O_2 Diffusion through Collagen Scaffolds at Defined Densities: Implications for Cell Survival and Angiogenic Signalling in Tissue Models

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Abstract— **The success of any biomaterial for tissue engineering is dominated by its mechanical properties and ability to support nutrient diffusion. Collagen scaffolds are ideal candidates due to their ability to immerse cells in a biomimetic nano-fibrous matrix. We have established O_2 diffusion coefficients through native, dense collagen scaffolds at two tissue-like densities, with and without photo-chemical crosslinking, by adapting an optical fibre-based system for real-time core O_2 monitoring deep within collagen constructs. The high diffusion coefficients of these collagen scaffolds, as well as their material properties, render them viable tissue engineering matrices for tissue replacement. Due to this O_2 diffusion through cell-seeded collagen type I scaffolds, natural gradients of O_2 form and cells in different locations are subject to varying levels of O_2. These gradients were controlled by varying cell density, as it was found that cell consumption of O_2 played a greater role compared to material diffusion in formation of such O_2 gradients. Potent angiogenic signaling molecules were upregulated at both the gene and protein level, particularly within the core of 3D scaffolds, where O_2 was low, but remained within physiological hypoxia. By incorporating phosphate-based dissolving glass fibres into collagen constructs, as they are produced, it was possible to introduce channels throughout the construct in a gradual manner. Where channeled architecture was introduced to the 3D constructs, thus delivery of sustained O_2 to all cells even within the core, this upregulation of angiogenic factors was abolished. We can now engineer collagen type I scaffolds with varying density, varying degrees of crosslinking and various architectural features to control delivery of O_2 to all cells embedded within the construct.**

Keywords— **Collagen Scaffold, Biomimetic, Oxygen Diffusion, Oxygen permeability, Photo-chemical crosslinking, Phosphate-based dissolving glass fibres, Angiogenic Factors, Tissue-Constructs**.

I. Introduction

Within the fields of Tissue Engineering and Tissue Modelling, the formation of 3D cell-seeded constructs poses multiple problems of O_2, nutrient and lactate permeability both into and out of the 3D structure [1]. Given the nature of an engineered cell scaffold, a native vasculature does not exist, and therefore perfusive properties of the material are critical to take into consideration, especially with regards to the viability of cells within the core of 3D constructs. Development of synthetic polymer scaffolds have an advantage, whereby the architecture of a scaffold can be pre-determined, and porosity can be controlled to aid free-circulating culture media to supply cells throughout the 3D structure. However, synthetic scaffolds cannot mimic the mechanical and protein properties of native scaffolds. Collagen type I is an ideal biomimetic scaffold for engineering living tissues, as it is the predominant extracellular matrix protein within the body. It is difficult to control the architecture of this protein, as we do not manufacture it, instead we rely upon extraction of collagen from animal tissues, and then re-constitute it- or build it back up into a 3D tissue.

Due to the lack of vasculature, in a 3D cell-seeded constructs, natural gradients of O_2 exist from the surface to the core dependent upon perfusion of the O_2 and cell consumption of O_2 [2]. Therefore by manipulation of cell density or architecture of the collagen, these gradients can be manipulated, and in some experimental cases, provide good models of diffusion and varying effects of O_2 on cell behaviour. However, for the survival of a 3D tissue engineered model, an adequate, even and continuous supply of O_2 is critical viability of the core cells.

Here we describe a method to measure and model the diffusion of O_2 through collagen type I scaffolds at varying densities, with and without photo-chemical cross-linking. Once established we incorporated phosphate-based dissolving glass fibres into the structure, which dissolved over 24 hours to leave channels running through the 3D construct. We compare cell viability in constructs with and without channels, as well as regulation of angiogenic signals in constructs with and without channels. Our main hypothesis was that with that the incorporation of dissolving fibres increases supply of O_2 to core cells, and prevents them for up-regulating potent angiogenic signals, which are found when core cells are placed under defined levels of physiological and pathological hypoxia.

The aim being to produce 2 types of construct, the first where natural gradients of O_2 exist to control cell behaviour

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in a specific locations within the 3D construct, and the second, to created channeled structures to supply all cells within a scaffold with the same O_2.

II. Experimental Set-Up

Collagen Scaffold Preparation:

0.5 ml of 10X Eagles MEM solution (Gibco, Paisley, UK) was added to 4ml of rat-tail type I collagen (First Link) in 0.1M acetic acid, protein concentration = 2.035 mg/ml, and then neutralized with 5M NaOH, using the indicator colour changes from yellow to cirrus pink. Following setting and incubation, gels were routinely compacted by a combination of compression and blotting using layers of mesh and paper sheets [3,4].

O2 Diffusion Measure:

O_2 diffusion through acellular plastic compressed (PC) scaffolds was measured using fibre-optic oxygen probes in conjunction with an OxyLab pO_2 E^{TM} (Oxford Optronix, Oxford, UK). Constructs were submerged in solutions of Phosphate Buffered Saline (PBS) exposed to atmospheric air at 37^{0}C, giving a background O_2 level between 140-160mmHg. To measure de-oxygenation rates, the same construct was submerged into a different solution of PBS, which had Nitrogen gas bubbled through continuously to displace any O_2 in the system. All experiments were carried out in a 37^{0}C incubator. The oxygen diffusion coefficient has been established using the Ficks Law model [5].

Channelling:

Phosphate-based dissolving glass fibres were incorporated into the collagen scaffold. These dissolved over a period of 24 hours to leave channels which ran through the entire construct.

Cell Viability:

Quantitative cell viability was assessed using Live/Dead reduced biohazard viability/cytotoxity kit (Molecular Probes, L-7013) according to the manufacturer's protocol. SYTO® 10, a green fluorescent nucleic acid stain and dead Red (ethidium homodimer-2) and after capturing images, live/dead nuclei were counted to ascertain percentage viability. 5 random areas within constructs were visualized with confocal microscopy, and live/dead cell percentages determined (BioRad Radiance 2100, Carl Zeiss Ltd, Hertfordshire, UK).

Scanning Electron Microscopy:

Constructs were fixed in 4% paraformaldehyde (in 0.1M sodium cacodylate buffer) for 1 hour, then fixed in 1% tannic acid (in 0.05M sodium cacodylate buffer) for a further 1 hour. Samples were then dehydrated through an alcohol series to hexamethyldisilazane (HMDS) with air drying. Samples were then cut in various planes and gold-palladium sputter-coated, then viewed using a JEOL 5500LV SEM.

III. Results

Constructs were assembled by rolling the compressed collagen sheets to give 3D spiral configuration (figure 1). O_2 probes were inserted into the core of 3D spiralled collagen constructs to monitor the core levels as the O_2 tension of the external media was dropped from 21% to 0% (O_2 was displaced by bubbling with N_2).

The resulting O_2-time plot (figure 2) was used for mass transport modelling. It is clear from this figure that O_2 diffusion across the full thickness (1.1mm) of the construct was measurable and the collagen construct equilibrated to the O_2 tension of the external media. O_2 concentration changes were derived from a model based on the solution to Fick's second law, and matched to observational findings using 2 collagen matrix densities, with and without photo-chemical cross-linking. O_2 diffusion rates were high in single compressed (11%) native collagen construct, at 4.5 x 10^{-6} cm^2/s^{-1}. Increasing matrix density by approximately 3-fold (from 11 to 34% collagen) produced a fall in O_2 diffusion by ~62% to 1.7 x 10^{-6} cm^2/s^{-1} (Table 1). These data were charted alongside data previously published for cell viability in identical scaffolds (Table 1). A clear relationship between increased O_2 diffusion and increased cell viability can be seen in figure 5. This provides a good correlation of scaffold properties, density and diffusion coefficient with 24 hour cell viability.

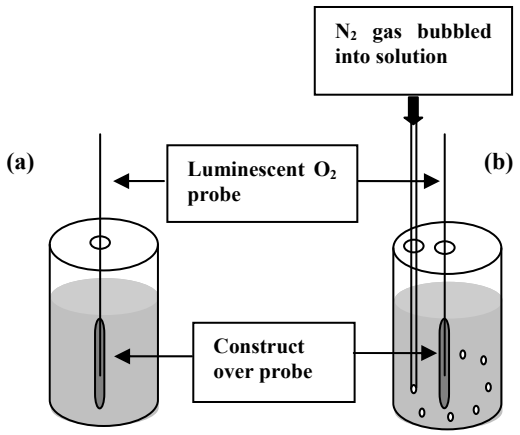

Fig. 1 **Diagrammatic representation of set-up for testing (a) oxygenation and (b) de-oxygenation of constructs.** The set-up was maintained at 37^{0}C, with constructs sealed at both end. N_2 was bubbled through the 0% O_2 solution for 30 minutes prior to testing

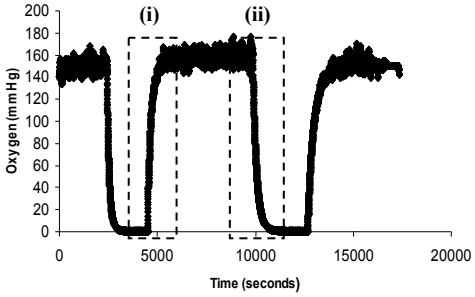

Fig. 2 A graph showing the oxygenation and de-oxygenation of a single compressed construct in either (i) normal PBS solution (140-160mmHg range), or (ii) PBS where N_2 is bubbled through (0mmHg)

Where PBDGF's were incorporated into cell-seeded scaffolds, after 24 hours, levels of O_2 increased in the core (fig. 3b). Resultant channels provided a means by which to deliver O_2 to the entire construct, even to cells within the core.

Table 1 **O_2 Diffusion coefficients of collagen materials at 2 densities.** The cell viability values denote the percentage of cell survival after 24 hours within such collagen constructs with standard deviation (*cell viability values for single compressed taken from: Cheema et al. 2008 [2])

Collagen type	Diffusion Coefficient	Cell viability (% viability at 24 hrs)
Single compressed- 11%	**$4.5 \times 10^{-6} \pm 2.1\%$ cm^2/s**	**90 ± 2.4% ***
Double compressed- 34%	**$1.7 \times 10^{-6} \pm 2.5\%$ cm^2/s**	**67 ± 4.1%**

IV. Discussion

The diffusion coefficient of O_2 through dense collagen scaffolds varies dependent upon density, however at 11% density collagen, the diffusion coefficient falls within the range of some native tissues, including small intestinal submucosa [6]. The O_2 monitoring system allows the diffusion coefficient of materials to be established due to the non-consumption of O_2 at the site of reading. These values are important to consider for survival of cells in scaffolds at early time points. It is clear then, that cell O_2 consumption gradients, which will develop as the cells/tissue grows, will play an increasingly important role in limiting effective delivery of nutrients to the core. The O_2 consumption by cells seeded within the 3D collagen scaffold results in the formation of O_2 gradients, where levels of O_2 are low within the core of 3D constructs.

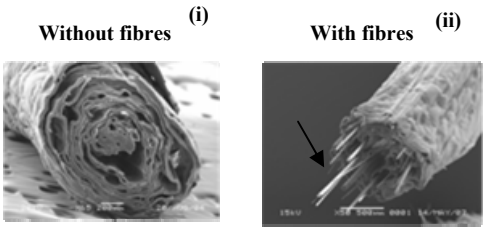

Figure 3a

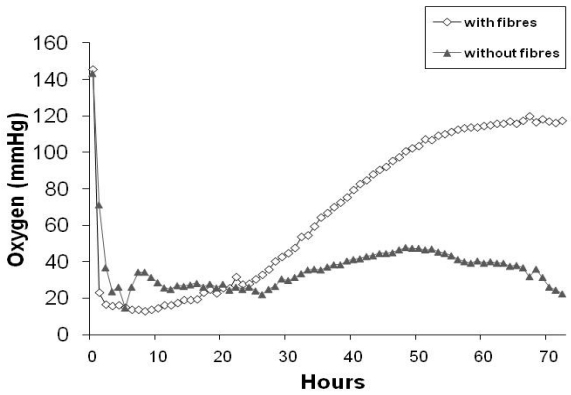

Figure 3b

Fig. 3 **(a) (i) SEM micrographs** of collagen constructs with and without glass fibres. **(ii)** Construct containing dissolving glass fibres, as the fibre dissolves continuous channels are left. . Arrow indicates fibre**(b) O_2 levels in the core of a cellular collagen construct with and without dissolving PBDGF.** By 24 hrs the fibres have completely dissolved, and levels of O_2 increase in the core, compared to control constructs without fibres, up to 3 days, n=1

It is well known that exposed to levels of physiological hypoxia can result in the upregulation of cell-generated angiogenic signaling [2], and that by the introduction of phosphate-based dissolving glass fibres, a channeled architecture can be introduced into the 3D scaffold. As O_2 is delivered to cells within the core, it is hypothesized that this signaling will be switched off.

For engineering of cell-rich tissues, the introduction of channeled features will help maintain a greater density of cells as nutrient limitations and O_2 limitation will not occur. Compared to matrix-rich tissues, i.e. tendon tissue

engineering, where the density of cells is much lower, such channeled features may not be necessary.

References

[1] Karande, T.S. Ong, J.L. Agrawal, C.M. (2004) 'Diffusion in muscloskeletal tissue engineering scaffolds: design issues related to porosity, permeability, architecture, and nutrient mixing'. *Ann. Biomed. Eng*. 32(12): 1728-1743.

[2] Cheema, U. Brown, R.A. Alp, B. MacRobert, A.J. (2008) 'Spatially defined oxygen gradients and VEGF expression in an engineered 3D cell model.' *Cell. Mol. Life Sci.* 65(1): 177-186.

[3] Brown, R.A. Wiseman, M. Chuo, C.B. Cheema, U. Nazhat, S.N. (2005) 'Ultrarapid Engineering of Biomimetic Materials and Tissues: Fabrication of Nano- and Microstructures by Plastic Compression.' *Adv. Funct. Mater*. 15 (11): 1762-1770

[4] Abou-Neel, E.A. Cheema, U. Knowles, J.C. Brown, R.A. Nazhat, S.N. (2006) 'Use of multiple unconfined compression for fine control of collagen gel scaffold density and mechanical properties.' *Soft Matter*. 2:986-992.

[5] Rong, Z. Cheema, U. Vadgama, P. (2006) 'Needle enzyme electrode based glucose diffusive transport measurement in a collagen gel and validation of a simulation model'. *Analyst.* 131(7): 816 – 821

[6] Androjna, C. Gatica, J.E. Belovich, J.M. Derwin, K.A. (2008) 'Oxygen diffusion through natural extracellular matrices: Implications for estimating "critical thickness" values in tendon tissue engineering'. Tissue Eng. Part A 14(4): 559-569.