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Plasma polymer and biomolecule modification of 3-D scaffolds for tissue engineering

Author(s), Corresponding Authors* David E. Robinson, Sameer Al-Bataineh, Brooke L. Farrugia, Andrew Micheltmore Allison J. Cowin, Tim R. Dargaville, Robert D. Short, Louise E. Smith* and Jason D. Whittle*

Dr. D.E. Robinson, Dr. S. Al-Bataineh, Dr. A. Micheltmore, Professor A. Cowin, Professor R.D. Short, Dr. L.E. Smith and Dr. J.D. Whittle

Mawson Institute, University of South Australia, Mawson Lakes, Adelaide, South Australia 5095, Australia.

E-mail: davidwardrobinson@outlook.com

E-mail: Sameer.Al-Bataineh@unisa.edu.au

E-mail: Andrew.Micheltmore@unisa.edu.au

E-mail: Allison.Cowin@unisa.edu.au

E-mail: Rob.Short@unisa.edu.au

E-mail: Louise.Smith@unisa.edu.au

E-mail: Jason.Whittle@unisa.edu.au

Dr. B.L. Farrugia, Dr. T.R. Dargaville

Tissue Repair and Regeneration Program, Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Avenue, Brisbane, Queensland 4059, Australia.

E-mail: b.farrugia@unsw.edu.au

E-mail: t.dargaville@qut.edu.au

Dr. A. Micheltmore, Dr. L.E. Smith, Dr. J.D. Whittle

School of Engineering, University of South Australia, Mawson Lakes, Adelaide, South Australia 5095, Australia

E-mail: Andrew.Micheltmore@unisa.edu.au

E-mail: Louise.Smith@unisa.edu.au

E-mail: Jason.Whittle@unisa.edu.au

Dr. D.E. Robinson, Dr. B.L. Farrugia, Professor A. Cowin, Dr. T.R. Dargaville, Professor R.D. Short, Dr. L.E. Smith and Dr. J.D. Whittle

Wound Management Innovation Cooperative Research Centre, West End, Queensland, 4101, Australia

E-mail: davidwardrobinson@outlook.com

E-mail: b.farrugia@unsw.edu.au

E-mail: Allison.Cowin@unisa.edu.au

E-mail: t.dargaville@qut.edu.au

E-mail: Rob.Short@unisa.edu.au

E-mail: Louise.Smith@unisa.edu.au

E-mail: Jason.Whittle@unisa.edu.au

Dr. B.L. Farrugia

Abstract

Plasma polymerization was used to coat a melt electrospun polycaprolactone scaffold to improve cell attachment and organization. Plasma polymerization was performed using an amine containing monomer, allylamine, which then allowed for the subsequent immobilization of biomolecules i.e. heparin and fibroblast growth factor-2. The stability of the plasma polymerized amine-coating was demonstrated by X-ray photoelectron spectroscopy analysis and imaging time-of-flight secondary ion mass spectrometry revealed that a uniform plasma amine-coating was deposited throughout the scaffold. Based upon comparison with controls it was evident that the combination scaffold aided cell ingress and the formation of distinct fibroblast and keratinocyte layers.

Keywords: Plasma Polymerization; Scaffold Modification, Glycosaminoglycan, Electrospinning, Skin Construct

Introduction

Skin replacements were among the first commercial tissue engineered (TE) products manufactured, recognising that chronic wounds are a major burden on healthcare systems worldwide. In developed countries, rapidly ageing populations and increased incidences of obesity and diabetes present a major challenge and it is estimated that in the USA 6 million chronic wounds are treated per annum (p.a.) at a cost of \$25Bn¹. Skin products are used for the treatment of wounds arising from injury (e.g. burns) and to aid healing in patients where normal healing is compromised, e.g. patients with non-healing diabetic foot ulcers. Lead products include Dermagraft, neonatal foreskin fibroblasts in a polyglactin mesh, and

Apligraf a living cell product containing both fibroblasts and keratinocytes seeded in a bovine collagen I matrix². As of 2014, these two major commercial products, Apligraf and Dermagraft had revenues of more than \$300M p.a.^{3, 4}.

In addition to wound healing, skin models have significant utility in the investigation of wound healing⁴⁻⁷. Such models provide an alternative to the extensive use of preclinical models (mice/pig) as animal testing becomes increasingly controversial and it incurs significant expense. Consequently, there has been substantial efforts made to develop better *in vitro* models⁸⁻¹⁵. Currently, laboratory skin models are typically based on de-epidermized dermis (DED) re-populated with primary fibroblasts and keratinocytes^{4, 16-18}. The DED retains the architecture for *in vitro* testing^{8, 19}. However, fabrication of these skin models is limited by the availability and variability of skin. Discards from cosmetic surgeries in the clinic are commonly used and there would be a significant advantage gained from the fabrication of skin models based upon synthetic scaffolds that could be cell-seeded.

Synthetic scaffold materials commonly used in TE products include polycaprolactone, and polyglycolide (poly(glycolic acid)) or polylactide (poly(lactic acid))^{20, 21}. Scaffolds are increasingly engineered to mimic the original architecture of the extracellular matrix (ECM)²²⁻²⁵. Consequently an understanding and tailoring of the required scaffold microstructural and surface chemical properties is a critical step to facilitating cell colonization and the production of the desired tissue, and ultimately a functional organ²⁶.

Whilst the effect of fibre diameter, porosity, topography and scaffold thickness on cell colonization and tissue growth have been investigated^{8, 27-29} little emphasis has been placed on modification of surface chemistry more on optimising the bulk properties of the polymer to achieve improved biomolecule binding^{20, 30-34}.

Ordinarily the low energy surfaces of synthetic (polymeric) materials are not conducive to cell colonization. Consequently, studies have shown that TE production can be aided by coating synthetic scaffold surfaces with biomolecules such as type 1 collagen, glycosaminoglycans (GAGs), chitosan³⁰⁻³², laminin α 3, β 3, γ 2 and the heparan sulphate proteoglycan, perlecan³³.

In the production of any cell-seeded scaffolds there are three significant issues; the uniform and adequate ingress of cells into the scaffold, cell proliferation within the scaffold, and in the case of co-culture, the organization of cell populations within the scaffold. A number of researchers have explored the use of plasma methods to modify poly caprolactone scaffolds⁶³⁻⁶⁶. In this paper, we explore the synergistic effect of using surface modification and biomolecule binding to enhance cell migration, proliferation and organization within a 3D synthetic scaffold. In³⁴ we showed (in 2-D culture) that use of a relatively inexpensive biomolecule (heparin) to “sequester” an expensive growth factor, fibroblast growth factor-2 (FGF-2), reduced the amount of GF used in culture (by up to 99%) and even reduced the need for serum. This approach is now extended to a 3-D scaffold, but herein our primary interest is in scaffold modification to improve cell ingress, proliferation and organization.

This paper describes a method for the production of a synthetic skin composite, starting from melt electrospun polycaprolactone (PCL) scaffolds³⁵. Plasma polymerization is employed to deposit a thin uniform and conformal layer over the entire internal surface of the PCL scaffold³⁶. The use of plasma polymerized coatings improves biomolecule binding^{37, 38} and more specifically binding of GAGs³⁹.

We use heparin as a surrogate of heparan sulphate (HS). HS is an important GAG biomolecule of the ECM^{40, 41} and can bind GF's including FGF-2. Our hypothesis is that a

heparin/FGF-2 surface will provide the necessary cues to cells to invade the scaffold, increase in number, and even help a mixed cell population re-organize.

The major challenges addressed in this work are the extension of the combined approach of plasma polymerization and followed by biomolecule coating already established in 2-D to 3-D scaffold materials and the evaluation of the impact of this technology in the production of skin composites. We explore cell attachment, ingress and proliferation and cell organisation of two different cell types, namely primary human keratinocytes and primary human dermal fibroblasts in co-culture within modified PCL scaffolds.

Materials and methods

Melt electrospun polycaprolactone (PCL) scaffolds

Melt electrospun scaffolds were fabricated using a direct writing method described previously in which a translating *x-y* stage is used to collect the fibres³⁵. Polycaprolactone (PCL) (Perstorp, $M_n \sim 41$ kDa, PDI = 1.78) pellets were placed into a 5 ml Luer lock syringe (Hamilton, USA) and heated to 60 °C in an oven overnight to remove any air bubbles from the melt. A blunt 23 G needle acting as the spinneret was attached to the syringe and placed into an electrical heating system heated to 65°C. The PCL melt was then electrospun using a collector distance of 15 mm, and flow rate of 20 μ l/h with a voltage of 10 kV applied to the spinneret. Writing with the PCL fibres was achieved by collecting on a grounded plate connected to a programmable *x-y* stage, controlled using Mach 3 software. The scaffold design consisted of a 10 mm x 10 mm square consisting of interlaced diagonal lines at a translational velocity of 12.5×10^{-3} m/s.

Scaffold characterisation

Scaffolds were visualized using scanning electron microscopy (Hitachi, TM3000) prior to surface modification. Samples were imaged under low vacuum to eliminate the need for a conductive coating at an accelerating voltage of 5kV. Images were recorded, and analysed using Image J (Version 1.45c, NIH) to determine both fibre diameter and interfibre distance (10 measurements per image).

Plasma polymerization and analysis

A schematic of the samples within the reactor is shown in *Figure 1a* and comprises a cylindrical stainless steel vacuum vessel with a diameter of 30cm and a volume of around 20 litres⁶². The scaffolds were suspended from a wire (*Figure 1b*) to aid the plasma coating throughout the scaffold. A schematic explaining how the scaffold was prepared for ToF-SIMS analysis is shown in *Figure 1c*. The reactor was evacuated to a base pressure better than 4×10^{-4} mbar, using a two-stage rotary pump with a liquid N₂ cold trap. A needle valve was used to control the flow of monomer vapour into the reaction chamber and the pressure in the reactor chamber with allylamine was 1.9×10^{-2} mbar.

Allylamine (AA) monomer (Sigma Aldrich) underwent several freeze-pump-thaw cycles prior to use to remove dissolved gases. A total monomer flow rate of ca. 5 sccm as measured by the method of Yasuda⁴² was used for all depositions and allowed to stabilize for a few minutes prior to deposition. Plasma was ignited using a radio frequency generator at 13.56 MHz and a power of 5W delivered via an automatic impedance matching network (Coaxial Power Systems Ltd, UK). The power reported herein is read from a dial power meter that is external to the plasma and the power given is only indicative of the actual power within the plasma⁴³. The process was allowed to run for 40 min at which point the power was switched

off and the chamber pumped back down to base pressure, resulting in a plasma polymerised allylamine (ppAAmm) surface.

X-ray photoelectron spectroscopy (XPS) analyses were carried out using a SPECS SAGE XPS system with a Phoibos 150 hemispherical analyser and a MCD-9 detector was used. Further details of operation can be found in^{34, 44, 62}. Samples for XPS analysis were collected on Al substrates.

Heparin incubation of ppAAm modified scaffolds

The ppAAm-modified scaffolds were incubated with heparin (sodium salt) (Sigma Aldrich, 18 kDa average MW) made up at 10 µg/ml in phosphate buffered saline (PBS). Scaffolds were incubated in 500 µl of the heparin solution for 18 hrs at ambient room temperature. Subsequently the solution was removed and the scaffold rinsed three times with PBS, once with ddH₂O, and left overnight to dry under a laminar flow hood.

Unmodified PCL, ppAAm coated PCL and ppAAm-heparin incubated PCL scaffolds were all cross sectioned and analysed by time-of-flight secondary ion mass spectrometry (ToF-SIMS).

Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS)

Analysis was performed using a Physical Electronics Inc. PHI TRIFT V nano-ToF instrument equipped with a pulsed liquid metal ⁷⁹Au⁺ primary ion gun (LMIG), operated at 30 kV. Surface analysis was performed using ‘unbunched’ Au₁ beam settings for imaging and ‘bunched’ Au₁ beam settings for mass spectrometry. Negative ToF-SIMS images were collected using a 500 x 500 µm² raster area and an acquisition time of 5 min. Negative ToF-SIMS spectra were collected from regions of interest (ROI) on individual fibres using a 5 x 5 µm² raster area and acquisition time of 1 min. Mass calibration of the spectra was carried out

using CH^- , C_2H^- , and Cl^- ions. Experiments were performed under a vacuum of 10^{-8} Torr or better and in the static mode to minimise possible effects arising from sample damage.

FGF-2 ELISA assay

A solution of heparin was made up at 10 $\mu\text{g}/\text{ml}$ in PBS and 100 μl was added per well to a ppAAm coated 96 well plate to give 1 $\mu\text{g}/\text{well}$. The plate was incubated with a lid to prevent evaporation for 18 hours, protected from light at room temperature, and was then washed twice with PBS and dried on a paper towel. Blocking solution (2% BSA/PBS) was added to each well to prevent non-specific binding of proteins and antibodies in later steps, and incubated at 37°C for 1 hour and then washed 3 times in PBS.

An FGF-2 (R&D systems cat: 450-14) calibration curve was established by 6 double dilutions of a stock solution of FGF-2 of 100 ng/well in 100 μl of PBS. The lowest concentration was 1.56 ng/well. FGF-2 was incubated at 37°C for 2 hours on heparin-coated microplate wells. Following incubation, these plates were washed twice with PBS and dried on a paper towel. Biotinylated polyclonal goat anti-human FGF-2 antibody (R and D Systems CAT: BAM233) was added at a concentration 0.025 $\mu\text{g}/\text{well}$ for 1h. Following another wash the presence of bound protein was detected by adding 100 μl of 0.1 $\mu\text{g}/\text{ml}$ ExtrAvidin™ - Alkaline Phosphatase (Sigma-Aldrich, Australia) for 30min. Colour was then developed using p-nitrophenylphosphate before being read at 405nm using a plate reader (BMG Labtech, UK).

Fibroblast and keratinocyte isolation and culture

Primary human dermal fibroblasts (fibroblasts) and primary human keratinocytes (keratinocytes) were isolated from surgical discards from cosmetic procedures. All patients gave informed consent for skin to be used for research through a protocol approved by the

Ethical Committee at The Queen Elisabeth Hospital and the University of South Australia Human Ethics Committee. Cells were isolated and expanded according to previously published protocols⁴. Keratinocytes were expanded on irradiated 3T3 cells in Greens medium (Dulbecco's modified eagles medium (DMEM) (Glutamax high glucose, Life Technologies, Australia) and Hams F12 medium (Sigma-Aldrich, Australia) in a 3:1 ratio supplemented with 10% (v/v) foetal calf serum (FCS) (AusGenex, Australia), 100 IU/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Australia), 0.625 µg/ml amphotericin B, 6.25 µg/ml adenine, 10 ng/ml epidermal GF, 1.36 ng/ml triiodothyronine, 5 µg/ml transferrin, 5 µg/ml insulin, 0.4 µg/ml hydrocortisone and 8.5 ng/ml cholera toxin (Sigma-Aldrich, Australia)) at 5% CO₂, 37°C and 95% humidity. Media was changed twice a week and keratinocytes were used between passages 1 and 3. Fibroblasts were expanded in fibroblast culture medium (FCM) (Dulbecco's Modified Eagles Medium (high glucose) (DMEM), 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin and 0.625 µg/ml amphotericin B at 5% CO₂, 37°C and 95% humidity. Media was changed twice a week and fibroblasts were used between passages 3 and 9.

Cell seeding and culture in scaffolds

PCL, PCL + ppAAm and PCL + ppAAm + Heparin (10 µg/ml) + GF (FGF-2 10 ng/ml) scaffolds were centered in an untreated 6 well plate and a seeding ring of 1 cm internal diameter was placed on top of the scaffolds. 1ml of FCM was placed around the scaffold to minimize cell loss from the scaffold⁴⁵. Fibroblasts (4×10^5) were seeded in 200µl of FCM into the centre of the rings and allowed to attach for 30 minutes. The centre of the ring was topped up with media to a total volume of 500µl. The media was changed carefully within the ring every 24 h for 4 days before the ring was removed. The media was then topped up around the well to 3 ml to cover the whole scaffold. The scaffolds were cultured for one week

in FCM to allow time for the fibroblasts to infill the scaffold. A resazurin assay was carried out to investigate the viable cell content in each of the modified scaffolds.

After this, the scaffolds were washed in PBS before the seeding ring was replaced and each scaffold was seeded with 4×10^5 primary keratinocytes in Greens medium into the centre of the seeding ring. The cells were allowed to attach for 30 min and then the well was topped up to 3ml of Greens medium once more. The cells were maintained in culture for 4 days after which the seeding ring was removed and the scaffolds were raised to an air liquid interface on a metal support grid in a fresh 6 well plate. Culture continued for up to a further 3 weeks with media changes every 2 to 3 days.

Resazurin assay

A resazurin assay was used to measure the viable fibroblast content of the 3 types of scaffolds. Resazurin sodium salt (Sigma-Aldrich, Australia) was dissolved in PBS at a concentration of 110 $\mu\text{g/ml}$ and filter sterilised to form a stock solution. 10% (v/v) resazurin stock solution in cell culture medium forms the working solution. The media was removed from the wells and the scaffolds moved to a 24 well plate for the initial fibroblast attachment investigations. 500 μl of the 10% resazurin solution was added to each well plus one blank well. For scaffolds that were to be used for seeding keratinocytes, the assay was carried out in a 6 well plate and 3 ml of the 10% (v/v) resazurin stock was required. In both cases the cells were incubated for 2 h at 37°C in a 5% CO_2 atmosphere. The blue resazurin is irreversibly reduced by enzymes within metabolically active cells into the fluorescent pink resorufin compound. At this point 200 μl of the solution was removed to a 96 well plate and the fluorescence read at λ_{ex} 544nm and λ_{em} 590nm on plate reader (BMG Labtech, UK).

Preparation for cryo-sectioning

After incubation the samples were gently washed with PBS to remove any non-adherent cells and fixed with 4% formaldehyde in PBS overnight at 4°C. The samples were then washed 3 times with PBS to remove any traces of fixative. The scaffolds were cut into quarters using a scalpel blade and one quarter was mounted in optimal cutting temperature solution (OCT) and stored at -80°C. The scaffolds were mounted into the viscous OCT solution in a plastic mould and immediately plunged into liquid nitrogen using a pair of tweezers. The samples were then stored until ready to be cryo-sectioned. 15 µm thick sections were cut at -12°C. Each section was transferred to a Super-Frost Plus slide (Thermo scientific).

Immunohistochemistry staining with DAPI and pan-cytokeratin-FITC

Following sectioning slides were stained with DAPI (4',6-Diamidino-2-Phenylindole, Dilactate) (Molecular Probes, Life Technologies, Australia) for detection of cell nuclei and FITC-conjugated pan-cytokeratin antibody (Sigma Aldrich F3418) was used to stain keratins. The sections were washed for 30 s in dH₂O to remove excess OCT mounting fluid. The samples were treated for 10 min with 0.1% triton X to permeabilise the cell membranes, before washing 3 times in water. The slides were then blocked for non-specific staining 1 h with 2% PBS-BSA, before washing 3 times in PBS. Samples were then incubated with DAPI (1:100) and anti-pan-cytokeratin (1:50) for 1 h at room temperature whilst being protected from light, followed by washing 3 times with pure water to remove any salt crystals, and mounted. Samples were imaged using a Nikon AR confocal microscope running NIS-Elements AR.

Statistical analysis

ANOVA with Tukey multiple comparison test was performed to analyse the statistical difference between various conditions in which fibroblasts were cultured (mean $n=3 \pm SD$) (3 experiments for each in duplicate for each condition each time), and to determine whether the

average cell viability was affected by the presence of bound biomolecules. The number of cells at each condition was compared with tissue culture plastic as a control within the same time period at the same serum concentration. GraphPad Prism 6 was used to perform the ANOVA tests and the statistical difference was marked as: significant *($P < 0.05$), ** ($P < 0.01$); very significant *** ($P < 0.001$) or extremely significant **** ($P < 0.0001$).

Results

Characterisation of PCL scaffolds - morphology

Melt electrospun scaffolds were characterised by electron microscopy (EM) prior to surface modification and subsequent *in vitro* experiments. A representative EM image of the electrospun scaffolds (*Figure 1d*) shows fibres were uniform in morphology and free from beading. By electrospinning in direct writing mode using an *x-y* plane moving collector the scaffold fibres were deposited periodically with only minor deviation from the pattern programmed to the *x-y* stage. Similar observations have been reported for direct written melt electrospun scaffolds³⁵. The fibre diameter and pore size (interfibre distance) of the scaffolds were determined using image analysis software and found to range from 21 to 43 μm with an average of $30.6 \pm 3.7 \mu\text{m}$ (mean $\pm$ S.D.) for the fibre diameter, and 18.8 to 147.2 μm , with an average of $67.8 \pm 27.8 \mu\text{m}$ (mean $\pm$ S.D.) for the interfibre distance (*Figure 1e-f*).

Characterisation of modified scaffolds – XPS

XPS spectra of ppAAm surfaces for both unwashed and samples washed (18 h in H_2O), were consistent with previously published data having N/C ratios of 0.25 before washing and 0.21 after washing⁴⁶. The deposited film contained carbon and nitrogen, elements present in the starting monomer, with the addition of some oxygen (6%) (*Figure S1*). The presence of oxygen in ppAAm coatings is common, and is the result of either residual air and water

within the reactor chamber, or the reaction of trapped radicals in the plasma polymer film with atmospheric oxygen and water on removal from the reactor⁴⁷. The surfaces produced and used in this work contain similar chemistries to those previously shown to contain positive charges and bind GAGs in a functional orientation⁴⁸⁻⁵⁰.

Characterisation of modified scaffolds –ToF-SIMS

The data in *Figure 2a-c* show negative ToF-SIMS images for PCL scaffolds (untreated), after modification with a thin film of ppAAm (PCL/ppAAm) and after incubation of PCL/ppAAm with heparin (PCL/ppAAm/heparin), respectively. Fragment ions originating from the chemical structure of PCL [CO_2H^- (m/z 45), $\text{C}_6\text{H}_9\text{O}_2^-$ (m/z 113) and $\text{C}_6\text{H}_{11}\text{O}_3^-$ (m/z 131)], ppAAm [CN^- (m/z 26) and CNO^- (m/z 42)] and heparin [SO_2^- (m/z 64) and SO_4^- (m/z 96)] were initially identified from negative ToF-SIMS spectra (0-150 m/z) (*Figure S2, ESI*). These negative ions were then mapped on the cross section of the scaffolds. The reason for imaging the cross section of the modified scaffolds was to determine whether the ppAAm coating and heparin binding were uniform throughout the scaffold materials. The total intensity of CO_2H^- , $\text{C}_6\text{H}_9\text{O}_2^-$ and $\text{C}_6\text{H}_{11}\text{O}_3^-$ fragments were high in the image of the untreated PCL scaffolds (*Figure 2a*). However, the total intensity of SO_2^- and SO_4^- fragments were as expected at the noise level demonstrating the absence of sulphur in the chemical structure of PCL. Furthermore, the presence of low intensity of CN^- and CNO^- fragments is attributed to contaminants, but also we believe that a large contribution to the total intensity comes from $\text{C}_2\text{H}_2\text{O}^-$ and C_2H_2^- fragments (have very close mass to CN and CNO , respectively). After coating the PCL scaffolds with a thin film of ppAAm (*Figure 2b*), the total intensity of CN^- and CNO^- fragments increased substantially and the total intensity of CO_2H^- , $\text{C}_6\text{H}_9\text{O}_2^-$ and $\text{C}_6\text{H}_{11}\text{O}_3^-$ fragments decreased. As expected, the total intensity of SO_2^- and SO_4^- fragments remained very low. Incubation of PCL/ppAAm scaffolds with heparin resulted in binding of

heparin molecules on the ppAAM surface by means of electrostatic interaction⁵⁷. Heparin binding was demonstrated by the substantial increase of the total intensity of SO_2^- and SO_4^- fragments (*Figure 2c*). These results demonstrate that successful surface modifications were achieved throughout the scaffolds with first a thin film of ppAAM and then the subsequent binding of heparin molecules. Uniform GAG coating throughout the scaffold is important for the subsequent capture of FGF-2.

FGF-2 ELISA

In *Figure 3a*, the binding of FGF-2 to ppAAM-coated and heparin modified 96 well plates is shown. Prior to the addition of FGF-2, heparin was adsorbed to the allyl amine plate surfaces from 100 μl of a 10 $\mu\text{g/ml}$ solution in PBS for 18 hours. FGF-2 was bound at a range of concentrations between 100ng/well and 1.5ng/well to heparin-coated allylamine-modified 96 well plates.

The ELISA data show FGF-2 was bound at all concentrations; this is an important prerequisite step to establish that a hep/FGF-2-coated surface has been produced, where the heparin facilitates the subsequent binding of FGF-2. Subsequently in the scaffold work FGF-2 was bound at concentration of 10ng/ml.

Seeding of modified scaffolds – fibroblast attachment

Figure 3b shows the results of a resazurin assay used to measure the initial attachment of viable fibroblasts at 1h on the 3 scaffolds - the PCL scaffold and PCL/ppAAM and heparin/FGF-2 modified scaffolds. When fibroblast cells were seeded the results indicate that initial cell attachment to the modified scaffolds increased compared to the basic PCL with each modification, PCL/ppAAM (at $P < 0.05$) and hep/FGF-2+ ($P < 0.01$). However, at this

time point the difference in cell viability between the PCL/ppAAm and heparin/FGF-2 was not significant (at $P < 0.05$).

Cells that had fallen through the scaffold (to the well bottom) during incubation were recovered and counted to find the initial seeding efficiency. This count was used to construct the data shown in SI-3 (cell number attached to each of the scaffolds at 1 h). The heparin/FGF-2 surface was found to be significantly more efficient in promoting the initial attachment of fibroblasts compared to the other two surfaces (1 and 2) ($P < 0.01$ and < 0.05 , respectively).

The data in SI-3 indicate that the PCL and PCL/ppAAm samples did not retain a significantly different numbers of cells from each other. However the number of cells attached to the PCL/ppAAm/heparin scaffold was significantly higher than on the unmodified ($p < 0.01$) and AAm modified ($p < 0.05$) scaffold.

Seeding of modified scaffolds – long term viability

Figure 3c shows the results of a resazurin viability assay after 2 weeks fibroblast culture in the 3 different scaffolds. These data show the marked effect that scaffold surface modification had upon final viable cell content, *i.e.* on cell proliferation, over the culture period. The lowest performing surface was the unmodified PCL scaffold. The resazurin assay shows that in the ppAAm modified scaffold the final viable cell content was significantly greater (at $P < 0.05$) than in the PCL scaffold. Additional modification with heparin and FGF-2 further increased the fibroblast viable cell content, *i.e.* increased cell proliferation over the unmodified control ($P < 0.01$). The hep/FGF-2 surface also outperformed the PCL/ppAAm surface ($P < 0.05$).

Co-culture of primary keratinocytes and fibroblasts in scaffolds

Figure 4 shows the formation of dermal and epidermal layers after 1, 2, and 3 weeks co-culture of primary fibroblasts and primary keratinocytes on the PCL, PCL/ppAAM and hep/FGF-2 scaffolds. The green staining is obtained using a specific antibody staining for pan-cytokeratin and is specific to keratin containing cells which in this system are the keratinocytes. The paler green staining indicated by arrow “A” indicates keratinocytes with a more cuboidal, basal morphology; arrow “B” indicates flattened keratinocytes with a corneocyte morphology; and “C” indicates fibroblasts in the dermis. The red stars indicate fibres of the PCL scaffold, and there is also some green staining observed around some of the PCL fibres due to auto-fluorescence. The blue nuclear stain (DAPI) counter stains for both keratinocytes and fibroblasts. In control scaffolds, seeded with fibroblasts only, no keratin staining was observed. This is as expected, because of the specificity of pan-cytokeratin stain to keratinocyte cells (*Figure SI4, ESI*).

Based on the confocal images in *Figure 4*, at week 1, it is evident that the DAPI staining is more intense in the modified scaffolds, i.e. PCL/ppAAM and heparin/FGF-2 scaffolds. This trend continued through week 2 and by week 3, this difference is very clear. Based upon cells that only exhibit DAPI staining, it is evident that both surface modifications improved fibroblast proliferation and resulted in more fibroblast “infilling” of the scaffolds.

The pan-cytokeratin staining (keratinocytes) also indicates different degrees of cell organization in the 3 scaffolds at different time points - into a fibroblast populated lower dermal layer, and a keratinocyte populated upper stratified epidermis. At week 1 there is clear ingress of keratinocytes and fibroblasts into the PCL/ppAAM and heparin/FGF-2 scaffolds. In the PCL scaffold, there is a lower degree of cell ingress and little organization. Unfortunately there does not appear to be many viable, basal keratinocytes remaining. The vast majority appear to have differentiated and are without a nucleus. In PCL this trend continues in weeks

2 and 3. Some organization is seen by week 3, however there are few basal / proliferative keratinocytes visible. In the PCL/ppAAm scaffold, at week 3, there still appears to be little tissue organization i.e. no separation between the dermis and epidermis. Even after three weeks keratinocytes are seen throughout the entire structure. Whilst these appear to be viable keratinocytes i.e. they still retain their nuclei there is very little organisation within the tissue. In the heparin/FGF-2 scaffold at 3 weeks the pan-cytokeratin staining shows organization of the keratinocyte cells and fibroblasts cells, and the development of an organized, stratified epidermis; this is suggestive that keratinocytes that had invaded the scaffold interior at week 1 have reorganized over the following 2 weeks.

Discussion

The production of cell-seeded 3-D TE constructs presents significant manufacturing challenges in terms of efficiency (time and number of steps) and quality (reproducibility, cell colonization and organization of different cell types). In the context of engineered skin products, it has been shown by the groups of MacNeil and Upton that sterilised acellular dermis can be used to produce reconstituted skin^{51, 52} for both clinical and research use. However, there are issues using acellular dermis, mainly around the availability of skin, the processing steps and reproducibility of the final product.

Synthetic scaffolds offer advantages in terms of manufacturability and reproducibility, and potentially cost, but typical scaffold materials employed are selected on availability and ease of processing, and often have less than ideal surface energetics for cell attachment and ingress (migration) and cell proliferation^{20, 31-33, 60, 61}.

The objectives of this work were threefold; firstly, to use plasma polymerization to engineer (on an electrospun 3-D PCL scaffold) a surface that could passively bind a glycosaminoglycan (GAG) – in this example heparin⁵⁰; secondly, to use this surface to

sequester a GF (herein, FGF-2)³⁴; and finally, to use the heparin/FGF-2 surface to stimulate cell attachment, ingress and proliferation. Plasma polymerization is now widely used to modify materials, and the equipment is available in many laboratories. The second and third steps (biomolecule binding) can be readily integrated into the work flow during cell seeding and are relatively easy to perform. Steps two and three do not rely on complex coupling chemistries and both GAGs and GFs can be passively adsorbed. Previously it has been shown on 2-D surfaces that passively bound GAGs can bind proteins such as osteoprotegerin, versican, and tissue inhibitor of metalloproteinase 4 (TIMP-3)⁴⁸. Recently published data has also demonstrated that FGF-2 can be bound functionally to adsorbed heparin and can subsequently affect cell proliferation over a longer time frame than when added to solution³⁴. In extended cell culture we show that these heparin/FGF-2 surfaces have the potential to significantly enhance the efficiency of production of TE constructs, but we have yet to demonstrate translation in 3-D cultures.

The electrospun PCL scaffold was 400-500 μm thick, with an average fibre diameter of 30.6 μm and an average inter fibre distance of 67.8 μm . Consequently, under the plasma operating conditions employed the plasma will not ignite within the scaffold, and a sheath (a region deplete of electrons) will be established around the scaffold⁵³. Plasma coating of fibres occurs by reactive species diffusing from the plasma into the scaffold. Therefore it was important to confirm that a uniform coating throughout the scaffold was achieved. The imaging ToF-SIMS data shown in *Figure 2b* mapping ions characteristic of ppAAm (CN^- and CNO^-), confirmed that this objective was realised. The stability of the plasma coating was established by a simple washing procedure monitoring the surface N/C, which showed only a modest reduction (SI-1)⁵⁴. The addition of a thin plasma polymer layer results in a defined chemistry applied to the surface of the fibres. Whilst it is possible that there may be some slight changes

to the material properties of the surface, the plasma polymerization process does not affect the bulk material properties⁶².

The subsequent uniform coating of the plasma coated surfaces by the passive adsorption of heparin was confirmed by *imaging* ToF-SIMS data (*Figure 2c*) using the SO_2^- and SO_3^- ions. These are the only ions unique to heparin, and signals of these ions are not particularly strong in (films of) heparin (see SI- 2) which explains the comparatively faint ion image.

The heparin-modified scaffolds were subsequently used to bind FGF-2, with the objective that surface bound FGF-2 would in the scaffold improve the proliferation and organisation of fibroblast and keratinocytes cells. The functionality of FGF-2 bound by this method was confirmed in microtitre plates (2-dimensional) using an ELISA (*Figure 3a*).

The benefit of the coating procedure in terms of enhancing initial fibroblast attachment is shown in the cell viability assay in *Figure 3b* and in SI-3 (cell number) with both these datasets showing the same trend. However, in *Figure 3b* (resazurin) the difference between the PCL/ppAAm surface was significant (<0.05) over PCL, whilst in SI-3 (cell number) no significance was seen. (The heparin/FGF-2 surface outperformed PCL on both of these measures). These data indicate that compared to the PCL, not only is there an increased number of cells (ESI-3) on the hep/FGF-2 scaffolds, but also that these cells are more metabolically active (*Figure 3*).

The differences between the 3 scaffolds at 1 hour translates into differences in fibroblast proliferation at 2 weeks. In terms of cell viability, the ppAAm treatment continues to (significantly) outperform the uncoated PCL scaffold. The addition of heparin and FGF-2 to the ppAAm further significantly increased the total number of viable cells in this scaffold, above the PCL/ppAAm, and well above the PCL scaffold. However, viable cell content (*Figure 3c*) only implies cell ingress (and proliferation) in scaffolds and does not provide any

direct information on the spatial position of cells within the scaffolds, nor in the case of the fibroblast/keratinocyte co-culture, any information on cell organization. DAPI staining of fibroblasts cultured (alone) in the scaffolds for 4 weeks confirmed the complete ingress of these cells into the scaffold (SI-4).

The overall objective of this study was to produce a cell seeded scaffold that had the features expected of a skin composite. Two weeks after seeding with fibroblasts, keratinocytes were added and the 3 scaffolds were cultured for a further 1, 2, and 3 weeks. The scanning laser confocal images in *Figure 4* show that in all cases after one week there is very little separation between the fibroblasts and the keratinocytes. With no surface modification, in PCL after three weeks a keratinocyte rich (pan-cytokeratin positive) epidermal layer is visible as a distinct layer. With the addition of the plasma polymer, heparin and FGF-2 cell ingress remains enhanced, but after three weeks there are two distinct layers forming, a keratinocyte rich epidermal layer and a fibroblast rich dermal layer. Additionally, the keratinocyte layer shows signs of forming a stratified epithelium with both viable (basal) and differentiated corneocytes visible.

This work constitutes a further piece of evidence on the utility of plasma polymerization and passive biomolecule binding to create new (superior) surfaces for stimulating cell growth. The applicability of our findings are that they can be applied in the production of 3-D scaffolds for a broader range of tissues organs, as the plasma polymer coating and biomolecule may be matched to the cell type(s) of interest. Here and elsewhere it is shown that ppAAm surfaces support the migration and attachment of fibroblasts⁵⁵. Previously, we have shown that in 2-D culture that plasma polymerised acrylic acid surfaces support keratinocyte migration and growth at a level comparable to collagen-coated surfaces^{56, 57}. In Bullett et al.³⁸ and Whittle et al.³⁷ it is also shown that plasma polymerized surfaces can be

used to bind a range of different biomolecules that are significant in terms of supporting cell attachment.

The architectures of polymer scaffolds produced to date^{22, 58-61} are not as close to native tissue as the architecture preserved in DED models that are currently employed. Therefore, the need for improved tissue composites in this respect is an obvious one. The techniques developed in this research could in part help solve this problem and improve upon what is already available. There is an ongoing research effort with polymers and hydrogels scaffolds to include ECM biomolecules i.e. collagen and hyaluronic acid to improve the quality of the totally synthetic skin composites currently available. Alternatively, improved scaffold architectures fabricated from currently available (FDA approved) engineering materials may be optimized for cells culture by plasma polymerization and the subsequent passive binding of selected biomolecules.

Conclusions

A simple, rapid and potentially cost-effective protocol is developed to prepare scaffold surfaces to enhance cell attachment, migration, proliferation and organization in the production of cell-seeded scaffolds. The protocol is demonstrated by forming a skin composite with primary human dermal fibroblasts and primary keratinocytes in a polymeric 3-D scaffold, where these cells organise to form an epidermis and dermis. Our approach involves the fabrication of an electrospun PCL scaffold that is plasma polymerized to enable the assembly of a biological surface comprising a GF-laden extracellular matrix. Our data indicate that the advantages of this strategy are greater cell ingress and proliferation, and better cell organization in the formation of a skin composite over a 5 week period.

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Figure caption

Figure 1a, Schematic of the scaffolds mounted for coating, 1b; placement of scaffolds within the reactor for coating and 1c; scaffold preparation for TOF-SIMS analysis. Figure 1d-f, Characterisation of fibre diameters (d) and inter fibre spaces (e) of melt spun PCL scaffolds (mean $\pm$ S.D, n=3). (f) Representative SEM image of melt electrospun scaffold. Scale bar = 500 μ m

Figure 2, Negative ToF-SIMS images taken using the unbunched mode. Total negative ion images and images for the total intensity of $[\text{CO}_2\text{H}^-]$ (m/z 45), $\text{C}_6\text{H}_9\text{O}_2^-$ (m/z 113) and

$\text{C}_6\text{H}_{11}\text{O}_3^-$ (m/z 131)], $[\text{CN}^-$ (m/z 26) and CNO^- (m/z 42)] and SO_2^- (m/z 64) and SO_4^- (m/z 96)] were acquired from (1) PCL, (2) PCL/ppAAm and (3) PCL/ppAAm/hep cross sections. Scale bar = 100 μm .

Figure 3a. ELISA concentration curve for FGF-2 binding to heparin at 1 $\mu\text{g}/\text{well}$ on a ppAAm plate, (mean $\pm$ SD, n=3).

Figure 3b. Resazurin assay after 1hr fibroblasts incubation (mean $\pm$ SD, n=3).

Figure 3c. Resazurin assay after 2 weeks fibroblasts culture (mean $\pm$ SD, n=3).

Figure 4. Formation of dermal and epidermal layers after 1, 2 and 3 weeks co- culture of primary fibroblasts and primary keratinocytes on unmodified PCL scaffolds, PCL scaffolds coated with ppAAm and ppAAm coated PCL scaffolds decorated with heparin and FGF-2. Immunohistochemistry images were taken using a confocal laser scanning microscope. Green staining is specific antibody staining for pan-cytokeratin with the blue nuclear stain DAPI as a counter stain. The paler green staining indicated by arrow A) indicates keratinocytes with a more cuboidal, basal morphology, B) indicates flattened keratinocytes with a corneocyte morphology and C) indicates fibroblasts in the dermis which are only stained with DAPI as keratinocytes are negative for keratins. Scale bars are 100 μm .

The table of contents entry should be fifty to sixty words long (max. 400 characters), and the first phrase should be bold.

Adsorption of appropriate biomolecules is crucial for the development of high quality tissue engineered constructs. Here plasma polymerization is used to modify the surface of a 3D melt electrospun polycaprolactone scaffold such that heparin and therefore growth factors can be immobilized. This surface modification resulted in the production of a synthetic human skin equivalent with distinct epidermal and dermal layers.

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Author/s:

Robinson, DE;Al-Bataineh, SA;Farrugia, BL;Michelmore, A;Cowin, AJ;Dargaville, TR;Short, RD;Smith, LE;Whittle, JD

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