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## The in vitro characterization of a gelatin scaffold, prepared by cryogelation and assessed in vivo as a dermal replacement in wound repair

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### ABSTRACT

A sheet gelatin scaffold with attached silicone pseudoepidermal layer for wound repair purposes was produced by a cryogelation technique. The resulting scaffold possessed an interconnected macroporous structure with a pore size distribution of  $131 \pm 17 \mu\text{m}$  at one surface decreasing to  $30 \pm 8 \mu\text{m}$  at the attached silicone surface. The dynamic storage modulus ( $G'$ ) and mechanical stability were comparable to the clinical gold standard dermal regeneration template, Integra<sup>®</sup>. The scaffolds were seeded in vitro with human primary dermal fibroblasts. The gelatin based material was not only non-cytotoxic, but over a 28 day culture period also demonstrated advantages in cell migration, proliferation and distribution within the matrix when compared with Integra<sup>®</sup>. When seeded with human keratinocytes, the neoepidermal layer that formed over the cryogel scaffold appeared to be more advanced and mature when compared with that formed over Integra<sup>®</sup>. The in vivo application of the gelatin scaffold in a porcine wound healing model showed that the material supports wound healing by allowing host cellular infiltration, biointegration and remodelling. The results of our in vitro and in vivo studies suggest that the gelatin based scaffold produced by a cryogelation technique is a promising material for dermal substitution, wound healing and other potential biomedical applications.

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### 1. Introduction

Acute trauma, genetic skin disorders and chronic wounds may result in skin loss, with burns and scalds being major contributors to rapid, extensive, deep (i.e. full thickness) wounds with substantial areas of skin damage, frequently without the possibility of skin regeneration. These often cannot be successfully treated with common routine surgical skin grafting techniques [1], and can be life threatening. Alternative life-saving approaches in the treatment of extensive full thickness wounds, where autologous skin grafts are not available, include the use of cultured autologous keratinocytes and bioengineered skin substitutes [2,3], with significant

progress being made recently in the development, marketing and clinical use of these products [2–8].

The clinical “gold standard” material for dermal substitution in full thickness wounds is considered to be the collagen based Integra<sup>®</sup> [9–11], which is a safe and effective burns treatment. However, its high cost and long biointegration time could be preventive factors for many patients. The main component of Integra<sup>®</sup>, collagen, is the natural and most abundant component of the extracellular matrix (ECM) of skin [12]. Gelatin is a product of collagen denaturation and hydrolysis, and is chemically similar to collagen in many ways [13]. It is cheap, biocompatible, biodegradable, non-immunogenic and widely used in clinic as, for example, wound dressings, implantable antibiotic carriers, vascular stent modifying material, as well as for neurosurgical applications [14–17].

The cryogelation technique described here can be used to produce robust, highly porous materials with large interconnected pores and thick polymer walls. The pore walls provide the flexibility, as well as the mechanical and osmotic stability of the scaffold

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[18–20]. The technique does not require the use of any organic solvents and allows the porosity of the scaffold to be controlled, and therefore tuned, for a particular application [21]. In general, cryogels produced in an aqueous solution have large pores of up to 100–150  $\mu\text{m}$ , suitable for the infiltration and proliferation of cells [18,22–24]. The cryogels have also shown shape memory, as they can be repeatedly dried and swollen returning to their original, hydrated shape [25]. The majority of the water in the cryogel is nonbound or weakly bound, and does not strongly interfere with the bioprocesses within the cryogel, making the cryogel an attractive material for biomedical applications [23]. All of these features make cryogels promising for use as dermal regeneration scaffolds [18,22].

In our previous work [18] we assessed and characterized mixed gelatin fibrinogen cryogel matrices for their potential suitability in wound repair. The purposes of this study were to synthesize, by cryogelation, macroporous gelatin scaffolds in a sheet form, to apply a nonporous silicone pseudoepidermal layer to mimic a bilayered skin structure and to assess the resulting prototype constructs for wound repair potential. The methods of assessment included the *in vitro* characterization of the porosity and structure, and the mechanical, cytotoxic, bioconductive, biosynthetic, proinflammatory and biotransformation properties of the scaffolds when seeded with human skin fibroblasts or keratinocytes. They further included the *in vivo* preclinical assessment in a large animal (porcine) wound healing model.

The gelatin cryogel dermal regeneration scaffold was compared with the clinically proven Integra® Dermal Regeneration Template, which is widely used in clinic for extensive burns management [9–11].

## 2. Materials and methods

### 2.1. Production of gelatin and gelatin–silicone cryogel sheet scaffolds

Glutaraldehyde (GA), 0.4% w/v final concentration (Sigma, UK), was added to an aqueous solution of fish skin gelatin (GL), 6% w/w (Sigma, UK), and the solution was frozen in a  $-12\text{ }^{\circ}\text{C}$  alcohol cooling bath (Julabo F34) for 20 h in a 2 mm thick glass mould ( $6.5 \times 8.5\text{ cm}$ ). The gelatin glutaraldehyde (GL–GA) sheets were thawed at room temperature, and the residual glutaraldehyde was washed out with excess of water. The residual glutaraldehyde groups in the gelatin matrix were inactivated by adding 50 ml of 0.5 M glycine per 10 g of cryogel (Fisher Scientific, UK) in sodium phosphate buffer (0.1 M, pH 8.4 (Sigma, UK). After overnight washing with excess deionized water, the samples were stored in 30% ethanol at  $+4\text{ }^{\circ}\text{C}$ .

To prepare the samples with a nonporous silicone pseudoepidermal layer, the gelatin and glutaraldehyde solution was poured over a layer of an nonporous medical grade silicone (S) rubber membrane (Silatos silicone sheeting, 0.12 mm thick, ATOS Medical AB, Sweden) and placed in Julabo cooling chamber at  $-12\text{ }^{\circ}\text{C}$ . Two thicknesses of cryogel scaffolds were produced – 1 and 2 mm – and the cryogels with a silicone layer were labelled GL1–GA–S and GL2–GA–S, respectively.

In this study, GL–GA, GL1–GA–S and GL2–GA–S cryogels and Integra® were tested in a native state as produced for most experiments; however, for dynamic storage/loss moduli testing and the assessment of human primary keratinocyte growth in scaffolds, the silicone layer was removed to mimic the clinical situation and obtain relevant results.

The commercially available Integra® Dermal Regeneration Template™ (new generation, stored in phosphate-buffered saline (PBS), Integra LifeSciences Corporation, Plainsboro, NJ, USA) served as a control for these studies. It consists of bovine collagen and shark

glycosaminoglycan sponge attached to a removable silicone outer layer.

### 2.2. Cryogel and Integra® scaffold characterization by confocal laser scanning microscopy (CLSM)

All scaffold materials were placed under a confocal laser scanning microscope (Leica TCS SP5 with Leica Application Suite Advanced Fluorescence software, LAS AF) following staining with fluorescein isothiocyanate (Sigma, UK) to view the structure of the materials as described previously [18,26]. The images of top and bottom surfaces plus a cross-section of triplicate samples for each material were analysed using ImageJ software (<http://rsb.info.nih.gov/ij/>) to assess the porosity, mean pore size, pore size distribution and wall thickness [23].

### 2.3. Cryogel and Integra® scaffold characterization by scanning electron microscopy (SEM)

Scaffold samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, washed with 0.1 M sodium cacodylate buffer  $\times 3$ , dehydrated in ethanol and air dried overnight. After drying, specimens were sputter coated with palladium and examined using a JEOL JSM-6310 scanning electron microscope.

### 2.4. The mechanical properties of GL–GA and GL–GA–S cryogel scaffolds and Integra®

The mechanical properties (dynamic storage modulus  $G'$  and dynamic loss modulus  $G''$ ) were assessed by rheometry using a ThermoHaake RheoWin Pro 2.90 rheometer. Sinusoidally oscillating stress of small amplitude within the linear viscoelastic region is applied to the sheet. The resulting strain was measured for both amplitude and phase lag, and compared with the input stress (oscillating frequency sweep test at  $20\text{ }^{\circ}\text{C}$ , 5.0 Pa and 0.1–100.0 Hz) to obtain the characteristic moduli: storage modulus  $G'$  and loss modulus  $G''$ .

### 2.5. Scaffold sterilization for *in vitro* and *in vivo* studies

Gelatin cryogel scaffolds and Integra® were sterilized by incubation with penicillin ( $1000\text{ units ml}^{-1}$ )/streptomycin ( $1000\text{ }\mu\text{g ml}^{-1}$ )/Fungizone ( $6.25\text{ }\mu\text{g ml}^{-1}$ ) in Hank's balanced salt solution (HBSS) (all from Gibco Invitrogen, UK) for 10 days at  $37\text{ }^{\circ}\text{C}$ , 5%  $\text{CO}_2$ . Samples were washed extensively with HBSS. Sterility was confirmed by the lack of bacterial/fungal/yeast growth after further incubation in fibroblast growth medium for 3 days.

For *in vivo* studies, scaffolds were double wrapped with 3 ml of PBS (Sodium Phosphate Dibasic, Sigma Aldrich, UK) and sterilized commercially with gamma irradiation at 25 kGy (Isotron Ltd., UK). Prior to implantation, scaffolds were washed with sterile normal saline (Isotonic 0.9% Sodium Chloride Solution, Baxter S.A., UK).

### 2.6. Human primary fibroblasts growth in GL–GA and GL–GA–S cryogel scaffolds and Integra®

All materials were assessed following the introduction of primary human dermal fibroblasts (SKF276; from University of Brighton cell bank, funded by DTI programme MPP4.5). These cells were cultured in fibroblast medium (Dulbecco's modified Eagle medium, low glucose, supplemented with 10% heat-inactivated foetal bovine serum, Gibco Invitrogen, UK) at  $37\text{ }^{\circ}\text{C}$ , 5%  $\text{CO}_2$  humidified atmosphere.

For transfer to the scaffold materials, the cells were grown in 6-well plates (Greiner Bio One, UK) until 95–100% confluent. To

each well, 1 cm × 1 cm squares of scaffold materials, hydrated in growth medium, were placed on the cell layer and allowed to attach for 8 h. The orientation of the scaffold material was with the nonporous silicone layer uppermost (where a silicone surface was present) or the small pore size surface up and with the larger pore size surface down, in contact with the cell layer. Each cell-seeded scaffold sample was then transferred to a new well and floated in growth medium to prevent attachment to the plastic. Scaffolds were incubated over a 28 day period and samples removed for assessment.

### 2.7. Human primary keratinocytes growth in GL-GA and GL-GA-S cryogel scaffolds and Integra®

As a standard test model, a multilayered epidermal model was constructed on culture well inserts by StratiCELL (Belgium) using human primary keratinocytes isolated from the skin samples of a pool of donors (minimum of three). After a differentiation period of 14 days at 37 °C, 5% CO<sub>2</sub> these cells form layers that exhibit the main molecular markers of keratinocyte differentiation and comprise basal, spinous and granulous living cell layers, as well as a compact and functional stratum corneum. The formed epithelial layer, comprising the cell layers, is then examined by ELISA for proinflammatory cytokine release, as well as histologically for cellular damage.

Using a modification of this standard protocol, the human primary keratinocytes isolated from the skin samples of a pool of a minimum of three donors were instead seeded directly onto the GL-GA cryogel (smaller pores surface topmost) and Integra®, in which the silicone layer was removed prior to cell seeding. The silicone layer was removed to mimic the spraying of keratinocytes in clinic, where the silicone layer remains on the Integra® until the scaffold is infiltrated with cells from the wound bed and ready for epithelial treatment. After a differentiation period of 14 days at 37 °C, 5% CO<sub>2</sub> the scaffolds were then examined by ELISA for proinflammatory cytokine release and assessed histologically for cellular damage.

### 2.8. Characterization of fibroblast proliferation within cryogel and Integra® scaffolds by MTT assay

Fibroblast proliferation within cryogel scaffolds and Integra® was measured indirectly by assessing the mitochondrial activity of living cells by Thiazolyl Blue Tetrazolium Bromide assay (MTT) (Sigma, UK). The working solution was prepared by diluting 5 mg ml<sup>-1</sup> MTT filtered stock solution in fibroblast growth medium 1:10. MTT working solution (500 µl per well) was added to triplicate scaffolds and incubated at 37 °C, 5% CO<sub>2</sub> for 3 h. The MTT was subsequently removed and DMSO added to the cell layer, any converted dye in the cells being solubilized with the DMSO (500 µl per well). The solubilized dye was transferred to a 96-well plate and the absorbance measured at 490 nm.

### 2.9. Characterization of fibroblast migration and proliferation within cryogel and Integra® scaffolds by CLSM

The method for characterization of fibroblast migration and proliferation within cryogel scaffolds described earlier by Allan et al. [26] was used, with modifications. Briefly, for the direct detection and quantification of cells within scaffolds, materials were fixed in neutral buffered 4% w/v formal saline, washed with PBS, cross-sectioned with a scalpel and immersed into propidium iodide nuclear stain solution (10 µg ml<sup>-1</sup>, Invitrogen, UK). Stained samples were examined with CLSM (Leica TCS SP5 with LAS AF) at an objective magnification of ×10 (the excitation and emission wavelengths were 543 and 610–640 nm, respectively). Samples

were analysed at the cell-seeded surface and transection surface of each scaffold in triplicates. Three areas in each of triplicate samples were selected randomly and 20 optical sections were taken to produce a z-stack of images of 1550 µm × 1550 µm × 100 µm.

ImageJ software was used to assess the depth of cell migration and cell numbers within matrices by quantifying the cellular nuclei by converting the image z-stacks to 8-bit monochromatic images and using the automatic Particle Analyser or manual Cell Counter plugin as described previously [26].

### 2.10. Characterization of cryogel and Integra® scaffold toxicity

Characterization of cryogel and Integra® scaffolds toxic effects on reconstituted human epidermis was performed by the cell viability MTS assay (group 1) and histologically (group 2).

Whole sterilized scaffolds (groups 1 and 2) were cultured in medium with the preformed StratiCELL epidermal model without direct contact and incubated at 37 °C, 5% CO<sub>2</sub> for 42 h. In group 1 the treated and multilayered epidermal model was transferred into MTS solution (300 µl per well) and incubated at 37 °C, 5% CO<sub>2</sub> for 90 min. The epidermal tissues were then removed and 200 µl aliquots were transferred to a 96-well plate for absorbance measurement at 490 nm.

Group 2 scaffolds were evaluated histologically, following fixation in 4% neutral buffered formal saline and paraffin embedding. Paraffin sections (6 µm) were stained with haematoxylin and eosin (H&E).

### 2.11. Characterization of inflammatory response to cryogel and Integra® scaffolds

The supernatant from the reconstituted human epidermis incubated with scaffolds was collected after 42 h to test for markers of inflammation. The presence of inflammatory marker interleukin IL1-α in the tissue supernatants was analysed with an ELISA detection kit (Pierce) according to the manufacturer's procedure.

### 2.12. Assessment of in vitro epithelialization potential of cryogel and Integra® scaffolds

Small circular pieces of the scaffolds (8 mm diameter) with the silicone removed were transferred to well inserts, orientated such that the removed silicone layer would have been at the top. After an incubation period of 30 min at room temperature, a suspension of keratinocytes was dispensed onto the scaffolds, which were subsequently incubated at 37 °C and 5% CO<sub>2</sub>. After 72 h the samples were raised to an air/liquid interface. Tissue culture was continued in this manner for 14 days. At 14 days, scaffolds were fixed in 4% neutral buffered formal saline. Paraffin sections (6 µm) were stained with H&E.

### 2.13. Assessment of cryogel and Integra® scaffolds in in vivo preclinical environment

The pig was chosen due to the structural and physiological similarities between porcine and human skin [27,28]. A porcine skin full thickness wound healing model with six square wounds (5 cm × 5 cm each), created on the back of the male Large White/Landrace crossbred pig (×3), was used to assess cryogel scaffolds in in vivo preclinical environment. Wounds were created on the dorsolateral side of each animal, three per side, and grafted randomly in each animal tissue regeneration scaffold (GL-GA/GL-GA-S *n* = 6 each, Integra® *n* = 3, nongrafted wound controls *n* = 3) using aseptic techniques. Standard materials used normally in clinic were used to complement the scaffolds; TelfaClear (Kendall) and standard gauze dressing. To assess the scaffolds'



biointegration, punch tissue biopsies 6 mm in diameter were taken at days 4 and 9, with final full wound excision biopsy on day 14. After fixing biopsies in 10% neutral buffered formal saline for 7 days, morphometric histological assessment of 5  $\mu\text{m}$  paraffin embedded tissue sections stained with H&E was performed. Animal experiments were approved by a local ethical review committee and carried out according to the Animals Scientific Procedures Act (1986) under all the required licences.

#### 2.14. Statistical analysis

Statistical analysis was performed in PASW Statistics 18 (IBM SPSS North America, USA) software package. The data were analysed statistically using a one-way analysis of variance with pairwise multiple comparisons of experimental groups (Tukey test). The differences between groups were considered to be statistically significant at  $p < 0.05$ .

### 3. Results and discussion

#### 3.1. Morphology and microstructure of GL–GA and GL–GA–S cryogel scaffolds and Integra®

Both SEM with dehydrated (Fig. 1) and CLSM with natural hydrated state (Fig. 2) gelatin cryogel scaffolds revealed a supermacroporous anisotropic structure, with smaller pores at the surface directly adjacent to the glass mould and larger pores at the opposite surface, exposed to the air. In GL–GA–S scaffolds, the surface with smaller pores was adjacent to the pseudoepidermal silicone layer. This surface with smaller pores served as the upper surface of a

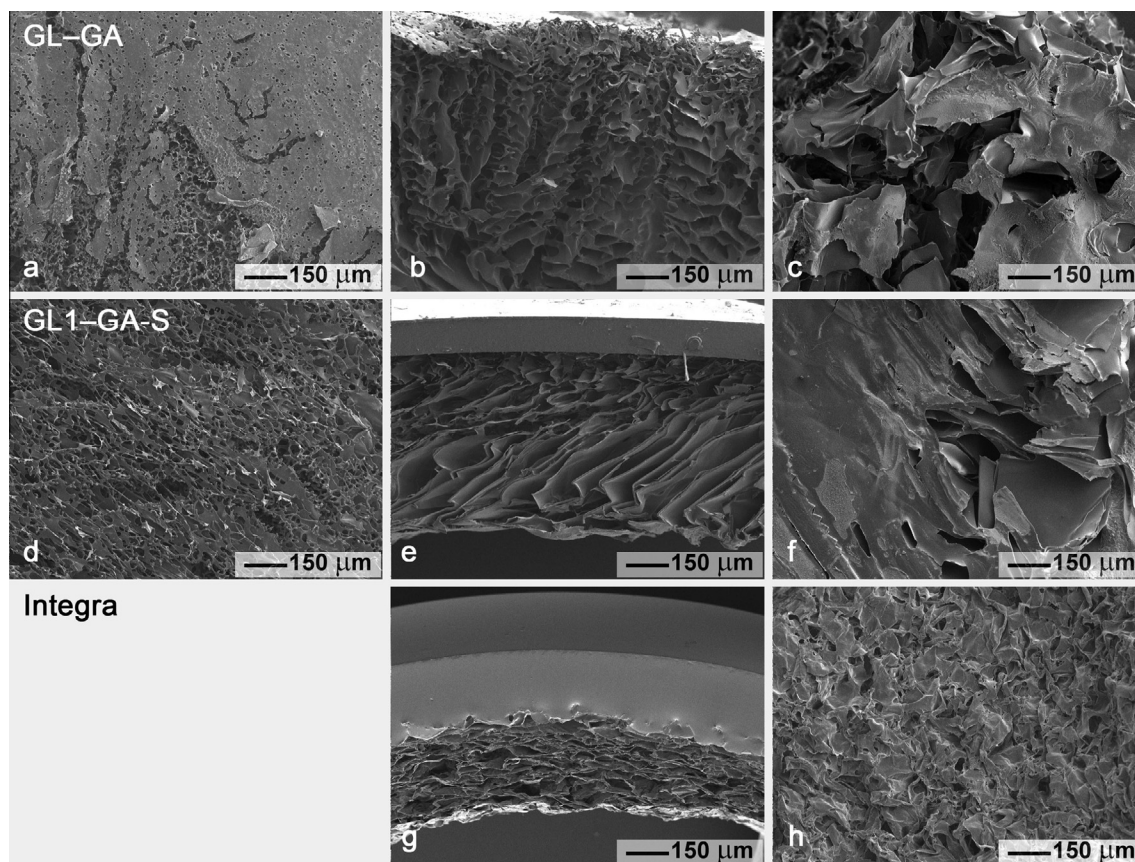
dermal scaffold. The surface with larger pores was treated as the lower surface in both GL–GA and GL–GA–S scaffolds, representing the surface that would be applied to a wound bed when used clinically. This is similar to the structure of previously described cryogels [29–31].

The materials are comparable to that of Integra®; however, whereas Integra® has uniform pores, the cryogel's anisotropic structure could be superior for clinical applications (Figs. 1 and 2).

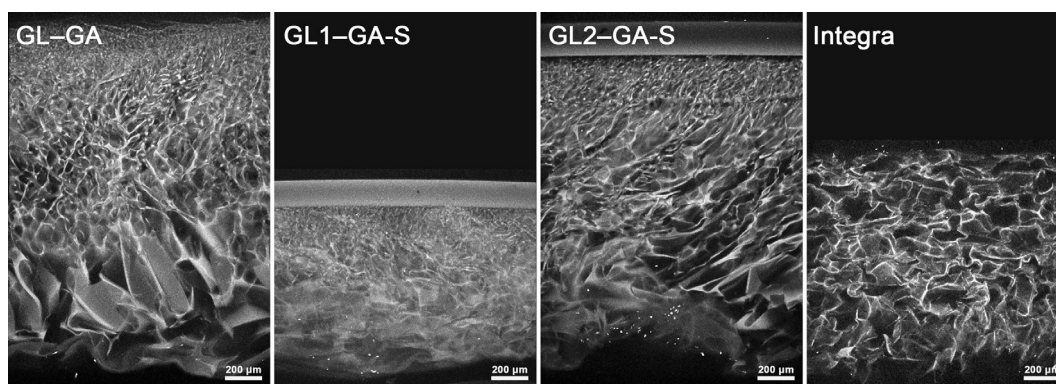
The mean of each measured parameter, including scaffold thickness, pore size and porosity, measured at the upper surface, cross-section and lower surface of the scaffold in a hydrated state ( $n = 3$ ), is summarized in Table 1.

As can be seen from Table 1, all cryogel scaffolds possess a well-defined porosity gradient, with smaller pores at one side (30–61  $\mu\text{m}$ , SD = 8–16) and larger pores at the bottom (76–131  $\mu\text{m}$ , SD = 1–17), unlike Integra®, where mean pore size (92  $\mu\text{m}$ , SD = 17) did not have a defined gradient across the thickness of the material. This anisotropic arrangement, with smaller pores put at the upper surface of the wound, could be of potential benefit for clinical use, allowing keratinocyte application (by spraying) to form a continuous epithelial layer without cells falling or quickly migrating through to the large pores of the scaffold, placed on the wound bed. Keratinocytes, which are trapped within a dermal regeneration scaffold, are reported to form cysts, which eventually migrate upwards to form an epithelial layer [29]; however, this process can delay wound healing.

An additional benefit of placing the larger pores of the material on the wound bed is increased flexibility to adjust to the roughness and curvature of the wound surface. The failure of skin substitute biomaterials to adjust to the wound surface often results in suboptimal healing [33]. The porosity, a measure of the void space in a



**Fig. 1.** Scanning electron microphotographs of the upper (a, d), cross-section (b, e, g) and lower (c, f, h) surfaces of without silicone layer GL–GA, with silicone layer GL1–GA–S cryogel and Integra® scaffolds. Top view of GL1–GA–S cryogel scaffold (d) shown with the silicone layer removed. Bar: 150  $\mu\text{m}$ .



**Fig. 2.** Cross-section microphotographs of GL-GA, GL1-GA-S, GL2-GA-S and Integra® scaffolds visualized in the hydrated state with confocal laser scanning microscopy. Bar: 200 µm.

**Table 1**

Distribution of mean pore size, wall thickness, porosity and surface area in the scaffolds.

		GL-GA	GL1-GA-S	GL2-GA-S	Integra®
Scaffold thickness (µm)	Overall	2072 ± 249	1178 ± 172	1756 ± 336	1244 ± 88
Mean pore size (µm)	Upper surface	30 ± 8	61 ± 16	48 ± 1	97 ± 10
	Cross-section	96 ± 10	– <sup>a</sup>	90 ± 6	92 ± 17
	Lower surface	115 ± 27	76 ± 1	131 ± 17	82 ± 11
Porosity (%)	Upper surface	90 ± 1	89.1 ± 0.9	89.4 ± 0.8	95.4 ± 0.8
	Cross-section	85.7 ± 0.5	– <sup>a</sup>	88.2 ± 0.9	96.3 ± 0.9
	Lower surface	76 ± 1	85 ± 2	86 ± 3	94.7 ± 0.2

<sup>a</sup> Due to the thinness of the GL1-GA-S sample, it was not possible to find and assess a cross-section with uniform pore size.

material, was lower in cryogels (76–90%) when compared with Integra® (94–95%) due to the thicker walls of cryogel scaffolds in the areas with larger pores, which might potentially limit the number of cells accommodated by the scaffold. However, to fill in the large voids, cells rely on an attachment to the pore walls to produce ECM and to proliferate. Cells attach to pore walls with integrins and other adhesion receptors, and successfully repair or regenerate tissue in vivo via ligation and traction-induced signaling [34,35].

### 3.2. Mechanical properties of GL-GA/GL-GA-S cryogel scaffolds and Integra®

The dynamic storage moduli and dynamic loss moduli of the scaffolds are shown in Fig. 3. The storage moduli (which may be thought of as stiffness) of the thicker GL2-GA-S scaffold and Integra® were indistinguishable, but differed from GL-GA scaffold and the thinner GL1-GA-S scaffold in the range of 0.1–1 Hz. This can probably be explained by the differences in the micromorphology of the cryogel scaffolds and Integra®, as well as by the presence of a stiffer silicone layer in the GL2-GA-S and Integra® matrices. Stiffer scaffold materials have been shown to potentiate fibroblast motility and proliferation [36–41]. The GL1-GA-S scaffold, although supplied with a silicone layer, was less stiff than the other scaffolds, which is probably related to the sample's decreased thickness. However, more importantly, in the physiological range, 1–10 Hz, there was no statistically significant difference between all the assessed materials. This suggests that the gelatin cryogel sheets with a silicon layer are as durable and easy to handle as clinically proven Integra®. Environmental variables acting on the cells within the ECM, such as surface topography, density, distribution and rigidity of the ECM, are crucial for cell adhesion, migration, proliferation and biosynthetic activity [35,37–40]. Based on the demonstration of the similar mechanical properties of Integra®

and gelatin cryogel matrices, we expected similar cellular behaviour within the matrices.

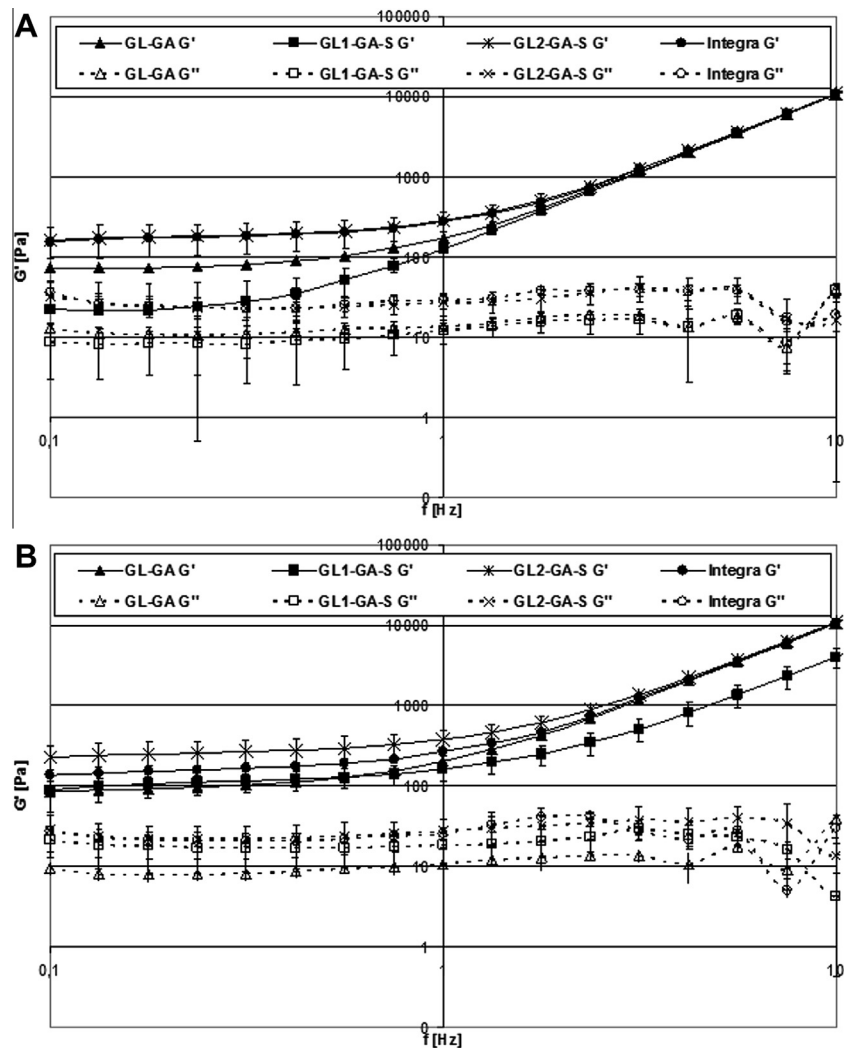
After the scaffolds had been incubated with normal primary human dermal fibroblasts for 28 days, the thinner GL1-GA-S cryogel scaffold was observed to have reduced stiffness (Fig. 4); there were no significant differences between the other scaffolds.

The reduced mechanical integrity of the GL1-GA-S scaffold is probably related to its smaller initial thickness combined with the initial stages of scaffold biomodification. This observation enables us to speculate that the GL1-GA-S scaffold could be more quickly modified and degraded in the in vivo environment, when compared with the other scaffolds. However, it is rather difficult to extrapolate in vivo biodegradation rates from in vitro data as the wound environment is complex, with an array of pro-synthetic factors, such as cellular and humoral components (e.g. fibroblasts and TIMP cytokines), as well as cellular and humoral factors aimed at matrix biodegradation (macrophages and matrix degradation enzymes) [41,42]. Interpretation is also limited to bulk observations because the material is multiphasic, consisting of the matrix, the mobile fluid phase and the cellular phase, so comparisons reveal only bulk behaviour.

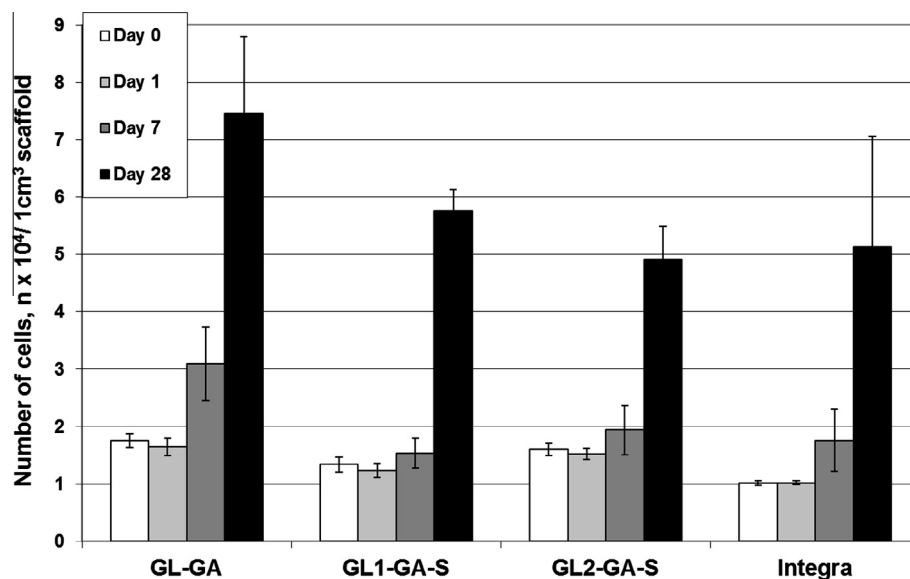
For the remaining scaffolds, differences in the mechanical properties of native and cell-modified materials were not statistically significant. This could be beneficial in vivo; the balance between remodelling of the scaffold while allowing the wound healing process to complete is an important consideration in scaffold design [2,3,6,33,40].

### 3.3. Fibroblast migration and proliferation within GL-GA/GL-GA-S cryogel scaffolds and Integra®

The MTT assay allows assessment of scaffold toxicity, but it has also been successfully used by researchers to quantify the number of cells seeded and proliferating within scaffolds [43,44]. In our experiments, the MTT assay revealed the mitochondrial activity



**Fig. 3.** The dynamic storage modulus ( $G'$ ) and dynamic loss modulus ( $G''$ ) of GL-GA, GL1-GA-S, GL2-GA-S and Integra® scaffolds (A) prior to seeding (day 0) and (B) postseeding (day 28) with normal primary human dermal fibroblasts. Oscillating frequency sweep test values, mean  $n = 3$ ; error bars represent SD.



**Fig. 4.** Proliferation of normal primary human dermal fibroblasts within GL-GA, GL1-GA-S, GL2-GA-S and Integra® scaffolds over 28 days in vitro. MTT assay; values are the number of cells  $\times 10^4$  per  $1\text{ cm}^3$  of scaffold, mean  $n = 3$ ; error bars represent SD. \*Statistically significant difference at  $p < 0.05$ .



of normal primary human dermal fibroblasts seeded into cryogel scaffolds: the measurements at days 0, 1, 7 and 28 indirectly show a gradual increase in cell numbers over the course of the experiment (Fig. 4). A higher number of cells was observed at day 28 in the silicone layer free GL-GA scaffold compared with the silicone-covered Integra®, GL1-GA-S and GL2-GA-S scaffolds, and this was statistically significant ( $p < 0.05$ ). No statistical significance was observed between cell numbers in the Integra®, GL1-GA-S and GL2-GA-S scaffolds.

The MTT assay only reveals the total number of cells within the scaffold; it does not allow measurement of the cellular distribution, crucial for characterization of the performance of potential skin substitute materials. Penetration of scaffold materials by host cells is a basic requirement for the important initial step of scaffold colonization, leading to successful constructive ECM remodelling, neodermis formation and consecutive wound healing [12]. Direct visualization of cells within scaffolds using confocal microscopy (Fig. 5) has confirmed our MTT findings of active cellular proliferation within matrices; moreover, it has shown crucial differences between cryogel scaffolds and Integra®. The distribution of normal primary human dermal fibroblasts within GL-GA, GL1-GA-S, GL2-GA-S and Integra® scaffolds after 28 days in vitro are shown in Fig. 6. Taking into account the differences between absolute depth of cell migration due to the different scaffold thicknesses, Table 1 (where GL-GA  $\approx$  GL2-GA-S  $\approx$  2 mm and GL1-GA-S  $\approx$  Integra®  $\approx$  1 mm), some trends were observed. In all gelatin cryogel scaffolds (with and without a silicone pseudoepidermal cover) cells were evenly distributed across the entire thickness of the scaffold, whereas in Integra® cells were predominantly located at the bottom part of the scaffold (Figs. 5 and 6). Another CLSM finding was that silicone-free GL-GA scaffolds were more favourable for cellular proliferation, as silicone-covered cryogel scaffolds GL1-GA-S, GL2-GA-S and Integra® had fewer cells within the matrices compared with the number of cells within the GL-GA scaffold, which corresponds with our MTT cell quantification results.

The pseudoepidermal silicone cover associated with the material seemed to slow down cellular migration and proliferation within the cryogel scaffolds. It is known that the structure, biochemical and biophysical composition of the extracellular matrix regulates cellular behaviour [45]; however, in this case GL-GA and GL1-GA-S/GL2-GA-S were similar in all aspects apart from the pseudoepidermal silicone layer. It can be speculated that the

silicone cover limited O<sub>2</sub>/CO<sub>2</sub> transfer, nutrient supply or medium passage through the scaffold, thus affecting the proliferation rate.

### 3.4. Characterization of cryogel and Integra® scaffold toxicity

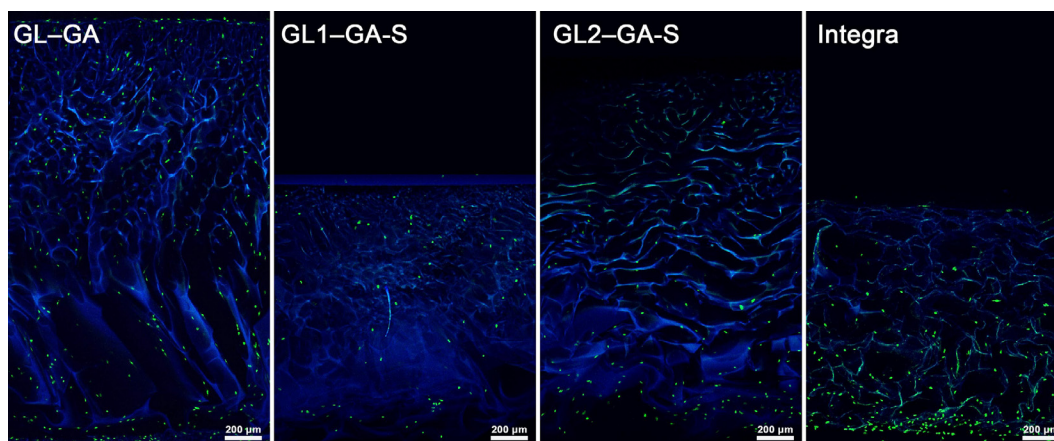
The MTS assays were carried out in culture medium, with scaffolds that had been cultured with a multilayered human epidermal model for 42 h. No significant cytotoxic effects were observed. Cell viability was not reduced below the 80% threshold in any of the samples: GL-GA =  $108 \pm 2\%$ , GL1-GA-S =  $102 \pm 2\%$ , Integra® =  $103 \pm 3\%$ , compared with the cell culture control of  $100 \pm 10\%$ .

These findings were also confirmed by histological observations, with there being no signs of toxic effects (i.e. keratinocyte necrosis, cell shrinkage, vacuolization, membrane rupture, loss of junction attachments between cells or epithelial layer detachment). These results suggest that the scaffolds are not toxic, and further in vivo characterization in a wound environment was assumed to be safe for regenerative purposes.

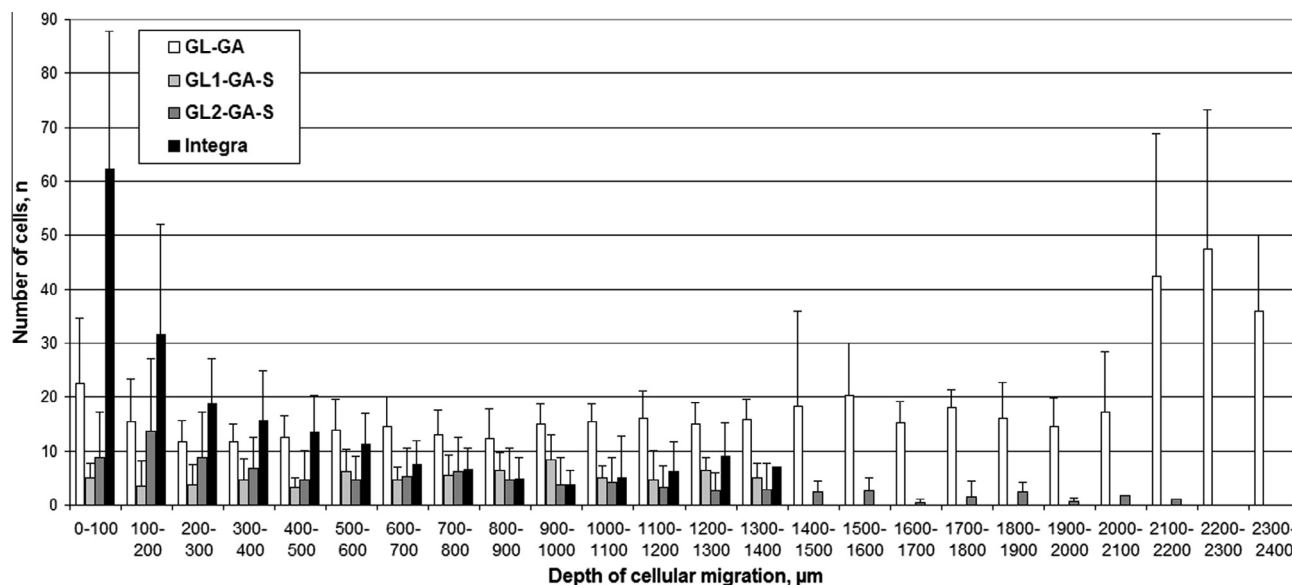
### 3.5. In vitro epithelialization potential of cryogel and Integra® scaffolds

After 17 days in vitro (Fig. 7), a continuous epithelial layer formed over the GL-GA cryogel matrix, which seemed to be more structured and mature when compared to the epithelial islands formed over Integra®. Although continuous, the epithelial layer over the cryogel matrix was immature. A stratum corneum-like layer was formed over the whole surface; however, nucleated cells were observed in this layer, indicating that the cornification processes was not complete within the given time frame. It is worth noting that the infiltration of keratinocytes through the whole thickness of the cryogel scaffold did not occur, unlike in Integra®, where cellular cysts were observed in some cases in the bottom part of the scaffold, which potentially might delay wound healing as keratinocytes would be forced to migrate upwards in vivo [29].

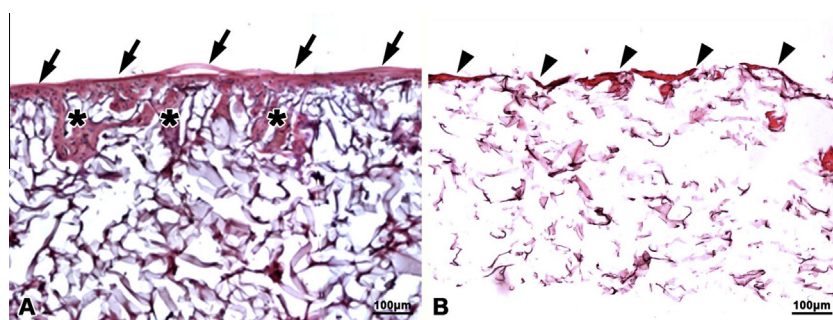
This can be explained by the presence of smaller pores in the superficial layer of the cryogel matrix ( $30 \pm 8 \mu\text{m}$ ) and larger pores in Integra® ( $97 \pm 9 \mu\text{m}$ ), which could potentially increase the dwell time at the surface and allow cellular attachment with subsequent stratification. This, however, did not preclude keratinocytes from migrating into the pores (\*) of the topmost layers of cryogel scaffolds (Fig. 7). This is a natural phenomenon, seen during wound healing process in vivo as well as in in vitro skin models, and is associated with many factors, including the lack of an underlying



**Fig. 5.** Cross-section microphotographs of GL-GA, GL1-GA-S, GL2-GA-S and Integra® fibroblast seeded scaffolds at day 28 in culture. CLSM, propidium iodide staining: blue, scaffold; green, cell nuclei. Bar: 200  $\mu\text{m}$ .



**Fig. 6.** Normal primary human dermal fibroblasts distribution within GL-GA, GL1-GA-S, GL2-GA-S and Integra® scaffolds after 28 days in vitro. Mean  $n = 9$ ; error bars represent SD.



**Fig. 7.** Epithelialization of GL-GA cryogel and Integra® scaffolds. After 17 days in vitro, a continuous epithelial layer formed over the GL-GA cryogel matrix (A, arrows) which seemed to be more structured and mature when compared with epithelial islands (arrowheads) formed over the Integra® (B). Keratinocyte migration (\*) into the pores of the topmost layer of GL-GA scaffold was also evident. H&E. Bar: 100  $\mu\text{m}$ .

basement membrane [46,47], which was not present in assayed cryogel scaffolds and Integra®.

### 3.6. Characterization of inflammatory response to cryogel and Integra® scaffolds

GL-GA, GL1-GA-S and Integra® matrices were exposed to a multilayered human epidermal model for 42 h to assess the inflammatory potential of the scaffolds. IL1- $\alpha$  production, as a pro-inflammatory cytokine, which plays a crucial role in wound healing [29,48,49], was measured in the supernatant ( $n = 3$ ) and was as follows: GL-GA  $1 \pm 15 \text{ pg ml}^{-1}$ ; GL1-GA-S  $14 \pm 29 \text{ pg ml}^{-1}$ ; Integra®  $9 \pm 11 \text{ pg ml}^{-1}$ . GL1-GA-S cryogel scaffolds induced the highest IL1- $\alpha$  production in the test, probably due to the silicone rubber type; however, the results did not differ statistically from the other scaffolds, and were similar to controls ( $5 \pm 7 \text{ pg ml}^{-1}$ ). This indicates that the assessed gelatin scaffolds are not likely to cause an inflammatory response and can be used for in vivo application without inducing an excessive inflammatory reaction.

### 3.7. In vivo biointegration potential of cryogel and Integra® scaffolds

Most studies investigating potential new skin substitutes appear to end at the in vitro stage. Some authors [6] express the concern

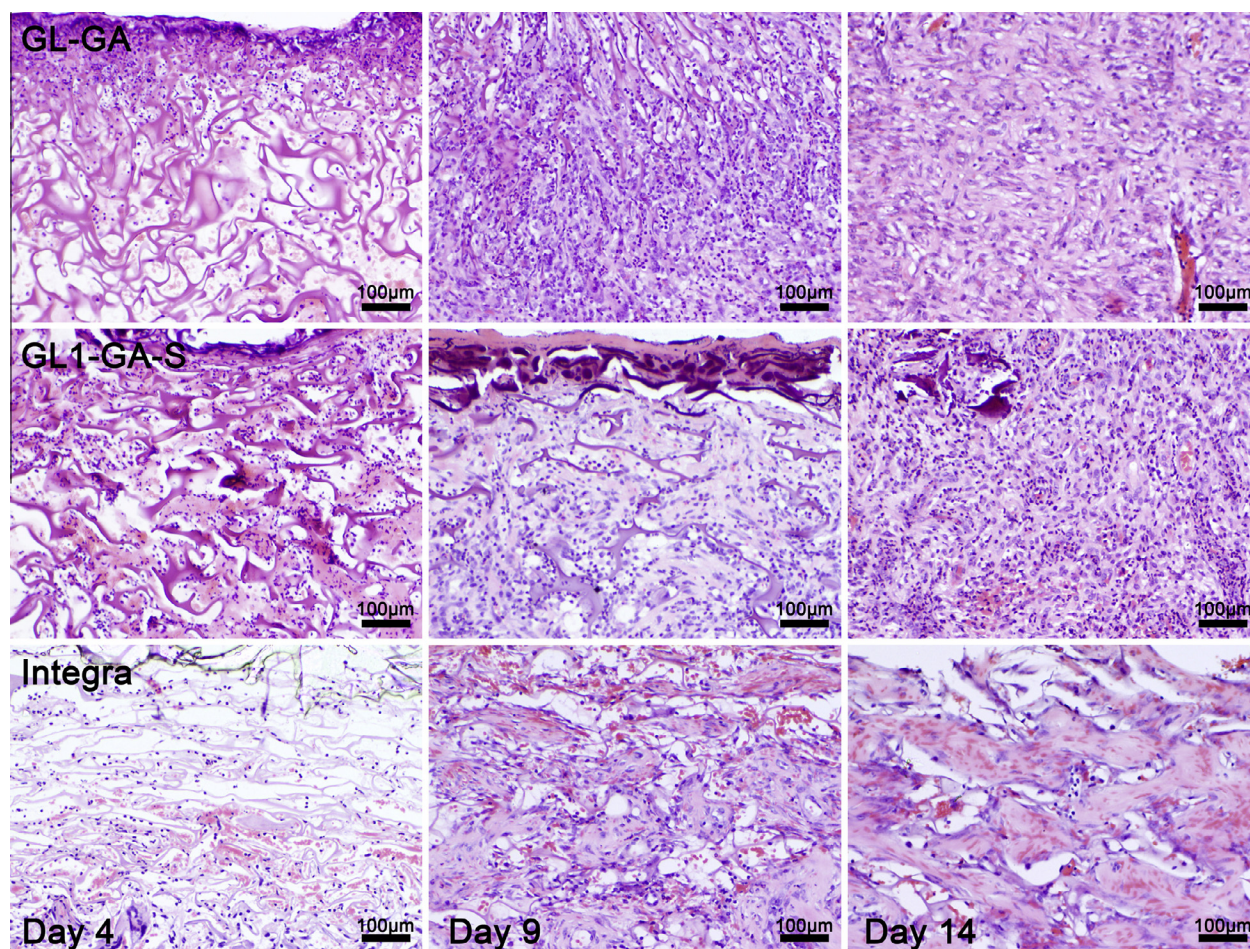
that there is a lack of information regarding the translation of in vitro results into predictions for in vivo applications, and in vivo studies are rare. Generally, it is widely accepted that preclinical in vivo studies are crucial for a complete material characterization prior to the clinical application in patients, as good in vitro results do not necessarily predict successful in vivo wound healing capabilities [50]. We have chosen a well-established domestic pig full thickness wound healing model [51] for the assessment of gelatin cryogel scaffolds, the pig being chosen due to the structural and physiological similarities between porcine and human skin [27,28].

Histological sections of implanted in vivo matrices GL-GA, GL1-GA-S and Integra® at days 4, 9 and 14 are shown in Fig. 8. At day 4, all the matrices were observed in the wound, the scaffold structure did not seem to be altered significantly and all the scaffolds were populated by host cells, predominantly neutrophils; however, there was neither new extracellular matrix formation, nor pronounced neocollagen deposition visible within the matrix structure.

By day 9, a significant fibrovascular tissue host cellular influx was seen in all scaffolds, including inflammatory cells such as neutrophils and macrophages, the spongy structure was filled with cellular and extracellular matter, and cryogel matrices seemed to be partially biotransformed and degraded.

By day 14, a clear difference between the Integra® and cryogel matrices was observed. The Integra® scaffold was still present





**Fig. 8.** In vivo application of GL-GA, GL1-GA-S and Integra® scaffolds in a porcine wound healing model, and their biomodification at days 4, 9 and 14. H&E. Bar: 100 µm.

and filled with host cells, fibroblasts and neutrophils, with a seemingly organized newly formed extracellular matrix. However, gaps without any cellular or extracellular matrix were also visible. Gelatin cryogel scaffolds, on the other hand, were completely bio-transformed; there was no visible structured network of the original matrix, and only occasional remnants of the scaffold could be seen. The space was completely filled with cells – predominantly fibroblasts, neutrophils and macrophages – and newly formed extracellular matrix, which resembled the typical structure of granulation tissue neodermis. The inflammatory stage was greatly resolved in the cryogel matrices, especially the GL1-GA-S scaffold, whereas greater numbers of neutrophils and macrophages were observed in the Integra® scaffold.

Scaffolds were implanted in the freshly excised wounds for a period of 14 days; however, at the first wound examination procedure on day 4 it was noted that the silicone layer had peeled off the cryogel matrices. In contrast, the silicone layer on the Integra®, however stayed in place, and is known to detach from the matrix when biointegration of the scaffold is complete [52,53]. It is unlikely that this was the case with the GL1-GA-S scaffold, as it was histologically proved that its integration was not complete. The collagen scaffold of Integra® is embedded into the silicone layer (Fig. 1g), whereas the method of manufacture of the gelatin cryogel matrix with a silicone layer does not allow such an embedding. An early silicone layer detachment indicates poor bonding between the gelatin scaffold and the silicone layer, which needs to be addressed in future studies.

After the silicone layer had detached from the GL1-GA-S scaffold, the scaffold was subjected to the dressings and external envi-

ronment influences in the same manner as the GL-GA scaffold, whereas the Integra® remained covered with a pseudoepidermal silicone layer for 14 days. This probably influenced the integration process and determined the differences in histological observations between the cryogel matrices and the Integra®, as well as the insignificant differences seen between the integration of the GL-GA and GL1-GA-S matrices.

It is known that, when Integra® loses its silicone cover prematurely, the granulation tissue ingrowth is uncontrollable, and results in suboptimal restoration [52]. As the presence of cells is the main factor for extracellular matrix production and degradation [53], the observed difference in cell numbers in silicone-covered and noncovered matrices – in this instance, the higher number of cells in the cryogel matrices – would probably result in different degradation speeds of the assayed matrices, which would be higher in the gelatin matrices lacking a pseudoepidermal layer. The higher cellular load, as well as the cellular composition within the cryogel matrices, where predominantly inflammatory cells were seen even at day 14, could possibly be attributed to a reaction to the dressing material. The nature of the scaffold material – collagen in the Integra® and gelatin in the cryogel scaffolds – presumably also played a significant role in biointegration and resulted in different biointegration patterns.

Although Integra® and gelatin cryogel scaffolds were found to be mechanically similar in our experiments, gelatin is a product of collagen denaturation and hydrolysis, and consists of peptides and proteins, where molecular bonds between individual collagen strands and hydrogen bonds which stabilize the collagen helix are



broken down [13]. This probably explains the quicker degradation of the gelatin matrix over the collagen matrix.

In other in vivo porcine implantation studies with prostheses coated with gelatin or collagen, the gelatin coating was found to degrade faster [54]. Integra® is reported to degrade at a clinically relevant pace and stays in the wounds for some time even when epithelialization from an applied epithelial source is complete [32]. Rapid and complete degradation of biological ECM scaffolds, and subsequent generation of bioactive molecules, are crucial for the constructive remodelling of ECM [2,12]. This is a very delicate balance, the assessed gelatin cryogel scaffolds being faster in cellular recruitment and speed of remodelling than Integra®, though neoECM formation is less structured and organized when compared with native dermis or Integra neomatrix. Based on our in vitro results, we speculate that if the pseudoeplidermal silicone layer had remained attached to the gelatin matrix for longer it could have better regulated the cellular loading and biomodification speed in the gelatin cryogel scaffolds, and, as a consequence, could have improved the neoECM deposition and its organization.

#### 4. Conclusions

Sheet gelatin scaffolds were produced by means of a cryogelation technique for wound repair purposes. The resulting scaffolds possessed a macroporous ordered structure, with interconnected uniform pores of up to 130 µm, with stiffness and mechanical stability comparable to the clinically accepted and proven dermal regeneration template Integra®. When seeded in vitro with human primary dermal fibroblasts or keratinocytes, cryogel polymers were not only noncytotoxic, but also demonstrated advantages in migration, proliferation and the distribution of fibroblast cells over a 28 day culture period when compared with Integra®. The neoepidermal layer formed over the cryogel scaffolds also appeared to be more advanced and mature when compared with that formed over Integra®. In vivo application of sheet gelatin scaffolds demonstrated that the material is capable of timely host cellular infiltration, biointegration and remodelling to support wound healing. According to the results of our in vitro and in vivo studies, sheet gelatin based scaffolds produced by the cryogelation technique are a promising material for dermal substitution, wound healing and other biomedical applications.

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#### Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 5, 7, 8, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at <http://dx.doi.org/10.1016/j.actbio.2014.03.027>.

#### References

- [1] Papini R. Management of burn injuries of various depths. *BMJ* 2004;329:158–60.
- [2] MacNeil S. Progress and opportunities for tissue-engineered skin. *Nature* 2007;445:874–80.
- [3] Shevchenko RV, James SL, James SE. A review of tissue-engineered skin bioconstructs available for skin reconstruction. *J R Soc Interface* 2010;7:229–58.
- [4] Clark RA, Ghosh K, Tonnesen MG. Tissue engineering for cutaneous wounds. *J Invest Dermatol* 2007;127:1018–29.
- [5] Horch RE, Kopp J, Kneser U, Beier J, Bach AD. Tissue engineering of cultured skin substitutes. *J Cell Mol Med* 2005;9:592–608.

- [6] van der Veen VC, Boekema BK, Ulrich MM, Middelkoop E. New dermal substitutes. *Wound Repair Regen* 2011;19(Suppl. 1):s59–65.
- [7] Zhong SP, Zhang YZ, Lim CT. Tissue scaffolds for skin wound healing and dermal reconstruction. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 2010;2:510–25.
- [8] Almeida LR, Martins AR, Fernandes EM, Oliveira MB, Corrello VM, Pashkuleva I, et al. New biotextiles for tissue engineering: development, characterization and in vitro cellular viability. *Acta Biomater* 2013;9:8167–81.
- [9] Heimbach DM, Warden GD, Luterman A, Jordan MH, Ozobia N, Ryan CM, et al. Multicenter postapproval clinical trial of Integra dermal regeneration template for burn treatment. *J Burn Care Rehabil* 2003;24:42–8.
- [10] Yannas IV, Orgill DP, Burke JF. Template for skin regeneration. *Plast Reconstr Surg* 2011;127(Suppl. 1):537–48.
- [11] Muller CS, Schiekofer C, Korner R, Pfohler C, Vogt T. Improved patient-centered care with effective use of Integra(R) in dermatologic reconstructive surgery. *J Dtsch Dermatol Ges* 2013;11:537–48.
- [12] Badylak SF. The extracellular matrix as a biologic scaffold material. *Biomaterials* 2007;28:3587–93.
- [13] Ward AG, Courts A. *The Science and Technology of Gelatin*. London, New York: Academic Press; 1977.
- [14] Choi YS, Lee SB, Hong SR, Lee YM, Song KW, Park MH. Studies on gelatin-based sponges. Part III. A comparative study of cross-linked gelatin/alginate/hyaluronate and chitosan/hyaluronate sponges and their application as a wound dressing in full-thickness skin defect of rat. *J Mater Sci-Mater Med* 2001;12:67–73.
- [15] Kharitonova KI, Rodiukova EN, Simonovich AE, Stupak VV. The experimental and clinical use of gelatin sponges with kanamycin and gentamycin for the prevention and treatment of suppurative complications in neurosurgery. *Zh Vopr Neirokhir Im N N Burdenko* 1990;9:–11.
- [16] Yang J, Woo SL, Yang G, Wang J, Cui L, Liu W, et al. Construction and clinical application of a human tissue-engineered epidermal membrane. *Plast Reconstr Surg* 2010;125:901–9.
- [17] Nakayama Y, Nishi S, Ueda-Ishibashi H, Matsuda T. Fabrication of micropored elastomeric film-covered stents and acute-phase performances. *J Biomed Mater Res A* 2003;64:52–61.
- [18] Dainiak MB, Allan IU, Savina IN, Cornelio L, James ES, James SL, et al. Gelatin-fibrinogen cryogel dermal matrices for wound repair: preparation, optimisation and in vitro study. *Biomaterials* 2010;31:67–76.
- [19] Jurga M, Dainiak MB, Sarnowska A, Jablonska A, Tripathi A, Plieva FM, et al. The performance of laminin-containing cryogel scaffolds in neural tissue regeneration. *Biomaterials* 2011;32:3423–34.
- [20] Bolgen N, Plieva F, Galaev IY, Mattiasson B, Piskin E. Cryogelation for preparation of novel biodegradable tissue-engineering scaffolds. *J Biomater Sci Polym Ed* 2007;18:1165–79.
- [21] Lozinsky VI, Galaev IY, Plieva FM, Savina IN, Jungvid H, Mattiasson B. Polymeric cryogels as promising materials of biotechnological interest. *Trends Biotechnol* 2003;21:445–51.
- [22] Savina IN, Dainiak M, Jungvid H, Mikhalovsky SV, Galaev IY. Biomimetic macroporous hydrogels: protein ligand distribution and cell response to the ligand architecture in the scaffold. *J Biomater Sci Polym Ed* 2009;20:1781–95.
- [23] Savina IN, Gun'ko VM, Turov VV, Dainiak M, Phillips GJ, Galaev IY, et al. Porous structure and water state in cross-linked polymer and protein cryo-hydrogels. *Soft Matter* 2011;7:4276–83.
- [24] Savina IN, Tomlins PE, Mikhalovsky SV, Galaev IY. Characterization of macroporous gels. In: Mattiasson B, Kumar A, Galaev IY, editors. *Macroporous Polymers: Production Properties and Biotechnological/Biomedical Applications*. Boca Raton, FL: CRC Press; 2009. p. 211–35.
- [25] Savina IN, Cnudde V, D'Hollander S, Van Hoorebeke L, Mattiasson B, Galaev IY, et al. Cryogels from poly(2-hydroxyethyl methacrylate): macroporous, interconnected materials with potential as cell scaffolds. *Soft Matter* 2007;3:1176–84.
- [26] Allan IU, Shevchenko RV, Rowshanravan B, Kara B, Jahoda CA, James SE. The use of confocal laser scanning microscopy to assess the potential suitability of 3D scaffolds for tissue regeneration, by monitoring extra-cellular matrix deposition and by quantifying cellular infiltration and proliferation. *Soft Matter* 2009;7:319–41.
- [27] Wollina U, Berger U, Mahrle G. Immunohistochemistry of porcine skin. *Acta Histochem* 1991;90:87–91.
- [28] Sullivan TP, Eaglstein WH, Davis SC, Mertz P. The pig as a model for human wound healing. *Wound Repair Regen* 2001;9:66–76.
- [29] Tripathi A, Kathuria N, Kumar A. Elastic and macroporous agarose–gelatin cryogels with isotropic and anisotropic porosity for tissue engineering. *J Biomed Mater Res A* 2009;90(3):680–94.
- [30] Lozinsky VI, Damshkaln LG, Bloch KO, Vardi P, Grinberg NV, Burova TV, et al. Cryostructuring of polymer systems. XXIX. Preparation and characterization of supermacroporous (spongy) agarose-based cryogels used as three-dimensional scaffolds for culturing insulin-producing cell aggregates. *J Appl Polym Sci* 2008;108(5):3046–62.
- [31] Fatima MP, Galaev IY, Mattiasson B. Production and properties of cryogels by radical polymerization. In: Mattiasson B, Kumar A, Galaev IY, editors. *Macroporous Polymers: Production Properties and Biotechnological/Biomedical Applications*. Boca Raton, FL: CRC Press; 2009. p. 23–47.
- [32] Jones I, James SE, Rubin P, Martin R. Upward migration of cultured autologous keratinocytes in Integra artificial skin: a preliminary report. *Wound Repair Regen* 2003;11:132–8.

- [33] Burke JF, Yannas IV, Quinby Jr WC, Bondoc CC, Jung WK. Successful use of a physiologically acceptable artificial skin in the treatment of extensive burn injury. *Ann Surg* 1981;194:413–28.
- [34] Ghosh K, Pan Z, Guan E, Ge S, Liu Y, Nakamura T, et al. Cell adaptation to a physiologically relevant ECM mimic with different viscoelastic properties. *Biomaterials* 2007;28:671–9.
- [35] Li S, Guan JL, Chien S. Biochemistry and biomechanics of cell motility. *Annu Rev Biomed Eng* 2005;7:105–50.
- [36] Grinnell F. Fibroblasts, myofibroblasts, and wound contraction. *J Cell Biol* 1994;124:401–4.
- [37] Grinnell F. Fibroblast mechanics in three-dimensional collagen matrices. *J Bodyw Mov Ther* 2008;12:191–3.
- [38] Rhee S. Fibroblasts in three dimensional matrices: cell migration and matrix remodeling. *Exp Mol Med* 2009;41:858–65.
- [39] Berrier AL, Yamada KM. Cell–matrix adhesion. *J Cell Physiol* 2007;213:565–73.
- [40] Evans ND, Oreffo RO, Healy E, Thurner PJ, Man YH. Epithelial mechanobiology, skin wound healing, and the stem cell niche. *J Mech Behav Biomed Mater* 2013;28:397–409.
- [41] Kondo T, Ishida Y. Molecular pathology of wound healing. *Forensic Sci Int* 2010;203:93–8.
- [42] Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. *Physiol Rev* 2003;83:835–70.
- [43] Kasoju N, Bhonde RR, Bora U. Preparation and characterization of Antheraea assama silk fibroin based novel non-woven scaffold for tissue engineering applications. *J Tissue Eng Regen Med* 2009;3:539–52.
- [44] Zhou J, Xu C, Wu G, Cao X, Zhang L, Zhai Z, et al. In vitro generation of osteochondral differentiation of human marrow mesenchymal stem cells in novel collagen–hydroxyapatite layered scaffolds. *Acta Biomater* 2011;7:3999–4006.
- [45] Kolacna L, Bakesova J, Varga F, Kostakova E, Planka L, Necas A, et al. Biochemical and biophysical aspects of collagen nanostructure in the extracellular matrix. *Physiol Res* 2007;56(Suppl. 1):S51–60.
- [46] Harrison CA, Heaton MJ, Layton CM, Mac Neil S. Use of an in vitro model of tissue-engineered human skin to study keratinocyte attachment and migration in the process of reepithelialization. *Wound Repair Regen* 2006;14:203–9.
- [47] Raja Sivamani K, Garcia MS, Isseroff RR. Wound re-epithelialization: modulating keratinocyte migration in wound healing. *Front Biosci* 2007;12:2849–68.
- [48] Gillitzer R, Goebeler M. Chemokines in cutaneous wound healing. *J Leukoc Biol* 2001;69:513–21.
- [49] Hu Y, Liang D, Li X, Liu HH, Zhang X, Zheng M, et al. The role of interleukin-1 in wound biology. Part II. In vivo and human translational studies. *Anesth Analg* 2010;111:1534–42.
- [50] Middelkoop E, van den Bogaerd AJ, Lamme EN, Hoekstra MJ, Brandsma K, Ulrich MM. Porcine wound models for skin substitution and burn treatment. *Biomaterials* 2004;25:1559–67.
- [51] Shevchenko RV, Sibbons PD, Sharpe JR, James SE. Use of a novel porcine collagen paste as a dermal substitute in full-thickness wounds. *Wound Repair Regen* 2008;16:198–207.
- [52] Dantzer E, Braye FM. Reconstructive surgery using an artificial dermis (Integra): results with 39 grafts. *Br J Plast Surg* 2001;54:659–64.
- [53] Eckes B, Nischt R, Krieg T. Cell-matrix interactions in dermal repair and scarring. *Fibrogenesis Tissue Repair* 2010;3:4.
- [54] Hirt SW, Aoki M, Demertzis S, Siclari F, Haverich A, Borst HG. Comparative in vivo study on the healing qualities of four different presealed vascular prostheses. *J Vasc Surg* 1993;17:538–45.