

***In vivo* characterisation of a novel bioresorbable polydactide-co-glycolide) tubular foam scaffold for tissue engineering applications**

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Abstract

Poly(lactide-co-glycolide) (PLGA) foams of tubular shape were assessed for their use as soft-tissue engineering scaffolds *in vitro* and *in vivo*. Porous membranes were fabricated by a thermally induced phase separation process of PLGA solutions in dimethylcarbonate. The parameters investigated were the PLGA concentration and the casting volume of solution. Membranes produced from 5 wt/v% polymer solutions and a 6 ml casting volume of polymer solution were selected for fabricating tubes of 3 mm diameter, 20 mm length and a nominal wall thickness of 1.5 mm. Scanning electron microscopy revealed that the structure of the tubular foams consisted of radially oriented and highly interconnected pores with a large size distribution (50-300 µm). Selected tubes were implanted subcutaneously into adult male Lewis rats. Although the lumen of the tubes collapsed within one week of implantation, histological examination of the implanted scaffolds revealed that the foam tubes were well tolerated. Cellular infiltration into the foams, consisting mainly of fibrovascular tissue, was evident after two weeks and complete within eight weeks of implantation. The polymer was still evident in the scaffolds after eight weeks of implantation. The results from this study demonstrate that the PLGA tubular foams may be useful as soft-tissue engineering scaffolds with modification holding promise for the regeneration of tissues requiring a tubular shape scaffold such as intestine.

1. Introduction

Tissue engineering represents a multidisciplinary approach for the replacement of damaged tissue by regenerating new tissue *in vitro* or *in vivo* as a biological substitute [1]. Development of novel biomaterials and fabrication of scaffolds from these materials is an essential step toward being able to engineer tissues from different anatomical locations in the body [1,2].

Different strategies can be adopted for tissue engineering of lost, injured or diseased tissue but the tissue of interest usually determines the approach taken. These include implantation of freshly isolated or cultured cells onto an implanted acellular scaffold, implantation of tissues grown on scaffolds *in vitro*, or implantation of acellular scaffolds to support *in situ* tissue regeneration [2]. For all of these approaches, the scaffold must provide a three-dimensional (3-D) structure that will support the in-growth of tissue into the structure, resulting in neotissue that has similar properties to the tissue that is to be replaced.

Materials used to fabricate scaffolds for soft-tissue engineering are usually selected to provide temporary structures that exhibit adequate mechanical and biological properties. The use of biodegradable scaffolds avoids the need for a permanent implant made of an engineered material remaining in the tissue. Numerous bioresorbable materials have been investigated as scaffolds for tissue engineering and tissue repair, including naturally occurring [3,4] and synthetic polymers [5-9], as well as porous bioactive ceramics and glasses [10] and polymer/ceramic composites [11,12]. Advantages of using synthetic scaffolds include the ability to precisely engineer the material's composition and micro- and macrostructure. This allows tailoring of scaffold properties, thus enabling the incorporation of optimal conditions for cell survival, proliferation, and subsequent tissue formation [6].

Synthetic bioresorbable polymers have been increasingly used as tissue engineering scaffolds during the past 10 years, particularly polylactide (PLA), polyglycolide (PGA) and their copolymers [5-9]. These materials have demonstrated promising results in clinical use in other applications such as resorbable surgical sutures and meshes or in drug delivery systems [5]. Limited options are available for tissue engineering scaffolds suitable for engineering tissues containing a luminal structure. In fact, there has been relatively limited previous research on developing macro-tubular synthetic biodegradable constructs for tissue engineering applications [13-22] in

comparison with other scaffolds architectures. These previous studies have mainly focused on developing scaffolds for vascular tissue engineering [14,15] but applications in other areas requiring tubular scaffolds such as peripheral nerve, long bone and intestine have been considered [17-22].

This study evaluates tubular foam scaffolds fabricated from poly(D,L-lactide-co-glycolide) (PLGA) co-polymers for use in the engineering of tubular tissue constructs such as trachea, small intestine and oesophagus, and specifically the ability of the scaffold to support *in situ* tissue regeneration that might produce a tubular construct suitable for seeding with other cell types, such as surface epithelial cells.

2. Materials and methods

2.1. Materials

PLGA foams were fabricated following a thermally induced phase separation process, also termed freeze-drying, which has been described in detail elsewhere [7,8]. Briefly, a given amount of 75:25 poly(D,L-lactide-co-glycolide) (PLGA RG756, Boehringer-Ingelheim, Germany), with inherent viscosity of approximately 0.6dl/g was dissolved in dimethylcarbonate (99%, Acros) under magnetic stirring overnight to yield 5 and 15wt/v% polymer solutions. The solution was filtered through a 0.45 μm Acrodisc nylon filter (GelmanSciences, USA) and cast onto a Petri dish (50 mm in diameter). The covered Petri dish was transferred into a 600 ml lyophilisation flask and frozen for 2h into liquid nitrogen. The flask was then connected to a vacuum pump (10^{-2} Torr) and the solvent was sublimated at -10°C for the first 48 h, and then at 0°C for additional 48 h. The residual solvent was then removed at room temperature until the foam reached a constant weight. Different polymer concentrations and casting volumes of solutions were assessed for their suitability to produce porous membranes for the fabrication of tubular constructs, as reported in Table I. The membrane selected for use in this study, that contained the largest pores whilst retaining its structure, was fabricated from 5 wt/v % polymer solution and a 6-ml casting volume (membrane no. T1 in Table I). The remaining membranes exhibiting different pore structures are being considered in parallel studies for other soft-tissue applications requiring tubular constructs [23].

Table 1 Characteristics of the membranes fabricated by the thermally induced phase separation process

Membrane	Polymer concentration (wt/v%)	Casting volume	Thickness (mm)	Flexibility
T1	5	6 ml	1.5-2	+
T2	5	8 ml	2.5-3	+
T3	15	6 ml	1.5-2	\pm
T4	15	8 ml	2-2.5	\pm

Tubular foam constructs were fabricated by rolling the polymer membranes into a tube. The opposing edges were joined by slowly dissolving in chloroform and pressing the edges together. This resulted in a tube with a lumen approximately 3 mm in diameter and walls approximately 1.5-2 mm thick. The surface topography of the membrane differed between the top and bottom surfaces due to the polymer casting techniques employed in this study, as discussed elsewhere [23]. The top surface of the membrane was less porous than the bottom surface. The tubes were rolled so that the top surface of the membrane became the luminal surface of the tube. This configuration was chosen since it would be most beneficial in providing a luminal surface for the seeding and localisation of other cell populations, such as epithelium. Fig. 1 shows a scanning electron microscopy (SEM) micrograph of the typical microstructure of the tubular constructs used in the present study. The figure shows that tubular pores highly oriented in radial direction are present. This porous structure is typical of foams prepared by the phase separation method [7,8,12]. The porosity is high ($>93\%$), as determined by mercury pycnometry in similar scaffolds in previous studies [24]. From the porosimetry measurements, the pore volume was calculated to be $11.5\text{ cm}^3/\text{g}$. Moreover the porosity structure comprises two distinct pore sizes, macropores of $\sim 100\text{ }\mu\text{m}$ average diameter and interconnected micropores of $10\text{-}50\text{ }\mu\text{m}$ diameter. A more detailed characterisation of the pore structure of the tubular foams has been presented elsewhere [23,25]. In principle, all the fabricated foams have sufficient pore volume and pores of given controlled size and orientation for cell proliferation as required in tissue engineering scaffolds.

2.2. Methods

2.2.1. Characterisation and *in vivo* studies

Implantation studies of the PLGA foams were performed on inbred adult male Lewis rats weighing 250-350 g in compliance with the Animals (Scientific Procedures) Act 1986. All the animals were fed on commercial standard

pelleted rat diet. Rats were anaesthetised with Hypnorm 0.4 ml/kg (fentanyl citrate and fluanisone) and diazepam 5 mg/kg. Three foam scaffolds that had been sterilised by ultraviolet light were placed into three subcutaneous pockets on the ventral aspect of one rat for each time-point to be studied and closed with 3/0 Mersilk® sutures (Ethicon®). Rats were kept under standard laboratory conditions until sacrificed at 7, 14, 28, 42 and 48 days, when the foam was removed.

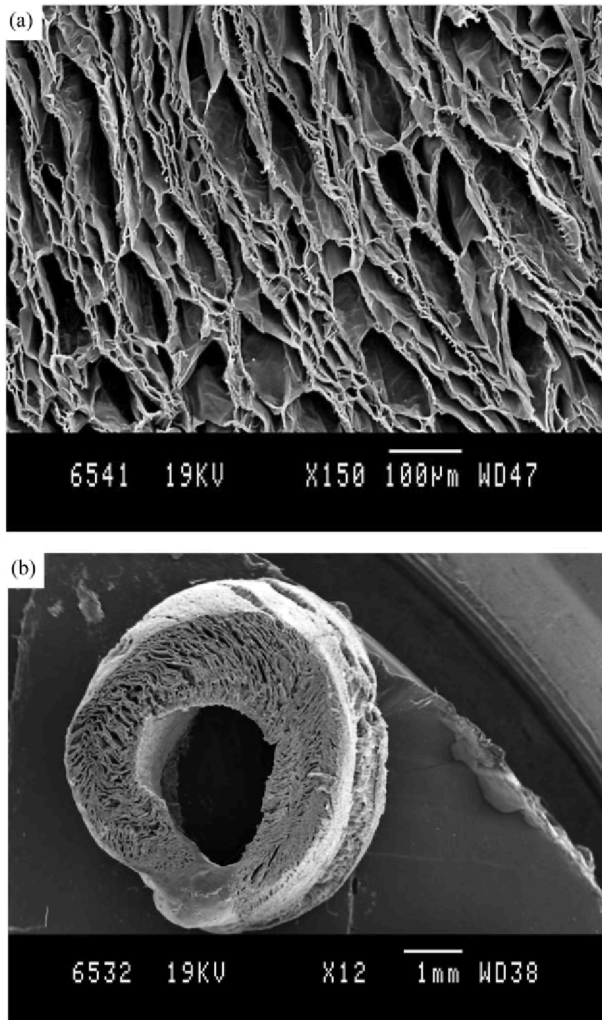


Figure 1 Scanning electron microscopy of the foam polymer selected for assessment of porosity and pore structure. (a) Cross-section of the membrane at mid-height shows the random pore size and high-degree of porosity within the membrane. (b) A tubular structure was formed by rolling the membranes and dissolving the opposing edges with chloroform.

2.2.2. Histological examination of implanted foam scaffolds

For light microscopy the tissue containing the cellularised foams was fixed in 10% buffered formalin (Fig. 2). During embedding into paraffin-wax, the foams were orientated so that a cross-section of the foam would be cut during sectioning for histological examination. Five-micrometre tissue sections were cut and stained with haematoxylin and eosin or haematoxylin and van Gieson stain, the latter demonstrates the deposition of connective tissue in which collagen stains red.

3. Results

Cross sections of the cellularised tubular scaffolds were used for histological examination using light microscopy. At one week after implantation the tubular structure of the foam scaffold was not maintained, with the lumen becoming compressed (Fig. 3(a)).

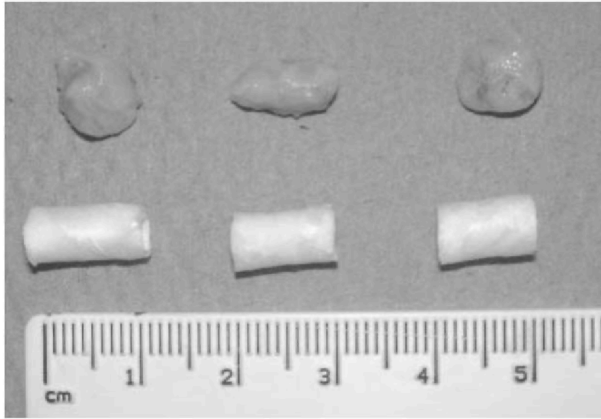


Figure 2 An example of the cellularised scaffolds (top) after implantation for eight weeks compared with scaffolds before implantation (bottom).

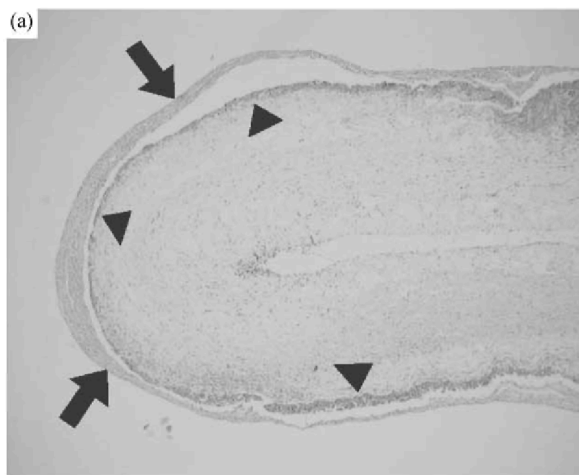
The foams were already loosely encapsulated with fibrous tissue; however this did not extend into the foam (Fig. 3(b)). Inflammatory cells had migrated into the foam from the surrounding tissue and were present throughout the thickness of the scaffold wall to the lumen (Fig. 3(c)). These cells consisted primarily of polymorphonuclear leukocytes and macrophages.

After two and four weeks of implantation, in-growth of fibrovascular tissue into the scaffold from surrounding tissue was evident (Fig. 4(a)). Collagen, demonstrated by haematoxylin and van Gieson staining, was present throughout the cellularised areas of scaffold (Fig. 4(b)). Blood vessels were present at the leading front of the infiltrating tissue. Small, non-nucleated cells, likely to be platelets, preceded these vessels. The structure of the scaffold remained intact in regions that had not yet become cellularised, whilst polymer was still evident in areas of the foam that had become cellularised (Fig. 4(c)).

After six and eight weeks of implantation, the foam scaffolds were completely infiltrated by fibrovascular tissue (Fig. 5(a)). Connective tissue was deposited throughout the thickness of the scaffold wall and a small amount was also present in the lumen of the tube (Fig. 5(b)). The cellularised scaffolds were infiltrated with a dense vascular network (Fig. 5(c)). The polymer that the scaffolds were constructed from still was still evident at eight weeks (Fig. 5(d)).

4. Discussion

The current study examines the feasibility of using a polylactide-co-glycolide foam of tubular shape as scaffold for use in the tissue engineering of tubular tissues, such as intestinal tissue. Foam scaffolds made by the thermally induced phase separation process were chosen for testing because their composition and porosity can be tightly controlled, enabling the scaffolds to be optimised for a particular tissue engineering application [7,8]. This technique is also favoured for the fabrication of porous conduits for nerve regeneration [22,26]. In the case of tubular scaffolds for soft-tissue engineering, the foam needs to have a high degree of porosity to allow rapid tissue infiltration, whilst having enough matrix material to retain its structure.



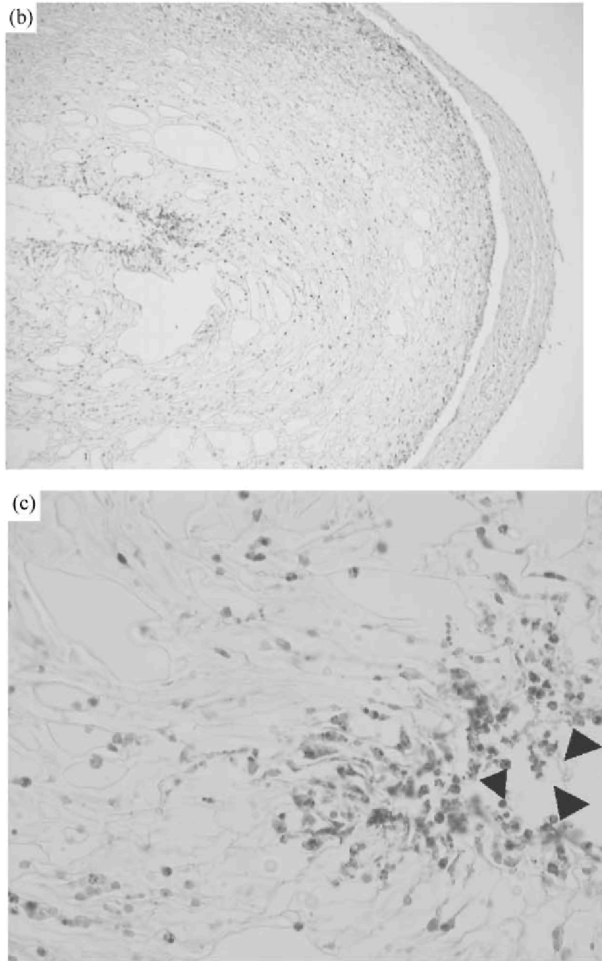
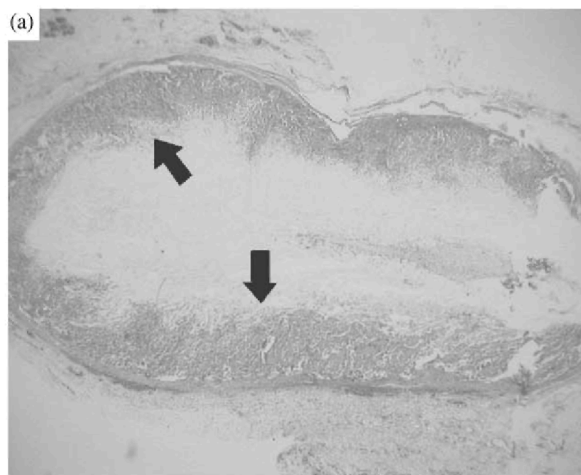


Figure 3 (a) A cross-section of a scaffold one week after implantation. A loosely attached sheath of connective tissue is present around the periphery of the foam scaffold (arrows). Connective tissue cells are present at the edge of the scaffold (arrowheads) but have not yet migrated into the foam. (b) Haematoxylin and van Gieson staining shows no positive staining for connective tissue in the foam. (c) Inflammatory cells are scattered throughout the thickness of the foam through to the lumen of the scaffold (arrowheads). ([a] haematoxylin and eosin, original magnification x 50; [b] haematoxylin and van Gieson x magnification, original magnification x 100; [c] haematoxylin and eosin, original magnification x 400.)



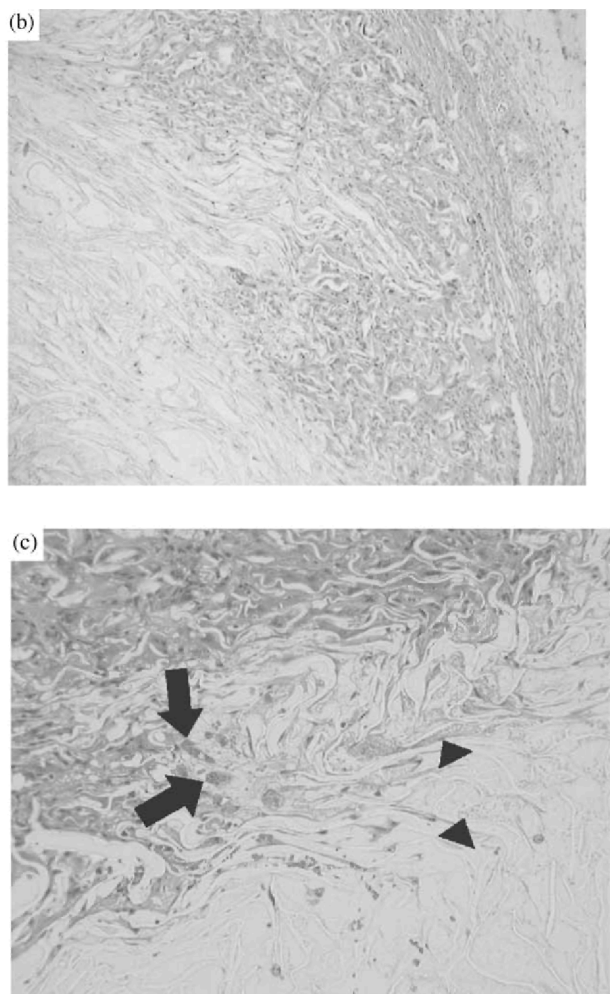


Figure 4 (a) A cross-section of a scaffold four weeks after implantation. Cells from the surrounding tissue have infiltrated approximately halfway through the wall of the foams (arrows). (b) The cellularised area of the foams stain positively with haematoxylin and van Gieson showing that connective tissue (pink) has been deposited. (c) Blood vessels (arrows) are evident at the leading front of the infiltrating tissue, preceded by platelets (arrowheads). ([a] haematoxylin and eosin, original magnification x 25; [b] haematoxylin and van Gieson, original magnification x 100; [c] haematoxylin and eosin, original magnification x 200.)

The foams used in the current study were fabricated as membranes before rolled into tubular constructs. The compressive strength of the tubes was not measured in this investigation but values are available for cylindrical PLGA foams made by the same technique and exhibiting similar porosity: 10 MPa [24]. Before implantation, the scaffolds were able to maintain their tubular structure, even after the tubes were cut to length with a razor blade, during which they were momentarily compressed. However, one week after implantation the tubular structures had become compressed reducing the volume of the lumen. After four weeks, the lumen had started to become filled with connective tissue deposits.

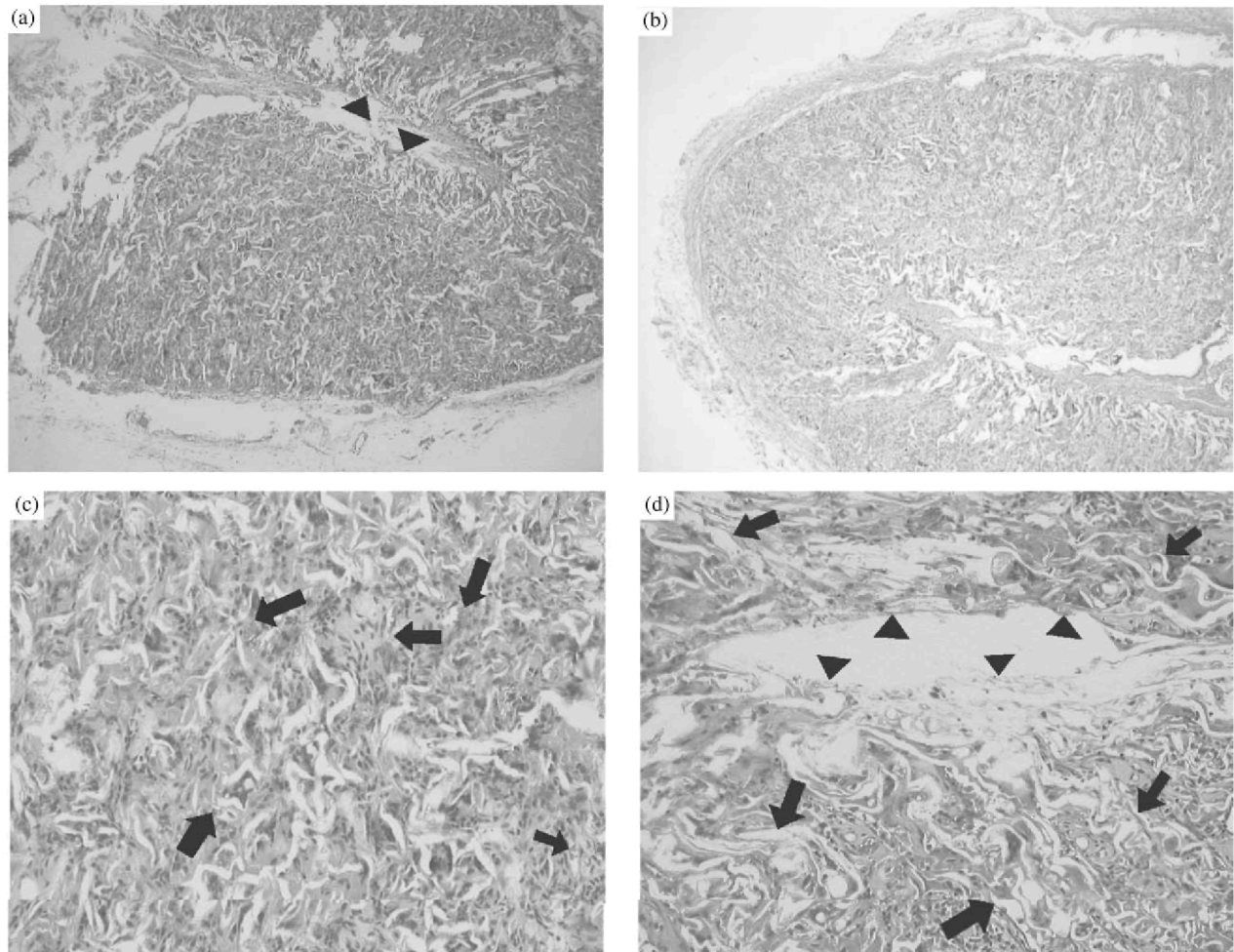


Figure 5 (a) A cross-section of a scaffold six weeks after implantation showing tissue infiltration of the foam through to the scaffold lumen (arrowheads). (b) Connective tissue (pink) is deposited throughout the wall of the scaffold, (c) Blood vessels (arrows) are evident throughout the tissue infiltrate. (d) The polymer foam (arrows) from which the scaffolds were constructed is still visible at eight weeks despite the scaffolds being cellularised through to the lumen (arrowheads). ([a] haematoxylin and eosin, original magnification x 50; [b] haematoxylin and van Gieson, original magnification x 50; [c] haematoxylin and eosin, original magnification x 200; [d] haematoxylin and eosin, original magnification x 200.)

One aim of future use for these scaffolds is to seed the luminal surface of the cellularised scaffold with other cell types, such as epithelial cells. This may be difficult to achieve with the location used in the current approach if the lumen becomes compressed. This problem might be overcome by changing the location of the implant from the ventral aspect, where it is likely to be compressed in the current model, to either the dorsal aspect or even the abdominal cavity, where it is less likely to be exposed to compressive forces. Alternatively, a more rigid scaffold could be fabricated using a different composition of polymer (lactide/glycolide copolymer ratio) or a composite material made of the degradable polymer and a stiff bioresorbable (e.g. inorganic) phase as filler [12,23]. Other possibility for extending the structural integrity of the tubular scaffolds after implantation, without changing the material itself, would be to create a more sophisticated porous structure, for example, with graded porosity in order to optimise the pore volume required from the biological and mechanical point of views. Issues related to scaling-up the tubes to consider larger constructs remain to be investigated, in particular in relation to their mechanical competence. In principle, the technique introduced in this paper can be scaled up without difficulties in order to prepare tubular scaffolds of larger diameters, lengths and wall thicknesses.

The pore structure of the foams enabled infiltration of cells and blood vessels from surrounding tissue fairly rapidly, with complete cellularisation occurring before the foams had completely degraded (Figs. 3-5).

The presence of inflammatory cells within the lumen of the scaffolds at one week (Fig. 3) is not surprising since the implantation of any foreign body into immunocompetent tissues will elicit an inflammatory response. A similar reaction has been observed with the implantation of non-woven polymer fibres of PGA used to support intestinal epithelial cell growth [27]. The inflammatory response may also be due in part to the release of acid degradation products from the polymer from which the scaffolds are constructed, an effect that has been described in the literature [4,5,7-9]. The acute inflammatory response seen after one week of implantation did not affect the long-term biocompatibility of the implanted scaffolds, as seen by the complete infiltration of

fibrovascular tissue after eight weeks of implantation.

Rather than being a detrimental effect, this inflammatory response may be of some benefit because leukocytes that have migrated into the scaffold will release a plethora of growth factors that will lead to further tissue infiltration. The essential role of macrophages in wound repair is clearly demonstrated in models where the repair rate is reduced by the administration of anti-macrophage serum [28,29].

Cells resembling platelets were also observed at the leading front of the infiltrating tissue (Fig. 4). Platelets are considered to be important mediators in tissue remodelling and repair through the release of important growth regulatory peptides, such as platelet-derived growth factor (PDGF) and transforming growth factor- β (TGF- β). During wound healing processes PDGF accelerates tissue repair, increasing deposition of extracellular matrix and granulation tissue formation [30,31]. TGF- β may also be important in tissue repair, as it is a multifunctional peptide that controls cell proliferation, differentiation and deposition of connective tissue [32]. Many of the growth regulatory peptides important in tissue repair and remodelling are likely to be of equal importance in tissue engineering processes such as neotissue formation.

5. Conclusions

The successful production of porous PLGA tubular foams by the thermally induced phase separation process has been shown. These scaffolds demonstrated good biocompatibility when tested *in vivo* by implanting subcutaneously in adult male Lewis rats for periods of up to eight weeks. The foams were rapidly cellularised suggesting they may be of use in biomedical applications where tubular constructs are required. Current research focuses on the optimisation of the tubular scaffolds to support luminal intestinal epithelial cell growth.

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