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Characterisation of a collagen membrane for its potential use in cardiovascular tissue engineering applications

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In this study, Biomend®, a collagen membrane conventionally used in the regeneration of periodontal tissue, is investigated for its possible use in the field of cardiovascular tissue engineering. A key requirement of most potential tissue engineering scaffolds is that degradation occurs in tandem with tissue regeneration and extra cellular matrix remodelling. To this end, it is crucial to understand the degradation mechanics and mechanisms of the material and to investigate the practicability of using Biomend® as a possible scaffold material. With this in mind, methodologies for the initial characterisation of the scaffold material were determined. The mechanical properties of Biomend® samples, subjected to various degrees of hydration and enzymatic degradation, were examined primarily through tensile testing experiments. The effects of enzymatic degradation were monitored quantitatively, by observing weight loss, and visually, by studying micrographs. Cell adhesion and viability were of primary concern. Confocal laser scanning microscopy was employed to illustrate endothelialisation on the surface of this collagen membrane. Fluorescence microscopy was used to visualise cell viability on the membrane surface. These images, coupled with assays to measure cell activity, suggest that Biomend® is not a suitable substrate to allow endothelialisation. In summary, this collagen membrane has suitable mechanical properties with the potential to control its degradation rate. However, since poor endothelial cell viability was observed on the membrane, it may not be suitable for use in cardiovascular tissue engineering applications.

1. Introduction

Coronary artery bypass grafting (CABG) is the most common method of replacing diseased blood vessels. However, this method may not always be possible due to previous extraction or disease of suitable autologous vasculature. Furthermore, conventional CABG may result in a diametrical mismatch at the anastomoses, particularly in the case of small diameter vascular grafts (<6 mm), leading to thrombus formation and/or intimal hyperplasia. Prosthetic grafts have shown some clinical success in larger vessels, however in small grafts the induced foreign-body inflammatory response is more likely to lead to vessel occlusion. Tissue engineering has the potential to provide tailor made blood vessels with the compositional, structural and physiological requirements to replace any diseased blood vessel.

In combination with choosing cell source, appropriate signalling and *in situ* incorporation, scaffold selection is paramount in most tissue engineering applications. As well as providing a suitable environment for allowing the adherence of a confluent endothelial layer, the ideal tissue engineered vascular graft (TEVG) scaffold should provide a platform for natural cellular remodelling and vasoactivity which occurs *in vivo*,

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coupled with providing sufficient mechanical integrity and compliance. Essentially, the scaffold should mirror the behaviour of the extra-cellular matrix (ECM) found in a natural vessel or provide a basis from which an ECM can be remodelled.

Collagen is found in the native ECM and therefore provides an ideal substrate for cell adhesion and integration. Along with elastin, it is pivotal to the ECM's role of providing mechanical integrity [1]. It also has the ability to be resorbed, encouraging cellular remodelling and interaction. These factors make it suitable for applications as a scaffold for TEVGs.

Collagen based TEVGs have been investigated extensively over the past two decades. Weinberg and Bell first attempted to tissue engineer an artery by seeding cultured vascular cells in tubular constructs comprised of a collagen gel matrix [2]. However, due to the natural poor mechanical stability of the gels, a Dacron™ sleeve was incorporated into the vessel to provide additional strength. L'Heureux *et al*. [3] and Tranquillo *et al*. [4] have employed techniques in which the collagen matrix based vessel was contracted by seeded cells over a central non-adhering mandrel. Several other attempts have been made to refine this technique, such as changing the components of the cell culture medium [5, 6], varying the initial cell and collagen concentrations [7, 8], and promoting the desirable circumferential alignment of the developing extra-cellular matrix components (ECM) [7–11]. The latter has been shown to provide the most significant improvement in mechanical integrity, in particular through the preconditioning of the constructs in a physiological environment [10, 11]. However, none of these methods produce vessels with mechanical properties matching that of the native vessel, without incorporating a synthetic support sleeve [2, 12, 13]. The introduction of these synthetic non-biodegradable substrates negates much of the initial benefit of using a 'natural' scaffold, by inducing a foreign-body immune response. As a solution to this, Berglund *et al*. [14] have highlighted the prospect of using crosslinked collagen as a support sleeve in developing a TEVG. It is with this in mind that the potential incorporation of commercially available collagen membranes into vascular constructs is investigated.

In this study, Biomend®, a collagen membrane conventionally used in the regeneration of periodontal tissue, is investigated for its possible incorporation into a TEVG. It has the advantages of being FDA approved, easy to handle and has the potential for its mechanical properties and hence its degradation rates to be optimised. To this end, the scaffold has been characterised in terms of its mechanical properties, degradation, and cell adhesion and cell viability in order to determine its suitability.

2. Materials and methods

2.1. Materials

2.1.1. Membrane

Commercially available Biomend®, a guided tissue regeneration (GTR) membrane, conventionally used in periodontal applications is the material used in this study (General Medical, Wiltshire, UK). The membrane is composed of type I collagen derived from bovine achilles tendon crosslinked using glutaraldehyde [15] and sterilised by ethylene oxide gas [16].

2.1.2. Cells

Human umbilical vein endothelial cells (HUVECs) expanded from frozen vials (Cambrex, UK) were used in this study. Cells were incubated at 37 °C in an atmosphere of 5% CO_2 and 100% humidity. HUVECs were maintained in endothelial growth medium supplemented with EBM-2 bullet kit. Cells were grown to 70–80% confluence in 25 cm^2 tissue culture flasks (Sigma, UK) before being passaged using 0.025% Trypsin/0.01% EDTA (Sigma, UK). All experiments were performed on cells at passages 5–8.

2.1.3. Assays and fluorescent dyes

The following assays and dyes were used to examine cell viability in this study: Live/Dead® Viability/Cytotoxicity kit (Molecular Probes, UK), Fluorescent conjugates of phalloidin (Molecular Probes, UK), Hoechst Stain (Sigma, UK), Alamar Blue assay (Biosource Europe).

2.1.4. Enzymatic solution

In vivo, cells including inflammatory cells produce collagenase, an enzyme involved in the natural remodelling of the ECM. To mimic this environment, a physiological solution was prepared. Collagenase was purchased in lyophilised form (Sigma, UK) and then diluted to the desired concentration in a sterile phosphate buffer saline solution (PBS). Depending on the concentration of the enzyme, the solution can be used to simulate the breakdown of the membrane in an *in vivo* environment. Similar to Ma *et al*. [17], for the purpose of this study, the same concentration (500 μg/ml) was used in all experiments.

2.2. Methods

2.2.1. Visual examination

To examine the architecture of the membrane during degradation, scanning electron microscopy (SEM) was used to visually compare samples that had been immersed in the enzymatic solution to control samples. Micrographs were taken to illustrate the degrading mechanisms of the membrane. Samples were gold-coated in preparation for imaging.

2.2.2. Weight change experiments

To determine the swelling/degradation behaviour of the membrane, a mass uptake analysis was conducted. Weight change measurements were calculated using a 5 decimal point balance. Specimens were weighed accurately, immersed in either a PBS or enzymatic solution and incubated at 37 °C in an atmosphere of 5% CO_2 and 100% humidity. At time points from 5 min up to 2 h, samples were removed from the solution, rinsed twice in PBS and cooled in an ice bath to impede the enzymatic effect. Samples were then lightly patted with blotting paper to remove the excess liquid and weighed again to monitor the change in weight of the membrane over time.

2.2.3. Tensile test experiments

The mechanical properties of Biomend® were examined in air and in physiological solutions and also after accelerated degradation in an enzymatic solution. Standard tensile test experiments were carried out on Biomend® on a Zwick 2.5S Universal Tensile Tester with a 100 N load cell. Samples were cut into 30 mm $\times$ 4 mm strips and immersed in either a PBS or enzymatic solution and incubated at 37 °C in an atmosphere of 5% CO_2 and 100% humidity for a series of time intervals. After cooling in an ice bath, samples were patted dry with blotting paper to remove excess water. Sample width and thickness were recorded using a micrometer screw gauge. Samples were then carefully placed between non-slip grips. Experiments were performed with an initial gauge length of 15 mm and subjected to a constant strain rate of 10 mm/s. Load displacement curves were recorded for each specimen. Subsequent stress versus strain curves were generated for each specimen, from which values of Young's Modulus, Ultimate Tensile Strength and Strain at Failure were obtained.

2.2.4. pH change experiments

When a material begins to degrade, particles may diffuse into the surrounding environment. Hence, the pH of solutions in which the membrane was degrading was monitored to highlight any resultant cytotoxic effect of its degradation products. Samples were immersed in either a PBS or enzymatic solution and incubated at 37 °C in an atmosphere of 5% CO_2 and 100% humidity. pH readings were taken after 0, 5, 60, 120, 210, 270 and 480 min.

2.2.5. Cell viability studies

To assess the suitability of using this material as a potential scaffold, a cell viability study was conducted. Cellular metabolic activity was investigated using an Alamar Blue assay (Biosource Europe). Samples ($n = 6$) were cut into 1.18 cm diameter circles (1.1 cm^2 SA) to fit into 48-well plates (Nunc, UK). Cells were seeded into the wells at a density of 50,000 cells/cm^2 and incubated for 1, 3 and 7 days at 37 °C in an atmosphere of 5% CO_2 and 100% humidity in endothelial cell culture medium. An Alamar Blue assay was carried out after each time period.

Cell viability was assessed qualitatively using a Live/Dead® Viability/Cytotoxicity assay kit (Calcein AM and Ethidium homodimer-1). Using this kit, healthy cells stained green while the nuclei of dead cells stained red. Multiphoton Laser Microscopy was used to visualise cell morphology. Fluorescent conjugates of phalloidin (Molecular Probes, UK) were used to label the actin filaments in the cell cytoplasm while Hoechst (Sigma, UK) was used to label the cell nucleus.

3. Results and discussion

3.1. Quantification of enzymatic degradation effects

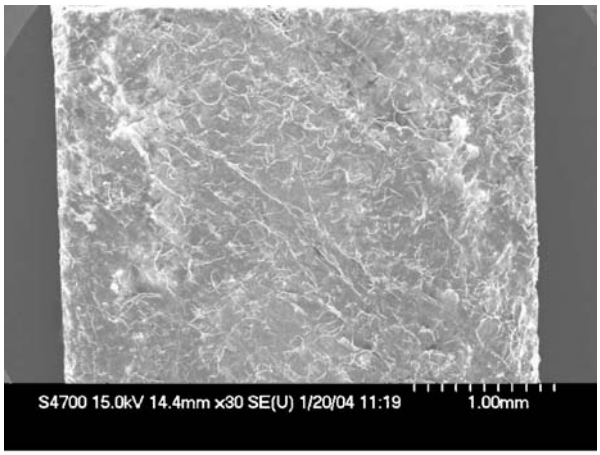
3.1.1. Microscopic change

SEM was used to examine Biomend® constructs that were exposed to different environmental conditions. Fig. 1(a), (b) and (c) show micrographs of the dry membrane, the membrane after 24 h in PBS, and after being in the enzymatic solution for 2 h, respectively.

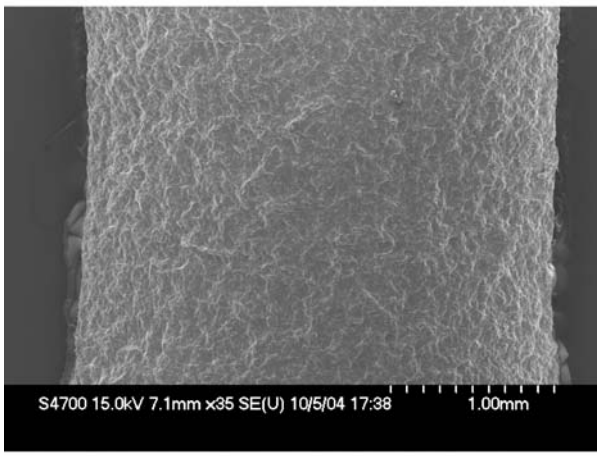
It is observed that the membrane has transformed from a uniform tightly packed structure (Fig. 1(a)) to a more uneven and porous one (Fig. 1(c)). However, the similarities between Fig. 1(a) and (b) indicate that the change in structure may be as a result of the enzymatic effect and not merely the hydrating effects of the PBS solution.

3.1.2. Weight change

In vivo, it takes up to 8 weeks for Biomend® to be fully adsorbed [16]. At the high concentration of collagenase (500 μg/ml) used in this experiment, the enzymatic effect is more catastrophic, causing a near instantaneous denaturing of the membrane. This is highlighted in the micrograph (Fig. 1(c)) taken after 2 h in the solution. After the initial swelling of Biomend® in the aqueous solution, the collagenase begins to take effect causing the subsequent loss in mass (Fig. 2). At time periods after 2 h the collagen membrane disintegrated. As a result, the mass was difficult to monitor accurately. In all experiments the disintegration of the membrane was found to be catastrophic at the high enzymatic concentration.



(a)



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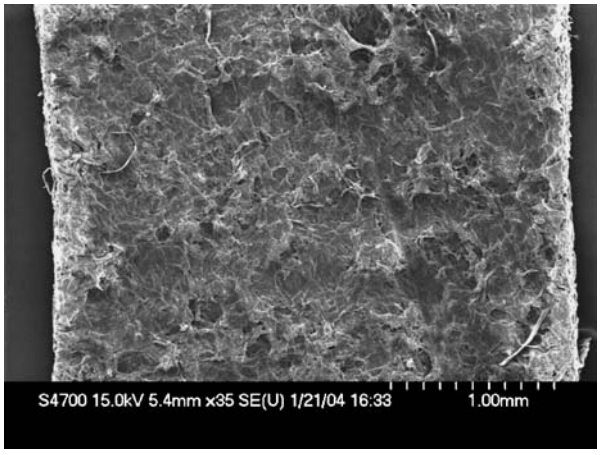


(c)

Figure 1 SEM micrographs of (a) dry Biomend®, (b) Biomend® after 24 h in PBS at and (c) Biomend® in a 500 μg/ml solution of collagenase (×30–35).

3.2. Characterisation of mechanical properties

3.2.1. Biomend in PBS solution

When Biomend® is placed into a saline solution, it becomes less stiff and more ductile. The longer it is left in the solution, the greater its capacity to be elongated without breaking. This is highlighted by the stress versus strain graph shown in Fig. 3(a).

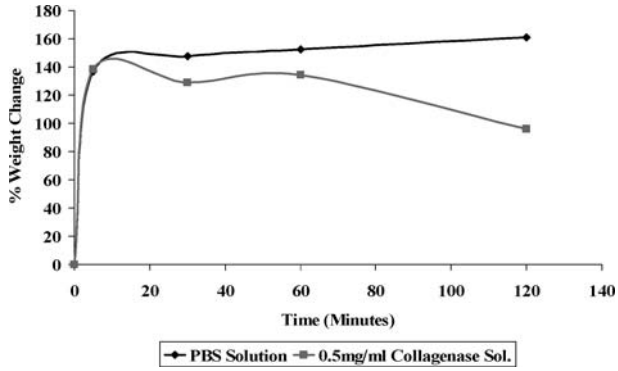


Figure 2 Comparison of the change in mass observed between Biomend® placed in an aqueous solution (PBS) and a solution containing 500 μg/ml collagenase in PBS.

3.2.2. Biomend in 0.5 mg/ml collagenase solution

When Biomend® is placed into a saline solution containing collagenase, an enzyme which helps breaks down collagen *in vivo* as part of the natural remodelling of tissue's extracellular matrix, a different trend is observed. In Fig. 3(b), it can be seen that over time the strain of failure decreases for the collagen, a reversal of tendency observed when the enzyme was not present. This complements what is seen visually in Fig. 1(c) and the trend observed in Fig. 2 where the breakdown and mass loss of the tightly packed fibrous structure is observed over time. As the fibres break up, the strength of the membrane is reduced. The change in environment has had a dramatic effect on the mechanical performance of the material.

3.3. Cytotoxicity studies

3.3.1. pH change

Fig. 4 illustrates that there are no significant changes in the pH of solutions containing degrading samples of Biomend® ($n = 6$) detectable by the sensitivity of the method used. Any such change would be indicative of potentially cytotoxic by-products being released by the membrane. While this type of investigation is merited, more detailed analysis can be provided by cell viability studies.

3.3.2. Cell viability studies

The results of the Alamar Blue assay are shown in Fig. 5 ($n = 6$). The lack of fluorescence produced by cells on Biomend® compared to tissue culture plastic controls indicate that there are few or no metabolically active

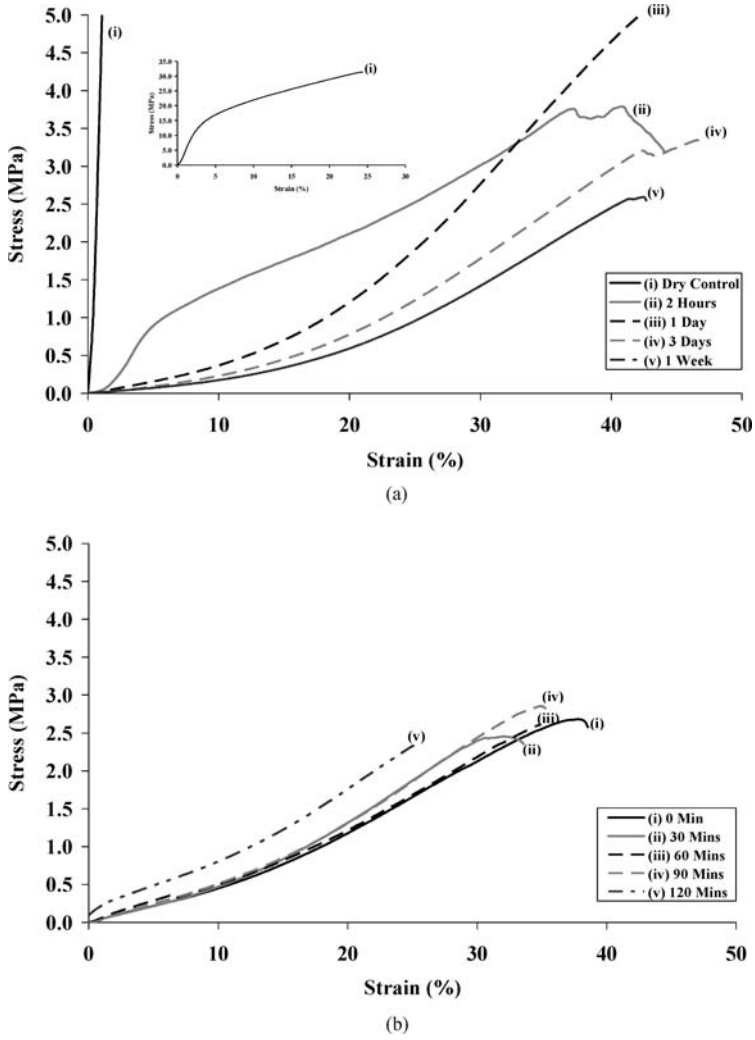


Figure 3 (a) Stress versus strain graphs of Biomend® samples immersed in a PBS solution for 0, 2, 24, 72 and 168 h [insert shows entire graph of the control]. (b) Stress versus strain graphs of Biomend® samples (pre-soaked in PBS solution) immersed in a 500 μg/ml collagenase in PBS solution for 0, 30, 60, 90 and 120 min.

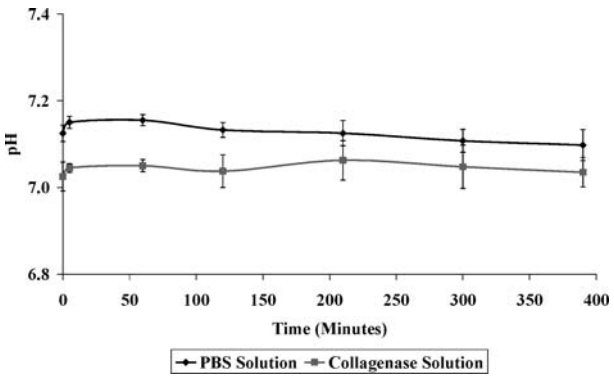


Figure 4 Comparison of pH measurements taken where Biomend® was left in PBS and enzymatic solutions for time periods up to 7 h.

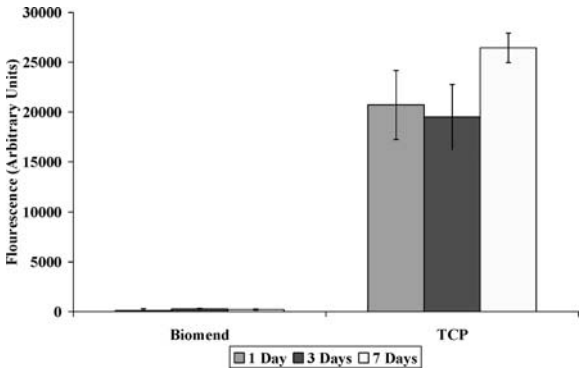


Figure 5 Alamar Blue Assay comparing the metabolic activity of HUVECs (50,000 cells/cm^2) seeded on Biomend® to those seeded on tissue culture plastic (Values shown are in arbitrary fluorescence units).

cells on Biomend® after 1, 3 and 7 days in supplemented endothelial cell medium. Fluorescence microscopy allows visualisation of the substrate and indicates if any cell attachment has occurred. The morphology of the HUVECs shown in Fig. 6 can be seen by staining their nuclei blue and actin filaments red. The shape and irregular positioning of the cells actin filaments indicate that the cells have not attached effectively. Fig. 7(a) shows

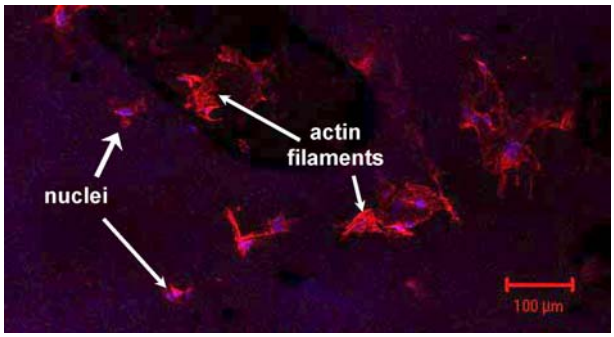
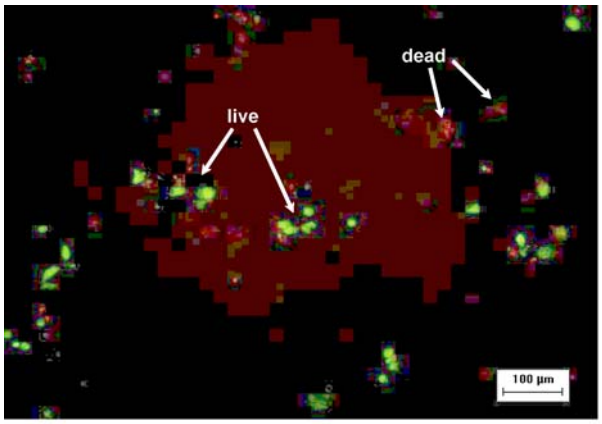
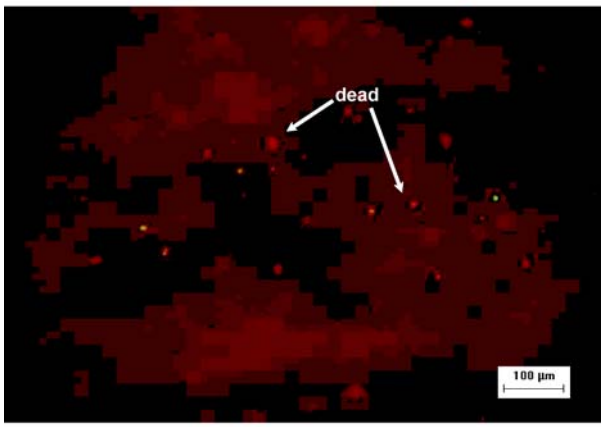


Figure 6 Picture of phalloidin and hoescht stained HUVECs cultured on Biomend® for 5 days showing the morphology of the cells on the surface.



(a)



(b)

Figure 7 Images of Live/Dead® Viability/Cytotoxicity assay used to stain live (green) and dead (red) HUVECs after (a) 1 and (b) 3 days on Biomend®.

a number of live cells (stained green) after 1 day of culture on Biomend®. However, by day 3 (Fig. 7(b)), fewer cells are attached to substrate and those that are, appear to be dead. By day 7, it is evident that there are no longer any cells attached to the substrate as neither of the dyes were taken up. These results substantiate the findings of the Alamar Blue assay, which indicated no cell activity.

4. Conclusions

From the initial results achieved in this study, it is evident that a collagen membrane offers the necessary mechanical integrity to potentially be incorporated into a tissue engineered vascular construct. However, direct comparison of these properties with the mechanical properties of other cardiovascular tissue engineering scaffolds in the literature is restricted due to different testing methodologies. Nevertheless, the membrane's strength has been shown to decrease as it degrades—a key requirement for its role as a support sleeve to a developing construct. pH monitoring and the ability for HUVECs to grow in the proximity of Biomend® suggest that there is no harmful by-products being eluted from the membrane in solution. In future studies it is conceived that cell attachment to the material could be improved by coating its surface with an adhesive glycoprotein such as fibronectin. However, the inability of these cells to successfully adhere and proliferate on the membrane, in its current form, casts doubt on its suitability for the proposed application in cardiovascular tissue engineering.

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