DESIGN OF NONWOVEN SCAFFOLD STRUCTURES FOR TISSUE ENGINEERING OF THE ANTERIOR CRUCIATE LIGAMENT

S.L. Edwards*†, W. Mitchell, J.B. Matthews**, E. Ingham**, S.J. Russell***

Department of Textile Industries* & School of Biochemistry and Molecular Biology**
University of Leeds, Leeds, LS2 9JT, UK
†E-mail: tex7sle@leeds.ac.uk

Abstract

This work is concerned with improving the design of textile scaffolds used to tissue-engineer anterior cruciate ligaments. Two important design criteria of a scaffold are internal structure and cell-fibre compatibility. This paper considers both of these criteria, providing a review of scaffold design and structural parameters, followed by experiments on the biocompatibility of various generic fibres.

In this paper, the influence of surface area to volume ratio and polymer morphology on cell-surface interactions is discussed, together with a consideration of the effect of pore-size and scaffold porosity on cell proliferation, migration and nutrient supply. Another structural factor discussed is the role of fibre orientation as a means of guiding and organising new tissue growth. It is possible to manipulate these scaffold parameters to produce a scaffold of optimal structural design for the tissue engineering of the anterior cruciate ligament.

A review of current scaffold types classified according to manufacturing method is presented. These manufacturing methods include solvent casting/particulate leaching, three-dimensional printing and fibre bonding. Scaffolds in fibrous form include woven, knitted, braided, embroidered and more recently nonwoven.

Biocompatibility tests performed by the authors study the reaction of fibroblast cells to the surface of different generic fibre types; including para-aramid, polyester, polypropylene, polyglycolic acid and viscose rayon. The results of these tests are discussed in relation to cell attachment and fibre morphology.

Keywords

anterior cruciate ligament, biocompatibility, scaffolds, tissue engineering

1. Introduction

Tissue engineering merges the fields of cell biology, engineering, materials science and surgery to fabricate new functional tissue using living cells and a matrix or scaffold [4]. It is concerned with the creation of biological substitutes designed to maintain, restore or improve the function of damaged tissues and organs [12].

In order to engineer a neo-tissue (or neo-organ) the following are required; a scaffold (either temporary or permanent) on which to generate the neo-tissue, appropriate cells or migrating host tissue from which to form the neo-tissue, culture medium for nutrient supply and waste removal and finally the optimum culturing conditions. These optimum culturing conditions can either be within the body (*in vivo*) or outside the body (*in vitro*), mimicking *in vivo* conditions.

Concerning the scaffold, it is the internal structure of the scaffold that helps determine the type of neo-tissue generated. Scaffold structural parameters such as pore size, porosity and fibre orientation dictate specific cellular activities, leading to tissue formation. By altering these structural parameters, it is possible to optimise the generation of tissues such as neo-ligaments, e.g. the anterior cruciate ligament (ACL).

It is the scaffold manufacturing method that dictates the properties of the scaffold, including structural parameters and scaffold architecture. Fibre bonding methods such as nonwoven technologies could make possible precisely engineered structures, with control over parameters such as fibre orientation and porosity. Other methods such as gas foaming and solvent casting/particulate leaching produce spongy scaffolds, with no means of orienting cells.

In addition to the scaffold structure, cell-substrate biocompatibility also influences cell attachment, leading to subsequent cell spreading, cell migration and often cell differentiation function [23]. Most tissue-derived cells (such as fibroblasts) are anchorage-dependent, and require attachment to a solid surface for viability and growth [23]. However, if the scaffold substrate is not found to be biocompatible, cell attachment will be poor and subsequent tissue generation may be inadequate. Substrate biocompatibility can be assessed through seeding the substrate with a known quantity of cells and monitoring for cell necrosis or proliferation.

2. Tissue Engineering Scaffolds

Tissue engineering scaffolds are three-dimensional structures that assist in the tissue engineering process by providing a site for cells to attach, proliferate, differentiate and secrete an extra-cellular matrix, eventually leading to tissue formation. In addition to optimising scaffold structure to ensure that such desired cellular activities occur, it is also possible to guide cells into forming a neo-tissue of predetermined, three-dimensional shape and size. Tissue engineering scaffolds can be either permanent or temporary in nature, depending on the application and the function of the neo-tissue. Temporary scaffolds are made from biodegradable polymers, such as polyglycolic acid, which degrade within the body to leave a purely biological neo-tissue [6]. Permanent scaffolds remain within the body, working with ingrown tissue to form a polymeric/biological composite [16].

3. Scaffold Structural Design Parameters

For a scaffold to function effectively, it must possess the optimum structural parameters, conducive to the cellular activities leading to neo-tissue formation; these include cell penetration and migration into the scaffold, cell attachment onto the scaffold substrate, cell spreading and proliferation and cell orientation. Such scaffold design parameters are now described with reference to these cellular activities.

Table 1. Ideal structural parameters of a tissue engineering scaffold

SCAFFOLD FUNCTION	SCAFFOLD DESIGN PARAMETER
Not to provoke inflammatory response or toxicity *in vivo*.	Must be biocompatible, non-toxic and non-carcinogenic.
To assist in the growth of three-dimensional tissue and organs.	Three-dimensional scaffold of specific shape.
Give way to a uniform high cell seeding density.	High porosity and high interconnectivity between pores.
To provide the appropriate surface for cell attachment, proliferation and differentiation of function.	Optimum polymer surface chemistry and topography
To allow significant cell surface interactions such as cellular attachment.	High surface-area to volume ratio.
To promote cell proliferation and migration leading to tissue growth throughout the scaffold.	Optimum pore size to allow for cell penetration, with high porosity and interconnectivity between pores.
To direct the orientation of cells, ECM and new tissue.	Correct fibre orientation within the scaffold.
To allow for the movement of nutrients and waste in and out of the scaffold.	High porosity and interconnectivity between pores.
The scaffold may degrade to leave only natural tissue.	Rate of degradation to match rate of tissue formation. Polymer degradation products must not be toxic or promote inflammation *in vivo*.
Possess sufficient structural integrity to retain shape *in vivo*, with enough mechanical strength to support developing tissue and withstand *in vivo* forces.	Scaffold should equal mechanical properties of developing tissue.

In order for neo-tissue formation to occur, cells must first be able to penetrate the scaffold sufficiently to give way to a high cell seeding density (cells/cm^2). This requires that the scaffold be highly porous, with a high surface-area to volume ratio. As most cells are anchorage-dependent and require attachment to a solid surface for viability and growth [23], a high surface area is also essential for high cell growth rates [15]. Research has shown that a scaffold porosity of at least 90% is ideal for specific scaffold-cell interactions, nutrient and waste diffusion and sufficient space for ECM regeneration within the scaffold [1].

Other structural parameters governing cell penetration and migration within the scaffold are pore-size [15] and pore interconnectivity. If the pore-size is too small, cells will be unable to initially penetrate the scaffold and subsequently migrate to other regions of the scaffold, producing uniform cell seeding throughout. If the pore-size is too large, cells will be unable to bridge the pore during cell proliferation, thus inhibiting effective neo-tissue generation.

It has been observed that fibre orientation in fibrous scaffolds can positively influence collagen-fibre orientation. Research found that when random and parallel oriented scaffolds were seeded with cells, collagen fibre orientation was increased in the parallel direction in the early stages of static *in vivo* culturing. However, it was found that with time the collagen fibres attained the same degree of fibre isotropy regardless of scaffold fibre orientation [3]. It can therefore be said that the rate of collagen organisation was enhanced with the use of oriented scaffolds. Some of the ideal design parameters, particularly relating to cell penetration and cell attachment, are summarised in table 1, based on previous studies [1, 4, 6, 10, 15, 18, 19, 20].

4. Scaffold Manufacturing Methods

The method used to manufacture a scaffold determines the key properties of that scaffold, such as porosity, pore-size, mechanical properties and three-dimensional shape [25]. When choosing the scaffold manufacturing method, it is important to take into consideration these desired scaffold properties, and to ensure that the method does not adversely affect these properties, e.g. mechanical characteristics or biocompatibility [15]. Another consideration is the use of high temperatures and harsh chemicals during scaffold manufacture, which can inhibit the incorporation of bioactive agents (e.g. growth factors) into the scaffold for drug delivery to the cells [1]. Different manufacturing methods produce scaffolds of different configurations: porous sponges (see figure 1), foams and fibrous scaffolds (see figure 2), some of these manufacturing methods are described below. Others not described include phase separation [22] and gas foaming [21].
The solvent casting/particulate leaching method uses particulate porogens to form sponge/foam-like scaffolds. This method involves dispersing the porogen (e.g. sodium chloride) into a polymer solution (e.g. PLLA/chloroform), casting the solution, evaporating off the solvent and finally leaching out the salt [17]. Advantages include manipulation of the scaffold pore-size and porosity by altering the salt particle size and concentration respectively; disadvantages include the time-consuming leaching step, which can significantly increase scaffold preparation time.

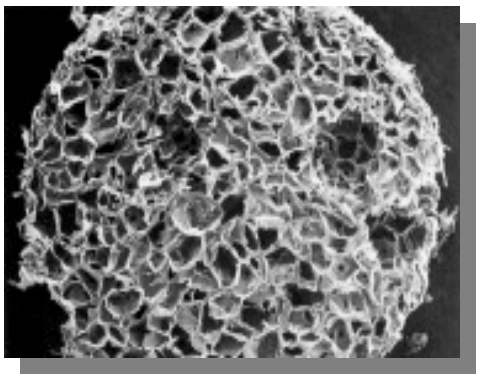


Figure 1. BD™ 3D OPLA® sponge scaffold [28]

Three-dimensional printing is a novel scaffold fabrication method, and can be used to produce complex-shaped three-dimensional scaffolds. This fabrication method works by inkjet-printing a binder onto layered polymer powder. Research has shown it is possible to form three-dimensional scaffolds, printing bars of PLLA powder then adding a solvent, namely chloroform, delivered from the ink-jet printer [7]. Varying printing parameters, enabling manipulation of the scaffold structure, include solvent

flow rate, print speed, line spacing and layer thickness. Disadvantages include the low milling yield (30%) of the PLLA granules and manual positioning of the polymer powder bed, a time-consuming action [7].

Figure 2. SEM of nonwoven PGA scaffold

Biodegradable suturing fibre was the first material used in scaffold fabrication. These scaffolds were manufactured to form felts and scaffolds for tissue regeneration, but failed due to lack of structural stability [5]. Present-day conventional fibre-bonding methods include embroidery [9], weaving, knitting & braiding, and more recently nonwoven manufacture [14] and composites. The obvious advantage of fibrous scaffolds over foam and sponge scaffolds is the large surface-area to volume ratio and, in general, greater opportunities for manipulating scaffold structure and mechanical properties.

5. Biocompatibility Testing of Generic Fibre Types

Cells attach to substrates via cell-surface receptors, which interact with proteins adsorbed onto the surface of the substrate [8]. These proteins are adsorbed from either the surrounding serum (culture medium or biological fluid) or secreted by the cells themselves [23]. With cells interfacing with these proteins, adhesive proteins are said to act as bridging molecules between the cells and substrate [11].

Research has found that low to moderately hydrophilic polymers support a high fraction of fibroblast cells [13]. In agreement with these findings, it has been observed that cell adhesion [via adsorbed protein] appears to be maximised on those surfaces with intermediate wettability [23]. Although the hydrophobic polymers PTFE, PP, and PET have been noted to support limited cell attachment [13], other hydrophobic polymers such as polystyrene have demonstrated low cell attachment. It is thought that cell attachment to non-polar polymers, such as polystyrene, differs, possibly due to the crystallinity of the polymer, with poor cell adhesion being associated with amorphous materials [13].

Other substrate properties thought to enhance cell adhesion are positively charged surfaces [23] and grooved surface topographies, which may be due to an increase in surface area [26].

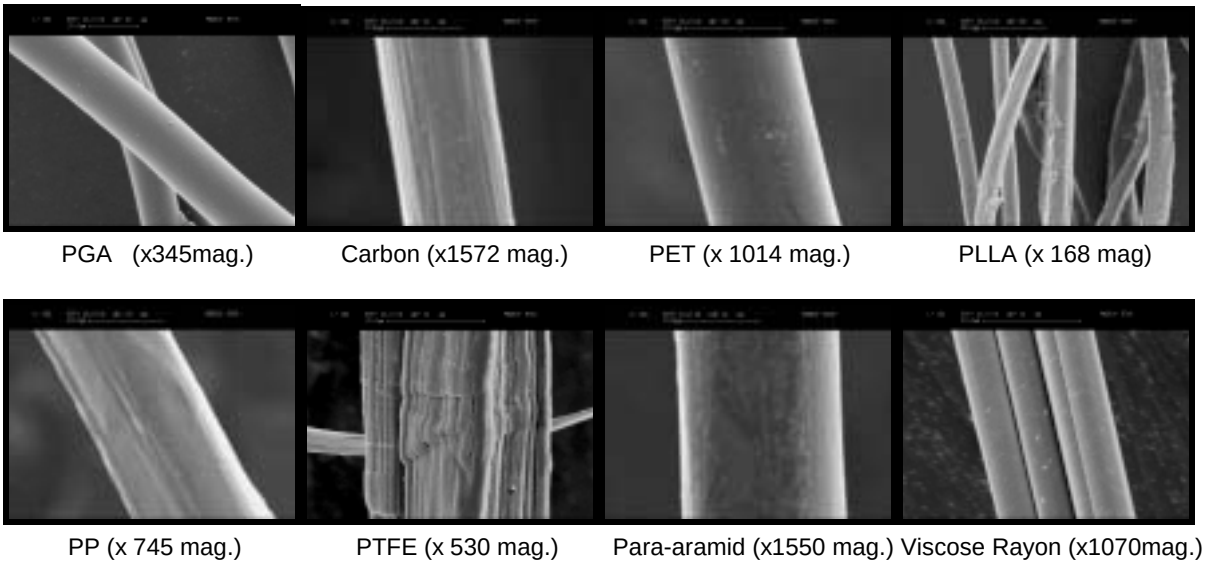
Biocompatibility has been defined as the ability of a material, prosthesis, artificial organ, or biomedical device to perform with an appropriate host response in a specific application [2]. In the case of tissue engineering, the scaffold's biocompatibility is a function of the material from which the scaffold is constructed. Two properties that influence cell-substrate compatibility are substrate surface chemistry and surface topography. On this basis, the present experimental research was designed to assess cell-fibre compatibility on a range of generic fibres. Biocompatibility tests were conducted to study the reaction of mouse fibroblast cells (L929) to a range of generic fibre types. Viable cell attachment results were quantified 24 and 72 hours after cell seeding.

5.1 Materials

Eight polymers were evaluated in this study: staple fibre polypropylene (PP) (Drake, UK), polyester (PET) (Dupont, USA), viscose rayon (Acordis, Germany), para-aramid (industry sourced), carbon (Soficar, France), polytetrafluroethylene (PTFE) (Lenzing, Austria), poly-L-lactic acid (PLLA) (Cellon, Luxembourg) and polyglycolic acid (PGA) (Cellon, Luxembourg).

5.1.1 Scanning Electron Microscopy

Since surface topography is one of the parameters that are thought to affect cell adhesion, the fibres were evaluated by SEM. The images are illustrated in Figure 3.



PGA (x345mag.) Carbon (x1572 mag.) PET (x 1014 mag.) PLLA (x 168 mag)

PP (x 745 mag.) PTFE (x 530 mag.) Para-aramid (x1550 mag.) Viscose Rayon (x1070mag.)

Figure 3. SEM images illustrating the surface topography of generic fibres used in the biocompatibility experiment

The surface topography of the PGA, para-aramid, PET and PLLA fibres was smooth. The PP fibre was found to have shallow, irregular grooves on its surface, and the carbon fibre to have slightly more regular, pronounced shallow grooves. The viscose rayon had wide striations along its length, which is a characteristic of the fibre due to wet spinning. The most irregular fibre surface was associated with the PTFE fibre, which possessed deep ridges.

5.2 Experimental Method

5.2.1 Fibre Scouring and Disinfecting

Prior to conducting the biocompatibility tests, any fibre finish that may have been present was removed using a filter-sterilised (0.2µm) non-ionic synthetic detergent (Croscour, Eurodye-ctc, UK), which had been diluted using sterile water (Miza Pharmaceuticals, UK). Fibres were immersed in sterile 70% (v/v) ethanol solution for one hour to disinfect the fibres prior to testing. All procedures were performed under aseptic conditions.

5.2.2 Testing Apparatus

Scoured and disinfected groups of fibres of a pre-determined total surface area were placed into plastic microbiological culture wells for testing. Washed (7x, ICN Biomedicals, Inc., USA) and sterilised stainless steel gauze (Expamet, UK) was used to position and retain the fibres beneath the cell suspension level to ensure effective cell seeding. Aseptic techniques were used throughout.

5.2.3 Cell Seeding

Mouse fibroblast cells (L929, passage15) were suspended in 3 ml of Dulbecco's Modified Eagles Medium (DMEM) (Sigma-Aldrich Co., Ltd., UK), supplemented with 10% (v/v) Foetal Calf Serum (Gibco Brl, Life Technologies Ltd., New Zealand), L-Glutamine (Sigma-Aldrich Co., Ltd., UK) and Penicillin and Streptomycin (Sigma-Aldrich Co., Ltd., UK). Cell suspension (3mls), containing 4.93×10^6 cells/ml, was seeded over the fibres at a cell seeding density of 2×10^4 cells/cm^2.

Cells seeded into tissue culture-treated wells acted as a positive control. Other controls used included sample controls (fibres without cells) and a background control (medium only). Samples and controls

were gassed with 5% CO^2 in air and incubated at 37°C for 1 day (n=4) and 3 days (n=4), after which time ATP-LITE™ (Perkin Elmer™ Lifesciences, USA) assays were conducted.

5.2.4 ATP

Assessment of cell attachment to fibres was conducted using the ATP-LITE™ assay, measuring the level of ATP activity as an indication of viable cell attachment to fibres.

Cell-seeded fibres were removed from culture wells, and the fibres gently rinsed using Dulbecco's Phosphate Buffered Saline (PBS) (Sigma-Aldrich Co., Ltd., UK) to remove any non-adherent cells. Washed fibres were transferred to clean culture wells for cell lysing: 1 ml of fresh supplemented DMEM culture medium was added to each well, followed by 0.5ml of Mammalian Cell Lysis Buffer (Perkin Elmer™ Lifesciences, USA). The wells were agitated for 5 minutes. Lysed cell solution was transferred to a 96-well Optiplate (150 µl/well), to which a further 50 µl/well ATP Cell Substrate Solution (Perkin Elmer™ Lifesciences, USA) was added and agitated for 5 minutes.

For the background control, 2 ml of the cell medium was removed from the well and 0.5 ml of the Mammalian Cell Lysis Buffer was added. The remainder of the experimental method was followed. For the positive control cell, suspension was removed from the wells and the wells rinsed with PBS to remove any non-adherent cells. 1ml of fresh supplemented DMEM culture medium was added, and the remainder of the experimental method followed. An assessment of viable cell attachment using the ATP-LITE™ assay was conducted.

A two-way analysis of variance (two-way ANOVA) and a calculation of the minimum significant difference was used to determine a statistically significant increase in cell attachment relative to that occurring on the viscose rayon fibre following equivalent incubation periods.

5.3 Results

The results of *in vitro* fibroblast cell attachment to a range of generic fibre types, differing in fibre properties, are illustrated in figure 4.

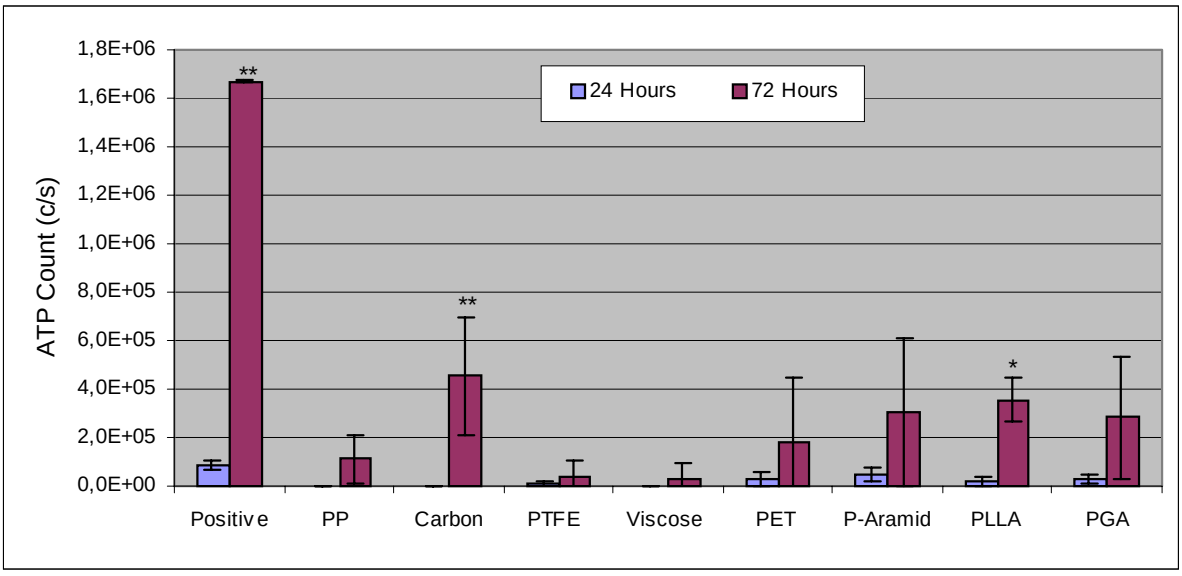


Figure 4. Graph illustrating fibroblast cell attachment to a range of generic fibre types both 24 hours and 72 hours after cell seeding (± 95% confidence limits). Results have been adjusted for surface area. (* = 0.05, ** = 0.01, significantly greater cell attachment compared to viscose rayon)

5.3.1 (24 Hours) Post Cell-Seeding

It was found that PP displayed no cell attachment 24 hours after cell seeding. Compared to the PP, a general trend could be observed in which cell attachment numbers increased with the following fibres

respectively: viscose rayon, carbon, PTFE, PLLA and PGA, with polyester and the para-aramid fibres displaying the greatest levels of cell. However this did not reach statistical significance.

5.3.2 (72 Hours) Post Cell-Seeding

The relative quantities of cells attached to the generic fibre types had altered considerably after 72 hours in culture. Viscose rayon had the lowest number of attached cells. Increasing levels of cell attachment were found for the PTFE, PP, PET, PGA, para-aramid and PLLA respectively, with the greatest level of cell attachment observed for the carbon fibre. It should be noted that the number of viable cells attached to the fibres 72 hours after cell seeding was greater than after 24 hours, due to cell proliferation. Statistical analysis has revealed there to be significantly higher numbers of attached cells to the carbon fibre ($p=0.05$) and the PLLA ($p=0.01$), compared to the fibre with lowest cell attachment at 72 hours (viscose rayon).

These observed results support the preliminary biocompatibility tests carried out by the authors, and are supported by research in which fewer fibroblast cells were found to attach to PET fibres, when compared to para-aramid 72 hours after cell seeding [27].

5.4 Conclusion and Discussion

In the early stages of cell attachment, cell-substrate interactions are likely to be a function of the substrate surface chemistry [27], with optimum cell attachment to surfaces with low to moderate levels of hydrophilicity [13]. This was found to be the case for these experimental results, 24 hours after cell seeding, with the most hydrophobic polymers supporting the lowest levels of cell attachment. With increasing hydrophilicity, cell attachment was seen to increase; this was true for all fibres tested except for the viscose rayon, which may have been too hydrophilic. Research has found some highly hydrophilic polymers to support low levels of cell attachment [13]. Differences in initial cell attachment to the hydrophobic fibres may be accounted for by their relative surface topographies, with PP having shallow grooves and PTFE having deep ridges (which may have increased the relative surface area for cell attachment).

Subsequent cell attachment and proliferation 72 hours after cell seeding may be due less to the wettability of the substrate and more due to its topographic nature. Contrary to the cited literature observation [26], it appears that cell numbers at 72 hours were higher on the smoother fibres (e.g. Carbon) as oppose to grooved fibres (e.g. PTFE). In order to fully understand these results, other properties of the fibres used need to be characterised and taken into consideration in further experiments.

Summary

In this paper, the relationship between some of the cellular activities which lead to tissue regeneration and scaffold structural parameters have been discussed. Leading on from this, several scaffold manufacturing methods have been described, with the possible advantages and disadvantages of each. Finally the biocompatibility of scaffold substrates has been investigated, testing a range of generic fibre types for viable cell adhesion at 24 hours and 72 hours post-cell seeding. This paper can be summarised in the following points:

- Tissue engineering scaffolds are three-dimensional structures that assist in the tissue engineering process by providing a site for cells to attach, proliferate, differentiate and secrete extra-cellular matrix, eventually leading to neo-tissue formation.

- For a scaffold to function effectively, it must possess the optimum structural parameters, conducive to the cellular activities leading to neo-tissue formation; these include cell penetration and migration into the scaffold, cell attachment onto the scaffold substrate, cell spreading and proliferation and cell orientation.

- The method used to manufacture a scaffold determines the key properties of that scaffold, such as structural parameters, mechanical properties and three-dimensional shape.

- Biocompatibility tests conducted found no significant difference in viable cell attachment between tested fibres, 24 hours after cell seeding. However, a general trend was observed, with increasing cell attachment on increasingly hydrophilic fibres.
  A significant difference in cell attachment was observed 72 hours after cell seeding between the fibre with the least cell attachment (viscose rayon) and the carbon and PLLA fibres.

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