Three-dimensional hypoxic culture of human mesenchymal stem cells encapsulated in a photocurable, biodegradable polymer hydrogel: A potential injectable cellular product for nucleus pulposus regeneration

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ABSTRACT

Nucleus pulposus (NP) tissue damage can induce detrimental mechanical stresses and strains on the intervertebral disc, leading to disc degeneration. This study demonstrates the potential of a novel, photo-curable, injectable, synthetic polymer hydrogel (pHEMA-co-APMA grafted with polyamidoamine (PAA)) to encapsulate and differentiate human mesenchymal stem cells (hMSC) towards a NP phenotype under hypoxic conditions which could be used to restore NP tissue function and mechanical properties. Encapsulated hMSC cultured in media (hMSC and chondrogenic) displayed good cell viability up to day 14. The genotoxicity effects of ultraviolet (UV) on hMSC activity confirmed the acceptability of 2.5 min of UV light exposure to cells. Cytotoxicity investigations revealed that hMSC cultured in media containing p(HEMA-co-APMA) grafted with PAA degradation product (10% and 20% v/v concentration) for 14 days significantly decreased the initial hMSC adhesion ability and proliferation rate from 24 hrs to day 14. Successful differentiation of encapsulated hMSC within hydrogels towards chondrogenesis was observed with elevated expression levels of aggrecan and collagen II when cultured in chondrogenic media under hypoxic conditions, in comparison with culture in hMSC media for 14 days. Characterization of the mechanical properties revealed a significant decrease in stiffness and modulus values of cellular hydrogels in comparison with acellular hydrogels at both day 7 and day 14. These results demonstrate the potential use of an in vivo photo-curable injectable, synthetic hydrogel with encapsulated hMSC for application in the repair and regeneration of NP tissue.

1. Introduction

Changes in the composition and properties of nucleus pulposus (NP) can cause changes in the mechanical stresses and strains and physiological loads applied, which subsequently result in disc degeneration and back pain [1]. Biochemical changes that occur within damaged NP tissue include: a decrease in large proteoglycans (PG), an increase in collagen I and an increase in the ratio of small to large PG, resulting in a more fibrous NP tissue [1]. The NP is also avascular, is thought to have a high concentration of lactic acid [2] and is moderately acellular, with only 5000 cells mm^{-3}

compared with the annulus fibrosus, which has $\sim$9000 cells mm^{-3} [3]. Moreover, cells in the NP rely solely on diffusion of nutrients from adjacent endplates, and therefore natural repair of damaged NP tissue is a difficult and inefficient process [3].

Recently, many researchers have attempted to combine the effect of tissue engineering strategies with stem cell biology to act as a potential therapy to restore the function and properties of NP tissue. Cells in NP are of a rounded morphology and resemble typical chondrocytes located in articular cartilage [4]. Owing to the limited availability of NP cells within the actual tissue [5], an alternative cell type may be required. Stem cells such as bone marrow-derived human mesenchymal stem cells (hMSC) offer great therapeutic opportunities, given their characteristics of multipotential differentiation capacity, including chondrogenic lineages. HMSC are also immunopriviliged [6] and have the ability to expand into adequate cell numbers relevant for NP tissue purposes.

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Hydrogels are a popular choice as biomaterial scaffolds for tissue engineering applications because of their hydrated nature and ability to mimic the natural structure of in vivo tissue environments. Hydrogels provide properties such as biocompatibility, a flexible method of formation, anticipated physical characteristics, the provision of structural integrity to tissue constructs, essential structural and compositional similarities to extracellular matrix (ECM) and an extensive framework, which provides cellular proliferation and survival, making them the ideal candidate for cell delivery and to function as a temporary support for NP tissue regeneration [7]. There have been a limited number of studies attempting to encapsulate hMSC and drive their differentiation towards a NP lineage. Of the studies that do exist, natural polymers have been the popular choice, including alginate [8,9], hyaluronan gel [10], collagen [11], thermoresponsive hyaluronic acid [12] and chitosan-glycerophosphate [5]. Natural polymers pose limitations such as batch variation in tissue and immune responses [13]. For this reason, recent attempts have included incorporating or using synthetic polymers through alternative fabrication techniques such as photo-crosslinking for the production of hydrogels. Puramatrix™ is an example of a commercial, synthetic hydrogel that encapsulates hMSC [10]. The advantage of using synthetic polymers over natural polymers [14] is the ability to control and tailor the mechanical properties as well as dictate degradation profiles to match the rate of matrix production.

Benefits of using photopolymerization over the reversible nature of conventional chemical and ionic crosslinking techniques [13] include short reaction times, minimal heating, not requiring organic solvents, and relatively easy tuning of the materials to provide the desired mechanical properties and swelling behaviour [15]. Photocrosslinking can result in hydrogels that could promote optimal performance for NP tissue regeneration and temporary disc height support. Attempts to use photocurable hydrogels for application in NP tissue engineering have included: a two-phase hydrogel; N-methacrylate glycol chitosan and star-poly-ε-caprolactone-co-D,L-lactide triacrylate [16] and carboxymethylcellulose [13,17], where encapsulated NP cells demonstrated good cell viability and successful maintenance of the NP phenotype. Furthermore, chondrocytes have also been encapsulated and cultured in photocurable hydrogels produced from polyethylene glycol [18] and degradable lactic acid in polyethylene glycol [19]. Articular cartilage foetal cells, interstitial cartilage foetal cells and bone foetal cells have all also been investigated within a Tween-20 methacrylate T3 and N-vinyl-2-pyrrolidone photocurable hydrogel [20]. However, one study has also attempted to encapsulate and drive MSC differentiation towards NP phenotype using a photocurable hydrogel fabricated from polyethylene glycol diacrylate (PEGDA). The study demonstrated successful differentiation and good expression of GAG and Coll II [21].

Enhancing differentiation ability of hMSC towards an NP cell type can be made possible by combining the effects of hMSC exposure to low levels of oxygen and mimicking the three-dimensional (3-D) ECM environment through hydrogels. Native NP tissue is hypoxic and, therefore, much of the metabolic energy is acquired through the glycolytic pathway. Hence, hypoxic conditions can heavily influence the differentiation of hMSC towards an NP cell phenotype [8]. Furthermore, it is well known that culturing stem cells [22] under hypoxic conditions significantly enhances their differentiation ability [9,23,24]. Therefore, mimicking the native hypoxic conditions may prove beneficial for differentiating hMSC towards a NP cell type in combination with additional cues, chemically and structurally. This cell culturing method has been attempted by Stoyanov et al. [9], where the effect of hypoxia, chondrogenic growth factors and co-culture with bovine NP cells was investigated on hMSC differentiation to NP cells when encapsulated in alginate beads [9].

This study investigates the effectiveness of encapsulating bone marrow-derived hMSC in a novel 3-D photo-curable, biodegradable synthetic polymer hydrogel, polyhydroxyl ethyl methacrylate-co-N-(3-aminopropyl) methacrylamide grafted with polyamidoamine (p(HEMA-co-APMA) g PAA). In addition to hMSC encapsulation within hydrogels, it is hypothesized that the combined effects of hypoxia and chondrogenic media culture conditions will encourage the differentiation ability of hMSC towards a NP cell-like phenotype.

2. Materials and methods

2.1. Recovery, isolation and expansion of bone marrow-derived hMSC

Fresh human bone marrow aspirate (purchased from Lonza, UK) was recovered, isolated and expanded as described previously by Pittenger et al. [6]. Briefly, hMSC media consisted of DMEM, 10% fetal bovine serum (FBS; Lonza, UK), 1% L-glutamine (L-Glut; Lonza, UK), 1% non-essential amino acids (NEAA; Lonza, UK) and 1% penicillin and streptomycin, and incubation during the whole process took place in a hypoxic (2% O_2), SCI-Tive workstation (RUSKINN Technology, The Baker Company). Once recovered, isolated and expanded hMSC were tryspinized and cryopreserved in liquid nitrogen for future use.

2.2. Preparation of photo-curable hydrogel solution

The p(HEMA-co-APMA) g PAA polymer was prepared as described previously [25]. Hydrogels were prepared at 13.5% (w/v) concentration by dissolving the required amount of polymer in phosphate buffered saline (PBS). A 50% (w/v) Irgacure 2959 (prepared using DMSO) solution was added to the polymer solution at 1 vol.% of the total polymer solution. The polymer solution was briefly vortexed and sterile filtered before use.

2.3. hMSC encapsulation in hydrogels and hypoxic culture

The hMSC (Passage 3) were expanded using hMSC media under 2% O_2 conditions. Cell seeding solutions were prepared at a density of 200,000 cells/50 µl of hMSC media per hydrogel. Cell seeding solution was combined with the polymer solution (pre-sterile filtered), homogeneously mixed, and 300 µl volume was pipetted into a well of a sterile 48-well plate (Corning, UK). Cells and hydrogel solution were exposed to UV light using a longwave UV lamp (Black-ray B-100AP, high intensity, 100 W/365 nm) for 2.5 mins from a distance of 10 cm. Acellular hydrogels were photo-crosslinked using the same protocol, but without cells, as shown in Fig. 1. Two-dimensional controls were also used where hMSC were seeded at 100,000 cells well^{-1} without UV exposure. Encapsulated hMSC in p(HEMA-co-APMA) g PAA hydrogels and controls (tissue culture plastic (TCP)) were cultured in either hMSC media or chondrogenic media (consisting of DMEM, 1% FBS, 1% NEAA, 1% L-glut, 0.1 µM dexamethasone, 50 µM ascorbic acid, 40 µg ml^{-1} L-proline, 1% sodium pyruvate and 10 ng ml^{-1} TGF-β3) and incubated at 2% O_2. Media changes took place every 2–3 days.

2.4. Genotoxicity effects of UV exposure on hMSC

The hMSC seeded at a density of 100,000 cells well^{-1} were exposed to UV light for 0, 2.5, 10 and 20 mins. Cells were then cultured in hMSC media and incubated at 2% O_2 for 24 hrs. The media were removed, and cells were washed with PBS and fixed with 1% methanol-free formaldehyde (prepared in PBS) at 0 °C for 15 mins. Fixative was aspirated off, and the cells were immersed in 70% ethanol for 2 hrs at −20 °C. Cells were washed twice in PBS and
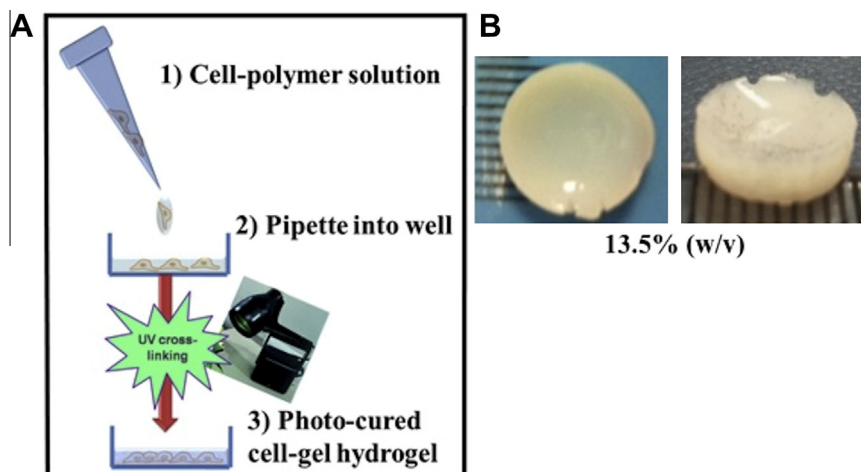
Fig. 1. Schematic showing the process of photo-curing of hMSC encapsulated within p(HEMA-*co*-APMA) g PAA hydrogels: (A) procedure of 3-D hMSC encapsulation in polymer to produce hydrogel; (B) representative image of a 13.5% (w/v) photo-cured hydrogel with dimensions 1 cm Ø and 3 mm thick).

immersed in 0.2% Triton-X-100 (prepared in 1% w/v PBS solution) for a further 30 mins. Cells were then incubated overnight in 1% bovine serum albumin (BSA) containing 5 μg ml^{-1} mouse anti-human phospho-histone monoclonal antibody (Ser139; Millipore, UK) at 4 °C. Finally, cells were washed twice with PBS and incubated with Alexa Fluor 633 goat anti-mouse IgG conjugated secondary antibody (1:100) for 30 mins at room temperature. Cells were also counterstained with DAPI (1 μg ml^{-1}; prepared in PBS) for 5 mins. Samples were imaged using a confocal microscope.

2.5. Cell viability: live/dead staining

Cell viability was investigated using a live/dead stain (FLUKA Analytical, Sigma–Aldrich, UK) at day 7 and day 14 time points. Media were removed from all cell–hydrogel samples and TCP controls, washed twice with PBS and stained with calcein-AM and propidium iodide solution as per the manufacturer's instructions. Samples in staining solution were incubated at 37 °C for 15–20 mins. The staining solution was removed, samples were washed twice with PBS and immersed in PBS before imaging. Imaging was performed with a confocal microscope using 490 nm/515 nm (excitation/emission) for calcein-AM and 535 nm/617 nm (excitation/emission) for propidium iodide.

2.6. Proliferative activity

Cell proliferation of hMSC cultured in p(HEMA-*co*-APMA) g PAA hydrogels and in the monolayer was evaluated using Alamar blue solution. Media (hMSC or chondrogenic) were removed at specific time points (24 hrs, day 7 and day 14), washed with PBS (for 5 mins) and immersed in 10% Alamar blue solution (prepared in hMSC media) for 90 mins and incubated at 37 °C. Fluorescence intensity was analysed at 570 nm/585 nm (excitation/emission) using the Synergy II plate reader (Biotek, UK).

2.7. Immunocytochemistry of multipotent expression and chondrogenic expression markers

Media were removed from hMSC encapsulated in p(HEMA-*co*-APMA) g PAA hydrogels and expanded on a monolayer in both media (hMSC and chondrogenic) for 7 and 14 days. Samples were washed twice with PBS and fixed using 100% ethanol for 30 mins, at room temperature. Samples were washed again with PBS, and non-specific proteins were blocked with 2% BSA (prepared in

PBS) for 1 hrs at room temperature. Samples were washed with PBS before primary antibody treatment with: goat anti-human aggrecan G1-IGD-G2 domains (20 μg ml^{-1}; R&D Biosystems, UK), mouse anti-human collagen II (Coll II, 1 mg ml^{-1}; Abcam, UK), mouse anti-human THY-1 (CD90, 1 mg ml^{-1}; Millipore, UK) and mouse anti-human STRO-1 (1 mg ml^{-1}; Millipore, UK) and incubated overnight at 4 °C. Primary antibodies were carefully removed, and the samples were washed with PBS and coated in relevant secondary antibodies: for Coll II and Stro-1 (donkey anti-mouse northern lights IgG; 10 μg ml^{-1}, NL557, R&D Biosystems), Thy-1 (goat anti-mouse northern lights IgM; 10 μg ml^{-1}, NL493, R&D Biosystems) and aggrecan (rabbit anti-goat IgG, Abcam, UK) and incubated at 37 °C for 2 hrs. Secondary antibody solution was removed, samples washed with PBS twice and stored in PBS, before imaging using a confocal microscope (Olympus Fluoview, FV5-PSU).

2.8. Cytotoxicity of p(HEMA-co-APMA) g PAA degradation products

The hMSC (20,000 cells well^{-1}) were plated and cultured in hMSC media containing; 0%, 5% and 10% (v/v) of degraded p(HEMA-*co*-APMA) g PAA polymer solution up to day 14 in physiological normoxic conditions. Media (with 0%, 5% and 10% degraded p(HEMA-*co*-APMA) g PAA polymer solution) were prepared by adding the relevant volume of degraded polymer solution in hMSC media prepared as stated in Section 2.3, as a total concentration of the polymer in media. Media changes took place every 3 days. At time points (24 hrs, day 7 and day 14) media were removed from cells, washed with PBS (twice) and immersed in 0.5 mg ml^{-1} MTT assay solution (prepared in serum-free media) for 4 hrs at 37 °C. MTT solution was gently removed, and samples were immersed in DMSO and incubated for 30 mins at 37 °C. Samples were homogenously mixed and aliquoted into a 96-well plate and absorbance read at 570 nm (Synergy II plate reader, Biotek).

2.9. Dynamic testing of mechanical property of acellular and cellular hydrogels

The hMSC were encapsulated in 13.5% (w/v) p(HEMA-*co*-APMA) g PAA hydrogels and cultured in both media types (hMSC and chondrogenic) up to day 14; in parallel, acellular hydrogels were also cultured in PBS under hypoxic conditions. Media changes took place every 2–3 days. At time points (day 7 and day 14) media/PBS were removed from the samples, and their mechanical properties

were characterized using the BOSE 3200 Series II test instrument and dynamic mechanical analysis (DMA) software (Bose Corporation, Electroforce Systems Group, Eden Prairie, MN, USA). The mechanical properties (dynamic stiffness and elastic modulus) of the samples were characterized under 5% compression at 1 Hz frequency.

2.10. Acellular hydrogel degradation under compression

Acellular p(HEMA-co-APMA) g PAA hydrogels were produced as previously described. Hydrogels were then exposed to a mechanical stimulation regime of uniaxial compression of 5% strain at 1 Hz for 900 cycles (15 mins) for 5 days a week, using the BOSE 3200 Series II test instrument, and then incubated back into PBS at 37 °C. The swelling behaviour and the weight loss of these hydrogels were evaluated at days 0, 7, 14, 28 and 56, with $n = 3$ repeats. At each time point, the swollen hydrogels were removed from the PBS and blotted gently to remove excess PBS. Hydrogels were washed in distilled water followed by ethanol 50% (v/v) and pure ethanol for an hour in each, and dried overnight from ethanol under a reduced pressure of 0.01 mbar. The swelling ability and weight loss were calculated using the following equations:

$$\text{Swelling degree } \% = (W_s - W_0) \times 100/W_0 \tag{1}$$

$$\text{weight loss } \% = (W_0 - W_d) \times 100/W_0 \tag{2}$$

where W_0, W_s and W_d are the initial, swollen and dried weight, respectively.

2.11. Statistical analysis

Average and standard deviations were calculated from three repeats on each sample, except for Alamar blue quantification data, where triplicate readings were performed for each repeat. Error bars on graphs represent one standard deviation (SD) in both positive and negative orientations. Data were tested for normality and a one-way ANOVA/Kruskall–Wallis test was performed, followed by a Tukey's post hoc test to determine the significance of origin for all data in Figs. 2, 3, 6 and 8. Significance levels were defined as: $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. Genotoxicity evaluation of UV exposure on hMSC

Expression of ϒH2αX was evaluated 24 hrs after hMSC were exposed to 0, 2.5, 10 and 20 mins of UV during initial seeding. The obvious increase in expression of ϒH2αX within hMSC with prolonged duration of UV exposure was visualized via immunocytochemistry. Quantification of ϒH2αX expression (performed by counting the number of ϒH2αX expressing cells in comparison with DAPI stained cells per field image using Image J) demonstrated significant increases between time points. Insignificant differences were observed between hMSC exposed to 0 mins and 2.5 mins of UV light. hMSC exposed to 2.5 mins of UV light resulted in 10% of hMSC expressing ϒH2αX (Fig. 2). However, 10 mins and 20 mins of UV exposure to hMSC resulted in a significant increase in the number of cells expressing ϒH2αX with a 1.5-fold and 7-fold increase, respectively (Fig. 2).

3.2. Cell viability and proliferation of encapsulated hMSC cultured under hypoxic conditions

The hMSC encapsulated within p(HEMA-co-APMA) g PAA hydrogels were cultured using two different media, hMSC and chondrogenic, up to day 14 in hypoxic conditions. Cell viability observations showed a majority of viable cells at day 7 and day 14, which confirmed acceptable biocompatibility and an appropriate 3-D environment that supported cell growth. Importantly, live/dead analysis also provided observations on morphological changes. It was witnessed that encapsulated hMSC within p(HEMA-co-APMA) g PAA hydrogels cultured in hMSC media exhibited typical hMSC morphology, which was fibroblastic and spindle-like. However, when cultured in chondrogenic media, a totally different morphology was observed (Fig. 3A). Cells appeared to be much more rounded and larger in size. Quantification of cell proliferation (Alamar blue) demonstrated a significant increase in cell numbers from day 7 to day 14 between all sample types (TCP and hydrogels in hMSC and chondrogenic media) (Fig. 3B). At day 14, significantly greater number of cells (1.185-fold increase) was observed on TCP when cultured in hMSC media in comparison with cells on TCP in chondrogenic media. However, among the hydrogels, a significant difference in cell numbers was not observed when cultured in either hMSC or chondrogenic media.

3.3. Multipotent and chondrogenic marker expression

The hMSC encapsulated in p(HEMA-co-APMA) g PAA hydrogels were cultured in both hMSC and chondrogenic media up to day 14 in physiological normoxia (2% O_2). Multipotent marker expression was evaluated using immunocytochemistry, which demonstrated, as expected, positive expression for Stro-1 and Thy-1(CD90) of hMSC encapsulated in p(HEMA-co-APMA) g PAA gels and when cultured on TCP in hMSC media at both day 7 and day 14 (Fig. 4). However, positive expression of both Stro-1 and Thy-1(CD90) was also witnessed when hMSC were encapsulated in pHEMA-APMA-PAA gels and also cultured on TCP in chondrogenic media at both day 7 and day 14 (Fig. 4).

Chondrogenic marker expression was evaluated by detecting expression of collagen II and aggrecan. As expected, the negative controls (primary antibody staining without secondary and vice versa) showed no expression of either collagen II or aggrecan. Despite not being a strong signal, expression of collagen II and aggrecan was visible for hMSC encapsulated in p(HEMA-co-APMA) g PAA hydrogels and cultured on TCP in hMSC media at day 7 and day 14 (Fig. 5). However, hMSC (cultured on TCP) in chondrogenic media displayed increased levels of collagen II and aggrecan in comparison with samples cultured in hMSC media at both time points (day 7 and day 14). Furthermore, increasing levels of collagen II and aggrecan were also witnessed from day 7 to day 14 in both hydrogels and TCP samples in chondrogenic media (Fig. 5). Interestingly, a change in cell morphology was also observed in hMSC cultured on TCP in chondrogenic media in comparison with hMSC media at both time points (day 7 and day 14), where cells appear with a rounded morphology (in chondrogenic media) and fibroblastic-like morphology in hMSC media. Furthermore, it is apparent that aggregates or condensation-like features are formed at day 14 in hMSC encapsulated hydrogels cultured in chondrogenic media, as shown in Figs. 4C, D and 5C, D.

3.4. Evaluation of cytotoxicity of p(HEMA-co-APMA) g PAA degradation products

Live/dead staining observations demonstrated the delay in initial adhesion and spreading of hMSC to TCP with increasing p(HEMA-co-APMA) g PAA monomer concentration in culture media after 24 hrs in culture (Fig. 6A). After a 14-day culture period, live/dead staining showed an increase in cell numbers within the observed field, with retention of hMSC morphology (typically fibroblastic) in all three media conditions. Quantification of cell
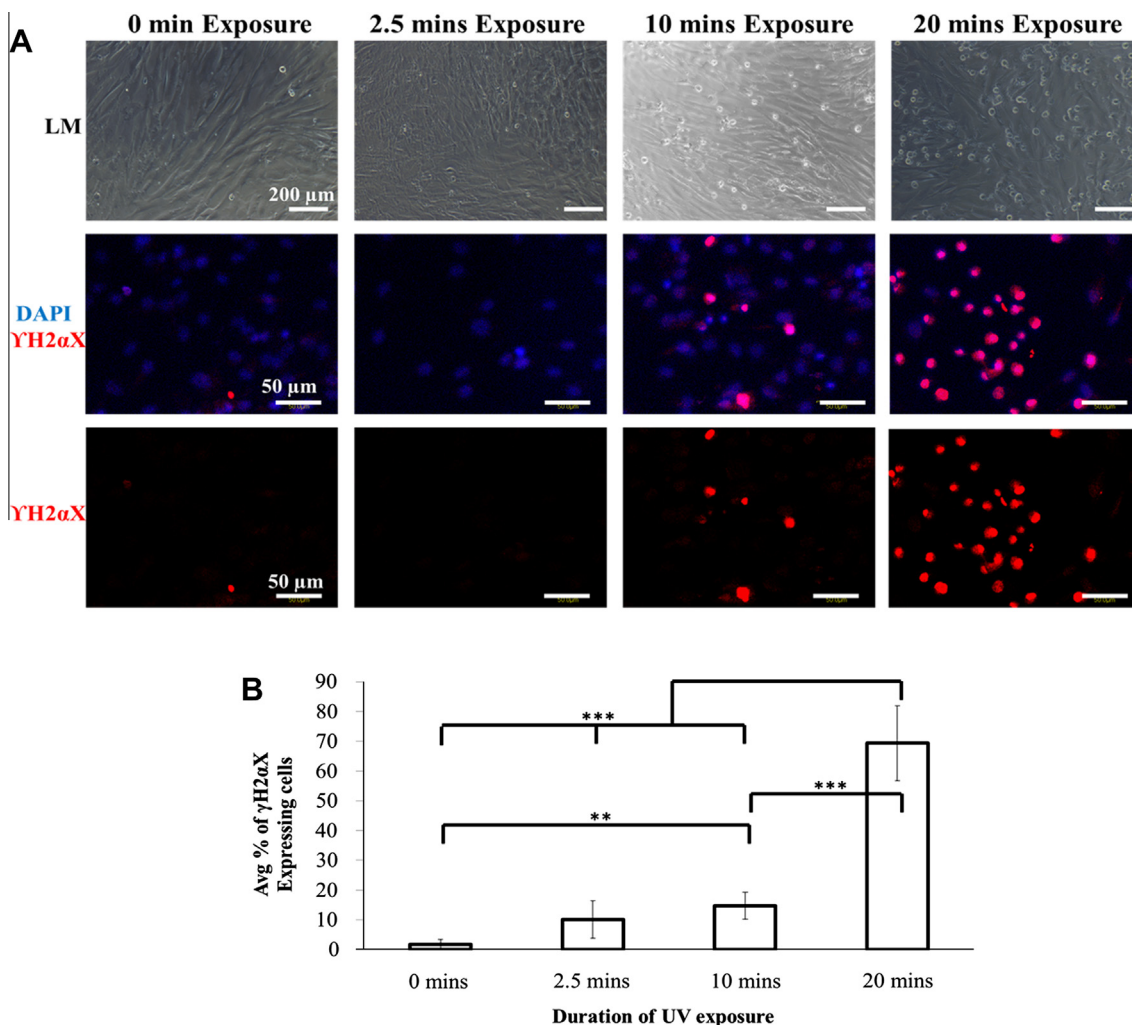
Fig. 2. Effect of UV exposure on hMSC DNA damage evaluated by the expression of ϒ-H2AX: (A) Top row: light microscopy images representing cell morphology and adhesion (scale bar = 200 μm); Middel and bottom rows: confocal immunofluorescence microscopy images of hMSC stained for ϒ-H2AX after exposure to 0, 2.5, 10 and 20 min to UV and then cultured on TCP for 24 h in hMSC media; (B) quantification of the number of rounded hMSC 24 h after being exposed to different exposure times of UV. Values indicate average % of ϒ-H2AX expressing cells and error bars indicate SD of $n = 3$ repeats. Data were tested for normality and a one-way ANOVA/Kruskall–Wallis test was performed followed by a Tukey's post hoc test. Significance levels are indicated according to the legend $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

viability was evaluated using MTT assay (Fig. 6B). Observations included an increase in hMSC numbers with increasing time of culture in all three media conditions (0%, 5% and 10%). However, a significantly greater number of hMSC were observed at day 7 in the control media in comparison with hMSC cultured in 5% (v/v) and 10% (v/v) of p(HEMA-co-APMA) g PAA monomer in media. A decrease in cell numbers of 6.33% and 10.6% was observed in 5% (v/v) and 10% (v/v) pHEMA-APMA-PAA monomer in media, respectively, at day 7, in comparison with cells cultured in control media. A significant decrease in cell numbers was also observed at day 14 with increasing concentration of p(HEMA-co-APMA) g PAA monomer in culture media. A 19% decrease in cell numbers was observed in 5% (v/v) p(HEMA-co-APMA) g PAA monomer in media compared with cells cultured in control media, and a 11.5% decrease in cell numbers was observed in 10% (v/v) p(HEMA-co-APMA) g PAA monomer in culture media.

3.5. Degradation profile under mechanical stimulation and mechanical property characterization of acellular and cellular hydrogels

The degradation behaviour of acellular hydrogels was determined by evaluating their swelling behaviour (water uptake) and weight loss, using the parameters calculated in Eqs. (1) and (2).

Observations revealed, as expected, an increase in water uptake from day 0 at 0% to a 467% increase at day 56 (Fig. 7A). As expected, an increase in water uptake was a result of an observed weight loss of these hydrogels, from 0% at day 0 to a 24.945% increase in weight loss (Fig. 7B).

Acellular hydrogels (cultured in PBS) and hMSC encapsulated hydrogels (cultured in hMSC and chondrogenic media) cultured under hypoxic conditions were characterized for their mechanical properties at day 7 and 14. Observations revealed a significant decrease in dynamic stiffness of cellular hydrogels at day 7 cultured in hMSC media (0.994 MPa; $p < 0.05$) and in chondrogenic media (0.929 MPa; $p < 0.05$) in comparison with acellular hydrogels kept in PBS also at day 7 (0.1272 MPa). Further significant decreases in stiffness were observed at day 14 of cellular hydrogels cultured in hMSC media (0.0775 MPa; $p < 0.01$) and in chondrogenic media (0.0692 MPa; $p < 0.01$) in comparison with acellular hydrogels (0.1198 MPa) (Fig. 8A).

Similar observations were also found for the elastic modulus values where there was a significant decrease in the modulus of cellular hydrogels at day 7, cultured in hMSC media (2.73 kPa; $p < 0.001$) and in chondrogenic media (2.7 kPa; $p < 0.001$) in comparison with acellular hydrogels cultured in PBS also at day 7 (4.23 kPa). Significant decreases were also observed in cellular
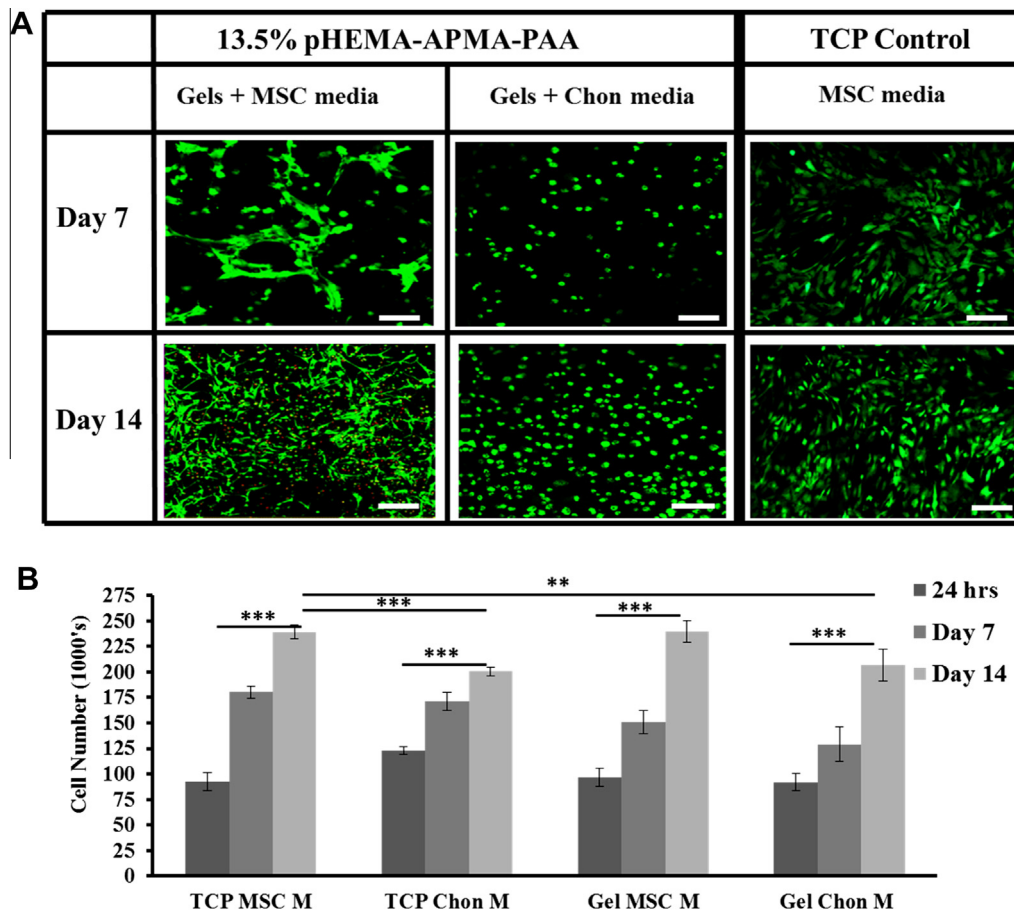
Fig. 3. Cell viability of encapsulated hMSC within p(HEMA-*co*-APMA) g PAA hydrogels: (A) live/dead staining of encapsulated hMSC expanded in both hMSC media and chondrogenic media up to day 14 (scale bar = 200 μm); (B) quantification of cell viability using Alamar blue. Values indicate average number of viable cells and error bars indicate SD for triplicate readings of three repeats for each sample type. Data were tested for normality and a one-way ANOVA/Kruskall–Wallis test was performed followed by a Tukey's post hoc test. Significance levels are indicated according to the legend $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

hydrogels at day 14, cultured in hMSC media (2.6865 kPa; $p < 0.01$) and in chondrogenic media (2.5168 kPa; $p < 0.01$) in comparison with acellular hydrogels cultured in PBS at day 14 (3.7 kPa) (Fig. 8B).

4. Discussion

Disc degeneration occurs as a result of changes in the biochemical process within NP cells. Changes in metabolic activity result in a decrease in matrix production and an increase in matrix breakdown with changes in the ratio of keratin sulfate to chondroitin sulfate, causing a decrease in proteoglycan content [26]. This ultimately has an impact on the fixed charged density of NP tissue, which decreases from 0.28 meq ml^{-1} to 0.18–0.20 meq ml^{-1}, resulting in a decrease in swelling properties and loss of disc height [26]. Biochemical changes usually translate into biomechanical changes such as alterations in stresses and strains, which lead to further distortion and damage to the intervertebral disc (IVD). The emerging therapy must allow tissue regeneration and complete recovery of the NP tissue both biochemically and biomechanically [26].

Currently, there are two in situ curable, polymers (Prosthetic Intervertebral Nucleus, Disc Dynamics, Minnetonka and Biodisc, Cryolife, Kennesaw) under development, and which have entered clinical trials. However, these therapies are only limited to restoring the height of the disc, but do not actually promote any neo tissue matrix secretion and regeneration [26]. Injectable,

in situ polymer hydrogels are of great interest for use in IVD repair, specifically targeting the restoration of the disc height and regeneration of NP tissue through minimally invasive surgery. Injecting stem cell (MSC) suspensions into NP tissue has demonstrated survival and ability to differentiate into chondrocytes [27], although issues have been reported, such as leakage of MSC into the disc surroundings, causing osteophyte formation and no improvement in disc height [28]. Therefore a preferred option may be to encapsulate MSC into a carrier, such as a hydrogel, to protect cell leakage, but also to provide the correct structural environment to support MSC expansion and appropriate differentiation [2]. Although nanofibrous substrates (poly-ʟ-lactic acid) have demonstrated an ability to increase the proliferative capacity of NP cells and enhance matrix secretion (GAG and collagen type II); a study by Feng et al. [29,30] explored the application of PLLA nanofibrous scaffolds in the support of rabbit MSC differentiation towards NP tissue under hypoxic conditions with TGF-β in media. Despite demonstrating successful differentiation by the up-regulation of typical markers, including aggrecan, collagen II and sox-9, this study did not combine an important feature of in vivo NP tissue, hydration, which hydrogels are able to do, while providing other advantages such as injectability and introduction via minimally invasive surgery [29,30].

Of the few studies that have attempted to use a photo-polymerization technique to fabricate hydrogels for NP tissue engineering, the present study is also an example where a novel, synthetic polymer hydrogel, p(HEMA-*co*-APMA) g PAA, is modified to provide relevant mechanical properties, increased swelling ability,
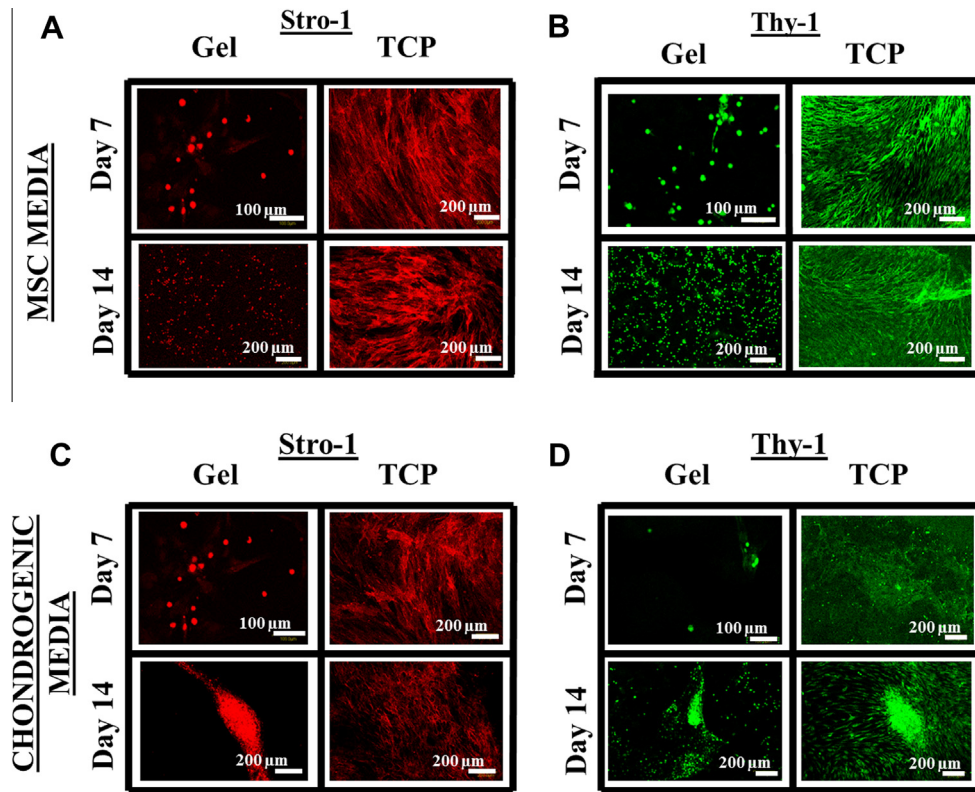
Fig. 4. Multipotent marker expression (Stro-1 and Thy-1) of hMSC encapsulated in p(HEMA-*co*-APMA) g PAA hydrogels and cultured on TCP in both hMSC and chondrogenic media up to day 14. Scale bar = 100–200 μm (see individual images for specific scale).
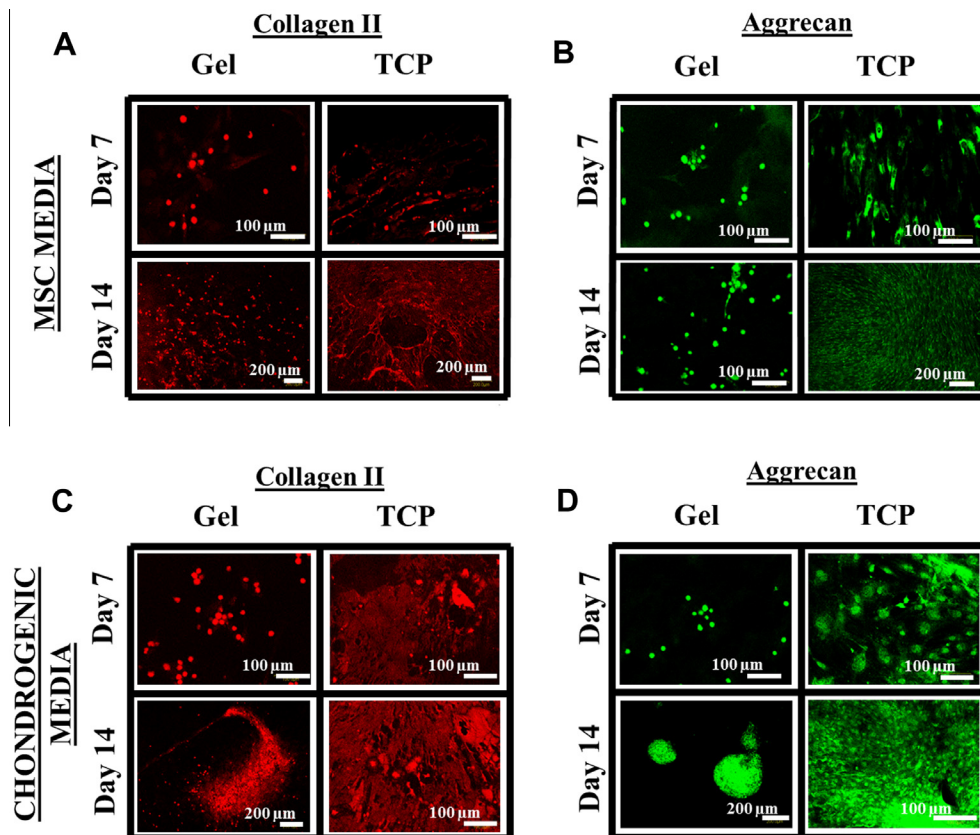

Fig. 5. Chondrogenic marker expression (collagen II and aggrecan) of hMSC encapsulated in pHEMA-APMA-PAA hydrogels and cultured on TCP in both hMSC and chondrogenic media up to day 14. Scale bar = 100–200 μm (see individual images for specific scale).
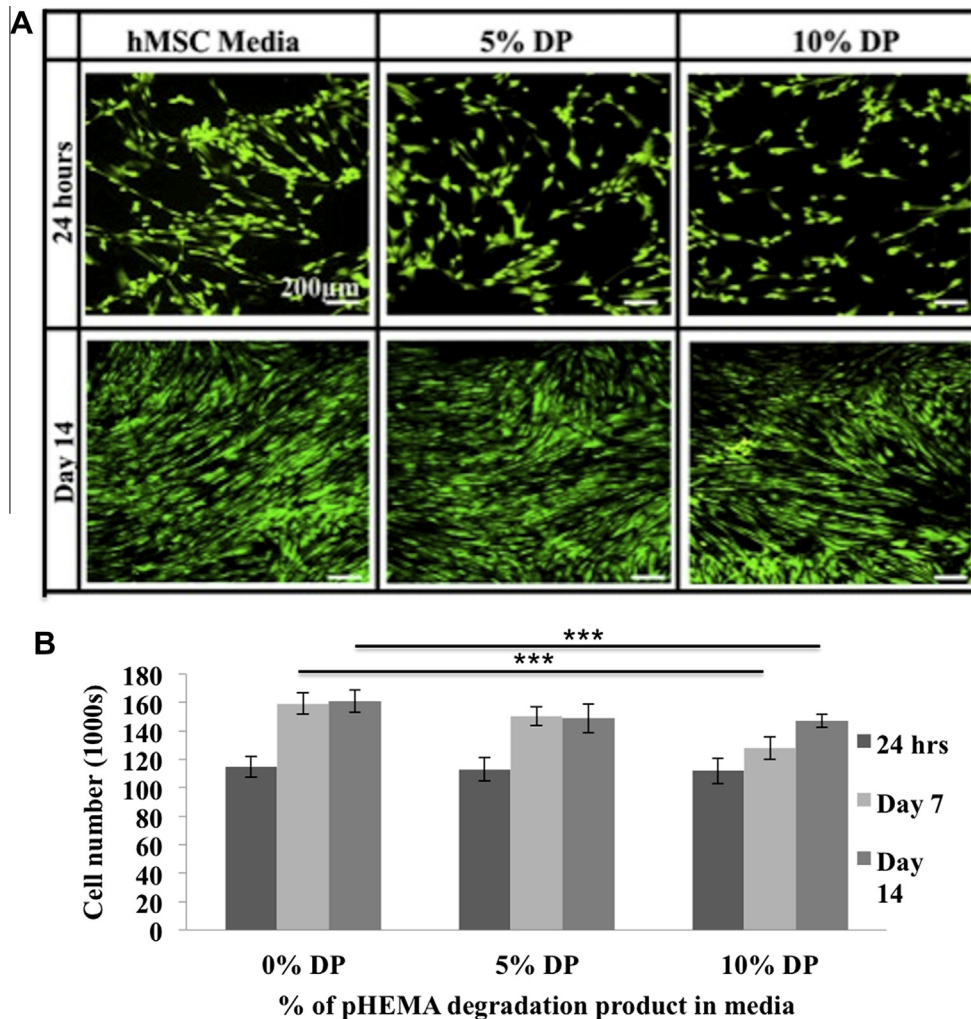
Fig. 6. Evaluation of cytotoxicity effect of p(HEMA-*co*-APMA) g PAA monomers on hMSC expansion. (A) Live/dead staining of hMSC cultured on TCP in control media, 5% of degradation product (DP) in hMSC media and 10% DP in hMSC media (scale bar = 200 μm). (B) Quantification of cell viability (MTT assay) during culture of hMSC in the different media up to day 14. Values indicate average number of viable cells, and error bars indicate SD for triplicate readings of three repeats for each sample type. Data were tested for normality and a one-way ANOVA/Kruskall–Wallis test was performed followed by a Tukey's post hoc test. Significance levels are indicated according to the legend: [*]$p < 0.05$, [**]$p < 0.01$ and [***]$p < 0.001$.

increased biocompatibility and is able to be photo-crosslinked by combining Irgacure 2959 in the polymer solution. Irgacure 2959 has proved to be cytocompatible [20] and has been used in previous investigations, where photo-curable hydrogels were produced from alginate, fibrin and carboxymethylcellulose [13]. In these studies, cells (NP cells and IVD cells of bovine origin) were encapsulated and cultured in vitro, and displayed good cell viability up to day 7, and pericellular deposition of chondroitin sulfate. The present study also demonstrated good cell viability and no toxic effect of Irgacure 2959 or the polymer hydrogel itself up to 14 days in culture, with successful differentiation of hMSC into chondrocyte-like cells using chondrogenic media indicated by increased expression of aggrecan and collagen II.

Studies that have used UV for photo-curing of their polymer hydrogels have all appeared to expose their cells and allow total crosslinking to occur within 2–3 mins, though some studies have used longer exposure times of up to 10 mins [31,32]. These studies, along with the present study, have confirmed no significant changes or damage to the cells as a result of UV exposure, validated by cell viability assays. Furthermore, many of the studies used the same parameters for UV energy, which is the Blak-Ray lamp (Model B100AP) at an intensity of ∼10 mW cm^{-2}, which were the exact parameters used in the present study for a maximum UV exposure time of 2.5 mins, which is also considered clinically acceptable for in vivo applications [31]. However, there are serious implications with using UV curable hydrogels, such as the risk of genotoxicity effects of UV exposure to encapsulated hMSC, which could result in genetic material modification and damage to DNA. DNA double strand breaks can induce the expression of ϒH2αX, an early detection marker of DNA damage [33,34]. The present authors investigated the effect of increasing UV exposure duration on hMSC DNA damage. It was observed that UV exposure to hMSC beyond 10 mins resulted in the detection and accumulation of ϒH2αX; however, insignificant differences were observed between no UV exposure and 2.5 mins of UV exposure, thus suggesting clinically acceptable levels of UV for hMSC encapsulation in UV cured hydrogels without causing any DNA damage [33,34]. Furthermore, as the synthetic polymer hydrogel is expected to degrade over time, it was proposed to determine the effects of accumulated degraded by-products on cell viability. It was observed that an increase in polymer degradation product in the media at 5% and 10% (w/v) concentration might affect cell proliferation to some extent, as evidenced by the slight decrease in cell numbers observed after 7 and 14 days of treatment with the degradation products. However, the main component of this hydrogel is the pHEMA backbone, which is considered highly
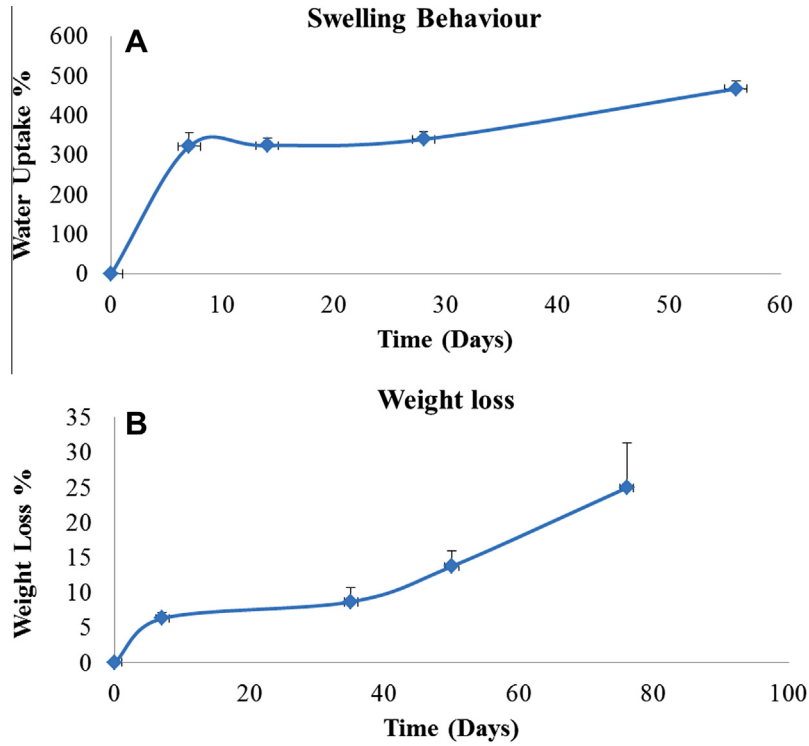
Fig. 7. Degradation profile of acellular hydrogels under mechanical stimulation determined by evaluation of: (A) water uptake; (B) weight loss.
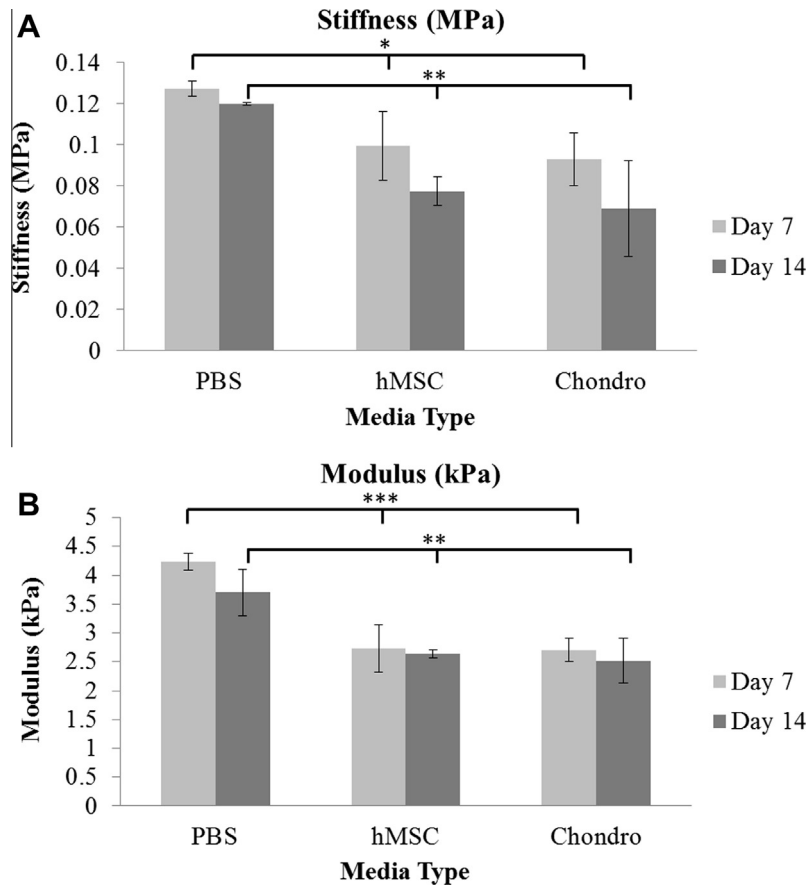

Fig. 8. Characterization of mechanical properties: (A) stiffness; (B) modulus values of both acellular and cellular hydrogels at day 7 and day 14 in culture. Values indicate average stiffness or modulus values, and error bars indicate SD of $n = 3$ repeats. Data were tested for normality and a one-way ANOVA/Kruskall–Wallis test was performed followed by a Tukey's post hoc test. Significance levels are indicated according to the legend: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

biocompatible and is commonly known for its use in soft contact lenses as a successful clinical product with no toxic effects during degradation [35]. Current clinical trials also include the investigation of pHEMA applications in ophthalmic surgery, dentistry and controlled drug delivery [36]. The other component of the hydrogel is PAA; degradation products of PAA also have a long history of biocompatibility and non-toxicity to various cells [37] and have been investigated for their use in many cellular activities, including mediating the efficient transfection of cells in culture [38,39]. A recent systematic study on the cytotoxicity of the degradation products of PAA copolymerized with PHEMA segments was assessed by Martello et al. [40] showing good biocompatibility. With this evidence in mind, it can be anticipated that the polymer hydrogel should show minimal cytotoxicity. Further to this, the hydrogel has been designed to degrade with degradation products <20 kDa (limit for renal filtration), which permits the ability to excrete these polymer by-products through the metabolic pathway efficiently, thus preventing localized accumulation of hydrogel by-products even in long term [40]. However, this proof of concept would have to be further validated by performing future in vivo experiments, which would be long term, where the cytotoxicity effects would be investigated in detail.

The advantages of using photo-curing as a method of hydrogel production include: short reaction times (as demonstrated in the present study; ~2.5 mins); minimal heating; no organic solvents; and the convenience of tuning the mechanical properties and swelling behaviour of materials [15]. Using natural polymers, it is very difficult to fine-tune their mechanical properties and swelling behaviour, similar to native NP tissue, which is soft, extremely hydrated and has an elastic modulus ranging between 3 and 6 kPa [15,41]. This study has shown that the novel synthetic p(HEMA-*co*-APMA) g PAA hydrogel can encapsulate hMSC in situ and support their growth and differentiation to cartilage-like cells. It has been well reported that stem cell differentiation can be affected by the mechanical properties of substrates supporting cell attachment and growth [42–45]. It is therefore anticipated that the polymer hydrogel used in the current study with mechanical properties similar to native NP tissue would not only function as a temporal mechanical support at the implantation site, but also facilitate the differentiation of encapsulated stem cells towards a phenotype similar to NP cells. This statement is further strengthened by a previous study that also proposes a similar hydrogel to function appropriately as a scaffold for NP regeneration [46].

Furthermore, it is promising to note an insignificant decrease in mechanical properties even after 14 days' degradation of acellular hydrogels in PBS. However, once hMSC were encapsulated in hydrogels and cultured in hMSC or chondrogenic media, a significant decrease in stiffness and modulus was also observed after 14 days in comparison with acellular hydrogels. The reason for such behaviour is likely to be a result of polymer degradation occurring through hydrolysis. It is very unlikely that the degradation was accelerated through enzyme-induced degradation as a result of incorporating biological material into the hydrogel, as no peptide crosslinker was presented in the polymer used in the current study. The influence of cells encapsulated within the hydrogel and the presence of serum on the degradation rate of hydrolytically biodegradable PLA and PEG-PLA-based gels has also been reported [47]. In addition, as no dramatic difference was noticed in medium containing 10% (hMSC media) and 1% (chondrogenic) FBS, the effects of proteases present in serum could be excluded. It has been demonstrated that PAA degradation is pH dependent, with a very significant increase in hydrolysis rate at reduced pH [48]. Considering that the cellular hydrogel was cultured in a hypoxic environment, it was anticipated that the increased lactate production as the consequence of anaerobic metabolism [49] could cause acidification in the immediate area of the cells and accelerate the

rate of hydrolysis and degradation, and the reduction of mechanical properties compared with acellular samples. As it has been confirmed that degenerative IVD have a more acidic ECM environment [50], it could be hypothesized that the rate of polymer hydrogel degradation in vivo could exceed the rate of neo matrix formation, and therefore further fine-tuning may be required to slow down polymer degradation to compensate for the slow rate of matrix production. Furthermore, a population of cells may be in a state of quiescence once encapsulated, as reported previously by Fedorovich et al. [51], which could explain the slower rate of matrix production. In any case, such a decline in the mechanical properties could be compensated by adjusting the polymer concentration; future work would be required to validate such changes in cell viability and differentiation, as these activities can also be affected by substrate stiffness [42].

The combined effects of hydrogel environment, hypoxia and chemical media on hMSC differentiation towards a chondrocyte-like cell were investigated in this study. It has been reported previously that hypoxic culture of hMSC encapsulated in alginate beads with chondrogenic media containing growth and differentiation factor-5 enhanced chondrogenic differentiation, but co-culture with bovine NP cells had little effect [9]. However, this study is the first to use a photocurable hydrogel in combination with hypoxic and chondrogenic media in culture, which also demonstrated successful differentiation of hMSC towards a chondrocyte-like lineage investigated by typical markers, collagen II and aggrecan, as used previously by others [9]. The precise differentiation of hMSC towards an NP cell type could have been further validated by evaluating the expression of additional markers such as HIF-1. In addition, the ratio of PG to collagen II could also have been quantified, which has recently been revealed to be different between NP tissue (27:1) and articular cartilage (2:1) [52]. However, the application of this hydrogel and study was to provide a biomaterial that would act as an encapsulation cell carrier, to provide mechanical support, not only to the cells, but also to the surrounding tissue in vivo and, more importantly, to provide a means of delivering the cells in a minimally invasive procedure to the target site, where photo-curing of the hydrogel would take place in vivo rather than prior to implantation. Thereafter, the present authors anticipate that the differentiation process of hMSC towards NP tissue would solely be reliant on cues signalled from the native in vivo tissue environment itself.

It is believed that, by mimicking the structural environment and the hydrated state of NP tissue using the hydrogel, and by providing the typical growth factors in chondrogenic media such as TGF-β and the hypoxic environment, which induces transcriptional changes in hMSC, it was possible to elicit an increased expression of aggrecan and collagen II as a result of outside-in and inside-out signalling between the cells and their environment, and would eventually lead to a subsequent secretion and production of NP-like tissue matrix.

In summary, this study has demonstrated the successful 3-D encapsulation, expansion and differentiation of bone marrow-derived hMSC in novel, synthetic polymer p(HEMA-*co*-APMA) g PAA hydrogels under hypoxic conditions. The combined effects of hydrogel structure, hypoxic conditions and chemical cues have all contributed towards promoting the differentiation of hMSC towards a chondrogenic cell lineage. Owing to short curing times via photo-crosslinking and relevant mechanical properties, this hydrogel has good potential as an injectable therapy, which is minimally invasive for in vivo NP tissue replacement.

5. Conclusion

This study investigated the potential for a novel, photo-curable synthetic polymer p(HEMA-*co*-APMA) g PAA for the 3-D

encapsulation, expansion and differentiation of bone marrow-derived hMSC under hypoxic conditions. This study confirmed good cell viability, polymer biocompatibility and little effect on genotoxicity as a result of UV exposure to encapsulated cells within hydrogels. Furthermore, the 3-D encapsulation within the hydrogel network, chemical and hypoxic cues supported their differentiation towards a chondrogenic lineage with elevated levels of collagen II and aggrecan. Characterization of mechanical properties of the hydrogels deciphered appropriate stiffness and modulus values of acellular hydrogels for the application in NP tissue repair. This injectable, photocurable hydrogel shows good potential for future in vivo NP applications, which can not only restore the disc height with relevant mechanical properties, but also has the opportunity to deliver therapeutic agents such as hMSC.

Acknowledgements

Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 1–7 are difficult to interpret in black and white. The full color images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2014.04.027.

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