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The effect of alginate, hyaluronate and hyaluronate derivatives biomaterials on synthesis of non-articular chondrocyte extracellular matrix

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Cartilage engineering consists of re-constructing functional cartilage by seeding chondrocytes in suitable biomaterials *in vitro*. The characteristics of neocartilage differ upon the type of biomaterial chosen. This study aims at determining the appropriate scaffold material for articular cartilage reconstruction using non articular chondrocytes harvested from rat sternum. For this purpose, the use of polysaccharide hydrogels such as alginate (AA) and hyaluronic acid (HA) was investigated. Several ratios of AA/HA were used as well as three derivatives obtained by chemical modification of HA (HA-C18, HA-C12$^{2.3}$, HA-C12$^{2.5}$-TEG$^{0.5}$). Sternal chondrocytes were successfully cultured in 3D alginate and alginate/HA scaffolds. HA retention in alginate beads was found to be higher in beads seeded with cells than in beads without cells. HA-C18 improved HA retention in beads but inhibited the chondrocyte synthesis process. Cell proliferation and metabolism were enhanced in all biomaterials when beads were mechanically agitated. Preliminary results have shown that the chondrocyte neo-synthesised matrix had acquired articular characteristics after 21 days culture.

1. Introduction

Articular cartilage lesion repair is a major preoccupation in surgery because of the tissue poor healing capacity *in vivo*. Several methods have been studied to promote cartilage repair among which cartilage tissue engineering [1–3] that consists of re-implanting cell seeded biomaterials. Cartilage specific cells (chondrocytes) were shown to preserve their phenotype for up to 8 months when cultured in 3D alginate scaffolds [4]. A study previously published by our group in 2003 investigated the use of alginate and hyaluronic acid beads to grow articular chondrocytes [5]. The study established that mechanical stress applied on scaffolds through agitation increased both the cell proliferation and more moderately the extracellular matrix (ECM) synthesis. Excessive load on articular cartilage may result in injuring the extracellular matrix. However, chondrocytes present in normal joints respond favourably to mechanical stress [6] by proportionally remodelling the cartilage to the level of stress. It was observed that load-bearing cartilage areas are thicker than non-load bearing ones. Articular and non-articular chondrocytes do not have the same functions in the body and therefore do not produce the same extracellular matrix. The concept that cells from non-weight bearing cartilage could be used for re-implantation in articular areas has been introduced with the surgical technique ACI (Autologous Chondrocyte Implantation). Several studies have demonstrated that non-articular chondrocytes inserted in articular lesions gradually synthesised articular ECM [7, 8]. For instance, rat xiphisternum chondrocytes could be used for first investigations. Harvesting these cells is non lethal and enables the use and re-implantation of autonomous cells thus avoiding immune reactions.

Mechanical forces applied on articular cartilage during movement can be mimicked using mechanical devices *in vitro*. Chowdhury and her group simulated compression and shear constraints with a well-characterised cell-straining apparatus and found that stimulated chondrocytes proliferate and produce more ECM than non stimulated cells [9]. A simple technique based on bead shock developed by Gigant-Huselstein was further investigated in our study. Chondrocyte proliferation was shown to improve when the cell-seeded beads were agitated, probably due to better mass

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transport [5]. Proteoglycan synthesis was also stimulated by mechanical agitation.

Alginate beads have been commonly used to encapsulate chondrocytes [10, 11] due to their capacity to preserve the chondrocytes' phenotype [12–14]. Hyaluronic acid has very good viscoelastic properties helping lubrication and shock absorption. Additionally, this polysaccharide is commonly used in cartilage engineering with the aim to facilitate the chondrocyte proliferation [15] and anchorage through the HA specific receptor on the chondrocyte membrane. Alginate and hyaluronic acid were associated in order to combine the alginate gel forming ability with HA biological and rheological properties. Controversial results can be found in the literature regarding the alginate/HA association. On one hand, alginate-HA mixtures were shown not to have significant effect on chondrocyte growth and metabolic activity [16] while on the other hand older studies indicated that HA had a positive effect on chondrocyte metabolic activity [17]. HA derivatives (HA-C18), HA-$C12^{2.3}$ and HA-$C12^{2.5}$-$TEG^{0.5}$ [18, 19] were synthesized with the aim to introduce hydrophobic interactions between HA chains that might favour HA retention in the beads.

The current work aimed at investigating HA retention in beads composed of alginate/HA and alginate/HA amphiphilic derivatives as well as monitoring cell proliferation and metabolic activity in several bead compositions. Finally, the possibility of using non-articular chondrocytes for articular cartilage repair was examined. The challenge lied in recreating the extracellular matrix composition using the optimum biomaterial and mechanical agitation.

2. Materials and methods

2.1. Materials

Sodium alginate (AA) medium viscosity extracted from *Macrocystis pyrifera*, calcium chloride, papain, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium citrate, L-cystein, hyaluronate lyase from *Streptomyces hyalurolyticus*, protease type XIV bacterial from Streptomyces *griseus* were obtained from Sigma Aldrich (France). Dulbelcco's Modified Eagle's Medium (D-MEM/F12), Fetal Bovine Serum (FBS), EDTA, HEPES buffer, penicillin-streptomycin, L-glutamine, amphotericin B, trypsine-EDTA, and sterile PBS were all purchased from Gibco Life Technologies (NY, USA). Hyaluronic acid was obtained from Acros Organics (Geel, Belgium). Collagenase B was purchased from Roche (Germany), sterile NaCl 0.9% from Braun (France) and sodium acetate from *Merk* (Damstadt, Germany). Radioactive products (^{3}H-thymidine and $Na_2^{35}SO_4$) were purchased from Perkin Elmer (Boston, USA). HA derivatives were chemically modified in our laboratories using native HA [18, 19]. Tetraethylene glycol di-tosilate (TEG) was bought from Fluka, Switzerland.

2.2. Methods

2.2.1. Chondrocyte isolation and culture

Xiphisternum cartilage was harvested from male Wistar rats (250–275 g) (Charles River, France) and washed with sterile 0.9% NaCl supplemented with 0.1% (v/v) penicillin-streptomycin (NaCl/pen-strep). Sequential enzyme digestion was performed by first incubating the cartilage in NaCl/pen-strep solution containing 0.15% (w/v) protease at 37 °C for 2 h. In the resulting solution, culture medium and 0.2% (w/v) collagenase B were added. The mixture was then left at 37 °C overnight [20]. The following day the tubes were centrifuged and the cell pellet washed with culture medium. The cells were counted before seeding 10^6 cells per culture flask. The cells were cultured using DMEM/NutMix F12 medium supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine, 0.7% (v/v) penicillin-streptomycin, 0.1% (v/v) amphotericin B. Culture medium was changed three times a week and the cells trypsinised when confluent. The chondrocytes were centrifuged and washed and the resulting cells were counted before mixing with polymer solutions.

2.2.2. Alginate, alginate/HA and alginate/HA derivatives bead fabrication

Alginate/HA (AA/HA) beads were made using 18/2, 16/4, and 14/6 (w/w) ratios of alginate and HA respectively. Before sterilisation HA molecular weight was 1.4 million Daltons. This molecular weight was found to be lower after sterilisation; around 700,000 Da (verified by size exclusion chromatography/multi-angle laser light scattering SEC-MALLS). Prior to mixing, HA was chemically modified to form HA-C18 (covalent fixation of 1% 18 carbon chains), HA-$C12^{2.3}$ (2.3% of 12 carbon chains) and HA-$C12^{2.5}$-$TEG^{0.5}$ (2.5% 12 carbon chains and 0.5% TEG). HA derivatives molecular weight was found to be around 10^6 Da before chemical modification. Molecular weight could not be determined after chemical modification but was expected to be similar.

Medium-viscosity sodium alginate solution, alginate/HA or alginate/HA derivative solutions were prepared in NaCl. Prior to mixing with NaCl, the polymer powders were weighed, distributed in glass tubes and autoclaved. The polymer suspension was homogenized for 24 h before being slowly extruded through an 18-gauge needle into a 100 mM calcium chloride solution to form beads. The beads were left for 10 min to gel in $CaCl_2$ and rinsed twice with sodium chloride. The beads were then washed once with culture medium containing an additional 5 mM HEPES (bead medium) to remove excess calcium chloride. Beads were collected in 50 mL sterile plastic tubes then transferred 10 by 10 in 15 mL sterile plastic tubes. The tubes were left at rest at 37 °C in 10 mL bead medium for 3 days before being used.

20 and 10 g/L polymer concentrations were used in our study. We distinguished "study A" when 20 g/L solutions were made from "study B" when 10 g/L solutions were used.

Study A: 20 g/L polymer concentration beads

Beads were either made with 20 g/l pure alginate or three ratios of alginate/HA progressively lowering the

amount of alginate in the beads (18/2, 16/4, 14/6). The same ratios were also used to make AA/HA-C18 mixtures. HA-C18 synthesis was previously described in [18, 19]. Beads were made as previously described.

Study B: 10 g/l polymer concentration beads

Beads were either made with 10 g/l pure alginate or ratios of alginate/HA (8/2), alginate/HA derivatives (8/2). HA derivatives HA-C12 and HA-C12-TEG synthesis is described in [18, 19]. Beads were made as previously described.

2.2.3. Chondrocyte culture in alginate and alginate/HA beads

The polymer solution was poured on the chondrocyte pellet (see part *a*) to prepare a mixture containing 3×10^6 cell/mL. The cell suspension was extruded through an 18-gauge needle into a 100 mM calcium chloride [21–23]. As previously described, the beads were left for 10 min to gel in $CaCl_2$ and rinsed with sodium chloride. The beads were then washed with culture medium containing HEPES (bead medium).

2.2.4. Measurement of HA release using Capillary Zone Electrophoresis (CZE)

Hyaluronic acid release from beads was measured by capillary zone electrophoresis (Beckman-Coulter P/ACE™ QMC system). 10 beads were digested separately with hyaluronate lyase (100 U/mL) at 37 °C in 0.02 M acetate buffer (pH 6) for 72 h [24]. Beads were digested at 7, 14, 21 and 35 days culture for study A and at 21 days culture for study B. The amount of HA in the digest was measured with CZE. This amount was first normalized using the beads' wet weight. The percentage HA released was estimated by comparing the amount of HA found in beads at day 7, day 14, day 21 and day 35 with the amount found at day 0 (equivalent to 0% HA released). This form of measurement allowed us to quantify the amount of free HA at different periods of time.

Mixture B composed of 4-hydroxy-benzoic acid (0.72 mM), (4-hydroxyphenyl)-acetic acid (0.66 mM) from Beckman Coulter (USA) was used as an internal standard and electrophoresis separation was carried out using borate/boric acid buffer in 50 cm effective length uncoated silica capillary tube (75 μm I.D., 375 μm O.D.). PIC® Reagent A (Waters corporation, Milford, USA) was used to facilitate separation [24].

25 to 250 μg/mL HA concentrations were used to make standard curves using the method described above. Usual electrophoregrams showed three peaks corresponding to HA hydrolysis products: Tetrasaccharides (T), Hexasaccharides (H) and Octasaccharides (O) (Fig. 1).

2.2.5. Histological procedure

Beads were fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer and 10 mM $CaCl_2$ at pH 7.4 for 4 h at room temperature. Samples were then placed at 4 °C in 0.1 M sodium cacodylate buffer with 50 mM $BaCl_2$ (pH 7.4) overnight. Beads were dehydrated with alcohol and toluene then embedded in paraffin wax. 5 μm thick sections were cut from the specimens and dyed [14]. Bead structure was observed following staining with toluidine blue (Tissue-Tek® DRS™ Sakura).

2.2.6. Cell viability by MTT assay

MTT test was used to measure the chondrocyte viability in bead. The process is based on mitochondrial dehydrogenase activity that transforms soluble yellow tetrazolium salt to blue formazan. The optical densities measured are proportional to the number of cell alive. The tests are performed on 96-wall plates with one bead in each well. Culture medium (100 μL) and 25 μL MTT solution (5 mg/mL in PBS) were added in each well and incubated at 37 °C in 5% CO_2 for 4 h. Then the medium is discarded and replaced by lysis buffer (SDS-di-methylformamide). Beads were incubated at 37 °C overnight before reading the wells optical density (OD) at 550nm using a Thermo Labsystems Multiskan EX [25].

2.2.7. Measure of chondrocyte proliferation and proteoglycan metabolism

At days 6, 13, 20 and 34, culture medium was changed with DMEM medium containing 5 μCi/mL ^{3}H-thymidine and 10 μCi/mL $Na_2{}^{35}SO_4$. After 24-h incubation (static or agitated conditions) the medium was discarded and the beads were transferred to 24-well plates (2 beads per well). Wells were washed 6 to 7 times with 0.9% sodium chloride, then each bead was transferred to individual vials and dissolved using citrate-EDTA buffer followed by cell digestion with a papain based buffer [5]. The vials were incubated at 60 °C overnight. Aliquots were counted using 4.5 mL scintillation liquid (Ultima Gold, Packard, USA) in a scintillation counter (Beckman, USA).

2.2.8. Mechanical activation technique

A rotating agitator 10 °/tridimensional Polymax 1040 (Fisher Bioblock Scientific, France) was used to stimulate the cells embedded in polymer beads. The agitator was set at 30 cycles per minute at 37 °C (1 Hz). 10 beads from original stock were transferred in sterile 15 mL conical tubes with 10 mL medium. The tubes were attached horizontally on the agitator plate and agitated when needed.

2.2.9. RNA extraction and Reverse Transcriptase (RT) for real time PCR

30 beads were dissolved in 55 mM sodium citrate/50 mM EDTA at pH 6.8 for 10 min. The tubes were centrifuged to separate cells and alginate gel. The cell pellet was washed with PBS before RNA extraction. RNA extraction was realised using RNeasy Mini Kit (Qiagen) modified with an additional DNase step. After extrac-

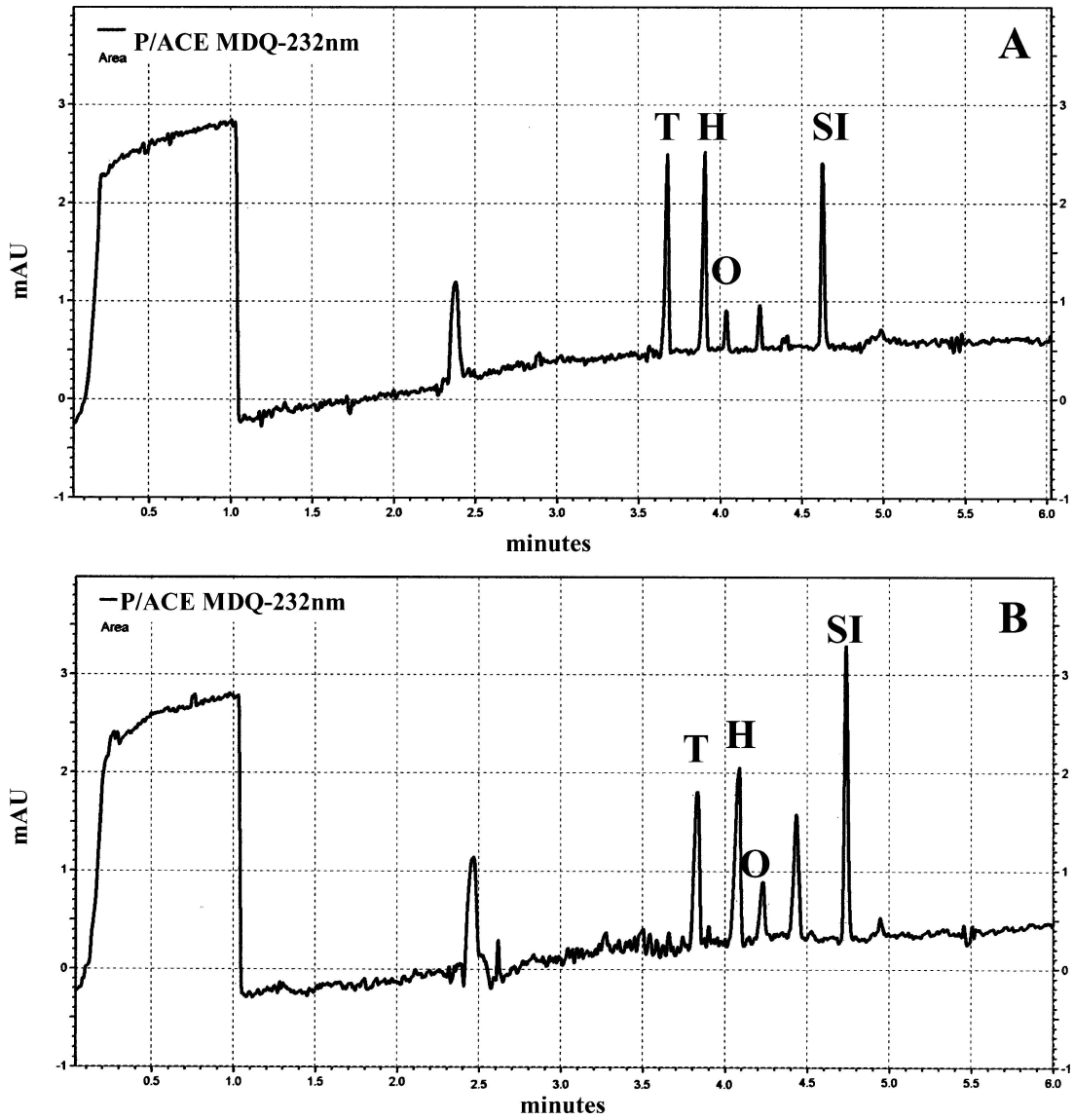

Figure 1 Hyaluronic acid electrophoregram obtained by capillary zone electrophoresis. Beads were hydrolyzed with hyaluronate lyase (100 U/mL) at 37 °C in 0.02 M acetate buffer (pH6) for 72 h. A: electrophoregram obtained with 200 μg/mL commercial HA (standard point). B: electrophoregram obtained from random bead sample. HA hydrolysis results in 3 oligosaccharides: T: tetrasaccharide; H: hexasaccharide; O: octasaccharide.

tion RT was performed on 1 μg of RNA using Omniscript RT Kit (Qiagen) (2 μl buffer 10X, 2 μl dNTP 5 mM, 1 μL oligo(dT) primer (Invitrogen), 1 μL Omniscript reverse transcriptase and RNase-free water. Real time PCR was performed using QuantiTect^{TM} SYBR® Green PCR (Qiagen). Amounts of mARN are compared with amounts obtained from standard curves then normalised with RP29 mARN (housekeeping gene).

3. Results and Discussion

3.1. HA release from alginate and alginate/hyaluronic acid beads

The release of hyaluronic acid from beads made with and without chondrocytes was investigated as a function of time and in several biomaterials. Results are shown in Figs 2 (AA/HA), 3 (AA/HA-C18) and 4 (HA-C12$^{2.3}$ and HA-C12$^{2.5}$-TEG$^{0.5}$).

In AA/HA and AA/HA-C18 beads (Figs 2 and 3), release was found to depend on the presence of chondrocytes in the beads. Beads that did not contain cells liberated about 30% more HA (in most cases) than beads that contained cells. The highest difference was found at 35 days culture for AA/HA (18/2) beads as 76.5 ± 5.8% HA was released from beads without chondrocytes compared to 23.8 ± 11.8% HA released from beads with chondrocytes (Fig. 2A).

In Fig. 2, HA leakage from cell-free beads (white blocks) increased until day 14 and then remained roughly stable. At all times but day 35 release rose with the amount of HA initially present in the beads (2(A)–(C)). HA release profiles from beads containing cells (black blocks) were found less homogenous. Release did not seem to follow a logical time pattern. Results were also found eclectic when releases were compared between the different AA/HA concentrations. At day 7, release progressed with the decrease of initial HA while it was the opposite at day 14. After 14 days, release was found quite stable for all compositions. Although it seems that passive diffusion may be involved in the liberation of HA from cell-free beads, it appeared that

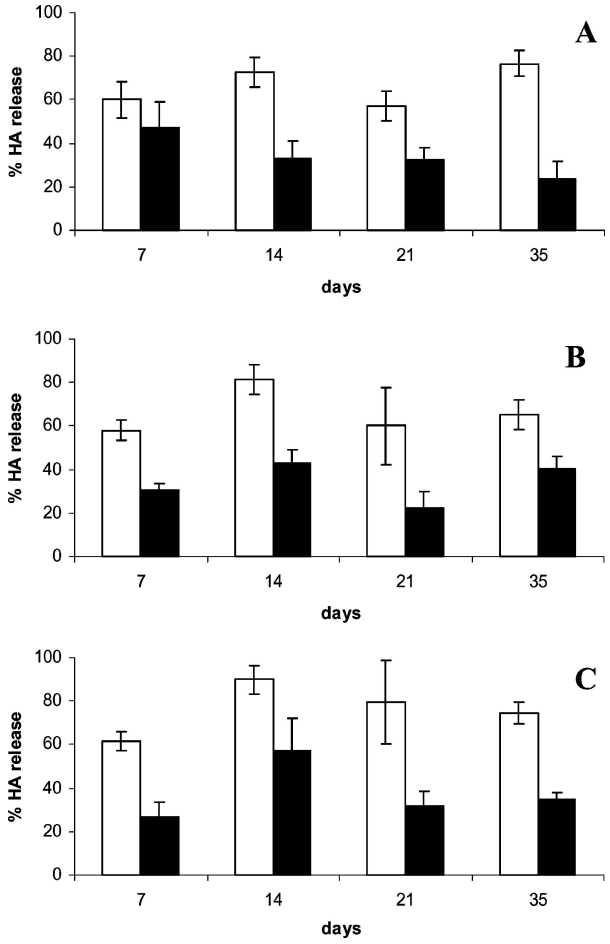

Figure 2 Hyaluronic acid release diagrams from several compositions of alginate/HA beads. Beads were digested with hyaluronate lyase for 72 h and content analysed using capillary electrophoresis. A. release from AA/HA (18/2) beads. B. Release from AA/HA (16/4) beads. C. Release from AA/HA (14/6) beads. Standard deviations were calculated from the mean value obtained from 10 bead samples.□ polymer beads not containing chondrocytes. ■Polymer beads containing chondrocytes.

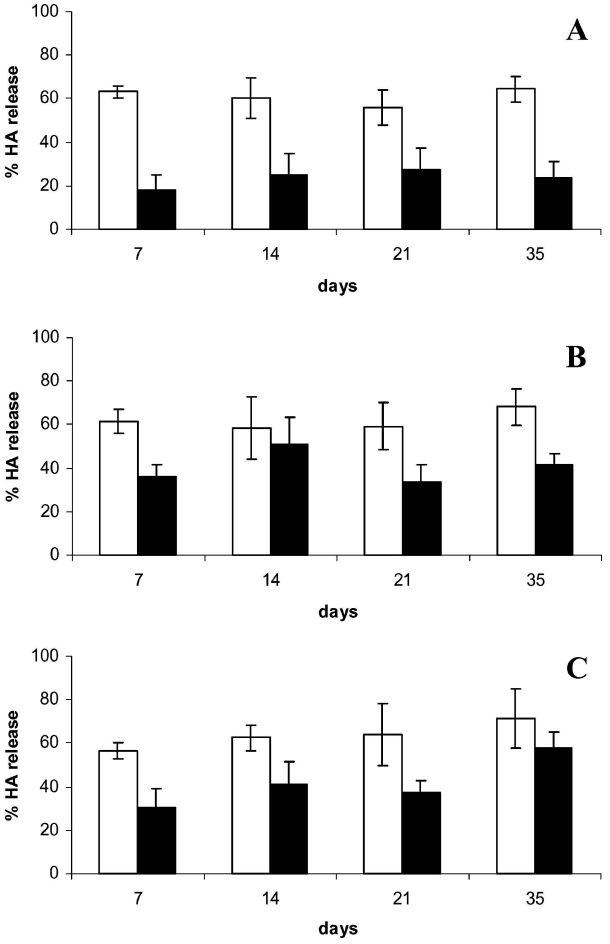

Figure 3 Hyaluronic acid release diagrams from several compositions of alginate/HA-C18 beads. Beads were digested with hyaluronate lyase for 72 h and content analysed using capillary electrophoresis. A. release from AA/HA-C18 (18/2) beads. B. Release from AA/HA-C18 (16/4) beads. C. Release from AA/HA-C18 (14/6) beads. Standard deviations were calculated from the mean value obtained from 10 bead samples. □ polymer beads not containing chondrocytes. ■ Polymer beads containing chondrocytes.

another phenomenon was involved in the release of HA from beads containing cells.

Chondrocytes increased HA retention in beads made of HA-C18 (Fig. 3) especially for AA/HA-Cl8 beads. However, release from cell-free beads did not augment with HA concentration or time. Release was found to be around 60% in all conditions. Similar conclusions could be drawn for HA released from beads containing cells. About 23% was freed from AA/HA-C18 (18/2) (Fig. 3A), about 40% from AA/HA-C18 (16/4) and 43% from (14/6) (Fig. 3B and C). HA release seemed to increase with the increase of initial amount of HA in the beads.

Fig. 4 shows data obtained with 3 biomaterials of total polymer concentration 10 g/L (AA/HA, AA/HA-$C12^{2.3}$, AA/HA-$C12^{2.5}$-$TEG^{0.5}$) at day 14 and 21. Results from beads containing no cells (Fig. 4A) and beads containing cells (Fig. 4B) were compared. In both Figs (A and B), HA release augmented with time. Cells only seemed to improve HA retention at day 21 for all biomaterials. The most retention was observed with AA/HA materials.

It is now accepted that HA binds to cells such as chondrocytes via receptors like CD44 (cluster determinant), RHAMM (receptor for hyaluronate mediated motility) and ICAM-1 (intracellular adhesion molecule) [26–28]. CD44 has been identified as a glycoprotein expressed by chondrocytes that functions as hyaluronic acid receptor at the chondrocyte cell surface [29]. It is therefore understandable that the presence of chondrocytes in alginate/HA beads would increase HA retention in 3D scaffolds. Indeed, an increase of about 30% retention could be observed in both Figs 2 and 3 and this regardless of the initial HA concentration.

In a comparable study, Lindenhayn [30] noticed that HA retention was increased with the amount of alginate in the beads (corresponding to a decrease in HA concentration). His explanation essentially relied on the porosity of the material. Fig. 5 shows micrographs of alginate and AA/HA (top line), AA/HA-C18 (middle line) and AA/HA-$C12^{2.3}$, HA-$C12^{2.5}$-$TEG^{0.5}$ (bottom line). The bead porosity was observed by optical microscopy. Pictures were magnified 4 times and pores could only be seen for beads composed of AA/HA-C18, AA/HA-$C12^{2.3}$, HA-$C12^{2.5}$-$TEG^{0.5}$ even when pictures were magnified up to 40 times. Structure of AA/HA-C18 (16/4 and 14/6) bead and HA-$C12^{2.5}$-$TEG^{0.5}$ bead especially appeared unorganised with very large pores.

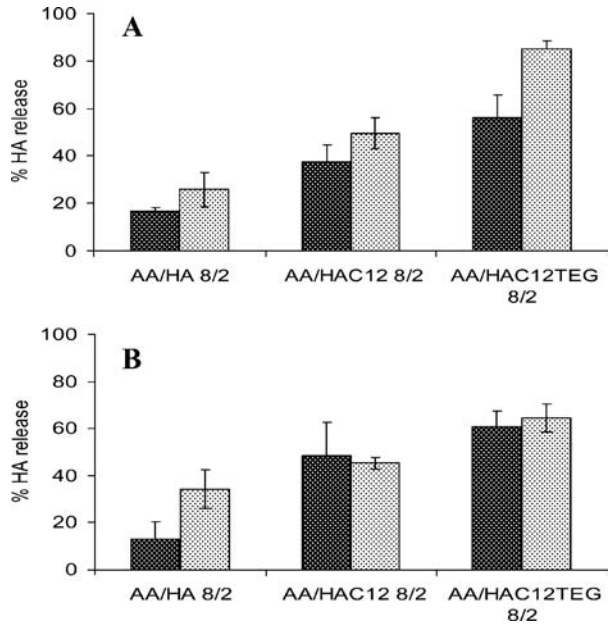

Figure 4 Hyaluronic acid release diagrams from several compositions of alginate/HA beads (10 g/L). Beads were digested with hyaluronate lyase for 72 h and content analysed using capillary zone electrophoresis. A. release from AA/HA (8/2) beads, AA/HA-$C12^{2.3}$ (8/2) beads, AA/HA-$C12^{2.5}$-$TEG^{0.5}$ (8/2) beads not containing chondrocytes. B. release from AA/HA (8/2) beads, AA/HA-$C12^{2.3}$(8/2) beads, AA/HA-$C12^{2.5}$-$TEG^{0.5}$beads containing chondrocytes. Standard deviations were calculated from the mean value obtained from 6 beads samples. ■ Day 14. ▒ day 21.

These observations are concomitant with results obtained in Fig. 3 as release was increasing with HA content in the case of AA/HA-C18 beads. Additionally, HA-$C12^{2.5}$-$TEG^{0.5}$ beads were shown to release more HA than AA/HA-$C12^{2.3}$.

Porosity very likely plays an important role in HA leakage but some other parameters like HA fixation on CD44 receptors may contribute to HA retention in alginate/HA beads. However, other parameters such as ionic exchange, viscosity and polymer miscibility might affect HA release.

Chemical modifications were introduced in our polymers with the aim to investigate the possibility of replacing the alginate by a biological agent (HA) while maintaining the polymer rigidity (alkyl chains C18 and C12, TEG cross-links). For some materials the polymer concentrations were lowered from 20 to 10 g/L due to alginate and HA-C12 immiscibility at 20 g/ L (Fig. 4). When compared to AA/HA beads, the chemical modifications involved in AA/HA-C18 did not significantly enhance HA retention in the beads regardless of the initial amount of HA in the beads. In Fig. 4, it even seemed like chemical modifications and cross-links had a detrimental effect on HA retention compared to unmodified alginate/HA. It is possible that immiscibility might lead to the formation of structural micro domains which could result in further leakage. This hypothesis was envisaged but could not be investigated in our study. Contrary to what was expected, HA-C18 or HA-$C12^{2.5}$-$TEG^{0.5}$ did not improve HA retention in beads.

3.2. Influence of bead composition on chondrocyte proliferation and metabolism

Chondrocyte proliferation and proteoglycan synthesis were measured in several biomaterials for 35 days. Results on chondrocyte proliferation and proteoglycan synthesis for each biomaterial are shown in Table I. A possible link between chondrocyte growth and the amount of HA remaining in the bead was investigated (Table I). Proliferation was measured by counting ^{3}H-thymidine incorporation (DPM) in DNA during cell division in individual beads. The total number of cells alive in one scaffold was estimated using MTT tests. The resulting optical densities are proportional to the number of live chondrocytes per bead. The number of DPM found per scaffold was normalised by the optical density. This form of measurement allowed us to compare results between several samples but did not

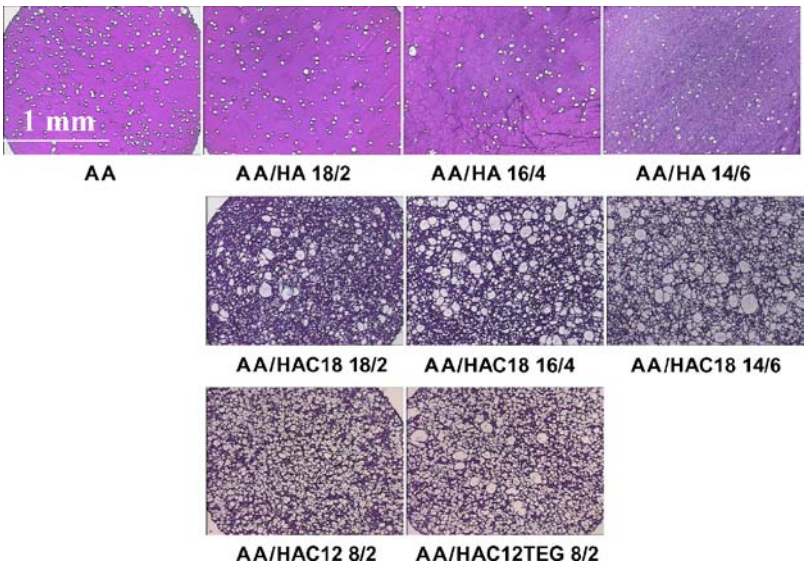

Figure 5 Microscopic images of several types of beads fixed using histological procedure and coloured with blue toluidine (magnification: ×4). Top line. AA and AA/HA beads. Middle line. AA/HA-C18 beads. Bottom line. AA/HA-$C12^{2.3}$(8/2) and AA/HA-$C12^{2.5}$-$TEG^{0.5}$ beads.

TABLE I Chondrocyte proliferation and proteoglycan metabolism measured in several types of beads. Results are expressed as DPM/OD ($\times 10^3$), standard deviations are calculated from the mean data ($n = 10$). For each biomaterial, 10 beads were counted individually for radioactivity and MTT tests were performed on 10 other beads from the same batch

Proliferation ($\times 10^3$ DPM/OD)	AA (20)	AA/HA (18/2)	AA/HA (16/4)	AA/HA (14/6)	AA/HA-C18 (18/2)	AA/HA-C18 (16/4)	AA/HA-C18 (14/6)	AA (10)	AA/HA (8/2)	AA/HA-C12	AA/HA-C12-TEG
Day 7	**11.5 ± 1.4**	7.3 ± 1.3	**9.4 ± 1.7**	5 ± 1	5.1 ± 0.6	4.6 ± 0.8	3.4 ± 1.3				
Day 14	**25.3 ± 5.3**	23.2 ± 4.6	**25.8 ± 1.4**	20.8 ± 2.7	12.3 ± 2.8	6.5 ± 0.5	5.3 ± 1				
Day 21	7 ± 0.8	5.8 ± 1.5	**10 ± 1.3**	15.9 ± 2.7	2.3 ± 0.5	3.1 ± 0.6	4.4 ± 0.9	**59.5 ± 6.4**	**50.7 ± 7.8**	12.9 ± 3.5	7.9 ± 1.7
Day 35	**13.4 ± 2.7**	7.5 ± 1.3	**33.7 ± 5.9**	12.6 ± 2.4	8.9 ± 1.9	9 ± 2.7	12.2 ± 1.7				
Metabolism ($\times 10^3$ DPM/OD)	AA (20)	AA/HA (18/2)	AA/HA (16/4)	AA/HA (14/6)	AA/HA-C18 (18/2)	AA/HA-C18 (16/4)	AA/HA-C18 (14/6)	AA (10)	AA/HA (8/2)	AA/HA-C12	AA/HA-C12-TEG
Day 7	**19.4 ± 2.6**	**15.4 ± 1.5**	14.1 ± 1.9	10.8 ± 1.3	5.5 ± 0.7	4.1 ± 0.5	3.7 ± 0.5				
Day 14	**40.2 ± 7**	25.9 ± 2.9	**34.3 ± 3.6**	22.7 ± 3	12.7 ± 2.6	7.6 ± 1.5	4.1 ± 0.8				
Day 21	**20.1 ± 3.2**	14 ± 2.1	**18.9 ± 2.5**	16.2 ± 1.7	14.8 ± 2.8	9.3 ± 2.4	4.7 ± 0.8	**76.8 ± 9.1**	**46.2 ± 6.2**	33.6 ± 5.6	15.2 ± 3
Day 35	**18.7 ± 2.6**	9.3 ± 2.4	**15.4 ± 2.9**	9.4 ± 1.5	6.2 ± 1.7	4.9 ± 1.3	4.8 ± 1.7				
HA Remaining (μg)		AA/HA (18/2)	AA/HA (16/4)	AA/HA (14/6)	AA/HA-C18 (18/2)	AA/HA-C18 (16/4)	AA/HA-C18 (14/6)		AA/HA (8/2)	AA/HA-C12	AA/HA-C12-TEG
Day 7		39.6 ± 8.7	**89.7 ± 4.6**	**141.8 ± 13.4**	36.11 ± 3.13	49.5 ± 4.1	91.3 ± 12.1				
Day 14		50.5 ± 6	73.4 ± 7.7	**83.1 ± 29**	32.1 ± 4.3	38.1 ± 9.5	**76.8 ± 13.3**				
Day 21		51.1 ± 4.5	**99.7 ± 9.3**	**132.6 ± 13.2**	32.1 ± 4.5	51.6 ± 6.4	82.4 ± 7.7		39.6 ± 5.8	18.4 ± 1	14.4 ± 3
Day 35		57.3 ± 6	**76.9 ± 7.2**	**126.7 ± 6.18**	33.6 ± 3.4	45.4 ± 3.8	55.2 ± 10				

TABLE II Chondrocyte gene expression measured in several types of beads in static and dynamic conditions at 21 days culture. Data were measured by RT-qPCR and normalized with RP 29 gene

Day 21	Static	Agitated	Agitated/Static
COLLAGEN I			
AA	7.38	23.47	–
AA/HA (16/4)	2.31	39.54	–
AA/HA-C18 (16/4)	6.59	14.94	–
COLLAGEN II			
AA	0.24	1.60	–
AA/HA (16/4)	6.81E-02	3.36	–
AA/HA-C18 (16/4)	0.38	1.90	–
AGRECAN			
AA	1.55E-03	4.04E-03	–
AA/HA (16/4)	ND	7.45E-03	–
AA/HA-C18 (16/4)	1.41E-03	3.41E-03	–

permit to determine the cell cycles. Metabolism estimation consisted in measuring the sulphate incorporation in the ECM proteoglycans (DPM). This number was divided by the optical densities derived from the MTT tests.

Manipulations were conducted in parallel on 20 and 10 g/L polymer beads. However, due to cost and synthesis difficulties, cell proliferation and metabolism were only measured at day 21 in AA/HA-$C12^{2.3}$ and AA/HA-$C12^{2.5}$-$TEG^{0.5}$. Additionally, it was shown that results were not significantly different past 14 days culture. Due to experimental differences, data obtained for materials used to make 20 g/L beads could not be compared with data obtained with 10 g/L polymer beads.

Our data all showed really high levels of proliferation and metabolism at day 14 that decreases afterwards. These values derive from the fact that we could not distinguished the cycle in which the cells were in. Cells that have replicated and then incorporated ^{35}S might or might not have undergone mitosis. Scaffolds in which cells have divided would give a MTT optical density reading that would be twice the reading obtained in scaffolds where cells have not yet divided. According to DA Lee [31] human chondrocyte cell cycle took at least 5 days and most cells had not undergone mitosis after 14 days. This was consistent with our results that showed that the ratio DNA/live cells increased until day 14 and decreased after that, possibly indicating that cells have divided between day 14 and day 21. However, it is not possible to indicate precisely the different steps of cell cycle in this paper.

Cells proliferated best in alginate and alginate/HA (16/4) scaffolds. Proliferation measured in AA/HA-C18 was about twice less compared to proliferation found in alginate or alginate/HA beads. Similarly, chondrocyte proliferation was found to be highest in alginate (10 g/L) and alginate/HA (8/2) beads than in beads containing chemically modified HA (AA/HA-$C12^{2.3}$ and HA-$C12^{2.5}$-$TEG^{0.5}$). Proteoglycans synthesis was also optimum in alginate and alginate/HA beads (20 and 10 g/L). When considering alginate/HA (20 g/L) scaffolds, cell proliferation and metabolism did not seem to depend on the HA initial amount in the beads. However, proliferation and metabolism decreased with the increase of initial HA-C18 in the beads. Based on these findings, it was hypothesised that HA-C18 had an inhibitory effect on the cells. To explain this hypothesis the amount of HA remaining in the beads from day 7 to day 35 were calculated (Table I(C)). Data showed that beads that had the highest initial amount of HA-C18 also had the highest amount of HA-C18 remaining after 7, 14, 21 and 35 days. To summarise, native HA might have a beneficial effect on chondrocyte proliferation and metabolism although it was not seen in our study. HA-C18 was found to have an inhibitory effect on cell functions. Chemically modified and cross-linked polymers did not improve these cell functions either.

The reasons why such inhibition happened could not be explained at this point of the study. However, our data clearly indicated that it was essentially due to the addition of C18 hydrophobic chains rather than the presence of HA. An average of 84.4 ± 12.5 μg HA remained in the AA/HA (16/4) beads (all times mixed). This amount was comparable to 74.7 ± 17.7 μg HA-C18 that was left in AA/HA-C18 (14/6) beads. Assuming that the whole HA molecule had an effect on cell functions the data obtained for proliferation and metabolism would be comparable in both cases. However, cell proliferation and proteoglycan synthesis were found much lower at all times in AA/HA-C18 (14/6) beads than in AA/HA (16/4). This observation could be repeated when comparing AA/HA (18/2) and AA/HA-C18 (16/4) beads (Table I).

Several studies have shown that chondrocyte proliferated with the increasing amount of HA in biomaterials [30, 32, 33] but some other work have demonstrated that HA did not influence chondrocyte proliferation [16, 34]. Our research agreed with these latter showing that HA did not have a significant impact on cell proliferation when compared to results found with alginate beads. HA-C18 was found to give much lower cell proliferation compared to either AA or AA/HA beads indicating an inhibiting effect due to hydrophobic C18 chains. C18 chains improved HA retention in beads (Fig. 3) probably due to the additional rigidity given to the beads. However, the general synthesis mechanism was affected. AA/HA-$C12^{2.3}$ and HA-$C12^{2.5}$-$TEG^{0.5}$ were not found to improve cell functions or HA retention in the beads despite their theoretical properties. Further work is being carried out to investigate the reasons of such unexpected material behaviour.

3.3. Effect of mechanical stimulation on chondrocyte proliferation and metabolism

Encouraged by results published in our previous publication [5] and with the aim to boost cell proliferation and metabolism in priority, the effect of mechanical stimulation was investigated on polymer beads. Results are shown in Fig. 6 for 20 g/L beads made of AA, AA/HA (16/4) and AA/HA-C18 (16/4). In static conditions, chondrocyte proliferation and metabolism were found satisfactory when AA/HA (16/4) were

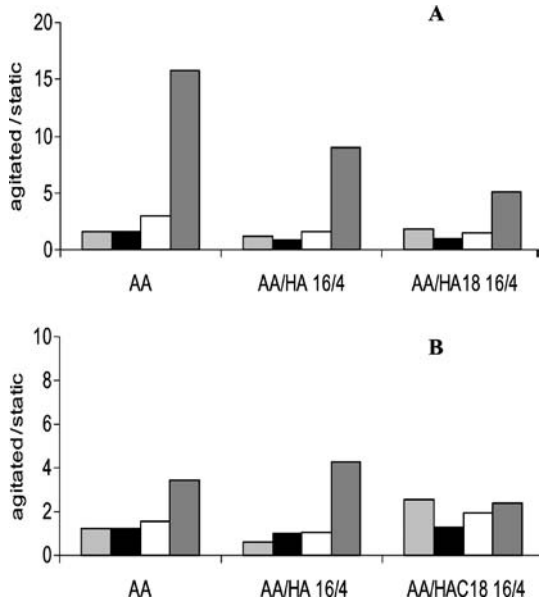

Figure 6 Comparison of chondrocyte proliferation and proteoglycan synthesis obtained in static and dynamic conditions. Incorporation and metabolism were measured by radioactive counting of ^{3}H-DNA and ^{35}S-proteoglycan. Beads were either left in static positions or agitated for 48 h before analysis. Data were plotted as ratios DPM (static)/DPM (agitated). A. Chondrocyte proliferation. B. Chondrocyte metabolic activity. ▒ Day 7. ■ Day 14. □ Day 21. ▒ Day 35.

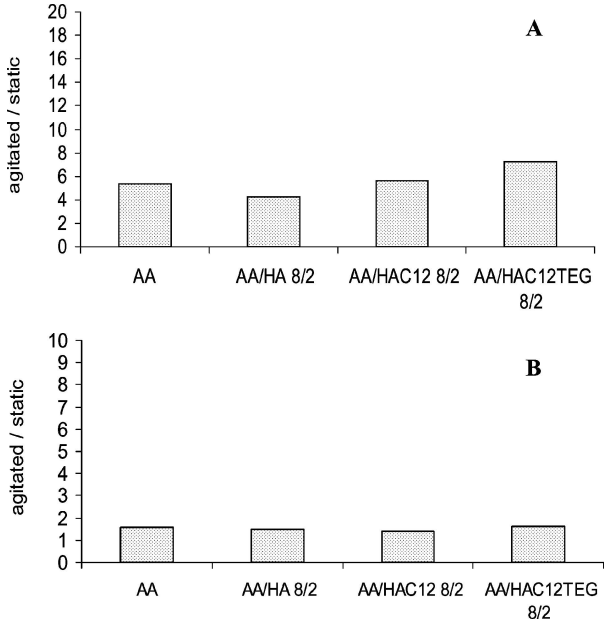

Figure 7 Comparison of chondrocyte proliferation and proteoglycan synthesis obtained in static and dynamic conditions in several types of material. Incorporation and metabolism were measured by radioactive counting of ^{3}H-DNA and ^{35}S-proteoglycan. Beads were either left in static positions or agitated for 48 h before analysis. Data were plotted as ratios DPM (static)/DPM (agitated). A. Chondrocyte proliferation. B. Chondrocyte metabolic activity. ▒ Day 21.

used. Therefore, we chose this composition for further experiments. Mechanical stimulation enhanced both cell proliferation and metabolism in all the materials at day 35. No significant effect could be seen before 35 days culture. Cell proliferation was multiplied 16 times when grown in alginate scaffolds. The addition of HA or HA-C18 diminished the stimulating effect (×8.5 AA/HA; ×4.2 AA/HA-C18). The effect of mechanical stimulation on proteoglycan synthesis is less obvious but still positive. The mechanical stimulating effect obtained by Gigant *et al*. was found to be around ×100 on chondrocyte proliferation and x5 on proteoglycan synthesis. Although results are comparable for metabolism, cell proliferation was not increased as much in our study. However several differences exist between the two studies. For instance, articular chondrocytes were used by Gigant-Huselstein [5] and the cell density was about one third less than in this study. Intrinsic chondrocyte properties as well as space availability have played a role in the diminution of mechanical stimulation. The inhibitory effect of AA/HA-C18 scaffolds was confirmed in this experiment. Chondrocytes in cartilage utilise mechanical signal in conjunction with other factors to regulate their metabolic activity. The chondrocytes receive the mechanical signals through the cell deformation and complex biological and biophysical interactions with the ECM. It has been advanced that a change in cell shape serves as a regulator of chondrocyte metabolism. In our system on top of fluid movement it is possible that both hydrostatic pressure and mechanical deformation occur. Cell proliferation and proteoglycan metabolism would therefore be monitored via complex interactions between the ECM and the presence of ionic species. It is possible that the hydrophobic domains prevent the signals induced by mechanical stimulation to circulate between cells and therefore inhibit the mechanical effect.

Fig. 7 shows data obtained with 10 g/L beads made of AA, AA/HA, HA-C12$^{2.3}$ and HA-C12$^{2.5}$-TEG$^{0.5}$. Proliferation was found to be highest in alginate and AA/HA-C12$^{2.5}$-TEG$^{0.5}$ beads. Results (not shown) indicated that after agitation HA was less released from AA/HA-C12$^{2.5}$-TEG$^{0.5}$ (45% released) beads than from any other materials (69% released for AA/HA-C12 and 54% for AA/HA (8/2). Conclusions drawn from Table I data indicated that proliferation and metabolism were related to the amount of HA left in the beads. It is then possible to envisage that cell proliferation and metabolism depend on the presence of HA in the beads. In dynamic conditions, the bead stability was increased with the addition of TEG which resulted in HA retention and enhancement of proliferation and metabolism. The hypothesis that the presence of hydrophobic domains that could stop the signal propagation was not confirmed in this experiment.

4. Conclusions

In this study we demonstrated that it was possible to grow non articular chondrocytes in several biomaterials based on alginate and hyaluronic acid. Biomaterials resulted from the replacement of alginate by a polymer with biological properties (hyaluronic acid). HA was also modified by grafting alkyl chains on the HA backbone and cross-linking with TEG. In theory, the rigidity that was lost by the diminution of alginate in scaffolds was regained by the introduction of hydrophobic

interactions between the alkyl chains and TEG cross-links. The chondrocytes growth and metabolic activities depended on the presence of alginate and native HA. However, the amount of HA did not seem to have a great influence on the cell activities. The introduction of C18 chains increased the retention of HA in the scaffolds but inhibited the general cell synthesis program. Unexpectedly, AA/HA-$C12^{2.3}$ and AA/HA-$C12^{2.5}$-$TEG^{0.5}$ did not increase HA retention in scaffolds and therefore did not show any beneficial effect on cell biology. Mechanical stimulation based on beads' shock was shown to be a simple method to improve cell proliferation and metabolism. AA/HA-$C12^{2.5}$-$TEG^{0.5}$ scaffolds retained more HA than other materials (10 g/L polymer constructs) in dynamic conditions. The cell proliferation was then found comparable in AA/HA-$C12^{2.5}$-$TEG^{0.5}$ and in alginate beads.

The final goal of our research will be to verify that non articular chondrocytes can gain articular characteristics when cultured in 3D scaffolds. Preliminary studies on chondrocyte gene expression were conducted by RT-qPCR (Table II). In static conditions, collagen I genes were predominantly found at days 21 and 35. It could be explained by the fact that in our culture technique, chondrocytes were initially grown in monolayer. At that stage, chondrocytes dedifferentiated in fibroblastic type cells while they over-expressed type I collagen [35] instead of collagen II and agrecan. Dynamic conditions increased all gene expression at both days 21 and 35. It is interesting to notice the cumulative stimulation provided by the presence of HA and dynamic conditions on expression of extracellular matrix genes such as collagen II and agrecan. Mechanical loading played an important role in gene expression but HA seemed to be an important factor to accelerate ECM gene expression. The expression of collagen II and agrecan indicated that articular like extracellular matrix components were forming [36]. Promising results indicated that in our culture conditions it might be possible to restore articular chondrocyte phenotype using non articular chondrocytes.

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