Development of Macroporous Chitosan Scaffolds for Eyelid Tarsus Tissue Engineering

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Received: 30 October 2018/Revised: 11 June 2019/Accepted: 13 June 2019/Published online: 26 July 2019
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Abstract

BACKGROUND: Reconstruction of large eyelid defects remains challenging due to the lack of suitable eyelid tarsus tissue substitutes. We aimed to evaluate a novel bioengineered chitosan scaffold for use as an eyelid tarsus substitute.

METHODS: Three-dimensional macroporous chitosan hydrogel scaffold were produced via cryogelation with specific biomechanical properties designed to directly match characteristics of native eyelid tarsus tissue. Scaffolds were characterized by confocal microscopy and tensile mechanical testing. To optimise biocompatibility, human eyelid skin fibroblasts were cultured from biopsy-sized samples of fresh eyelid skin. Immunological and gene expression analysis including specific fibroblast-specific markers were used to determine the rate of fibroblast de-differentiation *in vitro* and characterize cells cultured. Eyelid skin fibroblasts were then cultured over the chitosan scaffolds and the resultant adhesion and growth of cells were characterized using immunocytochemical staining.

RESULTS: The chitosan scaffolds were shown to support the attachment and proliferation of NIH 3T3 mouse fibroblasts and human orbital skin fibroblasts *in vitro*. Our novel bioengineered chitosan scaffold has demonstrated biomechanical compatibility and has the ability to support human eyelid skin fibroblast growth and proliferation.

CONCLUSIONS: This bioengineered tissue has the potential to be used as a tarsus substitute during eyelid reconstruction, offering the opportunity to pre-seed the patient's own cells and represents a truly personalised approach to tissue engineering.

Keywords Eyelid fibroblasts · Macroporous scaffold · Chitosan · Hydrogel · Eyelid reconstruction

1 Introduction

Eyelid reconstruction remains among the most challenging areas of oculoplastic reconstructive surgery and the most common indication following skin cancer removal. In

Australia, skin cancer is the most prevalent cancer and excision remains the mainstay of treatment. The eyelid is involved in 10% of cases and functional reconstruction remains an ongoing challenge which can significantly impact the quality of life of those affected [1]. Large full thickness lid defects require reconstruction of both the anterior and posterior lamella. The posterior lamella includes the palpebral conjunctiva and a highly specialised tissue called the tarsus [2]. The tarsus provides both support and structural form, making it an essential component of the eyelid's function and physical appearance. Natural tarsus is a specialised tissue which consists of fibroblast cells surrounded by an extra-cellular matrix (ECM) with types I and III collagen, as well as aggrecan [3]. Among the

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most challenging aspects of eyelid reconstruction is construction of a tarsal substitute due to its delicate and multi-layered tissue composition.

Presently there are no tarsal substitutes which can fully replace or replicate the functions of native tarsal tissue, therefore a new solution is required to achieve better functional outcomes for patients [4, 5]. Tissue engineering represents the future of regenerative medicine and has huge potential to advance our current reconstructive techniques by producing highly specialised and personalised tissue replacements. To the best of our knowledge, there are only two previous studies that investigated the use of polymeric scaffolds for tarsal repair [6, 7]. These studies used acellular polymer scaffolds as synthetic tarsal substitutes in rats and rabbits, and found that they were successful in supporting eyelid reconstruction and fibroblast growth. However, the authors noted inflammatory tissue responses within the first 2 weeks of implantation, which developed into a milder chronic inflammation thereafter. Importantly, these studies were conducted prior to published data on the normal biomechanical properties of human tarsus tissue, and the scaffolds used by Gao et al. [7] were much stiffer than native tarsus tissue.

Having established the biomechanical properties of fresh human tarsal tissue [8], in this study we report the development of a novel bioengineered chitosan hydrogel scaffold designed biomechanically mimic human tarsus tissue, onto which we were able to successfully seed human eyelid fibroblast cells. Naturally-derived polymers such as chitosan have excellent potential as tissue engineered scaffolds for soft tissues due to their high water content and biomimetic properties [9–13]. Chitosan is derived from chitin, which is primarily found in the exoskeletons of crustaceans, and modified via alkali deacetylation, rendering it biodegradable in mammals. It can be degraded by endogenous enzymes (e.g., lysozymes) *in vivo*. Chitosan scaffolds are particularly applicable in tarsus engineering owing to their chemical similarities to glycosaminoglycan (GAG) molecules found in the native extra-cellular matrix of tissues. Although hydrogel scaffolds tend to be relatively soft due to their high water content, their strength and stability can be enhanced through chemical cross-linking [14, 15]. To improve biocompatibility, we established culture of human eyelid skin fibroblasts using biopsy-sized tissue and successfully seeded these cells onto our novel scaffold to create a truly personalised tissue construct which has the potential to be utilised as a tarsus tissue substitute during eyelid reconstruction.

2 Materials and methods

Chitosan (medium molecular weight 190–310 kDa, degree of deacetylation 85%), glycine, and phosphate buffered saline (PBS) were purchased from Sigma Aldrich, Sydney, Australia. Acetic acid (AnalaR, BDH) was used to dissolve the chitosan. Analytical grade sodium hydroxide (NaOH) and ethanol were purchased from Chem Supply, Adelaide, Australia. Glutaraldehyde (GTA, 25 w/w%) (Ajax Finechem, Adelaide, Australia) was used as a crosslinker. Ethylene glycol (Ajax Finechem, Adelaide, Australia) was diluted by 50% with water to be used in water baths operating below 0 °C. All solutions were made with ultrapure water purified to a resistivity of 18.2 MΩ in a Millipore water filtration system (Millipore, Victoria, Australia).

NIH 3T3 mouse fibroblasts (ATCC CCL-92, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, Sydney, Australia) supplemented with 10% bovine calf serum (Scientific Life, Victoria, Australia), 4 mM glutamine and 100 U/mL penicillin–streptomycin (Sigma Aldrich, Sydney, NSW, Australia). Cells were detached from culture plates using 0.25% trypsin (Gibco Life Technologies, Auckland, New Zealand).

Resazurin (Sigma Aldrich, Sydney, NSW, Australia) dye was dissolved in PBS to make up 440 μM Alamar blue assay, which was filtered through a 0.22 μm syringe filter (Millipore, Melbourne, Australia) and used for assessing cell proliferation. Confocal imaging of cell seeded scaffolds was performed using CellMask™ Deep Red Plasma membrane stain (ThermoFisher Scientific, Adelaide, SA, Australia) followed by DAPI- 4',6-diamidino-2-phenylindole (DAPI, Abcam, UK).

Cryogelation was used to produce three dimensional (3D) macroporous chitosan hydrogel scaffolds with varying weight/volumes by cooling solutions of the polymers to below the solvent freezing point under controlled conditions. The mixture was thus separated into a water-rich phase that crystallised and a polymer-rich phase with an increased polymer concentration that gelled to form the walls of the resulting scaffold. A crosslinking agent (glutaraldehyde) was added to stabilise the structure before it was returned to room temperature, when the water in the pores melted and the porous scaffold was obtained, as shown schematically in Fig. 1. The pore size and internal architecture can be regulated by controlling the processing parameters including mould geometry, freezing rates, chitosan and cross-linker concentrations [14–18].

Chitosan solutions of different concentrations (3 and 5 w/v%) were prepared by dissolving chitosan in 4 mL of 5 v/v% acetic acid at 37 °C for 24 h. To these solutions,

Fig. 1 Schematic illustrating the steps involved in scaffold synthesis

1 mL of freshly prepared GTA (0.04 or 0.1 v/v% in water) was added, mixed and centrifuged at 4000 RPM for 5 min to remove air bubbles. The final chitosan solutions were injected into either a cylindrical or rectangular mould. The cylindrical moulds were 28 ml polypropylene screw cap containers (Techno Plas, Adelaide, SA, Australia) with a diameter of 27 mm. The rectangular moulds comprised two glass microscope slides (ThermoFisher Scientific, Adelaide, SA, Australia) separated by a 3D printed acrylic polymer spacer (Objet Eden 260 V, 60 × 15 × 1.6 mm) held together with clear adhesive tape and sealed in plastic to prevent water ingress. The moulds were chilled in a water bath (Alpha Ra 12 Lauda, Melbourne, Australia; containing 50% ethylene glycol) set at -20°C for 24 h. The frozen and crosslinked scaffolds were then neutralized overnight at room temperature, mixing at 120 rpm on an orbital shaker (Zhicheng, All Lab Scientific, Victoria, Australia) in excess 3 M NaOH. Scaffolds were washed (3 ×) in fresh water for 10 min under orbital shaking at 180 rpm. Scaffolds were then washed in excess PBS for 1 h (at 180 rpm) followed by excess 0.1 M glycine in PBS (at 180 rpm) for 4 h to cap any unreacted GTA. Scaffolds were stored at 4°C in PBS until use.

Confocal microscopy was utilized for observation of the pore morphology in the hydrated and swollen scaffolds. Thin sections were cut from the surface and centre of the scaffolds, mounted on microscope glass slides and observed using a Nikon A1R confocal microscope. Scaffolds were kept hydrated with PBS. A 20 × objective lens and 488 nm laser were used for image acquisition and the emission spectra were collected at 500–550 nm, exploiting the scaffolds' intrinsic fluorescence. Pore size calculations were performed using Fiji and ImageJ software (National Institute of Health, Bethesda, MD, USA). To avoid possible bias, a nine-lined horizontal grid was placed over the images and the pores along the overlaid lines were

measured [15]. A minimum of 100 pores per sample were analysed and the mean and standard deviations (s.d.) reported for each fabrication condition.

Tensile tests were carried out using an Instron Microtester 5848 (Instron, Melbourne, Victoria, Australia) with a temperature controlled water bath (BioPuls, Instron, Melbourne, Victoria, Australia), under the same operating conditions as previously described for tarsus tissue [8]. Scaffolds made using rectangular moulds were mounted between two pneumatic clamps and lowered into the water bath containing PBS at 37°C . The average width and thickness of each scaffold were determined from five equally spaced calliper measurements along the scaffold length. The samples were held for 10 min at a tensile pre-load of 50 mN before extension at a rate of 1%/s to a maximum of 30% extension. Dynamic cycling was performed with a data collection rate of 167 Hz over 15 cycles. The raw data from the 15th cycle was used to calculate the mechanical properties via a custom-written MATLAB function (MATLAB R2014b, The MathWorks Inc., Natick, MA, USA). The initial and final moduli were calculated from the initial linear region and the final linear region of the stress–strain curve, respectively, using a moving linear fit with the highest coefficient of determination [8]. The elongation was calculated from the point of intersection of the two moduli tangents.

Initial cell compatibility studies were conducted using NIH 3T3 fibroblast cells as a model cell line, as fibroblasts are a key component of tarsus tissue. Circular samples were cut from the rectangular scaffolds using a 5 mm hole punch and soaked in 80% ethanol overnight for disinfection, followed by washing (3 ×) in sterile PBS. Scaffolds were soaked in supplemented DMEM overnight to allow saturation of scaffold pore walls with nutrients and proteins from the media. This step was introduced as our previous studies showed that cell proliferation could be adversely

affected by nutrient and protein sequestration by chitosan hydrogels [15]. Scaffolds were then transferred to sterile gauze and gently squeezed to remove excess DMEM from the pores, and placed in sterile Corning 96 well plates (Sigma Aldrich, Sydney, Australia). This was done to utilize the hydrogel's swelling properties and capillary action to improve cell infiltration throughout the interconnected scaffold pores.

To observe whether cells could attach and survive on chitosan hydrogel scaffolds, 100,000 cells per 100 μ L of supplemented DMEM were aliquoted onto the scaffolds and incubated for 2 h (at 37 °C, 5% CO₂) to allow cell infiltration and attachment in 96 well plates. Each scaffold well was then topped up with a further 100 μ L of supplemented DMEM and incubated for 24 h.

After incubation, the scaffolds were sectioned with a sterile scalpel. A live dead assay, CellMask™ Deep Red stain, was diluted 100 times from stock in supplemented DMEM and 100 μ L was added to each of the sectioned scaffolds and incubated at 37 °C for 1 h. Scaffolds were then washed with sterile PBS to remove excess dye before adding 100 μ L of 1% GTA solution to each well for fixation and incubating at 37 °C for 4 min. The solution was removed and the scaffolds were washed with sterile PBS (3 $\times$). To stain cell nuclei, 100 μ L of DAPI dye, (diluted 1000 times from stock in PBS) was added to each well and incubated at 37 °C for an hour and then scaffolds were again washed in PBS.

Imaging was achieved using a Nikon A1R confocal microscope with a 20 $\times$ objective lens, exciting at 640 nm for CellMask™ Deep Red labelled cells, 405 nm for DAPI labelled cell nuclei, and 488 nm for the scaffolds. Composite images of the scaffolds and fluorescently labelled cells were overlaid and stitched together using imaging processing software (Fiji) [19].

For cell proliferation studies, scaffolds were disinfected, swelled in supplemented DMEM and blotted on sterile gauze, as described previously. Cells (5000 per 100 μ L media) were added to each test scaffold. Scaffolds were incubated for 2 h in a humidified incubator at 37 °C, 5% CO₂ to allow cell attachment to the scaffold walls. Control wells contained either cells alone (5000 cells per 100 μ L of supplemented DMEM), media alone (100 μ L) or scaffolds plus media (100 μ L). Finally, all the wells were topped up with 100 μ L of supplemented DMEM each and incubated for 24 h.

The proliferation of the 3T3 fibroblast cells was measured using the Alamar blue assay. To Each well 20 μ L of Alamar blue dye was added and incubated for 4 h to allow the dye to be reduced by metabolically active cells. From each well 100 μ L of the dye + DMEM mixture was transferred to wells of an opaque Whatman 96 well plate (Sigma Aldrich, Australia). The spent dye and DMEM

mixture were removed and the scaffolds were washed in PBS. Then 200 μ L of fresh supplemented DMEM was to the scaffolds and controls and the plate incubated for another 24 h. Fluorescence readings were measured using an Infinite 200 Pro fluorescent plate reader (Tecan, Mannedorf, Switzerland) with excitation and emission wavelengths of 545 nm and 590 nm, respectively. Readings for Alamar blue dye in media alone were subtracted from the signal for each well. This process was repeated at 48 and 72 h.

In this preliminary study, establishment of eyelid skin fibroblast culture was achieved using human eyelid skin removed for various surgical procedures which would otherwise have been discarded. Patient consent was obtained and this study was approved by the Royal Adelaide Hospital ethics committee.

After removal of a piece of tissue for histological analysis via fixation in 10% neutral buffered formalin, the remaining sample was divided into 1–2 mm cubes which were first washed in sterile phosphate-buffered saline (PBS; 137 mM NaCl, 5.4 mM KCl, 1.28 mM NaH₂PO₄, 7 mM Na₂HPO₄; pH 7.4) and then immersed in orbital fibroblast (OF) culture medium (Dulbecco's modified eagle medium plus low glucose; DMEM with 5.5 mM glucose; supplemented with 100 U/mL penicillin/streptomycin, 2 mM L-glutamine and 5% heat-inactivated foetal bovine serum) in a 30 mm diameter petri dish. Four pieces of tissue were incubated in each dish at an orientation of 90° to each other. Sterile borosilicate glass 13 mm diameter coverslips were applied vertically onto the tissue pieces in order to encourage migration of fibroblasts. The tissue was then allowed to grow at 37 °C for 5 days, undisturbed. At this point, glass coverslips were removed and the medium changed. After a further 5 days, tissue pieces were removed and the medium changed again. At this point, fibroblasts will have migrated from tissue onto the dishes. Within a further 4 days the fibroblasts typically reached confluence and were passaged into 25 cm² culture flasks (passage number 1). After reaching confluence again, cells were then placed into 75 cm² culture flasks and thereafter cells were split at a ratio of 1:4, typically reaching confluence within 1 week each time.

It is well known that primary cells de-differentiate in culture with increasing passage. It was imperative, therefore, that for the purposes of transplantation, fibroblasts derived from healthy patient tissue were not propagated through too many passages *in vitro*, lest they lost their defining characteristics. The rate of fibroblast de-differentiation *in vitro* was determined by growing cells of increasing passage number to confluence on 13 mm diameter borosilicate glass coverslips and then assessing these for expression of normal protein markers of fibroblasts by immunocytochemistry.

For single-label immunocytochemistry, cells were fixed in neutral buffered formalin for 10 min. Subsequent to fixation, cells were washed three times in PBS, exposed to PBS containing 0.1% triton X-100 detergent (PBS-T) for permeabilisation of membranes for 15 min and then pre-incubated in PBS containing 3.3% (v/v) normal horse serum (PBS-HS). Incubation overnight with primary antibodies that label all cells (α -tubulin, Abcam, Melbourne, Australia; 1:5000), fibroblasts (anti-vimentin, DAKO, Mulgrave, Australia; 1:1000; anti-fibroblast surface protein, Sigma Chemical Company, Castle Hill, Australia; 1:1000) or myofibroblasts (α -smooth muscle actin, Abcam, Melbourne, Australia; 1:1000), to confirm that cells had not differentiated, was followed by washing with PBS and labelling with appropriate biotinylated secondary antibodies (Vector Laboratories, Abacus ALS, Brisbane, Australia; 1:250 in PBS-HS), followed by streptavidin-AlexaFluor 488 or streptavidin-AlexaFluor 594 (Molecular Probes, Invitrogen, Mulgrave, Australia; 1:500 in PBS-HS). Following this, cell nuclei were counter-labelled by incubating coverslips with DAPI (500 ng/mL in PBS). Visualisation was via confocal fluorescence microscopy.

For double-label immunocytochemistry, visualisation of one antigen was achieved as described above using a 3-step procedure (primary antibody, biotinylated secondary antibody, streptavidin-conjugated AlexaFluor 488 or 594), while the second antigen was labelled via a concurrent 2-step procedure (primary antibody, secondary antibody conjugated to AlexaFluor 594 or 488; the “opposite” fluor-tag to that used for the first antigen).

Bioengineered chitosan scaffolds were prepared as discs by using a 5 mm diameter Stiefel biopsy punch (Smithkline Beecham Ltd, Slough, UK). Scaffold discs were placed in 96 well culture plates (Sarstedt, Adelaide, Australia) and soaked in 70% (v/v) ethanol for 8 h and exposed to UV lighting to ensure sterilisation before being washed three times in PBS and then incubated over-night in OF culture medium to allow saturation. For cell attachment, fibroblasts were sub-cultured onto scaffolds by a standard trypsinisation procedure at a density of 1×10^5 cells/mL and left to grow for up to 7 days. These scaffolds with cultured cells were fixed in neutral buffered formalin at appropriate times and characterised by SEM imaging and immunocytochemistry.

Statistical analyses were performed using Minitab 17 (Minitab Inc., State College, PA, USA). Mechanical data and cytotoxicity results were assessed using analysis of variance (ANOVA). Post-hoc data analysis was performed using Tukey’s method. Data were reported as statistically significant at $p < 0.05$.

3 Results

The pore morphologies of the rectangular and cylindrical shaped scaffolds were characterised using confocal microscopy, revealing their highly porous, interconnected pore architectures (Fig. 2). Pore size analysis showed a mean pore size of 9–19 μm at the scaffold surfaces and 24–52 μm at the scaffold centres across the various conditions (Figs. 3, 4). As expected, a distribution of pore sizes was obtained at the different fabrication conditions, with a tendency towards larger pores at the centres of the scaffolds than at their surfaces [14, 16]. This is attributed to the slower rates of cooling inside the scaffolds than at their surfaces, allowing time for larger solvent crystals to form during the cryogelation process. The mould geometry also affects the pore morphology, due to its impact on the heat transfer rates within the cryogelling mixture, with more elongated pores obtained at the centres of the scaffolds produced in cylindrical moulds and more random pore morphology obtained from the rectangular moulds. Thus, the pore sizes and shapes in the cryogelled scaffolds can be regulated both through the polymer concentration and the heat transfer conditions. Previous studies suggested that a pore size of 15–160 μm is sufficient for fibroblast growth and proliferation [9], so the scaffolds fabricated here with internal interconnected pores of sizes of 24–52 μm are expected to be suitable for fibroblast culture.

The mechanical properties of the human tarsus tissue were an initial modulus, final modulus and elongation of 0.14 ± 0.1 MPa, 1.7 ± 0.6 MPa and $16 \pm 2\%$, respectively. The scaffolds with the closest mechanical properties to the tarsus tissue were those produced with 5% chitosan and crosslinked with 0.1% GTA, which showed a mean initial modulus, final modulus and elongation of 0.17 ± 0.01 MPa, 0.62 ± 0.1 MPa, and $22.1 \pm 0.02\%$, respectively. These values were comparable to the mechanical properties of the tarsus, particularly when considering that the scaffolds were tested without cells inside the pores whereas the tissue includes cells closely bound to their ECM.

The interactions of the chitosan cryogel scaffolds with fibroblast cells were assessed initially by observation of the attachment, survival and proliferation of 3T3 fibroblast cells in the scaffolds *in vitro*. Confocal images of the scaffolds after 24 h of cell culture revealed metabolically active cells distributed across the scaffold surface and within the scaffold (Fig. 5). The appearance of cells in clusters, too large for passive entry, suggest proliferative capacity with incorporation into the chitosan scaffolds.

The 3T3 fibroblasts proliferated over 72 h in culture, as shown by a statistically significant increase in the Alamar blue dye fluorescence (Fig. 6). These observations of cell

Fig. 2 Confocal images of the centre and surface of 3% and 5% weight/volume chitosan scaffolds with 0.04% GTA in either rectangular or cylindrical moulds. Scale bars: 100 μm

Fig. 3 Mean pore sizes of 3% (dark grey) and 5% (light grey) chitosan scaffolds with 0.04% GTA compared across fabrication mould shape and pore location (mean $\pm$ S.D., $n > 100$). Tensile testing of rectangular 3% and 5% chitosan scaffolds cross-linked with either 0.04% or 0.1% GTA was performed to characterise them in terms of initial (low strain) modulus, final modulus and elongation of the scaffolds. The initial modulus values increased significantly with increasing chitosan concentration (by ANOVA). The final modulus also increased with the chitosan concentration and with GTA concentration (for the 5% chitosan scaffolds), as would be expected as the polymer content and extent of crosslinking of the hydrogel increases. There were no significant differences in the elongation values of the scaffolds

penetration, survival and proliferation in the scaffolds further indicate their potential as supports for fibroblast growth. Thus, they were next assessed for interactions with primary human orbital fibroblasts, as desired for tarsus tissue engineering.

From initial cell harvesting, the orbital skin fibroblasts typically reached the stage of confluent passage 2 culture after approximately 3 weeks *in vitro*. All cells in passage 1

and passage 2 labelled with alpha-tubulin, which co-labelled with vimentin. Vimentin in turn, co-labelled with fibroblast surface protein (Fig. 7). None of the cells labelled with alpha-smooth muscle actin (α -SMA), suggesting they had not yet become fibrotic or myofibroblastic. However, cells passaged through greater numbers demonstrated reduced expression of fibroblast-specific markers.

After establishing cultures in which all cells expressed fibroblast markers and none had differentiated into myofibroblasts (Fig. 7), it was decided to limit scaffold-seeding experiments to cells at passage numbers 1 and 2. Some cells were then either seeded onto 3% or onto 5% chitosan scaffolds or, concurrently, onto normal coverslips, as growth controls. We found that our both the 3% and the 5% chitosan scaffolds were successful in supporting growth and proliferation of fibroblasts (Fig. 8). The control cells growing concurrently on coverslips were more numerous at both 1 and 7 days *in vitro*, however.

4 Discussion

Our study represents the first step towards a truly personalised bioengineered eyelid tarsus tissue substitute. We have created a 3D polymeric chitosan scaffold which is biomechanically similar to the native tarsus and is able to support the growth of human fibroblasts derived directly from the target tissue using clinically-applicable, biopsy-sized samples of eyelid tissue.

Tissue engineering aims to restore pathologically altered tissue architecture by using bioengineered scaffolds and transplanted cells. Chitosan is a partially deacetylated derivative of chitin [20], the primary structural polymer in

Fig. 4 Mean **A** tensile moduli and **B** elongation values of 3% chitosan (dark grey) or 5% chitosan (light grey) crosslinked with 0.04% GTA (solid) or 0.1% GTA (striped) (mean $\pm$ S.D., * $p < 0.05$, $n = 3$)

Fig. 5 Confocal images of 3T3 murine fibroblast cells after 24 h of growth on rectangular 3% chitosan, 0.04% GTA scaffolds. Merged image projection of the scaffold cross-section (left), scaffold cross-

section (middle) and scaffold surface (right). Scale bars: **A** 100 μ m, **B**, **C**: 25 μ m. Green: chitosan, blue: DAPI-stained cell nuclei, red: mitochondria stained with Deep Red

arthropod exoskeletons, and has been widely investigated in tissue engineering, particularly in the fields of orthopaedics and wound healing [12, 21]. It was approved for widespread use in 2005 as part of the HemCon bandage (HemCon Medical Technologies, Portland, Oregon) which is made from ChitoClear chitosan (Primex, Siglufjörður, Iceland) and creates an antibacterial barrier upon applications [22]. Chitosan demonstrates multiple favourable characteristics for tissue engineering, including the ability to be moulded into various porous structures and intrinsically antibacterial nature [12, 13]. Animal studies have

demonstrated minimal inflammatory reactions when implanted, with successful collagen deposition within the pore spaces [23]. To create a scaffold with the desired biomechanical properties, pore size, orientation and volume fraction, as well as chitosan concentration and degree of cross-linking were carefully calibrated. We found that an average internal pore size of $25 \pm 17 \mu$ m was able to successfully support the growth of human orbital skin fibroblasts *in vitro* which would likely translate to ongoing proliferation *in vivo*.

Fig. 6 Fluorescent intensity of the Alamar blue assay for 3T3 murine fibroblast growth on 3% chitosan, 0.04% GTA scaffolds over 24, 48 and 72 h (mean $\pm$ SD, * p < 0.05, n = 6)

There have been only two previous studies investigating the use of bioengineered scaffolds as a tarsal substitute, although both pre-dated our study on the normal biomechanical properties of tarsus tissue [8], which was the first

of its kind. Zhou et al. [6] studied polyhydroxyalkonate scaffolds implanted in rats, and compared with commercial acellular dermal matrices (ADM) and blank defect controls. The resultant scaffolds demonstrated a macropore size of 5 μ m, which is significantly smaller than our pore sizes, and there were no available biomechanical studies. When examined histologically, the bioengineered scaffolds elicited changes suggestive of an acute, followed by chronic inflammatory response, which contrasted with minimal inflammatory changes around the ADM and blank defect controls. Gao et al. [7] produced scaffolds composed of poly(propylene fumarate)-*co*-2-hydroxyethyl methacrylate (PPF-HEMA) that were characterised based on mechanical and degradation properties. The tensile moduli of the scaffolds were reported to be in the range 5–14 MPa, distinctly higher than those of tarsus tissue and the chitosan scaffolds produced here. Rabbits were used as models to study *in vivo* degradation and biocompatibility of the scaffolds. Histological examination showed a mild, yet more severe inflammatory response than the control ADM group. Fibrous encapsulation was also observed to be

Fig. 7 Immunological and gene expression analysis of cultured orbital skin fibroblasts at passage 1 and 2 with all cells co-labelling with α -tubulin and vimentin or fibroblast surface protein and

vimentin (both standard markers for fibroblasts). Note that cells do not label for α -SMA). Magnification, $\times$ 100. Scale bar equals 200 micrometers

Fig. 8 Demonstration of attachment and growth of human orbital skin fibroblasts on either glass coverslips (A, D) or 3% chitosan (B, E) or 5% chitosan (C, F) scaffolds. Cells were able to attach, as shown after 1 day *in vitro* (DIV; A–C) and also proliferate, as shown after 7 days (D–F). Cells were labelled with an antibody against α -tubulin (red) and counter-labelled with DAPI (blue). Magnification, $\times 400$. Scale bar equals 50 micrometers

thicker in the PPF-HEMA group than the ADM control group.

To the best of our knowledge, there has yet to be a study investigating the use of eyelid skin fibroblast culture in tissue engineering. We used similar culture techniques as previously described with lung fibroblasts derived from lung tissue from scleroderma patients at autopsy [14, 17], and were successful in establishing and maintaining fibroblast culture using biopsy-sized samples of fresh eyelid skin. Although we found that cells cultured beyond passage 2 had started to de-differentiate, previous studies with lung fibroblasts utilised cells between the second and fourth passages [24]. Furthermore, none of our cells in early passages stained for alpha-smooth muscle actin, a marker suggestive of fibrosis. However, Ludwicka et al. [25] found that the lung fibroblasts expressed some markers of human smooth muscle differentiation and partially attributed this to their slightly differing biologic behaviour. Previous studies have demonstrated that fibroblasts from differing origin and anatomical location exhibit varying phenotypes and characteristics [26, 27]. One study comparing fibroblasts cultured from pre-tarsal eyelid skin and presternal skin found that the dermal fibroblasts isolated from the two locations differed in their mechanical properties and response to inflammatory cytokines and growth factors [28]. This highlights the importance of harvesting fibroblasts from the target tissue in order to ensure the similarity of the resultant tissue engineered construct. We anticipate direct applicability in clinical practice with this technique, given the standard practice of taking a biopsy of the eyelid lesion with a small section of healthy surrounding skin prior to excision and reconstruction.

Chitosan has previously demonstrated minimal inflammatory responses when implanted in animal models, and

we hypothesize that the addition of pre-culturing patient's own cultured fibroblasts on the scaffold, allowing them to lay down their own ECM on the surfaces, will further reduce the risk of inflammation. We are currently working on further studies involving animal and eventually human studies to further investigate the suitability of this novel bioengineered tarsal substitute.

In summary, this bioengineered tissue possesses characteristics which, we believe, make it an ideal candidate matrix for use in eyelid reconstruction. By showing that 3T3 mouse fibroblasts successfully incorporate into the matrix and by further demonstrating that human eyelid fibroblasts also attach to and proliferate on these same constructs, we believe that in the future this may offer the possibility for reconstruction of an eyelid by a patient's own cells.

Acknowledgements Dr Sun is supported by a University of Adelaide Early Career Fellowship. This study was partly funded by a New Investigator Grant from the Ophthalmic Institute of Australia and an AVANT Doctor in Training Grant.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement The study protocol was approved by the institutional review board of the Royal Adelaide Hospital (R20131001 HREC/13/RAH/412).

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