Evaluation of NovoSorb™ novel biodegradable polymer for the generation of a dermal matrix
Part 2: In-vivo Studies

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Abstract

In previous work, the biocompatibility, safety and potential of three variants of NovoSorb™ (a family of novel biodegradable polyurethanes) as dermal scaffolds were determined *in-vitro.* This paper documents the subsequent *in-vivo* work. Subcutaneous implantation of the three candidate NovoSorb™ polymers in rats demonstrated no systemic toxic effects of the materials or their degradation products. The anticipated local foreign body reaction compared favourably with commercially-available medical sutures. Assessment of a three-dimensional polymer matrix followed. When engrafted onto a surgically-created full-thickness sheep wound, the non-cellular matrix integrated, healed with an epidermis supported by a basement membrane and was capable of withstanding wound contraction. The resistance to contraction compared favourably to a commercially-available collagen-based dermal matrix (Integra™). These results suggest that the NovoSorb™ matrix could form the basis of an elegant 2-stage burn treatment strategy with an initial non-cellular Biodegradable Temporising Matrix to stabilise the wound bed followed by the application of cultured composite skin.

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

Abbreviations: BTM, Biodegradable Temporising Matrix; GAG, glycosaminoglycan; CEA, cultured epithelial Autograft; PAS, Periodic Acid Schiff.

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Introduction

Although the split-skin graft remains the workhorse of the burn surgeon, it has limitations. As burn surface area increases, available donor site decreases. Eventually the point is reached when, even with maximal (reasonable) mechanical expansion, sufficient skin cannot be harvested to cover all debrided burn wounds and still achieve an acceptable functional result and cosmetic appearance. The number of re-harvests from a donor area is finite because each harvest (no matter how thin) includes a variable thickness of dermis which will not be replaced when donor site re-epithelialisation occurs. The surgeon must gauge correctly when a further re-harvest will result in a donor wound which will not heal spontaneously and promptly [1]. How frequently these limited re-harvests can occur is dependent on robust re-epithelialisation, itself a factor of the thickness of the harvested graft as well as the thickness of the remaining dermis at the site. It is seldom, if ever, that re-harvesting can be contemplated within 10 days. With the patient already susceptible to infection due to loss of mechanical restriction to

pathogen ingress and generalised immunocompromisation, the donor site extends the area of skin loss and inflicts further physiological insult.

The use of the split skin graft does not replace 'like with like'. Grafts are taken so that the donor site will re-epithelialise rapidly by proliferation, differentiation and migration of keratinocytes from both the wound edge and retained adenexal epidermal cell nests. Adenexal structures are not transported with a split-skin graft to the recipient site. Grafts thus have no means of auto-hydration and can dry and crack, causing itch. The thermoregulatory and sensory functions of grafted areas are also reduced. Thin grafts used to replace deep or full thickness skin loss cannot confer the same degree of robustness to mechanical, thermal or chemical insult, such that further injury requires less insult. Nor does providing a reduced thickness of elastic dermis often allow complete restitution of the supple envelope that uninjured skin provides to facilitate joint mobility and range. Without underlying dermal support, the junction between pieces of skin graft is often visible as a 'seam' and the sharp demarcation where graft meets normal skin is sometimes very obvious. Because the skin graft contains dermis, a donor site scar (however good) is always created since the dermis is not capable of regaining its pre-injury architecture. Finally, it is seldom possible to choose the donor site for its colour match to the recipient site, potentially creating mildly hypo- or hyper-pigmented skin grafts scars.

The property of split-skin graft which is most desirable (and thus far impossible to emulate in 'skin substitutes' is the speed of re-vascularisation and when one considers the speed with which split skin grafts "pink up" compared to the relative latency of neovascularisation; it becomes obvious that inosculation is occurring as the mechanism for the reestablishment of blood flow within grafts [2]. Neovascularisation should be expected to (and does) take much longer than inosculation since the angiogenetic process alone is responsible for a neovasculature to invade and supply a relatively thick structure. The vascularisation of Integra™ dermal matrix, for example, takes ~20 days in the acute (hot) burn setting but can take 4 to 5 weeks in reconstructive (cold) cases. This vascularisation can occur only from the wound bed since no vascular elements exist in Integra™. In developing a composite skin, either possessing cultured vessels or a means to rapidly establish a vasculature within the composite, must form part of the structural design.

Materials and methods

Production of polymer fibres and mats

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). Two commercially available sutures, Prolene™ (polypropylene, non-resorbable, Ethicon Inc., Piscataway, NJ, USA) and Monocryl™ (polyglecaprone; resorbable, Ethicon Inc., Piscataway, NJ, USA) were used as controls. These were used in the cytotoxicity and rat safety studies.

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. The fibres were orientated in such a way that the matrix resisted compression in one direction but not the other.

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

For the animal studies, the tissues collected were fixed in 10% neutral-buffered formalin then processed for paraffin embedding. 2µm sections were stained with haematoxylin and eosin (H&E) for histological analysis. Sections were also stained for a basement membrane with Periodic Acid Schiff (PAS). All animal tissue sections were examined by a veterinary pathologist who had no prior knowledge of the trial protocol or the nature of the implants.

Rat implantation procedure and analysis

Twelve male and 12 female Sprague Dawley rats, >12 weeks old and weighing 200-300g each were randomly assigned to groups by a weight-ordered distribution. A small incision was made in the flank. The subcutaneous fascia was blunt-dissected anteriorly and dorsally to approximately 2cm and the implant, a figure 8 BTM or control polymer, was inserted until completely covered by skin. The wound was closed with one or two stainless steel clips. All surgical procedures were performed under aseptic conditions using isoflurane in oxygen anaesthesia.

Body weights were recorded prior to implantation, daily for the week post-implantation, then weekly for the duration of the experiment. Clinical records were taken daily throughout the study. Six animals (3 male/3 female) from each study group were humanely killed at weeks 3, 6, 12 and 24. Terminal (fasting) blood samples were taken from anaesthetised animals via cardiac puncture, and analysed for haematological and biochemical parameters prior to the administration of 2-4mL of 4M potassium chloride into the heart. Results of these parameters were analysed using unpaired t-test and two-way ANOVA.

All animals were subjected to a comprehensive necropsy (defined as examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities, and their contents) by the Veterinary Pathologist. All implants were collected for histology at the above time points. When the actual implant could not be located, large sections of skin and subcutaneous tissue were collected from the area deep and anterodorsal to the implantation scar. The collected samples were processed for histological analysis.

Sheep implantation procedure and analysis

A total of 6 male (wethers) Merino sheep weighing 50-55kg each were used. The animals were housed individually in pens, in a controlled environment and exposed to a 12h/12h light/dark cycle for the first two weeks after implantation, after which they were kept in groups of three in sheltered outside pens exposed to ambient temperature and natural light.

Surgery was performed in two groups of three animals approximately three weeks apart under isoflurane in oxygen anaesthesia. The left and right flanks of each animal were shaved. Two test sites (5cm by 5cm) at least 5cm apart were created per flank along lines parallel to and equidistant (18cm) from the spine. The sites were infiltrated with 0.25% bupivacaine (Marcaine™) and 1:400,000 adrenaline (to facilitate haemostasis and afford post-operative analgesia) before full thickness skin squares were removed leaving the underlying panniculus carnosus intact, creating a strong model of wound contraction. The implants were applied and secured with 3/0 Prolene™ sutures. The wounds were dressed with Mepitel™ (Mölnlycke Health Care AB, Göteborg, Sweden), Cutilin™ (Defries Industries Pty Ltd, Keysborough, Aust) and held in place with Tubular-Net™ (Sutherland Medical Pty Ltd, Oakley, Aust). All animals were administered prophylactic penicillin for four days post-operatively.

Each animal received two test articles, BTM at 0° (transverse) and 90° (axial), as well as Integra™ dermal regeneration template (Life Sciences, New Jersey, USA). One of the test sites on the animals received no graft and was allowed to heal by secondary intention (granulation and contraction). The orientation of the BTM polymer refers to the orientation of the dominant fibre pattern. Materials were randomly allocated to ensure that each was used at least once in the four treatment sites. The silicone layer of the Integra™ was maintained in the first three animals but removed under aseptic conditions prior to placement on the wounds of the second group.

Measurement of the four vertices of each test site was taken using micrometer callipers on alternate days for the first two weeks, then every 3-4 days. The area of each test site was measured using a wound measurement system, Visitrak Digital and grid (Smith & Nephew Ltd). Photographic capture of the progress of the test sites accompanied these measurements with a scale appended. Prior to necropsy, the animals were humanely killed with a captive bolt to the skull. Wound/scar tissue samples were harvested under the supervision of the pathologist. Samples of skin (implant and 2cm surrounding tissue) were collected and processed for histological analysis. All results were examined by an independent veterinary pathologist.

The sheep study data was analysed as individual values by animal (clinical observations and physical examinations). Two-way ANOVA (where possible) was performed to ascertain if there were differences between the groups over time.

Results

BTM polymers caused no adverse local or systemic reactions in vivo

The possible local and systemic cytotoxic effect of the candidate BTM polymers and their degradation products *in-vivo* was investigated by implantation of 'figure of 8' polymer fibres subcutaneously in rats for 24 weeks. Throughout the experimental period the animals thrived and gained weight with no significant difference in weight between the groups. The rats displayed no behavioural change and there were no unscheduled deaths or cases of morbidity.

Analyses of blood samples collected at 3, 6, 12 and 24 weeks post-implantation showed all haematological parameters

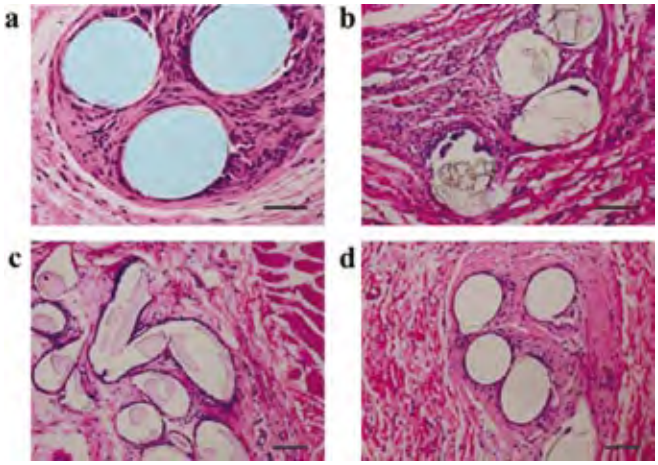

Figure 1. Histological analysis of polymer fibre implants in rats at week 3 post-implantation.

(a) Prolene™ 'spheroids' with blue-green hue seen for all inert control sections. (b) Tissue section containing BTM-1 polymers showing eosinophilic fragments within the spheroids. (c) Monocryl™ reactive control showing fragmented 'spheroid/ovoid' content. (d) Tissue section containing BTM-2 polymers showing non-staining spheroids with definite limiting membrane. Scale bar = 50µm.

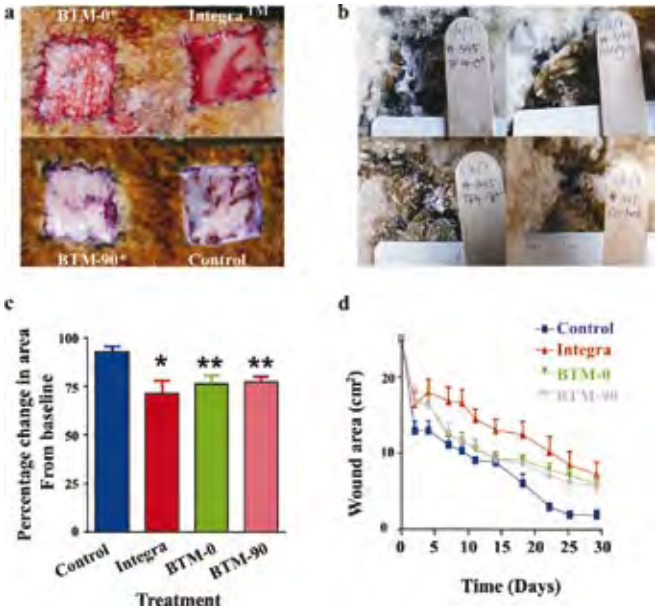

Figure 2. Contraction of full thickness wounds in sheep treated with BTM-2 matrices.

*(a) Full thickness wounds were surgically created on the back of sheep and grafted with either BTM-2 matrices or Integra™ or left un-grafted. (b) The wound sites as seen at day 29 when all sites had healed. (c) Mean+SEM change in wound/scar area (n=6 for days 7-28, n=2-5 for days 2-7). (d) Percentage change in wound/scar area at day 29 compared to baseline (n=6 for all data points), showing significant differences from the control wounds (*p<0.05).*

were within normal range. There were no clinically significant differences in blood biochemical parameters. Macroscopic analysis of all organs examined at necropsy showed that all were normal except for a degree of lung redness. There were no significant differences between the groups in any organ weights.

There was no evidence of infection or inflammation at the implantation wound site in any animal at any of the collection time points. By week 24, all wounds had healed, with only the faintest scarring detectable at the site. The histopathological appearance was remarkably similar in all 5 implant types. The BTM and control polymers were surrounded by a granulomatous reaction composed of epithelioid macrophages and multinucleated giant cells with variable fibrosis (Figure 1). Interestingly, Prolene™, appeared to elicit a more robust granulomatous reaction than the BTM polymers or Monocryl™ (Figure 1a).

The degradation rate of each polymer was faithfully reflected in the appearance of the material post-implantation. At week three, BTM-1 implants could not be detected macroscopically but eosinophilic comma-shaped fibre fragments surrounded by a non-staining (artefact) halo (Figure 1b). The time of BTM-1 fragmentation was similar to that of Monocryl™ which also began to degrade by 3 weeks (Figure 1c). BTM-2 implants showed no signs of degradation at week 3 and resisted the ingress of the stains, thus were seen as rows of spheroidal to oval, non-staining 'spaces', many with a definite limiting 'membrane' which is the outer surface of the polymer strand (Figure 1d). By the 12 and 24 week time-points, the BTM-2 implants were becoming difficult to discern macroscopically. All BTM-3 implants were located at weeks 3 and 6, most at week 12 and one at week 24.

Assessment of 3-D BTM as a dermal scaffold in a full-thickness wound model

The potential of a BTM-2 matrix to resist wound contraction and the quality of scaffold invasion by fibroblasts and blood vessels *in-vivo* was assessed in a sheep wound model.

By day 29, the two BTM and one Integra™ grafted sites as well as the ungrafted control site on all sheep had healed (Figure 2a and 2b). A graph of the change in wound area over this time illustrated that in the initial days following grafting,

all four sites showed a considerable decrease in area which then levelled out; with the exception of the untreated control which showed a further rapid wound contraction between day 15 and 20 (Figure 2c). This resulted in ~75% decrease in area in the treated sites which was significantly less compared to the ungrafted control at 92 ± 3% (Figure 2d). BTM-2 mats placed with the dominant fibre either transversely (BTM-0°) or axially (BTM-90°) showed no increased resistance in wound contraction in the dorsal-ventral and rostral-caudal axes.

The potential importance of a 'sealing' silicone layer in preventing wound contraction was also investigated by leaving the silicone layer on the Integra™ in the first group of sheep but removed in the second group. 2 out of 3 sheep grafted with intact Integra™ had infections which caused premature separation of the silicone layers. The silicone sheets were not removed and the infections were not treated. There were no significant differences in wound contraction between the two groups at the conclusion of the experiment, suggesting that the advantage of the silicone layer was limited and may inadvertently facilitate an increased incidence of wound infection.

Re-epithelialisation was observed in all wounds. The neo-epidermis appeared acanthotic and hyperkeratotic compared to adjacent normal epidermis (Figure 3a) and was intact in both the untreated control (Figure 3c) and the BTM-2 treated sites (Figure 3e) in spite of some superficial erosion in the latter. In contrast, the Integra™ treated sites showed extensive epidermal erosion with sometimes severe epidermal ulceration and secondary acute dermatitis. In these cases, there was an abundant accumulation of necrotic cellular debris heavily admixed with polymorphonuclear cells over the denuded epidermis. More importantly, Periodic Acid Schiff (PAS) staining revealed a well developed basement membrane at the dermal-epidermal junction of the BTM-2 grafted sites (Figure 3g).

The dermal tissue of the un-grafted control sites showed extensive reparative fibrosis (Figure 3c & 3d) compared with normal dermal tissue (Figure 3a & 3b) with an increased prominence of small blood vessels due to endothelial hyperplasia, perivascular oedema and lymphocytic cuffing.

In sections containing BTM-2, a large number of polymer fibres were observed in the subcutis, but fewer in the dermis and epidermis (Figure 3e). These polymers were surrounded by a granulomatous (foreign body) reaction of epithelioid macrophages (often attenuated) and multinucleated giant cells (Figure 3h). There was effacement of much of the dermis and subcutis by reparative collagenous connective tissue with elongated fibroblastic/fibrocytic nuclei embedded in

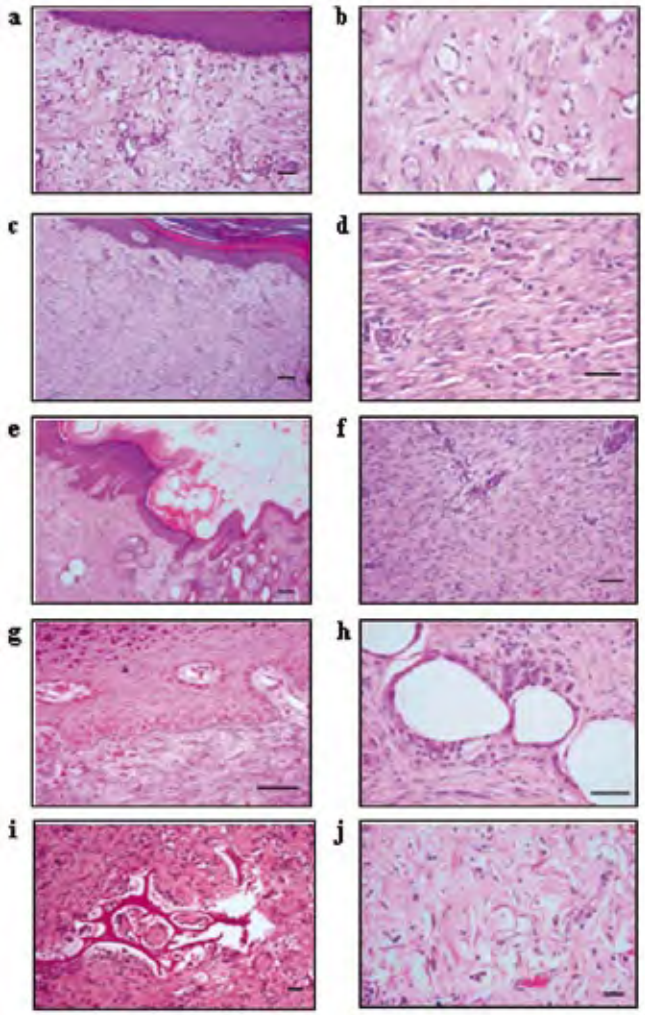

Figure 3. Histological analysis of wounds in sheep grafted with BTM-2 matrix.

Tissue collected from sheep engrafted with BTM-2 matrix was stained with H&E. (a) A section of normal sheep skin. (b) higher magnification of normal dermal tissue. (c) Section of the full-thickness wound left to heal by secondary intention showing a hyperkeratotic epidermis with dense fibrotic dermis. (d) Fibrosis of the ungrafted site. (e) Regenerated epidermis over fibrotic; normal epidermis is depicted to right of the picture. (f) Effacement of the dermis and subcutis by reparative collagenous connective tissue with elongated fibroblastic/fibrocytic nuclei embedded in collagen in polymer-containing sections. (g) Normal basement membrane between epidermis and polymer 'dermis'. (h) Granulomatous (foreign body) reaction of epithelioid macrophages and multinucleated giant cells around BTM-2 fibres. (i) Integra™ in skin sections showing eosinophilic, branching core distinguishing it from surrounding tissue. (j) Integra containing skin with loose fibrovascular penetration and collagen deposition. Scale bar = 50µm.

collagen (Figure 3f). Scattered throughout this fibrous tissue were capillaries and arterioles. The endothelial cells in the dermal tissue were frequently hypertrophied and hyperplastic. Inflammatory cells were often loosely distributed in replacement connective tissue, especially sub-epidermally where fibrosis was sometimes less dense; with actively proliferating fibroblasts and neovascularisation. Some fibrous tissue in this region appeared uniformly homogeneous and more hyalinised as indicated by high eosinophilic staining.

The Integra™ in the dermis and subcutis was clearly distinguishable from surrounding tissue as the branching, deeply eosinophilic core (Figure 3i). The lattice was often loosely invaded by fibroblasts and capillaries with variable and often loose collagen deposition compared to BTM-2 matrix (Figure 3j). It was also mildly infiltrated by a few inflammatory cells, principally lymphocytes and eosinophils. The Integra™ was occasionally attended by a few epithelioid macrophages and multinucleated giant cells, but this foreign body granuloma reaction was minimal. The dense collagenous connective tissue surrounding Integra™ was similar to that observed around BTM-2, extending from beneath the epidermis to the panniculus carnosus.

Taken together, these results demonstrated that BTM-2 matrix engrafted at the site of full-thickness wounds, exhibited mechanical robustness to wound contraction and allowed controlled development of dermal granulation tissue, the mechanism by which all dermal scaffolds work.

Discussion

In this study we investigated the biocompatibility of three NovoSorb™ variants for use as biodegradable temporising matrix in full thickness wounds as well as a scaffold for the generation of a skin composite.

The prior 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 assessment 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, as well as 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 groups of rats. 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 resorbed within 6 months, thus allowing further tissue remodelling. The degradation rate of BTM-2 (in-vitro and in-vivo in the rat model) fulfilled these criteria and was thus ideal for development into a 3-dimensional scaffold for the large animal wound study.

The sequential production of a covering epidermal tissue over the dermal composite with existing microvasculature will generate a material capable of replacing skin autografts. The use of such a composite skin mandates the consideration of protecting and preparing the excised burn wound whilst the composite is in culture (potentially 21 days). Given that the BTM-2 matrix integrated into the full thickness animal wounds in a manner comparable to Integra™ and was able also to restrain wound contraction to a similar degree, this material can also be used as an immediate non-cellular implant to temporise the wound. The observation that the BTM-granulation tissue supported the migration of keratinocytes from the wound edge to form a more robust epithelium and produced the appropriate signals to ensure the development of a normal basement membrane, provided further evidence that the BTM therapeutic model would work. Since it can be produced at a fraction of the cost of the Integra™, it could already represent an attractive treatment option.

The necessity of the silicone layer on Integra™ to further limit wound contraction was not firmly established in this study due to delamination of the silicone layer by pus when it was left on. Despite an increased incidence in wound infection observed in this study, it may still play an important role in sealing and protecting the debrided wound against moisture loss and should still be considered in the design of optimised BTM.

Conclusion

In this study, we demonstrated that the series of biodegradable polyurethanes created as candidates for generation of a dermal scaffold are biocompatible with no observable cytotoxic effect *in-vivo*. Implantation studies in both rat and sheep demonstrated that the inflammatory response and granulomatous reaction to the polymers were comparable to clinically used materials including sutures and the dermal regeneration template Integra™. 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 demonstrated that this (as yet un-optimised) matrix performed as well as Integra™ as a dermal scaffold in preventing wound contraction but allowing re-epithelialisation over the resultant dermal granulation tissue with normal basement membrane formation. We also demonstrated that the BTM-2 matrix can be used to create a rudimentary bi-layered composite skin which may eliminate our reliance on skin autografts. Our previous study suggested 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.

Acknowledgements

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Both the rat safety and sheep implantation studies were approved by the Animal Ethics Committee of IMVS and conducted in accordance with the IMVS standard operating procedures (SOP), and the OECD Principles of Good Laboratory Practice.

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