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Thermally triggered injectable hydrogel, which induces mesenchymal stem cell differentiation to nucleus pulposus cells: Potential for regeneration of the intervertebral disc

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ABSTRACT

There is an urgent need for new therapeutic options for low back pain, which target degeneration of the intervertebral disc (IVD). Here, we investigated a pNIPAM hydrogel system, which is liquid at 39 °C *ex vivo*, where following injection into the IVD, body temperature triggers gelation. The combined effects of hypoxia (5% O_2) and the structural environment of the hydrogel delivery system on the differentiation of human mesenchymal stem cells (hMSCs), towards an NP cell phenotype was investigated.

hMSCs were incorporated into the liquid hydrogel, the mixture solidified and cultured for up to 6 weeks under 21% O_2 or 5% O_2 where viability was maintained. Immunohistochemistry revealed significant increases in NP matrix components: aggrecan; collagen type II and chondroitin sulphate after culture for 1 week in 5% O_2, accompanied by increased matrix staining for proteoglycans and collagen, observed histologically. NP markers HIF1α, PAX1 and FOXF1 were also significantly increased where hMSC were incorporated into hydrogels with accelerated expression observed when cultured in 5% O_2.

hMSCs cultured under hypoxic conditions, which mimic the native disc microenvironment, accelerate differentiation of hMSCs within the hydrogel system, towards the NP phenotype without the need for chondrogenic inducing medium or additional growth factors, thus simplifying the treatment strategy for the repair of IVD degeneration.

1. Introduction

Low back pain (LBP) is a major cause of disability worldwide with over 80% of the population estimated to report LBP during their lifetime [1–3]. In the majority of cases, the aetiology of LBP is unknown; however, degeneration of the intervertebral disc (IVD) is believed to precede the clinical presentation in approximately 40% of cases [4,5]. The IVD is an avascular and aneural structure composed of three morphologically distinct regions: the cartilaginous endplates (CEP), which separate the IVD from the adjacent vertebra; the highly organized annulus fibrosus (AF) and the central gelatinous nucleus pulposus (NP), rich in collagen type II and proteoglycans (mainly aggrecan) [6,7]. Degeneration of the IVD is characterised by progressive changes in the extracellular matrix (ECM) including loss of proteoglycans and concomitant reduction in tissue hydration [7–10], alongside cellular changes including increased apoptosis [11] and senescence [12–14], together with decreased tissue cellularity and viability of remaining NP cells [12,15].

Current treatment strategies for LBP have been directed towards alleviating patient symptoms by lifestyle adjustments or surgical procedures to stabilize or remove the damaged tissue including discectomy and spinal fusion [16]; however current practices have been shown to have low efficacious outcomes and are often associated with altered spine biomechanics and accelerated degeneration in adjacent discs [17,18]. Future proposed treatments, driven by scientific research, have aimed to develop a

Abbreviations: LBP, low back pain; IVD, intervertebral disc; CEP, cartilagenous endplate; AF, annulus fibrosus; NP, nucleus pulposus; MSC, mesenchymal stem cell; hMSC, human mesenchymal stem cell; LCST, lower critical solution temperature; DMA, dynamic mechanical analysis; DLS, dynamic light scattering; HIF1α, hypoxia inducible factor 1 alpha; Foxf1, forkhead box F1; Pax1, Paired box protein 1; Runx2, runt related transcription factor 2; IHC, immunohistochemistry; XRD, X-ray Diffraction.

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biological therapeutic strategy, which addresses the underlying pathogenesis of IVD degeneration, with particular focus on the repopulation of functional and viable NP cells responsible for NP extracellular matrix synthesis and homeostasis [19]. From a clinical perspective the aim is to restore disc biomechanics and alleviate patient symptoms, thus the use of regenerative cells in combination with a biomaterial scaffold, in order to repopulate the NP and regenerate the matrix, whilst restoring disc height, appears to be an attractive strategy. One group of potential biomaterials are hydrogels which are three-dimensional, insoluble, crosslinked, hydrophilic polymer networks [20] which can potentially be used to deliver cells with regenerative capacity and provide mechanical support, facilitate cell growth, infiltration and differentiation [21,22]. Mesenchymal stem cells (MSC) are an attractive cell choice for transplantation since they can be extracted from multiple sources [23] and have the ability to differentiate into NP-like cells capable of producing an NP matrix rich in collagen type II and aggrecan [24–27]. Differentiation of MSCs to the NP phenotype may also be enhanced by culture in a hypoxic environment, native to the NP microenvironment [28]. Co-culture of MSCs and NP cells has also been shown to enhance NP cell differentiation, increase NP cell proliferation and restore the normal NP cell phenotype, thus providing anti-catabolic and anti-inflammatory effects [29–31].

The ideal biomaterial scaffold for MSC delivery would be a low viscosity, stimuli responsive hydrogel, which can be administered by minimally invasive injection followed by *in situ* gelation, to deliver cells and/or catabolic inhibitors, fills micro and macro fissures whilst restoring disc height. A variety of hydrogel scaffolds have been developed with the potential to be used for IVD repair [32]. Despite this, the gelation of many hydrogels requires the use of photocrosslinking or the addition of toxic cross- linking agents to induce gelation *in vivo* which can result in toxicity to the cells being delivered as well as the local tissues during delivery [27,33–37]. Other systems are too viscous for injection through fine bore needles, which may result in further damage to the surrounding AF and could accelerate rather than inhibit degeneration [38,39]. More recently thermosensitive injectable hydrogel systems have been investigated in pre-clinical studies, which enable the safe encapsulation of cells for NP regeneration [19,40–44]. However, differentiation of hMSC has not always been assessed [42] or the use of chondrogenic inducing medium [44] and/or the addition of growth factors [45] has been required to induce NP cell differentiation, thus adding both complexity and cost to the treatment strategy.

We have previously reported the development of a synthetic hydrogel delivery system which exploits the thermal phase transition of pNIPAM and its ability to form electrostatic interactions with Laponite® surfaces [46,47]. The polymerisation of NIPAM at the Laponite® surface above the lower critical solution temperature (LCST) in the globule conformation results in a stable low viscosity colloidal suspension; thus enabling the incorporation of cells and subsequent injection, via small bore needles (26 gauge), prior to them forming hydrogels *in situ* without the use of any additional, potentially toxic, reactive agents [46,47]. Upon cooling, the pNIPAM chains transform from the globule to the coil conformation, the polymer chains extend outwards, encapsulating the cells and forming bridging interactions with neighbouring clay platelets and entanglements with adjacent uncoiling polymer chains. This results in a crosslinked, three-dimensional hydrated polymer network, with incorporated cells, which does not re-liquefy at elevated temperature. We have previously demonstrated, through cell viability studies that cells could survive, adhere to the hydrogel surface, migrate through the hydrogel and deposit matrix [47].

This study tested the hypothesis that combined effects of hypoxia [5%O_2] and the structural environment of the pNIPAM-DMAc-Laponite® hydrogel delivery system could differentiate MSCs towards an NP cell phenotype without the need for additional chondrogenic inducing factors. The relative simplicity and clinical convenience of such a method could provide an effective and minimally invasive treatment platform for regeneration of the NP as a treatment strategy for IVD degeneration.

2. Materials and methods

2.1. Hydrogel synthesis

An exfoliated suspension of Laponite® clay nanoparticles (25–30 nm diameter, <1 nm thickness) (BYK Additives Ltd, Cheshire UK) was prepared by vigorous stirring of Laponite® (0.1 g) in deionised H_20 (9.0 ml) (18 mΩ) for 24 h. N-isopropylacrylamide 99% (NIPAM) (0.783 g) (Sigma, Poole UK), N,N′-dimethylacrylamide (DMAc) (0.117 g) (Sigma, Gillingham UK) and 2-2′-azobisisobutyro nitrile (AIBN) (0.009 g) (Sigma, Poole UK) were added to the suspension and stirred for 1hr. After passing the suspension through a 5–8 μm pore filter paper, polymerisation was initiated by heating to 80 °C and the reagents were allowed to react for 24 h. It was observed, that after heating the monomeric suspension to 80 °C, the transparent liquid transforms to a milky suspension which is comprised of a statistical copolymer with a composition of 1% Laponite, 7.83% pNIPAM, 1.17% DMAc and 90% water (by weight). Following 24 h the hydrogel suspension was cooled to 38–39 °C prior to cell incorporation. Further cooling of the polymeric suspension to 37 °C, i.e. below the LCST, resulted in rapid gelation (Supplementary Video 1) to a solidified hydrogel which was shown to be X-ray Diffraction (XRD) silent indicating complete dispersion of the Laponite® platelets [46].

2.2. Material characterisation

2.2.1. Dynamic light scattering (DLS)

DLS was performed using a Malvern Zetasizer Nano ZS instrument with a 4 mW He-Ne solid-state laser operating at 633 nm and RI detector at 173°. The instrument was equipped with an integrated Peltier temperature control device with accuracy of ±0.1 °C. The formation of pNIPAM-Laponite® liquid hydrogel was monitored by performing a polymerisation reaction in a quartz cuvette placed into the pre-heated instrument set at 80 °C.

2.2.2. Dynamic mechanical analysis (DMA)

DMA was conducted in triplicate using a PerkinElmer DMA8000 model in compression mode at 25 °C, applying a sinusoidal force with a 0.5 mm displacement at 10 Hz. Liquid pNIPAM-DMAc-Laponite® hydrogel were freshly prepared and solidified as a 4 mm thick sheet at room temperature (2 h), and a circular biopsy punch (4.5 mm i.d.) was used to remove cylindrical samples from the solid hydrogel. All sample dimensions were confirmed using digital callipers prior to measurement.

2.2.3. Dynamic viscosity

A glass capillary Ostwald viscometer was used to measure the dynamic viscosity of the liquid hydrogels. The absolute viscosity of the liquid was calculated using Eq. (1):

$$\eta_2 = \frac{\eta_{1\times}\rho_2 t_2}{\rho_{1\times} t_1} \quad (1)$$

where η_1 and η_2 are the absolute viscosities of water and sample respectively, t_1 and t_2 are time flow of water and sample respectively, and ρ_1 and ρ_2 are the density of water and sample, respectively. Flow times were measured using liquids equilibrated at