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RESEARCH ARTICLE

Chitosan-based injectable hydrogel as a promising in situ forming scaffold for cartilage tissue engineering

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Abstract

Chitosan-beta glycerophosphate-hydroxyethyl cellulose (CH-GP-HEC) is a biocompatible and biodegradable scaffold exhibiting a sol–gel transition at 37°C. Chondrogenic factors or mesenchymal stem cells (MSCs) can be included in the CH-GP-HEC, and injected into the site of injury to fill the cartilage tissue defects with minimal invasion and pain. The possible impact of the injectable CH-GP-HEC on the viability of the encapsulated MSCs was assessed by propidium iodide-fluorescein diacetate staining. Proliferation of the human and rat MSCs was also determined by MTS assay on days 0, 7, 14 and 28 after encapsulation. To investigate the potential application of CH-GP-HEC as a drug delivery device, the in vitro release profile of insulin was quantified by QuantiPro-BCA^{TM} protein assay. Chondrogenic differentiation capacity of the encapsulated human MSCs (hMSCs) was also determined after induction of differentiation with transforming growth factor β3. MSCs have very good survival and proliferative rates within CH-GP-HEC hydrogel during the 28-day investigation. A sustained release of insulin occurred over 8 days. The CH-GP-HEC hydrogel also provided suitable conditions for chondrogenic differentiation of the encapsulated hMSCs. In conclusion, the high potential of CH-GP-HEC as an injectable hydrogel for cartilage tissue engineering is emphasised.

Keywords: cartilage tissue engineering; chitosan injectable hydrogel; chondrogenesis; in vitro release profile; mesenchymal stem cells

Introduction

The choice of appropriate scaffold material for cell- and factor-delivery is crucial for the development of cartilage-engineered constructs. The materials should be complemented with biomimetic and extracellular matrix (ECM) components to provide biological cues in order to elicit specific cellular responses and direct new tissue formation (Fedorovich et al., 2007; Yu and Ding, 2008). Among various biomaterials, hydrogels have become increasingly attractive in cartilage tissue engineering (Hao et al., 2010; Spiller et al., 2011) and drug delivery (Fedorovich et al., 2007; Ta et al., 2008). In this regard, chitosan (CH)-based hydrogels are predominantly used for cartilage tissue engineering (Fedorovich et al., 2007; Spiller et al., 2011) because of their desirable biocompatibility and biodegradability, without generating toxic by-products or debris, and also their high water solubility (free of any organic solvents) (Jin et al., 2009; Bhattarai et al., 2010). As glycosaminoglycans (GAGs) may play a special role in modulating chondrocyte morphology, and their differentiation and function (Bhardwaj et al., 2011), biomaterials that are structurally analogous to GAGs, such as chitosan, seem suitable candidates for tissue engineering of cartilage (Roughley et al., 2006). Due to the cationic nature of CH, it is primarily responsible for entrapment of anionic GAGs by electrostatic interactions. This would lead to the

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retention and concentration of many biomolecules linked to GAGs (Shi et al., 2006). CH-based scaffolds can serve as a delivery device for chemokines to mobilise stem/progenitor cells to the injection site of the hydrogel in defected areas. Hoemann et al. (2005) showed that chitosan hydrogels can adhere to cartilage and reside within the full-thickness of the chondral articular defects after loading.

Although in vitro engineered biomaterial-assisted cell-based approaches have shown promising clinical results, there are setbacks limiting their routine applications in clinic. Necrotic core formation in engineered-tissues thicker than 100 μm (Ko et al., 2007; Thevenot et al., 2010; Naderi et al., 2011), the need for invasive surgery, inflammation, and subsequent infection are among these limitations. To overcome these challenges, in situ forming scaffolds have been the focus of many investigations recently (Lim et al., 2013; Vanden Berg-Foels, 2013). Chitosan can be induced to form injectable hydrogels either by a physical endothermal mechanism or by covalent cross-linking (Hoemann et al., 2007). In the latter procedure, chitosan in combination with glycerol phosphate (GP) and a cross-linking agent like hydroxyethyl cellulose (HEC), can undergo sol–gel transition at 37°C. Injectable in situ forming gels are highly desirable in the clinics as they can be introduced into the body with minimal invasive surgery using endoscopic or percutaneous procedures (Hoemann et al., 2005; Amini and Nair, 2012). Bioactive molecules and/or cells can be entrapped in situ, merely via a syringe injection of their aqueous solutions at target sites with capability of filling irregularly shaped defects of various dimensions (Yan et al., 2010). On the other hand, injectable hydrogels, may have great potential for use as cell recruiting biomaterials for various in situ tissue engineering applications (Vanden Berg-Foels, 2013). For instance, some recent studies have examined chemotactic recruitment of adult progenitor cells into the hydrogels which provided sustained SDF-1α release (Lim et al., 2013). Given these circumstances, use of in situ-forming hydrogels seems to be an attractive approach for cartilage tissue engineering (Ringe et al., 2012).

Chitosan hydrogels provide a temporary three-dimensional (3D) matrix that increases cell retention and survival within the harmful environment of injured tissues. Such a condition also reduces clustering and the poor distribution of transplanted cells that are limiting factors for efficacy of the autologous cell implantation (Dell'Accio et al., 2001; Huang et al., 2008).

Bioactive molecules can also be entrapped into the chitosan hydrogels to guide cartilage regeneration. Insulin is structurally analogous to IGF-1 (insulin-like growth factor 1), which binds to the IGF-1 receptor, and thus elicits similar effects on cartilage (Maor et al., 1993; Schmid, 1995; Gaissmaier et al., 2008). This suggests that insulin may be an appropriate and inexpensive alternative to IGF-1 as a growth factor to improve the cartilage tissue engineering and was thus chosen as a model protein (Kellner et al., 2001). Some have described cartilage tissue engineering approaches based on insulin release using atelocollagen scaffolds (Ko et al., 2011), chitosan particles (Malafaya et al., 2010) and poly(D,L-lactide-*co*-glycolide) (PLGA) microspheres (Andreas et al., 2011). Insulin is also a potent survival factor (Loeser and Shanker, 2000), which can prevent central necrosis, induce redifferentiation of dedifferentiated chondrocytes, and promote matrix accumulation (Ko et al., 2011).

Our objective has been to investigate in vitro features of CH-GP-HEC hydrogel essential for application in cartilage tissue engineering, such as thermo-sensitive in situ gelling, cytocompatibility, insulin release profile and chondrogenic differentiation of MSCs within this scaffold.

Materials and methods

Isolation of human/rat MSCs

The human bone marrow aspirates were received in accordance with the ethical committee of the Charité Universitätsmedizin Berlin. hMSCs were cultivated as described previously in Dulbecco's modified Eagle's medium (DMEM, Biochrom, Berlin, Germany) containing 2 ng/mL basic fibroblast growth factor (bFGF, Peprotech, Hamburg, Germany), 10% foetal bovine serum (FBS, Hyclone, Bonn, Germany), 100 μg/mL streptomycin and 100 U/mL penicillin (Biochrom), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)-buffered saline (Biochrom) and 2 mM L-glutamine (Biochrom) (Ringe et al., 2007; Binger et al., 2009). Medium was exchanged after 72 h and every 2–3 days. Reaching 90% confluence, cells were detached by the addition of phosphate-buffered saline (PBS, Biochrom) containing 0.05% trypsin-EDTA (Biochrom) and replated at 5,000–10,000 cells/cm^2. Characteristics of isolated hMSCs (morphology, flowcytometry patterns, standard differentiation assays) were confirmed as in previous work (Menssen et al., 2011). MSCs from passage 3 were used for the experiments.

Rat MSCs (rMSCs) were extracted from bone marrow of adult male Lewis rats after ether euthanasia. Cells were removed from femurs and tibias by washing the bone shaft into cultivation medium. Cells were filtered through a 100 μm sieve and placed in 75 cm^2 culture flasks at 3×10^5 cells/cm^2. Non-adherent cells were removed after 72 h by media replacement. Upon reaching 80–90% confluence, adherent cells were trypsinised, washed and placed into new flasks. The experiments were carried out in accordance with the Guidelines of the Animal Care of the Charité Universitätsmedizin Berlin and Ferdowsi University of Mashhad.

Injectable CH-GP-HEC hydrogel preparation and experimental design

Temperature-responsive CH-GP-HEC injectable hydrogels were prepared as reported by others (Hoemann et al., 2007; Ahmadi and de Bruijn, 2008; Yan et al., 2010; Kim et al., 2011), with some changes. Briefly, a chitosan solution was prepared by dissolving 150 mg of chitosan (Polysciences, Warrington, England, MW 1.5×10^4, degree of deacetylation 84%) in 9 mL 80 mM hydrochloric acid (Sigma–Aldrich, Taufkirchen, Germany). This solution was autoclaved and cooled to 4°C. A 10× GP stock solution (1.5 M) was prepared by dissolving 3.240 g β-GP (tissue culture grade, Sigma–Aldrich) in 10 mL deionised water. One millilitre of 1.5 M (10×) filter-sterilised (0.22 μm) disodium-β-GP was added dropwise to the stirred chitosan solution on ice. The resulting mixture was stirred for another 15 min under aseptic conditions. Separately, HEC (Sigma–Aldrich) was dissolved at 20 mg/mL in DMEM and filter sterilised. The cell pellet of MSCs was homogeneously resuspended in 1 mL HEC, and mixed with 4 mL chitosan-GP at 4°C (final concentration 4×10^5 cells/mL for viability and proliferation assays, and 1×10^7 cells/mL for chondrogenic differentiation study). Immediately, the thermo-gelling cell suspension was dispensed into 12-well tissue culture plates (500 μL/well for viability and proliferation assays, 250 μL/well in chondrogenic differentiation study). The gel was allowed to solidify for 30 min in a humidified air atmosphere with 5% CO_2 at 37°C. After gelation, each well was rinsed twice with fresh DMEM plus 10% FBS and finally each scaffold was covered with 2 mL expansion medium that was refreshed every 2–3 days. The final concentrations of chitosan, GP and HEC in CH-GP-HEC scaffold solution were 12 mg/mL, 120 mM and 4 mg/mL, respectively.

Viability and proliferation assays

The viability of hMSCs and rMSCs after entrapment in the hydrogel was measured using a fluorescence assay that simultaneously determines live and dead cells using fluorescein diacetate (green) and propidium iodide (red), respectively (both from Sigma–Aldrich). The cell-encapsulating hydrogels were exposed to the probes for 30 min at 37°C, and cells were examined with an Olympus fluorescence microscope equipped with a digital camera.

The CellTiter 96® AQueous One Solution Cell Proliferation Assay was used for cell quantification (Promega, Mannheim, Germany). In brief, freshly prepared MTS reaction mixture was diluted in standard medium at 1:5 (MTS: medium) volume ratio at the indicated time intervals and was added to the wells with the cell-loaded hydrogel and incubated at 37°C under air plus 5% CO_2 for 3 h. Absorbance at 490 nm of the supernatant was measured in duplicate with a microplate reader (BIO-TEK Instrument, Germany) using Gene5 software. Viability and proliferation of hMSCs were measured from three donors and rMSCs from two donors in triplicate at 0, 7, 14 and 28 days. A blank sample of chitosan hydrogel was used as control.

In vitro release study

For preparation of factor-loaded CH-GP-HEC hydrogels, human recombinant insulin (Sigma–Aldrich) as model chondrogenic differentiation factor was diluted in HCl (0.1 M). One hundred microlitres of the protein solution was mixed homogeneously with the CH-GP-HEC solution. Each insulin-loaded hydrogel was placed in a well of a 12-well microplate and covered with 1 mL PBS. The hydrogels reached a final concentration of 100 μg and 1,000 μg insulin per 500 μL gel per well. In vitro release was measured in five replicates under physiological conditions (pH 7.4, 37°C, humidified atmosphere). Non-loaded (blank) hydrogels were used as controls. The release medium at each time-point (12 h and days 1, 2, 3, 4, 5, 6, 7 and 8) was completely replaced by 1 mL fresh PBS and stored at −20°C until protein quantification using QuantiPro™-BCA assay kit (Sigma–Aldrich). For the quantification, 150 μL of each of the standards, controls (supernatant of non-loaded hydrogels), and samples were placed in triplicate in a 96-well microplate and 150 μL of working reagent was added to each well. The plate was mixed thoroughly on a plate shaker for 30 s, incubated at 37°C for 3 h, and cooled to room temperature before absorbance was measured at 562 nm with a microplate reader (BIO-TEK Instrument, Germany). The control readings were subtracted from the samples. Standard curves were prepared by known concentrations of insulin.

Chondrogenesis of encapsulated hMSCs in 3D hydrogels

Three groups were included in the chondrogenesis study; the first group was composed of hydrogels encapsulating hMSCs that were cultured with control medium [high glucose DMEM, 20 mM HEPES and 100 μg/mL penicillin/100 U/mL streptomycin, 0.1 μM dexamethasone, 1 mM sodium pyruvate, 0.17 mM ascorbic acid 2-phosphate, 0.35 mM L-proline (all from Sigma–Aldrich), and insulin, transferrin and selenium (ITS+ premix) (Becton Dickinson, Bedford, England)]. In the second group, the hMSC encapsulating hydrogels were cultured with standard chondrogenic induction medium [control medium + 10 ng/mL transforming growth factor β3 (TGF-β3) (Peprotech)]. The third blank control group with non-loaded hydrogels was cultured with the standard chondrogenic induction medium.

hMSCs from three different donors (all at passage 3) were encapsulated at a concentration of 1×10^7 cells/mL hydrogel. Each experiment was conducted with three replicate and culture duration was 28 days. Briefly, pellets of hMSCs were

resuspended in 100 μL control media and loaded into the sol form of the hydrogels. The cell-containing hydrogel solutions were dispensed into 12-well tissue culture plates (250 μL/well) and allowed to gel at 37°C for 30 min. Two millilitre of the respective cell culture medium was added to each well. The cell-encapsulated hydrogels were cultured for 2 and 4 weeks in a humidified air atmosphere at 37°C with 5% CO_2. The culture medium was changed every 2–3 days until the end of experiments. Chondrogenic differentiation was examined by histological analysis and biochemical quantification of proteoglycans and GAGs.

Histological analysis of proteoglycans/GAGs production

For histological analysis, hydrogel samples at 1, 14 and 28 days from the three experimental groups were rinsed with PBS and embedded in Tissue-Tek® (OCT) compound, snap-frozen in liquid nitrogen and stored at −20°C. Twenty micrometres serial sections from frozen samples were mounted on Superfrost-Plus microscope slides and dried for 24 h at room temperature. After fixation with methanol/acetone (1:1), Alcian Blue/Nuclear Fast Red and Safranin O stainings were used to stain cryosections and detect proteoglycan synthesis as an indicator of cartilage ECM production. Since control hydrogels are stained with Fast Green, this nuclear counterstain was omitted during Safranin O staining. To assess cell morphology and distribution, cryosections were stained with Mayer's haematoxylin.

Biochemical quantification of sulphated GAGs

After 1, 14 and 28 days of culture, samples from all three groups of the chondrogenic differentiation experiment were collected, put on filter paper to drain surface water and their wet weight was taken. The same weight of each hydrogel was digested for 16–18 h at 60°C using 1 mL/sample papain solution (300 μg/mL papain in 100 mM sodium phosphate buffer with 10 mM EDTA-disodium salt and 10 mM L-cysteine, pH 6.5; Sigma–Aldrich) to release sulphated GAGs of hydrogels into the solution. GAG concentration was assayed spectrometrically from triplicate samples of the papain digests using DMMB (Sigma–Aldrich) dye-binding assay (Farndale et al., 1986). The content of sulphated GAGs in the culture medium supernatants was quantified without papain digestion. As GAGs are stable in the media for at least 2 weeks under the culture conditions, supernatant medium of one week (4 mL) at the end of each indicated time from 1 to 28 days was collected after every exchange of medium and stored at −20°C until the end of experiment (day 28). Media collected from all three groups were thawed at room temperature and biochemically analysed for GAG content similar to papain-digested samples with the DMMB assay.

In both experiments, 100 μL supernatant from papain-digested samples or culture medium supernatants were added to individual wells of a 96-well microplate, followed by addition of 150 μL DMMB solution (Hwang et al., 2007) [16 mg DMMB in 1l deionised water containing 3.04 g glycine (Sigma–Aldrich), 2.37 g NaCl and 95 mL of 0.1 M HCl]. In this assay, a red colour is generated upon the binding of DMMB to GAGs, which allows GAGs to be quantified by measuring the absorbance at 530 nm with a microplate reader (BIO-TEK Instrument). The GAG concentration was calculated via comparison with a calibration curve generated from standards of known quantities of chondroitin sulphate A (Sigma–Aldrich). OD readings of the blank hydrogels ($n = 4$) were used for background subtraction.

Statistical analysis

The SPSS v.21 statistical program was used to analyse the data. The data were expressed as mean ± SD. Significance of the data was considered statistically different at $P < 0.05$, using the one-way ANOVA and the Tukey test.

Results and discussion

Thermal gelation of the cytocompatible CH-GP-HEC solutions

Injectable and temperature-responsive CH-GP-HEC hydrogels are highly versatile with gelling times ranging from 5 min to several hours at 37°C. The CH-GP-HEC hydrogel used in this study gels after 20 min incubation in the incubator. However, this gelation time can be fine-tuned by varying the concentration of phosphate salts (Nair et al., 2007). The time of gelation is important in the clinical setting and 20 min is considered to be appropriate as it provides enough time to combine cells or therapeutic factors homogeneously into the chitosan sol before it becomes a viscose hydrogel in the defective site. GP concentration was 2.6% (w/v), which is necessary to bring chitosan pH and osmolarity to a cytocompatible level, and for sufficient neutralisation of amine groups of the chitosan to allow thermogelation by HEC at body temperature. Commercial-grade HEC contains glyoxal, a dialdehyde, which gives rise to covalent cross-linking of CH-GP-HEC hydrogels (Li and Xu, 2002; Hoemann et al., 2007). Because HEC helps to protect cells during gel formation (Roughley et al., 2006; Hoemann et al., 2007), MSCs were suspended first in HEC and then added to a combination of pre-prepared chitosan and β-GP before injection/incubation at 37°C.

Cell viability and proliferation inside the hydrogel

To assess the suitability of the CH-GP-HEC to support cell survival, hMSCs (Figure 1) and rMSCs (Figure 2) were

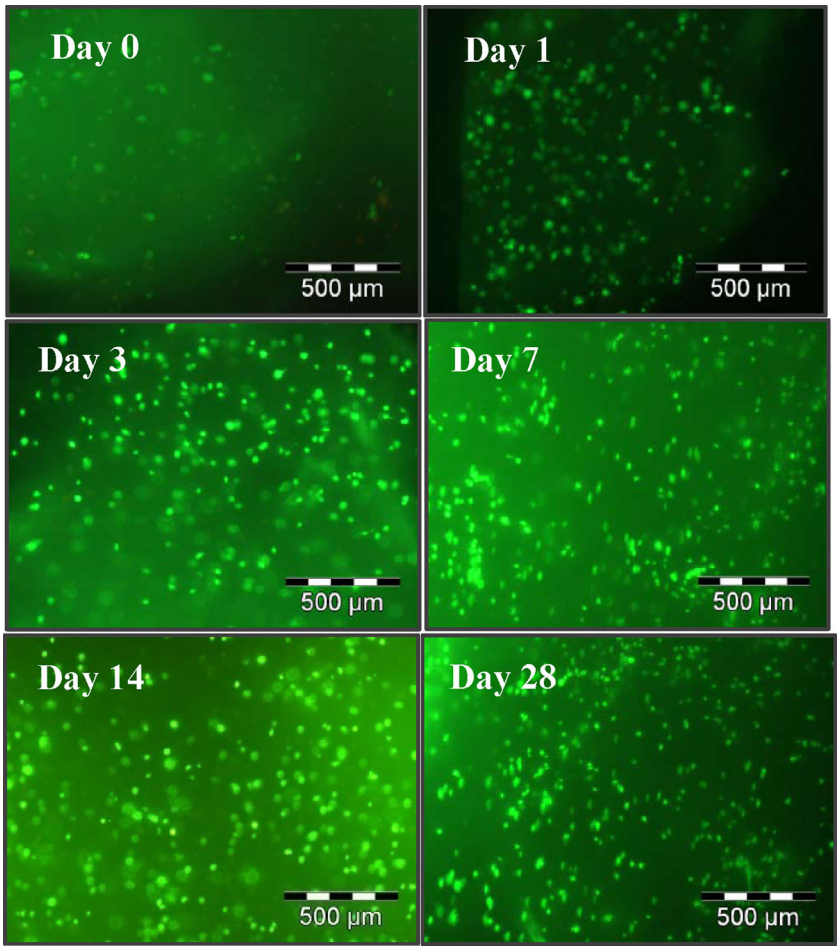

Figure 1 Live/dead fluorescent staining of encapsulated hMSCs with concentration of 2×10^5 cells in 500 μL CH-GP-HEC hydrogels after 0, 1, 7, 14 and 28 days in culture. Using PI-FDA staining live cells are indicated by green colour, while dead cells can be observed in red.

encapsulated in CH-GP-HEC hydrogels and viability monitored by propidium iodide-fluorescein diacetate (PI-FDA) staining of cell-hydrogel constructs over indicated time-points. Cell-free hydrogels were used as controls (Figure 2). Cells encapsulated inside CH-GP-HEC hydrogels were viable and maintained a round morphology. Consistently, Kim et al. (2011) showed that seeded cells have a spindle-like shape, but encapsulated cells have a round morphology inside chitosan gels (Kim et al., 2011). CH-GP-HEC hydrogels can maintain a good overall viability of MSCs for 28 days of culture, although the encapsulation process may still result in cell death at early time-points (day 0; immediately after encapsulation).

In the next step, proliferation of encapsulated human and rat MSCs in chitosan hydrogel was monitored in expansion media at different time-points over 28 days by MTS assay (Figure 3A and B). Both cells showed significantly higher

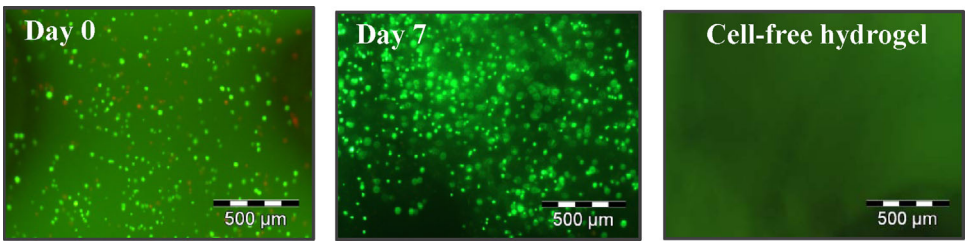

Figure 2 PI-FDA staining of rMSCs seeded at 2×10^5 cells in 500 μL scaffold per well in 12-well plates. Directly after seeding, some dead cells (red) can be observed. This may result from the encapsulation process of cells into the scaffold and the long experimental time. At day 7, the majority of cells were alive within the scaffold (green).

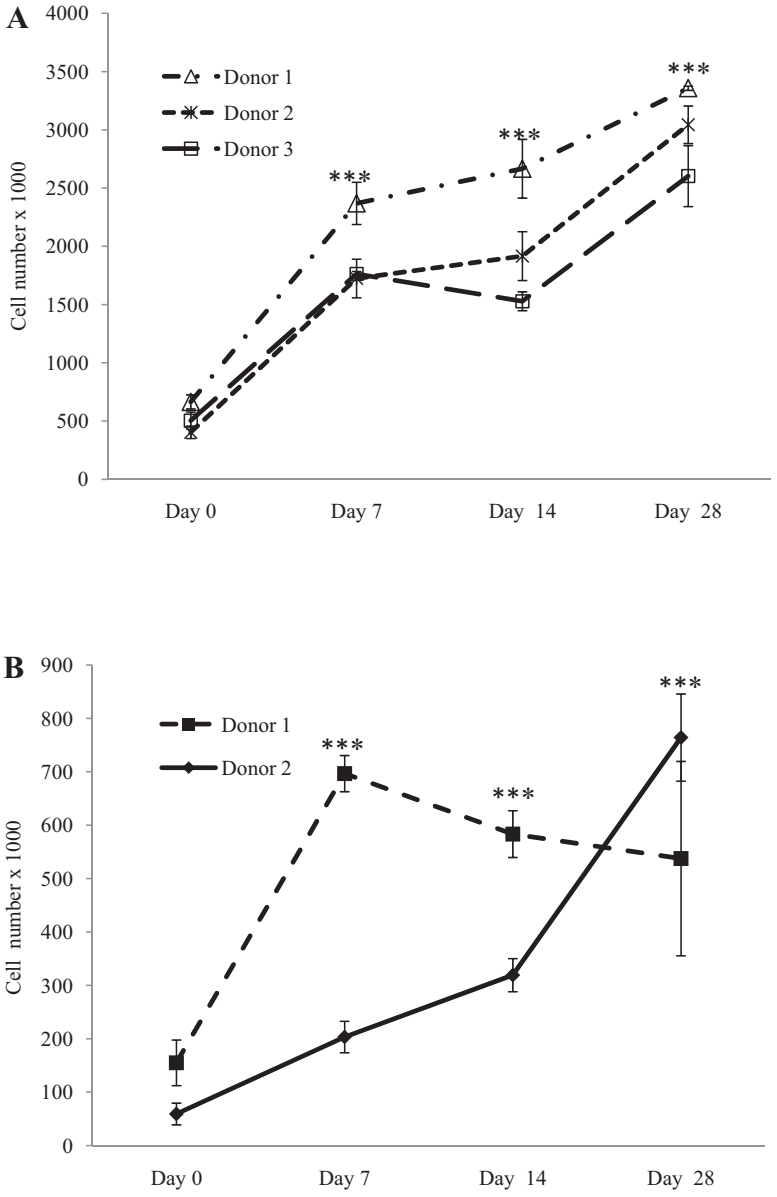

Figure 3 Number of encapsulated hMSCs (A) and rMSCs (B) in 500 μL of CH-GP-HEC hydrogels after 0, 7, 14 and 28 days in expansion media. Proliferation of cells was measured by MTS assay. Blank hydrogel was used as control for background subtraction. Results are represented as means ± SD (n = 3). *** denotes significant difference at all time points as compared to day 0 of culture ($P < 0.001$).

metabolic activity at day 7–28 compared to day 0 ($P < 0.001$), indicating that the cells could grow and proliferate within CH-GP-HEC hydrogels. However, changes in cell number were inconsistent for rMSCs (Figure 3B) compared to hMSCs (Figure 3A). A possible explanation for this is that MSCs from different species and donors were heterogeneous and had variable proliferation rates with time. Other possibilities may be inhomogeneous distribution of cells or non-optimal initial cell density so that after sometime cells face surface area limitation and undergo differentiation or cell death. However, fold change of cell number at day 28 compared to day 0 was significantly increased ($P < 0.001$) for all donors from both specious by ∼5-, ∼7.5- and ∼5-fold for hMSCs (donors 1, 2 and 3, respectively) and by ∼4- and ∼13-fold for rMSCs (donors 1 and 2, respectively). These results are consistent with results from Ahmadi and de Bruijn (2008) and demonstrate that chitosan hydrogels are not only non-toxic, but can stimulate MSC proliferation, representing a suitable biomaterial for cell encapsulation.

Chitosan hydrogels with high salt (GP) concentration produce ionic strengths unsuitable for cells and inhibit cell growth (Ahmadi and de Bruijn, 2008; Cho et al., 2008). In

order to reduce the cytotoxicity of chitosan-based injectable hydrogels and improve cell viability, some authors have used less toxic cross-linkers, such as genipin, glyoxal and HEC, responsible for the solidification with low concentrations of GP (Li and Xu, 2002; Hoemann et al., 2007; Moura et al., 2011; Wang and Stegemann, 2011).

Insulin release kinetics

The in vitro release of insulin from the CH-GP-HEC hydrogels was determined over 8 days, the cumulative release being shown in Figure 4. The CH-GP-HEC hydrogels containing a higher concentration (1,000 μg/500 μL scaffold) of insulin released significantly greater amounts compared with hydrogels containing a lower concentration (100 μg/500 μL scaffold) (Figure 4A) within the examination time. However, the percentage cumulative insulin release from the differently loaded gels showed no obvious difference (Figure 4B). For example, the CH-GP-HEC hydrogels containing a higher concentration of insulin (1,000 μg/500 μL scaffold) exhibited average cumulative release up to $60.7 \pm 1.6\%$ at day 2 similar to average cumulative release of lower concentration (100 μg/scaffold) up to $61.9 \pm 2.1\%$ at day 2. The results show that the initial loading concentration does not play an important role in the release profile of insulin from the CH-GP-HEC hydrogels; both concentrations had similar percentage release profiles (Figure 4B) and complete release was achieved after 8 days. Thus insulin

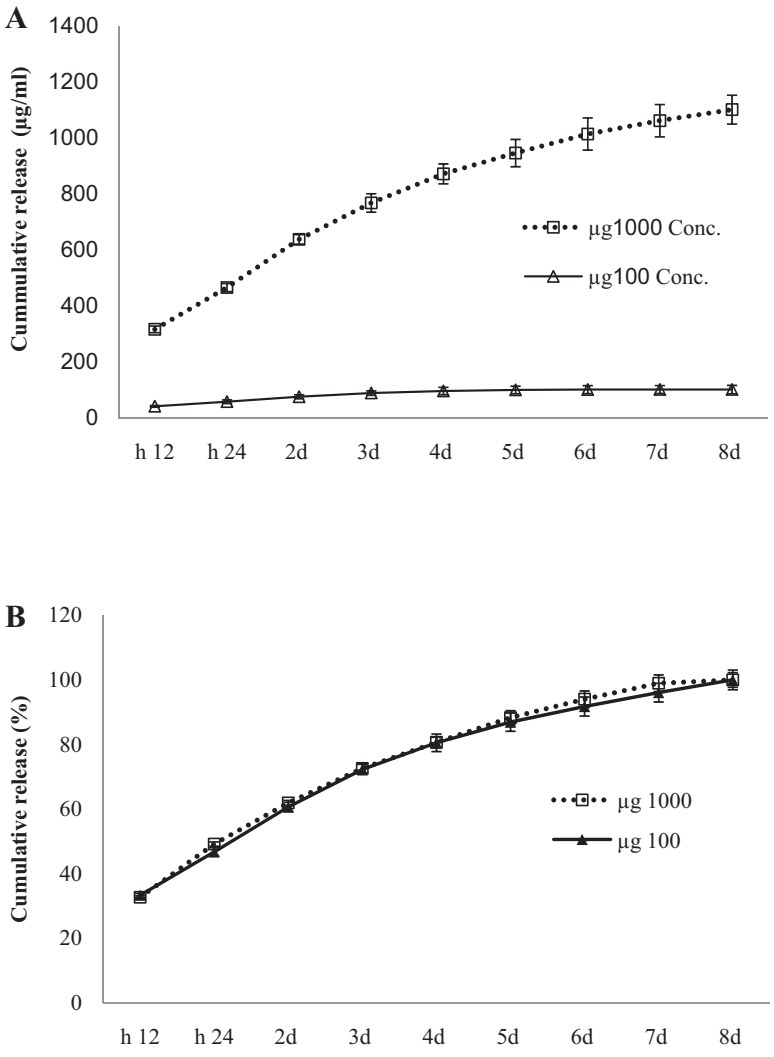

Figure 4 Cumulative in vitro insulin release from CH-GP-HEC hydrogel. (A) Cumulative released amount as micrograms of two different insulin loading concentrations per 500 μL hydrogel during indicated times. (B) Cumulative release percent. Results are presented as means ± SD of five replicates. No significant differences were observed between the release percentages of two different concentrations.

seems to have a sustained release from the CH-GP-HEC hydrogels over this period of time and is independent of the initial loading concentrations.

Chitosan-based delivery systems have gained popularity because of their ability to release drugs at a local target site in a controlled manner (Ruel-Gariepy et al., 2000; Bae et al., 2006; Yu and Ding, 2008; Bhattarai et al., 2010; Kim et al., 2010). Generally, drug release from biodegradable polymeric systems is governed by initial drug loading, diffusion, drug and polymer interactions, drug solubility and polymer degradation (Gunatillake and Adhikari, 2003; Bhattarai et al., 2010). In this study, the incorporation of insulin into CH-GP-HEC hydrogels showed a sustained release of insulin over 8 days. This is in contrast with the findings of other studies (Ruel-Gariepy et al., 2000; Wu et al., 2006) as it demonstrated that insulin encapsulation into a CH-GP-HEC formulation allows a prolonged release of drugs. Interestingly, percentage insulin release was not affected by initial drug loading. Conversely, Ruel-Gariepy et al. (2004) found that the initial paclitaxel loading substantially affected the release percentage from chitosan-GP hydrogel, 92% cumulative release for the 6.4 mg/mL-loaded gel rather than a 43% cumulative release for the 64 mg/mL-loaded gel after one month (Ruel-Gariepy et al., 2004).

Due to the high water content of hydrogels, their release mechanisms are different from other drug delivery systems comprised of less hydrophilic or hydrophobic polymers. Some studies have predicted that the release of an active agent from a hydrogel, as a function of time, is controlled by diffusion, swelling or chemical reactions (Amsden, 1998; Bhattarai et al., 2010; Lamberti et al., 2011). Considering the nature of hydrogels and their high water content, the mechanism behind the insulin release seems to be concentration gradient-driven diffusion. In this manner, the insulin was incorporated into the aqueous environment of the CH-GP-HEC hydrogels and its in vitro release was governed by its diffusion from the gel matrix. However, other studies suggest that the delivery of drugs and growth factors from injectable chitosan hydrogels is not only initially by diffusion, but later by degradation of the hydrogel (Ruel-Gariepy et al., 2000; Nair et al., 2007; Bhattarai et al., 2010). However, we did not observe obvious degradation of CH-GP-HEC hydrogels in vitro after 8 days (data not shown). In addition, the initial release rate can be controlled by molecular weight, degree of deacetylation of chitosan and concentration of GP (Ruel-Gariepy et al., 2000; Bae et al., 2006; Wu et al., 2006; Nair et al., 2007). Li and Xu (2002) investigated in vitro release profiles of CH-GP in combination with three different concentrations of HEC, showing that the release mechanism of drugs from CH-GP-HEC hydrogels is independent of HEC content in the gels and is governed by Fickian diffusion, although HEC is one of the principle components of the network formation (Li and Xu, 2002).

We explored the possibility of the CH-GP-HEC hydrogel for delivery of insulin as a potent factor that prolongs the cell survival and enhances the matrix production of chondrocytes. Indeed, the feasibility of using the CH-GP-HEC gels as a stem cell carrier vehicle as well as an insulin delivery vehicle has been shown. Others found insulin to enhance strongly the redifferentiation of chondrocytes, which inevitably dedifferentiate during cell expansion, to increase the cell viability in the middle part of the constructs and induce the in vivo cartilage regeneration (Ko et al., 2011). In our previous study, we showed that the insulin released from PLGA microspheres effectively promoted cell viability and matrix synthesis (Andreas et al., 2011). Another study suggests the efficacy of insulin in increasing the growth rate and glycosaminoglycan fraction of engineered cartilage (Kellner et al., 2001; Malafaya et al., 2010).

Chondrogenic differentiation of the encapsulated MSCs

The chondrogenic induced cell-CH-GP-HEC hydrogel constructs were examined by histological methods to identify the location of the entrapped cells and the distribution of the retained proteoglycans. The Alcian Blue/Nuclear Fast Red stainings were used to detect the anionic GAG chains of the proteoglycans (Figure 5A). Additionally, Safranin O staining was performed (Figure 5B) to confirm the results. A non-induced construct as a control experiment showed less or minimum staining for proteoglycans/GAGs in chondrogenic control medium (without TGF-β3) at day 28 (Figure 5C). Analysis of the cell-encapsulating hydrogel by haematoxylin staining revealed a relatively uniform distribution of cells throughout the scaffold (Figure 5D). Consistent with our data, others have shown that hydrogels allow uniform encapsulation of cells (Hoemann et al., 2005; Amini and Nair, 2012; Rao et al., 2012).

The CH-GP-HEC hydrogel supported GAG production. Histological staining for proteoglycans showed the differences in the matrix deposition over time, more visible at day 14, indicating the time in which MSCs started to differentiate towards chondrocytes. The GAG density in the scaffold increased considerably from day 14 to 28 (Figure 5A and B). The data suggest that MSCs encapsulated in CH-GP-HEC hydrogel expressed elevated levels of proteoglycans compared to the control group, indicating that addition of TGF-β3 shifted the MSCs toward the chondrogenic phenotype. Others have demonstrated enhanced MSC chondrogenesis following delivery of TGF-β3 from alginate microspheres within hyaluronic acid hydrogels in vitro and in vivo (Bian et al., 2011). Even transient exposure (7 days) to a very high level of TGF-β3 (100 ng/mL) improved chondrogenesis of MSC-loaded hyaluronic acid hydrogel (Kim et al., 2012). Hence, it is reasonable to conclude that CH-GP-HEC constructs with a sustained release profile of 8 days may be

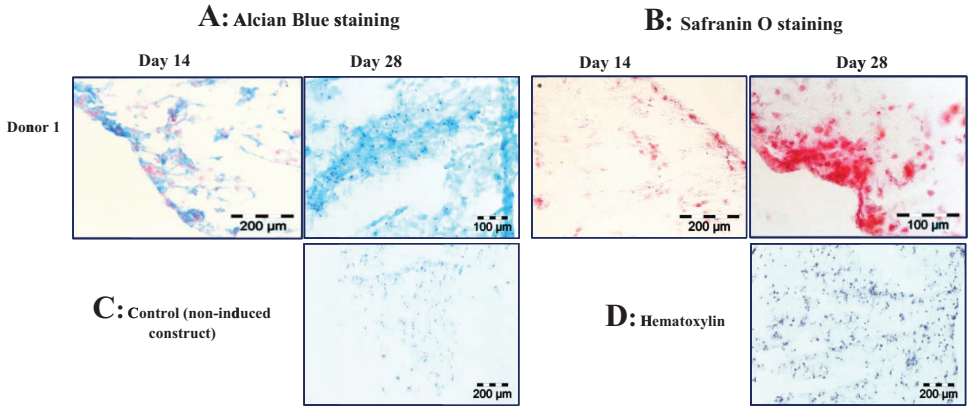

Figure 5 Histological staining of hydrogels. Staining of proteoglycans/GAGs produced by hMSCs encapsulated in CH-GP-HEC hydrogel with Alcian Blue/Nuclear Fast Red (A) and Safranin O (B) cultured in chondrogenic induction medium on days 14 and 28. Cryosections prepared from constructs cultured in control medium without TGF-β3 at day 28 were used as non-induced control, stained with Alcian Blue/Nuclear Fast Red (C). Haematoxylin staining (D) was performed for observation of cell distribution.

used as scaffolds to transiently induce chondrogenesis of encapsulated MSCs by incorporating TGF-β3 and/or insulin for cartilage engineering. Administration of insulin into atelocollagen hydrogel enhances the in vivo cartilage regeneration (Ko et al., 2011).

Biochemical analyses of the constructs showed significant accumulation of ECM (assessed as GAG) in thermo-sensitive chitosan hydrogels at days 14 and 28 compared to control and to day 1 (Figure 6). Increased GAG content indicates that encapsulated hMSCs maintained their differentiated phenotype. Irrespective of GAG release into the medium, a

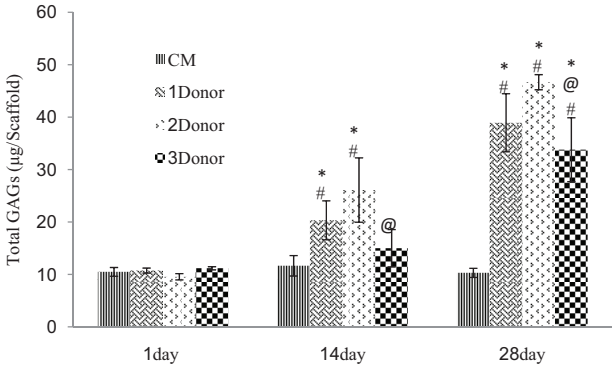

Figure 6 Total GAG content present in the supernatant of constructs after papainase digestion of hydrogels. Normalisation of samples was done by wet weight of the constructs at each indicated time point of culture from 3 different MSC donors (n = 3). Blank hydrogel digest was used as control for background subtraction. CM assigned for chondrogenic control medium. Data are represented as means ± SD (n = 3). **#** denotes significant difference relative to CM at each time point ($P < 0.001$). * denotes significant difference of each donor compared with day 1 of culture ($P < 0.001$). @ denotes significant difference of donor 3 relative to donor 2 at same time point ($P < 0.05$).

statistically significant increase in GAG accumulation in the gels occurred between 14 and 28 days for all three donors. However, there is a significant difference between donor 3 relative to donor 2, but not to donor 1 (Figure 6), indicating MSC-donor dependency of results. Similarly, heterogeneity of MSCs originated from different donors has already been reported (Karp and Leng Teo, 2009). Increase of proteoglycan content has gained particular note, because it has been shown to be important for functional compressive and mechanical properties (Kisiday et al., 2002; Mizuno et al., 2006), although the role of collagen type II is more prominent (Huang et al., 2008). It is important to consider that a low cell seeding density of 1×10^7 cells/mL was used here to specifically examine the effect of the biomaterial on cell behaviour. Typically, cell-encapsulating hydrogel constructs for cartilage tissue engineering are seeded with 20×10^6 to 60×10^6 cells/mL (Huang et al., 2008; Hao et al., 2010; Kim et al., 2012). Thus, our low initial seeding density may have contributed to the low levels of ECM production (Hoemann et al., 2005).

Visual observations showed no obvious changes (shrinkage or swelling) of CH-GP-HEC hydrogels with time (data not shown). Many hydrogels suffer from shrinkage or swelling in solution that finally lead to structural disruptions (Li and Xu, 2002; Bhattarai et al., 2010; Bertolo et al., 2012). Our results were consistent with other reports (Hannouche et al., 2007; Kurdi et al., 2010) indicating that CH-GP-HEC hydrogels are stable in solution and keep their structural integrity for 4 weeks in vitro. After 28 days, cellular controls also remained translucent while the cell-loaded constructs had an opaque appearance.

Consistent with retained GAGs, release of GAGs into medium significantly increased over 28 days (Figure 7). Only ~26% of the proteoglycans produced by MSCs was retained by the constructs at day 28 and remaining proteoglycans

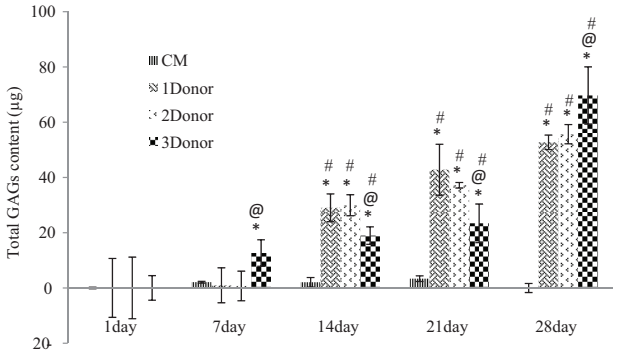

Figure 7 Total net GAGs present in culture media from three different MSC donors (each in triplicate) at the end of each time period. Blank hydrogel supernatant was used as control for background subtraction. CM assigned for chondrogenic control medium. Data are represented as means ± SD. # denotes significant difference relative to CM at each time point ($P < 0.001$). * denotes significant difference of each donor compared with day 1 of culture ($P < 0.001$). @ denotes significant difference of donors relative to others at same time point ($P < 0.05$).

(~74%) were released into the culture medium. The specific hypothesis of our study was that the cationic chitosan would form an ideal environment in which large quantities of newly synthesised anionic proteoglycan/sulphated-GAG could be entrapped in the hydrogel. In contrast to Roughley et al. (2006), cell-encapsulating CH-GP-HEC hydrogels showed that the majority of proteoglycans produced by encapsulated MSCs were released into the culture medium rather than retained within the hydrogels. A possible explanation could be that the cationic chitosan in our formulation (CH-GP-HEC) may be fully neutralised by GP (Hoemann et al., 2007). Irrespective of the mechanism of retention, which may result from chemical or physical properties of the hydrogel (Roughley et al., 2006), it remains to be established whether a proteoglycan concentration similar to that observed in vivo can ever be obtained. In the present work, proteoglycan contents of the scaffold (Figure 6) never reached that of native tissue. Prolonging the culture period and increasing cell density may improve the proteoglycan content (Kisiday et al., 2002; Hoemann et al., 2005).

Regarding potential applications, autologous MSCs can be recovered from the patient's bone marrow or adipose tissue, expanded, chondrogenically differentiated and combined with the hydrogel to repair cartilage defects (Moreau and Xu, 2009). MSCs provide an ideal cell source for cartilage engineering because they are relatively easy to access, can differentiate into chondrogenic cells, possess little to no immunogenic and tumorigenic ability, and their use is not complicated by ethical and legal controversies (Nejadnik et al., 2010; Tang et al., 2012; Maumus et al., 2013). MSC-laden hydrogels can achieve tensile properties that are comparable to chondrocyte-seeded constructs (Huang et al., 2008), confirming the utility of this alternative cell source in cartilage tissue engineering.

The development of scaffolds is a central topic in MSC-based cartilage engineering. To date, hydrogels and other polymeric systems have been examined for cell delivery and cartilage engineering (Hannouche et al., 2007; Park et al., 2007; Hao et al., 2010; Bian et al., 2011). The application of hydrogels as 3D scaffolds has recently gained attention because they can mimic key features of the ECM, such as their 3D nature and high water content. A wide range of polymers can be used for their fabrication, including (but not limited to), collagen-derived scaffolds (i.e., atelocollagen and collagen sponges), gelatin hydrogels, alginate hydrogels, hyaluronic acid hydrogels and chitosan hydrogels (Hoemann et al., 2005; Roughley et al., 2006; Hannouche et al., 2007; Park et al., 2007; Cho et al., 2008; Tan et al., 2009; Hao et al., 2010; Bian et al., 2011; Ko et al., 2011; Kim et al., 2012). Consistent with others (Lahiji et al., 2000; Roughley et al., 2006; Hao et al., 2010), our results show that CH-GP-HEC hydrogels can support chondrogenic differentiation. Richardson et al. (2008) studied the temperature-sensitive hydrogel CH-GP, seeded with hMSCs and cultured for 4 weeks in standard medium. They demonstrated differentiation of MSCs to a phenotype which showed similarities to both articular chondrocytes and nucleus pulposus cells, suggesting that MSC-seeded CH-GP gels could be used clinically for the regeneration of human intervertebral disc. The development of an injectable, biodegradable hydrogel composite of oligo(poly(ethylene glycol) fumarate) (OPF) with encapsulated rabbit MSCs and gelatin microparticles loaded with transforming growth factor-β1 for cartilage tissue engineering applications has been described (Park et al., 2007). Their results indicate that encapsulated rabbit MSCs remain viable over the culture period and differentiate into chondrocyte-like cells, thus suggesting the potential of OPF composite hydrogels as part of a novel strategy for localised delivery of stem cells and bioactive molecules. Tan et al. (2009) examined the potential of the injectable in situ forming biodegradable chitosan-hyaluronic acid based hydrogel for cartilage tissue engineering. They found that this composite hydrogel supported cell survival and retained chondrogenic morphology. Additional experiment demonstrated enhanced chondrogenic differentiation of murine embryonic stem cells in hydrogels with glucosamine, an amino monosaccharide found in chitin, glycoproteins and GAGs, such as hyaluronic acid, chondroitin sulphate and heparin sulphate (Hwang et al., 2006). Hannouche et al. (2007) demonstrated that in alginate-poly glycolic acid/MSCs constructs, cell growth phase and the chondrogenic differentiation of MSCs occurred during the first 3 weeks. In addition, cells remained round in the hydrogel, thickness of alginate-polyglycolic acid (PGA)/MSCs constructs increased substantially over time, no

shrinkage was seen, and composite hydrogel-PGA scaffold supported the in vitro growth of implantable cartilaginous structures.

Conclusion

To date, many new materials and medical strategies are being developed to aid the regeneration of damaged cartilage tissue**.** Apart from mimicking GAG structure of cartilage ECM, chitosan-based injectable hydrogels support cell survival and proliferation of MSCs and differentiation towards cartilage-like tissue. Thus, this type of hydrogel may fill cartilage defects with encapsulated MSCs that can be applied during arthroscopic procedures. This potential is critical for the optimisation of MSCs as a viable alternative to chondrocytes for cartilage engineering. Our data also show that CH-GP-HEC hydrogels have the potential to support MSC and/or chondrogenic factor delivery. However, further work is needed to understand the effect of CH-GP-HEC hydrogel on production of type I and II collagen by MSCs and to estimate tensile properties, compressive and mechanical strength of the engineered-cartilage in comparison with the native tissue. Since the in vitro cell culture is a closed static system and different from the dynamic circulation in vivo, animal studies will be needed to investigate the efficacy of cartilage regeneration of the CH-GP-HEC hydrogel-MSC system.

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