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Procedia Engineering 59 (2013) 247 – 254

Procedia Engineering

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3rd International Conference on Tissue Engineering, ICTE2013

Bio-mechanical Properties of Novel Bi-layer Collagen-Elastin Scaffolds for Heart Valve Tissue Engineering

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Abstract

Collagen and elastin are two major components of the extracellular matrix of heart valves. This work examines bi-layer scaffolds made of collagen and elastin for potential use in the tissue engineering of heart valves, by investigating the effects of the layered structure on the mechanical and biological properties. The scaffolds showed anisotropic bending moduli depending on the loading directions (lower when with curvature than against curvature), which mimicked the characteristic behaviour of the native heart valves. The interaction of cardiosphere-drived cells with the scaffolds was characterized by scanning electron microscopy and multiphoton microscopy, and an asymmetrical cell distribution was observed. The bi-layer scaffolds have represented a forwarding step to the tri-layer scaffolds that more closely resemble the native heart valves.

Selection and peer-review under responsibility of the Centre for Rapid and Sustainable Product Development, Polytechnic Institute of Leiria, Centro Empresarial da Marinha Grande.

Keywords: heart valve; scaffold; collagen; elastin; cardiosphere-derived cells; mechanical properties.

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doi:10.1016/j.proeng.2013.05.118

1. Introduction

Heart valves control the unidirectional flow of blood, for the approximately 3 billion cardiac cycles in one's life [1], and thus play an indispensible role in the cardiovascular system. With heart valve diseases being a major health issue (directly causing 21,386 deaths in the US in 2010 [2]), researchers have long been seeking a curative and long-lasting method of heart valve replacement. Tissue engineering, which involves cell culturing in vitro on a scaffold and subsequent implantation of the cell-scaffold composite into the patient for tissue regeneration, is currently considered the most promising way of finding a suitable heart valve substitute [3] .

A native heart valve (taking the aortic valve for example) consists of three distinct layers: fibrosa is on the aorta side of the valve leaflet, with the extracellular matrix (ECM) substance consisting of mainly collagen; ventricularis is on the ventricle side of the valve leaflet and is predominantly comprised of elastin; between the fibrosa and the ventricularis is the spongiosa, which is rich in glycosaminoglycans (GAGs). Also, a native heart valve is mechanically anisotropic due to the layered structure. It has been discovered that, the heart valve leaflet is naturally curved towards the ventricularis side, and is easier to bend towards the ventricularis side, referred as 'With Curvature' (WC), than towards the fibrosa side, referred as 'Against Curvature' (AC), as is shown in Fig 1. Merryman et al reported the bending modulus of 492kPa WC and 703kPa AC of porcine aortic valves [4].

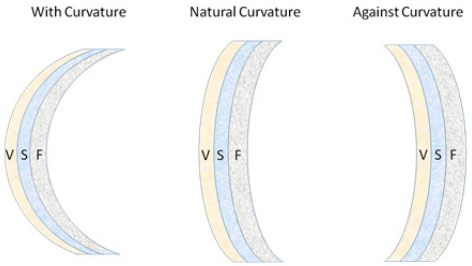

Fig 1. Schematic graph of the bending anisotropy of native heart valve. V, S and F stand for ventricularis, spongiosa and fibrosa respectively. [1]

As the scaffold is acting as an analogue of the ECM of the tissue and is crucial to the tissue engineering process, the scaffolds for tissue engineered heart valves (TEHVs) should both compositionally and mechanically mimic the anisotropic structures of the native heart valve, in addition to the general requirements of biocompatibility.

At the same time, a suitable cell source is also essential to achieve a viable TEHV. Cardiosphere-derived cells (CDCs) are an endogenous stem cell population present in the adult heart and are able to differentiate into the cell types of the three major cardiac lineages [5]. Therapeutic efficacy has been established in animal models [6, 7], and a clinical trial is currently on-going [8], demonstrating that CDCs are a promising candidate for heart tissue regeneration.

This work, therefore, investigated the scaffolds made of collagen and elastin, the two major ECM materials in native heart valves. Mechanical and biological characterizations were carried out on the novel scaffolds with a bi-layer structure which had a collagen-rich layer resembling the fibrosa and an elastin-rich layer resembling the ventricularis. The results presented served as a forwarding step for the preparation of scaffolds with a tri-layer structure that would even more closely mimic the ECM of a native heart valve. This tri-layer structure made of collagen, GAGs and elastin will be presented in the next paper.

2. Materials and Methods

2.1. Scaffolds Preparation

Type I Collagen (from bovine Achilles tendon) and elastin (from bovine neck ligament) were obtained from Sigma-Aldrich Ltd., UK. A mixture of collagen and elastin with the desired collagen-elastin ratio (100%-0%, 50%-50%, 20%-80%) was made into a suspension in 0.05 M ethanoic acid (pH 3.2) [9]. All the suspensions were of the same total concentration of 1% wt/v.

The suspension was then cast into polytetra-fluoroethylene (PTFE) moulds, and was subsequently frozen at a constant temperature of -20˚C in a freezer. To produce bi-layer structures in the scaffolds, casting was performed twice, i.e. a lower layer was cast and frozen first, and then followed by the upper layer. The volume of the suspension for each layer was controlled so as to give an equal thickness. After freezing, the samples were placed in a Christ I-5 freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) for 24 hours in a vacuum of 0.05 mbar, removing the ice crystals by sublimation and leaving the porous solid scaffolds.

2.2. Mechanical Properties

Three-point bend tests were conducted using a DMA7 (PerkinElmer, MA). The samples were of 40mm × 12mm × 5mm rectangular geometries and were loaded at a cross-head speed of approximately 0.5mm/min with a span of 20mm during the test (see Fig 2). For every type of scaffolds, bend tests were conducted in both two directions (WC and AC). All samples were tested dry at room temperature.

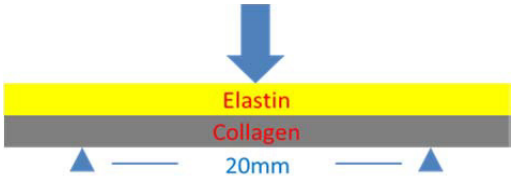

Fig 2. Schematic graph of three-point bend test on bi-layer scaffold

Data were reported as means, with the standard deviations as the errors. One-way analysis of variance (ANOVA) followed by Tukey's test was used to compare groups of data, with a probability value of 95% ($p < 0.05$) to determine statistical significance. At least 6 samples of each group were tested.

2.3. CDCs Culture on Scaffolds

CDCs were isolated from adult Sprague Dawley rats (Harlan) and expanded in accordance with previously published protocols [7, 10, 11]. Briefly, explanted rat hearts were minced, plated on fibronectin-coated petri dishes with complete explant medium (CEM), and then incubated at 37ºC with 5% CO_2. After approximately a week, cells grown out from the explants were isolated and re-plated in poly-d-lysine-coated 24 well plates with cardiosphere growth medium (CGM). Cardiospheres then formed after 2–3 days, and were harvested by mechanical trituration and plated in CEM in fibronectin-coated flasks to culture for CDCs.

For CDCs seeding, the original scaffolds were sectioned into 1mm thick pieces (see Fig 3), and were sterilized by three 30-minute washes with 100% ethanol, followed by three 30-minute washes with Dulbecco's phosphate buffered saline (DPBS). Then they were immersed in CEM in 24 well plates. CDCs (50000 cells per scaffold) were seeded on to the scaffolds and cultured for 7 days at 37ºC with 5% CO_2.

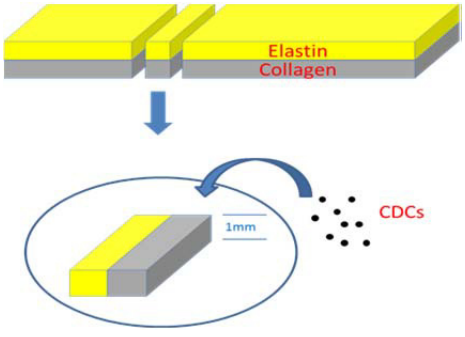

Fig 3. Schematic graph of seeding CDCs onto the bi-layer scaffold

2.4. Scanning Electron Microscopy

Scaffold and cell morphologies were characterized using scanning electron microscopy (SEM) JSM840F (JEOL Ltd) at an accelerating voltage of 4kV. Non-seeded scaffolds were sectioned with a sharp razor blade, and the cross sections were examined. CDCs-seeded scaffolds were fixed at Day 7 in 4% paraformaldehyde (PFA, Sigma-Aldrich) at 4ºC for overnight, then dehydrated through exposure to a gradient of alcohol followed by hexamethydisilazane (HMDS, Sigma–Aldrich), and finally air-dried [12]. All specimens were coated with 3nm thick platinum by a 208HR sputter coater (Cressington Scientific Instruments Ltd) before observation.

2.5. Multiphoton Microscopy (MPM)

Multiphoton microscopy was used to characterize the distribution of cells on the scaffolds, as well as the distinguishing the scaffold materials in different layers.

The scaffolds were imaged using a multiphoton microscope (Radiance 2100MP, Bio-Rad Laboratories Ltd) [13]. The collagen and elastin were auto-fluorescent by second-harmonic generation (SHG) and two-photon excitation (TPE) respectively [14]. The cells were stained with rhodamine phalloidin.

All images were obtained from an excitation wavelength of 800 nm. The signals from collagen, elastin and cells were collected with ~400nm, ~520nm, and ~560nm band-pass filters respectively, and were shown blue, green and red respectively on the images.

For each image, a Z stack was taken, from the surface of the scaffold to the deepest level where signals could be detected, with a step size of 2.55µm. The images were then reconstructed by ImageJ (National Institutes of Health, USA) as projections in the Z direction.

3. Results and Discussion

The SEM images (Fig 4) show that the bi-layer scaffolds have well connected interfaces, and similar microstructures on both layers.

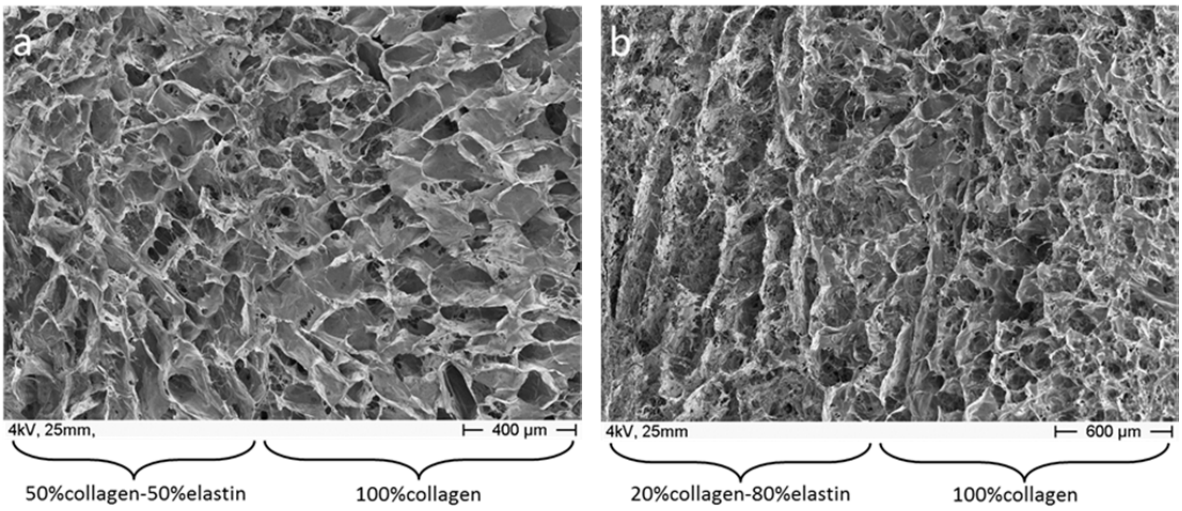

Fig 4. SEM micrographs of the interfaces of (a) 100% collagen / 50% collagen - 50% elastin bi-layer scaffold, and (b) 100% collagen / 20% collagen - 80% elastin bi-layer scaffold.

Mechanical characterization shows that, like the single-component scaffolds that were previously studied [15], bi-layer scaffolds also exhibit two-stage load-deflection curves during bending (see Fig 5). The effective bending modulus of the scaffold is calculated from the slope (δP/δD) of the load-deflection curve, with the equation

$E_{bend} = (\frac{\delta P}{\delta D})\frac{l^3}{48I}$, where P is the load, D is the deflection, l is the span distance, and I is the second moment of area. The E_{bend} for each of the two distinct regions on the curve is obtained (hereby addressed as E_{bend1} and E_{bend2} respectively), and the results are listed in Table 1.

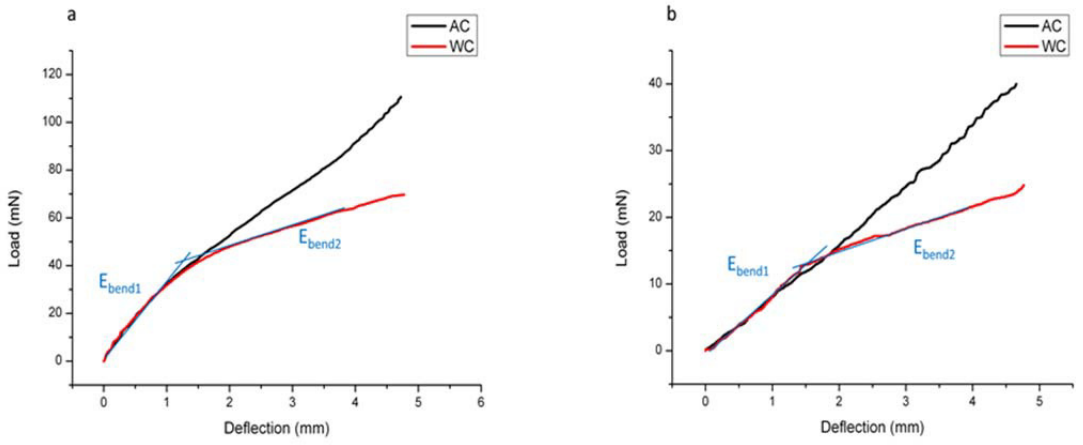

Fig 5. Load-deflection curves of (a) 100%collagen / 50%collagen-50%elastin bi-layer scaffold and (b) 100%collagen / 20%collagen-80%elastin bi-layer scaffold bent in WC and AC directions. The slopes of the first and the second stages on the curves are indicated, and the corresponding effective bending moduli are labeled respectively.

Table 1. Effective bending moduli of bi-layer scaffolds. a Denotes significant difference ($p<0.05$) in bending modulus between WC and AC.

Bi-layer Scaffolds	100%collagen/ 50%collagen-50%elastin		100%collagen/ 20%collagen-80%elastin	
	WC	AC	WC	AC
E_{bend1}(kPa)	70.7±8.5	73.5±4.8	20.7±5.0	20.7±3.8
E_{bend2}(kPa)	21.9±4.1[a]	39.3±3.6[a]	9.6±2.0[a]	18.1±1.4[a]

We can see that the bi-layer scaffolds have almost the same initial bending modulus (E_{bend1}) regardless in which direction they are bent. However, from the second bending stage, the scaffolds show a marked difference between the E_{bend2} in the WC (bent towards the collagen-elastin layer) and the AC (bent towards the collagen layer) directions. This then leads to a difference in the final deflection depending on which direction (WC or AC) the scaffold is loaded, which is considered as the bending anisotropy, as shown in Fig 5.

The bending property of the bi-layer scaffolds is a complex summary of the different mechanical properties of each layer. If we assume that the neutral axis rests at the layer interface of the scaffold when in bending, the effective bending modulus in WC direction will then take contributions from the compressive modulus of the collagen layer and the tensile modulus of the collagen-elastin layer, whereas the effective bending modulus in AC direction will result from the compressive modulus of the collagen-elastin layer and the tensile modulus of the collagen layer. Because each layer has different tensile and compressive moduli [15], a difference between the effective bending moduli in WC and WC directions is then achieved.

In the real situations, the neutral axis will initially rest within the lower layer, which position can be obtained by Eq.1:

$$E_{ten,lower}(h-h_1)^2 = E_{comp,lower}\left(h_1-\frac{h}{2}\right)^2 + E_{comp,upper}\left[{h_1}^2-\left(h_1-\frac{h}{2}\right)^2\right] \quad (1)$$

where h is the total thickness of the scaffold, h_1 is the distance from the neutral axis to the upper surface, $E_{ten.lower}$ and $E_{comp.lower}$ are the tensile and compressive moduli of the lower layer, and $E_{comp.upper}$ is the compressive modulus

of the upper layer. The initial effective bending modulus (E_{bend1}) of the bi-layer scaffolds, therefore, takes into account of the properties of both layers, as is shown in Eq.2.

$$E_{bend1} = 4E_{ten.lower}\frac{(h-h_1)^3}{h^3} + 4E_{comp.lower}\frac{\left(h_1-\frac{h}{2}\right)^3}{h^3} + 4E_{comp.upper}\frac{{h_1}^3-\left(h_1-\frac{h}{2}\right)^3}{h^3} \quad (2)$$

Eq.2 predicts that the same bi-layer scaffold gives similar E_{bend1} in WC and AC bending conditions. However, Eq.2 will fail to represent the bending behaviour of the bi-layer scaffold after a critical deflection around 1mm, due to a membrane effect that is caused by the elongation of the tensile region. Since the two edges of the scaffold on the span are restrained from horizontal displacement, the elongation of the tensile region will become more and more visible as the deflection increases, and will produce membrane force that acts against the load. This will make the E_{bend2} more largely dependent on the tensile modulus of the layer in tension and hence will lead to the anisotropy in E_{bend2}.

The membrane effect is observed to be important in the native heart valves [16], and is also the reason for the higher bending stiffness in the AC direction (especially when there is interaction between the cells and the ECM) [4]. This behaviour of the native heart valves is essential in ensuring the valves' prompt opening in systole, and in preventing sharp reverse curvature that may cause flexure fatigue in the valve leaflets [17]. Therefore, the bending anisotropy of the bi-layer scaffolds that resembles the property of the native heart valves would be desirable for producing mechanically qualified TEHVs.

After 7 days' culture, CDCs show good proliferation on both layers of the scaffolds, as can be seen from the SEM image (Fig 6). However, previous research has demonstrated that the CDCs favour collagen more than elastin when proliferating [15]. This might be due to the presence of different signaling pathways for the cell receptors on collagen and elastin [18, 19]. Moreover, the less resilient elastin is more susceptible to the cell contraction, and this is likely to have caused shrinkage of the scaffold, which, in turn, limits the space for the cell proliferation.

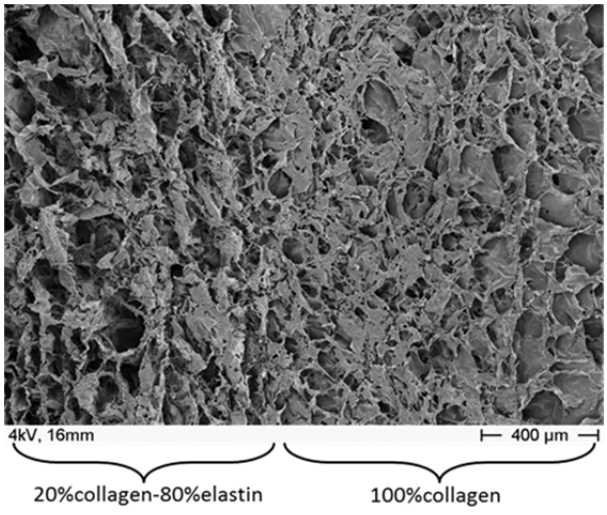

Fig 6. SEM micrograph of CDCs on 100% collagen / 20% collagen - 80% elastin bi-layer scaffold

A preliminary analysis of the multiphoton microscopy (MPM) images of the bi-layer scaffold gives further insights to the actual distribution of the cells on the scaffolds. The MPM image suggests that there are fewer cells, as well as more volume shrinkage on the collagen-elastin layer, in accordance with the fact that the cells respond differently to the different extracellular matrices, giving an asymmetry in cell distribution through the scaffolds. Although further mechanical tests on scaffolds seeded with cells are needed, we believe that the cells will help to increase the mechanical properties of the scaffolds [20], and more importantly, the demonstrated asymmetrical cell distribution within the bi-layer scaffolds will further contribute to the anisotropic behaviours due to different

extents of cell contraction in each layer. Having now provided an initial assessment of the collagen-elastin bi-layer scaffolds, which proves the mechanical and biological capabilities of layered structures, our next step is to create a tri-layered composite scaffold that more closely mimics the native heart valve.

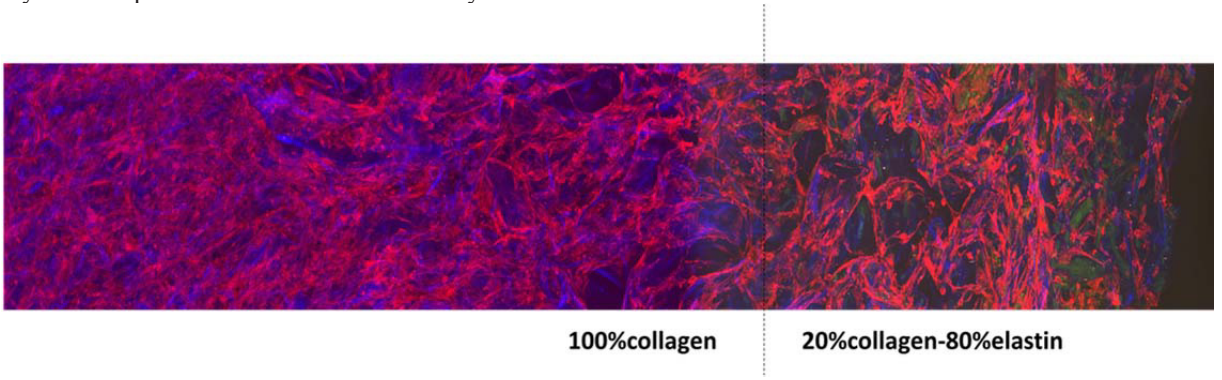

Fig 7. MPM micrograph of CDCs on 100% collagen / 20% collagen - 80% elastin bi-layer scaffold. Collagen, elastin, and CDCs are shown in blue, green and red respectively.

4. Conclusion

Collagen-elastin scaffolds with novel bi-layer structures were fabricated with well-connected interfaces. The difference in the composition in different layers introduced to the scaffolds not only an anisotropic bending behaviour that mimicked the property of a native heart valve, but also a controllability over the cell distribution throughout the scaffold. Both outcomes bode well for a mechanically and biologically qualified tissue engineered heart valve. More importantly, this work represents a step forward from our previous work on collagen TEHVs scaffolds [15, 21-23], to the final stage of the tri-layer TEHVs scaffolds.

Acknowledgements

This study was funded by China Scholarship Council-University of Oxford Scholarship, and by a British Heart Foundation Studentship. The authors would like to acknowledge Prof. Chris Grovenor and Prof. Kieran Clarke for providing the laboratory facilities. Mr. Shaoyang Yeh and Prof. Zhangfeng Cui are thanked for assistance on the multiphoton microscopy.

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