Integration of an ultra-strong poly(lactic-co-glycolic acid) (PLGA) knitted mesh into a thermally induced phase separation (TIPS) PLGA porous structure to yield a thin biphasic scaffold suitable for dermal tissue engineering

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Abstract

We aimed to capture the outstanding mechanical properties of meshes, manufactured using textile technologies, in thin biodegradable biphasic tissue-engineered scaffolds through encapsulation of meshes into porous structures formed from the same polymer. Our novel manufacturing process used thermally induced phase separation (TIPS), with ethylene carbonate (EC) as the solvent, to encapsulate a poly(lactic-co-glycolic acid) (PLGA) mesh into a porous PLGA network. Biphasic scaffolds (1 cm × 4 cm × 300 μm) were manufactured by immersing strips of PLGA mesh in 40 °C solutions containing 5% PLGA in EC, supercooling at 4 °C for 4 min, triggering TIPS by manually agitating the supercooled solution, and lastly eluting EC into 4 °C Milli-Q water. EC processing was rapid and did not compromise mesh tensile properties. Biphasic scaffolds exhibited a tensile strength of 40.7 ± 2.2 MPa, porosity of 94%, pore size of 16.85 ± 3.78 μm, supported HaCaT cell proliferation, and degraded in vitro linearly over the first ~3 weeks followed by rapid degradation over the following three weeks. The successful integration of textile-type meshes yielded scaffolds with exceptional mechanical properties. This thin, porous, high-strength scaffold is potentially suitable for use in dermal wound repair or repair of tubular organs.

Introduction

When designing and manufacturing scaffolds for dermal tissue repair, there are an array of biological and mechanical factors to consider. A challenging but critical provision for dermal scaffolds is a thin template material that is flexible, can withstand significant tensile loads, can support cells (delivery and integration with host tissue), and possesses a degradation profile that is compatible with wound healing [1, 2].

Electrospinning has been widely utilised in skin tissue engineering because of its capacity to generate thin fibrous scaffolds, having many of the desired properties [3]. Tensile properties of electrospun sheets can be enhanced by aligning fibres parallel to anticipated forces or by increasing the layers of fibres, but these solutions compromise properties along the perpendicular axis or yield dense fibre packing that can restrict cell penetration, respectively [4, 5]. Melt electrowriting (MEW) addresses some electrospinning limitations...
[6–8], however widespread adoption of MEW has been hampered by machine/software complexity, as well as the considerable time required for MEW scaffold fabrication [9].

Centuries of textile mass production have led to versatile processes that can yield meshes with a wide range of dimensions, and these processes offer greater scalability than more recent scaffold manufacturing processes, such as electrospinning [10]. There are now many degradable knitted meshes available commercially, and some are used in surgical tissue repair [10] of hernias, heart valves, ligaments, tendons, and skin [11, 12]. A knitted mesh structure is potentially ideal for dermal tissue repair because of the tuneable tensile properties, as well as compatibility of knit structures with suturing to adjacent tissue. A weakness of current biodegradable knit structures is that they are knitted from dense fibre bundles with large void spaces between the bundles, which are unlikely to support the formation of a continuous epithelial layer [13, 14]. Augmenting existing biodegradable meshes with finer secondary structures could provide support for the delivery and growth of cells across meshes and extend their utility in dermal and other tissue repair applications.

We sought to integrate a clinical grade 75:25 poly(lactic-co-glycolic acid) (PLGA) knit mesh (Biomedical Structures Ltd, Boston) into a porous PLGA structure to capture the mechanical strength of the knit mesh within a porous network, suitable for dermal tissue regeneration. To generate an interconnected porous polymer network, we utilised thermally induced phase separation (TIPS). TIPS scaffolds are commonly manufactured using organic solvents such 1,4-dioxane [15, 16] and tetrahydrofuran [17], which have melting points of 11.5°C–12°C and −108.44°C, respectively [18, 19]. A risk associated with casting a PLGA TIPS structure around an existing PLGA mesh is that the solvent may degrade the mesh. To minimise this risk, and to enable rapid manipulation, we selected ethylene carbonate (EC) as a solvent. While use of EC to manufacture PLGA TIPS scaffolds has been described [19, 20], the process has never been used in the manufacture of thin biphassic structures. We reasoned EC would be an ideal solvent for this specific manufacturing process as it requires relatively mild working conditions; with a melting temperature of 36.4°C [21], ability to be supercooled between 0°C to 10°C before freezing [22, 23], and high miscibility in water that would allow EC to be rapidly leached from thin scaffolds [19]. Furthermore, EC represents a suitable solvent for clinical application, as the health hazards of EC are relatively low, particularly when compared to commonly utilised TIPS solvents such as 1,4-dioxane [24] or tetrahydrofuran [25]. The lethal dose (LD50) of 1,4-dioxane, tetrahydrofuran and EC are 4200–5400 mg kg⁻¹, 1650 mg kg⁻¹, and 10 400 mg kg⁻¹, respectively [19, 24–26]. Herein, we outline a novel process for manufacturing a biphasic scaffold that encases a mechanically robust knit PLGA mesh within a porous PLGA structure generated using EC as the solvent and TIPS structure around an existing PLGA mesh

Materials and methods

PLGA and ethylene carbonate (EC) solution

A 5% weight/volume (w/v) PLGA in EC solution was generated by dissolving PLGA pellets having a lactide-glycolide ratio of 75:25 and molecular weight 66 000–107 000 (g/mol) (Sigma-Aldrich) in EC (99% anhydrous, Sigma-Aldrich). The polymer solution was agitated with a magnetic stir bar at 60°C until the PLGA pellets had completely dissolved, and then stored at −20°C until further use.

Preparation of PLGA mesh (PLGAmesh)

The PLGA mesh (PLGAmesh) utilised in this study was a custom-made product purchased from Biomedical Structures (Warwick, Rhode Island, USA). Individual sheets of PLGAmesh came in vacuum sealed sleeves and were stored at −20°C prior to use. Each original PLGAmesh sheet was 10.5 cm × 10.5 cm. For biphassic scaffold manufacture, strips of PLGAmesh 1 cm × 4 cm were cut from the sheet using scissors. These dimensions were selected based on allowing for mechanical properties characterisation [27–29], as well as efficient use of the mesh. Strips of PLGAmesh were stored at −20°C and protected from prolonged light exposure between all handling processes.

Biphassic scaffold manufacture

In this study we had three scaffolds, (1) PLGA mesh (PLGAmesh), (2) PLGA TIPS scaffold (PLGATIPS), or (3) the PLGA mesh encapsulate in a PLGA TIPS scaffold (PLGA mesh+TIPS). As the use of EC as a solvent has not been well characterised, during the optimisation process, we evaluated (1) the merits of inducing nucleation at either 24°C or 4°C, (2) the merits of degassing the PLGA + EC solution, (3) the use of polydimethylsiloxane (PDMS) or glass as the template material for scaffold fabrication, as well as (4) scaffold assembly temperature. Comparisons of these results and outcomes are provided in the supplementary data section, with a detailed description of the selected optimised process described in the results section.

The steps used in the optimised biphassic PLGA mesh+TIPS scaffold manufacture and characterisation process are represented schematically in figure 1(A). The 5% (w/v) PLGA + EC solution was transferred to a 25 ml vacuum filtration flask to eliminate gas from the solution. The flask was maintained at 55°C and the solution degassed for 30 min. Four strips of PLGAmesh (1 cm × 4 cm) were assembled on
glass microscope slides (Menzel Glaser) and placed on a 40 °C heating block. Following degassing of the polymer solution, 0.25 ml of 5% (w/v) PLGA + EC solution was carefully applied to each 1 cm × 4 cm knit PLGA<sub>mesh</sub> ensuring that the addition of the liquid solution displaced air from the mesh rather than trapping bubbles within the mesh. A pre-warmed (40 °C) glass slide was then placed on top of each PLGA<sub>mesh</sub> immersed in the PLGA + EC solution. The glass plate (including the four replica moulds) was transferred to a refrigerated centrifuge set at 4 °C. The centrifuge rotor had been removed, and the centrifuge only used for its refrigeration function. Scaffolds and moulds were permitted to quench at 4 °C for 4 min before nucleation and TIPS was manually triggered by agitation of the supercooled solution as shown in figure 1(B) and demonstrated in supplementary video 1, which is available online at stacks.iop.org/BF/12/015015/mmedia.

Immediately following triggering of TIPS through agitation of the supercooled PLGA + EC solution, four biphasic PLGA<sub>mesh</sub>+TIPS scaffolds were placed into a single glass beaker containing 1 l of pre-cooled (4 °C) Milli-Q water to leach the polymer-lean domain (EC phase). Leaching was performed for four days at 4 °C in a non-transparent container to ensure that the scaffold remained below the EC melt temperature, and to protect the PLGA from photodegradation. A large volume of Milli-Q water (1 l) relative to the total EC volume (1 ml) yielded a maximum EC content in the leaching bath of less than 0.1%. This dilution ratio was selected to minimise solvent
Morphological characterisation of the PLGA mesh and PLGA scaffolds

Morphological characterisation of the PLGA mesh, PLGA TIPS scaffolds and PLGA + TIPS biphasic scaffold was performed utilising a Hitachi TM3000 Environmental Scanning Electron Microscope (SEM) and Bruker Skyscan Ultra-high resolution micro-computed tomography (microCT) in accordance with the ASTM F2450-18 Standard Guide for Assessing Microstructure of Polymeric Scaffolds for Use in Tissue-Engineered Medical Products [31]. Morphological characterisation of PLGA TIPS and PLGA mesh + TIPS scaffold pore size and pore shape was determined through the individual measurement of pores (n = 20) on four replicates, respectively. Pore sizes on a single unmodified PLGA mesh specimen were assessed similarly (n = 22) for comparison. Analysis of the data was performed using ImageJ (NIH) software. Morphological characterisation was performed on freshly prepared scaffolds at time zero (T0), as well as at each specified time point in the degradation study.

The density of the fabricated biphasic scaffolds (\( \rho_d \)) was estimated by dividing mass by volume, in accordance with previous methods [17]. Porosity (\( \varepsilon \)) was determined using standard PLGA 75:25 polymer biomaterial density (\( \rho_p \)) [32, 33], as well as the respective calculated PLGA mesh, PLGA TIPS or PLGA mesh + TIPS density (\( \rho_f \)), in accordance with equation (1) below [34].

Equation (1). Porosity equation for PLGA mesh, PLGA TIPS and PLGA mesh + TIPS scaffolds

\[
\varepsilon = \frac{\rho_p - \rho_f}{\rho_p}
\]

Mechanical testing

Mechanical characterisation involved the determination of the ultimate tensile strength (UTS), elastic modulus, and failure strain. The tensile properties of the PLGA mesh, PLGA TIPS and PLGA mesh + TIPS scaffolds were quantified. Additionally, a control consisting of PLGA mesh encapsulated in EC solution containing no PLGA was also characterised to determine if the EC solvent processing step compromised mesh tensile properties (PLGA mesh + EC). Mechanical characterisation was performed utilising a 30 kN Instron universal tester and 500 N load cell, in accordance with the ASTM D3039/D3039M-17 Standard Test Method for Tensile Properties of Polymer Matrix Composite Materials [35]. Characterisation parameters utilised a gauge length of 20 mm and specimen strain rate of 50%/min, with a minimum of four replicates evaluated for each scaffold type. The effect of EC on the unmodified PLGA mesh was also qualitatively compared to the commonly used organic solvents dimethyl sulfoxide (DMSO) and 1,4-dioxane (see supplementary figure 1).

Degradation studies

The physical properties of PLGA mesh or PLGA mesh + TIPS scaffolds were characterised weekly following simulated degradation studies over 7 weeks. PLGA mesh, or PLGA mesh + TIPS scaffolds were individually weighed prior to initiation of the degradation study. Degradation was performed in individual sterile 50 ml sample containers containing 25 ml of phosphate buffered saline (PBS, GIBCO) supplemented with 0.1% Sodium Azide (Sigma-Aldrich). The PBS to scaffold mass ratio (100:1) was selected based on recommendations from the ASTM F1635-16 Standard Test Method for in vitro Degradation Testing of Hydrolytically Degradable Polymer Resins and Fabricated Forms for Surgical Implants [36]. The PBS volume was exchanged weekly, and the pH of the PBS solution was measured at each interval. Every week, four or more PLGA mesh or PLGA mesh + TIPS scaffold replicates were collected, rinsed and frozen (−20°C). Water/ice was removed via sublimation, and each scaffold was weighed to estimate PLGA mass loss. Dried scaffolds were stored at −20°C until further characterisation.

Biphasic scaffolds capacity to support cell culture

Biphasic PLGA mesh + TIPS scaffolds were cut into sections 0.5 cm × 1 cm for cytocompatibility characterisation. To wet and stabilise the scaffolds, each scaffold was placed into a 40 μm cell strainer (Corning) and 70% ethanol dripped through the scaffold. To ensure that this process wetted all surfaces and that air bubbles were eliminated the wetted scaffolds were individually transferred to 6-well tissue culture plates (NUNC) containing 10 ml of 70% ethanol in each well. The samples/solutions were then placed under vacuum for 30 min. Sinking of the specimens to the bottom of the specimen containers was assumed to indicate that entrained air had been eliminated. Following degassing, scaffolds were rinsed twice with PBS and then allowed to soak in PBS for 1 h to eliminate any residual ethanol.

To aid cell seeding onto scaffolds, polydimethylsiloxane (PDMS) moulds were fabricated to reduce the overall seeding area, as shown in supplementary figure 2. PDMS moulds were cast directly into fresh 6-well tissue culture plates around a single centrally placed sample tube of 14 mm diameter, which was used to form the cell seeding cavity. The depth of the moulds was 5 mm, yielding a cell seeding cavity with a volume capacity of ~770 μl. The 6-well tissue culture plates with the cast PDMS cell culture moulds were then sterilised with 70% ethanol according to a
protocol previously developed by our group [37]. Wetted biphasic PLGA_{mesh} and PLGA_{mesh}+TIPS scaffolds were coated with human fibronectin (Sigma-Aldrich F2006) to facilitate cell attachment [38]. Scaffolds were transferred aseptically to 6-well tissue culture plates (NUNC) containing the cell seeding PDMS moulds and 500 μl of 10 μg ml^{-1} fibronectin in PBS. The fibronectin was permitted to absorb to scaffolds overnight at 4 °C.

Fibronectin-coated biphasic PLGA_{mesh}+TIPS scaffolds were then seeded with either 2 × 10^{4}, 5 × 10^{4}, or 1 × 10^{5} HaCaTs suspended in 750 μl of cell culture medium, in replicate (n = 4). These scaffolds were characterised after day 1 and 5 of culture. Fibronectin-coated PLGA_{mesh} specimens were only seeded at the highest density of 1 × 10^{5} HaCaTs and analysed after day 5 of culture. To model HaCaT cell proliferation, control cell cultures with seeding densities of 1 × 10^{4}, 2 × 10^{4}, and 3 × 10^{4} cells in replicate (n = 4) were simultaneously established in a 24-well plate (NUNC) to provide a relative comparison of HaCaT proliferation on 2D surfaces versus the more complex scaffold surfaces. 2D HaCaT controls were characterised after day 1, 3, and 5 of culture. HaCaTs are an immortal keratinocyte cell line widely described in the literature [38–40] and used in similar scaffold characterisation studies [29, 39]. Cell culture medium contained DMEM-high glucose, plus 10% fetal bovine serum (FBS) and penicillin streptomycin (P/S) (all from ThermoFisher). Cells were permitted to attach to the scaffolds or 2D controls for 16 h at 37 °C in a humidified 5% CO_{2} incubator. Cultures were fed an additional 2 ml of culture medium on day 1 and day 3 of culture, where appropriate.

The number of HaCaT cells in each culture was estimated using the alamarBlue™ assay [41, 42]. Scaffolds were aseptically transferred to a new 24-well tissue culture plate containing 500 μl of fresh medium per well, and then 500 μl of fresh medium containing 6% (v/v) alamarBlue™ added to commence a 2-h incubation (3% (v/v) final alamarBlue™ concentration). AlamarBlue™ concentration and incubation time was based on previous work completed by our group [43]. Cell culture plates were placed in a 37 °C humidified 5% CO_{2} incubator for 2 h. Following incubation, 100 μl of supernatant was transferred from each well to a black 96-well plate (Corning) and the fluorescence intensity read using the BMG LABTECH FLUOstar Omega multidetection microplate reader. Replicate assays were performed on day 3 and 5 for 2D cell cultures, and day 5 for the scaffold cultures. Cell numbers were back calculated from the alamarBlue™ assay outcomes using an HaCaT cell standard curve (5 × 10^{2} to 1 × 10^{5} HaCaT per well in replicate (n = 4), in a 48 well plate).

To better understand the role fibronectin coating of scaffolds and medium serum supplementation played in HaCaT cell attachment, we conducted short-term cell attachment assays. Pieces (0.5 cm × 1.0 cm) of PLGA_{mesh}+TIPS scaffolds were either coated with fibronectin as described above, or left uncoated. Each scaffold was seeded with 2 × 10^{4} HaCaT cells in either serum-containing (+ FBS) or serum-free (-FBS) cell culture media, in (n = 2) replicate for each condition. Cells were permitted to attach for 4 h at 37 °C in a humidified 5% CO_{2} incubator.

**Fluorescence staining and confocal microscopy**

Following the cell expansion (alamarBlue™) assay and cell attachment assay, PLGA_{mesh}, PLGA_{mesh}+TIPS, and 2D control cultures were washed three times with PBS, fixed for 1 h at room temperature in 4% paraformaldehyde (Sigma-Aldrich), washed twice again and stored in PBS prior to fluorescence staining. For staining, cells on all scaffolds and in 2D cultures were permeabilised with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min and washed twice with PBS. Cells were stained with DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride, Sigma-Aldrich) to identify nuclei, and with Phalloidin-TRITC (Sigma-Aldrich) to identify F-actin in the cytoskeleton. Cell adhesion, migration and proliferation was then investigated and imaged using an Olympus FV3000 Confocal Microscope.

**Statistical analysis**

Data analysis for morphological characterisation (pore diameter), mechanical characterisation (UTS, elastic modulus, and failure strain), and degradation characterisation (change in pH and PLGA mass loss) involved the calculation of PLGA_{mesh} and PLGA_{mesh}+TIPS mean and standard deviation at each respective time point, where pertinent. Statistical analysis was performed utilising a Two-Way Analysis Of Variance (Two-Way ANOVA) with a 95% confidence interval. A p-value of <0.05 was accepted as statistically significant. The degree of statistical significance was denoted with asterisks as follows *** <0.001, ** <0.01, * <0.05, or as non-significant (ns) >0.05.

**Results**

**Manufacturing optimisation**

During manufacturing process optimisation, we identified that key variables were (1) exposing the system to a 4 °C quench temperature, (2) degassing the PLGA + EC solution, (3) the use of glass as the mould material to cast the biphasic PLGA_{mesh}+TIPS scaffolds, and (4) prewarming scaffold components and assembling the scaffold at 40 °C. Supplementary figure 3 contrasts the pore sizes formed in the TIPS scaffold when nucleation was triggered either at 4 °C or 24 °C. When nucleation was triggered at 24 °C after thermal equilibration the pore structure was heterogeneous, large closed pores formed, and scaffolds lacked the desired interconnected pore structure (supplementary
figures 3(C)–(J)). Large closed pores with limited interconnectivity are characteristic of TIPS scaffolds that undergo phase separation whilst in the metastable region of the temperature-composition phase diagram [44, 45]. When nucleation was initiated at 24 °C, it is likely that the PLGA + EC system was transitioning through the metastable region and subsequently TIPS proceeded via the thermodynamic mechanism of nucleation and growth (NG). By contrast, when TIPS nucleation was triggered after the scaffolds had been quenched at 4 °C for 4 min (supplementary figures 3(K)–(R)) the pore structure was highly interconnected, and the pore size was homogenous (further characterisation provided in figure 2 below). The preferred microporous and highly interconnected TIPS morphology produced when a 4 °C quench temperature was used is indicative of the thermodynamic mechanisms of spinodal decomposition (SD) [44, 45]. When SD occurs, a system will phase separate into polymer-rich and polymer-lean domains that yield a uniform and bi-continuous structure [44]. This is ideal, as it is the leaching of the polymer-lean domain (EC solvent) that yields the desired highly porous and interconnected structure. In these preliminary studies we also observed that when the PLGA + EC solution was not degassed and quenched at 4 °C (supplementary figures 3(K)–(N)), gas would come out of solution during the phase separation process forming bubbles that created voids in the TIPS scaffold. By contrast, when the PLGA + EC solution was degassed and quenched at 4 °C (supplementary figures 3(O)–(R)), few bubbles formed, and the TIPS scaffolds were continuous.

In our early work, we considered using PDMS as the casting material for the biphasic scaffolds. The advantage of using PDMS as a moulding material is that it can be easily shaped [46], and this shape could have been used to impart a superstructure on the biphasic scaffold. A weakness associated with using PDMS in this application was that its thermal conductivity is low (0.15 W · m⁻¹ · K⁻¹ [47]), and this functioned to insulate the PLGA + EC solution during the quenching process resulting in spatial temperature gradients and ineffective cooling. The pore size of TIPS scaffolds is influenced by the temperature at nucleation [17, 48], and the impact of temperature gradients in PDMS moulded scaffolds was visible (supplementary figure 4). When PDMS moulds were used, the pore structure was observed to vary across the width of a single scaffold (supplementary figure 5), as well as considerably between scaffolds (data not shown). Based on these observations we used glass to mould the biphasic scaffolds. The thermal conductivity of glass (1 W · m⁻¹ · K⁻¹ [49]) is approximately 6-fold greater than that of PDMS.

Through the optimisation process we identified that if the glass slide mould structure was pre-heated to 40 °C (EC melting temperature is 36.4 °C [21]), the PLGA + EC solution added, and the moulds immediately transferred to a 4 °C refrigerator, the PLGA + EC solution could be consistently supercooled and that the supercooled solution would be stable in the liquid phase for 5 min. This strategy conveniently enabled us to observe the cooling process and manually initiate TIPS by physically agitating the supercooled solution at 4 min (supplementary video 1). All subsequent work was conducted using glass moulds and nucleation triggered after 4 min of quenching at 4 °C.

**Morphological characterisation of PLGA scaffolds**

The PLGAmesh pore openings demonstrated directionality, with continuous fibre bundles that could be orientated either horizontally or vertically, as shown in figures 2(A) and (B). The pore shape of unmodified PLGAmesh was tear-drop, as shown in figures 2(A) and (B). The pore size diameter of the unmodified PLGAmesh was determined to be 508 ± 26.72 μm in the longest dimension (L), and 377 ± 24.37 μm in the shorter dimension (W). These parameters were set by Biomedical Structures Ltd (Boston, USA) at our request and could be tailored by modifying the knit or fibre bundle parameters.

The TIPS process yielded a continuous interconnected pore structure. PLGA TIPS scaffolds were found to exhibit highly homogeneous and microporous morphology, with uniform pore structures predominantly circular in shape. The pore size diameter of PLGATIPS scaffolds was characterised to be 15.78 ± 3 μm, as shown in figure 2(L) and supplementary figure 6. In the manufacture of PLGAmesh+TIPS scaffolds, figure 2(C) highlights the homogeneous and microporous formation of the TIPS structure, as well as the predominantly circular pore shape of the TIPS scaffold. MicroCT imaging (figure 2(D) and supplementary video 2) clearly shows that the mesh is uniformly encapsulated within the TIPS structure. A bubble that spontaneously formed in one scaffold offered the opportunity to view the pore structure within the biphasic PLGAmesh+TIPS scaffold (figure 2(E)). The TIPS pore structure at the core of the scaffold appeared larger than the pore structure on the surface where the PLGA + EC solution would have been in direct contact with the supercooled glass slide. Cross-sectional imaging of PLGAmesh+TIPS scaffolds confirmed the presence of larger pores towards the centre of the constructs (supplementary figure 7). Furthermore, cross-sectional imaging also revealed that in a PLGAmesh+TIPS Scaffold that underwent nucleation prior to the four-minute quench period had substantially larger pore sizes in the core of the scaffold compared to scaffolds that underwent the full 4 min quench period (supplementary figure 7). The larger pore size sometimes evident at the core of the scaffold indicated a likely thermal gradient through the depth of the scaffold. Previous TIPS studies have shown that when phase separation occurs at
Figure 2. Morphological characterisation of PLGA\textsubscript{mesh}, PLGA\textsubscript{TIPS}, and PLGA\textsubscript{mesh+TIPS} constructs. (A) Horizontal orientation of thread in knit mesh perpendicular to uniaxial tensile testing plane; (B) Vertical orientation of thread in knit mesh parallel to uniaxial tensile testing plane; (C) PLGA\textsubscript{mesh+TIPS} porous structure under 250X; (D) MicroCT images of PLGA\textsubscript{mesh+TIPS} scaffold; (E) PLGA\textsubscript{mesh+TIPS} scaffold with rare spontaneous bubble formed through porous TIPS structure showing the embedded PLGA\textsubscript{mesh}; (F)–(I) PLGA\textsubscript{mesh+TIPS} replicates highlighting reproducible morphology; (J)–(K) Variability in pore size diameter across PLGA\textsubscript{mesh+TIPS} replicates; (L) Morphological characterisation of PLGA\textsubscript{mesh}, PLGA\textsubscript{TIPS}, and PLGA\textsubscript{mesh+TIPS} mean volume, density, porosity, and pore size.

| Experimental Condition | Mass ± Std Dev. (g) | Volume ± Std Dev. (cm\(^3\)) | Density ± Std Dev. (g/cm\(^3\)) | Porosity ± Std Dev. (%) | Pore Size ± Std Dev. (μm) |
|-----------------------|---------------------|-------------------------------|---------------------------------|-------------------------|--------------------------|
| PLGA\textsubscript{mesh} | 0.020 ± 0.0005 | 0.07 ± 0.003 | 0.29 ± 0.014 | 77.72 ± 1.05 | (L) 508 ± 26.72 (W) 377 ± 24.37 |
| PLGA\textsubscript{TIPS} | 0.004 ± 0.0002 | 0.05 ± 0.01 | 0.076 ± 0.013 | 94.17 ± 0.99 | 15.78 ± 3.00 |
| PLGA\textsubscript{mesh+TIPS} | 0.029 ± 0.002 | 0.12 ± 0.02 | 0.24 ± 0.026 | 81.8 ± 1.99 | 16.85 ± 3.78 |
a higher temperature, the pore size is greater, while a lower temperature yields smaller pores [17, 48]. As such, it is likely that the temperature in the core of the scaffold was greater than the temperature on the surface, and therefore larger pores formed when the system underwent phase separation. This is consistent with the concept that pore size is affected by the solution temperature during phase separation [17, 48].

Figures 2(F)–(I) shows SEM images from four replicate PLGA_{mesh+TIPS} scaffolds, whilst the respective replicate pore sizes measured were quantitatively shown in figure 2(K). Replicates that exhibited the largest difference in morphology were selected for characterisation to ensure the broadest possible pore size variability was represented. The mean pore diameter across the four replicates was calculated to be 16.85 ± 3.78 μm (figures 2(F)–(I)). Whilst there was some variation between the replicate scaffolds, the pore size distribution was similar for all four samples. The pore size distribution obtained from the four replicates yielded a relatively narrow pore size range of 10 μm to 27 μm. Pore diameters of approximately 14 μm to 19 μm were the most common pore sizes measured, with approximately 55% of the pores comprising this range. Further characterisation of the TIPS scaffold pore shape was complicated by the interconnected nature of the construct, which yielded pores within pores (note that this interconnective pore structure is a desired outcome).

The table in figure 2(L) shows the relative porosity of the different scaffolds. The mean density of unmodified PLGA_{mesh} PLGA_{TIPS} scaffold, and the PLGA_{mesh+TIPS} biphasic construct was determined to be 0.29 g cm⁻³, 7.6 × 10⁻² g cm⁻³, and 0.24 g cm⁻³, respectively. The mean porosity of unmodified PLGA_{mesh}, PLGA_{TIPS}, and the PLGA_{mesh+TIPS} scaffold was also calculated and determined to be 77.7%, 94.2%, and 81.8%, respectively. The porosity calculated for our PLGA_{mesh+TIPS} scaffold was influenced by the assumption in both the density and porosity equations that the construct being measured was uniform in composition, density and porous fraction distribution. We observed through morphological characterisation that porosity was not evenly distributed, as we had fabricated a biphasic construct from scaffolds with distinctly different physical properties. As such, it was deemed reasonable to assume that the majority of the PLGA_{mesh+TIPS} Volume that cells could access would have a porosity of 94%, aligning with the TIPS portion of the construct. The porosity of the PLGA_{TIPS} scaffold was derived from a 5% mass/volume solution of PLGA in EC and expected to be approximately 95%. The calculated 94% porous PLGA_{TIPS} functioned to increase the porous fraction of the PLGA_{mesh} which was knitted from tight bundles of PLGA fibres.

**Mechanical testing and fibre orientation**

The PLGA_{mesh}, PLGA_{TIPS}, and PLGA_{mesh+TIPS} constructs were characterised mechanically. PLGA_{mesh} and PLGA_{mesh+TIPS} were also further characterised over a 7-week *in vitro* degradation study. The knitted mesh in the manufacture of the PLGA_{mesh} resulted in primary fibre bundles that run either vertically or horizontally, yielding a different ultimate tensile strength (UTS) depending on the bundle orientation. The mean UTS of PLGA_{mesh+TIPS} scaffolds was determined to be significantly different with respect to orientation of the PLGA_{mesh} fibre bundles. PLGA_{mesh+TIPS} with fibre bundles aligned vertically had a UTS at T0 of 40.7 ± 2.2 MPa, while those with vertical alignment had a UTS of 32.6 ± 6.6 MPa, as shown in figure 3(A). Perhaps counterintuitively, having the fibre bundles perpendicular to the tensile force yielded the greatest UTS. The influence fibre bundle had on UTS was quantified over the degradation study, and found to be significant (p < 0.005) for up to 2 weeks, after which the difference between the conditions was determined to be non-significant.

**Mechanical testing and influence of manufacturing process**

The mean ultimate tensile strength (UTS) for PLGA_{mesh}, PLGA_{TIPS}, and PLGA_{mesh+TIPS} Constructs was determined to be 39.1 ± 3.2 MPa, 0.21 ± 0.04 MPa and 40.7 ± 2.2 MPa respectively, as shown in figure 3(B). Statistical analysis indicated that the mechanical properties of PLGA_{mesh} and PLGA_{mesh+TIPS} were similar, suggesting that the addition of the PLGA_{TIPS} to the PLGA_{mesh} did not modify the UTS of the mesh. However, the PLGA_{TIPS} construct on its own was extremely fragile as shown by the negligible UTS. Thus, within the PLGA_{mesh+TIPS} biphasic scaffolds, it was the PLGA_{mesh} primarily responsible for the observed mechanical strength and tensile properties. We further characterised the mechanical properties of the PLGA_{mesh} after it was exposed to EC that did not contain PLGA, and measured the UTS (figure 3(B), condition PLGA_{mesh+EC}). These samples had an UTS of 38.3 ± 1.7 MPa, which was statistically similar to the unmodified PLGA_{mesh}. As the cumulative results of the PLGA_{mesh}, PLGA_{mesh+EC} and PLGA_{mesh+TIPS} yielded a similar UTS we concluded that our manufacturing process did not compromise the mechanical properties of the mesh.

**Tensile properties following degradation**

The UTS of both PLGA_{mesh} and PLGA_{mesh+TIPS} was determined to be the greatest at T0, as shown in figure 3(C), with the mean calculated to be 39.1 ± 3.2 MPa and 40.7 ± 2.2 MPa, respectively. At T0, PLGA_{mesh} and PLGA_{mesh+TIPS} scaffolds were mechanically similar. Following 1 week of degradation (T1), the UTS of the PLGA_{mesh+TIPS}
biphasic scaffolds was greater than the PLGA\textsubscript{mesh} (p = 0.003), where the mean UTS of PLGA\textsubscript{mesh} and PLGA\textsubscript{mesh+TIPS} was determined to be 30.64 ± 2.9 MPa and 36.71 ± 1.4 MPa, respectively.

Both PLGA\textsubscript{mesh} and PLGA\textsubscript{mesh+TIPS} scaffolds exhibited consistent loss of tensile strength from T0 to T4, after which the compromised structural integrity of PLGA\textsubscript{mesh} and PLGA\textsubscript{mesh+TIPS} rendered them incapable of undergoing accurate mechanical characterisation. PLGA\textsubscript{mesh} and PLGA\textsubscript{mesh+TIPS} underwent significant reduction in mechanical properties between T1 and T3.

The mean elastic modulus for PLGA\textsubscript{mesh} exhibited a slight increase in magnitude from T0 (43.43 ± 4.4 MPa) to T2 (49.73 ± 9.7 MPa), as shown in figure 3(D), after which a rapid reduction occurred, and specimens could not be measured accurately after T4 (11.84 ± 2.5 MPa). The mean elastic modulus for PLGA\textsubscript{mesh+TIPS} underwent similar changes in mechanical properties and exhibited an increase from T0 (48.69 ± 3.3 MPa) to T1 (49.38 ± 4.2 MPa), after which it significantly diminished by T4 (11.21 ± 1.8 MPa). The change in elastic modulus from T0 to T2 was not statistically significant for both

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**Figure 3.** Mechanical and degradation properties of PLGA\textsubscript{mesh} and PLGA\textsubscript{mesh+TIPS} with respects to time. (A) The ultimate tensile strength (UTS) of the PLGA\textsubscript{mesh+TIPS} biphasic scaffold was tested with the primary fibre bundles orientated horizontally or vertically (n = 4). (B) The UTS of the PLGA\textsubscript{mesh}, PLGA\textsubscript{TIPS}, PLGA\textsubscript{mesh+EC} and the PLGA\textsubscript{mesh+TIPS} were evaluated (n = 4). (C) The UTS of the PLGA\textsubscript{mesh} and PLGA\textsubscript{mesh+TIPS} measured and compared over 4 weeks of in vitro degradation (n = 4). (D) The elastic modulus calculated for the PLGA\textsubscript{mesh} and PLGA\textsubscript{mesh+TIPS} following degradation. (E) The failure strain calculated for the PLGA\textsubscript{mesh} and PLGA\textsubscript{mesh+TIPS} following degradation, and (F) the pH of the PBS solution was measured each week during of the degradation study (line graph, n = 4), and the percent mass lost at each week quantified (bar graph, n = 4). (G)–(K) are images of the PLGA\textsubscript{mesh} fibre bundles at each point on the degradation timeline, and (L)–(P) show the degradation of the TIPS network in the PLGA\textsubscript{mesh+TIPS} biphasic scaffold over the same timeline.
PLGAmesh and PLGAmesh+TIPS. The change in elastic modulus for both PLGAmesh and PLGAmesh+TIPS from T0 to T4 was not statistically significant between sequential timepoints; however, the overall reduction in modulus between T0 and T4 was statistically significant for both PLGAmesh and PLGAmesh+TIPS conditions (P < 0.001).

An inconsistent failure cascade was exhibited by a number of PLGAmesh and PLGAmesh+TIPS specimens (as demonstrated in supplementary figure 8). In these specimens, fibres or regions under disproportionate localised stress would undergo failure, resulting in a brief decrease in the load (N) on the construct without gross mechanical failure occurring. The strain at failure was therefore accepted as the strain at the UTS, as it was generally after this point that the gross failure cascade commenced to occur. Failure strain rate was calculated as a percentage of gauge length.

The failure strain for both PLGAmesh and PLGAmesh+TIPS underwent a reduction in magnitude from T0 to T4, as shown in figure 3(E). The mean failure strain for PLGAmesh exhibited relative linear decline from 117 ± 11.5% at T0 to 18 ± 2% at T4. The change in failure strain for PLGA mesh+TIPS exhibited similar linear decline from 102 ± 3% at T0, to 17 ± 3% at T4. Statistical analysis indicated that the difference in strain at failure between PLGAmesh and PLGA mesh+TIPS conditions at each respective time point was not significant.

Mass loss during degradation

Figure 3(F) displays the change in pH in the PBS degradation solution as the PLGA mesh or PLGA mesh+TIPS degraded and corresponding percent mass loss. The pH between the degradation solutions for the PLGA mesh and PLGA mesh+TIPS was similar over time. The pH in both solutions declined over the first 5 weeks (T0 to T5), and then plateaued until week 7 (T7). The similarity in pH behaviour suggested that both scaffolds had similar degradation chemistry, which is rational as the scaffolds were composed of a common material. This pattern of pH decline and degradation in vitro has been previously reported for the 75:25 PLGA blend [50], however at a comparatively slower rate. The initial wave of degradation in a 75:25 PLGA blend is the poly(glycolic acid) (PGA) which hydrolyses rapidly, followed by the poly(lactic acid) (PLA), which is more stable [51, 52].

The percent mass loss was also estimated for both PLGA mesh and PLGA mesh+TIPS scaffolds. Mass loss correlated with pH, with the pH reduction being associated with accelerated mass loss. While the pH of the degradation solutions was similar, the percent mass loss differed over time between the PLGA mesh and PLGA mesh+TIPS scaffolds. Over the first four weeks of degradation, the mass loss from scaffolds was minute and similar between the PLGA mesh and PLGA mesh+TIPS scaffolds. Subsequently, mass loss was more rapid for the PLGA mesh alone, with these samples being too fragile following 5 weeks of degradation (T5) to enable reliable mass quantification. By contrast, PLGA mesh+TIPS biphasic scaffolds were more robust and mass quantification could be carried out until week 7 (T7), at which time approximately 51% of the scaffold mass had been lost. These results are consistent with degradation patterns observed in similar previous studies performed in vitro [50].

Analysis of the images of the PLGA mesh and PLGA mesh+TIPS scaffolds (figures 3(G)–(P)) suggest that the fibre bundles had significantly degraded by week 5 (T5), but that the TIPS structure added modest support to the degrading fibre bundles. Fibre bundles in the PLGA mesh were seen to be fraying by week 4 (T4), and by week 6 (T6) the PLGA mesh underwent gross mechanical failure prior to, or upon, aspiration of the PBS degradation solution.

Characterisation of HaCaT growth on the PLGA mesh and PLGA mesh+TIPS biphasic scaffolds

To assess the potential of the PLGA mesh+TIPS biphasic scaffold to support HaCaT cell attachment and growth in vitro, we used the alamarBlue™ assay and microscopic analysis (figure 4). The alamarBlue™ assay indicated that cells were proliferating and increasing in number on the PLGA mesh+TIPS over time (from day 1 to day 5, figure 4(A)). We seeded scaffolds at three cell densities, LOW, MEDIUM, and HIGH. Representative images of LOW, MEDIUM and HIGH cell seeding densities are shown at day 1 (figures 4(C)–(E)) and day 5 (figures 4(F)–(H)). The LOW cell seeding density cultures underwent the greatest change in cell number, expanding to approximately 6.5 × 10⁴ cells, or 5.9-fold at day 5. The MEDIUM cell seeding density cultures also reached approximately 6.5 × 10⁴ cells, or 3-fold expansion. The HIGH cell seeding density had the least cell numbers at day 5, reaching an average of approximately 5.5 × 10⁴ cells, or 1.6-fold expansion. The estimated cell number at day 5 was statistically similar for LOW, MEDIUM and HIGH cell densities, suggesting that confluenacy had been reached. The difference in expansion observed between the three density conditions was likely due to changes occurring in the proliferation mode of the keratinocyte cell line (HaCaT). As keratinocytes reach confluency they transition from proliferative mode to a balanced mode, where proliferation is generally only triggered to backfill void areas introduced by damage to the epithelium, and we would expect proliferation to taper as cultures reached confluence [53]. The fibronectin-coated PLGA mesh facilitated HaCaT adhesion (figure 4(B)), however because of the large voids between fibre bundles, it was unable to support a continuous epithelial layer (figures 4(J) and (K)). The inability of keratinocytes/HaCaTs to traverse the significant pore sizes of the PLGA mesh and the reduced cell supportive capacity of the PLGA mesh is indicative
of the benefits the TIPS structure imparts on the biphasic scaffold.

Cell expansion results were mirrored by the 2D HaCaT cell cultures, see supplementary figure 9. The alamarBlue™ assay revealed that the LOW cell seeding density cultures reached approximately $1.03 \times 10^5$ cells or 10-fold expansion. The medium cell seeding density reached $1.21 \times 10^5$ cells or 6.5-fold expansion.
The high cell seeding density 2D cultures reached 1.53 x 10^5 cells or 6-fold expansion at day 3, and then dropped to approximately 1.27 x 10^5 cells at day 5. Like the 3D scaffold cultures, the 2D culture surfaces reached a maximum where they could no longer support further HaCaT cell expansion.

To confirm that surface-coating was necessary to support cell attachment and growth on scaffolds, we compared fibronectin-coated and uncoated scaffolds. Surface modification of PLGA scaffolds commonly involves either absorbing extracellular matrix molecules (ECM) or crosslinking of ECM cell-binding motifs onto PLGA surface [34]. The role of fibronectin coating, as well as medium serum supplementation, on HaCaT cell attachment on PLGA_{mesh+TIPS} scaffolds is shown in supplementary figure 10. Coating scaffolds with fibronectin resulted in significant numbers of HaCaT cells being attached and having a visibly spread morphology on scaffolds at 4 h when the culture medium was supplemented with or without FBS (supplementary figures 10(A), (B)). Supplementation of medium with FBS appeared to increase the total number of cells attached (supplementary figures 10(E)–(H)). Few HaCaT cells attached to uncoated scaffolds and appeared to have a rounded morphology, regardless of medium supplementation with FBS (supplementary figures 10(C), (D)). These data suggest that coating PLGA_{mesh+TIPS} scaffolds with fibronectin increases the number of cells attached to the scaffold and their spread area, consistent with previous reports [54, 55].

Discussion

In developing scaffolds for dermal wound repair there are a number of biological and mechanical variables that contribute to efficacy. Designing mechanically robust scaffolds that facilitate ease of handling by clinicians and can withstand the loads placed on this tissue will promote the likelihood of their uptake and use [56]. Critical design features include appropriate morphology, biocompatibility, mechanical resilience against typical in vivo environmental stresses [57], as well as degradation kinetics that can be tuned to align with typical wound healing cascades [58]. Whilst skin tissue engineering is not a new field, the capacity to combine the necessary morphological, mechanical, and degradation properties in a single construct remains challenging. Herein, we developed a scaffold manufacturing process that enabled the encapsulation of a PLGA knit structure into a highly porous PLGA_{TIPS} scaffold. The mechanical advantages of using knit structures are significant, capturing centuries of textile optimisation and scaled production technologies [11]. While TIPS scaffolds can be fragile, they yield a highly interconnected porous structure that is ideal in tissue regeneration applications. Using EC as a solvent, coupled with an optimised manufacturing process, we were able to assemble a porous PLGA TIPS structure around a PLGA mesh. The result was the reliable manufacture of a PLGA_{mesh+TIPS} biphasic scaffolds that exhibited consistent microporous and interconnected morphology, robust mechanical properties, and degradation kinetics conducive to wound healing applications. To our knowledge no group has previously described a viable method to encapsulate a PLGA mesh into a PLGA TIPS scaffold to generate a thin scaffold having similar strength and porous structure.

Our refined PLGA_{mesh+TIPS} biphasic scaffold indicated suitability for wound healing applications as it exhibited a porosity of 94%, as well as homogenous pore structures on its surface with an average pore size of 16.85 ± 3.8 μm. There are a number of factors that can influence the pore size diameter including quench temperature, quench period, quench rate, polymer molecular structure, polymer concentration, and aging time [59]. We maintained control over these conditions through optimisation of the protocol, and using this protocol were able to generate replicates with a relatively narrow pore size range of 10–28 μm. Whilst variability between replicates showed statistical significance, the common narrow pore size range across all the characterised replicates demonstrates that the primary features are uniform. Slight differences in pore size measurements can reflect limitations in being able to assign dimensions to pores with complex geometry. For example, many pores have a complex ‘pore within pore’ structure. Nevertheless, pore size variability could be further reduced through enacting greater control over thermodynamics of TIPS, through the use of thermocouples and heat exchangers. Keratinocytes, the predominant cell type in the epidermis, have shown preferential growth on highly porous and interconnected constructs [57], and pore sizes below 30 μm have been shown to support formation of a keratinocyte epithelial layer [13, 14]. Achieving a continuous and homogeneous epithelial layer is a primary objective in wound healing, as the restoration of barrier function represents a primary function of skin [60]. The PLGA_{mesh+TIPS} biphasic scaffold appears to be appropriately designed to achieve this primary objective.

We show that PLGA_{mesh+TIPS} scaffolds exhibited robust mechanical properties, suitable for the repair of wounded skin. The mechanical properties of human skin exhibit a broad range of variability due to the significant influence that biomechanical requirements of the region, age, direction of Langer lines, and method of testing can have [28, 61]. Nevertheless, widely accepted values for native human skin include a UTS of 21.6 ± 8.4 MPa, elastic modulus of 83 ± 34.9 MPa, and failure strain of 54 ± 17% [61]. The PLGA_{mesh+TIPS} scaffolds fabricated in this study were shown to exhibit a baseline UTS of 40.7 ± 2.2 MPa, elastic modulus of 48.7 ± 3.3 MPa, and failure strain of 102 ± 3%. In addition to satisfying the
biomechanical properties of native skin, the tensile strength of the PLGAmesh+TIPS biphasic scaffold was also observed to be considerably greater than commonly utilised dermal substitutes such as Integra™, rehydrated Alloderm™, and decellularised human dermal matrix, which exhibit a UTS of 0.26 ± 0.023 MPa [62], 21.2 ± 2.9 MPa [63], and 13.7 ± 0.26 MPa [64], respectively. Robust mechanics will facilitate ease of handling and usability in clinical applications, as well as prevent premature destruction when utilised in vivo [58].

The significant tensile strength of the PLGAmesh conferred ultra-strong tensile properties to the PLGAmesh+TIPS construct that would enhance its ability to withstand clinical handling, manipulation during use, and capacity to be sutured into the wound site. It was noted during the initial characterisation that the PLGAmesh exhibited a degree of anisotropy, dependant on the orientation of the fibre bundle with respect to the direction of tensile stress. Anisotropy in the PLGAmesh was determined to not impede the potential clinical efficacy or application of our PLGAmesh+TIPS construct, as it was calculated to exhibit 2 times the UTS of native skin in vivo when orientated perpendicular (40.7 ± 2.2 MPa), and 1.5 times the UTS of native skin in vivo when orientated in parallel (32.6 ± 6.6 MPa).

The reduced modulus, as well as increased failure strain, of PLGAmesh+TIPS was understood to be the result of the PLGAmesh construct containing geometric slack that could absorb and realign in response to load. The ability for the PLGAmesh to undergo deformation without destruction in the initial phases of mechanical characterisation is characteristic of knit mesh structures, and subsequently enabled the construct to undergo greater extension without the associated stress upon the construct [65, 66]. The specimens were also observed to undergo non-uniform destruction, with individual fibres under disproportionate stress undergoing isolated failure first. The localised failure of these high stress fibres often did not result in the failure of the gross construct, but rather facilitated realignment in the construct and redistribution of load. This subsequently influenced the constructs calculated modulus and strain at failure, and indicated that the PLGAmesh+TIPS exhibited greater deformation capabilities and subsequent elasticity compared to native skin in vivo.

Our data indicated that the PLGAmesh+TIPS biphasic scaffolds gradually lost tensile strength as they degraded over four weeks in vitro in PBS. While the presence of the TIPS structure modestly supported the PLGAmesh in the 5th week of the degradation study, neither the PLGAmesh nor the PLGAmesh+TIPS biphasic scaffolds retained sufficient mechanical integrity at week 5 to be accurately characterised using an Instron. SEM images of the PLGAmesh and PLGAmesh+TIPS structures revealed visible decay by week 4. The loss of mechanical integrity correlated with the decline in buffer pH, indicating the liberation of glycolic acid from hydrolysing co-polymer [51, 52]. The mechanical failure of the PLGAmesh and PLGAmesh+TIPS correlated with accelerated mass loss in weeks 4–6 of the assay. The rate of degradation for our PLGAmesh+TIPS constructs was determined to be more rapid than other scaffolds manufactured from a 75:25 PLGA blend [50, 67], and this was largely attributed to the influence of scaffold geometry on degradation, as well as the unknown monomer sequence of the PLGAmesh and raw PLGA pellets used [51]. It would also be expected that the rate of degradation would be incrementally faster in vivo due to biological factors [68]. This rate of degradation aligns with normal rates of dermal wound healing [60], and so it would be reasonable to anticipate that extracellular matrix deposited by cells contributing to tissue regeneration would compensate for the declining mechanical contributions of the scaffold.

The HaCaT keratinocyte cell culture assay highlighted the capacity for the PLGAmesh+TIPS structure to support HaCaT adhesion, proliferation, and the formation of a homogeneous epithelial layer. HaCaT proliferation was observed at LOW, MEDIUM, and HIGH cell seeding densities, and resulted in the formation of a highly confluent scaffold after 5 days of culture. The similarity in cell number at day 5 between the LOW, MEDIUM, and HIGH cell densities is an indication that the scaffolds became saturated and had reached their maximal cell carrying capacity in cell culture. Confocal analysis of the scaffolds validated that confluent HaCaT monolayers had formed on the scaffolds seeded with LOW, MEDIUM, and HIGH cell densities. While unmodified PLGAmesh also supported cell adhesion, unlike the PLGAmesh+TIPS, the mesh alone could not support formation of a homogeneous HaCaT keratinocyte monolayer by day 5. This was due to the significant pore sizes (508 ± 26.72 μm by 377 ± 24.37 μm) within the mesh, and the inability of keratinocytes to traverse pore diameters larger than 30 μm [13, 14].

There were a number of methodological insights generated through this study that may be useful to other researchers, and efforts to develop new scaffold manufacturing processes. Optimisation of our PLGAmesh+TIPS manufacturing methodology included (1) degassing the polymer/solvent solution, (2) utilising glass scaffold moulds, (3) and incorporating a 40 °C heating block upon which scaffold templates were pre-warmed and scaffolds were assembled, (4) transferring the assembled scaffold moulds to a 4 °C refrigerator for 4 min, and (5) manually stimulating phase separation by physically agitating the super-cooled PLGA + EC solution.

We found that degassing of the PLGA + EC solution was essential. Bubble formation in the PLGA + EC scaffolds generated via TIPS can occur because the solution is mixed vigorously during the preparation process, entraining air in the solution.
Simultaneous degassing of the PLGA + EC solution while heating minimises subsequent bubble formation during scaffold manufacture. In our hands, this led to a more consistent scaffold morphology.

EC is an elegant solvent for use in the manufacture of thin scaffolds where temperature can be controlled. In order to maximise control over solution temperature the scaffold mould should have a high thermal conductivity. Optimisation of the TIPS methodology was required to promote late-stage nucleation in our system. Late-stage nucleation is characteristic of systems that have achieved significant supercooling [69]. Industrial manufacturing processes would use heat exchangers and thermocouples to regulate scaffold manufacture cooling rates [19]. As we were reliant on control over the bulk environment only, we used thin glass sheets to form the mould for the biphasic scaffold. In early optimisation we trialled using PDMS as the moulding material, but it has a low thermal conductivity coefficient (0.15 W·m⁻¹·K⁻¹) relative to glass (1 W·m⁻¹·K⁻¹) [47, 49]. When PDMS moulds were used, a visible spatial gradient in pore size was observed, consistent with likely temperature gradients (see supplementary figure 5). By contrast, when glass was used as the mould, the pore size was uniform across the width of the scaffolds suggesting that temperature gradients were minimal. Glass was similarly advantageous in that its transparency aided the ability to monitor, image, and record the thermodynamics of supercooling and the TIPS scaffold fabrication process (supplementary video 1). Scaffold templates could be configured from other materials, provided they satisfied the necessary physical and surface properties as well as thermal conductivity.

Pre-heating all materials to 40 °C led to more consistent scaffold manufacture. EC has a melt temperature of 36.4 °C [21], and at 40 °C the PLGA in EC solution remains a stable liquid. By heating all components to 40 °C it was possible to assemble and manipulate the scaffold moulds without risk of spontaneous phase separation. The use of EC similarly benefited scaffold assembly with a reduced likelihood of PLGAmesh dissolution, as the assembly temperature of 40 °C remained below the 50°C–55 °C glass transition temperature of the 75:25 PLGA co-polymer [70].

Supercooling the PLGA + EC solution in a 4 °C refrigerator for 4 min enabled the desired porous morphology. The ability to achieve significant supercooling of the system, prior to nucleation and TIPS, results in highly porous and interconnected structures with micropores <30 µm, suitable for keratinocyte adhesion and proliferation [48, 69]. However, scaffold systems that are cooled below their melting temperature increasingly become less thermodynamically stable and seek to reduce their Gibb’s Free Energy by undergoing spontaneous nucleation and TIPS [69, 71]. In our hands, PLGA + EC solution reliably remained stable for 4 min in a 4 °C environment.

Manually stimulating phase separation by physically agitating the supercooled PLGA + EC solution gave us the capacity to consistently generate the described TIPS porous structure. The supercooled PLGA + EC solution is unstable, and in our hands the frequency of spontaneous nucleation and phase separation increased after 5 min at 4 °C. By selecting to initiate nucleation at 4 min, we were able to reliably control when phase separation occurred. When supercooled nucleation of the PLGA + EC solution could be easily triggered by mechanically compressing or agitation the glass moulds.

Conclusion

This paper describes a novel method to manufacture a biphasic scaffold from a PLGA knit mesh and porous PLGA TIPS scaffold (PLGAmesh+TIPS). Our protocol outlines important considerations to replicate and optimise this scaffold manufacturing process. Coupling knitted meshes with designs derived from the textile industry yields a thin biphasic scaffold having an UTS greater than most dermal scaffolds, and that is ideal for the stresses associated with dermal tissue repair. Our extensive evaluation of the impact of the scaffold processing method on the PLGAmesh and on the PLGAmesh+TIPS biphasic scaffold demonstrated that the UTS was unchanged by processing or by the addition of the TIPS porous scaffold. This important outcome demonstrated that pre-fabricated PLGA mesh can be manufactured using traditional textile methods, and then encapsulated in a porous PLGA+TIPS scaffold without compromising the integrity of the mesh. This biphasic scaffold provides an excellent platform for greater exploration in dermal wound healing, whilst our highly-manipulable methodology suggests broader capabilities for enhanced scaffold fabrication in bone, cartilage, skeletal, cardiac, and vascular organ repair.

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Statement of significance

The impressive mechanical and manufacturing properties of textiles could be better utilised in tissue engineering. Here we describe a novel biphasic scaffold manufacturing method that assembles a PLGA
thermally induced phase separation (TIPS) scaffold structure around a PLGA biodegradable textile mesh, without compromising the mechanical properties of the mesh. The addition of the continuous TIPS porous structure makes the mesh a functional scaffold. This is the first description of ethylene carbonate (EC) use as a solvent to assemble a PLGA TIPS scaffold around a PLGA mesh. The resulting thin single-material, biphasic scaffold has outstanding tensile properties, making it suitable for use in dermal or tubular organ repair.

Disclosures

The authors declare no conflict of interest.

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