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An interpenetrating network composite for a regenerative spinal disc application

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ABSTRACT

Severe degeneration of the intervertebral disc has an immensely debilitating effect on quality of life that has become a serious health and economic burden throughout the world. The disc plays an integral role in biomechanical movement and support within the spine. The emergence of tissue engineering endeavours to restore the structural characteristics and functionality of the native tissue. Hydrogels have been widely investigated as a candidate for regeneration of the gelatinous nucleus pulposus due to its architectural resemblance and fluid retention characteristics. However, hydrogels are often limited due to small compressive stiffness and tear resistance, leading to extrusion complications. Reinforcement of the hydrogel network using polymeric scaffolds may address these issues of inadequate mechanical properties and implant instability. This study investigates the potential of a carrageenan gel-infused polycaprolactone scaffold for nucleus pulposus tissue engineering. Mechanical properties were characterised using viscoelastic and poroelastic frameworks via microindentation. The incorporation of polymeric reinforcement within the gels increased material stiffness to that comparable to the native nucleus pulposus, however permeability was significantly greater than native values. A preliminary cell evaluation culturing NIH 3T3s over 21 days suggested the incorporation of polymeric networks also enhanced cellular proliferation compared to gels alone.

1. Introduction

Lower back pain is a growing health and economic burden in society, costing the US over $ 100 billion per annum in direct and indirect costs (Katz, 2006). Chronic back pain is often attributed to intervertebral disc abnormalities derived from trauma, ageing processes, metabolic disorders or degenerative disc diseases (Sharifi et al., 2014). Cases where neurological pain is debilitating and conservative treatments are insufficient, surgical intervention is required. Techniques involving decompression of the spine and fusion implants typically yield good short term clinical results with pain alleviation, however do not restore physiologic mobility.

Tissue engineering has opened a paradigm for intervertebral disc regeneration and the restoration of natural joint biomechanics. As intervertebral disc degeneration is often believed to originate from the central gelatinous component, the nucleus pulposus, hydrogels have been widely investigated as a potential replacement due to its structural and viscoelastic similarities. The gelatinous materials exhibit similar water retention and attraction properties as well as the characteristic long-range network order similar to the nucleus.

While gels resemble the natural nucleus pulposus on one level of molecular order, they lack the hierarchical structure and mechanical stability integral for good connectivity and cohesion with hard tissues. This is inevitably problematic *in vivo* due to leakages of biomolecules fundamental for localised regeneration and the inability to resist collapse in the spinal joint (Xu et al., 2010; Borges et al., 2011; Thorvaldsson et al., 2013). As a result, implants harnessing hydrogels place significant restrictions on implant performance and have known incidences of expulsion (Marcolongo et al., 2006; Martino et al., 2005; Goins et al., 2005; Korge et al., 2002; Husson et al., 2003).

Hydrogel-polymer composites have been explored, harnessing the structural and mechanical elements of both materials for nucleus pulposus regeneration (Gloria et al., 2007; Klara and Ray, 2002). Studies have focused on the integration of hydrogels with electrospun fibres to replicate the native extracellular matrix (Thorvaldsson et al., 2013; Strange et al., 2014). It was shown that the inclusion of nanofibers in hydrogels induced varying levels of reinforcement depending on fabrication methods. In addition, it was hypothesized that further reinforcement could be achieved by altering fibre alignment and gel concentrations (Thorvaldsson et al., 2013).

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Carrageenan is a naturally occurring anionic sulphated polysaccharide extruded from red seaweed of the Rhodophyceae family (Prajapati et al., 2014). Carrageenan has been investigated in biomedical applications for cell encapsulation and drug delivery (Garg et al., 2012; Popa et al., 2013, 2014). Types of carrageenan are determined by the number of sulphated groups with kappa (κ), iota (ι) and lambda (λ) possessing one, two and three sulphated groups per disaccharide unit respectively (Mihaila et al., 2013). Advantages of carrageenan over other hydrogels include its thermoreversible and ionic hydrogel characteristics which enable mild conditions for cell encapsulation and versatility in manufacturing and processing (Popa et al., 2014).

κ-carrageenan has the potential for tissue engineering applications due to its gelation properties, mechanical strength and its resemblance to natural glucosaminoglycans (Mihaila et al., 2013). *In vivo* screenings of an electrospun polycaprolactone (PCL)-carrageenan composite indicated that cells readily adhered to the scaffold (Basilia et al., 2008). In addition, carrageenan exhibits thixotropic characteristics which resemble nucleus behaviour as it can become fluidic when experiencing pressure, acting both as a fluid and a viscoelastic solid (Mihaila et al., 2013).

While carrageenan exhibits desirable mechanical and material similarities to the nucleus pulposus, it has not been considered for intervertebral disc regeneration. The present study evaluates the suitability of κ-carrageenan for the augmentation of a polymer scaffold to replicate connective tissue characteristics, with the gel functioning as a temporal incompressible phase in a nucleotomized spinal disc. Particular focus is placed on a regenerative non-injectable solution as a component of a total disc replacement, rather than an injectable hydrogel composite treatment, to provide a more continuous interface between the central nucleus and annulus fibrosus.

2. Methods

2.1. Sample preparation

Scaffold precursors were formed using sucrose crystals infused in a mixture of 4:1 acetone and PCL (M_n=80,000 g/mol; Perstrop Capa™ 6800, Australia) heated at 50 °C over night. Precursors were processed using particle leaching techniques and allowed to dry at room temperature. Polymer scaffolds were made to approximate dimensions of 18 mm×17 mm×11.5 mm and had an average porosity of 93.7 ± 0.2%.

Carrageenan gels (PT Forisa Nusapersada, Indonesia) were made to concentrations of 1%, 2% and 3% by stirring at over 85 °C for 5 minutes. Gel solutions were allowed to rest for one minute to allow air bubbles to escape and subside. Pure gels were made into varying geometries depending on test type by casting in moulds with a 40 mm diameter and 5 mm height, then cut to 5 mm and 10 mm diameters using a hole punch tool after allowing to set overnight.

Gel infusion within interpenetrating network (IPN) scaffolds was performed using a custom syringe system at 50 °C. Microscopy was used to verify infusion efficacy and consistency. The overall experimental setup is outlined in Fig. 1.

2.2. Mechanical testing

The mechanical properties of the gels and IPN scaffolds were characterised using microindentation. Indentation was carried out using an Instron 5544 universal testing frame (Canton, MA, USA) with a 5 N load cell. A spherical indenter tip of radius (R) 8 mm and 3 mm was used on gels and IPN scaffolds respectively to induce a maximum displacement (h_{max}) of 0.5 mm to conform to 5% strain as outlined in $\varepsilon = 0.2\sqrt{\frac{h_{max}}{R}}$ (Johnson, 1985). A small preload of 0.01 N was applied prior to a 10 second ramp to peak displacement. Viscoelastic responses were then examined over 300 s.

Gel samples of 40 mm diameter and 5 mm thickness and were secured to the plate using acrylonitrile and immersed in PBS. Prior to testing, infused gels were cut in half to a thickness of approximately 5.5 mm to ensure a flush gel-scaffold surface. 3–5 tests were carried out on 4 samples of each gel concentration and gel-infused scaffold. Viscoelastic indentation analyses were performed using an elastic-viscoelastic approach previously validated via numerical integration and finite element analysis (Mattice et al., 2006; Strange and Oyen, 2012; Qiang et al., 2011).

Succinctly, time dependent mechanical properties were analysed via the principle of elastic-viscoelastic correspondence using a Bolzmann hereditary integral formulation based on an elastic Hertzian contact solution (Mattice et al., 2006). As seen in Eq. (1), indentation load $P(t)$ was related to indentation displacement $h(t)$ and time t, assuming a constant Poisson's ratio v of 0.5.

$$P(t) = \frac{8\sqrt{R}}{3(1-\nu)} \int_0^t G(t-u)\left[\frac{d}{du} h^{\frac{3}{2}}(u)\right] du. \tag{1}$$

The function at which stress relaxes over time due to viscous effects in the material was assumed to take the form of a Prony series as expressed in Eq. (2). From this, the calculated parameters included the instantaneous modulus $[G_0 = 2G(0)(1+v)]$, equilibrium modulus $[G_\infty = 2G(\infty)(1+v)]$, and the viscoelastic ratio f ($f = \frac{G_\infty}{G_o}$), a measure of viscoelastic deformation. Viscoelastic ratios indicated the level of elasticity and viscosity exhibited by the material, where f=1 signifying a perfectly elastic material.

$$G(t) = C_0 + \sum_{i=1}^{2} C_i \exp(-t/\tau_i). \tag{2}$$

2.3. Permeability

Intrinsic permeability (k) was calculated using a custom rig to simulate fluid flow through a porous medium as described by Darcy's law (Eq. (3)) (Wang, 2000). Specimen dimensions were measured for thickness (L) and cross sectional area (A) then secured within the rig. 9 test samples were used with a diameter of 10 mm and an average thickness of 3.62 ± 1.44 mm. Pressure differences (ΔP) were measured with an applied flow rate of 0.1 mL/hr and 0.05 mL/hr (Q) of distilled water with a viscosity (μ) of 8.90×10^{-4} Pa s. Tests were allowed to equilibrate over 48 h.

$$Q = -\frac{kA\Delta P}{\mu L}, \tag{3}$$

2.4. Cell viability

Cellular evaluations of gels and gel-infused scaffolds were carried out using NIH 3T3 murine fibroblasts. Gel concentrations of 2% and 3% were used to compare cell mobility throughout the biomaterials. 36 samples were cut to dimensions of 5 mm×5 mm×3 mm using a scalpel blade and placed in a 48 well culture plate. Cells were seeded on top of samples with a density of 5×10^4 and incubated in a humidified atmosphere of 37 °C and 5% CO_2. Samples were cultured for 21 days in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin, with the culture medium changed every other day.

An AlamarBlue test was carried out to assess cell proliferation by adding 10% AlamarBlue solution in DMEM and incubated for 4 h at 37 °C. Absorbance was monitored using a fluorescence microplate reader (BMG Labtech Fluostar Optima Microplate reader; Ortenberg, Germany) at wavelengths of 544 and 590 nm. Absorbance of solutions were proportional to the cell metabolism and population in each