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Gelatin microparticles aggregates as three-dimensional scaffolding system in cartilage engineering

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Abstract A three-dimensional (3D) scaffolding system for chondrocytes culture has been produced by agglomeration of cells and gelatin microparticles with a mild centrifuging process. The diameter of the microparticles, around 10 μ, was selected to be in the order of magnitude of the chondrocytes. No gel was used to stabilize the construct that maintained consistency just because of cell and extracellular matrix (ECM) adhesion to the substrate. In one series of samples the microparticles were charged with transforming growth factor, TGF-β1. The kinetics of growth factor delivery was assessed. The initial delivery was approximately 48 % of the total amount delivered up to day 14. Chondrocytes that had been previously expanded in monolayer culture, and thus dedifferentiated, adopted in this 3D environment a round morphology, both with presence or absence of growth factor delivery, with production of ECM that intermingles with gelatin particles. The pellet was stable from the first day of culture. Cell viability was assessed by MTS assay, showing higher absorption values in the cell/unloaded gelatin microparticle pellets than in cell pellets up to day 7. Nevertheless the absorption drops in the following culture times. On the contrary the cell viability of cell/TGF-β1 loaded gelatin microparticle pellets was constant during the 21 days of culture. The formation of actin stress fibres in the cytoskeleton and type I collagen expression was significantly reduced in both cell/gelatin microparticle pellets (with and without TGF-β1) with respect to cell pellet controls. Total type II collagen and sulphated glycosaminoglycans quantification show an enhancement of the production of ECM when TGF-β1 is delivered, as expected because this growth factor stimulate the chondrocyte proliferation and improve the functionality of the tissue.

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1 Introduction

A three-dimensional (3D) arrangement of cells in an adequate substrate seems to be a requirement for the development and maintenance of the phenotype of hyaline cartilage chondrocytes [1]. Mature chondrocytes can be isolated from the cartilage tissue obtained in a biopsy of articular cartilage. When they are plated in monolayer culture on plastic substrates, they can grow to the cell numbers required for tissue regeneration but they loose their native phenotype showing clear changes in cell morphology and expression of type I collagen which is nearly absent in articular cartilage tissue [2]. The re-differentiation of these cells in 3D cultures is thus a subject of great interest for cartilage engineering.

A huge number of different 3D scaffolding systems have been proposed for cartilage engineering including sponges, fibber meshes, gels and microparticles [3] with varying materials of synthetic or natural origin and a variety of pore architecture. Among them, 3D arrangements obtained by fusion of microparticles as scaffolding blocks have been investigated [3]. The drawback of these systems is that global porosity is not high enough for cell survival, and the strong linkage between particles allows few spaces for production and accumulation of ECM components. On the other hand, the interest in developing injectable scaffolding systems yielded to explore a number of combinations of synthetic microparticles and "in situ" crosslinkable hydrogels [4–8].

Gelatin microparticles (Gel-MCP) have been explored extensively as drug or growth factor delivery systems. In particular they have been used for delivery of TGF-β1 or TGF-β3 growth factors in "in vitro" chondrogenic differentiation of bone marrow mesenchymal stem cells. TGF-β3 loaded Gel-MCP were mixed with cells to form a pellet by centrifugation, labelling as a modified pellet by Fan et al. [9]. A small amount of microparticles was used in that work in order to preserve the large cell to cell contact that favours chondrogenic differentiation of mesenchymal stem cells. Han et al. [10] used a mixture of gelatin and chitosan microparticles with the same purpose, while Park et al. [11] used a hydrogel to entrap both cells and Gel-MCP. A similar construct was proposed by Hu et al. [12] who introduced microparticles and chondrocytes in a chitosan hydrogel. Gelatin has been also used to functionalize microparticles made of synthetic biodegradable polyester such as polylactide/polyglycolide copolymers, PLGA, [13].

On the other hand Gel-MCP have been used as microcarriers for chondrocyte expansion in stirring bioreactors. It was shown that human nasal chondrocytes expanded on the surface and in the micropores of the microcarriers maintain the ability to differentiate to hyaline cartilage chondrocytes in pellet culture [14]. These microcarriers have been used in spinner flasks as well [15, 16]. Gelatin has been also used to fabricate macroporous scaffolds for cartilage engineering profiting the capacity of delivering growth factors from the scaffold itself [17]. Although the mentioned works show that gelatin is a suitable substrate for chondrocyte 3D culture none has proved to be the ultimate solution. Results obtained from tissue engineering procedures suggest that the proposed gelatin scaffolding systems may lead to a mixture of hyaline and fibrocartilage tissue.

In our work we explore the use of Gel-MCP not only as a growth factor delivery vehicle but also as a 3D scaffolding system. Dedifferentiated chondrocytes are mixed with microparticles at a Gel-MCP/cells ratio high enough to form a construct in which cells are surrounded by the gelatin as the scaffolding material. In addition in one of the experimental series Gel-MCP will be loaded with TGF-β1 which is delivered continuously during "in vitro" culture. It will be shown that Gel-MCP can agglomerate in situ by the action of the cells; the particles thus provide a 3D structural support for cells which is able to release relevant bioactive molecules at the same time.

2 Materials and methods

2.1 Preparation of unloaded and TGF-β1 loaded Gel-MCP

Crosslinked Gel-MCP encapsulating TGF-β1 growth factor were prepared by emulsification solvent extraction and crosslinking method [18]. Briefly, 1 g of gelatin (Scharlab, Spain) was dissolved in 10 mL of Dulbecco's phosphate buffer solution (DPBS) at 50 °C to a final concentration of 10wt. %. Subsequently, gelatin solution was cooled to 37 °C, mixed with the TGF-β1 (Sigma, Spain) solution (20 μg/mL) and homogenized. Then, the mixture was added into olive oil at a feeding rate of 1 mL/min under stirring at 1,500 rpm for 30 min. After this time, 10 mL of 20 mM genipin (Wako Chemical, Japan) solution was added into the biphasic system at a rate of 1 mL/min and maintained under stirring for 17 h. Finally, loaded crosslinked Gel-MCP were collected by centrifugation, washed several times in acetone to remove residual olive oil and lyophilised. The loaded microparticles were stored at −20 °C until further use. Unloaded crosslinked Gel-MCP were also prepared and used as reference. The percentage yield of the lyophilized TGF-β1 loaded Gel-MCP crosslinked with genipin was calculated dividing the weight of microparticles obtained by initial weight of gelatin used. The unloaded Gel-MCP were obtained using the same procedure, without add TGF-β1 in the gelatin solution.

2.2 Determination of crosslinking degree

Ninhydrin (NHN) assay [19] was used to determine the percentage of free amino groups remaining in the Gel-MCP after crosslinking reaction. Ninhydrin (Sigma-Aldrich, Spain) solution was freshly prepared on the day of the assay dissolving 0.8 g of NHN and 0.12 g of hydrindantin (Sigma-Aldrich, Spain) in 40 mL of ethylene glycol (Sigma-Aldrich, Spain). This solution was then mixed with lithium acetate buffer (4 M, pH 5.2) to prepare the working reagent. For the assay, 0.5 mg of lyophilized Gel-MCP (uncrosslinked and crosslinked with genipin) was mixed with 0.5 mL of working reagent in eppendorf tubes. Then, the tubes were immediately capped, shaken and heated to

100 °C in a water bath for 30 min to allow the reaction to proceed. The solution was then cooled down to room temperature, diluted with 5 mL of 50 % isopropanol and vortexed for 15 s in order to oxidise the excess of hydrindantin. The absorbance of each solution was measured at 570 nm using a UV spectrophotometer (Cecil CE9200, UK). The amount of free amino groups in the assayed microparticles was determined by a calibration curve of glycine ($n = 5$). The crosslinking degree was calculated according to the Eq. 1.

$$Crosslinking\,degree = \frac{\left[(NHN\ reactive\ amine)_{fresh} - (NHN\ reactive\ amine)_{fixed}\right]}{(NHN\ reactive\ aMine)_{fresh}} * 100 \quad (1)$$

where, "*fresh*" is the mole fraction of free NH_2 groups in the uncrosslinked and unloaded Gel-MCP and "*fixed*" is the mole fraction of free NH_2 groups remaining in the unloaded Gel-MCP after the crosslinked reaction with genipin.

2.3 Determination of encapsulation efficiency (EE)

The EE of the TGF-β1 loaded Gel-MCP crosslinked with genipin was expressed as the percentage of the TGF-β1 amount in the microparticles with respect to the initial TGF-β1 used. For that, 2 mg of TGF-β1 loaded Gel-MCP were swelled in DPBS and centrifuged at 15,000 rpm for 30 min, three times, in order to extract the TGF-β1 from the Gel-MCP ($n = 5$). The supernatant was extracted and the TGF-β1 was quantified by an enzyme-linked immunosorbent assay kit (R&D systems, USA).

2.4 Morphological analysis

The morphology of unloaded gelatin and TGF-β1 loaded Gel-MCP were examined by scanning electron microscopy (SEM) (Jeol JSM-5410, Japan). All samples were coated with a conductive layer of sputtered gold. The micrographs were taken at an accelerating voltage of 15 kV in order to ensure a suitable image resolution. Microparticle size distribution was determined analysing 10 different images, with around 30 microparticles in each one.

2.5 Water uptake studies

The water uptake of Gel-MCP was determined by immersion in distilled water at 37 °C for 24 h. The weight of the dry and swollen microparticles was measured and the water uptake (W) was calculated using the following equation:

$$W = \frac{(weight\ of\ swollen\ Gel - MCP - weight\ of\ dry\ Gel - MCP)}{weight\ of\ dry\ Gel - MCP} * 100 \quad (2)$$

Uncrosslinked microparticles were also prepared and were used as control in the water uptake studies ($n = 5$). The appearance of swollen crosslinked and uncrosslinked Gel-MCP were observed using an optical microscope (ECLIPSE-E6000, Nikon, Japan).

2.6 In vitro release studies

The in vitro release profile of TGF-β1 from Gel-MCP was examined over 14 days. The microparticles were dispersed into 1 mL of DPBS (pH 7.4). These dispersions were immediately placed in an orbital shaker water bath at 37 °C and were shaken at a frequency of 60 rpm. After 0.04, 0.08, 0.125, 0.16, 0.20, 1, 2, 4, 7, 10 and 14 days, the microparticle suspension was centrifuged at 5,000 rpm to collect the supernatant for analysis ($n = 8$). The amount of TGF-β1 in the supernatant was measured using an enzyme-linked immunosorbent assay kit (eBioscience, USA).

2.7 Cell isolating and seeding

Chondrocytes were isolated from human articular cartilage from the knee of a patient undergoing total knee arthroplasty by standard protocols [20] and in accordance with the Declaration of Helsinki of 1975 as revised in 1983, and approved by our local Ethical Committee. All subjects provided a written informed consent before their inclusion in the study. The chondrocytes used in this study were supplied by IMIM (Institut Municipal d'Investigació Mèdica, Barcelona, Spain). Cells were plated in culture flasks (Nunc, Spain) at high density in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) (both reagents supplied by Gibco, UK) and 50 μg/mL ascorbic acid (Sigma-Aldrich, Spain) at 37 °C in a 5 % CO_2 humidified atmosphere. The culture medium was renewed every 2-3 days. After 5 days, adherent cells were enzymatically harvested with tripsin/EDTA (Gibco, UK) and seeded on the Gel-MCP, as control sample, and TGF-β1 loaded Gel-MCP at cell density of 2.5×10^5 cells/tubes. To obtain the cell/Gel-MCP pellets (with and without TGF-β1), tubes that contained 20 mg of loaded or unloaded Gel-MCP and chondrocytes were centrifuged at 300 g × 5 min. Cell pellets without any sample were used as reference. The experiments were conducted for 3 weeks in chondrogenic media consisting of Dulbecco's high glucose modified Eagle medium with 1 % ITS + Premix (DB Biosciences, USA), 100 μg/mL sodium pyruvate (Gibco, UK), 1 % L-glutamine (Gibco, UK),

100 μg/mL penicillin–streptomycin (Lonza, USA) supplemented with 50 μg/mL ascorbic acid (Sigma-Aldrich, Spain). The culture medium was replaced three times/week.

2.8 Cell adhesion and morphology

Chondrocyte adhesion and morphology were investigated by scanning electron microscopy, SEM (Jeol JSM-5410, Japan). For this purpose, cell/gel-MCP pellets (with and without TGF-β1) and cell pellets samples were removed from the culture medium at different times (1, 7, 14 and 21 days), washed in PBS, fixed in 2.5 % glutaraldehyde, rinsed twice with PBS and dehydrated in series of ethanol solution (30, 50, 70, 90 % and absolute) for 15 min with final dehydration in absolute ethanol for 30 min. Finally, samples were dried at room temperature and sputter coated with gold using a Fisons Instrument Coater (Polaron SC 502, UK) before observation with SEM.

2.9 Cell viability

The viability of chondrocytes cultured for 1, 7, 14 and 21 days was determined using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay following manufacturer instructions (Promega, Spain). The samples were washed twice with PBS and incubated with fresh culture medium without phenol red and FBS containing MTS reagent (ratio 5:1) at 37 °C for 3 h in darkness. After the incubation period, the absorbance was quantified by spectrophotometry at 490 nm with a microplate reader (VICTOR3TM, PerkinElmer, USA). The background absorbance values obtained from unseeded gelatin microparticle pellets (with and without TGF-β1) were subtracted to the seeded pellets absorbance values to get the final viable cell number.

2.10 Immunofluorescence

Aggrecans and type I collagen were detected by immunofluorescence assays. Cell/Gel-MCP pellets (with and without TGF-β1) with 8 mm of diameter were fixed with formalin and sectioned in slices (~2 mm), washed with PBS twice and permeabilized with 0.1 % v/v Triton X-100 in PBS. The cell pellet of 1 mm of diameter was fixed and washed as previously described. Afterwards, all samples were incubated with 10 % fetal bovine serum in PBS blocking solution for 2 h at room temperature. Then, samples were incubated in the dilute primary antibody solution (Mouse anti-Human Aggrecan and Rabbit anti-human Type I Collagen, 1:50, from Invitrogen, Spain and Chemicon, Spain respectively) overnight at 4 °C, samples were washed again three times in PBS and then incubated with the respective secondary fluorochrome-conjugated antibody (1:200) for 2 h at room temperature in dark. Also, the formation of actin cytoskeleton was visualized using bodipy FL phallacidin (Invitrogen, Spain). Finally, samples were washed three times with PBS and mounted with a drop of mounting medium containing DAPI for nuclei staining (blue).

2.11 Biochemical analysis

The type II collagen secreted by the chondrocytes were determined quantitatively using type II Collagen Elisa kit (MD Bioproducts, USA) after pepsin digestion of cell/Gel-MCP pellets (with and without TGF-β1) and cell pellets ($n = 5$) according to the supplier's instructions. Using 500 μL of digested solution, GAG content was quantified following instructions provided by the manufacturer in the Blyscan_Sulfated Glycosaminoglycan Assay kit (Biocolor Ltd., UK). Bound dye values were quantified at 656 nm using a microplate reader (VICTOR3TM, PerkinElmer, USA).

2.12 Statistical analysis

Data were expressed as mean values ± standard deviation (SD) of the mean (mean ± SD) and analysed by one-way analysis of variance (ANOVA) and Student's t test. The level of statistical significance was set as $P < 0.05$.

3 Results

3.1 Characterization of Gel-MCP

The yield of Gel-MCP (with and without TGF-β1) was around 90 %. The crosslinking degree through NHN assay was determined in order to know the quantities of free NH_2 remaining in the Gel-MCP after the reaction with genipin. The obtained results indicate that a 74 % free NH_2 remain in the gelatin chemical structure.

The EE has a strong dependency with the preparation technique used to synthesize the microparticles. The TGF-β1 loaded Gel-MCP crosslinked with genipin retain more than 85 ± 1 % of the TGF-β1 initially used.

The morphology of Gel-MCP (with and without TGF-β1) was examined by SEM (Fig. 1). The microparticles showed a spherical morphology of all samples with slightly roughness surface without wrinkles or cracks. On the other hand, the microparticles have a homogeneous size, ranging from 6 to 24 μm in diameter with a mean diameter size in both cases (microparticles with and without TGF-β1) of 12 μm (see Fig. 1).

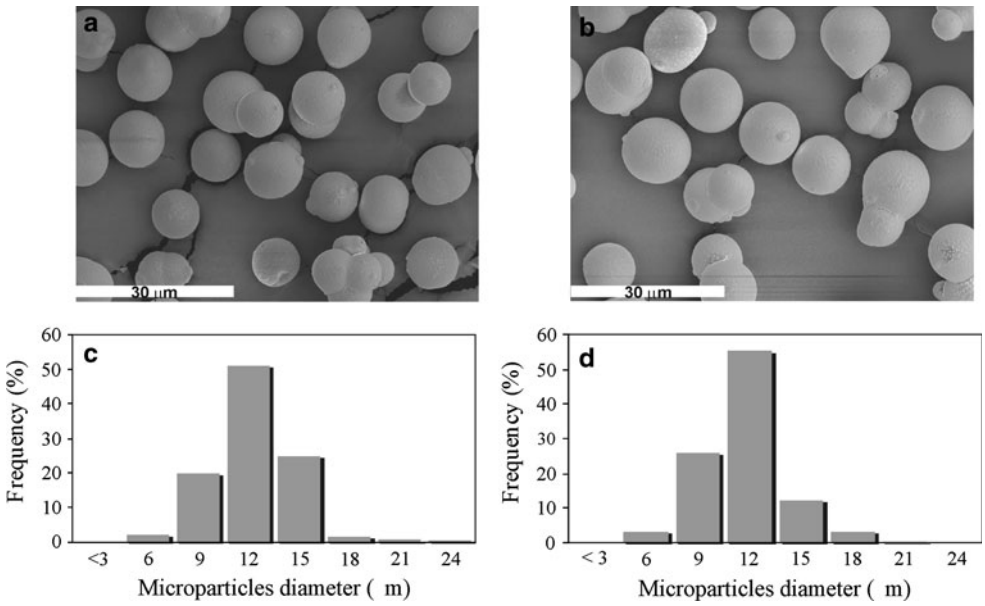

Fig. 1 Morphology and particle size distribution of crosslinked Gel-MCP without (**a**, **c**) and with (**b**, **d**) TGF-β1

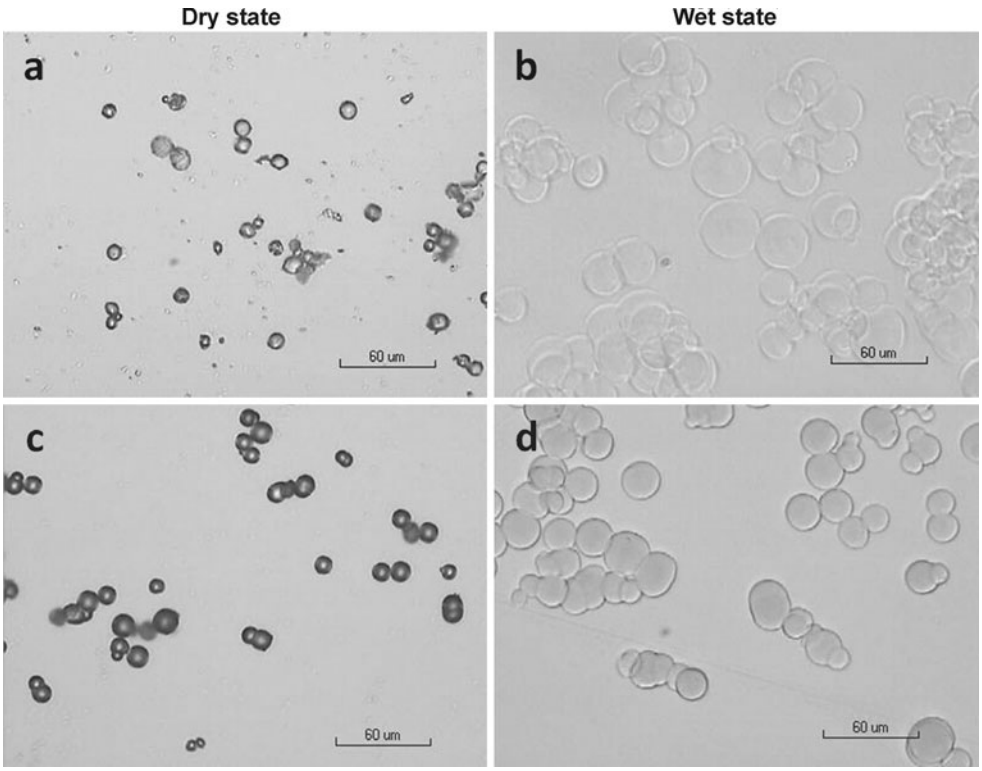

Fig. 2 Gross appearance of non-crosslinked (**a**, **b**) and crosslinked (**c**, **d**) Gel-MCP in dry and wet state

3.2 Water uptake experiments

Figure 2 shows the morphological appearance of the non-crosslinked (a,b) and crosslinked (c,d) Gel-MCP in dry and wet state. After 24 h, the water uptake of the crosslinked Gel-MCP (111.5 ± 12.5 %) significantly decrease compared with the values of the non-crosslinked ones (325.3 ± 11.1 %).

3.3 In vitro TGF-β1 release study

After the encapsulation process, 38.4 ng of TGF-β1 were loaded in 20 mg of Gel-MCP. The accumulative release profile of TGF-β1 from the Gel-MCP crosslinked with genipin over 14 days at 37 °C in D-PBS was represented in Fig. 3. TGF-β1 release pattern showed a burst until 24 h,

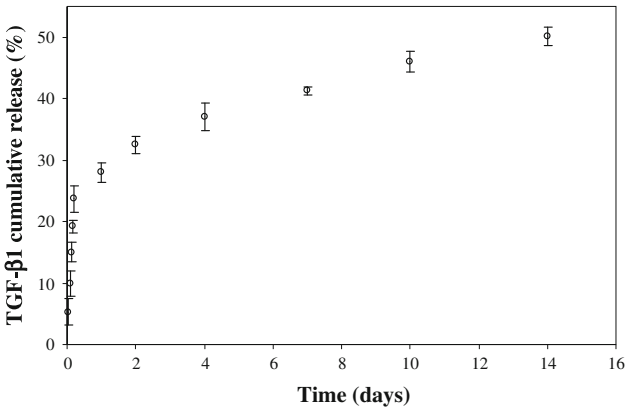

Fig. 3 Cumulative TGF-β1 release profile from Gel-MCP. *Error bars* represent means ± SD with $n = 8$

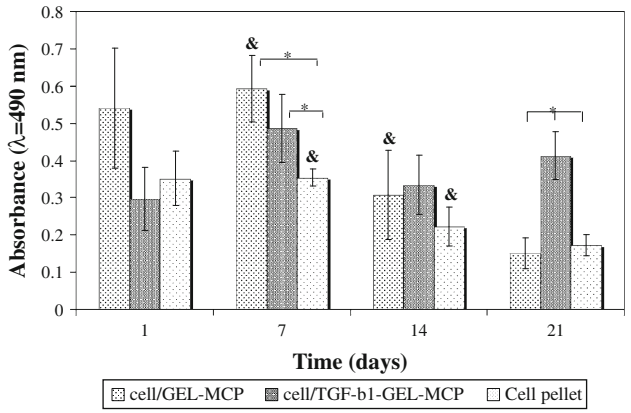

Fig. 4 MTS assay for cell/gelatin microparticle pellets (with and without TGF-β1) and cell pellets, used as reference. *Asterisks* denote significant differences between samples on the same culture day. *Ampersand* represent significant differences on different culture days. *Error bars* represent means ± SD with $n = 4$

where approximately 24 % of growth factor was released, followed by a slower release for the remaining 14 days. The cumulative release was 50 % of initial loading by day 14.

3.4 Chondrocytes and Gel-MCP (with and without TGF-β1) cultured in pellet

The cell viability of chondrocytes seeded in crosslinked Gel-MCP with and without TGF-β1 was examined by MTS assay. The obtained results after 24 h and 7, 14 and 21 days of culture show a consistent viability of the cell agglomerated with both, loaded and unloaded Gel-MCP (Fig. 4). Comparing the pellet of Gel-MCP with TGF-β1 and the cell pellet (used in this case as control sample), the cell viability was improved by the TGF-β1 released in the culture medium. That is way the number of viable chondrocytes remains almost constant after 21 days in the case of TGF-β1 loaded Gel-MCP while viability after 1 week significantly decreased in both, unloaded Gel-MCP pellet and in the cell pellet.

Chondrocyte morphology evaluated by SEM of both cell/microparticle pellets (microparticles with and without TGF-β1) and the control (cell pellet) (see Fig. 5) demonstrated that after 1 and 7 days of culture, the cells adhered very well on the surface of both types of microparticles (with and without TGF-β1), forming interparticle cellular bridges (indicated by white arrows). Cells have a round morphology and can be distinguished from Gel-MCP by the characteristic surface roughness. As a result, microparticles were bound together. A homogeneous layer of chondrocytes, microparticles and ECM can be seen after 14 days of culture, in cell-TGF-β1 loaded microparticle pellets. Some isolated cells emerge, with round morphology (Fig. 5). Cell aggregates were also observed in the constructs made with microparticles without TGF-β1. The chondrocyte pellets revealed an elongated morphology after 14 days of culture in chondrogenic medium.

The expression of type I collagen and aggrecans and the actin cytoskeleton formation were examined by confocal laser scanning microscopy (CLSM). Cell/Gel-MCP pellets with and without TGF-β1 and cell pellets (reference) were cross-sectioned in thick slices (∼2 mm). CLSM images after 14 and 21 days of culture are represented in Fig. 6. Nuclei were counterstained with DAPI (blue) and the expression of type I collagen and aggrecans was followed by Alexa-488 (green) and 647 (red) linked to the secondary antibody respectively. Positive expression of aggrecans (red) was observed mainly in the cell pellet (control material) and in the lesser extent in TGF-β1 loaded Gel-MCP pellet, localized around the cells after 14 days. After 21 days of culture, the aggrecans expression was positive for cell/Gel-MCP (with and without TGF-β1) pellet and cell pellet. In chondrocyte pellets, used as control, the secretion of type I collagen was clearly detected and predominantly on the surface of the cell pellet. On the contrary, in the cell/Gel-MCP (with and without TGF-β1) only a few cells, even after 21 days, showed a positive staining.

Actin cytoskeleton was clearly detected in the cell pellets after 14 and 21 days of culture in chondrogenic medium compared to the cell/Gel-MCP pellets, where only in some cells was observed.

Glycosaminoglycans and type II collagen are the most important components of ECM in native articular cartilage; their expression in 3D culture is a recognized criterion of chondrogenecity. The results obtained in our constructs are represented in Figs. 7 and 8 respectively. The differences in GAG synthesis among the different supports are not significant at 14 days, nevertheless at 21 days of culture the amount of GAG per 20 mg of Gel-MCP is significantly

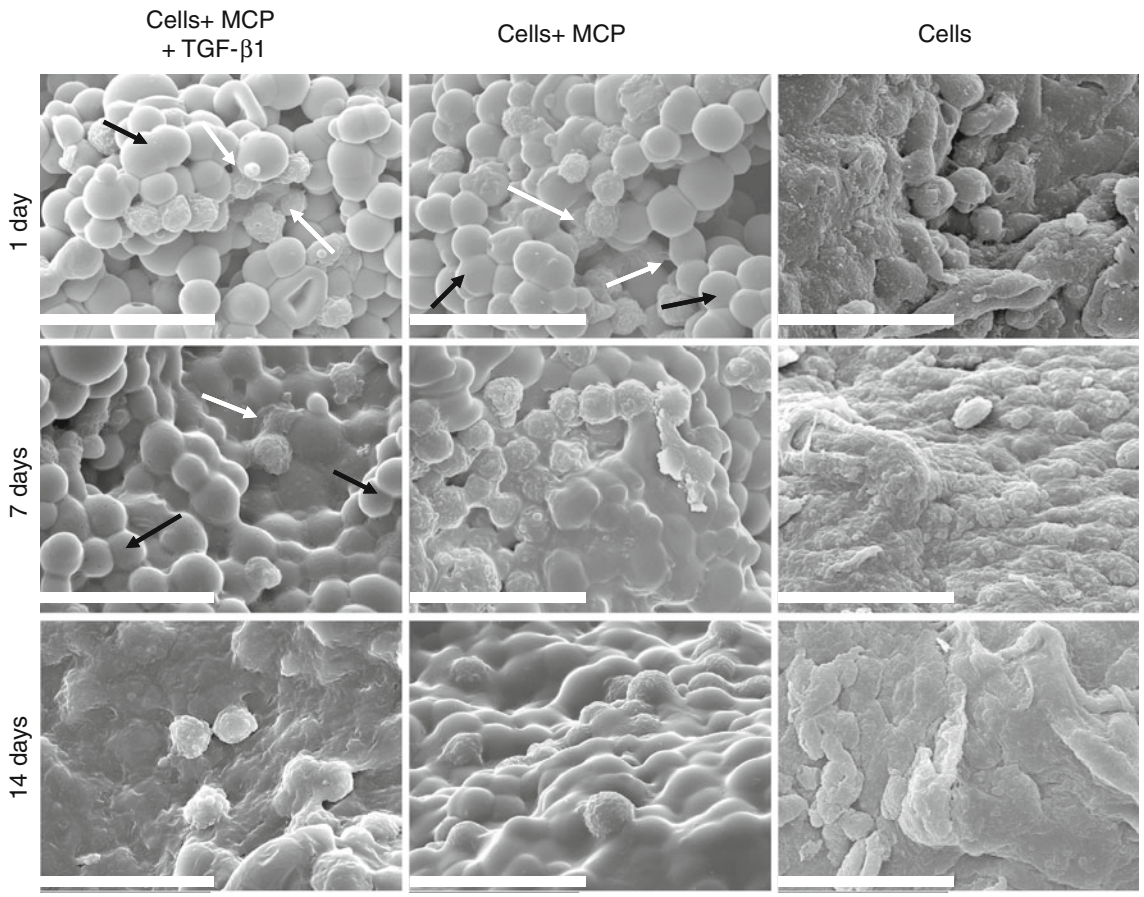

Fig. 5 Scanning electron microscopy images of chondrocytes seeded in TGF-β1 loaded Gel-MCP (*left column*), control sample (microparticles without TGF-β1) and cell pellet (*right column*) after 1, 7 and 14 days. Scale bars correspond to 40 μm. The *white arrows* indicate interparticle cellular bridges. The *black arrows* indicate the Gel-MCP

higher in loaded microparticles than in unloaded microparticles or in the pellet.

Type II collagen synthesis was significantly increased from 14 to 21 days ($P < 0.05$) for cell/TGF-β1 loaded Gel-MCP pellets being significantly higher than in the pellets or in unloaded Gel-MCP.

In cell pellets, GAG content clearly decreases with the culture time, while type II collagen deposition increase. This feature could be due to a partial dissolution of GAG as result of decrease of pellet size [21].

4 Discussion

Regeneration of articular cartilage with the technique of autologous chondrocyte implant, ACI, has proved to be able to produce newly formed cartilage tissue in the region of the cartilage defect although the evolution of the tissue with time present important drawbacks including the degeneration of the tissue to fibrocartilage with poor mechanical properties [22–24]. The implant of a mature chondrocyte pellet in the cartilage defect in a rabbit model also produced a cartilaginous tissue lacking the organization of hyaline cartilage and with very low elastic modulus after 3 months of implantation [25]. Culture of chondrocytes in a pellet in chondrogenic medium produce cells with round morphology with production of ECM with abundance of glycosaminoglycans and type II collagen, but expressing negative markers for hyaline cartilage chondrocytes as type I collagen [26–28]. The presence of a scaffolding system that supports cell adhesion and controls stress transmission to the cells can improve the performance of chondrocyte transplant to a cartilage defect. The strategy proposed in this work is somewhere in between the entrapping of chondrocytes in a gel such as alginate [29], poly (ethylene oxide) [30], oligo poly(ethylene glycol) fumarate [11, 31], poly (ethylene glycol dimethacrylate) [32, 33], silk [34] extensively explored in the literature and seeding the cells in a macroporous scaffold in which they adhere to the pore walls a situation which is not far from monolayer culture. The cell-microparticles suspension can be injected in the zone of the cartilage following the

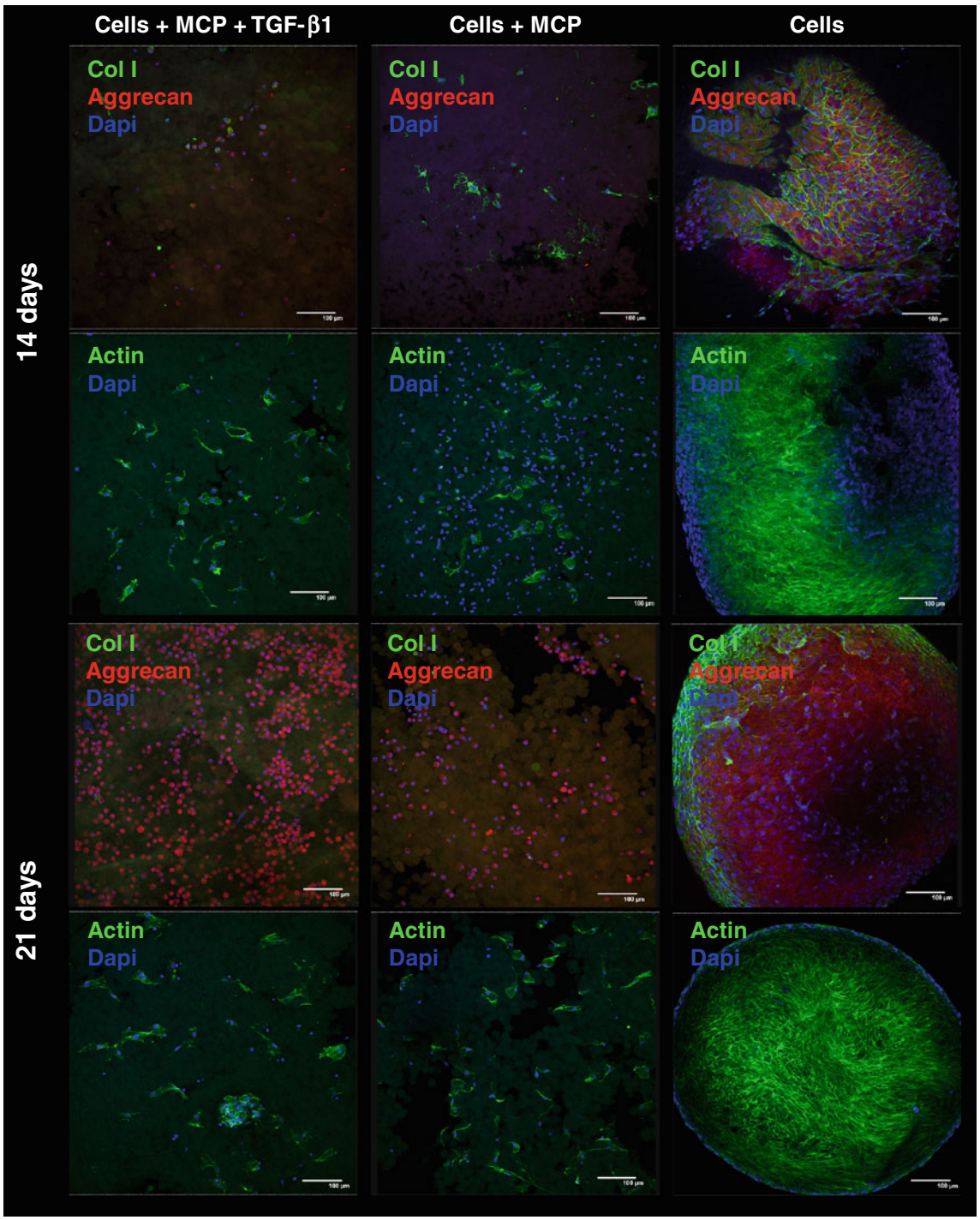

Fig. 6 Actin cytoskeleton (*green*) development and immunofluorescence staining for aggrecan (*red*) and type I collagen (*green*) of chondrocytes/gelatin microparticle pellets (with and without TGF-β1) and cell pellets cultured after 14 and 21 days in chondrogenic medium. The *scale bar* represents 100 μm for all images. The cells nuclei were counterstained with DAPI (*blue*) (Color figure online)

surgical approaches currently used in ACI practice, but adding the required support for cell attachment.

In this study, we crosslinked Gel-MCP with genipin, which was reported to be non cytotoxic and a safe crosslinking agent, compared to commonly used glutaraldehyde or formaldehyde crosslinkers [35]. Nevertheless, Wang et al. [36] recently reported a certain toxicity of genipin on chondrocytes and osteoblasts at high doses being its toxicity depended on doses but not on the time. Genipin has been used as a culture medium supplement to develop enhanced engineered cartilage [37]. Chitosan microparticles crosslinked with genipin were also used to culture

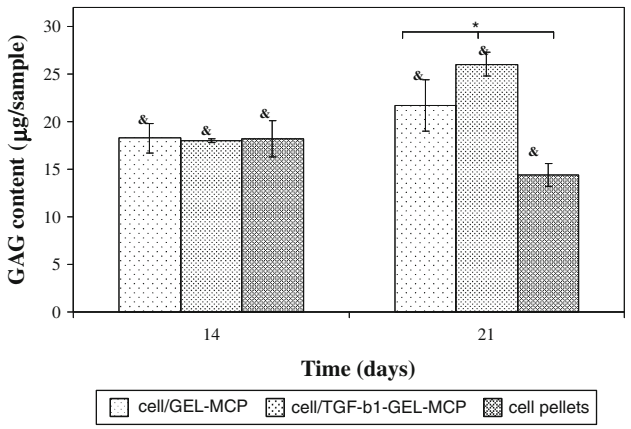

Fig. 7 GAG quantification assay after chondrocyte/gelatin microparticle pellets (with and without TGF-β1) and cell pellets cultured for 2 and 3 weeks in chondrogenic medium. *Asterisks* denote significant differences between samples on the same culture day. *Ampersand* represent significant differences on different culture days. *Error bars* represent means ± SD with $n = 5$

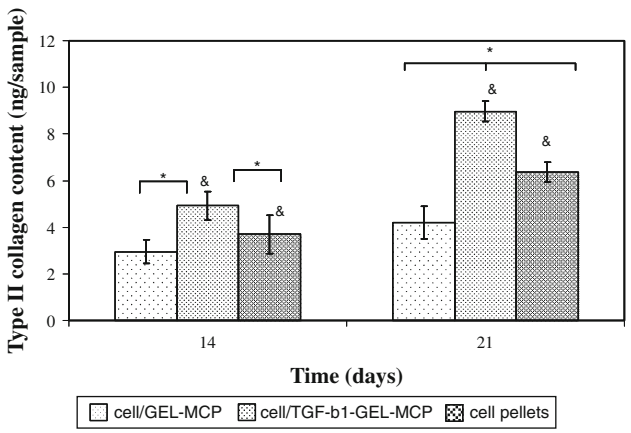

Fig. 8 Collagen II content after chondrocyte/gelatin microparticle pellets (with and without TGF-β1) and cell pellets cultured for 2 and 3 weeks in chondrogenic medium. *Asterisks* denote significant differences between samples on the same culture day. *Ampersand* represent significant differences on different culture days. *Error bars* represent means ± SD with $n = 5$

mesenchymal stem cells in osteogenic medium during 14 days, showing no signs of toxicity [18]. In addition, the results published by Solorio et al. [38] demonstrated the potential of Gel-MCP crosslinked with genipin to deliver growth factors locally to the cells.

The crosslinking reaction during the formation of microparticles results in a 26 % crosslinking degree. Crosslinking of already formed Gel-MCP can yield higher crosslinking density as reported in the [39, 40]. The strategy of this work was to prepare the water/oil emulsion in PBS (pH 7.4) at 37 °C using a mixture of biopolymer and growth factor as aqueous phase. In this way an ionic complex is formed between the acidic gelatin (isoelectric point 5.0) and TGF-β1 (isoelectric point 9.5) [41] to which genipin is added. It can be expected that some of the growth factor molecules be linked to gelatin by covalent bonds created by the crosslinker.

At 14 days the cumulative release of TGF-β1 is around 50 % (Fig. 3). Growth factor molecules fixed to the matrix by covalent bonds would be delivered only by support degradation at longer times. The significant differences in chondrocyte response between TGF-β1 loaded and unloaded microparticles supports that delivered growth factor is bioactive.

Recent studies by Catela et al. [42] demonstrated that TGF-β1 released from fibrin gels to which was covalently bonded was bioactive inducing chondrogenic differentiation of mesenchymal stem cells. Crosslinking considerably reduce water uptake of Gel-MCP and improves their stability in aqueous medium. Crosslinking density can be used to modulate the rate of sustained delivery of TGF-β1.

In this work we have shown that Gel-MCP agglomerated together with the expanded chondrocytes create a 3D environment, without the need of another gel embedding both Gel-MCP and cells as proposed in other works [10–12]. The construct is consistent and easily handleable, due to the strong adhesion between the ECM produced by the cells and the Gel-MCP. Interestingly enough, due to the Gel-MCP/cells ratio these microparticles acts as a 3D scaffold, favouring cell to matrix interaction with respect to cell to cell contacts and even, without growth factor supply, the behaviour of the seeded chondrocytes change significantly with respect to the cell pellet: increased cell viability, sharp diminution of type I collagen in the produced ECM, absence of actin stress fibers in most of the cells, and increased production of aggrecan. On the other hand, Gel-MCP may act as a mean for stress transmission to the cells. Acquisition and maintenance of the chondrocyte phenotype "in vivo" is highly addressed by dynamic compression loading to which articular cartilage is subjected. The transmission of these stresses to the cells in a pellet is quite different to the situation in healthy cartilage where cells are isolated in lacunae and protected by a quite hard tissue. Crosslinked Gel-MCP can be produced with an elastic modulus that can vary in a broad range, thus, it allows designing the best conditions to create the adequate environment with respect to compression load sustaining. On the other hand, we have shown that sustained delivery of TGF-β1 can be obtained from the cell/support agglomerate itself, thus low amounts of the growth factor can be delivered to the cells locally with small dissemination to the rest of the organism. The improvement of cell behaviour in the culture with TGF-β1 delivery is very clear, with increased viability and chondrogenic markers with respect to the culture in non-loaded microspheres.

5 Conclusions

Cell/Gel-MCP constructs allow culture and redifferentiation of mature chondrocytes previously expanded in monolayer culture. Cell adhesion to microparticles agglomerates the whole construct, which from the first day of culture maintains coherence and can be easily handled. TGF-β1 can be effectively included in the Gel-MCP during their preparation by a water–oil emulsion process. Crosslinking with genipin allows progressive delivery when the particles are immersed in culture medium. Chondrocytes culture in agglomerates with growth factor delivery show viability for longer times, increased production of ECM components characteristics of hyaline cartilage. The results obtained indicate that the developed gelatin-based microparticles could be used as an injectable scaffold to delivery chondrocytes through minimally invasive procedures, that will agglomerate in situ by the action of the cells; the particles are be able to provide mechanical and a 3D structural support for the cells and also act as a system able to release relevant bioactive molecules.

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