



## Development of biodegradable electrospun scaffolds for dermal replacement

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### ABSTRACT

Our objective is to develop a synthetic biodegradable replacement dermal substitute for tissue engineering of skin and oral mucosa. Our *in vivo* criteria were that candidate scaffolds should allow surrounding cells to migrate fully into the scaffolds, enabling vasculogenesis and remodelling without invoking a chronic inflammatory response. We examined a total of six experimental electrospun polymer scaffolds: (1) poly-L-lactide (PLLA); (2) PLLA + 10% oligolactide; (3) PLLA + rhodamine and (4–6) three poly(D,L)-lactide-co-glycolide (PLGA) random multiblock copolymers, with decreasing lactide/glycolide mole fractions (85:15, 75:25 and 50:50). These were evaluated for degradation *in vitro* up to 108 days and *in vivo* in adult male Wistar rats from 4 weeks to 12 months. *In vivo*, all scaffolds permitted good cellular penetration, with no adverse inflammatory response outside the scaffold margin and with no capsule formation around the periphery. The breakdown rate for each scaffold *in vitro* versus *in vivo* was similar, and an increase in the ratio of polyglycolide to polylactide correlated with an increase in breakdown rate, as expected. Scaffolds of PLLA were stable *in vivo* even after 12 months whereas scaffolds fabricated from PLGA 85:15 and 75:25 revealed a 50% loss of mass after 4 and 3 months, respectively. *In vitro* PLGA 85:15 and 75:25 scaffolds were able to support keratinocyte, fibroblast and endothelial cell growth and extracellular matrix production, with evidence of new collagen production after 7 days. In conclusion, the data supports the development of PLGA 85:15 and 75:25 electrospun polymer scaffolds as potential degradable biomaterials for dermal replacement.

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## 1. Introduction

Our aim is to develop biodegradable electrospun dermal scaffolds to replace donor human dermis or bovine collagen for 3D skin reconstruction for future clinical use. While there have been significant advances in expanding skin cells and delivering them to patients for the treatment of extensive skin loss due to burns injury or chronic wounds [1–5] one major challenge which remains is the production of 3D tissue engineered skin which has both an epidermal and a dermal layer. In the treatment of extensive full thickness burns where sufficient autologous skin grafts are not immediately available for the patient the dermal and epidermal layers are often replaced in two separate operations (as described

in Refs [6,7]). The majority of dermal replacement materials currently in use are based on scaffolds of bovine collagen (e.g. Integra [7] or human donor allografts [6]).

In the US, bovine collagen has been approved by the FDA for clinical use in tissue engineered products. For example Apligraf, which consists of bovine collagen and allogeneic keratinocytes and fibroblasts, is used for the treatment of chronic wounds [8]. Similarly Integra, which consists of bovine collagen covered by a silastic membrane to give a temporary epidermal type of barrier, is used as a dermal replacement material in the treatment of full thickness burns [7]. Bovine collagen, while extensively used and approved by the FDA, is not entirely risk free. In December 2003 there were press release reports of bovine spongiform encephalitis occurring in the US. Against this background avoiding the clinical use of bovine derived products should be considered in the future design of tissue engineered products.

Significant progress has also been made using donor skin which is decellularised and sterilised and then repopulated with

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the patient's own laboratory expanded keratinocytes and fibroblasts [5,9,12]. However, even when sourced from accredited skin banks, donor skin will always have a small but appreciable risk of viral transmission. There are also practical difficulties in sourcing donor skin and many patients and surgeons view donor skin as being acceptable only in extremely life threatening conditions. In many other conditions where it could benefit patients, e.g. contracture release and scar revision, it is not commonly used. In contrast, Integra is viewed as less of a risk, but requires a second operation to achieve epithelial cover with a thin split-thickness skin graft. A synthetic dermal matrix substitute which could also be seeded with the patients own keratinocytes and fibroblasts would decrease the risk of disease transmission for the patient and lead to a greater clinical uptake of tissue engineered skin and oral mucosa.

Of the various approaches for producing a dermal replacement, electrospun scaffolds are attractive for a number of reasons. Electrospinning can be used to produce a 3D open porous structure which approximates the structure of collagenous dermis. It is also possible to electrospin natural materials such as collagen and chitosan or synthetic materials such as poly-L-lactide (PLLA), polycaprolactone (PCL) and polyglycolic acid (PGA) [13]. Blends of both natural and synthetic materials too have been used [14]. The above materials have been approved by the FDA and used clinically for a number of years for a wide range of applications such as resorbable sutures, fracture plates and stents [15]. There is also considerable information on the degradation of PLLA and PGA which are hydrolysed into lactic and glycolic acids, respectively, and metabolised *in vivo* [16]. In general terms, copolymers of poly(D,L)-lactide-co-glycolide (PLGA) degrade at a faster rate than pure poly(D,L-lactide) (P(D,L)LA), but slower than pure PGA. Thus, degradation rate can be carefully controlled according to copolymer composition [13].

For the development of dermal replacement materials for clinical use in skin and oral mucosa tissue engineering, we suggest that these materials should degrade within a few months and be replaced by neo-dermis produced by the patients skin or oral mucosa cells. The degradation rate of a polymer scaffold and the manner of its degradation *in vivo* are the keys to achieve tissue reconstruction. As the environment encountered by a scaffold is chemically dynamic, non-equilibrium and multi-component a detailed examination of promising materials must be made. For electrospun scaffolds, fibre diameter, the interfibre space and the extent to which the scaffolds are infiltrated and vascularised influence the degradation rate *in vivo*. However, there is little work on the degradation rate of PLGA electrospun scaffolds *in vivo*. Accordingly, we report on the biodegradability of electrospun scaffolds in which we have varied the ratio of PGA to P(D,L)LA-co-PGA – referred to as PLGA polymers throughout the paper. We examined the breakdown of scaffolds under cell-free conditions *in vitro* and *in vivo* by subcutaneous implantation into rats. Data from this work were then used to select relevant scaffold materials to support both skin cell growth *in vitro* and the ability to synthesise production of extracellular matrix.

## 2. Materials and methods

### 2.1. Polymers

For this study four polymers were investigated; poly(L-lactide) (PLLA) ( $M_n \sim 99K$ ) (supplied by Fluka) and three poly(D,L-lactide-co-glycolide) (PLGA) copolymers with various ratios of lactide to glycolide (85:15 ( $M_w 50\text{--}75K$ ), 75:25 ( $M_w 66\text{--}107K$ ) and 50:50 ( $M_w 40\text{--}75K$ )). All the PLGA copolymers were from Sigma-Aldrich. The polymers were dissolved in dichloromethane (DCM) to form solutions of suitable viscosities for electrospinning; PLLA to form an 8% (wt/wt) solution, PLGA 85:15, 75:25 and 50:50 to form 24%, 20% and 25% (wt/wt) solutions, respectively. In addition, two variant materials were synthesised for studying the breakdown of scaffolds: (i) PLLA plus 1% (wt/wt) rhodamine and (ii) PLLA plus 10% (wt/wt) oligolactide (relative to the concentration of polymer) ( $M_w \sim 1500$ ). Both rhodamine and oligolactide were added to the PLLA solution prior to electrospinning.

### 2.2. Electrospinning conditions

Polymer solutions were loaded into 2 ml syringes fitted with blunt tipped stainless steel needles with an internal diameter of 0.8 mm (I&J Fisnar Inc.). The solutions were delivered at a constant feed rate of 3.5 ml/h using a programmable syringe pump (Aladdin 1000) and were electrospun horizontally with an accelerating voltage of 15 kV supplied by a high voltage power supply (Brandenburg, Alpha series III). Fibrous mats were collected on aluminium foil sheets (18 cm by 16 cm) wrapped around an earthed aluminium rotating collector (rotating at 300 rpm) 20 cm from the tip of the needle. Jet formation/stability was assisted by means of an aluminium focusing ring at a voltage of 15 kV, 5 mm behind the tip of the needle.

### 2.3. Implantation of scaffolds in animals

Adult male Wistar rats, 12 weeks of age were surgically implanted with scaffolds under general anaesthesia. Two implants were placed subcutaneously on the back of each animal, one either side of the midline. Four layers of scaffolds were placed together to achieve a thickness of 0.5–0.8 mm and then cut into 5 mm diameter discs using a tissue punch. Implants were sterilised in 70% ethanol and then washed with sterile PBS immediately prior to implantation.

### 2.4. Ex vivo preparation of scaffolds for histology and SEM and immunostaining

Animals were sacrificed at various time points using a humane method of euthanasia (cervical dislocation); the scaffolds were dissected from the rat to include surrounding soft tissue; Table 1 shows the time points examined and number of animals sacrificed. One sample per animal was placed in formalin prior to tissue processing; another sample was frozen and stored at  $-80^{\circ}\text{C}$  prior to processing for scanning electron microscopy. Formalin fixed samples were then processed conventionally to produce 4  $\mu\text{m}$  paraffin sections and then stained with haematoxylin and eosin. Histological sections were examined blind by a consultant histopathologist. For SEM, specimens were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 3 h at  $4^{\circ}\text{C}$ . Secondary fixation was carried out in 2% osmium tetroxide (aq) for 1 h. Samples were dehydrated in ascending grades of alcohol, freeze dried, bisected and mounted on 12.5 mm stubs. The samples were sputter coated with approximately 25 nm of gold and then examined using a scanning electron microscope (Philips/FEI XL-20 SEM) at an accelerating voltage of 10–15 kV.

### 2.5. Immunostaining of explanted scaffolds

The following primary antibodies were used: goat polyclonal anti-rat CD31 (Santa Cruz) which identifies endothelial cells; mouse monoclonal anti-rat CD68 (AbCam) which identifies macrophages and mouse anti-rat CD45RC (BD Pharmingen) which identifies lymphocytes. Formalin fixed samples were cut at 5  $\mu\text{m}$ , dewaxed, rehydrated and antigen retrieval performed for CD45 and CD31 by microwaving in 500 ml Tris and EDTA for 3 min. Sections were then incubated in 5% H<sub>2</sub>O<sub>2</sub> in methanol to block endogenous peroxidase and incubated with the primary antibodies with normal rabbit or horse serum for 1 h at room temperature at the following dilutions: CD68 1:300, CD45RC 1:150 and CD31 1:300. The sections were then washed in phosphate buffered saline and incubated with biotinylated

**Table 1**

Time course of ex vivo examination of scaffolds implanted in rats

Polymer	2 Weeks	4 Weeks	7 Weeks	3 Months	4 Months	5 Months	6 Months	9 Months	1 Year
PLLA	3	3							
PLLA + rhodamine	3	3							
PLLA + oligolactides	3	3	3			3			
PLGA 85:15	3	3	3	3	3	3			
PLGA 75:25	2	3	3				3		
PLGA 50:50	3	3	4					3	3

The table indicates the number of animals sacrificed at each time point.

secondary stage antibody (rabbit anti goat for CD31, and mouse anti-rat for CD68 and CD45RC) for 30 min and the reaction product was visualised by DAB. Sections were counterstained using Harris haematoxylin.

## 2.6. Assessment of fibre diameter

Three micrographs of random fields of view within the implant body were taken at  $\times 40$  magnification. The diameter of residual fibres within the sections was measured by capturing digital images, calibrated using a graticule and determined using software (SPOT 4.0.6, supplier).

## 2.7. Detection of fibre diameter by fluorescence microscopy

Scaffolds containing 1% rhodamine 4',6-diamidino-2-phenylindole (DAPI, 3 mM) were incubated with 4  $\mu$ m paraffin sections of PLLA + rhodamine for 1 h to stain nuclei. Sections were then analysed at  $\lambda_{\text{ex}} = 358 \text{ nm}/\lambda_{\text{em}} = 461 \text{ nm}$  for DAPI and at  $\lambda_{\text{ex}} = 580 \text{ nm}/\lambda_{\text{em}} = 650 \text{ nm}$  for rhodamine using a Leica DM-IRB inverted epifluorescence microscope.

## 2.8. MALDI

The use of MALDI ToF mass spectrometry to detect electrospun fibre breakdown using PLLA fibres dosed with 10% lactide oligomers ( $M_w \sim 1500$ ) will be reported elsewhere. We noted that the inclusion of these oligomers did not affect the spinning of the scaffolds, their appearance or the *in vivo* response to these scaffolds in any of the assessments reported in this manuscript.

## 2.9. In vitro degradation of scaffolds

Scaffolds were cut into 8 mm or 18 mm diameter discs using a tissue or biopsy punch. Implants were sterilised in 70% ethanol for 10 min and then washed three times with sterile Ringers solution immediately prior to study. Scaffolds were immersed in either 0.5 ml (for 8 mm samples) or 1.5 ml (for 18 mm) of Ringers solution and incubated at 37 °C. Ringers solution was initially replaced everyday for 4 days and then every 4 days thereafter up to 108 days. Samples were analysed by phase contrast microscopy and by SEM. Solutions were stored frozen for later chemical analysis (to be reported elsewhere).

## 2.10. Culture of normal human keratinocytes and fibroblasts

Normal human keratinocytes and fibroblasts were isolated and cultured as described previously [11]. Passage 1 keratinocytes and passage 3–7 fibroblasts were used. Keratinocytes were cultured in Green's medium: Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium in a 3:1 (v/v) ratio supplemented with 10% (v/v) foetal calf serum (FCS), 0.1  $\mu$ m cholera toxin, 10 ng/ml of epidermal growth factor (EGF), 0.4  $\mu$ g/ml hydrocortisone, 0.18 mM adenine, 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, 2 mM glutamine, 0.2  $\mu$ m triiodothyronine, 0.625  $\mu$ g/ml amphotericin B, 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin. Green's medium without serum was also used in some experiments. Fibroblasts were cultured in DMEM supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 0.625  $\mu$ g/ml amphotericin B, 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin. Serum-free DMEM was used in some experiments. A microvascular endothelial cell line (HuDMEC) was cultured as described previously [12] in EBM-2 medium with the appropriate microvascular additives (Cambrex Bio Science Walkersville, Walkersville, MD).

## 2.11. Preparation of tissue engineered skin constructs with PLGA scaffolds

Reconstructed skin was prepared as follows. 2 cm diameter scaffolds were sterilised in 70% ethanol for 10 min and washed three times in PBS and twice in medium. Scaffolds (PLGA 75:25 and 85:15) were then placed in 6-well plates and stainless steel rings with an internal diameter of 1 cm<sup>2</sup> were applied with pressure to one side of the electrospun scaffolds. Following this, cells were seeded to achieve a total of  $3 \times 10^5$  cells per scaffold. Thus for fibroblasts or keratinocytes as single cultures  $3 \times 10^5$  cells were used and for co-cultures  $1.5 \times 10^5$  each of fibroblasts and of keratinocytes were used to give a 1:1 ratio of cells and  $3 \times 10^5$  cells in total. Cells were seeded in a volume of 300  $\mu$ l of medium, and added to the upper surface of the constructs prior to being submerged for 18 h before being raised to an air-liquid interface on stainless steel grids (for keratinocytes and co-culture constructs). Constructs were incubated for either 7 or 14 days in Green's medium with or without serum. Culture medium was replenished twice a week.

## 2.12. Cell viability of skin composites by MTT-ESTA

Viable cell density was then assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) (Sigma-Aldrich, St Louis MO). Briefly, samples were washed three times in PBS and then incubated with MTT solution (0.5 mg/ml MTT in PBS, 1 ml per well of 24-well plate) for 45 min at 37 °C and in a 95% air/5% CO<sub>2</sub> environment. Intracellular dehydrogenase activity reduces MTT to a purple-coloured formazan salt. After 45 min, the unreacted solution was aspirated and the insoluble intracellular formazan product was solubilised and released from cells by

adding iso-propanol (0.5 ml per well of 24-well plate or 1 ml/cm<sup>2</sup> cultured tissue) and incubated for 10 min. The optical density at 540 nm was then measured using a plate reader spectrophotometer.

## 2.13. Quantification of collagen deposition in scaffolds by picosirius red staining

Quantification of collagen deposition on scaffold fibres was performed using a slight modification of the method described [17]. Briefly, constructs were grown for 7 or 14 days then washed three times in PBS and fixed in 4% formalin. Following three washes with PBS the constructs were stained for collagen deposition in a 0.1% solution of Sirius Red F3B (C.I. 35780, Direct Red 80, Sigma-Aldrich) in saturated picric acid for 18 h. As collagen is a basic protein, the sulphonate groups of the dye interact at low pH with the amino groups of the lysine and hydroxylysine, and the guanidine groups of arginine. Afterwards, the constructs were washed with tap-water until no more red colour was eluted. Phase contrast and polarised light digital micrographs were then taken. For a quantitative analysis, the stain from scaffolds was eluted with 500  $\mu$ l of a destain solution (0.2 M NaOH/methanol 1:1) for 15 min. The optical density at 490 nm was then measured using a plate reader spectrophotometer.

## 2.14. Assessment of electrospun construct contraction in vitro

Images of the scaffolds after MTT-ESTA and picosirius red staining were taken in 12-well plates alongside a scale bar to calibrate the image. The margin of the construct was traced and the diameter of the circular scaffolds was measured. The original diameter of the scaffolds (2 cm) was designated as 100% and all changes in diameter were expressed relative to this original measurement by image analysis over several days so that scaffold area versus time could be measured and results expressed relative to the original diameter of the scaffold.

## 2.15. Statistics

Student's unpaired t-test was used to compare fibre diameters at different times post implantation and also to compare the viability and collagen production of cells on the different scaffolds.

## 3. Results

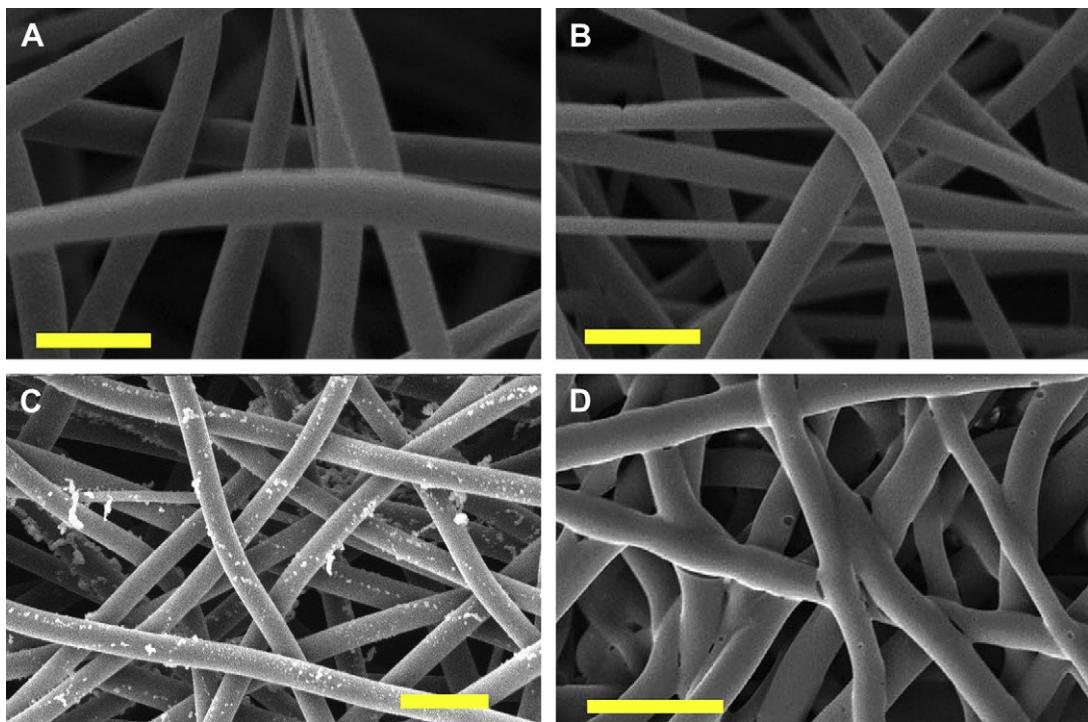
### 3.1. Physical characterisation and in vitro investigation of the electrospun scaffolds

#### 3.1.1. Electrospinning of scaffolds

Fig. 1 shows scanning electron micrographs of scaffolds prior to implantation. Electrospun mats were 0.1–0.2 mm in thickness and all scaffolds exhibited similar surface characteristics when viewed under SEM. Polymer electrospinning produced non-woven randomly arranged fibres with an average diameter of  $2.1 \pm 1.2 \mu\text{m}$  for PLLA (mean  $\pm$  SD),  $2.9 \pm 1.3 \mu\text{m}$  for PLLA plus lactide oligomers,  $2.7 \pm 0.5 \mu\text{m}$  for PLGA 85:15 scaffolds,  $4.5 \pm 1.4 \mu\text{m}$  for PLGA 75:25, and  $4.3 \pm 1.5 \mu\text{m}$  for PLGA 50:50 as summarised in Table 2. The pore size of all fibre mats ranged from 5 to 10  $\mu\text{m}$ , with occasionally pores of size greater than 20  $\mu\text{m}$ . From these it can be seen that the fibre diameter increased (essentially doubled) as the proportion of PGA increased to 25% or 50%. Thus these relatively uniform, randomly orientated scaffolds displayed initial fibre diameters between 2 and 5  $\mu\text{m}$ , with greater than 90% porosity. There was no obvious difference in physical appearance between scaffolds comprised of PLLA, PLLA plus oligomers or PLGA.

#### 3.1.2. In vitro degradation of scaffolds

The polymer scaffolds were then studied for their ability to degrade *in vitro*. Fig. 2 shows brightfield light microscopy images and Fig. 3 shows SEM images for scaffolds as they degraded over time in Ringers solution at 37 °C in a 95% air 5% CO<sub>2</sub> environment. The PLGA 85:15 scaffold appeared not to undergo any significant degradation over 108 days, whereas the PLGA 75:25 scaffold displayed no immediate physical changes until 80 days, but thereafter individual fibres were observed to merge together until day 108 when no visible fibres remained. In contrast PLGA 50:50 scaffold underwent immediate and rapid degradation as seen by the merging of individual fibres by day 8, which continued until day 24 when there were no visible fibres.



**Fig. 1.** SEM micrographs of electrospun PLLA (A), PLGA 85:15 (B), 75:25 (C), and 50:50 (D) scaffolds prior to implantation. The average fibre diameters were around 3 µm – the details for each are summarised in Table 2. 10 µm.

### 3.2. Investigation of the behaviour of cells in the scaffolds in vitro

#### 3.2.1. In vitro culture of primary human keratinocytes, fibroblasts and endothelial cells in PLGA electrospun scaffolds

PLGA 75:25 and 85:15 scaffolds were subsequently examined in further detail for their ability to support keratinocyte, fibroblast

and endothelial cell growth *in vitro*, based on their suitable degradation rates determined above (Figs. 4–8).

Figs. 4 and 5 show that the overall viability levels (assessed by MTT) after one week in serum-free single culture of fibroblasts or keratinocytes or co-culture of the two were not significantly different whether cultured in the presence or absence of serum (comparing Fig. 5A with C and Fig. 5B with D with one exception as noted in Fig. 5) where the metabolic activity in the absence of serum was significantly less without serum for keratinocytes after 14 days of culture. Interestingly the metabolic activity of the cells after 14 days was also not significantly greater than that seen at 7 days.

Fig. 5 also shows that metabolic activity in co-cultures of fibroblasts and keratinocytes was slightly greater than in keratinocytes alone (reaching significance in Fig. 5A) and significantly greater than in single cultures of fibroblasts in most comparison as shown in Fig. 5A–C.

Endothelial cells proliferated well on scaffolds in monoculture as shown in Fig. 6 indicating a significant increase in total cellular viability between days 7 and 14.

Fig. 7 shows SEM images of keratinocytes and fibroblasts co-cultured on a 75:25 PLGA scaffold for 10 days at an air–liquid interface indicating that there was a continuous layer of cells well attached to the upper surface of the scaffold while the fibres throughout the scaffold were thickly surrounded by cells which penetrated throughout the scaffold.

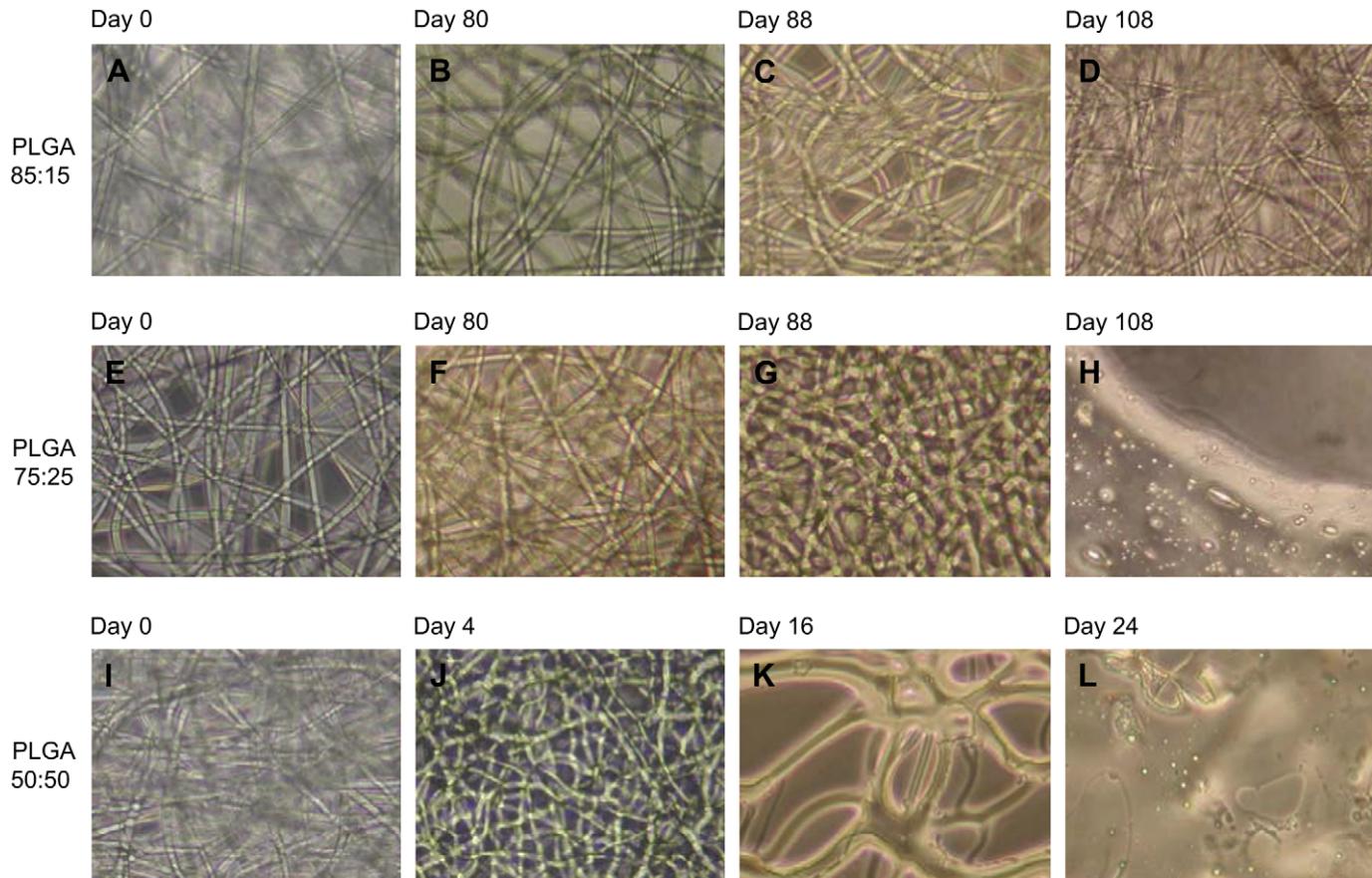
#### 3.2.2. Production of collagen by skin cells on PLGA 75:25 and 85:15 scaffolds

Skin cells grown on scaffold fibres (PLGA 75:25 and 85:15) showed intense staining with picrosirius red suggesting collagen synthesis (as shown in Fig. 4C and D and quantified in Fig. 8). Picrosirius red staining alone is not specific for collagen as it stains all basic proteins. However, the scaffolds were birefringent when the same material was examined under polarising light microscopy, indicative of the oriented molecular structure of collagen [17]. Collagen deposition was observed to be significantly greater in

**Table 2**  
Breakdown of electrospun scaffolds when implanted in rats

Polymer	Time point	Scaffold mass	Fibre diameter (µm)
PLLA + oligomers	Pre-implantation	++++	2.9 ± 1.4
	4 weeks	++++	2.9 ± 1.0
	7 weeks	++++	2.5 ± 0.9
	3 months	++++	2.2 ± 0.9
	5 months	++++	2.1 ± 1.0
	9 months	++++	2.1 ± 0.9
	12 months	++++	1.9 ± 0.8
P(D,L)LA-co-PGA 85:15	Pre-implantation	++++	2.7 ± 0.5
	4 weeks	++++	3.5 ± 1.4*
	7 weeks	++++	3.2 ± 1.5
	3 months	+++	2.2 ± 1.2
	4 months	+	1.6 ± 1.1
	5 months	+/-	N/A
	6 months	0	N/A
P(D,L)LA-co-PGA 75:25	Pre-implantation	++++	4.5 ± 1.4
	4 weeks	++++	5.9 ± 1.8*
	7 weeks	++++	5.3 ± 1.6
	3 months	++	3.5 ± 1.6
P(D,L)LA-co-PGA 50:50	Pre-implantation	++++	4.3 ± 1.5
	2 weeks	+++	6.5 ± 4.1*
	4 weeks	++	N/A
	7 weeks	++	N/A

The approximate mass of the explanted scaffolds was assessed as follows; implants of 75–100% of the original mass (taken to be ‘++++’) are indicated as ‘++++’; 50–75% of the original mass as ‘+++’; 25–50% of the original mass as ‘++’ and 0–25% of the original mass as ‘+’; no implant detected is indicated as ‘0’; this was assessed for two implants each for three animals and the mass given is the mean of all six implants; Fig. 4 shows examples of the explanted masses for these scaffolds; \*Indicates values significantly greater than the control fibre diameter.



**Fig. 2.** Light microscope images ( $\times 40$ ) of PLGA 85:15 (A–D), 75:25 (E–H), and 50:50 (I–L) electrospun polymers after immersion in Ringers solution at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  for various lengths of time indicated in the figure. As can be seen as the percentage of PGA increased the polymer fibres lost integrity faster.

co-cultures of cells and to increase significantly from week 1 to week 2 in most cases as detailed in Fig. 8. In a few comparisons (fibroblasts alone and fibroblasts plus keratinocytes grown on the PLGA 75:25 scaffold at 14 days) there was slightly less collagen produced when cells were cultured in serum-free media compared to the culture with serum. Although these results were statistically significant the overall picture in the absence of serum was very similar to that in the presence of serum.

### 3.2.3. Contraction of PLGA 75:25 and 85:15 electrospun scaffolds by skin cells

Both the PLGA 75:25 and 85:15 scaffolds are significantly contracted by fibroblasts but not by keratinocytes, as illustrated in Fig. 4 and detailed in Table 3. Contraction was also generally higher when cells were cultured in serum containing medium than in serum-free medium (Table 3). In co-cultures of keratinocytes and fibroblasts there was significantly less contraction seen than with fibroblasts alone, suggesting a suppression of fibroblast-induced contraction by the keratinocytes.

### 3.3. In vivo degradation of electrospun scaffolds upon subcutaneous implantation

#### 3.3.1. In vivo response of rats to scaffolds

All rats survived. There were no infections or loss of animals and no obvious response of the rats to the scaffolds that could be detected in terms of the animals' behaviour, weight gain or skin healing. All scaffolds remained in the connective tissue beneath the skeletal muscle layer and there was little or no evidence of disruption to surrounding structures at any time point (Fig. 9). There

was no macroscopic evidence of local or systemic upset; specifically there was no erythema or swelling at the sites of implantation of the scaffolds. There was no evidence of an acute inflammatory response.

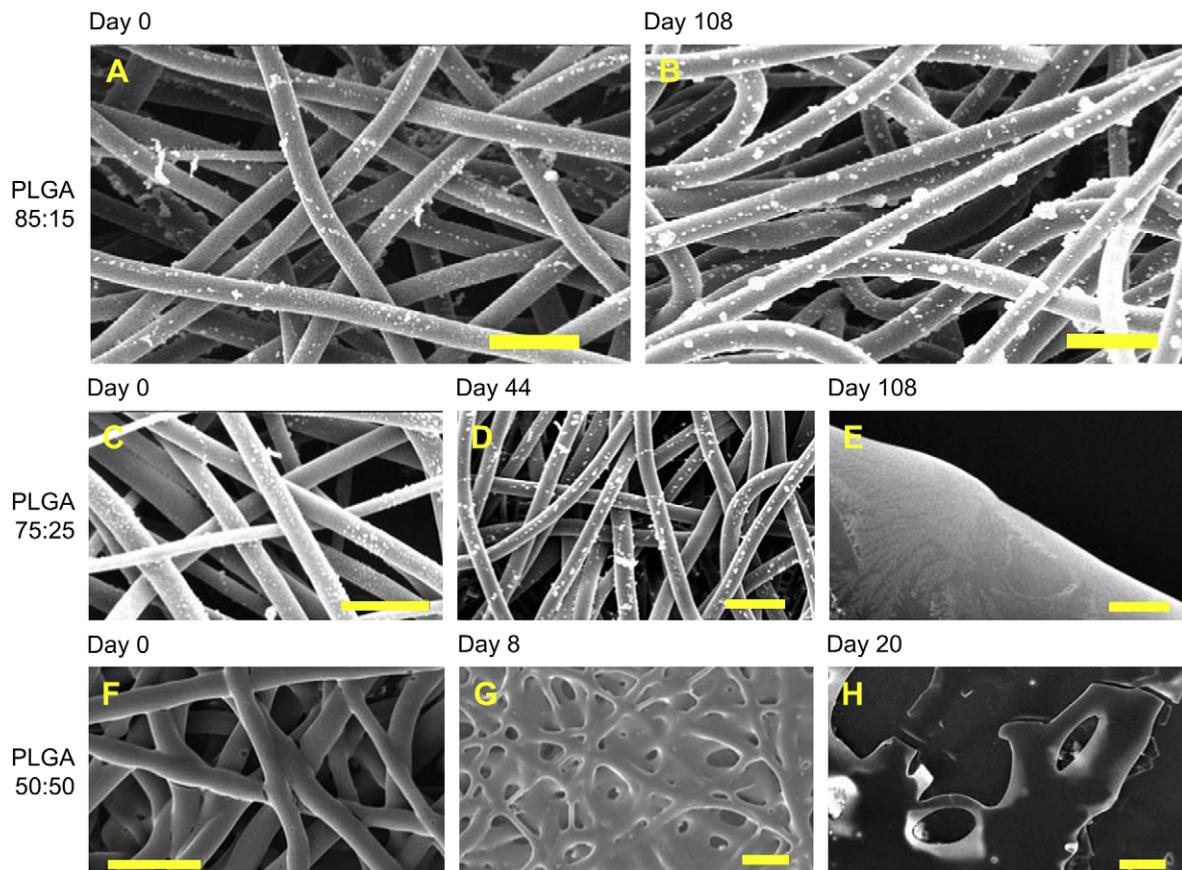
#### 3.3.2. Investigation of the in vivo degradation of the implanted scaffolds

Figs. 9–11 and Table 2 summarise the loss of implant mass and the change in fibre diameter with time seen in the explanted scaffolds. Individual scaffold fibres were clearly visible in the PLLA and PLLA plus oligomer scaffolds when viewed in both light microscopy (Figs. 9 and 11) and SEM (Fig. 10). When viewed under polarised light, fibres were birefringent and they appeared in cross section as ovoid fibres or occasionally as long strands. SEM analysis showed a close interaction between electrospun fibres and the surrounding cellular mass (Fig. 11). PLLA fibres had a degree of porosity on the surface of the fibres.

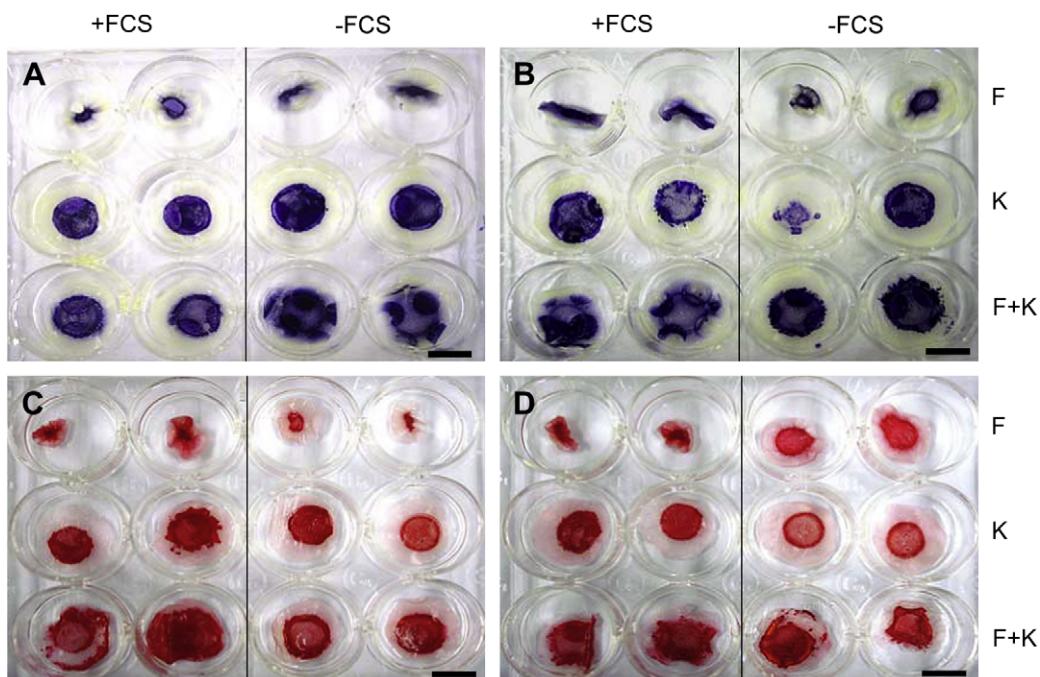
PLGA scaffolds did not polarise after processing for H&E and dissolved during the tissue processing (which uses organic solvents to remove the wax from sections) leaving empty spaces. However, fibres were clearly visible when viewed by SEM.

PLGA 50:50 fibres coalesced, with individual fibres merging into clumps of polymer. The latter was not visible in H&E sections but the spaces occupied by the polymer appeared as cell-free areas (Fig. 9), while in SEM, balls of polymer are clearly visible and contrast strongly with the surrounding tissue (Fig. 10).

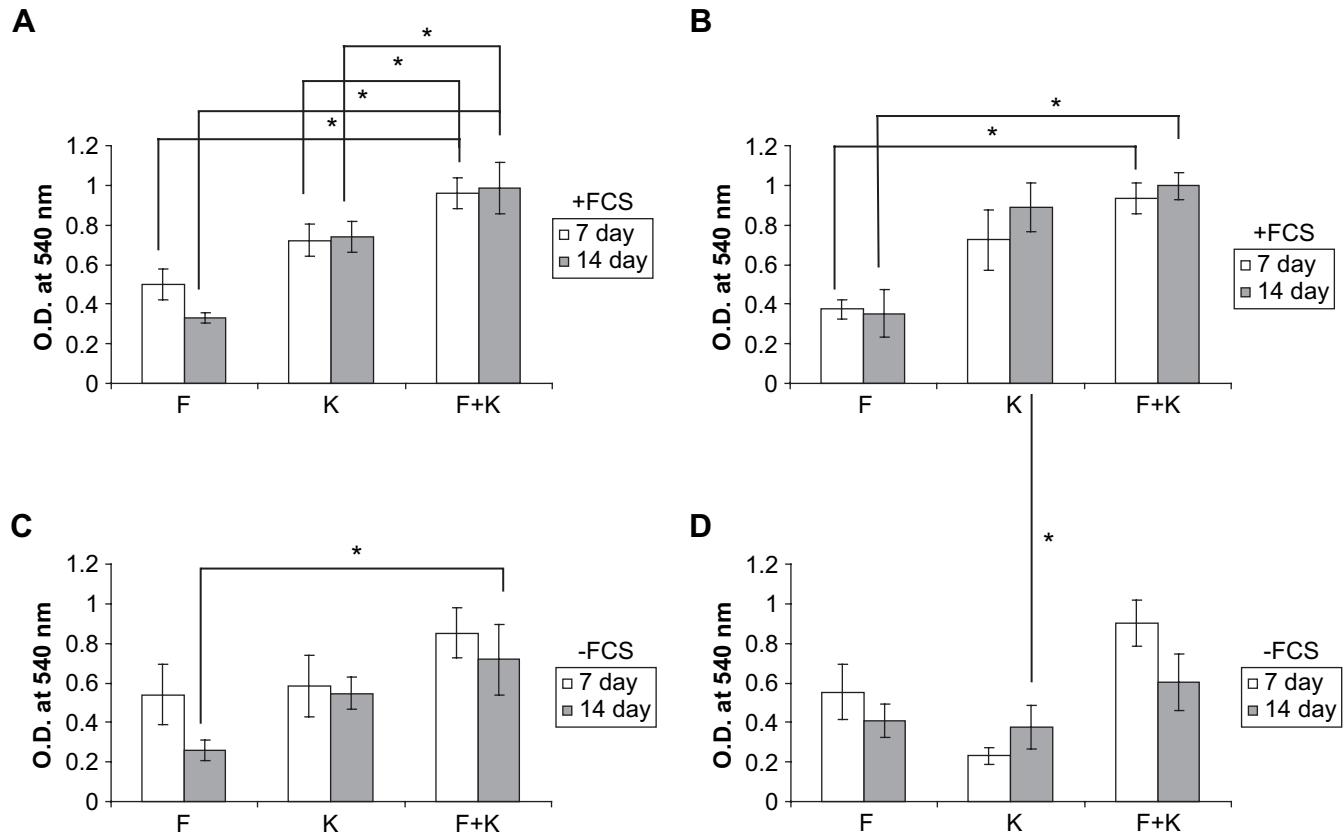
PLLA implants were detectable after one year, with the gross size of the implants very similar to that seen at 4 weeks (Fig. 9 and Table 2). Fibres of PLLA plus oligomers showed a very gradual reduction in size which was first detected at 7 weeks and continued



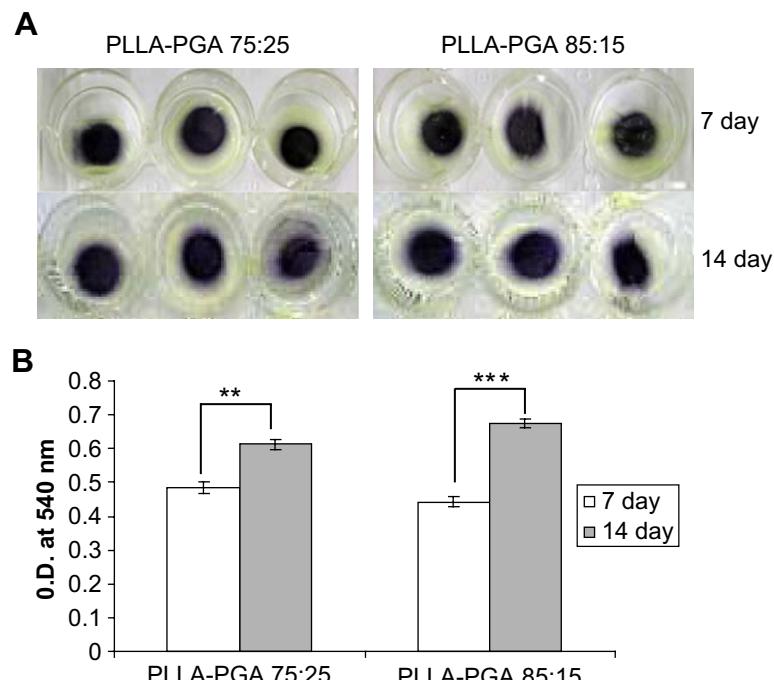
**Fig. 3.** SEM micrographs of electrospun PLGA 85:15, (A + B), 75:25 (C–E), and 50:50 (F–H) after being immersed in Ringers solution at 37 °C in 5% CO<sub>2</sub> for various lengths of time as indicated in the figure. Scale bar = 10 μm.



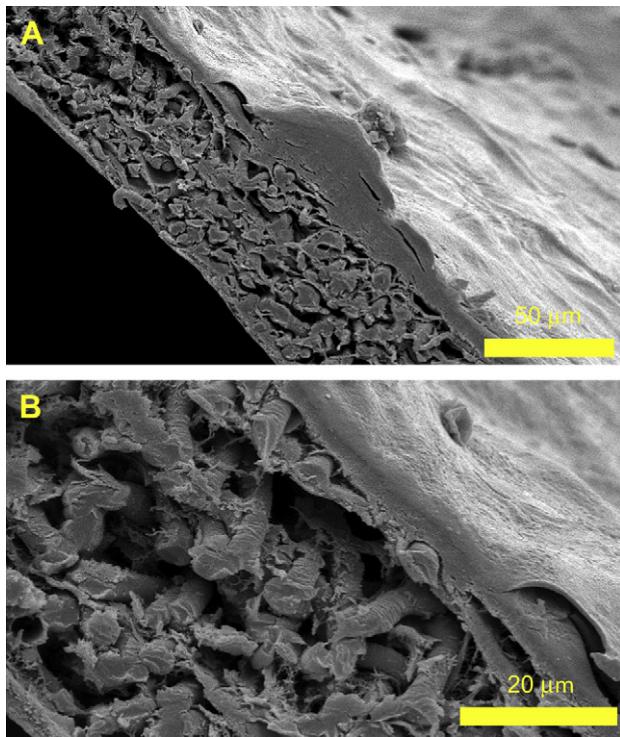
**Fig. 4.** Digital photographs of cellular viability (MTT) (A + B) and picrosirius red (C + D) staining of cells cultured on PLGA scaffolds in serum containing (+FCS) and serum-free (-FCS) conditions. Fibroblasts (F), keratinocytes (K) and co-cultures of both fibroblasts and keratinocytes (F + K) were seeded onto electrospun scaffolds of PLGA 75:25 (A + C) and PLGA 85:15 (B + D) and cultured for 14 days. Scale bar = 1 cm. (Quantitative results from these experiments are shown in Figs. 5 and 6 (for metabolic activity) and Fig. 8 (for collagen production).)



**Fig. 5.** Metabolic viability of single cultures and co-cultures of human dermal keratinocytes (K) and fibroblasts (F) in PLGA 75:25 (A and C) and PLGA 85:15 (B and D) electrospun scaffolds, under serum-free conditions (C and D) or serum containing conditions (A and B). Time points of 7 days (open columns) and 14 days (solid columns) were investigated. Values represent mean values  $\pm$  SEM ( $n = 3$ ). Values differing significantly from each other are linked by lines and the degree of significance is depicted as \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .



**Fig. 6.** Metabolic viability of monocultures of HuDMECs in PLGA 75:25 and PLGA 85:15 electrospun scaffolds. Digital photograph (A) and graph of cellular viability (B). Time points of 7 days (open columns) and 14 days (solid columns) were investigated. Values represent mean values  $\pm$  SEM ( $n = 3$ ). Values differing significantly from each other are linked by lines and the degree of significance is depicted as \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .



**Fig. 7.** SEM micrographs of keratinocytes and fibroblasts co-cultured on a PLGA 75:25 scaffold for 7 days. (A) shows SEM at  $\times 500$  magnification and B shows a section of this at  $\times 1500$  magnification. The upper surface shows a continuous layer of interconnected cells while the fibres themselves are closely covered by cells.

throughout the period of the study until at 12 months the average fibre diameter was 65% of the initial diameter. However, there was no overall loss of implant mass over the year of study.

PLGA 85:15 scaffolds underwent a progressive reduction in the size of the implant with approximately 50% loss of mass between 3 and 4 months and by 6 months only traces were found in two animals. This is shown for one of these scaffolds in Fig. 11J–M.

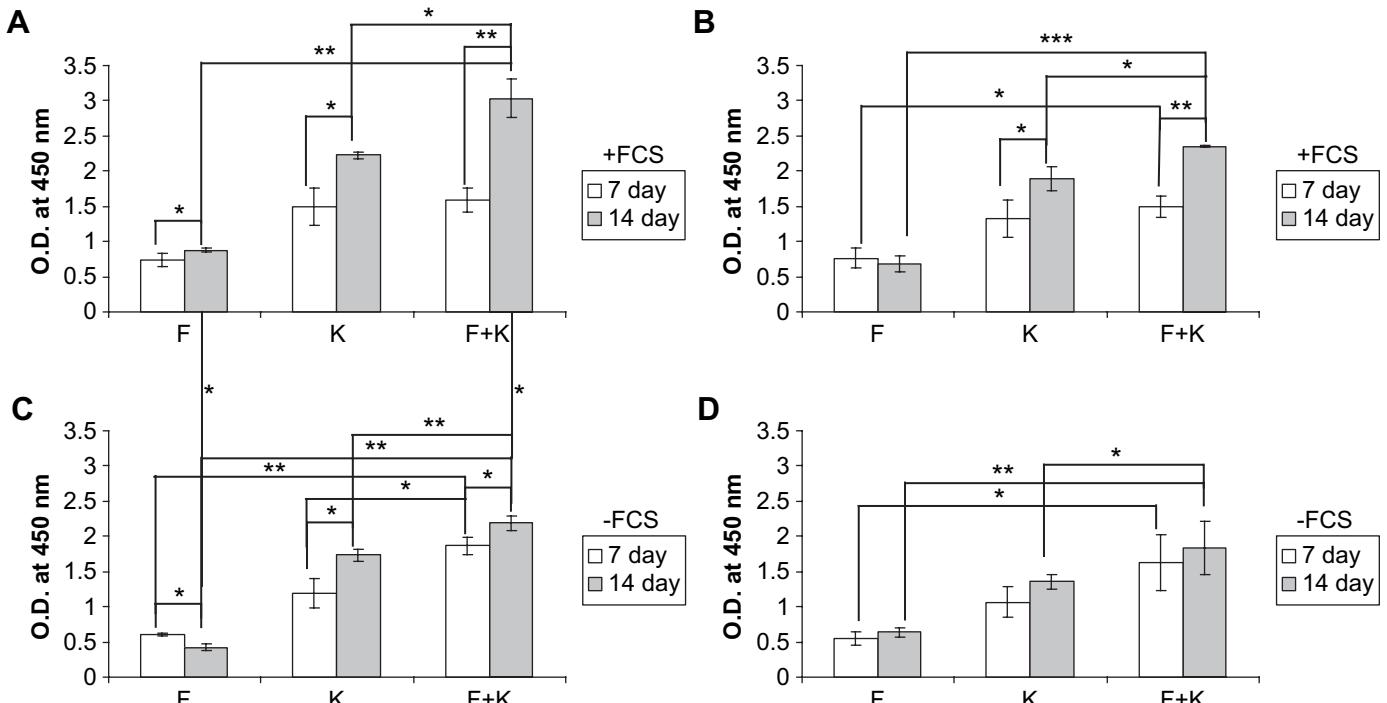
PLGA 75:25 scaffolds showed a 50% reduction in the size of the implants by 3 months and PLGA 50:50 scaffolds showed a 50% reduction in implant mass by 4 weeks. The reduction in implant mass with this particular scaffold was evident after only 2 weeks and relatively little remained at 7 weeks (the longest time point studied).

PLGA scaffolds initially increased in average fibre diameter post implantation. Fibres of PLGA 85:15 and 75:25 significantly increased in size by the 4-week time point and fibres of the 50:50 scaffold increased by the 2-week time point. Scaffold fibres then underwent a subsequent gradual decrease in average fibre diameter with the PLGA 85:15 and 75:25's average diameters being less than pre-implantation width after 3 months. As PLGA 50:50 fibres underwent coalescence within 4 weeks, it was impossible to measure fibre diameter beyond 2 weeks. Results are summarised in Table 2.

The use of rhodamine impregnated fibres to assess fibre location and breakdown was investigated but after 4 weeks of implantation all of the rhodamine had leached out of fibres into surrounding tissue. This was judged not useful for helping visualise scaffolds *in vivo* and was not studied further (results not shown).

### 3.3.3. Investigation of the cellular response to the implanted scaffolds

There was some variability in the degree of cellular penetration of granulation tissue and other cell types into the scaffolds. None of the PLLA scaffolds (0/3) had been completely penetrated by cells



**Fig. 8.** Collagen deposition measured using picrosirius red staining of single cultures and co-cultures of human dermal keratinocytes (K) and fibroblasts (F) in PLGA 75:25 (A and C) and PLGA 85:15 (B and D) electrospun scaffolds, under serum-free conditions (C and D) or serum containing conditions (A and B). Time points of 7 days (open columns) and 14 days (solid columns) were investigated. Values represent mean values  $\pm$  SEM ( $n=3$ ). Values differing significantly from each other are linked by lines and the degree of significance is depicted as \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

**Table 3***In vitro* contraction of electrospun scaffolds seeded with human skin cells<sup>a</sup>

% Contraction of original scaffold area (mean ± SEM, n = 3)								
PLLA:PGA				PLLA:PGA				
75:25		85:15		Serum		Serum-free		
Serum		Serum-free		Serum		Serum-free		
Week 1		Week 1		Week 1		Week 1		
Week 2		Week 2		Week 2		Week 2		
F	46.8 ± 2.9	54.2 ± 4.2	28.2 ± 10.9	50.0 ± 1.4	59.2 ± 3.6	62.5 ± 3.4	29.8 ± 7.2	38.8 ± 8.6
K	17.7 ± 11.8	1.3 ± 1.3	9.3 ± 9.3	2.0 ± 2.0	5.8 ± 4.6	0 ± 0	0 ± 0	0 ± 0
F + K	14.4 ± 11.0	3.5 ± 3.5	0 ± 0	0 ± 0	0 ± 0	4.2 ± 4.2	0 ± 0	6.1 ± 3.4
Statistical analysis <sup>b</sup>								
F:K	**	***	*	***	***	***	**	***
F:F + K	*	***	*	***	***	***	**	**
	Week 1		Week 2		Week 1		Week 2	
F – S:SF <sup>c</sup>	*	**	—	—	—	—	**	—

<sup>a</sup> Scaffold disks of 2 cm diameter were seeded with fibroblasts (F), keratinocytes (K) or co-cultures of both (F + K) in serum containing medium or serum-free (see materials and methods) and incubated for 1 or 2 weeks. Scaffold contraction is expressed as a percentage of the original scaffold area (n = 3).

<sup>b</sup> Statistical analysis was performed using Student's unpaired t-test. (n = 6 independent experiments in triplicate wells), \*p ≥ 0.05, \*\*p ≥ 0.01, \*\*\*p ≥ 0.001.

<sup>c</sup> Serum:serum-free.

after 4 weeks, however, all (3/3) of the PLGA 85:15, 75:25 and 50:50 scaffolds were completely penetrated as were 2 of the 3 PLLA + oligolactide scaffolds at 4 weeks. After 7 weeks all the scaffolds (except for one PLGA scaffold out of the 6 examined) were completely penetrated with cells.

At the gross histology level (H&E), as shown in Fig. 9, all samples looked very similar initially but clearly while PLLA scaffolds survived for more than 12 months, scaffolds with increasing concentrations of PGA disappeared with time. All scaffolds were able to support cellular in-growth (Fig. 9), becoming infiltrated by granulation tissue including fibroblasts and endothelial cells (as illustrated in Fig. 11 for scaffold PLGA 85:15).

Vascularisation could be clearly seen on H&E stained sections as illustrated in Fig. 11A, D and G for PLGA 85:15 scaffolds explanted after 4 weeks, 7 weeks and 3 months. This was confirmed by specific staining of endothelial cells with CD31 (results not shown).

The scaffold fibres also attracted numerous macrophages as identified by staining with CD68 and promoted the formation of foreign body type multinucleate giant cells, which were closely opposed to the fibres, and in some cases, completely surrounded them as shown in Fig. 11 (C,F,I and M). Even at the late stages, macrophages were present associated with the residual fibres.

There was little evidence of an immune response and only occasional lymphocytes were seen by immunostaining for CD45. At the early stages lymphocytes were scattered throughout but by three months occasional foci were seen (Fig. 11B, E, M and L) although large areas contained no lymphocytes at all. There was no evidence of plasma cells indicative of immunoglobulin production.

Looking at the eventual fate of the scaffolds extensive sectioning revealed very small fragments of material remaining at 6 months as illustrated in Fig. 10J and K. Fig. 11 shows only very occasional lymphocytes (L) but numerous macrophages (M) associated with these last few scraps of scaffold at 6 months.

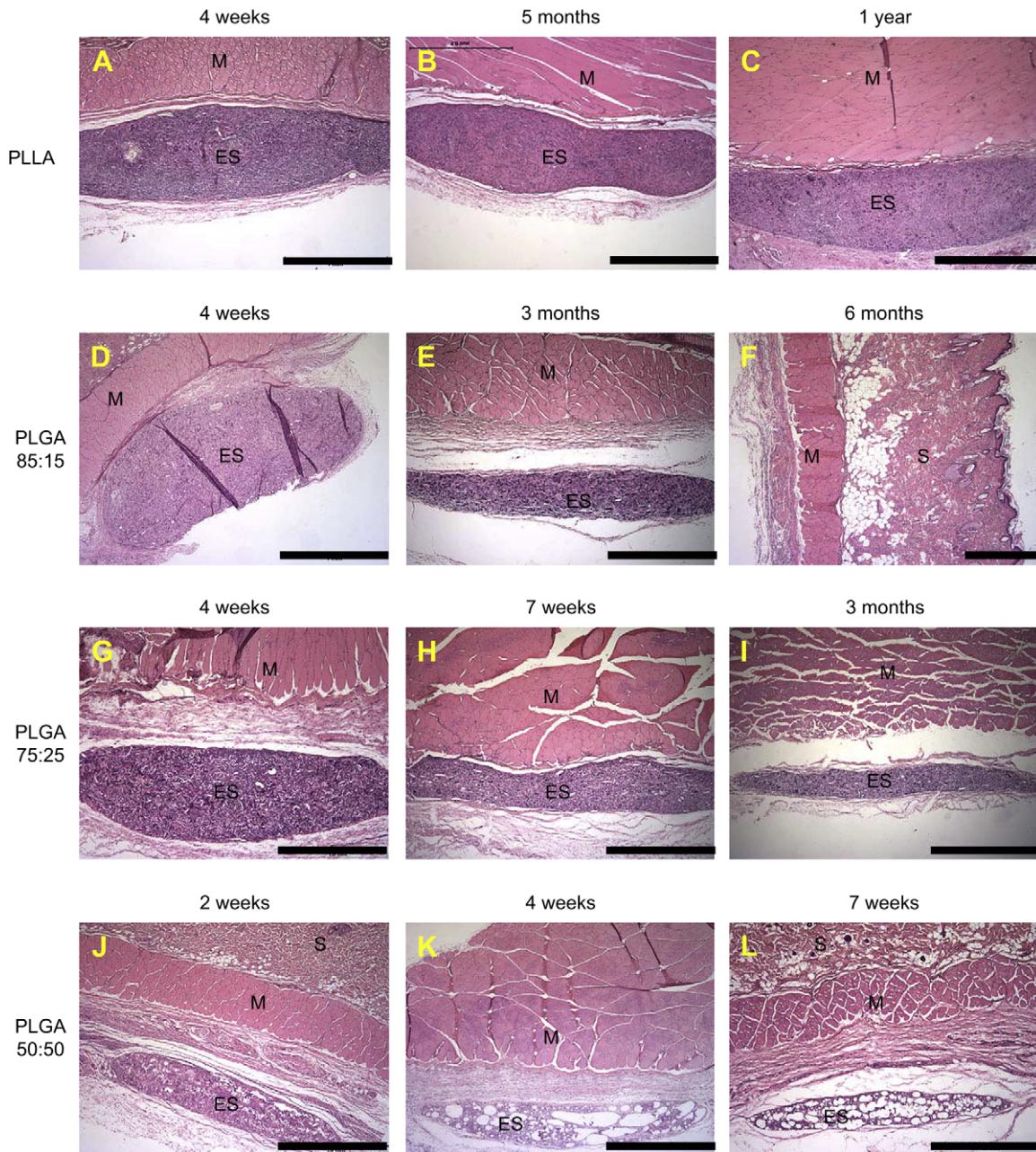
#### 4. Discussion

The aim of this study was to develop biodegradable electrospun scaffolds as candidate materials to replace the use of human donor dermis or bovine collagen for skin reconstruction purposes. In brief, this study shows that it was possible to produce scaffolds composed of PLGA copolymers with fibre diameters of around 2–5 μm and over 95% porosity. Extensive *in vitro* studies showed skin cells readily attached and grew within these scaffolds, co-cultures of keratinocytes and fibroblasts were capable of organising spatially in the scaffolds when cultured at an air–liquid interface, they

produced collagen and also showed evidence of epithelial/mesenchymal interactions with respect to cell viability, matrix production and the ability of the cells to contract the scaffolds. When implanted cell-free into rats, scaffolds were readily infiltrated by cells and blood vessels. Scaffolds did not provoke an aggressive inflammatory response and increasing the ratio of PGA to PLA increased the rate of breakdown of the scaffolds both *in vivo* and *in vitro* in a predictable manner.

In the beginning of this work our specifications were that any scaffold for clinical use must support cellular in-growth, both *in vivo* and *in vitro*, it must permit neovascularisation *in vivo*, it must not provoke an aggressive inflammatory response *in vivo* and finally, it must degrade at a predictable rate being replaced by a new matrix produced by the host cells. Of the three PLGA scaffolds studied, the 50:50 scaffold lost structural strength by two weeks, the 75:25 scaffold lost approximately half of its mass by 3 months and the 85:15 scaffold lost half of its mass after 4 months. By 5–6 months there was little PLGA 85:15 material detectable. In contrast, the PLLA scaffold remained largely intact after one year *in vivo* with some slight reduction in fibre diameter over this period. Accordingly, we suggest that the PLLA degrades too slowly for a dermal skin replacement, whereas the PLGA 50:50 material would be too fast. However, the PLGA 85:15 and 75:25 scaffolds would appear to be very promising candidates, with approximately 50% loss of mass by 4 and 3 months, respectively.

In terms of tissue engineering and regenerative medicine, the majority of scaffolds which have been used clinically are derived from natural matrices (see [18] for a recent review of this area). Depending on how these are processed, these can work extremely well as cell-free scaffold materials for small defects. Natural scaffolds such as allografts can also be used effectively for clinical use [19,20] to provide a neo-dermis for burns patients onto which cultured cells can then be grafted. Boyce et al. [1] have shown that bovine collagen can be seeded with autologous keratinocytes and fibroblasts and used in the treatment of extensive burns injuries. Similarly, we have previously shown that a sterilised acellular allograft can be used to make reconstituted skin [12] and oral mucosa [21]. However, there are two major challenges in working with any natural scaffold – sourcing and preparing them. Scaffolds are usually derived from human or bovine or porcine sources and prepared (processed to become cell-free and sterilised) so that they do not present an appreciable disease risk to the patient. Processing of the materials in such a way that their behaviour is predictable when introduced into the body is a bigger challenge than it might seem at first sight.



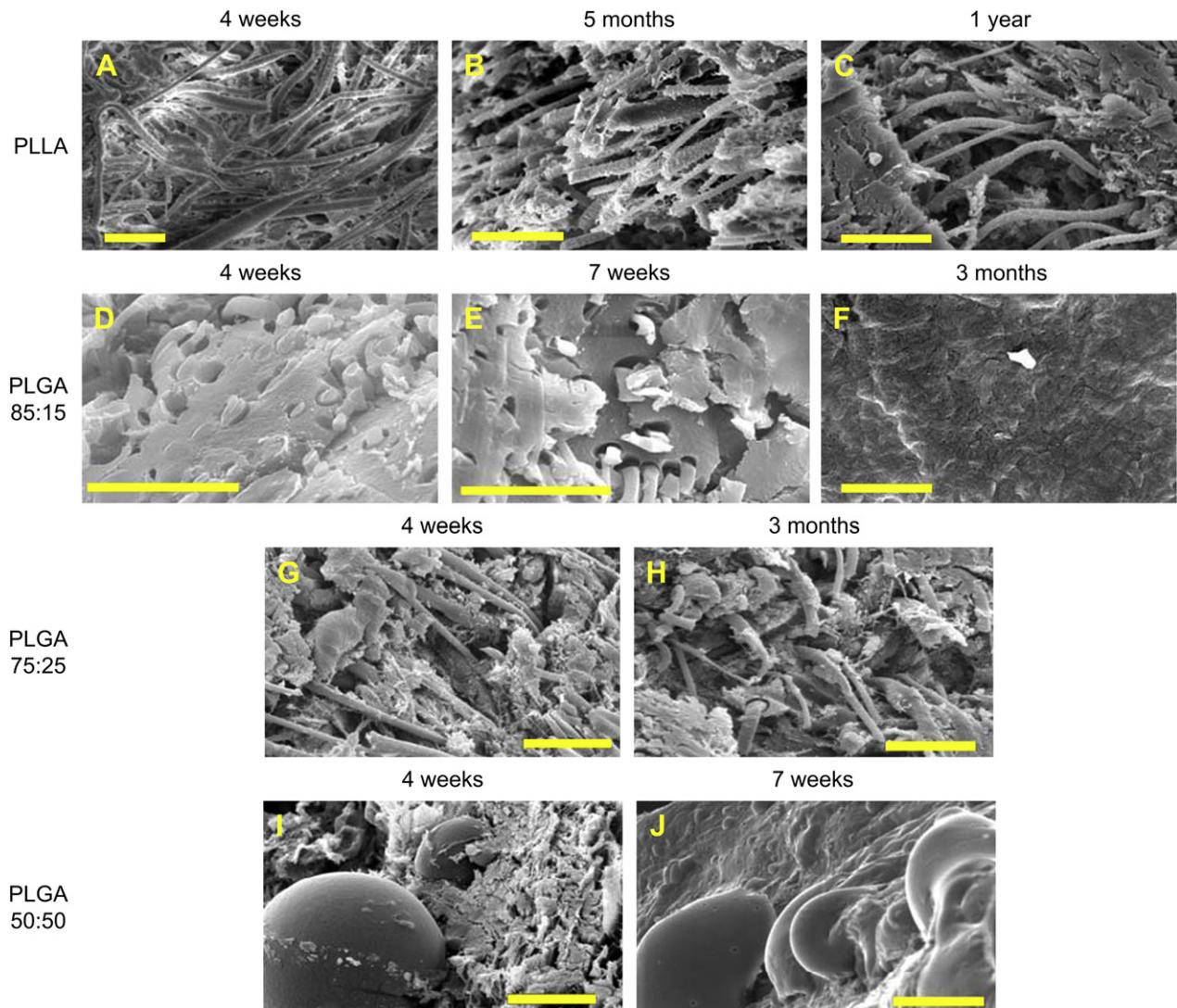
**Fig. 9.** Light microscope H&E images ( $\times 4$ ) of PLLA (A–C), PLGA 85:15 (D–F), PLGA 75:25 (G–L) following implantation into the flank of adult male Wistar rats at the time points indicated (2 weeks to 1 year). Implanted scaffold has been labelled as (ES), with underlying muscle (M) and skin (S). Scale bar = 1 mm.

To achieve structural strength, natural scaffolds are frequently crosslinked but the degree of crosslinking can create problems. Crosslinking also occurs during protocols that sterilise natural matrices. In our experience ethylene oxide [10] and peracetic acid [22] both decreased dermal pliability when used to sterilise human donor dermis for clinical use. Recent thinking in this area suggests that scaffolds based on natural matrices which are strongly cross-linked (for example with glutaraldehyde) to the extent that the host cells cannot break them down will provoke a chronic inflammatory response [23], often ending up with encapsulation and failure of the implant material. This may be mild in the case of implant material used in for example hernia repair (where fibrosis of the material may occur) but could be catastrophic if used in a critical structure such as a heart valve replacement.

Natural scaffolds which are modified to only a modest degree to achieve appropriate mechanical strength appear to be incorporated

into the body after a process of remodelling by the immune system led by the macrophages as discussed by Badylak [18]. Mantovani et al. [24] point out that macrophages are capable of responding to materials (living and non-living) introduced into the body by acquiring a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype.

Badylak [18] suggests that the former response to materials will result in a cytotoxic inflammatory response whereas the latter will result in a more constructive tissue remodelling response. Following on from this is the premise that any scaffold to be implanted must be capable of being remodelled and replaced by the body's own extracellular matrix tissues. Accordingly, it should be possible to design synthetic electrospun scaffolds composed of biodegradable/bioresorbable materials which can act as temporary tissue supports and guides, providing these can ultimately be replaced in a process of constructive remodelling.



**Fig. 10.** SEM micrographs of PLLA (A–C), PLGA 85:15 (D–F), 75:25 (G–H), 50:50 (I–J) following implantation into the flank of adult male Wistar rats at the time points indicated (4 weeks to 1 year). Scale bar = 20  $\mu$ m.

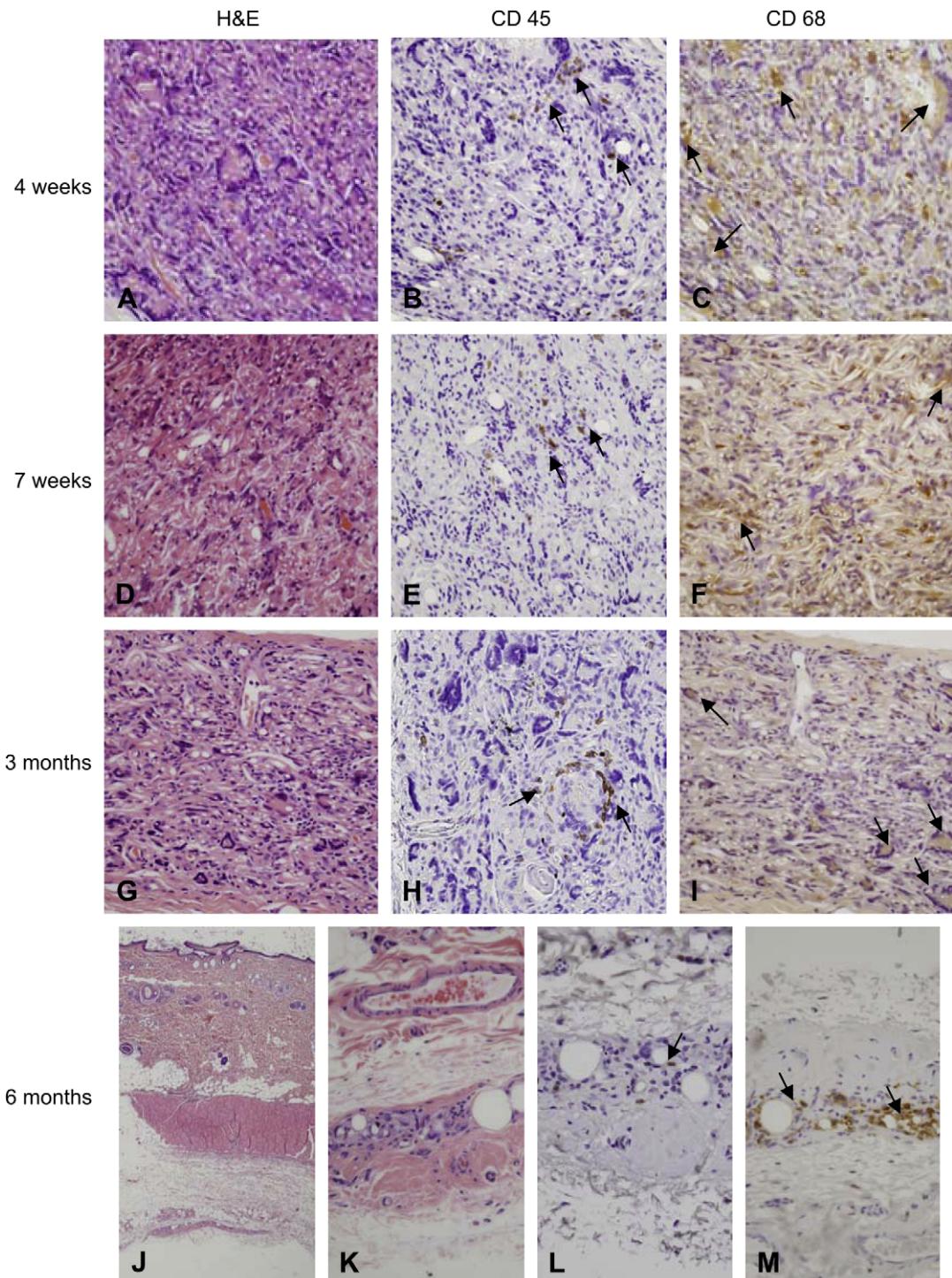
There is a wealth of literature reporting on the biocompatibility of PLLA and PGA (for a review see [25]). It has been determined that PGA itself is not immunostimulatory but rather the hydrolysis of PGA to glycolide (*via* glycolic acid) does produce an inflammatory response due to the local accumulation of glycolide [26]. PLLA takes longer to degrade than PGA due to its hydrophobic methyl group on the polymer backbone and higher degree of crystallinity in its structure. Studies of implanted PLLA have shown that the material may still be detectable after 5 years *in vivo* depending on the form of the PLLA implant [27,28].

With electrospinning, one achieves a very high surface area for a very low density of material. The electrospun mats produced in this study have a very high void area in excess of 90%, with the overall weight of the implanted material being extremely low. The physical structure of the scaffold is likely to have a major influence on how these materials degrade in addition to any consideration of the nature of materials alone. Over the last 15 years there have been an increasing number of studies of electrospun scaffolds (for a review of this area see [29]). To progress work in this area, however, it is necessary to consider not only the polymer chemistry and physical attributes of the scaffolds, but how the body responds to implanted scaffolds *in vivo* and how efficient scaffolds will be in supporting skin cells *in vitro* (and ultimately *in vivo*). To the best of

our knowledge there are few studies that combine an investigation of electrospun scaffold breakdown with an investigation of the tissue response to them *in vivo* and also the ability of these scaffolds to act as tissue engineered scaffolds *in vitro*. All of this information is vital for the progression of electrospun scaffolds towards the clinic.

To review the results obtained in this study – the rate of breakdown of these scaffolds reflected the composition of the scaffolds as expected. The higher the percentage of glycolide to lactide in the copolymer, the faster one would expect scaffolds to break down *in vivo* and this was indeed the case. A scaffold of 50:50 broke down very rapidly (within a few weeks) compared to one composed of 75% lactide and 25% glycolide which showed something like a 50% loss of mass at 3 months. Looking at the rate of breakdown of the PLGA 85:15 scaffold, this lost around half its mass by 4 months and had disappeared almost completely by 5–6 months. This suggests a gradual slow rate of breakdown of fibres and then perhaps an accelerated loss. The increasing percentage of glycolide present in the polymer was associated with a thicker fibre diameter post-spinning. Post implantation these fibres increased in volume initially – almost certainly taking up water before they then progressively decreased in diameter.

Xinhua et al. [13] give a detailed explanation of this breakdown in terms of thermally induced crystallisation of oligomeric



**Fig. 11.** Histology and immunohistochemistry of explanted PLGA 85:15 electrospun scaffold. The figure shows histology and immunohistochemistry of scaffolds explanted at 4 weeks (A, B, C), 7 weeks (D, E, F), 3 months (G, H, I) and 6 months (J, K, L and M). Panels A, D, G, J and K show H&E histology. The magnification for all panels except J is  $\times 40$ . In J ( $\times 4$  magnification) a small piece of residual scaffold is shown deep in this skin section. Higher power ( $\times 40$ ) views of this material are shown in sections K, L and M. Panels A, D, G, J and K show H&E staining. Panels B, E, H and L show the presence of lymphocytes ( $\uparrow$ ) confirmed by staining for CD45RC. Panels C, F, I and M show a vigorous macrophage response confirmed by CD68 immunoreaction, with areas of interest highlighted using arrows.

fractions/degraded material at early time points. After initial rapid crystallisation, as the glass transition temperature is close to body temperature (and tissue culture incubators) the crystallisation appears to gradually increase over time until a point where it then begins to reduce until it is then lost and the material completely degrades. With the 50:50 scaffolds large crystalline balls of polymer were seen in the SEMs of the implanted scaffolds which suggest

a dramatic crystallisation of oligomeric fractions of degraded material. The extent of this fibre merging and subsequent breakdown had adverse effects on the tissue morphology and as the rate of breakdown was so rapid *in vivo* we suggest this is unlikely to be a useful substrate for a dermal replacement material.

The immune response of the rats to the implanted scaffolds was similar throughout. There was clear evidence of neovasculature as

seen both by H&E and confirmed by CD31 staining for endothelial cells. The host response was predominantly macrophage-dominated with little evidence of lymphocytic infiltration at any of the time points studied. This would be consistent with a vigorous foreign body response in which this material is resorbed by the macrophages without provoking a major immune involvement.

We selected two scaffolds for an *in vitro* examination of their ability to support skin cells asking whether one could culture the cells satisfactorily and whether there was any evidence of cells starting to produce a new matrix to replace the scaffolds and any evidence of keratinocyte and fibroblasts interacting effectively on the scaffolds. The results show that in terms of overall viability, both scaffolds supported keratinocytes, fibroblasts and endothelial cells. Interestingly, as we have reported in other publications, when cells were in co-culture it was possible to get as good results in the absence of serum as in the presence of serum. We have found this to be the case whenever keratinocytes are cultured with fibroblasts or endothelial cells with fibroblasts [30] strongly suggesting that a dialogue between the fibroblasts and the other cells enables cells to supply each other with relevant mitogens which are normally obtained in the foetal calf serum. This is a clear indication of a route to avoid the use of bovine serum for future clinical use.

Encouragingly, we also found clear evidence of new collagen production, with a greater amount produced when keratinocytes and fibroblasts were cultured together, compared to separate monocultures. This supports the finding that scaffolds permitted a dialogue to occur between the two cell types as would normally be the case *in vivo*. It was interesting that endothelial cells also did well on the scaffolds on their own, again another encouraging sign for neovascularisation. Certainly *in vivo* the scaffolds became well vascularised and there was no evidence of necrosis or encapsulation, both of which would have been strong contraindications for considering the scaffolds for future clinical use.

*In vitro* the results support the scaffolds being appropriate for skin reconstruction as the co-culture of skin cells on the PLGA 75:25 scaffold confirmed that cells achieved spatial organisation with fibroblasts penetrating into the scaffold and wrapping around the fibres and keratinocytes forming a well attached continuous barrier layer on the upper surface of the scaffold. This is similar to an earlier study we reported for keratinocytes and fibroblasts (and endothelial cells) co-cultured on non-biodegradable electrospun polystyrene scaffolds under similar air-liquid culture circumstances [30]. In this earlier study, which showed a similar spatial organisation of cells to that shown in Fig. 7, keratinocytes were positively identified by immunostaining for cytokeratins and the use of an air-liquid culture interface was found to be essential to induce keratinocytes to become organised into a barrier layer, hence the same conditions were used in the current study.

Skin cells also altered the overall mass of the scaffolds, apparently contracting them. Fibroblasts on their own produced significant contraction of these scaffolds, keratinocytes on their own did not contract the scaffolds to any great degree but reduced the contraction of the scaffolds seen when co-cultured with fibroblasts on the scaffolds. Again this is further evidence of an epithelial/mesenchymal dialogue and suggests that contraction of these scaffolds by skin cells may not prove to be a major problem *in vivo*. We have found contraction of tissue engineered skin based on a natural dermal scaffold – sterilised allodermis – can be a considerable problem [31–34]. Contraction of conventional split-thickness skin grafts is also a major concern as recently discussed from this laboratory [35], accordingly this will definitely be one area for further investigation prior to progressing this to clinical application but these preliminary results in this area look very promising.

In summary we conclude that both the PLGA 75:25 and 85:15 electrospun scaffolds have fulfilled the initial criteria we posed for

development as synthetic dermal replacement scaffolds for clinical use.

## Acknowledgements

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