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THE COMPOSITION OF HYDROGELS FOR CARTILAGE TISSUE ENGINEERING CAN INFLUENCE GLYCOSAMINOGLYCAN PROFILES

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Abstract

The injectable and hydrophilic nature of hydrogels makes them suitable candidates for cartilage tissue engineering. To date, a wide range of hydrogels have been proposed for articular cartilage regeneration but few studies have quantitatively compared chondrocyte behaviour and extracellular matrix (ECM) synthesis within the hydrogels. Herein we have examined the nature of ECM synthesis by chondrocytes seeded into four hydrogels formed by either temperature change, self-assembly or chemical cross-linking. Bovine articular cartilage chondrocytes were cultured for 14 days in Extracel®, Pluronic F127 blended with Type II collagen, Puramatrix® and Matrixhyal®. The discriminatory and sensitive technique of fluorophore-assisted carbohydrate electrophoresis (FACE) was used to determine the fine detail of the glycosaminoglycans (GAG); hyaluronan and chondroitin sulphate. FACE analysis for chondroitin sulphate and hyaluronan profiles in Puramatrix® closely matched that of native cartilage. For each hydrogel, DNA content, viability and morphology were assessed. Total collagen and total sulphated GAG production were measured and normalised to DNA content. Significant differences were found in total collagen synthesis. By day 14, Extracel® and Puramatrix® had significantly more total collagen than Matrixhyal® (1.77±0.26 μg and 1.97±0.26 μg *vs.* 0.60±0.26 μg; $p<0.05$). sGAG synthesis occurred in all hydrogels but a significantly higher amount of sGAG was retained within Extracel® at days 7 and 14 ($p<0.05$). In summary, we have shown that the biochemical and biophysical characteristics of each hydrogel directly or indirectly influenced ECM formation. A detailed understanding of the ECM in the development of engineered constructs is an important step in monitoring the success of cartilage regeneration strategies.

Keywords: Glycosaminoglycans, cartilage tissue engineering, chondrocytes, hydrogels, hyaluronan, chondroitin sulphate, fluorophore-assisted carbohydrate electrophoresis.

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Introduction

The injectable and hydrophilic nature of hydrogels makes them suitable candidates for cartilage tissue engineering (Zheng *et al*., 2009). Hydrogels can not only encapsulate cells but also maintain both cell viability and phenotype. Furthermore, they have the potential to support neocartilage formation (Passaretti *et al*., 2001, Saim *et al*., 2000). Hydrogels can be formed from or blended with cartilage extracellular matrix (ECM) components by methods such as temperature change, chemical cross-linking and self-assembly (Vinatier *et al*., 2006, Van Tomme *et al*., 2008). Several studies have examined chondrocyte behaviour in one type of hydrogel. Monroy *et al*. (2007) and Saim *et al*. (2000) examined chondrocyte behaviour in the temperature-sensitive gel, Pluronic F127. They demonstrated that Pluronic F127 could be successfully used to encapsulate chondrocytes and facilitate *in vivo* cartilage formation. More recent work by Lee and Park (2009) showed that Pluronic F127 could also be blended with ECM components to improve cell adhesion and ECM production. A major disadvantage to this method is that the ECM component can leach out of the hydrogel since it is not anchored to the Pluronic F127. One way to overcome this is to cross-link or chemically modify the ECM component or the surrounding hydrogel to reduce or prevent leaching. The ECM component, hyaluronan (HA), can be cross-linked or chemically modified in a variety of ways. For example, HA can be thiol-modified and bound to other ECM components without significantly reducing its activity (Prestwich, 2008). This approach has been used to develop novel hydrogels. One such hydrogel, Extracel®, can be formed by adding polyethylene glycol cross-linker to a mixture of thiol-modified HA and thiol-modified gelatin. Extracel® has not yet been used to culture chondrocytes. However it has been seeded with mesenchymal stem cells and implanted into rabbits where it has been found to form *in vivo* cartilage (Liu *et al*., 2006). All of the studies described herein illustrate the exciting potential of hydrogel but there is clearly a need for more definitive characterisation of the ECM produced within different hydrogel formulations.

Articular cartilage is composed of chondrocytes that organise the collagens, proteoglycans, glycosaminoglycans (GAGs) and non-collagenous proteins into a highly organised ECM (Buckwalter and Mankin, 1998). An ECM without GAGs would be drastically compromised since their biological functions include tissue hydration, resistance to compressive load, intracellular signalling, protein up-take, and cell migration (Prabhakar and Sasisekharan, 2006). GAGs are long

chains of repeating disaccharides that are negatively charged and variably sulphated. With the exception of HA, GAGs are synthesised covalently bound to core proteins to form proteoglycans. In adult human and bovine cartilage, 50-80% of GAGs are chondroitin sulphate (CS) chains which comprise the repeating disaccharide unit; glucuronic acid and N-acetylgalactosamine. Glucuronic acid monosaccharides are unsulphated. N-acetylgalactosamine monosaccharides can be sulphated on carbon positions 4 (C4S or chondroitin-4 sulphated) and/or 6 (C6S or chondroitin-6 sulphated). HA accounts for 1-10% of GAGs in adult human and bovine articular cartilage. HA is composed of the repeating disaccharide unit; glucuronic acid and N-acetylglucosamine. Neither monosaccharide is sulphated. Studies, including our own, have shown that as a function of development, age and disease the GAGs in adult human and bovine cartilage exhibit many changes in their chain length, composition and sulphation (Buckwalter *et al*., 1994, Plaas *et al*., 2001, Sharma *et al*., 2007, Bayliss *et al*., 2000, Bayliss *et al*., 1999, Lauder *et al*., 1998). All of the changes in CS and HA are thought to be initiated by the chondrocytes in response to their altered microenvironment. However the functional significance of these changes has yet to be elucidated.

To date, few studies have fully characterised the behaviour of chondrocytes seeded into hydrogels formed by different methods. Most studies report total ECM production rather than definitive molecular ECM information. This detailed information would improve our understanding of how the biochemical characteristics of a particular hydrogel impacts upon ECM formation. Mouw *et al*. (2005) employed more sophisticated methods to evaluate their engineered cartilage constructs to show how different biomaterials directly or indirectly influenced ECM production. In this study we have examined the nature of ECM synthesis by chondrocytes seeded into four different hydrogels formed by either temperature change, chemical cross-linking or self-assembly. We have assessed cell characteristics and metabolic activity. In addition, we have used the sensitive and discriminatory technique of fluorophore-assisted carbohydrate electrophoresis (FACE) to reveal differences in CS and HA fine structures.

Materials and Methods

Isolation of bovine chondrocytes

Full depth articular cartilage was dissected from the articulating surface of the trochleal humerus of four 18-month-old cows. For each humerus, chondrocytes were isolated and seeded into the four hydrogel formulations. Chondrocyte isolation was performed as previously described (Wang *et al*., 2008). Briefly, diced cartilage was sequentially digested with 700 U/ml Pronase E™ (Sigma, St. Louis, MO, USA) for 1 h, then 200 U/ml collagenase XI (Sigma) and 0.1 mg/ml DNase 1 (Sigma) for 16 h. Chondrocytes from the supernatant were strained through a 70-μm cell sieve, washed in Complete Culture Medium (CCM), composed of Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) foetal calf serum (Invitrogen Gibco; www.invitrogen.com), 2.5 μg/ml Fungizone™ (Sigma), 50 μg/ml gentamycin (Sigma) and 62 μg/ml ascorbic acid (Sigma) and centrifuged at 750*g*. The chondrocytes were washed three times before seeding directly into hydrogels.

Hydrogel culture

Freshly isolated bovine chondrocytes were seeded into 150 μl hydrogels at 4 million cells/ml. For each formulation, one hydrogel from each of the four isolations was cultured on transwell inserts (Becton Dickinson Labware, Oxford, U.K.; 10 mm inserts with 0.45 μm pore size and pore density 1 x 10^8 pores/cm^2) in 24 well plates for 0, 1, 3, 5, 7 or 14 days. During the culture period the CCM was replaced every third day. All spent culture media were stored at -20°C for analysis. Four replicates of blank hydrogels (negative controls) were also prepared.

Each hydrogel formulation was prepared in accordance with the manufacturer's instructions. To prepare the Extracel® hydrogel (Glycosan Biosystems Inc., Salt Lake City, UT, USA), equal volumes of thiol-modified HA and thiol-modified gelatin were mixed with the chondrocytes. Cross-linker (1% w/v) was added to the HA and gelatin mixture at a final ratio of 1:3. Extracel® hydrogel was incubated at 37°C for 60 min to enable gelation and then 1 ml of CCM was added. The second hydrogel, Pluronic F127 (30% w/v, Sigma), was mixed with bovine nasal cartilage Type II collagen (20% w/v, Sigma) at 4°C to form a Pluronic-Collagen blend. Chondrocytes were mixed directly with this blend and warmed to 37°C for 90 mins to enable gelation. The third hydrogel, Puramatrix® (3DM Medical Technology Inc., Cambridge, MA, USA), is a peptide composed of 16 amino acid residues (Zhang *et al*., 1993). When exposed to physiological salt conditions the peptide forms a hydrogel, which can support ECM production (Yamaoka *et al*., 2006). To prepare the Puramatrix® hydrogel a stock (1%) Puramatrix® was diluted in 20% (v/v) sucrose solution to a concentration of 0.5%. Chondrocytes were suspended in 10% (v/v) sucrose solution and mixed with the diluted Puramatrix® to give a final concentration of 0.25%. The Puramatrix® hydrogel was incubated at 37°C for 60 min to allow gelation and then 1 ml of CCM was added. Matrixhyal®, the fourth hydrogel used in the study, was a kind gift from Hyaltech Ltd (Edinburgh, U.K). This novel hydrogel comprised high molecular weight HA entrapped within a lightly cross-linked acetylated chitosan at neutral pH (Hyaltech Ltd). Matrixhyal® was directly mixed with the chondrocytes and then cultured at 37°C with 1 ml of CCM.

Cell viability

Chondrocyte viability was assessed by Trypan blue exclusion before seeding into the hydrogels and with Live/Dead Double Staining (BioChemika, Sigma) (Yoshida *et al.,* 1998) after 0, 1, 3, 5, 7 and 14 days in culture. The Live/Dead stained viable cells green with calcein-AM and non-viable cells red with propidium iodide. The live/dead working solution was prepared by mixing 10 μl of solution A (Calcein-AM) and 5 μl of solution B (PI) solution with 5 ml Phosphate Buffered Saline (PBS). Hydrogels were

washed with PBS for 3 min first and then mixed with 1ml of assay solution and incubated at 37°C for 15 mins. The gel was placed on a glass slide and viewed under a fluorescence microscope. The fluorescence was detected with 490 nm excitation for simultaneous monitoring of viable and dead cells. With 545 nm excitation, only dead cells could be observed. Two hundred cells were counted in three separate regions of each hydrogel (4 replicates). The viability was assessed by dividing the number of viable cells to the total number of counted cells.

Sample preparation for assays

During the culture period, spent culture media were collected for analysis. The Extracel®, Pluronic-Collagen and Puramatrix® hydrogels were disrupted mechanically by pipeting. The Matrixhyal® hydrogel was digested with 100 U/ml hyaluronidase (bovine testes, Sigma) for 6 hr at 37°C. Spent culture media or hydrogels were digested with 125 μg/ml Proteinase K (Sigma), in 100 mM ammonium acetate, pH 7.0 at 60°C, for 4 hours. Blank hydrogels (negative controls) were prepared in the same manner.

Assessment of cell number

Cell number was assessed at 0, 1, 3, 5, 7 and 14 days by Picogreen® fluorescent DNA quantification (Molecular Probes, Eugene, OR, USA) as previously described (Wang *et al.,* 2008). Fluorescence was read against a standard curve of calf thymus DNA (Sigma). The previously reported value of 7.7 pg of DNA per chondrocyte was used to approximate cell number in the hydrogels (Kim *et al.,* 1988).

Determination of total collagen production

Total collagen production was determined at 0, 5, 7 and 14 days using the hydroxyproline assay as described by Brown *et al.* (2001). Total collagen was not detectable until after 3 days in culture (unpublished observations). Standards (0-10 μg/ml) of hydroxyproline (Sigma) were prepared in distilled water. All reagents were procured from Sigma. Samples and standards were hydrolysed in 10 M hydrochloric acid at 110°C for 16 h. On cooling, the hydrolysates were partially neutralised with 1M sodium hydroxide. Aliquots (50 μl) of each sample and each standard were added to a 96-well plate. Chloramine-T (100 μl) and then Ehrlich's reagent (100 μl) were added to each well. The plate was sealed and incubated for 45 mins at 65°C. Absorbance at 540 nm was read on a Labsystems Multiskan® Multisoft microplate reader (Labsystems, Helsinki, Finland). A multiplication factor of 7.2 was used to convert total hydroxyproline to total collagen (Cortivo *et al*., 1981).

Determination of total sulphated glycosaminoglycan (sGAG) production

Total sGAG production was determined at 0, 1, 3, 5, 7 and 14 days using the 1,9-dimethylmethylene blue (DMMB) dye assay as described by Farndale *et al.* (1986). All reagents were obtained from Sigma. 4 X DMMB solution (32 mg DMMB, 1.52 g glycine, 1.19 g NaCl, 47.5 ml, 0.1 M HCl, pH 3.0) was prepared. Standards (0-200 μg/ml) of bovine tracheal chondroitin sulphate (Sigma) were prepared in distilled water. Aliquots (50 μl) of each sample and each standard were added to a 96-well plate. DMMB (200 μl/well) were added to all wells and the plate was immediately read at 530nm on a Wallac Multilabel Counter 1420 (Victor Wallac/Perkin Elmer, Waltham, MA, USA).

Hyaluronan (HA) and chondroitin sulphate (CS) profiling by fluorophore-assisted carbohydrate electrophoresis (FACE)

FACE was performed as previously described (Sharma *et al.,* 2007; Wang *et al.,* 2008) for Pluronic-Collagen, Puramatrix® and Matrixhyal® but not for Extracel®. The heavily thiol-modified HA component of Extracel® inhibited the activity of the GAG-specific enzymes (data not shown). GAGs were ethanol precipitated from Proteinase K digests from each hydrogel at day 14. Aliquots from each hydrogel containing >5 μg sulphated GAG (as determined by the DMMB dye assay) were digested with GAG-specific enzymes (Seikagaku) in 100 μl of 50 mM ammonium acetate buffer (Sigma). Briefly, HA was digested into disaccharides (ΔDiHA) using 100 mU/ml of hyaluronidase (*Streptococcus dysgalactiae)* for 1 h at 37°C. CS was digested into disaccharides (chondroitin-6 sulphate, ΔDi6S; chondroitin-4 sulphate, ΔDi4S; unsulphated chondroitin, ΔDi0S) with 100 mU/ml of chondroitinase ABC (cABC) for 3 h at 37°C. Sulphation of CS was confirmed by incubation of cABC-digested samples with 100 mU/mL of chondroitin-4ase and/or chondroitin-6ase for 12 h at 37°C. After digestion, buffer was evaporated and digestion products were fluorescently tagged for 16h at 37°C with 15 μl of 0.1M 2-aminoacridone (Sigma) and reduced with 1.0M sodium cyanoborohydride (Sigma). After fluorotagging, 10 μl of 25% glycerol were used to quench excess sodium cyanoborohydride.

All reagents for electrophoresis were purchased from Sigma. Samples and standards were run on a 0.75 M Tris-0.5 M Borate pH 7.0 resolving gel (10 cm x 10 cm x 0.5 cm) with 150 mM Tris-Borate pH 8.8 running buffer for 80 min at 4°C. Gels were placed on a transilluminator light box fitted with a 312-nm light source. Fluorescent images were captured using a High CCD Camera (UVP, Cambridge, U.K.) and the mean pixel density for each product band was quantified using LAB WORKS Software. For each gel, FACE product bands were identified by their co-electrophoresis with a range of pre-defined fluorotagged saccharide standards (25-250 pmol). For comparison, native bovine articular cartilage (250 μg wet weight) obtained from the humerus was also analysed by FACE.

Statistical analysis

For each hydrogel, a sample size of four was used. Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was carried out using a 2-way ANOVA with type of hydrogel and days in culture as independent variables and cell number, GAG and collagen production as dependent variables. Cell number, GAG and collagen production were compared between the four gel types at

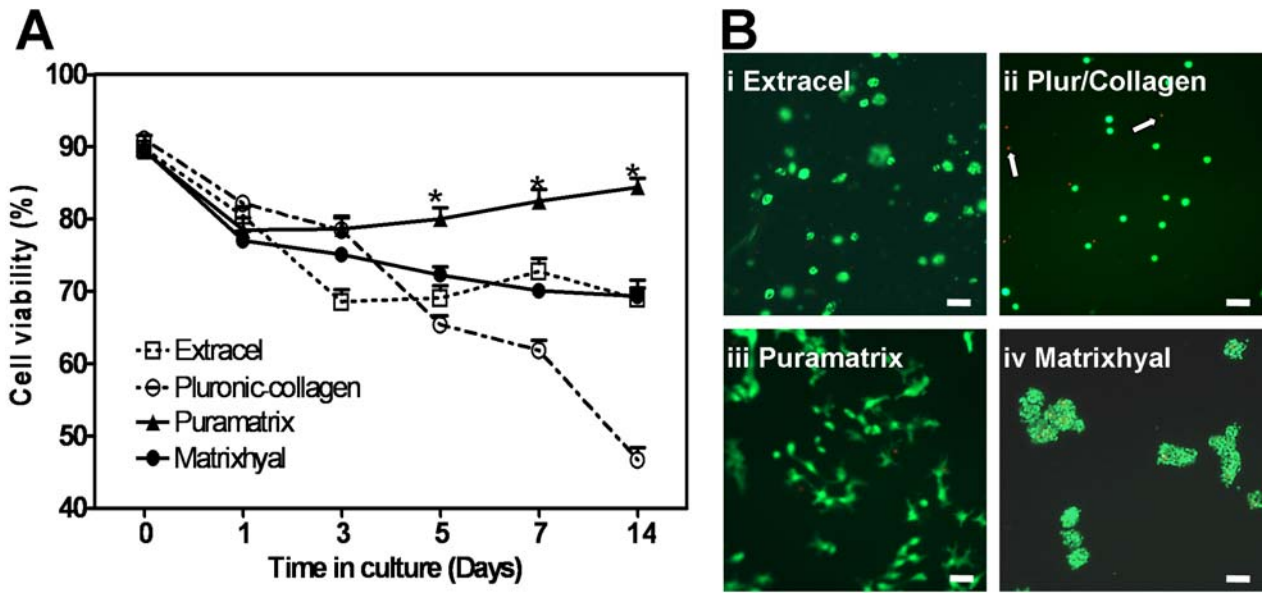


Fig.1 (A) Chondrocyte viability as assessed by Live/Dead Double Staining. Live cells stained green and dead cells stained red. Two hundred cells were counted in three separate regions of each hydrogel (4 replicates). These data are expressed as a percentage. (B) Representative interior of hydrogel stained with fluorescent Live/Dead Double Staining at day 14 day. The red fluorescence and arrow indicate the locations of cell nuclei. Bars = 100μm. Asterisks indicate significant (*p<0.05) increase in cell number *vs.* all other hydrogels at the same time point.

two specific time points (7 and 14 days) with p values derived from the full ANOVA model. *P* values <0.05 were considered significant. The Benjamin-Liu step down procedure was used to adjust for multiple comparisons (Benjamini and Liu, 1999).

Results

Cell viability and number

Prior to seeding, chondrocytes had good viability (>95%) as assessed by Trypan blue exclusion. Cell viability as assessed by Live/Dead Double Staining (Fig. 1A), morphology (Fig. 1B) and number (Fig. 2) for the encapsulated chondrocytes varied depending upon their ECM microenvironment. In general, manipulation of chondrocytes during seeding into each hydrogel had a slightly negative effect upon their viability, which decreased from 95% to 90%. Cell viability within Puramatrix® initially decreased between day 0 and day 1 but then steadily increased thereafter. At day 14, cells cultured in Puramatrix® appeared to be well spread and were uniformly distributed (Fig. 1Biii). During the 14-day experiment, cell number increased two-fold (*p<0.05, Fig. 2iii). During the 14-day culture period, cell viability in both Matrixhyal® and Extracel® decreased by 20%. At day 14, cells cultured in Extracel® displayed predominantly a rounded morphology and were uniformly distributed in clusters (Fig. 1Bi). Cell number remained constant throughout the time course (Fig.2i). By contrast, cells cultured in Matrixhyal® were more randomly distributed and appeared to be clustered in larger islands (Fig. 1Biv). Cell number increased three-fold during the time course (**p<0.001, Fig.2iv) however cell viability was determined to be at least 70% by day 14. Although cells in Pluronic-Collagen were uniformly distributed and appeared rounded in their morphology (Fig. 1Bii) less than 50% were viable at the end of the 14 day culture period.

Total collagen production

For each time point we present total accumulated collagen content (Fig. 3A) and total accumulated collagen content normalised to total DNA content (Fig. 3B). Puramatrix® accumulated the most collagen of the four hydrogel formulations over the 14 day culture period (Fig. 3Aiii, *p<0.05 for days in culture). Collagen/DNA increased significantly in all four hydrogels from initial seeding to day 14 (Fig. 3B, *p<0.05 for days in culture). At day 7 no significant differences in collagen/DNA were found between the four hydrogels, but at day 14 Extracel® and Puramatrix® had significantly more collagen/DNA than Matrixhyal® (1.77±0.26 μg/DNA and 1.97±0.26 μg/DNA *vs.* 0.60±0.26 μg/DNA; **p<0.001).

Total sulphated GAG production

Fig. 4 illustrates the total amount of GAG accumulated and released into the surrounding media. For each time point we present total accumulated GAG content (Fig. 4A) and total accumulated GAG content normalised to total DNA content (Fig. 4B). All of the hydrogels accumulated sulphated GAG over the 14 day culture period. Matrixhyal® accumulated the most GAG of the four hydrogel formulations over the 14 day culture period but it also lost the most GAG to the surrounding media (Fig. 4Biv, p<0.05 for days in culture). At day 7 and day 14, Extracel® had significantly more GAG/DNA than the other

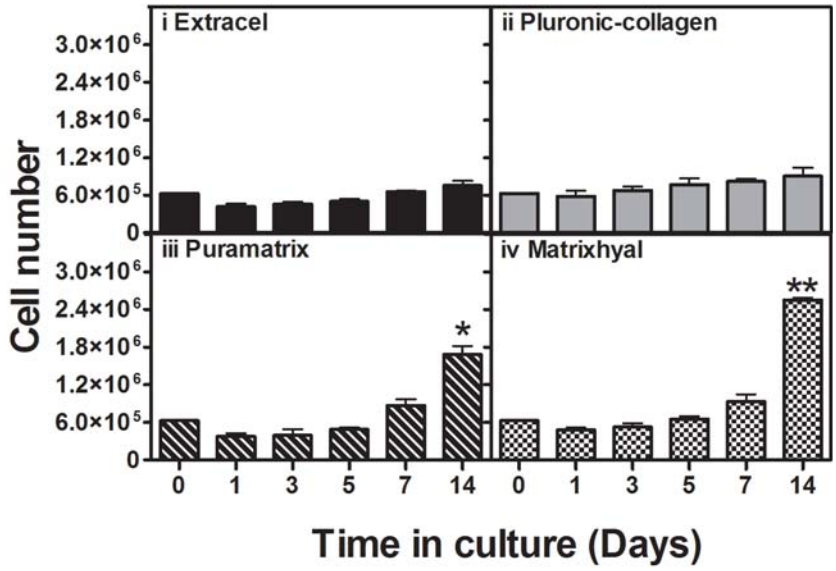
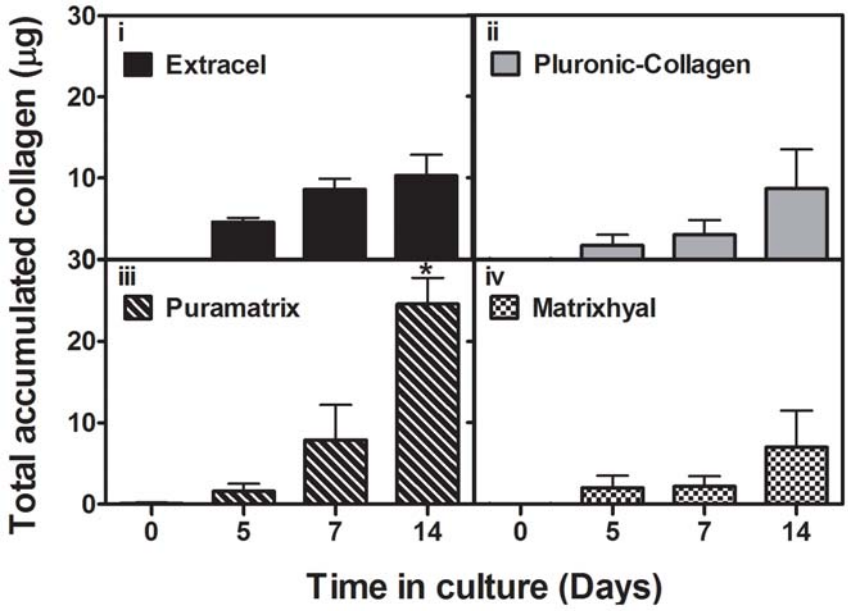


Fig.2 Chondrocyte cell number as assessed by Picogreen® dye. Cells were cultured in each hydrogel for two weeks. The initial seeding density was 600,000 cells per 150 µl hydrogel (4 replicates). Asterisks indicate significant (*p<0.05, **p<0.001) increases in cell number *vs.* all other hydrogels at the same time point.



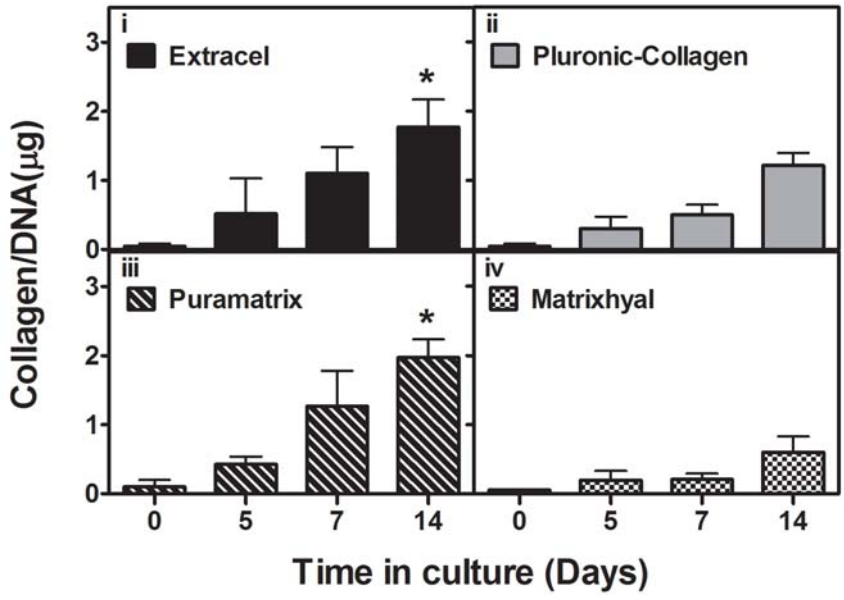


Fig.3 (A) Total collagen accumulated within the four hydrogel formulations. (B) For each hydrogel, the total collagen accumulated (n=4) was normalised to the DNA content. Asterisk indicates significant (*p<0.05) increase in collagen production *vs.* all other hydrogels at the same time point.

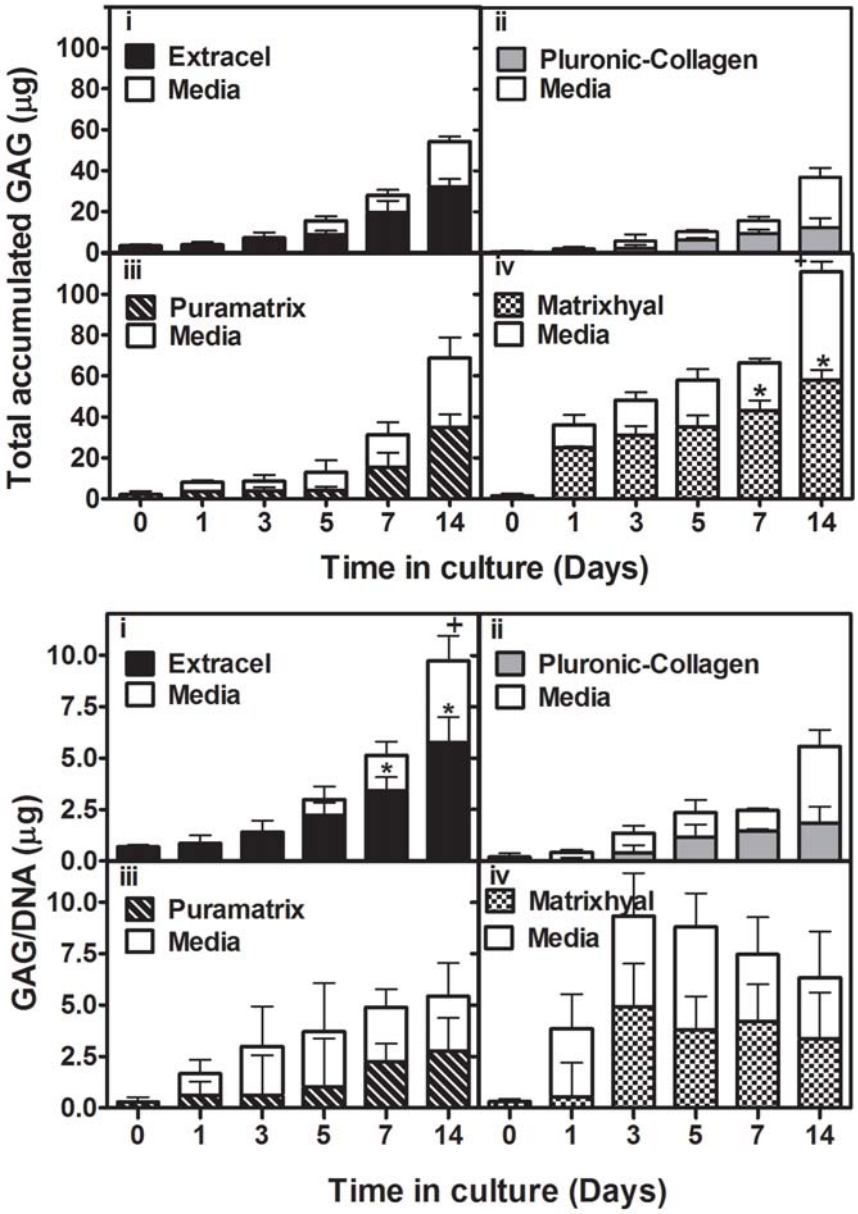


Fig.4 (A) Total sulphated glycosaminoglycan accumulated within the four hydrogel formulations and the media. (B) For each hydrogel and media, the total GAG accumulated (n=4) were normalised to the DNA content. Asterisk indicates significant (*p<0.05) increase in GAG production vs all other hydrogels at the same time point. Plus sign indicates significant (+p<0.001) loss of GAGs to media *vs*. some other hydrogels at the same time point.

three hydrogels (Fig. 4B, p<0.05 for days in culture). None of the hydrogels retained all of the GAGs during the time course. At day 7 there was no significant difference with respect to GAG/DNA loss to the media. At day 14 Extracel® lost significantly more GAG/DNA to the surrounding media than Puramatrix® (3.8±0.52 µg *vs*. 1.5±0.52 µg; p<0.01).

HA and CS profiling by FACE

Fig. 5 is a representative FACE gel showing the co-electrophoresis of a known concentration of pre-defined fluorotagged saccharide standards alongside hyaluronidase and cABC digestion products obtained from Puramatrix® (Fig. 5). Two image exposures were captured. The first exposure (Fig.5A) was used for quantitation as it had all of the pixels within a linear 12-bit depth range to provide baseline data. The second exposure (Fig. 5B) had an over-saturated pixel intensity allowing identification of less abundant structures. Lane 1 is the negative control. Lane 2 shows HA disaccharides (ΔDiHA) after hyaluronidase digestion. Lane 3 shows the CS disaccharides (ΔDi0S, ΔDi4S and ΔDi6S) after cABC digestion. The CS disaccharides were digested using chondroitin-4ase (Lane 5) or chondroitin-6ase (Lane 6) to confirm the presence of sulphation.

The FACE data for HA and CS are summarised in Fig. 6. Total CS and HA in the native bovine cartilage preparation were determined to be 22.47±1.04 pg/cell and 0.74±0.34 pg/cell, respectively. The total CS comprised ΔDi6S (54.74%), ΔDi4S (31.39%), and ΔDi0S (13.87%). For Puramatrix®, CS and HA were determined to be 1.95±0.26 pg/cell and 0.18±0.04 pg/cell, respectively. The total CS comprised ΔDi6S (45.81%), ΔDi4S (35.61%), and ΔDi0S (18.58%). For the Pluronic-Collagen, CS and HA were determined to be 0.52±0.21 pg/cell and 0.38±0.15 pg/cell, respectively. The total CS comprised ΔDi6S (23.73%), ΔDi4S (14.08%), and ΔDi0S (62.19%). For Matrixhyal®, CS and HA were determined to be 0.34±0.25 pg/cell and 0.09±0.02 pg/cell, respectively. The total CS comprised ΔDi6S (11.49%), ΔDi4S (58.00%), and ΔDi0S (30.51%).

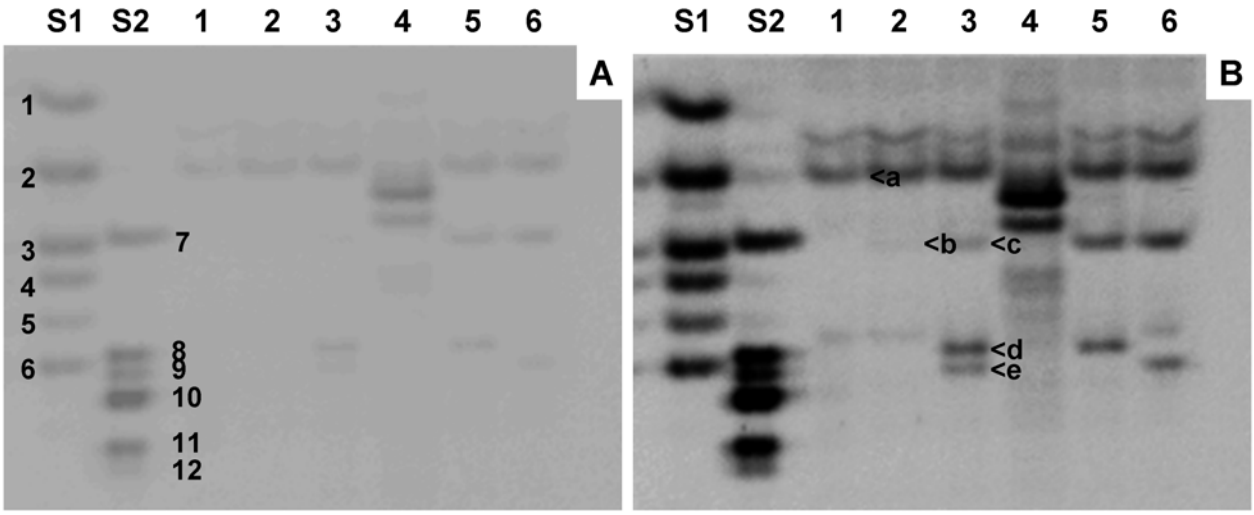


Fig.5 Representative FACE gel for analysis of HA and CS of the Puramatrix® hydrogel. The first exposure (A) was used for quantitation to provide baseline data. The second exposure (B) had oversaturated pixel intensity to allow identification of less abundant structures. Lanes S1 and S2 contain predefined fluorotagged saccharide standards. S1: Glucose (1), Nacetyl galactosamine (GalNAc) (2), ΔDi0S (3), Nacetyl galactosamine6 sulphate (GalNAc6S) (4), Nacetyl galactosamine4 sulphate (GalNAc4S) (5) and 4/6sulphated Nacetyl galactosamine (GalNAc4,6S) (6). S2: DiHA (7); ΔDi6S (8), ΔDi4S (9); dermatan sulphate disaccharides ΔDi2S (10), ΔDi4,6S (11) and Di2,6S (12). Samples were digested with Streptococcus dysgalactiae hyaluronidase (lane 2), cABC (lane 3), cABC + mercuric ion treatment (lane 4), cABC + chondroitin4ase (lane 5) and cABC + chondroitin6ase (lane 6). Lane 1 is the negative control. Arrows indicate the migratory position of Glucose (a), ΔDiHA (b), ΔDi0S (c), ΔDi6S (d), and ΔDi4S (e).

Discussion

This study was designed to examine the influence of different hydrogels on ECM synthesis over a 2 wk culture period. It is important to understand chondrocyte behaviour and ECM synthesis in different hydrogels since this may provide essential detail to inform future cartilage tissue engineering strategies. So far, few studies have quantitatively compared ECM production in hydrogels formed using different methods and different polymers. We investigated a range of hydrogels formed by temperature change, self-assembly and chemical cross-linking. Our study presents several notable findings pertaining to the influence of the components within four hydrogel formulations on ECM production. We show that cell morphology and viability differ between the hydrogels. We demonstrate that gross biochemical analyses indicate that the hydrogels support ECM formation but detailed biochemical analyses show that the ECM components do not closely match components found in native cartilage.

Pluronic gelation occurred in response to increasing temperature, Puramatrix® gelation occurred via self-assembly, whilst Extracel® and Matrixhyal® gelation occurred via different methods of chemical cross-linking. All four of the hydrogels studied supported chondrocyte *in vitro* culture to different degrees. At day 0, chondrocytes were distributed homogenously within each hydrogel (data not show). By day 14, clear differences in the morphology of the cells in each hydrogel were noted. Cells cultured in Extracel® were evenly distributed within small islands. Cells cultured in Matrixhyal® formed even larger islands. Together, these findings suggest that both gels have a loose structure and large pore size. Cells cultured in Puramatrix® were more fibroblastic in appearance than the other three hydrogels. Puramatrix® has been reported to promote cell adhesion and contain large pores (Yamaoka *et al*., 2006). Thus bovine chondrocytes exposed to this hydrogel would be expected to adhere to the nanofibres as well as migrate through the large pores between the nanofibres. Cells cultured in Pluronic-Collagen were uniformly distributed and appeared rounded in their morphology, but their cell viability decreased from 70% to 40% by the end of the 14 day culture period. This decrease in cell viability could be due to one or all of three reasons. Firstly, it may be due to the high concentration (30%) of Pluronic F127 used in our study. Concentrations at or above 20% have been shown to result in decreased cell viability (Roberts *et al*., 2005, Khattak *et al*., 2005). Secondly, studies have shown (Brunet-Maheu *et al*., 2009, Roberts *et al*., 2005) the importance of frequent media replenishment. We replenished the culture media every third day but this may not have been frequent enough to maintain viability. Thirdly, previous work has suggested that some cell types have better viability than other cell types when cultured in Pluronic F127 (Khattak *et al*., 2005).

Following their encapsulation, the surrounding microenvironment of the hydrogel would directly or indirectly influence the behaviour of the chondrocyte. This would result in the differential expression of ECM components and/or organisation of the surrounding ECM components (Urban, 1994). Hydrogels with a larger porosity would be expected to loose newly synthesised

GAGs to the surrounding media. In our study, there were significant differences in the production and retention of newly synthesised ECM components within the four hydrogels. Both Extracel® and Puramatrix® had the greatest accumulation of ECM. The raw data showed that chondrocytes cultured in Matrixhyal® produced the most GAG but the large porosity of the gel could not prevent more than half of the GAGs leaching out into the surrounding media. Puramatrix® accumulated the most collagen. It also accumulated a large amount of GAG but it was noted that GAG synthesis within this hydrogel appeared to increase significantly after day 7. Puramatrix® does not contain any cartilage ECM components but its nanofibrils, water content and pore size are similar to those found in native cartilage (Zhang *et al*., 1993). These data may suggest that chondrocytes need to be cultured in Puramatrix® for longer that 14 days for the production of ECM. Extracel® contained significantly more collagen/DNA and GAG/DNA than all of the other gels. Extracel® comprises modified HA and gelatin, both of which have previously been shown to increase the chondrogenic potential of chondrocytes (Goodstone *et al*., 2004; Petterson *et al*., 2009). Both Extracel® and Puramatrix® facilitated ECM synthesis whilst preserving the high cell viability and low cell proliferation. Pluronic-Collagen accumulated very little GAG and collagen.

This study revealed considerable quantitative differences in the disaccharide composition of the accumulated GAGs. FACE detected and quantitated saccharides derived from GAGs in the picomolar range. Of the three hydrogels assessed by FACE, only Puramatrix® had a disaccharide profile which closely matched the disaccharide profile for native articular cartilage (Fig.6). Puramatrix® accumulated significantly more ΔDi6S and ΔDi4S disaccharides than Matrixhyal® or Pluronic-Collagen (**p<0.001). In terms of CS, there are some similarities between Puramatrix® and native bovine cartilage. The ΔDi6S:ΔDi4S ratio for Puramatrix® was determined to be 1.28±0.11 which is similar to the ratio found in the native bovine cartilage (Lammi *et al*., 2004). During adulthood, chondrocytes produce CS chains with more C6S than C4S (Plaas *et al*., 2001). The functional implication of increased CS6 in adult native cartilage is unclear. There is indirect evidence that it may alter the binding of nutrients (Nakatani *et al*., 2007) and growth factors (Asada *et al*., 2009). This could have a downstream effect on chondrocyte migration and chondrogenic potential. Pluronic-Collagen and Puramatrix® had significantly more ΔDiHA and ΔDi0S disaccharides than Matrixhyal® (p<0.05). High concentrations of HA are generally found during adulthood and development (Holmes *et al*., 1990). Our results suggest that Puramatrix® provided a suitable environment in which chondrocytes could produce more relevant levels of specific GAGs. This work has shown that the 3D scaffold microenvironment clearly influences the behaviour of the chondrocyte. It also highlights the importance of closely monitoring ECM production in different scaffolds.

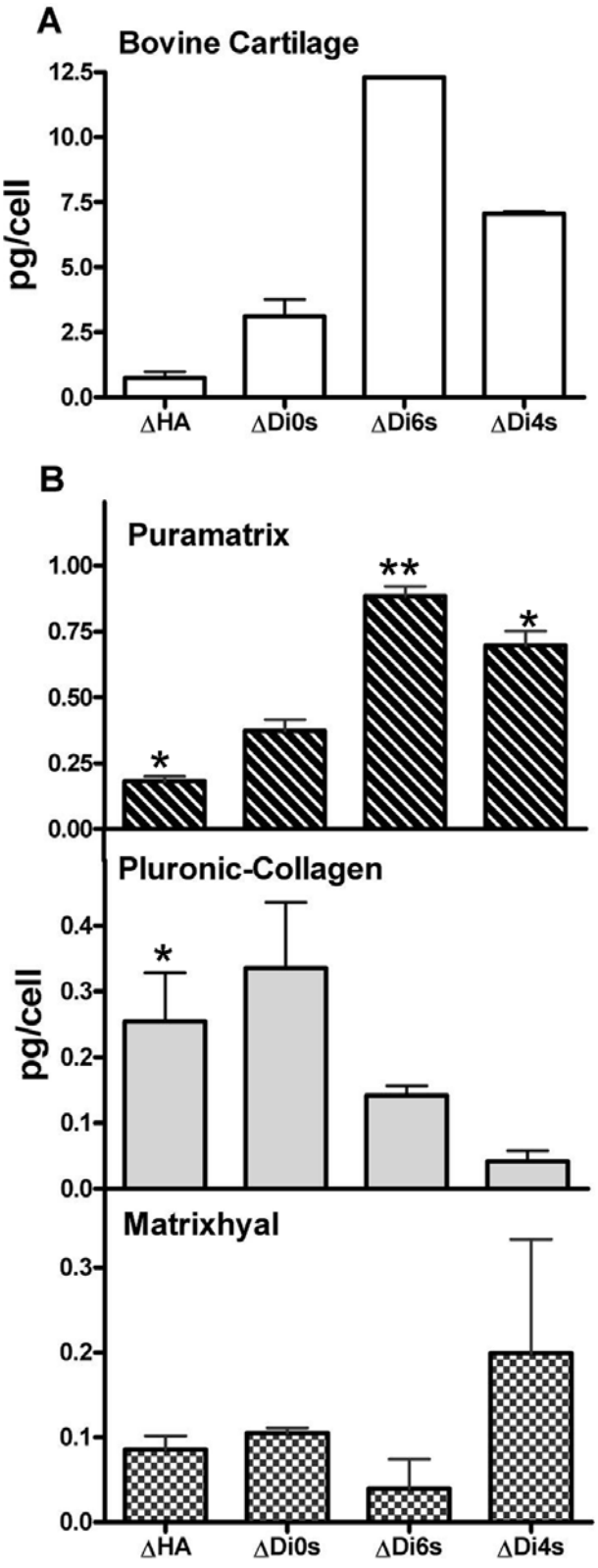


Fig.6 Summary of FACE data for HA and CS. The concentrations of the HA and CS disaccharides derived from Puramatrix®, PluronicCollagen and Matrixhyal® as assessed by FACE. Values for native articular cartilage are included for comparison. Values are plotted as pg/cell. Each bar represents the mean ± SEM (n=4). Asterisks indicate significant (*p<0.05, **p<0.001) increases in disaccharide concentration *vs*. all other hydrogels.

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Discussion with Reviewer

Reviewer II: What do the authors believe is more important, a disaccharide profile similar to native cartilage or the total amount of GAG produced?

Authors: We believe that both are equally important. GAGs have important biological functions such as tissue hydration. The functions of cartilage with little or no GAGs will be drastically compromised so it is crucial that we achieve high GAG levels in the hydrogel. GAG fine structure varies throughout native cartilage. We know that GAGs form a gradient in specific areas of the cartilage allowing cell signalling and cell migration to occur.