investigated normal human dermal fibroblast and keratinocyte from the HaCaT cell line attachment to electrospun poly L-lactic acid fibres of various diameters.\textsuperscript{49}

Limitations to the study included the absence of immune response and blood supply to the skin model. Nevertheless, observations made with regard to skin integration are more relevant compared to those made in for example small rodent models, due to numerous
differences in tissue architecture and the fact that wounds in small rodents heal primarily through contraction, as opposed to re-epithelisation, such as in humans. Moreover, not all the implants withstood the routine processing for the sectioning process for the histological study. This could mean that some of the results observed by microscopic analysis of H&E stained cross-sections could have been caused by sectioning artefacts and don’t necessarily represent the cellular behaviour at the implant interface.

This study focussed on skin integration around the percutaneous device exit site. In order to study whether the increased skin integration creates a seal against bacteria it could be of interest to challenge the exit site with bacteria and study bacterial infiltration into the scaffolds and dermis.

PCL has a quick degradation rate in vivo ranging from 2-5 years, ultimately it would be necessary to manufacture the scaffolds from a material which is non-degradable in vivo, such as polyurethane. However, PCL was chosen for this study as it is well characterised and highly amenable to MEW and the primary aim of this study was to evaluate the feasibility of the pre-seeding approach.

The reality that commercial and clinically available pre-seeded dressings and subcutaneous implants have been utilised in patients, demonstrate that pre-seeding percutaneous devices may be viable. However, the fibroblast layer does not appear to prevent epidermal marsupialisation in all cases. This may be due to donor variability or indicate that the pre-seeding approach is not sufficiently robust and reproducible. An alternative approach could consist of coating the scaffolds with synthetic ECM components or growth factors to encourage more robust seeding and initial growth of pre-seeded fibroblasts.

Conclusions
This study outlines a novel tissue engineering inspired approach to encourage skin integration with percutaneous devices. Our aim was to determine whether MEW modified tubes, pre-seeded with dermal fibroblasts, have the ability to encourage skin integration in a reconstructed HSE model. The performance of pre-seeded tubes, in terms of skin integration and mechanical stability, was compared to MEW modified un-seeded tubes and solid PCL tubes. An obvious disadvantage of the pre-seeding strategy is that for translation to the clinic it requires taking a patient biopsy then culturing the patient’s cells on the scaffolds for a period of days prior to implantation. To address this, future work will investigate ways of achieving the same integration by shortening culture times (e.g. higher seeding density) or using the minimum viable biological components via ECM or growth factor coatings without cells.

Nonetheless, the MEW modified surface encourages skin integration in the HSE model and has the potential to reduce micro-pistoning around percutaneous devices. The results further demonstrate the ineffectiveness of the solid PCL tubes only to allow for epidermal attachment and improve mechanical stability. However, whether epidermal downgrowth or upgrowth is better to create a tight biological seal against bacteria around the implant exit site needs to be assessed in further experiments.
Conflicts of Interest

There are no conflicts to declare.

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**Figures**

**Figure 1**: Structural observation of the MEW scaffolds and tubular scaffolds using SEM. (A) MEW scaffold with a 45° lay-down pattern; (B) Scaffold – PCL tube construct; (C) Interface of the PCL tube and scaffold after heat clamping and (D) Laser cut edge of the scaffold. Scale bar denotes 1000 µm for A, B and D and 100 µm for C.
Figure 2: Morphological evaluation of the cells on the tubular scaffolds, 4 days after seeding, using CLSM and SEM. (A) CLSM micrograph of the tubular scaffolds on the tubular scaffolds on the day of insertion into the HSE show fibroblasts infiltrating the pores, with cell nuclei present in the voids, indicated by white arrows and (B) migration along the fibres. Tubular scaffolds were stained with TRITC conjugated Phalloidin to visualise actin filaments (red) and DAPI to visualise cell nuclei (blue). Fibroblast attachment to the fibres and cell sheet formation was assessed using SEM. (C) Micrographs show points of cell attachment throughout the entire thickness of the scaffold, labelled with black arrows and (D) that the cells start forming cell sheets within the pores after 4 days of culture. Scale bar denotes 100 µm.
**Figure 3:** Cell viability for all three biological replicates. Representative images of (A) biological repeat #1, (B) biological repeat #2 and (C) biological repeat #3 on the scaffolds on the day of insertion into the human skin equivalent. Cells were visualised using live/dead cell staining and images captured using a CLSM. Green = live, red = dead. Scale bar 250 µm.

**Figure 4:** Quantitative analysis of cell proliferation on the tubular scaffolds. Cell nuclei were visualised with DAPI after 4 and 7 days of cell culture and counted using Fiji software. Results show a significant difference (*p= 0.04) between the day 3 and day 7 timepoint for biological repeat #3 and between biological repeat #2 and biological repeat #3 at day 7 (*p= 0.005). Significance was determined by 2-way ANOVA and Sidak’s multiple comparison test.
**Figure 5**: Representative micrographs of cross-sections of the implants in the HSE stained with H&E. The top row shows the PCL only implants after 3, 7 and 12 days with the epidermis growing downwards (3 and 7 days) and upwards (12 days) along the implant. The asterisk denotes the PCL tube separated from the tissue. The second row shows the un-seeded scaffolds at 3, 7 and 12 days. No fibroblasts can be observed on the scaffolds and the epidermis tends to grow downwards in parallel to the implant. The bottom row shows the pre-seeded scaffolds after 3, 7 and 12 days. Fibroblasts are present on the outer layer of the scaffolds and the epidermis is growing upwards in parallel to the scaffolds. Scale bar denotes 1000 µm.
Figure 6: Cellular behaviour at the implant-skin interface observed using light microscopy. (A) Un-seeded scaffold in the HSE stained with H&E after 12 days of culture showing the epidermal cell – scaffold interconnection and (B) shows the highlighted segment in A, with black asterisks showing PCL fibres in the epidermis. (C) Pre-seeded scaffold in the HSE stained with H&E after 7 days of culture. (D) shows the highlighted section in C, with black asterisks pointing to PCL fibres, black arrowheads to cell nuclei and the white arrowheads to ECM deposited by the fibroblasts. Scale bar denotes 1000 µm for A and C and 100 µm for B and D.
Figure 7: Overview of epidermal migration along the implants for all samples at all time points, based on visual assessment of H&E stained cross-sections visualised on a light microscope.
**Figure 8:** Maximum pull-out force in Newtons of pre-seeded, un-seeded and PCL implants inserted into the HSE. Data are presented as the mean pull out force in Newtons +/- SD (pre-seeded n=14; both un-seeded and PCL samples n=15). Statistical significance was determined by one way ANOVA and Tukey’s test and accepted where: * p<0.05.