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RESEARCH ARTICLE

Enriching a cellulose hydrogel with a biologically active marine exopolysaccharide for cell-based cartilage engineering

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Abstract

The development of biologically and mechanically competent hydrogels is a prerequisite in cartilage engineering. We recently demonstrated that a marine exopolysaccharide, GY785, stimulates the *in vitro* chondrogenesis of adipose stromal cells. In the present study, we thus hypothesized that enriching our silated hydroxypropyl methylcellulose hydrogel (Si-HPMC) with GY785 might offer new prospects in the development of scaffolds for cartilage regeneration. The interaction properties of GY785 with growth factors was tested by surface plasmon resonance (SPR). The biocompatibility of Si-HPMC/GY785 towards rabbit articular chondrocytes (RACs) and its ability to maintain and recover a chondrocytic phenotype were then evaluated *in vitro* by MTS assay, cell counting and qRT–PCR. Finally, we evaluated the potential of Si-HPMC/GY785 associated with RACs to form cartilaginous tissue *in vivo* by transplantation into the subcutis of nude mice for 3 weeks. Our SPR data indicated that GY785 was able to physically interact with BMP-2 and TGF*β*. Our analyses also showed that three-dimensionally (3D)-cultured RACs into Si-HPMC/GY785 strongly expressed type II collagen (COL2) and aggrecan transcripts when compared to Si-HPMC alone. In addition, RACs also produced large amounts of extracellular matrix (ECM) containing glycosaminoglycans (GAG) and COL2. When dedifferentiated RACs were replaced in 3D in Si-HPMC/GY785, the expressions of COL2 and aggrecan transcripts were recovered and that of type I collagen decreased. Immunohistological analyses of Si-HPMC/GY785 constructs transplanted into nude mice revealed the production of a cartilage-like extracellular matrix (ECM) containing high amounts of GAG and COL2. These results indicate that GY785-enriched Si-HPMC appears to be a promising hydrogel for cartilage tissue engineering.

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1. Introduction

Articular cartilage (AC) is an avascular connective tissue containing a unique cell type, the chondrocyte, which synthesizes an abundant and highly hydrated extracellular

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matrix (ECM) (Steinert *et al*., 2007), mainly composed of type II collagen and aggrecan (Demoor *et al*., 2014). Unfortunately, AC has a poor capacity for self-repair and injuries to AC are thus irreversible and may lead to long-term joint degeneration (Gomoll and Minas, 2014). Tissue-engineering strategies involving the combination of cells, scaffolds and bioactive agents have emerged to build functional new tissue in order to replace damaged cartilage (van Osch *et al*., 2009; Vinatier et al., 2009a). The ideal scaffold material for cartilage tissue engineering should be one that closely mimics the natural cartilage environment in a structural, mechanical and biofunctional way (Bonzani *et al*., 2006; Griffith and Naughton, 2002). Several studies have demonstrated that chondrocyte functions differ in two-dimensional (2D) and three-dimensional (3D) systems (Brodkin *et al*., 2004; Mukaida *et al*., 2005), with a progressive dedifferentiation of chondrocytes in 2D monolayer culture, whereas this phenomenon is prevented by 3D culture (Dehne *et al*., 2010; Mukaida *et al*., 2005). Given that articular cartilage is a tissue subject to considerable stress, to mimic the environments of cartilage tissue in structural terms, the fundamental structure of a scaffold must therefore be a 3D system. In this context, an injectable, self-setting silated hydroxypropyl methylcellulose (Si-HPMC)-based hydrogel has been developed previously (Bourges *et al*., 2002a, 2002b). This Si-HPMC is steam sterilizable, injectable and self-sets at physiological pH by a crosslinking reaction involving the condensation of silanol groups (Fatimi *et al*., 2008). This crosslinking process without an exogenous reticulating agent is usually associated with excellent biocompatibility. This cellulose-based hydrogel, once reticulated, is composed of only 2% dry polymer and 98% water, a composition that mimics the high hydration of articular cartilage ECM. Although the mechanical properties of Si-HPMC (compressive modulus around 2.9 kPa) are distinct from those of articular cartilage (compressive modulus around 30 kPa (Discher *et al*., 2009)), it offers a 3D environment in which the cells can grow and produce cartilage ECM tissue. Cellulose-based hydrogels such as Si-HPMC are very stable *in vivo* (Laib *et al*., 2009), giving 3D support for the growth of newly formed tissue over a long period. Finally, Si-HPMC hydrogel derived from plant cellulose is likely to be safer than animal-derived hydrogels, which can exhibit risks of unconventional pathogen transmission. In a previous study, this Si-HPMC hydrogel was demonstrated to be a suitable matrix for the 3D culture of chondrocytes *in vitro* (Vinatier *et al*., 2005) and the production of cartilage *in vivo* (Vinatier *et al*., 2007).

Biological sulphated polysaccharides, such as sulphated glycosaminoglycans (GAGs), are major components of articular cartilage ECM. GAGs are implied in many biological processes, such as cytokinesis, cell proliferation, differentiation, migration, tissue morphogenesis, organogenesis and wound repair (Yamada and Sugahara, 2008). GAGs can bind many different classes of proteins, ranging from growth factors, e.g. FGFs, VEGFs, PDGF, glial cell-derived neurotrophic factor (GDNF) and HGF, cytokines such as interleukins, interferon, PF4 and RANTES, to metabolic enzymes such as lipoprotein lipase and structural proteins such as laminin and fibronectin, among many others (Casu and Lindahl, 2001). GAGs would control the diffusion of the growth factors to establish protein gradients or act as a repository of growth factors that could be sequestered from cells and released at an appropriate time (Zcharia *et al*., 2005). The actions of biologically active polysaccharides are largely dependent on their molecular structure, in particular their molecular size and varying size of repeating unit features, osidic residues, linkage and degree of sulphation.

Natural polysaccharides derived from marine prokaryotes offer a significant structural chimiodiversity, with novel and striking biological properties (Guezennec, 2002). In addition, natural GAG-mimetics can be chemically customized and produced in large amounts with relatively simple and reproducible processes, making them potentially suitable as bioactive agents for medical applications. Among the large number of prokaryotic species capable of producing GAGs (Guezennec, 2002), *Alteromonas infernus* has been shown to produce a branched high-molecular weight polysaccharide, GY785 ($\sim 10^6$ g/M). This exopolysaccharide (EPS) is unique, with no known analogue. Low-molecular weight (GY785 DR) and low-molecular weight oversulphated (GY785 DRS) derivatives of GY785 are described as 'heparin-like' compounds. These GY785 derivatives have been shown to exhibit some anticoagulant properties (Matou *et al*., 2005). Interestingly, they have also been recently been described to positively influence the chondrogenic differentiation of adipose-derived stromal cells (Merceron *et al*., 2012). In order to develop a biologically and biomechanically competent hydrogel for cartilage tissue engineering, we proposed to associate the branched high-molecular weight polysaccharide GY785 (0.67% w/v) with Si-HPMC scaffold.

The aim of the present study was thus to investigate whether enrichment in GY785 EPS improves the potential of Si-HPMC hydrogel for engineering cartilage. In this attempt, we first assessed the growth factor-interacting properties of GY785, then the cytocompatibility of Si-HPMC/GY785 was assessed with regard to articular chondrocytes. Second, the potential of Si-HPMC/GY785 to support the maintenance of a chondrocytic phenotype *in vitro* and to produce cartilaginous matrix in subcutaneous pockets of nude mice were investigated.

2. Materials and methods

2.1. Materials

Hydroxypropyl methylcellulose (HPMC) E4M® was purchased from Colorcon-Down Chemical (Bougival, France). GY785 EPS was produced by Seadev-FermenSys SAS (Plouzané, France) and sterilized using ethylene oxide by IONISOS (Gien, France). Hyaluronidase, actinomycin-D, trypsin–EDTA, type II collagenase

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(290 U/mg), HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulphonic] and isopropanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hanks' balanced sodium salt (HBSS), Dulbecco's modified Eagle's medium (DMEM; 4.5 g/l glucose), penicillin–streptomycin, L-glutamine, bone morphogenetic protein-2 (BMP-2), Trizol®, DNase I, Taq DNA polymerase and reverse transcriptase superscript II were obtained from Invitrogen (Paisley, UK). Methyl tetrazolium salt (MTS) was purchased from Promega (USA). A live/dead viability/cytotoxicity kit assay was obtained from Invitrogen. Fetal calf serum (FCS) was obtained from Dominique Dutscher (Brumath, France). Cell-culture plastics were purchased from Corning (VWR, France). New Zealand rabbits were obtained from Grimaud Frères (Roussay, France). Monoclonal antibody against type II collagen was purchased from Oncogene (San Diego, CA, USA). Monoclonal antibody against type I collagen was purchased from MP Biomedicals (Illkirch, France) and monoclonal antibody against aggrecan was obtained from Thermo Scientific Pierce Protein Biology Products (Rockford, IL, USA). TGFβ1 was obtained from PeproTech (London, UK). DIG RNA labelling kit was acquired from Roche Diagnostics (Mannheim, Germany). All other chemicals were from standard laboratory suppliers and were of the highest purity available.

2.2. Surface plasmon resonance

Experiments were carried out on a Biacore 3000 instrument (Biacore, Uppsala, Sweden). TGFβ1 and BMP-2 were covalently immobilized to the dextran matrix of a CM5 sensor chip (Biacore), as recommended by the manufacturer, at a flow rate of 5 µl/min. Binding assays of GY785 (0.03125, 0.0625, 0.125, 0.25, 0.50 and 1 µg/ml) were performed in 10 mM HEPES buffer, pH 7.4, containing 0.15 M NaCl and 0.005% P2O surfactant (HBS-P buffer, Biacore) and dissociation was monitored for 15 min. Regeneration was achieved with NaOH (4.5 mM) after each cycle. The resulting sensorgrams were fitted using BiaEval 4.1 software (Biacore) and, for dissociation constant (K_d) calculations, a GY785 molecular weight of 1 400 000 g/M was used.

2.3. Synthesis of Si-HPMC/GY785 hydrogel

The Si-HPMC was synthesized by grafting 0.5% of silicium (by weight) onto HPMC (E4M®) in heterogeneous medium, as previously described (Bourges *et al.*, 2002a, 2002b). Si-HPMC powder (3% w/v) was solubilized in 0.2 M NaOH under constant stirring for 48 h. The solution was then sterilized by steam (121°C, 30 min). For the production of Si-HPMC/GY785, sterile GY785 polysaccharide was mixed with the sterile Si-HPMC (3% w/v) basic solution at the concentration of 10 mg/ml (1% w/v) in Si-HPMC. The resulting Si-HPMC/GY785 (3/1% w/v) mixture was left under mild rotatory stirring for 12 h to dissolve the GY785. To allow the formation of a reticulated hydrogel, one volume of Si-HPMC (3% w/v) or Si-HPMC/GY785 (3%/1% w/v) solution was mixed with 0.5 volume of 0.26 M HEPES buffer, as previously described (Rederstorff *et al.*, 2011; Vinatier *et al.*, 2005). The final products consisted of hydrogels (pH 7.4) containing Si-HPMC (2% w/v), with or without GY785 (0.67% w/v), respectively named Si-HPMC and Si-HPMC/GY785. In the same manner, a hydrogel was produced with HPMC in order to obtain a Si-HPMC/HPMC (2/0.67) hydrogel, used as a control of macromolecular incorporation of GY785 for the rheological experiments.

2.4. Animals and surgical procedures

All animal handling and surgical procedures were conducted according to European Community guidelines for the care and use of laboratory animals (DE 86/609/CEE) and with the agreement of the Pays de la Loire Ethical Committee. Experiments were performed according to good laboratory practice (GLP) at the Experimental Therapeutics Unit of the University of Nantes.

2.5. Isolation of rabbit articular chondrocytes (RACs)

Rabbit articular cartilage was harvested from euthanized 5 week-old New Zealand White rabbits and RACs were isolated by enzymatic digestion, as described previously (Ghayor *et al.*, 2000). The suspended RACs were plated (P0) at a density of 5×10^4 cells/cm^2 in a 75 cm^2 culture flask with culture medium (DMEM 4.5 g/l glucose, 10% FCS, 100 UI/ml penicillin and 100 µg/ml streptomycin). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and the culture medium was changed every 2–3 days. Cultured RACs were passaged up to four times. RACs from passages 1, 3 and 4 were frozen in Trizol® for subsequent real-time PCR analysis.

2.6. Cytocompatibility of Si-HPMC/GY785 hydrogel

Briefly, RACs were allowed to attach in 24-well plates at a final density of 1×10^4 cells/cm^2. After 24 h, the culture medium was removed and 500 µl Si-HPMC/GY785 was added to each well. The samples were incubated at 37°C for 1 h before adding 1 ml culture medium. As a control, RACs were also cultured without Si-HPMC/GY785 and Si-HPMC (CT) or in the presence of actinomycin-D (5 µg/ml), an inhibitor of RNA polymerase (Vinatier *et al.*, 2007), which was used as a potent inducer of cell death. After 1, 2 and 3 days, the hydrogels and culture media were removed and cell viability was measured, using an MTS assay as described elsewhere (Vinatier *et al.*, 2005). The results were expressed as relative MTS activity compared to control conditions (cells cultured without Si-HPMC/GY785 and Si/HPMC).

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Cell proliferation was evaluated by Trypan blue exclusion dye experiments under the conditions described above. After each indicated time, the hydrogel and culture medium were removed and the cells were detached by adding trypsin/EDTA for 2 min. The suspended cells were transferred into fresh culture medium and counted after Trypan blue staining. The results were expressed as total number of living cells/well.

2.7. 3D culture of rabbit articular chondrocytes (RACs)

RACs, freshly isolated or dedifferentiated (P2), were gently mixed with Si-HPMC/GY785 or Si-HPMC hydrogel, prepared as described previously, at a density of 1×10^6 cells/ml. Hybrid constructs were then seeded in 12-well plates (1.5 ml/well) and incubated at 37°C in 5% CO_2. After 1 h of incubation, 2 ml culture medium (DMEM 4.5 g/l glucose, 10% FCS, 100 UI/ml penicillin and 100 μg/ml streptomycin) was added to each well, and this medium was changed every 2–3 days. Cell viability was evaluated after 3 weeks. RAC phenotypes and the production of sulphated glycosaminoglycans (GAG) and type II collagen were assessed after 3 weeks.

Cell viability in the three-dimensional (3D) cultures was visualized using a live/dead cytotoxic assay, as previously described (Rederstorff *et al*., 2011). RACs imaging was performed using a confocal laser-scanning microscope (Nikon D-Eclipse C1).

Hybrid constructs associating RACs and hydrogels at 3 weeks were fixed in formol over 1 h and embedded into 2% agarose solution before embedding in paraffin. Serial sections of each paraffin block (5 μm thickness) were made, processed for histological staining with alcian blue and immunostained for type II collagen (anti-rabbit type II collagen 1:100), as described elsewhere (Merceron *et al*., 2011). Immunopositive cells showed brown staining with type II collagen antibody.

For real-time PCR analysis, total RNA was extracted using Trizol reagent, in accordance with the manufacturer's instructions. After DNase I digestion, RNA was quantified using a UV-spectrophotometer (Nanodrop NND-1000, Labtech, France); 1 μg RNA/sample was reverse-transcribed using a Superscript III Kit in a total volume of 20 μl. Complementary DNA (cDNA) was amplified in a total volume of 25 μl PCR reaction mix containing 12.5 μl Brillant® SYBR® Green Master Mix (1×) and 30 nM SYBR Green reference dye. The sequence and concentration of each primer set are shown in Table 1. Real-time PCR was carried out in a MX3000P® real-time PCR system (Stratagene) under the following conditions: 10 min at 95°C, followed by 40 cycles of 30 s at 60°C and 30 s at 72°C, as previously described (Merceron *et al*., 2010). The efficiency and specificity of each primer set was confirmed with standard curves of cycle threshold (*C*T) values vs serial dilution of total RNA and melting profile evaluation. Cycle thresholds were normalized to *GAPDH* to control for cDNA differences. The results are reported as fold change in gene expression relative to control conditions.

2.8. *In vivo* tissue formation

2.8.1. Implantation

To investigate whether constructs associating RACs with Si-HPMC/GY785 or Si-HPMC allow the *in vivo* formation of cartilaginous tissue, constructs were implanted into subcutaneous pockets of 4 week-old nude mice (Swiss Nude Mice, Janvier, France). 5×10^5 freshly isolated RACs were gently mixed with 250 μl Si-HPMC/GY785 or Si-HPMC hydrogel prior to crosslinking and implanted subcutaneously into nude mice as previously described (Vinatier *et al*., 2007). Si-HPMC/GY785 and Si-HPMC without cells were also implanted and used as negative controls. Each condition was tested in quadruplicate and 12 animals were implanted (two implants/animal). After 3 weeks, the mice were sacrificed and the implants were recovered and processed histologically, as described earlier. In addition, serial sections of each paraffin block (5 μm thickness) were immunostained for type I collagen (anti-rabbit type I collagen, 1:100)(Merceron *et al*., 2011) and aggrecan (anti-rabbit aggrecan, 1:100). Immunopositive cells showed brown staining with type I collagen antibody. Rabbit growing bone was used as a positive control for type I collagen immunostaining.

2.8.2. Type I collagen riboprobe preparation

Total RNAs from MC3T3-E1 cells were extracted with Trizol. Total RNA (1 μg) was reverse-transcribed for 60 min at 42°C, using 200 U Superscript II. An equivalent of 100 ng total reverse-transcribed RNA was used for PCR, using the following primers containing the T3 or T7 promoter sequences: Col1a1-T3 forward primer, 5′-GAGAATTAACCCTCACTAAAGGGGAGCGGAGAGTACTG GATCG-3′; and Col1a1-T7 reverse primer, 5′-GAG-TAATACGACTCACTATAGGGGGTGGAGGGAGTTTACAC GA-3′, localized on exons 48 and 50, respectively. The T3- and T7-tailed 598 bp-long ^{2}PCR fragment was purified and used as template to synthesize sense and antisense digoxigenin-11-UTP-labelled RNA probes that were prepared using a DIG RNA labelling kit, according to the manufacturer's instructions.

2.8.3. In situ hybridization

Tissues were fixed in neutral buffered formalin, embedded in paraffin and sectioned at 4 μm. Non-radioactive *in situ* hybridization was adapted from Moorman *et al*. (2001). Briefly, sections were deparaffinized, treated with 10 μg/ml proteinase K for 15 min at 37°C, followed by three washes with PBS–Tween 0.1% (PBST). The sections

Table 1. Sequences of primer pairs, gene bank accession numbers used for real time RT-PCR analysis and size of PCR products

Gene	GeneBank No.	Forward primer	Reverse primer	Amplicon size (bp)
Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	NM_001882253	5′-agaacgggaagctggtcat-3′	5′-ttgatgttggcgggatct-3′	70
Aggrecan (*ACAN*)	L38480	5′-gaggatggcttccaccagt-3′	5′-tggggtacctgacagtctga-3′	61
Type I collagen chain α1 (*Collα1*)	D49399	5′-agcgatggtcctccaggt-3′	5′-gccagggtaaccacgttct-3′	63
Type II collagen chain α1 (*Col2α1*)	D83228	5′-acagcaggttcacctataccg-3′	5′-cccacttaccggtgtgtttc-3′	60

were then refixed for 20 min in 4% paraformaldehyde dissolved in PBS and washed three times in PBST for 5 min before being acetylated (0.1 M triethanolamine with 0.25% acetic anhydride) for 10 min. The sections were prehybridized for 1 h at 60°C and then hybridized overnight at 60°C. After hybridization, the sections were rinsed in 2× SSC, pH 4.5, washed three times for 30 min at 60°C in 50% formamide/2× SSC, pH 4.5, followed by three 5 min washes in PBST. Probe bound to the section was immunologically detected using a sheep anti-digoxigenin Fab fragment covalently coupled to alkaline phosphatase and NBT/BCIP as chromogenic substrate, essentially according to the manufacturer's protocol (Roche).

2.9. Rheological characteristics

Si-HPMC, Si-HPMC/GY785 or Si-HPMC/HPMC (used as a control of macromolecular incorporation), 1 ml, was allowed to reticulate in 12-well plates. The samples were incubated at 37°C for 1 h before adding 2 ml culture medium (DMEM supplemented with 10% FCS, 1% penicillin–streptomycin and 1% L-glutamine). The plates were maintained at 37°C in a humidified atmosphere and with a change of medium every 2 days. After 3 weeks, oscillatory measurements were performed at 25°C on Si-HPMC, Si-HPMC/GY785 or Si-HPMC/HPMC, using the Haake MARS rheometer (ThermoHaake®, Germany) with titanium plate geometry [20 mm diameter (PP20 Ti) plate with 3 mm gap] with a home-made device for direct measurement inside the 12-well plates. We applied a 0.2 N normal force on the hydrogels before the measurements. The complex viscosity (η^*) was determined as a function of stress under conditions of linear viscoelastic response in the range 0.1–5 Pa, at a constant oscillation frequency (ω) of 1 Hz. The storage (G') and loss (G'') moduli were measured within the linear viscoelastic region.

2.10. Statistical analysis

Results are expressed as mean ± standard error (SE) of the mean of triplicate determinations. Comparative studies of means were performed using one-way ANOVA followed by a *post hoc* test, with statistical significance set at $p < 0.001$.

3. Results

3.1. Interactions between GY785 and growth factors

GAGs of the extracellular matrix are well known to modulate the activity of a large panel of biological factors. Whether an exogenous GAG, such as GY785, may interact with chondrogenic factors has, however, not yet been determined. To address this issue, we embarked on a set of surface plasmon resonance experiments with two chondrogenic factors, TGFβ1 and BMP-2. To investigate whether these growth factors can specifically interact with GY785, quantitative measurements of their potential physical interactions were performed by surface plasmon resonance analysis. Growth factors were immobilized on the chip and increasing concentrations of GY785 polysaccharide were injected over the chip surface. The results, expressed in response units, were recorded for each analyte concentration and K_d was calculated. Our biacore data indicate that GY785 polysaccharide was able to strongly bind immobilized TGFβ1 and BMP-2. The binding affinity of GY785 for BMP-2 was about 10-fold higher than for TGFβ1, with respective K_d of 2.27×10^{-8} and 5.08×10^{-7} M. These results indicate that GY785 can specifically bind BMP-2 and TGFβ1.

3.2. Cytocompatibilty of Si-HPMC/GY785 hydrogels

To determine whether or not the addition of GY785 to Si-HPMC hydrogel was cytotoxic, the viability and proliferation of primary rabbit articular chondrocytes (RACs) cultured in contact with Si-HPMC/GY785 were examined through measurement of MTS activity and cell counting after 24, 48 and 72 h of culture. Si-HPMC hydrogel was used as a positive control. The results (Figure 1A) show that Si-HPMC and Si-HPMC/GY785 did not affect the MTS activity of RACs at 24 h. After 48 h of culture, the presence of Si-HPMC and Si-HPMC/GY785 slightly reduced the MTS activity of RACs by 20% and 30%, respectively. However, after 72 h there was no significant difference between the MTS activity of RACs cultured in the presence or in the absence of the two different hydrogels. On the contrary, actinomycin-D treatment reduced the MTS activity of RACs by nearly 70% after just 24 h of treatment.

Cell-counting experiments (Figure 1B) produced similar results. After 72 h, no significant difference in cell

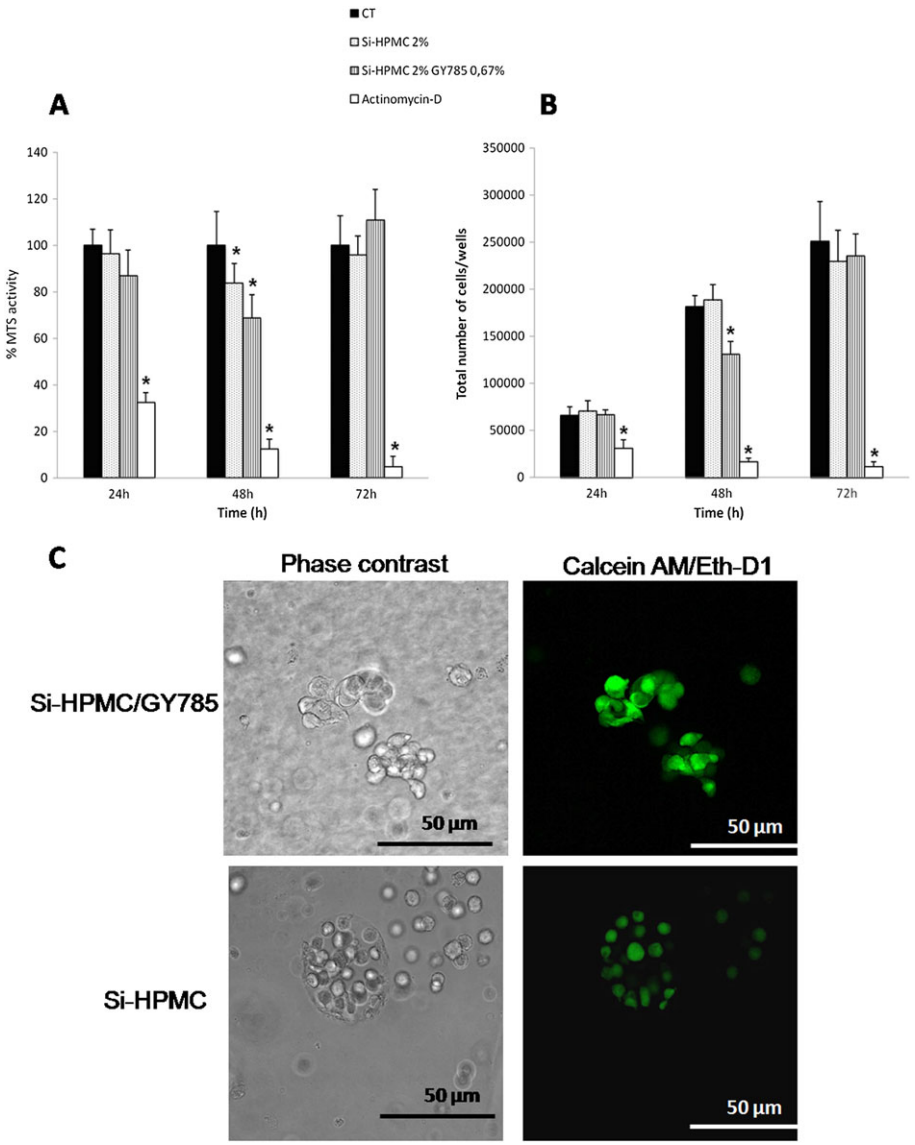

Figure 1. Cytocompatibility of Si-HPMC/GY785 with RACs in 2D and 3D culture. Rabbit articular chondrocytes (RACs) were cultured in 2D on culture plates over 72 h without hydrogel (positive control, CT) or with actinomycin-D (5 µg/ml; negative control) or with Si-HPMC (2/0) or Si-HPMC/GY785 (2/0.67). (A) Viability was assessed by MTS activity, as indicated in Materials and methods; the results are expressed as relative MTS activity compared with the respective controls; *$p < 0.001$ compared to control conditions. (B) Proliferation was assessed by scoring the cells after trypan blue staining; the results are expressed as the total number of cells/well; *$p < 0.001$ compared with the respective controls. (C) RACs were cultured in 3D for 3 weeks in Si-HPMC/GY785 (2/0.67) or Si-HPMC (2/0) prior to staining with calcein AM and EthD-1. Viability was visualized using confocal microscopy, as indicated in Materials and methods; scale bar = 50 µm. [Colour figure can be viewed at wileyonlinelibrary.com]

number was observed between cells cultured in contact with Si-HPMC/GY785 and cells cultured in control conditions or in contact with Si-HPMC alone.

Taken together, these results indicate that GY785 incorporated to Si-HPMC has neither stimulatory nor adverse effects on RACs viability and proliferation compared to Si-HPMC hydrogel.

To investigate whether the presence of GY785 within Si-HPMC influences its 3D cytocompatibility, the viability of RACs in 3D culture in an Si-HPMC/GY785 hydrogel was visualized using confocal microscopy after 3 weeks of culture (Figure 1C). Cells were stained with calcein AM and EthD-1, which respectively label living cells green and dead cells red. As shown in Figure 1C, RACs 3D-cultured in Si-HPMC/GY785 were green and organized in nodular structures, as observed with RACs cultured in Si-HPMC alone. The absence of red fluorescence indicates the absence of dead cells in the Si-HPMC/GY785. These results show that RACs retained their viability and organized into nodular structures when 3D-cultured in Si-HPMC/GY785 hydrogel, indicating that GY785 incorporation did not modify the 3D cytocompatibility of Si-HPMC.

3.3. Maintenance of chondrocyte phenotype in 3D cultures

To assess whether GY785 enrichment could act on the ability of Si-HPMC hydrogel to maintain a chondrocytic phenotype in 3D culture, RACs were cultured for 3 weeks,

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either in 2D without hydrogels or in 3D in Si-HPMC/GY785 hydrogel, or in Si-HPMC alone as a control. The expression of transcripts coding for type I collagen (dedifferentiation marker), type II collagen and aggrecan were evaluated using real-time PCR (Figure 2A). 2D-cultured RACs showed an expression of type II collagen and aggrecan 10- and 14.3-fold lower, respectively, than for primary RACs (P0). RACs cultured in 3D in Si-HPMC/GY785 expressed levels of type II collagen and aggrecan transcripts that were significantly higher, of approximately five- and 19-fold, respectively, when compared to 2D RACs and 2.7- and three-fold, respectively, as compared to Si-HPMC 3D-cultured RACs. Likewise, RACs cultured in 3D in Si-HPMC alone exhibited an expression of type II collagen and aggrecan two- and seven-fold higher than primary RACs. Whereas the expression of type I collagen in 2D RACs (Figure 2A) was more than three times higher than that of RACs (P0), 3D-cultured RACs in Si-HPMC/GY785 exhibited a barely detectable increase in type I collagen expression. RACs cultured in 3D in Si-HPMC did not show any increase in the expression of type I collagen as compared to primary RACs (P0).

These results show that GY785 enrichment of Si-HPMC hydrogel seems to increase its ability to maintain a high level of type II collagen and aggrecan mRNA synthesis. These results, taken as a whole, also

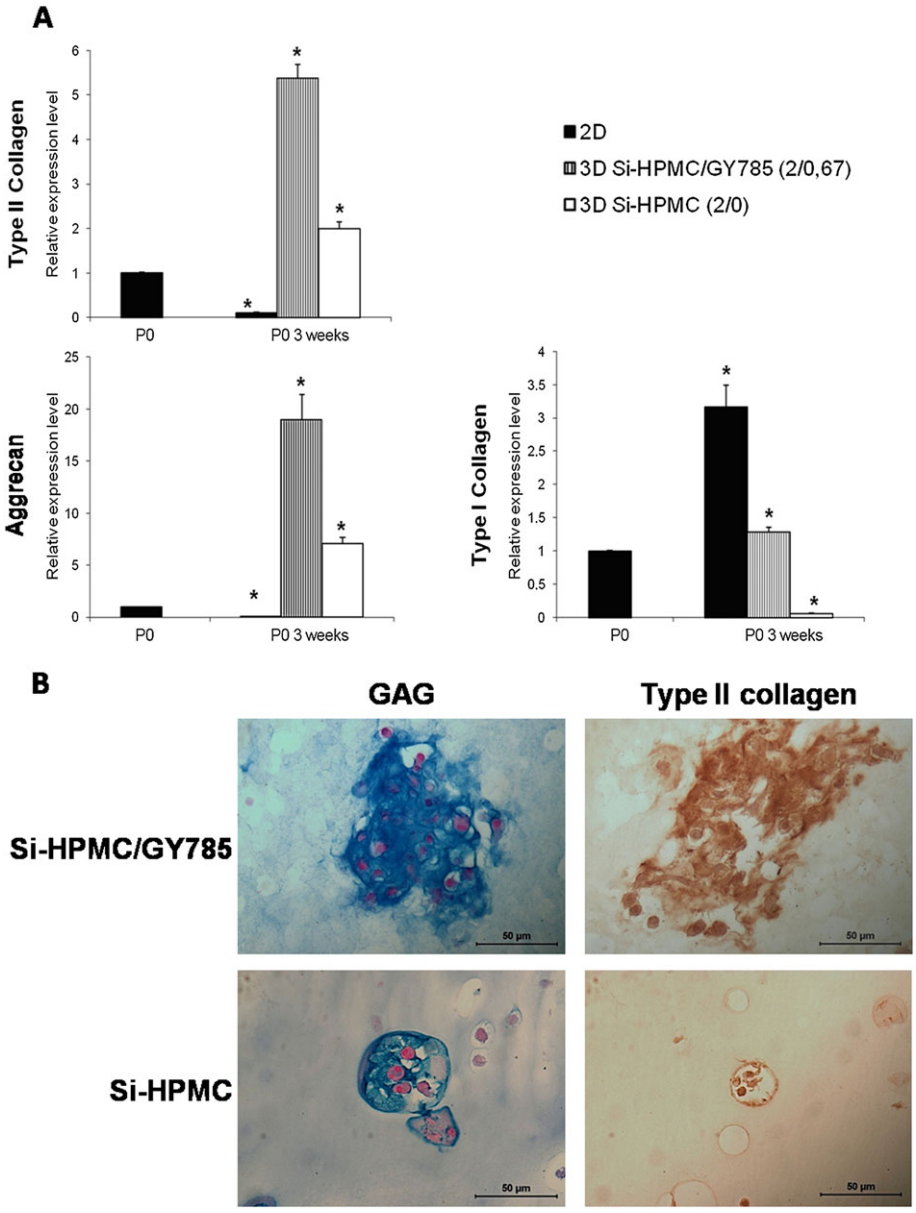

Figure 2. The effects of a 3D culture on the RACs phenotype. (A) Maintenance of chondrocyte phenotype in 3D culture. Total RNA was purified from freshly isolated RACs (P0), from RACs cultured in 2D and RACs cultured in 3D in Si-HPMC/GY785 or Si-HPMC for 3 weeks (P0 3 weeks). Expression of the chondrocytic markers (type II collagen and aggrecan), as well as the dedifferentiation marker (type I collagen), were investigated using real-time PCR, as indicated in Materials and methods; the results are expressed as relative expression levels compared to the control conditions (P0). (B) Cartilaginous matrix production by RACs in 3D culture. Rabbit articular chondrocytes were cultured for 3 weeks in 3D in Si-HPMC/GY785 (2/0.67) and Si-HPMC (2/0). Alcian blue staining for glycosaminoglycans (GAGs) and immunohistochemical type II collagen stainings were carried out, as detailed in Materials and methods; the samples were observed with a light microscope; scale bar = 50 µm. [Colour figure can be viewed at wileyonlinelibrary.com]

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demonstrate the capability of Si-HPMC/GY785 hydrogels to maintain a differentiated chondrocyte-like phenotype in RACs.

To further investigate the ability of Si-HPMC/GY785 to maintain a differentiated chondrocytic phenotype, the effect of 3D culture in Si-HPMC/GY785 on sulphated GAGs and immunostaining for type II collagen were performed on RACs cultured in 3D in Si-HPMC/GY785 or Si-HPMC for 3 weeks. Under these conditions (Figure 2B), and despite Alcian blue-positive staining of cell-free GY785 containing Si-HPMC (data not shown), both hydrogels exhibited positive Alcian blue staining and type II collagen immunostaining. These results show an accumulation of sulphated GAG and type II collagen in the ECM surrounding the RACs nodules formed in 3D culture in Si-HPMC/GY785 hydrogels, as observed with Si-HPMC alone. However, Si-HPMC/GY785 hydrogel allows the formation of the broadest Alcian blue and type II collagen-positive RACs nodules compared to Si-HPMC hydrogel.

Taken together, these results indicate that Si-HPMC/GY785 hydrogel supported the 3D culture of functionally competent RACs able to produce type II collagen and sulphated GAG.

3.4. Dedifferentiation of RACs in 2D cultures and redifferentiation in 3D cultures

It is well known that dedifferentiated articular chondrocytes are able to retrieve a chondrocytic phenotype when replaced in a suitable 3D environment (Chen *et al*., 2003; Miot *et al*., 2005; Vinatier *et al*., 2007). We therefore sought to evaluate whether this phenomenon also occurs with RACs initially cultured in 2D and thereafter placed in 3D in Si-HPMC/GY785 hydrogels. For this experiment, freshly isolated RACs were first dedifferentiated by culturing them into monolayer for four passages. Expressions of transcripts coding for type I and II collagen and aggrecan were evaluated using real-time PCR (Figure 3A). When the RACs were passaged, they showed an approximately four-fold decrease in type II collagen expression at passage 2 (P2) and were 16-fold decreased at P4 as compared to the primary RACs (P0). Likewise, aggrecan expression exhibited a three-fold and 4.5-fold decrease after passages 2 and 4, respectively (Figure 3A; P2, P4). In contrast, the expression of the type I collagen transcript increased dramatically about seven- and 10-fold after 2 and 4 passages, respectively, in monolayer culture (Figure 3A). These results show that the RACs underwent a dedifferentiation process as early as passage 2 (P2).

Therefore, to investigate whether Si-HPMC/GY785 allows the recovery of a chondrocytic phenotype, dedifferentiated RACs P2 were placed for 3 additional weeks in either 3D culture in Si-HPMC/GY785 or Si-HPMC or in 2D culture. Real-time PCR analysis revealed that, after 3 weeks, the RACs cultured in 3D in Si-HPMC/GY785 exhibited a 28-fold increase in type II collagen expression and a 75-fold increase in aggrecan expression, as compared to the RACs P2 (Figure 3B). On the contrary, the RACs cultured in 2D for the same additional duration failed to exhibit any significant increase in type II collagen and aggrecan transcripts. Interestingly, expression of the dedifferentiation marker type I collagen after 3 weeks in 3D culture in Si-HPMC/GY785 was 3.4-fold less than that measured in the RACs P2 (Figure 3B). 2D RACs exhibited a two-fold increase in type I collagen expression as compared to RACs P2. When compared to the control hydrogel (Si-HPMC), the RACs cultured in Si-HPMC/GY785 exhibited a two-fold increase in expression of the transcripts coding for type II collagen and aggrecan. These results suggest that the recovery of the chondrocytic phenotype is improved in Si-HPMC/GY785 hydrogel compared to Si-HPMC hydrogel alone.

3.5. *In vivo* tissue formation

To investigate whether the addition of GY785 within Si-HPMC hydrogel makes possible the formation of cartilaginous tissue *in vivo*, constructs associating freshly isolated RACs with Si-HPMC/GY785 or Si-HPMC hydrogels were implanted subcutaneously into nude mice. After 3 weeks, histological examination (Figure 4A) showed the formation of chondroid nodules with RACs associated with Si-HPMC/GY785 as well as with the control hydrogel (Si-HPMC). Nodules formed by RACs were positively stained with Alcian blue (Figure 4A), suggesting the production of an ECM containing GAGs. In addition, these nodules were positively stained for aggrecan and type II collagen (Figure 4A). In contrast, type I collagen immunostaining remained barely detectable in both conditions (Figure 4B, left and middle panels). As a control, rabbit growing bone exhibited an intense type I collagen immunostaining (Figure 4B, right panel)

As an additional control, Si-HPMC and Si-HPMC/GY785 alone showed neither the formation of nodular structures nor the presence of an ECM, despite a positive Alcian blue staining in cell-free Si-HPMC/GY785 hydrogel (data not shown).

To confirm the absence of type I collagen immunostaining, *in situ* hybridization for type I collagen transcripts were then performed. The mouse *Col1a1* riboprobe was used and was demonstrated to hybridize specifically with the rabbit *Col1a1* transcripts (Figure 4C). *In situ* hybridization indicated that mRNA coding for type I collagen remains at a barely detectable level in GY785-reinforced Si-HPMC and Si-HPMC hydrogels (Figure 4C, left and middle panels). As a control, *in situ* hybridization for type I collagen in rabbit growing bone was found to be intensely positive (Figure 4C, right panel).

These results indicate that Si-HPMC enriched with GY785 makes possible the formation of cartilaginous tissue containing type II collagen and aggrecan *in vivo* with freshly isolated RACs.

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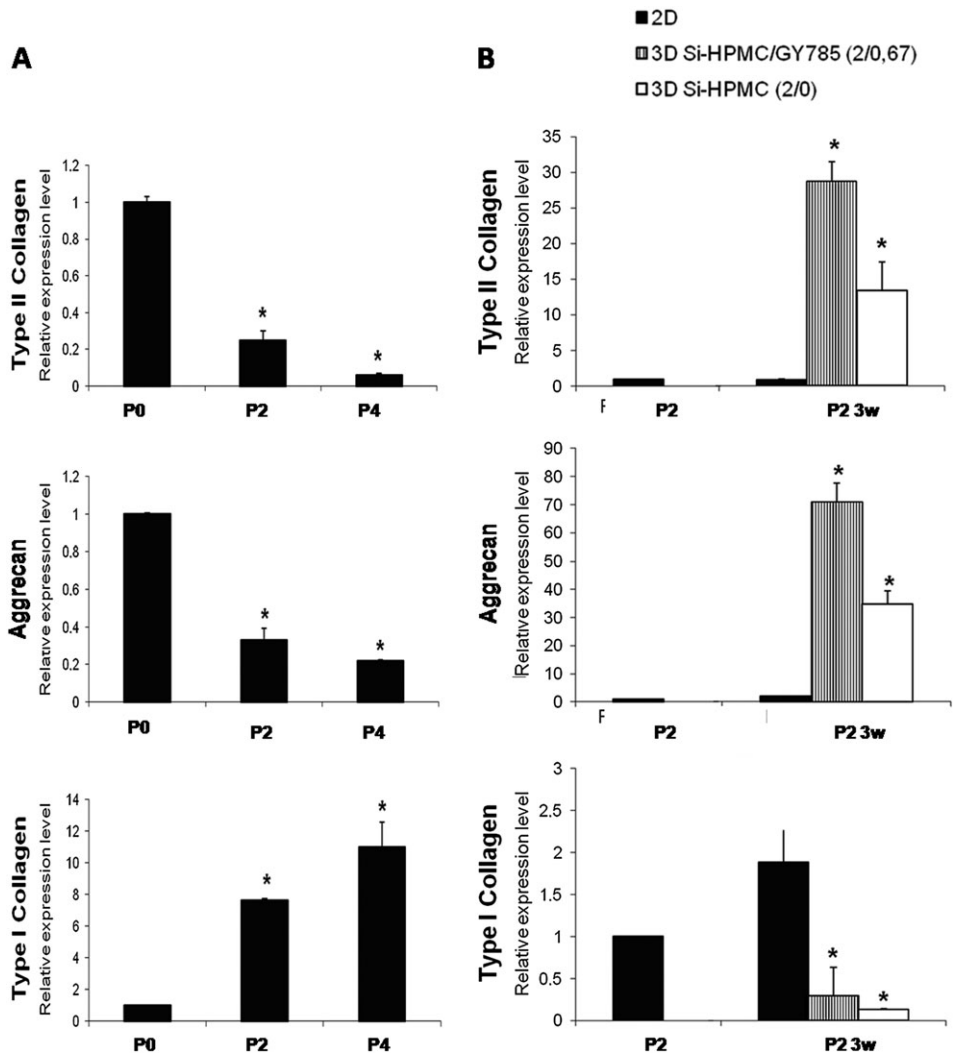

Figure 3. Recovery of a chondrocytic phenotype in dedifferentiated RACs by 3D culture. (A) Dedifferentiation of RACs in monolayer culture. Total RNA was purified from freshly isolated RACs (P0), from RACs cultured in 2D for 2 (P2) and 4 (P4) passages. Real-time PCR analysis for type II collagen and aggrecan, as well as type I collagen transcripts, were performed using the primers and conditions detailed in Materials and methods; the results are expressed as relative expression levels compared to the control conditions P0; *$p < 0.001$ compared to RACs (P0). (B) Redifferentiation of RACs in 3D culture. Total RNA was purified from dedifferentiated RACs at passage 2 (P2) and from dedifferentiated RACs cultured for an additional 3 weeks (P2 3w) in 3D in Si-HPMC/GY785 (2/0.67) and Si-HPMC (2/0). Real-time PCR analysis for type II and I collagen transcripts, as well as aggrecan, were carried out using the primers and conditions detailed in Materials and methods; the results are expressed as relative expression levels compared to the control conditions (P2); *$p < 0.001$ compared to P2

3.6. Rheological characteristics

To evaluate whether GY785 incorporation modified the viscoelastic properties of Si-HPMC, an oscillatory measurement was performed to evaluate the elastic modulus (G') and viscous modulus (G'') (Figure 5). Si-HPMC/GY785 showed a G' of ca. 723 ± 171 Pa and a G'' of ca. 96 ± 37 Pa. Si-HPMC and Si-HPMC/HPMC exhibited the same G', with values of ca. 398 ± 65 and 324 ± 52 Pa, and a G'' of ca. 28 ± 6 and 22 ± 5 Pa, respectively.

These results indicate that Si-HPMC/GY785 hydrogel has higher stiffness than Si-HPMC and Si-HPMC/HPMC, whereas Si-HPMC and Si-HPMC/HPMC have similar stiffness.

4. Discussion

Today, regenerative medicine is moving towards the development of less and less invasive surgical techniques, with the aim of reducing morbidity and hospitalization time. From this point of view, injectable hydrogels appear to be promising (Cushing and Anseth 2007). The main challenge in developing new hydrogels for cartilage tissue engineering is to allow the viability and differentiation of cells within the hydrogel.

Marine-based polysaccharides (Colliec-Jouault *et al.*, 2004) that exhibit structural analogies with glycosaminoglycans (GAGs) are well acknowledged as molecules influencing cell proliferation, differentiation and adhesion (Chiu *et al.*, 2011). GAGs have long been known to be able

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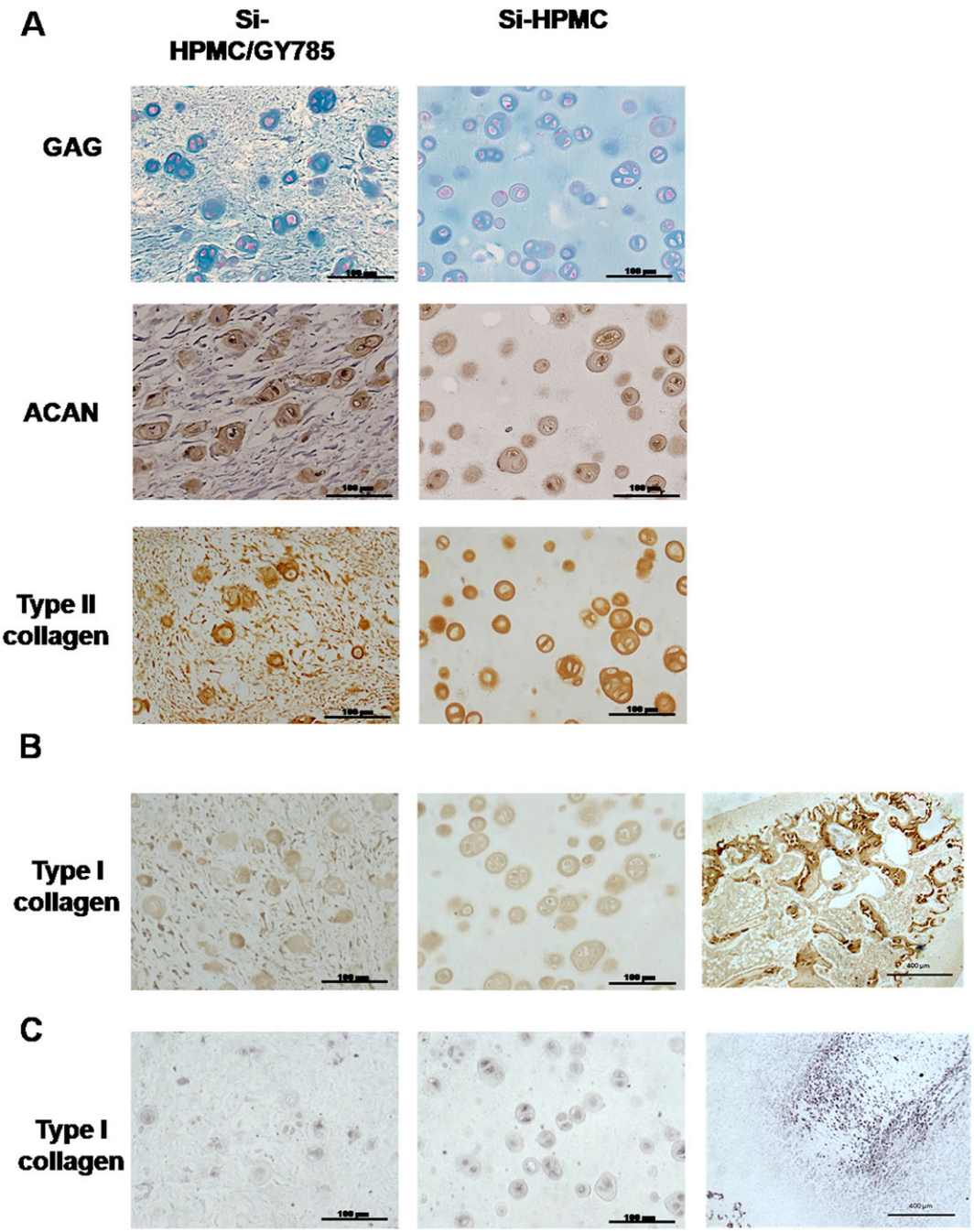

Figure 4. Histochemical analysis of tissue-engineered cartilage; RACs associated with Si-HPMC/GY785 (2/0.67) or Si-HPMC (2/0) were implanted subcutaneously into nude mice and analysed 3 weeks later. (A) Histological sections were stained, as described in Materials and methods, for the presence of GAGs (Alcian blue), aggrecan (ACAN) and type II collagen (immunostaining); samples were observed with a light microscope; scale bar = 100 μm. (B) Histological sections were stained, as described in Materials and methods, for the presence of type I collagen (immunostaining). The samples were observed with a light microscope; scale bar = 100 μm. A positive control (right panel) was performed with rabbit growing bone; scale bar = 400 μm. (C) Histological sections were processed for *in situ* hybridization and hybridized with *col1A1* riboprobe, as described in Materials and methods; scale bar = 100 μm. A positive control (right panel) was performed with rabbit growing bone; scale bar = 400 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

to bind and regulate the biological activity of a large number of growth factors through the modulation of their storage, stabilization and degradation in the matrix (Jackson *et al*., 1991; Vlodavsky *et al*., 2006). To determine whether TGFβ1 or BMP-2, two major chondrogenic growth factors, can physically interact with the marine polysaccharide GY785, surface plasmon resonance experiments were performed. Interestingly, our Biacore data demonstrate the existence of a high affinity between GY785 and the chondrogenic TGFβ1 and BMP-2. These data therefore establish a strong rationale for testing our hypothesis that GY785 could be used to enrich scaffolding biomaterials, and improves their potential for engineering cartilage. In this attempt, and to propose a 3D scaffolding hydrogel capable of supporting regeneration of functional cartilage, a self-setting cellulosic hydrogel (Si-HPMC),

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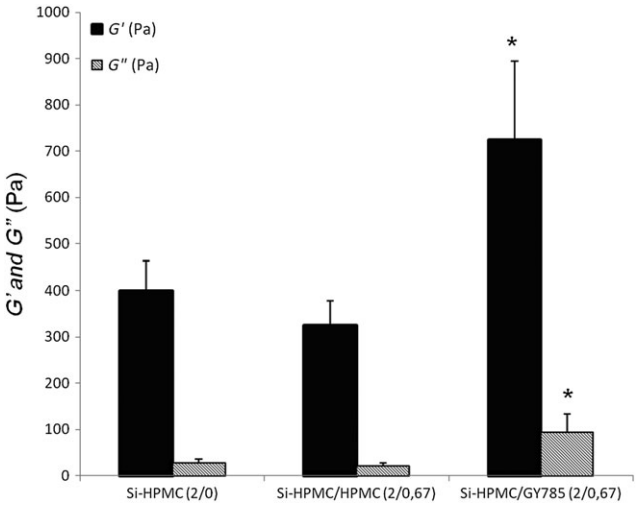

Figure 5. Oscillatory measurements. Oscillatory measurements of the different hydrogels [Si-HPMC, Si-HPMC/HPMC (2/0.67) and Si-HPMC/GY785 (2/0.67)] after 3 weeks of storage at 37°C in a humid environment. Measurements (n = 3) were made directly in 12 multi-well culture plates, using a ThermoHaake® (Germany, Mars™, Titanium plate PP20Ti) instrument with the following parameters: gap ≈ 3 mm; normal force 0.2 N; 1 Hz; 0.1–5 Pa; at 25°C. The conservative modulus G' and loss modulus G'' are shown in Pa; *$p < 0.001$ compared with the respective controls, Si-HPMC and Si-HPMC/HPMC

widely used for cartilage tissue engineering, was chosen (Portron *et al*., 2013; Vinatier et al., 2009b). A prerequisite to the use of hydrogels for *in vivo* experiments is to evaluate their non-cytotoxicity. We were therefore first interested in examining the *in vitro* cytotoxicity of Si-HPMC/GY785 with rabbit primary articular chondrocytes (RACs). We thus focused on a cytotoxicity test with direct contact with RACs (ISO 10993-5: Biological evaluation of medical devices, Part 5: test for *in vitro* cytotoxicity). Our data indicate that the Si-HPMC/GY785 hydrogel is cytocompatible with respect to RACs, as evidenced by estimation of mitochondrial dehydrogenase activity (MTS assay) and cell counting (Figure 1). In addition, dual-fluorescence staining, using calcein AM and EthD-1 reagents, revealed that RACs 3D-cultured in Si-HPMC/GY785 hydrogel retained their viability and formed nodules (Figure 1). These results indicated that the Si-HPMC/GY785 hydrogel makes possible the 3D culture of RACs without altering their viability and proliferation.

Another characteristic of Si-HPMC hydrogel, shared with many other biomaterials used for cartilage tissue engineering, is its ability to maintain the chondrocytic phenotype in 3D culture (Vinatier *et al*., 2005). Indeed, it is well known that maintaining or recovering a chondrocytic phenotype in a scaffolding hydrogel is a key point for cartilage tissue-engineering strategies. Dedifferentiation of chondrocytes, evidenced by a concomitant decrease in type II collagen and aggrecan expression and an increase in type I collagen expression, effectively occurs during *in vitro* 2D monolayer expansion (Brodkin *et al*., 2004; Schnabel *et al*., 2002). In our hands, and according to this dedifferentiation process, type II collagen and aggrecan expressions were downregulated, along with passages in RACs, whereas that of type I collagen expression increased. However, culturing chondrocytes in 3D scaffolds has been shown to not only prevent, but even to reverse, this phenomenon, allowing dedifferentiated chondrocytes to recover their phenotype (Vinatier *et al*., 2005). Accordingly, the expressions of type II collagen and aggrecan are maintained in 3D cultures in Si-HPMC, with and without GY785, highlighting their ability to maintain a chondrocyte-like phenotype *in vitro*. Moreover, the GY785 enrichment of Si-HPMC increases ca. two- to three-fold the expression level of type II collagen and aggrecan when compared to Si-HPMC alone. These results were further confirmed by the production of GAG and collagen type II by 3D-cultured RACs in the Si-HPMC/GY785 hydrogel, which appears higher than in Si-HPMC.

In addition, and of particular interest for cell-based cartilage tissue engineering, Si-HPMC hydrogel also induces the recovery of a chondrocytic phenotype of previously dedifferentiated RACs (P2), as evidenced by the increased expression of type II collagen and aggrecan, while expression of type I collagen is decreased. Further, GY785 enrichment of Si-HPMC hydrogel improves this recovery of chondrocytic phenotype, as evidenced by the two-fold increase in type II collagen and aggrecan expression as compared to Si-HPMC alone. It therefore seems reasonable to speculate that Si-HPMC/GY785 not only makes possible the 3D culture of phenotypically stable chondrocytes but also allows the recovery of such a chondrocytic phenotype in a more efficient manner than Si-HPMC alone. This result strongly suggests that GY785 positively alters the biological activity of Si-HPMC hydrogel.

To address the *in vivo* potential of Si-HPCM/GY785 as a new hydrogel for cartilage engineering, we secondly embarked on a preliminary animal experiment in a well-documented model of tissue engineering, the subcutis of nude mice. Hybrid systems associating chondrocytes (RACs) with Si-HPMC/GY785 were implanted into subcutaneous sites in the back of nude mice. Si-HPMC associated with RACs was used as a control. Three weeks after implantation, constructs associating RACs with Si-HPMC/GY785 showed the formation of nodules with an ECM containing sulphated GAGs, aggrecan and type II collagen. Interestingly, type I collagen expression at both the mRNA and protein levels remained barely detectable, suggesting that the cells probably do not undergo any dedifferentiation process.

Given that GY785 polysaccharide is a high-molecular weight macromolecule, incorporating GY785 into Si-HPMC hydrogel certainly modified its mechanical properties. To address this issue, the rheological properties of Si-HPMC/GY785, Si-HPMC and Si-HPMC/HPMC hydrogels were determined, and indicated that G' was higher than G'' (Figure 5) for the three hydrogels, thereby confirming that these hydrogels are solid after 3 weeks. The G' of Si-HPMC/GY785 is approximately two-fold higher than those of Si-HPMC or Si-HPMC/HPMC, showing that enrichment of Si-HPMC with GY785 increased its stiffness (Rederstorff *et al*., 2011). The storage modulus

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G' was also linked to the network density in polysaccharide hydrogels (Moe *et al*., 1992). In our study, the G' value revealed a higher network density with the adjunction of GY785 polysaccharides (Figure 5). The increased stiffness and network density in GY785-enriched Si-HPMC may explain the enhancement of the chondrocyte phenotype in this hydrogel compared to Si-HPMC alone. Indeed, these mechanical parameters have previously been shown to influence chondrocyte phenotype (Brodkin *et al*., 2004; Sanz-Ramos *et al*., 2013) as well as direct mesenchymal stem cell differentiation (Discher *et al*., 2005; Engler *et al*., 2006).

5. Conclusions

In conclusion, our results show that GY785 physically interacts with some chondrogenic factors. Si-HPMC doped with GY785 is cytocompatible and competent for the 3D culture of chondrocytes, capable of producing cartilage ECM proteins *in vitro*. GY785-enriched Si-HPMC interestingly supports the recovery of a chondrocytic phenotype in dedifferentiated articular chondrocytes. *In vivo*, this doped hydrogel allows the synthesis of a cartilage-like ECM by chondrocytes in the subcutis of nude mice. This site is, however, rather far from an articular cartilage situation, due to the lack of resident endogenous growth factors. Regarding the growth factors-interacting properties of GY785, the preclinical relevance of GY785-doped hydrogel will be given further attention in adapted animal models of articular cartilage defects, where growth factors, notably TGFβ, are found in large amount (Goldring *et al*., 2006).

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Conflict of Interest

The authors have declared that there is no conflict of interest.

References

Bonzani IC, George JH, Stevens MM. 2006; Novel materials for bone and cartilage regeneration. *Curr Opin Chem Biol* **10**(6): 568–575.

Bourges X, Weiss P, Coudreuse A *et al*. 2002a; General properties of silated hydroxyethylcellulose for potential biomedical applications. *Biopolymers* **63**(4): 232–238.

Bourges X, Weiss P, Daculsi G *et al*. 2002b; Synthesis and general properties of silated-hydroxypropyl methylcellulose in prospect of biomedical use. *Adv Colloid Interface Sci* **99**(3): 215–228.

Brodkin KR, Garcia AJ, Levenston ME. 2004; Chondrocyte phenotypes on different extracellular matrix monolayers. *Biomaterials* **25**(28): 5929–5938.

Casu B, Lindahl U. 2001; Structure and biological interactions of heparin and heparan sulfate. *Adv Carbohydr Chem Biochem* **57**: 159–206.

Chen G, Sato T, Ushida T *et al*. 2003; Redifferentiation of dedifferentiated bovine chondrocytes when cultured *in vitro* in a PLGA–collagen hybrid mesh. *FEBS Lett* **542**(1–3): 95–99.

Chiu LH, Chen SC, Wu KC *et al*. 2011; Differential effect of ECM molecules on re-expression of cartilaginous markers in near-quiescent human chondrocytes. *J Cell Physiol* **226**(8): 1981–1988.

Colliec-Jouault S, Zanchetta P, Helley D *et al*. 2004; Les polysaccharides microbiens d'origine marine et leur potentiel en thérapeutique humaine. *Pathol Biol* **52**(3): 127–130.

Cushing MC, Anseth KS. 2007; Materials science. Hydrogel cell cultures. *Science* **316**(5828): 1133–1134.

Dehne T, Schenk R, Perka C *et al*. 2010; Gene expression profiling of primary human articular chondrocytes in high-density micromasses reveals patterns of recovery, maintenance, re- and dedifferentiation. *Gene* **462**(1–2): 8–17.

Demoor M, Ollitrault D, Gomez-Leduc T *et al*. 2014; Cartilage tissue engineering: molecular control of chondrocyte differentiation for proper cartilage matrix reconstruction. *Biochim Biophys Acta* **1840**(8): 2414–2440.

Discher DE, Janmey P, Wang YL. 2005; Tissue cells feel and respond to the stiffness of their substrate. *Science* **310**(5751): 1139–1143.

Discher DE, Mooney DJ, Zandstra PW. 2009; Growth factors, matrices, and forces combine and control stem cells. *Science* **324**(5935): 1673–1677.

Engler AJ, Sen S, Sweeney HL *et al*. 2006; Matrix elasticity directs stem cell lineage specification. *Cell* **126**(4): 677–689.

Fatimi A, Tassin JF, Quillard S *et al*. 2008; The rheological properties of silated hydroxypropylmethylcellulose tissue engineering matrices. *Biomaterials* **29**(5): 533–543.

Ghayor C, Herrouin JF, Chadjichristos C *et al*. 2000; Regulation of human *COL2A1* gene expression in chondrocytes. Identification of C-Krox-responsive elements and modulation by phenotype alteration. *J Biol Chem* **275**(35): 27421–27438.

Goldring MB, Tsuchimochi K, Ijiri K. 2006; The control of chondrogenesis. *J Cell Biochem* **97**(1): 33–44.

Gomoll AH, Minas T. 2014; The quality of healing: articular cartilage. *Wound Repair Regen* **22**(suppl 1): 30–38.

Griffith LG, Naughton G. 2002; Tissue engineering – current challenges and expanding opportunities. *Science* **295**(5557): 1009–1014.

Guezennec J. 2002; Deep-sea hydrothermal vents: a new source of innovative bacterial exopolysaccharides of biotechnological interest? *J Ind Microbiol Biotechnol* **29**(4): 204–208.

Jackson RL, Busch SJ, Cardin AD. 1991; Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. *Physiol Rev* **71**(2): 481–539.

Laib S, Fellah BH, Fatimi A *et al*. 2009; The *in vivo* degradation of a ruthenium-labelled polysaccharide-based hydrogel for bone tissue engineering. *Biomaterials* **30**(8): 1568–1577.

Matou S, Colliec-Jouault S, Galy-Fauroux I *et al*. 2005; Effect of an oversulfated exopolysaccharide on angiogenesis induced by fibroblast growth factor-2 or vascular endothelial growth factor *in vitro*. *Biochem Pharmacol* **69**(5): 751–759.

Merceron C, Portron S, Masson M *et al*. 2011; The effect of two- and three-dimensional cell culture on the chondrogenic potential of human adipose-derived mesenchymal stem cells after subcutaneous transplantation with an injectable hydrogel. *Cell Transplant* **20**(10): 1575–1588.

Merceron C, Portron S, Vignes-Colombeix C *et al*. 2012; Pharmacological modulation of human mesenchymal stem cell chondrogenesis by a chemically oversulfated polysaccharide of marine origin: potential application to cartilage regenerative medicine. *Stem Cells* **30**(3): 471–480.

Merceron C, Vinatier C, Portron S *et al*. 2010; Differential effects of hypoxia on osteochondrogenic potential of human adipose-derived stem cells. *Am J Physiol Cell Physiol* **298**(2): C355–364.

Miot S, Woodfield T, Daniels AU *et al*. 2005, Effects of scaffold composition and architecture on human nasal chondrocyte redifferentiation and cartilaginous matrix deposition. *Biomaterials* **26**(15): 2479–2489.

Moe ST, Draget KI, Skjåk-Bræk G *et al*. 1992; Temperature dependence of the elastic modulus of alginate gels. *Carbohydr Polym* **19**(4): 279–284.

Moorman AF, Houweling AC, de Boer PA *et al*. 2001; Sensitive nonradioactive detection of mRNA in tissue sections: novel application of the whole-mount *in situ* hybridization protocol. *J Histochem Cytochem* **49**(1): 1–8.

Mukaida T, Urabe K, Naruse K *et al*. 2005; Influence of three-dimensional culture in a type II collagen sponge on primary cultured and dedifferentiated chondrocytes. *J Orthop Sci* **10**(5): 521–528.

Portron S, Merceron C, Gauthier O *et al*. 2013; Effects of *in vitro* low oxygen tension preconditioning of adipose stromal cells on their *in vivo* chondrogenic potential: application in cartilage tissue repair. *PLoS One* **8**(4): e62368.

Rederstorff E, Weiss P, Sourice S *et al*. 2011; An *in vitro* study of two GAG-like marine polysaccharides incorporated into injectable hydrogels for bone and cartilage tissue engineering. *Acta Biomater* **7**(5): 2119–2130.

Sanz-Ramos P, Mora G, Vicente-Pascual M *et al*. 2013; Response of sheep chondrocytes to changes in substrate stiffness from 2 to 20 Pa: effect of cell passaging. *Connect Tissue Res* **54**(3): 159–166.

Schnabel M, Marlovits S, Eckhoff G *et al*. 2002; Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. *Osteoarthr Cartilage* **10**(1): 62–70.

Steinert AF, Ghivizzani SC, Rethwilm A *et al*. 2007; Major biological obstacles for persistent cell-based regeneration of articular cartilage. *Arthritis Res Ther* **9**(3): 213.

van Osch GJ, Brittberg M, Dennis JE *et al*. 2009; Cartilage repair: past and future – lessons for regenerative medicine. *J Cell Mol Med* **13**(5): 792–810.

Vinatier C, Bouffi C, Merceron C *et al*. 2009a; Cartilage tissue engineering: towards a biomaterial-assisted mesenchymal stem cell therapy. *Curr Stem Cell Res Ther* **4**(4): 318–329.

Vinatier C, Gauthier O, Fatimi A *et al*. 2009b; An injectable cellulose-based hydrogel for the transfer of autologous nasal chondrocytes in articular cartilage defects. *Biotechnol Bioeng* **102**(4): 1259–1267.

Vinatier C, Magne D, Moreau A *et al*. 2007; Engineering cartilage with human nasal chondrocytes and a silanized hydroxypropyl methylcellulose hydrogel. *J Biomed Mater Res A* **80**(1): 66–74.

Vinatier C, Magne D, Weiss P *et al*. 2005; A silanized hydroxypropyl methylcellulose hydrogel for the three-dimensional culture of chondrocytes. *Biomaterials* **26**(33): 6643–6651.

Vlodavsky I, Abboud-Jarrous G, Elkin M *et al*. 2006; The impact of heparanese and heparin on cancer metastasis and angiogenesis. *Pathophysiol Haemost Thromb* **35**(1–2): 116–127.

Yamada S, Sugahara K. 2008; Potential therapeutic application of chondroitin sulfate/dermatan sulfate. *Curr Drug Discov Technol* **5**(4): 289–301.

Zcharia E, Zilka R, Yaar A *et al*. 2005; Heparanase accelerates wound angiogenesis and wound healing in mouse and rat models. *FASEB J* **19**(2): 211–221.