Introduction

One of the cell sources for the regeneration of articular cartilage with tissue engineering techniques are autologous chondrocytes. In the autologous chondrocytes implantation technique (ACI), a small number of autologous cells are obtained by enzymatic digestion of a small portion of healthy cartilage. These chondrocytes are expanded “in vitro” to obtain around 30 million cells that are injected in the site of the cartilage defect covered by a layer of inverted periosteum.\textsuperscript{1,2} Expansion in monolayer culture tends to dedifferentiate chondrocytes. When cells adhere to the substrate developing focal adhesion and a subsequent actin cytoskeleton they start proliferating but change their characteristic round morphology in hyaline cartilage and the expression of characteristic hyaline cartilage genes, in particular they produce type I collagen instead or in addition of type II collagen which is the predominant collagen form in the extracellular matrix of articular cartilage.\textsuperscript{3,4} Much research effort has been dedicated to find the culture conditions that allow chondrocyte proliferation without changing its phenotype and in this sense it has been reported that the substrate material plays a role at least in retarding chondrocyte dedifferentiation.\textsuperscript{3}

Delivery of some growth factors such as TGF$\beta$, or other signalling factors also modify the chondrocyte behaviour in monolayer culture while culture in 3D supports such as in high density pellets, micromass or in gels such as alginate hydrogels seems to preserve the round morphology and expression of characteristic chondrogenic markers but suppresses proliferation.\textsuperscript{5–9}

In any case the key question, which remains unanswered, is if previously expanded chondrocytes are able to generate hyaline cartilage when implanted in the zone of the defect alone or seeded into a 3D scaffold. Actually, clinical experience shows that the initial beneficial effect of the ACI operation, with generation of cartilage tissue and subjective improvement with pain reduction, is followed in many cases by tissue degeneration by reasons that are not well known. This is similar to what happens when subchondral bone is injured below the cartilage defect allowing bleeding and the formation of a fibrin clot that acts as a way for pluripotential mesenchymal bone marrow stem cells migration and differentiation to chondrocytes. The tissue formed has the characteristics of fibrocartilage with poor mechanical properties.\textsuperscript{10–13}

The use of chitosan in cartilage engineering has been explored by many groups. Different modifications of chitosan have been developed to make it water soluble at pH = 7 and easily crosslinkable to entrap chondrocytes.\textsuperscript{14–18} Chondrocytes are viable in these hydrogels and maintain the round morphology. Gels containing chitosan as a component also show the ability to differentiate mesenchymal stem cells (MSCs) “in vivo” to produce viable chondrocytes. The regenerative effect of chitosan solution injected in an articular
cartilage of a rat knee was studied by Lu et al.19 Chitosan microparticles have been explored as carriers to deliver growth factors during culture.20–24 Chitosan scaffolds can be easily prepared by freeze-drying techniques that allow production of pores in the range from some tens to a few hundred microns with high porosity. This method allows combining chitosan with proteins or polysaccharides such as collagen (or gelatin) or hyaluronan to mimic the natural extracellular matrix in articular cartilage.18,21,24–28 Larger, well interconnected pores can be formed in the scaffold by combining freeze drying with the presence of a porogen in the form of polymeric microparticles.29 On the other hand, chitosan can be used in the form of a coating of the walls of a macroporous scaffold made of a different polymer such as polylactide.30

In this study, we explore the behaviour of human articular chondrocytes, previously expanded in monolayer, in a 3D environment formed by chitosan microparticles. This situation was called a modified pellet by Fan et al. who produced a pellet by centrifuging a suspension containing MSCs and gelatin microparticles.31 The size of the microparticles, in the order of magnitude of the cells size should allow the expansion of the pellet when chondrocytes produce extracellular matrix and a certain capacity of cell clustering and lacunae formation. In our work, several cellular parameters and chondrocytic markers were evaluated in order to explore cell viability, proliferation and differentiation of primary chondrocytes mixed with chitosan microparticles in a chondrogenic medium. A similar procedure was followed by Han et al. using a mixture of gelatin and chitosan microparticles.22 Lu et al. used porous chitosan microparticles coated by collagen nanofibers to culture articular chondrocytes.32 Lao et al. used PLLA microparticles coated with chitosan and reported better performance of the chitosan coated microparticles than PLLA alone.33 In this way, it is expected that cells be surrounded by chitosan in a 3D environment some way intermediate between being entrapped in a hydrogel (in which cell to cell contact is absent and no mobility or displacement of the neighbourhood is possible except by matrix degradation) and a macroporous scaffold in which cells can adhere to the pore walls and behave as in a monolayer without a true 3D environment.34 This approach was studied in a recent work using gelatin microspheres as scaffolding materials,35 the essential difference of the use of chitosan microparticles as a scaffolding system is that whereas cell adhesion to gelatin surfaces was strong, producing, even at very short times of culture, a mechanically consistent cell-material construct, cell adhesion to chitosan is poor as we will show below maintaining a continuously mobile environment. The size of the microparticles, in the order of magnitude of the cells size, should allow the expansion of the pellet when chondrocytes produce extra-cellular matrix and a certain capacity of cell clustering and lacunae formation.

Materials and methods

Materials

Ultrapure Protasan™ chitosan base, UP B 80/20, (molecular weight: 125–350 kDa, deacetylation degree: 80–89%) was purchased from NovaMatrix/FMC Biopolymer (Norway). All chemicals (with purity >99%, without further purification) were purchased from Sigma Aldrich (Valencia, Spain). MilliQ water was used in this study.

Chitosan microparticles production

Chitosan solutions with concentration of 1 wt% were prepared dissolving chitosan powder in 0.1 M acetic acid. Chitosan solutions were spray dried using a laboratory scale spray dryer (Büchi Mini Spray Dryer B-290, Büchi Labortechnik AG, Switzerland). Air was used as the drying medium. The processing conditions were as follows: inlet temperature 150 °C, aspiration rate 80%, air flow 600 NL h⁻¹ and solution feed rate 5 mL min⁻¹. The outlet temperature was between 69–71 °C. These conditions were selected following preliminary experimentation. The chitosan acetic microparticles were removed from the collection vessel and dispersed in ethanol under magnetic agitation at room temperature. Then, sodium hydroxide solution (0.1 M) was added dropwise and the microparticles were kept under agitation during 16 h in order to neutralize the chitosan and restore the amine groups. Finally, the microparticles were collected by centrifugation and washed several times with water until pH = 7. Chitosan microparticles were sterilized in ethanol 70% v/v and stored until used at 4 °C.

Characterization of microparticles morphology, size and zeta potential

The morphology of chitosan microparticles was examined by scanning electron microscopy (SEM) (Jeol JSM-5410). Samples of microparticles 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 by Laser light scattering using a Mastersizer 2000 (Malvern Instrument, UK). Average particle size was expressed as the volume mean diameter ($D_{4.3}$). Polydispersity was given by a span index, which was calculated by $(D_{0.9} - D_{0.1})/D_{0.5}$ where $D_{0.9}$, $D_{0.5}$ and $D_{0.1}$ are the particle diameters determined respectively at the 90th, 50th and 10th percentile of undersized particles. The zeta potential of microparticles was measured by a Malvern Zeta analyzer (Nano-ZS 90, Malvern Instrument, UK) with ultrapure water as solvent (pH = 7 at 25 °C). These measurements were run at least three times with independent microparticle batches.

Swelling experiments

The swelling degree, $w$, was determined gravimetrically using an analytical balance with very high precision, 10⁻⁵ g, (Mettler Toledo). Certain amounts of microparticles were first weighed inside an Eppendorf tube and 1 mL of phosphate buffer solution was added to each tube. Dynamic swelling experiments were performed at 37.0 ± 0.1 °C during 48 h. After this time, the samples were centrifuged at 10 000 rpm during 5
min and weighed again. The swelling degree, \( \psi \), is expressed as the amount of water per unit mass of dry chitosan microparticles.

**Chondrocyte isolation**

Human articular cartilage was obtained from osteoarthritic knee joints after prosthesis replacement. Cartilage was extracted from the finest conserved region of the osteoarthritic knee although it cannot be considered as normal cartilage. The study was conducted in accordance with the 1975 Declaration of Helsinki, as revised in 1983, and approved by our local Ethics Committee. All patients submitted written informed consent before their inclusion in the study. Cells were obtained according to the protocol described in ref. 36. Briefly, cartilage was dissected from subchondral bone, finely diced and then washed with Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 100 U penicillin, 100 µg streptomycin (Biological Industries) and 0.4% fungizone (Gibco). The diced cartilage was incubated for 30 min with 0.5 mg mL\(^{-1}\) hyaluronidase (Sigma-Aldrich) in a shaking water bath at 37 °C. The hyaluronidase was subsequently removed, and 1 mg mL\(^{-1}\) pronase (Merk, VWR International SL) was added. After 60 min incubation in a shaking water bath at 37 °C, the cartilage pieces were washed with supplemented DMEM. After removal of the medium, digestion was continued by addition of 0.5 mg mL\(^{-1}\) of collagenase-IA (Sigma-Aldrich) in a shaking water bath kept at 37 °C overnight. The resulting cell suspension was filtered through a 70 µm pore nylon filter (BD Biosciences) to remove tissue debris. Cells were centrifuged and washed with DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen SA). Finally, the cells were cryopreserved in liquid nitrogen with DMEM containing 20% FBS and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) until use, or plated in tissue culture flasks for immediately chondrocyte culture.

Cells were expanded in DMEM supplemented with 10% FBS and 50 µg mL\(^{-1}\) ascorbic acid and then were seeded on biomaterial in the third subculture.

**Specimen preparation**

For pellet preparation, the resuspended cells from culture flasks were transferred to a 1.5 mL centrifuge tube (0.8–1 × 10^6 cells per tube) to which culture medium was added until the total volume of 1 mL was reached. The cell suspension was centrifuged for 4 min at 1200 rpm. The resulting pellet was cultured with DMEM supplemented with 10% FBS and 50 µg mL\(^{-1}\) ascorbic acid the first day and then chondrogenic medium was used until the end of the experiment. The chondrogenic medium consisted in DMEM supplemented with 1% of insulin-transferrin selenium (ITS), 10 ng mL\(^{-1}\) of TGF-β1 and 50 µg mL\(^{-1}\) ascorbic acid.

Chitosan microparticles were sterilized with 70% ethanol overnight and conditioned in phosphate buffer solution (PBS) 24 h before chondrocyte seeding. Chitosan microparticles were mixed with the resuspended cells from culture flasks in a sterile tube at a ratio of 5–10 mg of chitosan microparticles for 0.8–1 × 10^6 cells. Culture protocol of chitosan specimens was performed under the same conditions as pellet culture. The experiment was conducted during 28 days of culture and specimens were analysed at two (T0), seven (T7), fourteen (T14) and twenty eight (T28) days after seeding. The chondrogenic medium was changed every 3 days.

**Cell viability assay**

Cell viability in chitosan and pellet cultures was monitored by the MTT test (Roche Diagnostics GmbH) at 2, 7, 14 and 28 days of culture. MTT assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. The resulting coloured solution was quantified using an ELISA reader (A550). Cell pellet samples were used as a positive control of the experiment. Data from each independent experiment were normalised to sample at T0 culture time. Each experiment was repeated a minimum of five times using different chondrocyte populations.

**Cell proliferation**

Cell proliferation was assessed by means of double stranded deoxyribonucleic acid (dsDNA) quantification over culture time. In this manner, at T0, T7, T14 and T28, cell–chitosan microparticles constructs were resuspended in 0.5 mL of bi-distilled water and stored at −80 °C until use. For PicoGreen analysis, the samples were sonicated for 100 s and vortexed before fluorescent measurements according to the Quan-iT PicoGreen dsDNA reagent manufacture’s protocol (Invitrogen).

**Histology**

The ability of chondrocytes seeded in chitosan microparticles to synthesise glycosaminoglycan (GAG) was monitored by Alcian blue staining, counterstained using Mayer's haematoxylin and then analysed by optical microscopy. Briefly, the specimens were embedded in optimum cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek), cryosectioned (10 µm thick) and fixed in acetone for 10 min at 4 °C before GAG staining.

**Immunohistochemistry**

Immunohistological analysis was used to detect the synthesis of type I and type II collagen, the expression of S-100 (a chondrocyte differentiation marker), and Ki-67 (a proliferation marker). At 2, 7, 14 and 28 days post-seeding, the specimens were embedded in OCT, cryosectioned (10 µm thick), and fixed in acetone for 10 min. Sections were incubated for 1 h at room temperature with a 1 : 100 dilution of type II collagen antibody (Calbiochem), 1 : 1000 dilution of type I collagen antibody (Sigma), 1 : 100 dilution of anti-human Ki-67 antibody (DakoCytomation), and pre-diluted S-100 antibody (DakoCytomation). Antigen–antibody complexes were incubated using a 1 : 100 dilution of Polyclonal Goat anti-mouse conjugated with HRP or Goat anti-rabbit conjugated with HRP (in the S-100 detection) and detected colorimetrically with DAB (3,3′-diaminobenzidine). Finally, samples were counterstained with Mayer’s haematoxylin and analyzed by optical microscopy.
Results

Preparation and characterization of chitosan microparticles

The spray-drying process provides a good control over particle size and morphology. In our work, using the high performance cyclone resulted in the collection of high yields of spray-dried microparticles (the yields ranged from 75% to 85%). The morphology of the microparticles showed a spherical geometry with rough surface and diameters in the range from 1 to 5 μm (Fig. 1).

The physical characteristics of the chitosan microparticles are listed in Table 1. Spray-dried chitosan microparticles are positively charged, with a mean microparticle diameter of 2.14 ± 1.12 μm. Chitosan is a hydrophilic polysaccharide and absorbs high amounts of water. The swelling degree of chitosan microparticles was determined in order to have a characterization of their size in cell culture experiments. As expected, the chitosan microparticles absorbed four times their weight in water.

The appearance of swollen chitosan microparticles (figure not shown) reveals that the microparticles keep their spherical morphology. The average diameter of swollen chitosan microparticles is around 10 μm, five times larger than in the dry state.

Cell culture, viability and proliferation

The results of MTT analysis (cell viability) and DNA-quantification (cell proliferation), both normalized to T0, are shown in Fig. 2.

MTT results show that the number of viable cells in the cell–chitosan microparticles constructs is maintained up to 28 days of culture in a way similar to pellet culture. In the case of proliferation results, cell number seeded in the chitosan microparticles slightly increases from two weeks of culture, which is corroborated by the Ki-67 marker that proves a small part of the cells in culture are proliferative (brown nuclei in Fig. 3, some of them have been shown by arrows).

Histology and immunohistochemistry

Cells cultured in the presence of chitosan microparticles show a sustained glycosaminoglycan production as revealed by Alcian blue staining, which is concentrated around the cells (Fig. 4). Besides, cells were positive for S100, an intracellular calcium binding protein found in all chondroid tissues (Fig. 4).

Immunohistological staining of type I and type II collagen also showed that both markers were abundantly present in the chondrocyte–chitosan microparticles constructs (Fig. 5).

It seems that the presence of type I collagen in chitosan cultures increases with culture time with a more clear formation of collagen fibres around the cells at 28 days. This behaviour is different to that of the pellets in which type I collagen production is very clear at the beginning of culture but at long culture times it seems that the presence of this protein in the core of the pellet is residual while cells in the periphery continue expressing actively type I collagen as they would do in a monolayer culture. On the other hand, type II collagen was clearly detected at 7 and 14 days in chondrocyte–chitosan microparticles constructs and in the chondrocyte pellets. At 28 days of culture, type II collagen is reduced in chitosan specimens while it can be observed in the form of fibrils in the core of the pellets.

Table 1

| Physical properties of spray-dried chitosan microparticles |
|--------------------------|---------|---------|---------|---------|
| Size (μm)                | D4.3   | D0.1   | D0.9   | Span   |
|                         | 2.14   | 1.12   | 3.36   | 1.12   |
| Zeta potential ± SD (mV)| 27 ± 5 |         |         |         |
| Swelling degree ± SD (%)| 410 ± 24|        |        |        |

Fig. 2 Viability (A) and proliferation (considering T0 = 100%) (B) of chondrocytes seeded in CHT microparticles at 2, 7, 14 and 28 days. Cell pellets were used as control in A.

Fig. 3 Ki-67 immunostaining of chondrocytes seeded in CHT microparticles after 7, 14 and 28 days of culture. Cell pellets were used as control.
Discussion

An agglomerate of cells in supporting microparticles is an interesting three-dimensional arrangement for cell culture. The situation simulates some way the disposition of cells in cartilaginous tissues, surrounded by extracellular matrix and without cell to cell contact. The advantage with respect of cell embedding in a hydrogel network is that microparticles allow support remodelling while cells produce their own extracellular matrix, which is not possible in the case of the hydrogel unless simultaneous support degradation takes place. The formation of the cell-support construct depends very much on cell-support adhesion. It was shown that bone marrow mesenchymal stem cells agglomerated with around 200 micrometer diameter chitosan microparticles were able to agglutinate them with their own extracellular matrix but only if the microparticles surface were modified with an Argon plasma treatment to improve cell adhesion. Consistent chondrocytes–gelatin microparticles constructs can be also obtained due to the good adhesion of the cells with the microparticles surfaces. In this work, the main feature in the chondrocyte culture in the presence of chitosan microparticles with a diameter in the range of 10 microns is the lack of cell-support aggregation. At any time, the cell-chitosan microparticles constructs remain unbundled. In this environment, the chondrocytes are viable with a reasonable survival at 28 days of culture. Hence, chitosan microparticles could act as a mobile scaffolding material for cell culture in a three-dimensional environment and even be suitable to be injected into articular injury.

Interestingly, a part of the cells are proliferative in this environment when cultured with a chondrogenic culture medium, as proven by Ki-67 staining, although cell number seems to remain essentially constant. One explanation would be the certain degree of cell mortality detected in the chitosan cultures which, on the other hand, is common to any standard cell culture. The way in which the culture proceeds does not allow quantification of extracellular matrix production since a large part of the biosynthesized components is lost in the culture medium and no coherent tissue is formed. The histology pictures showing the expression of chondrogenic markers (Fig. 4 and 5) were taken spreading samples from the well content on the microscope slides and subjecting them to the histological or immunochemical preparation.

The literature shows that mature chondrocytes expanded in monolayer can recover, at least to some extent, the phenotype of hyaline cartilage chondrocyte when cultured in three dimensional supports such as encapsulated in gels, in micromasses or in pellets, in adequate culture medium. In these supports, the cells interact with the material in its surroundings or with other cells situated all around it. This fact seems to be essential to maintain the round form of the chondrocyte and seems to be also crucial for differentiation. In the support formed by chitosan microparticles, cell-material interaction is very weak but three-dimensionality is maintained. The immunohistochemistry analysis shows that chondrocytes cultured in this microparticulate construct produce characteristic components of hyaline cartilage at least up to 14 days culture. Nevertheless, at longer culture times, production of type I collagen seems to increase, and fibrils of this protein are formed around the cells. A minimum
type II collagen is also detected at 28 days culture but no fibrils were observed. It is not expected that a consistent tissue can be formed with this environment since GAGs produced can be easily lost in the culture medium but the results obtained shows the suitability of this supporting material if it is combined with a macroporous scaffold that could provide the required mechanical reinforcement of the construct. In fact cells seeded in a macroporous scaffold can adhere to the pore walls and behave as in a monolayer culture; seeding cells plus microparticles construct could combine true three-dimensionality with the adequate biomechanical environment for the cells. Certainly the role of microparticulate gels as a supporting mobile system can be explored with other materials or combinations of materials allowing intermediate situations between chitosan and gelatin that could be considered the extreme situations with very weak and quite strong cell-material interaction.

Conclusions

Chondrocytes are viable up to 28 days of culture in an environment created by chitosan microparticles with diameters around 10 microns. The very poor adhesion between cells and supporting microparticles maintains full capacity of construct remodelling during tissue regeneration. Cell–chitosan specimens produce specific articular cartilage markers up to 14 days of culture in chondrogenic medium. However, an important production of type I collagen is shown mainly at 28 days. Proliferation is residual and cell number remains nearly constant. The 3D environment created by microparticles allows modulating cell–support interaction allowing support remodelling during formation of new extracellular matrix and could be combined with a mechanical support provided by a macroporous scaffold in cartilage engineering.

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