Evaluation of NovoSorb™ novel biodegradable polymer for the generation of a dermal matrix

Part 1: In-vitro Studies

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Abstract

Dermal skin substitutes can be used to overcome the immediate problem of donor site shortage in the treatment of major skin loss conditions such as burn injury. In this study the biocompatibility, safety and potential of three variants of NovoSorb™ (a family of novel biodegradable polyurethanes) as dermal scaffolds were determined in-vitro. All three polymers exhibited minimal cytotoxic effects on skin cells allowing human keratinocytes, dermal fibroblasts and microvascular endothelial cells to grow normally in co-culture. Assessment of a three-dimensional polymer matrix followed. A rudimentary composite skin was created with the sequential culturing of dermal fibroblasts and keratinocytes within the matrix. Furthermore, the polymeric matrix provided a scaffold for the guided formation of a cultured microvasculature. These results prompt further investigation in-vivo to assess their safety in biological systems and to elucidate their interaction with the wound environment.

Key words: Dermal matrix; Polymer; Wound healing; Burn injury; Biodegradable polyurethane

Abbreviations: MVECs, microvascular endothelial cells; TCP, tissue culture plastic; BTM, Biodegradable Temporising Matrix; GAG, glycosaminoglycan; 5-DTAF, 5-Dicholorotriazynlfluorescein; CEA, Cultured Epithelial Autograft; Cal AM, Calcein AM; EthD-1, Ethidium homodimer-1; PBS, phosphate buffered saline; PECAM, platelet endothelial cell adhesion molecule.

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Introduction

One strategy to attain success in the management of extensive skin loss conditions, such as major burns, lies in the development and utilisation of skin substitutes. Over the past three decades, a range of biosynthetic skin substitutes have emerged. Since the development of techniques to culture keratinocytes [1], cultured epithelial autografts (CEA) in the form of epidermal sheets [2-5] or epidermal cell suspensions [6] have been used in the treatment of burns. However, without a dermal component, the fragile CEAs fail to establish proper anchorage and formation of a basement membrane which is fundamental to resilience to shear. Thus grafts often blister leading to increased inflammation and scarring. The use of pre-confluent suspensions of keratinocytes is scientifically unproven in any controlled trial.

The dermis makes up 95% of the thickness of skin and is the key to skin suppleness and mobility. In many units, a range of biosynthetic substitutes have succeeded cadaver skin as temporizing dressings to maintain and stimulate the debrided burn bed until definitive split-skin graft becomes available to effect wound closure. These replacements are generally synthetic matrices with or without 'actives' such as

collagen/peptide fragments or glycosaminoglycans (GAG) [7-9] or comprised of a non-degradable polymer scaffold with an extracellular matrix laid down by monoclonal fibroblasts [10,11]. Some materials like Integra™ and Alloderm™ act as a scaffold for the infiltration of host fibroblasts and the in-growth of host blood vessels, becoming integrated into the wound site.

The development of the dermal matrix strategy 'buys time'. Ultimately, it is desirable to have a bi-layer composite skin comprising both dermal and epidermal components, replacing the need for split-thickness autografts. Apligraf™ is one such material. Initially intended for use in full-thickness burns, it was only trialled as a biological dressing over split-thickness skin grafts [12]. There has been only one reported case of successful treatment of full-thickness burn (to the foot) since its introduction [13]. Boyce et al. [14] have reported more success with a collagen based dermal-epidermal composite, but due to the cost and complexity of manufacture, the use of this substitute has been limited.

Almost all of the currently available skin substitutes use biological materials of allogenic or xenogenic origin. The development, regulation, manufacture and marketing have been expensive, with this expense transferred to the end-user. Even in first world economies, the enormous costs incurred have restricted the use of these skin substitutes to major burn injuries and difficult reconstructive surgery. The high cost has created interest in synthetic polymer technology. Fiscal arguments are not the only impetus for this exploration as synthetic polymers have the added advantage of customised design to enable properties such as molecular elution or fluid absorption to effect better wound healing.

Biodegradable polyurethanes are of great interest for medical application because of their variable mechanical properties, biocompatibility and structural versatility. They are currently used in bone [15,16] and cartilage [17-19] applications as well as nerve regeneration [20]. For burns, polyurethane-based dressings have been shown to be superior to hydrogels [21-23]. Keratinocytes have also been successfully cultured on polyurethane membranes [24-26] demonstrating that polyurethane may be developed as a delivery system for CEA. However, the use of biodegradable polyurethane as a dermal scaffold has not been explored.

NovoSorb™ represents a family of novel polyurethanes. A sub-class of this family is biodegradable, designed and constructed to degrade by hydrolysis to naturally occurring and biologically-tolerated products. The aim of this in-vitro work was to assess the biological compatibility of three NovoSorb™ variants in the cell culture environment and their ability to act as cellular scaffolds for the in-vitro production of composite 'skin'. One of the candidate polymers was shown

to be ideal for development as a Biodegradable Temporising Matrix (BTM).

Materials and methods

Production of polymer discs, fibres and mats

To form discs, the polymers were cured between two glass plates to form flat, nonporous films which were then cut into discs of ~1cm in diameter and placed into 24-well tissue culture plates.

Polymer fibres were produced by curing the polymers prior to extrusion (using a twin screw reactive extrusion process) into polymer fibrils ranging from 60-100µm in diameter. 10cm lengths of each polymer fibre were wound around two parallel rods spaced 1 cm apart in a 'figure of 8' conformation and tied in the centre. This configuration provided a 'dumb-bell' of length 10mm and width 5mm (at the two widest points).

After the initial biocompatibility testing, the most suitable candidate polymer (BTM-2) was spun into 3-dimensional porous mats. These matrices comprised of 6 layers of fibres at 90° orientation and of 0.65mm in thickness with intervening pores of 200 - 500µm in diameter.

All materials were sterilised with 25KGy a-irradiation prior to use in experiments.

In-vitro degradation study

BTM polymers in the figure-8 conformation were placed in individual glass vials containing 20mls of phosphate buffered saline (PBS; 0.1M and 2mg/ml sodium azide) and incubated at 37°C. At various time points, the vials were removed from the incubator and cooled to room temperature before the PBS solution was tipped off for determination of molecular mass changes (M_n) using Gel Permeation Chromatography (GPC) with dimethylformamide as the eluent. Changes in physical properties of polymer fibres such as breakage or change of the 'figure of 8' shape were observed and recorded at each time point.

Cell culture

Adult human keratinocytes, dermal fibroblasts and microvascular endothelial cells (MVECs) were expanded in tissue culture flasks. These cells were cultured in keratinocyte basal medium (Lonza, Switzerland) supplemented with 5µg/ml insulin, 0.5µg/ml hydrocortisone, 10ng/ml epidermal growth factor and 50µg/ml bovine pituitary extract, Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% normal human serum and Endothelial Growth Media (EGM-2MV; Lonza, Switzerland) respectively.

Cell experiments were performed in 24-well plates with triplicate wells set up for every condition. Cells were seeded at the following densities in all experiments: keratinocytes - $2.5x10^4$ cells/cm^2; fibroblasts - $1x10^4$cells/cm^2; MVECs - $1.5x10^4$ cells/cm^2.

For the growth of skin cells on scaffolds, cells at the specified densities were seeded into wells containing 8mm x 8mm matrices in 2mls of the appropriate medium. At various time points (e.g. Days 7, 14 and 21), the resultant constructs were harvested for analysis. For the generation of a bi-layer skin substitute, dermal fibroblasts were seeded over BTM scaffolds and cultured for 7 days prior to seeding of keratinocytes. The constructs were then kept in submerged culture for a further 4 days before harvesting for analysis.

All cells were cultured in humidified incubators at 37°C with 5% CO_2. Media changes were performed every 2-3 days. Whenever multiple plates were used for one experiment, these were carried in parallel and handled in a similar manner.

Cell viability/cytotoxicity assay

For the cytotoxicity assay, skin cells (at the densities stated in the cell culture section) were added to wells containing the candidate BTM fibre bundles in 2ml of media. When untreated tissue culture plastic (TCP) control wells reached 100% confluence (generally 3-4 days after seeding) the set of plates were assayed using a Live/Dead fluorescence viability kit (Molecular Probes). Briefly, wells were washed twice with PBS before incubation at 37°C for 10 minutes in 0.5ml of two fluorescent markers (2mM of Calcein AM and 1mM EthD-1 in Hank's Buffered Salt Solution). The fluorescence intensities were then measured using a microplate fluorometer (FLUOstar Optima).

To standardise the results, TCP control wells containing mainly live (untreated) and dead (killed by 10 minute treatment with 70% ethanol) cells of the same type and seeding density as the treatment wells, stained with single dyes only, were included in every set of experiments. The percentages of live and dead cells were calculated from the fluorescence readings according to the manufacturer's instructions.

Results were expressed as the relative cell viability of the individual experimental treatment to the TCP control. The student t-test was used to ascertain any differences in the results, with p-values <0.05 considered statistically significant.

Histological, microscopic and immunofluorescence analysis

In-vitro cell cultures were monitored and photographed everyday (CX40, Olympus). Cultures were also stained

with an array of fluorescent markers including Calcein AM; Ethidium homodimer-1; 5-Dicholorotriazynlfluorescein (5-DTAF, 2µM; Sigma-Aldrich) for collagen; propidium iodide (Sigma-Aldrich) for nuclear staining; FITC-conjugated anti-CD31 antibody (1:100; BD Pharmingen) for endothelial cell edge delineation, and analysed by laser scanning confocal microscopy. All fluorescent markers were incubated with the cells for 30-60 minutes and washed with PBS before viewing.

2ESULTS

Candidate BTM polymers

Twelve series of polymers (approximately 70 in total) were created and their physical (thermal transitions by differential scanning calorimetry) and mechanical properties (tensile testing by tensometer) were assessed. This process resulted in the selection of three candidate Biodegradable Temporising Matrix (BTM) polymers designated BTM-1, BTM-2 and BTM-3 for further analysis.

The degradation rate of the polymers was determined. Figure 1 demonstrates that BTM-1 degraded most rapidly, losing over 50% of its molecular mass after 7 days. BTM-2 exhibited a modest degradation rate losing 50% of its molecular mass around 90 days and ~90% by 6 months, while BTM-3 had only lost ~30% of its molecular mass at the end of 6 months. All polymers retained their 'figure-8' conformation throughout the study, but BTM-1 became soft and easily breakable with little pressure by 3 weeks.

BTM polymers were non-cytotoxic and provide suitable cell growth surfaces

Initial evaluation of the cytotoxicity of the candidate polymers was carried out in-vitro by culturing keratinocytes, dermal fibroblasts and microvascular endothelial cells (MVECs) in the presence of, but not on, the polymer fibres. These cell

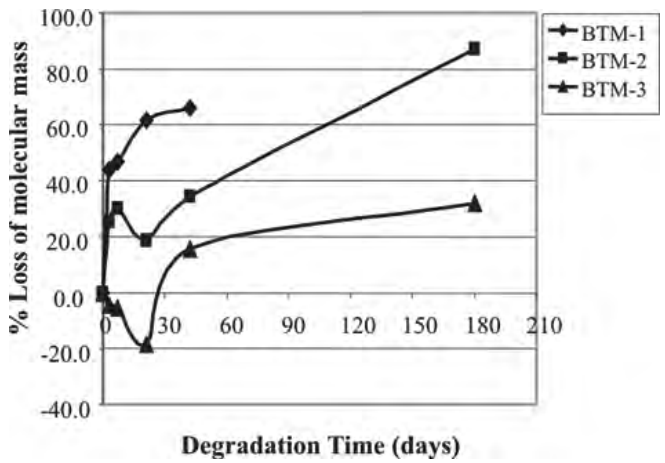

Figure 1. Change in percentage of molecular mass of BTM polymers in degradation study.

types were included in the cytotoxicity analysis because they are the cells which will be used in the eventual construction of a vascularised bi-layered skin substitute.

Daily monitoring of the cells indicated that there were no differences in cell morphology between the treatment wells and the tissue culture plastic (TCP) control. When the TCP control wells reached 100% confluence, the percentage of viable cells under each condition was determined using the fluorescent cell viability assay which detects viable and dead cells by simultaneously measuring two well known parameters of cell viability; intracellular esterase activity and plasma membrane integrity, using calcein AM (Cal AM) and ethidium homodimer (EthD-1). Figure 2 demonstrates that all three BTM polymers exerted minimal cytotoxic effects on skin cells. Specifically, BTM-1 and BTM-2 were mildly cytotoxic to keratinocytes but not to dermal fibroblasts or MVECs, reducing the viability of keratinocytes by ~10% to 91.9 ± 0.8% and 89.5 ± 3.1%. BTM-3 caused no significant keratinocyte cell death, but was cytotoxic to dermal fibroblasts and MVECs reducing the viability for these cells to 81 ± 2.2% and 77.4 ± 2.6% respectively. The two clinically used sutures, Monocryl™ (polyglecaprone) and Prolene™ (polypropylene), caused ~20% cell death in MVECs while having no significant effect on the growth or viability of keratinocytes or dermal fibroblasts.

Physical examination of the polymers showed that BTM-1 fibres became fragile after 3-4 days in culture and broke apart when picked up with forceps. BTM-2 and BTM-3 and the control fibres remained firm and intact. This demonstrated that BTM-1 degraded considerably faster than the other two polymers, consistent with the in-vitro degradation data.

After the initial assessment of cytotoxicity, skin cells were plated onto flat, nonporous polymer discs and maintained

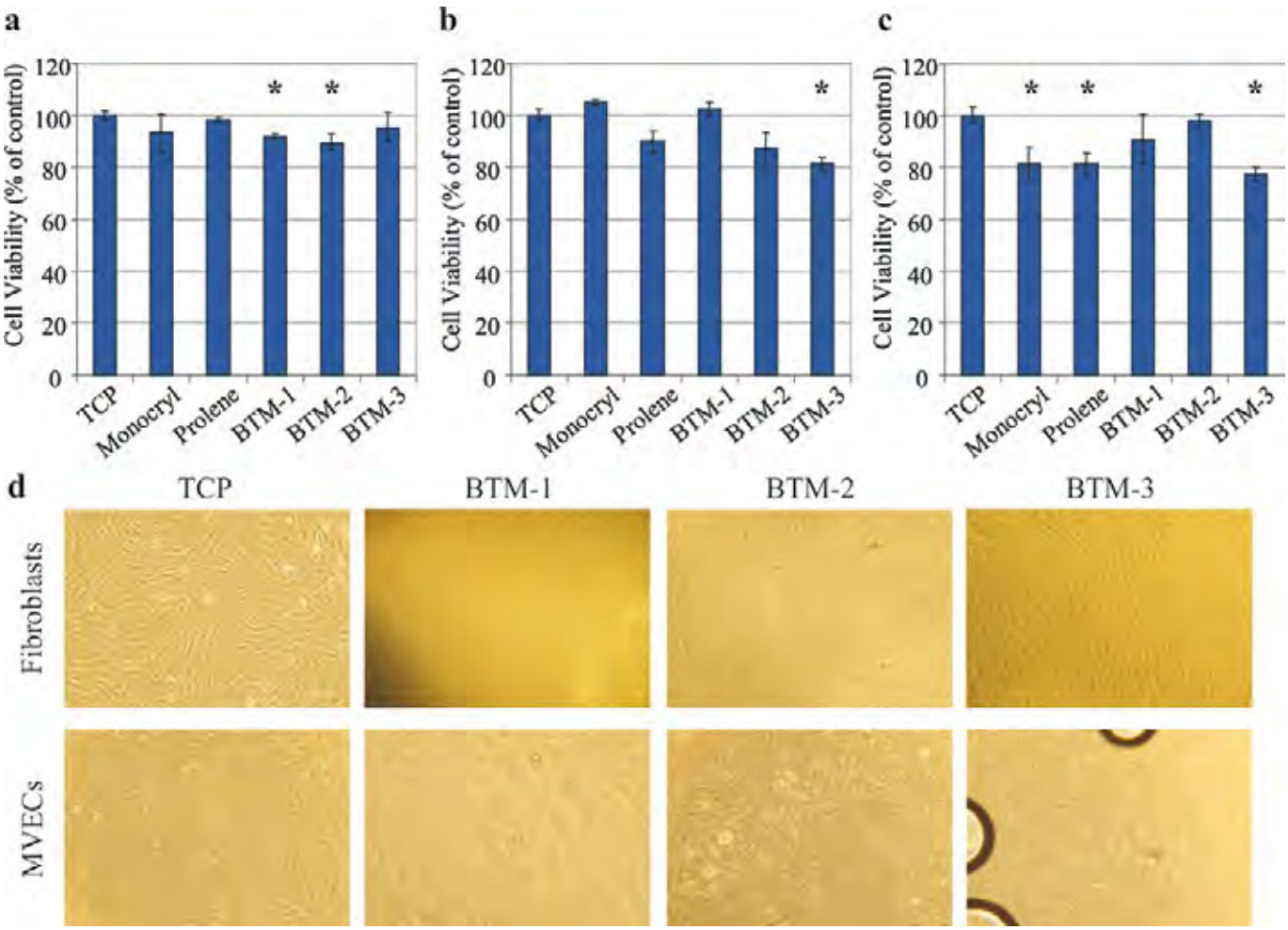

Figure 2. Growth of skin cells with the candidate BTM polymer fibres and films.

(a) Percentage of viable keratinocytes, (b) dermal fibroblasts and (c) microvascular endothelial cells cultured with candidate BTM polymer fibres assayed when 100% confluence was reached in one of the conditions. Results represent mean ± STDEV of three replicate wells. (d) Growth of fibroblasts and microvascular endothelial cells at day 4.

in culture to determine the attachment and growth of these cells. Dermal fibroblasts were shown to have proliferated to reach 100% confluence within 4 days on BTM-2 and BTM-3 comparable to their growth on TCP, but failed to grow on BTM-1 (Figure 2d). MVECs grew to 100% confluence within 4 days on all three polymer discs. It was observed that MVECs were scattered and spindle-shaped on the BTM polymers unlike their counterparts on TCP which were more spread out and rounded. This was a direct response to the change in growth surfaces and indicated that these cells were more migratory [27]. Keratinocytes exhibited limited growth capacity on the polymer discs forming clusters of cells (data not shown) and may require additional substrates such as laminin or collagen [28-30].

Growth of skin cells in 3-dimensional BTM matrix

The in-vitro biocompatibility data indicated that BTM-2, which degraded slowly over 24 weeks and supported the growth of keratinocytes, dermal fibroblasts and MVECs, was overall the best BTM candidate for the generation of the dermal scaffold. Hence, three-dimensional, porous mats were produced using BTM-2.

The ability of the dermal fibroblasts to infiltrate and populate the BTM-2 matrix in-vitro was investigated. Figure 3a shows that from as early as day 2, fibroblasts were able to attach to and spread out from the fibre junctions within the matrix. Throughout the culture period, fibroblasts continued to proliferate and migrate along the fibres as well as extending across the pores. By day 21 of culture, the majority of the small to medium pores were completely filled with cells (Figure 3b), and were shown by scanning electron microscopy (SEM) to comprise several layers of fibroblasts (Figure 3c). Some of the larger pores remained with a circular hole in the centre which the fibroblasts were unable to close even after 30 days of culture. These results were consistently observed in three separate experiments and demonstrated that the matrix needs to have consistently smaller pores for complete infiltration by fibroblasts.

When the fibroblast-BTM-2 dermal composite was subjected to staining with 5-DTAF, a fluorescent dye commonly used to stain for collagen in the cornea and cartilage [31,32], high levels of collagen was detected in the fibroblast sheets covering the spaces in the scaffold (Figure 3d). This was confirmed with Masson's Trichrome staining of 5µm paraffin sections of the construct (data not shown).

To assess the potential of generating a composite with pre-existing vascular structures, MVECs were also seeded over BTM-2 scaffold. After 4 days, a number of cells could be seen along and around the polymer fibres. At day 7, MVECs-BTM2

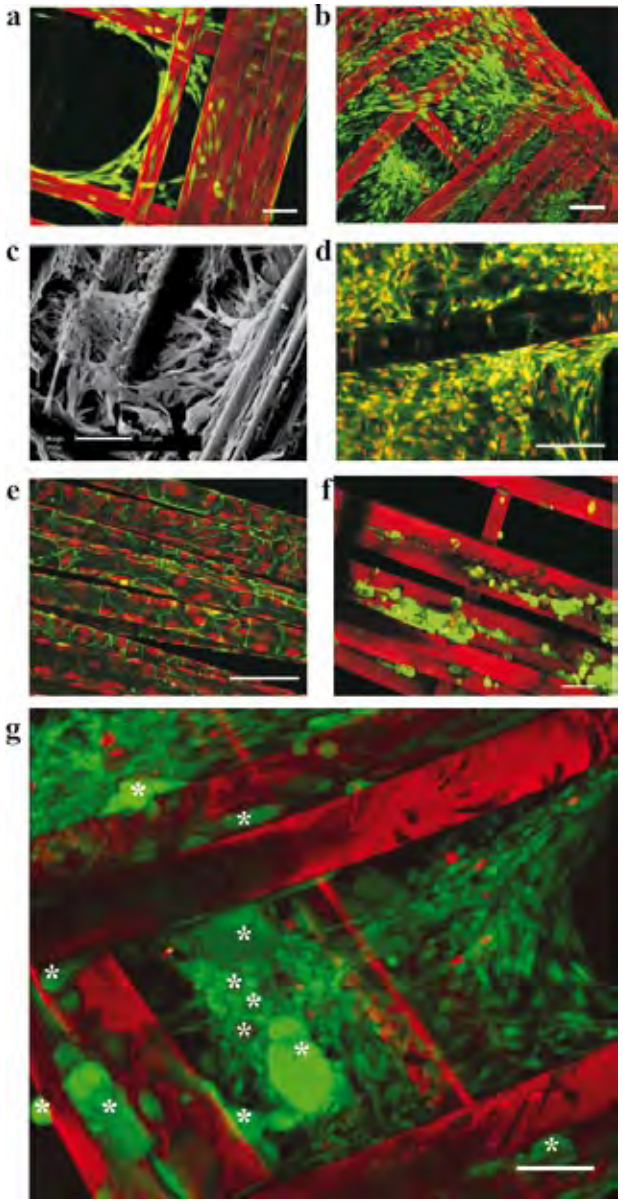

Figure 3. Growth of skin cells in BTM-2 matrix.
(a) Growth of fibroblasts at day 2-3 and (b) day 21 in the BTM matrix. The cells were stained green with Calcein AM while the polymer fibres were stained red non-specifically with EthD-1 (N=3). (c) Fibroblast infiltration into the BTM matrix examined using scanning electron microscopy. (d) Expression of collagen in fibroblast-BTM-2 constructs. Fibroblasts cultured in the BTM-2 matrices for 19 days were stained with 5-DTAF. Note the green collagen fibrils on the polymer fibre and the on the sheets of fibroblasts. (e) Growth of microvascular endothelial cells in BTM-2 filamentous matrix at day 7 as detected by FITC-conjugated anti-CD-1 antibody. These cultures were stained with propidium iodide which detected the presence of the fibroblasts (red), and anti-CD31, which detected the endothelial cells (green). (f) Growth of keratinocytes with BTM after 7 days. (f) Growth of keratinocytes over fibroblast infiltrated BTM-2 scaffolds. The fibroblasts and keratinocytes were both stained with the viability dye, Calcein AM (green), while the polymer fibres and some dead nuclei are stained red with EthD-1. The stars indicate the larger, flatter keratinocytes growing over the fibroblasts. Scale bar = 100µm.

constructs were stained for platelet endothelial cell adhesion molecule (PECAM/CD31), which showed that MVECs had surrounded all of the polymer fibres forming blood vessel like structures (Figure 3e). The same result was also observed at day 14.

Keratinocytes were also seeded over the BTM-2 scaffolds. Some cells were observed to attach to the polymers and proliferate over the culturing period, however, the growth of these cells was limited, similar to their growth on non-porous polymer discs. The cells also remain rounded but were viable as shown by staining with the viability dye, Calcein AM (Figure 3f).

One of our ultimate goals is to create a bi-layer skin substitute. To this end, keratinocytes were seeded over fibroblast-BTM constructs. Preliminary studies show that after 4 days of submerged culture, keratinocytes had attached and started to grow on the fibroblast-BTM-2 composite. In some areas, the keratinocytes had formed a monolayer (Figure 3g), indicating that the generation of an epithelialised skin substitute is possible.

Discussion

The advantages of using synthetic polymers such as biodegradable polyurethane as a dermal scaffold are low antigenicity and the potential for design with specific degradation profiles and desired mechanical properties. They can also be enriched with instructional molecules, such as growth factors, and designed to elute antimicrobial and analgesic agents to improve wound healing and comfort [33]. In this study, we investigated the biocompatibility of three NovoSorb™ variants to choose the best candidate for a biodegradable temporising matrix; both in full thickness wounds and as a scaffold for the generation of a skin composite.

The success of culturing keratinocytes, fibroblasts and MVECs to confluence in the presence of the polymer fibres was extremely suggestive that subsequent in-vivo work would be uneventful from a biocompatibility perspective. Indeed subcutaneous implantation of the BTM polymers in rats to assess the foreign-body reaction showed that it was no greater than a commonly used absorbable suture Monocryl™ and less than that generated by Prolene™, a suture frequently used to create permanent meshes for hernia repair, in addition to skin suturing. The implantation of the very rapidly degrading BTM-1 ensured that one group of rats would sustain an early bolus of polymer degradation products within the first three weeks. This group showed no clinically significant differences to the other treatment and control groups.

Ideally, a dermal scaffold should maintain its structural integrity for the first three months for guided infiltration of host cells and regeneration of the dermis, but completely degrade and are resorbed within 6 months, thus allowing further tissue remodelling. The degradation rate of BTM-2 fulfilled these criteria. Based on this, and on its biocompatibility profile in-vitro, we chose to develop BTM-2 into a 3-dimensional scaffold for further study.

Despite the BTM matrix being rudimentary and un-optimised, fibroblasts attached to the polymer fibres and proliferated to cover majority of the pores within the irregularly spun matrix, synthesising collagen to form a cultured dermal substitute. We were also able to show that while keratinocytes had limited growth on the naked matrix, they were able to form a monolayer over the fibroblast-containing scaffold, demonstrating that the extracellular matrix produced by the fibroblasts was inductive to keratinocyte attachment and growth and that generation of a composite skin is achievable.

In order to make clinical use of a cell-containing composite skin, it must become rapidly vascularised if death of the cellular components is to be avoided. Ideally, this should be as rapid as split-skin graft take (i.e. within ~48 hours) and by a process of inosculation rather than neo-angiogenesis. This cannot be achieved without an integrated microvasculature [34]. The observation that MVECS grew along and enveloped the BTM fibres in a manner similar to other guided vascular networks [35,36] indicates that a vascularised dermal substitute can be generated and may reveal exciting possibilities for clinical application in many other tissue-engineered organs requiring rapid vascularisation.

The sequential production of a covering epidermal tissue over a dermal composite with an existing microvasculature will generate a material capable of replacing skin autografts.

Conclusion

In this study, we demonstrated that the series of biodegradable polyurethanes created as candidates for generation of a dermal scaffold are biocompatible with minimal cytotoxic effect in-vitro. Of the three biodegradable polyurethanes tested, BTM-2 displayed the desired degradation profile for a dermal scaffold and was developed into a 3-dimensional porous matrix for further study. We also demonstrated that an un-optimised BTM-2 matrix can be used to create a rudimentary bi-layered composite skin which may eliminate our reliance on skin autografts. Our study suggests that a vascular structure can be generated by culturing MVECs in

the BTM-2 matrix, having potential in the generation of a 'pre-vascularised' composite skin and other tissue engineered organs.

Conflict of interest

NovoSkin is a joint venture established to investigate the role of NovoSorb in wound management applications between PolyNovo Pty Ltd (who own 80%) and Skin Pty Ltd (20%). A/Prof John Greenwood is a 50% stakeholder of Skin Pty Ltd.

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