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RESEARCH ARTICLE

Effect of double growth factor release on cartilage tissue engineering

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Abstract

The effects of double release of insulin-like growth factor I (IGF-I) and growth factor β1 (TGF–β1) from nanoparticles on the growth of bone marrow mesenchymal stem cells and their differentiation into cartilage cells were studied on PLGA scaffolds. The release was achieved by using nanoparticles of poly (lactic acid-co-glycolic acid) (PLGA) and poly(*N*-isopropylacrylamide) (PNIPAM) carrying IGF-I and TGF–β1, respectively. On tissue culture polystyrene (TCPS), TGF-β1 released from PNIPAM nanoparticles was found to have a significant effect on proliferation, while IGF-I encouraged differentiation, as shown by collagen type II deposition. The study was then conducted on macroporous (pore size 200–400 μm) PLGA scaffolds. It was observed that the combination of IGF-I and TGF-β1 yielded better results in terms of collagen type II and aggrecan expression than GF-free and single GF-containing applications. It thus appears that gradual release of a combination of growth factors from nanoparticles could make a significant contribution to the quality of the engineered cartilage tissue. Copyright © 2011 John Wiley & Sons, Ltd.

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1. Introduction

Articular cartilage can resist a significant amount of mechanical stress; however, it has a very limited self-repair capability upon suffering a trauma (Hunziker, 1999). Studies on cartilage degeneration have shown that aggrecan fragments are generated by aggrecanase action in the synovial fluids of healthy individuals and of patients with osteoarthritis, rheumatoid arthritis and acute knee injuries (Lohmander *et al*., 1993). Degraded aggrecan molecules can no longer protect the collagen fibres, which in turn undergo proteolytic degradation, and the result is articular cartilage degeneration (Sztrolovics *et al*., 1997).

Cartilage treatment by transplantation of autogenous or allogenous chondrocytes, or through the use of mesenchymal stem cells (MSCs), has several advantages over solid tissue transplantation or local debridement procedures. However, allogenous chondrocytes carry an inherent risk of an immune reaction, while autogenous chondrocytes lack a suitable donor site, and the need for large samples limit chondrocyte transfer from either (Worster *et al*., 2001).

Cartilage tissue engineering can offer a solution to this problem, but the selection of appropriate cell type, the fabrication of biocompatible and mechanically stable scaffolds and the amount and timing of growth factor delivery are highly crucial to obtaining satisfactory results. Worster *et al*. (2001) demonstrated that MSCs cultured with IGF-I

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were significantly more chondrogenic than when pretreated with TGF-β1, highlighting the importance of the role of growth factor supplementation.

As reported in the literature, there are two growth factors that play a major role in chondrocyte proliferation and differentiation. These are insulin-like growth factor I (IGF-I) and transforming growth factor β1 (TGF-β1) (Croucher and Russell, 1999). IGF-I is known to drive DNA synthesis in a number of cell types, including chondrocytes, and stimulates the chondrocytes in the serum (Kim *et al*., 2000; Elisseeff *et al*., 2001). In addition, it increases the synthesis of proteoglycan and collagen type II and thus assists cartilage formation (Loeser *et al*., 2003; Darling and Athanasiou, 2005). TGF-β1, on the other hand, is reported to enhance extracellular matrix (ECM) fabrication and stimulate cell proliferation (Guerne *et al*., 1994; Blunk *et al*., 2002). Thus, these two growth factors have significant and complementary activities in cartilage formation.

Since the times of bioavailability of these growth factors are not the same, scientists started testing combinations of growth factors released at different times to mimic this. Jaklenec *et al*. (2008) showed sequential release of bioactive IGF-I (as a mitogenic factor enhancing growth in adult cells, as well as aiding in embryonic growth and differentiation) and TGF-β1 (which induces chondrogenesis) from PLGA microsphere-based scaffolds. They reported that the ability of these scaffolds to release IGF-I and TGF-β1 sequentially was very useful in cartilage tissue engineering.

In other studies, IGF and TGF-β1 were released from poly(lactic acid-co-glycolic acid) (PLGA) and poly(*N*-isopropylacrylamide) (PNIPAM) nanoparticles, respectively, to enhance cartilage regeneration by mimicking the natural process of healing. Examples include the study of Lim *et al*. (2010), in which dual growth factor release from alginate (containing BMP-7) and polyion complex nanoparticles (containing TGF-β2) improved chondrogenesis. Similarly, the simultaneous delivery of dexamethasone and TGF-β3 from PLGA nanoparticles enhanced chondrogenesis significantly, both *in vitro* and *in vivo* (Park *et al*., 2009).

PNIPAM has been one of the most frequently studied polymers in biomedical applications. It shrinks after the lower critical solution temperature (LCST) of 32.5 °C and therefore is a good material to use as a responsive drug carrier. Since it expels its liquid contents at a temperature near that of the human body, PNIPAM has been investigated by many researchers for controlled drug delivery applications (Chung *et al*., 1999), in enzyme immobilization (Hamerska-Dudra *et al*., 2007), gene delivery (Twaites *et al*., 2005), drug delivery (Verestiuc *et al*., 2006), cell culture (Ozturk *et al*., 2009) and cell sheet engineering (Jeong *et al*., 2002; Cooperstein and Canavan, 2009). PLGA, on the other hand, is a synthetic polymer that is approved by the US Food and Drug Administration (FDA) for various biomedical applications and therefore is the most widely used polymer in biomaterials and tissue engineering (Kim and Mooney, 1998).

Chondroitin sulphate is an essential component of cartilage tissue, so its presence in the scaffold might lead to differentiation (Hardingham, 1998). Alginic acid is a linear block copolymer polysaccharide consisting of β-D-mannuronic acid and α-L-glucuronic acid residues joined by 1,4-glycosidic linkages. Dilute aqueous solutions of alginates form firm gels on addition of di- and trivalent metal ions by a cooperative process involving consecutive glucuronic residues in the α-L-glucuronic acid blocks of the alginate chain (Madan *et al*., 2009).

In this study, the effects of GF released from nanoparticles on cells seeded into 24-well plate tissue culture polystyrene (TCPS) and on three-dimensional (3D) PLGA scaffolds were determined. The nanoparticles were loaded into the scaffolds and entrapped in these locations by a coat of alginic acid, which was chosen because of its biocompatibility and biodegradability. For that purpose, PLGA scaffolds were first loaded with chondroitin sulphate to encourage the differentiation of cells by mimicking the cartilage tissue. After loading of the nanoparticles, cells were seeded and finally alginic acid was added and crosslinked to prevent the escape of the nanoparticles from the construct. Since alginic acid can be easily crosslinked in an aqueous Ca^{2+} solution, thus avoiding the use of organic solvents and any other chemical treatments that may harm and reduce bioactivity of the growth factors (Basmanav *et al*., 2008). Entrapment of both the cells and the nanoparticles under a thin coat of alginic acid is important, since this construct is planned for use in *in vivo* applications. The effects of these growth factors were studied by measuring proliferation, differentiation and immunohistochemistry.

2. Materials and methods

2.1. Isolation and culture of MSCs from bone marrow

Male Sprague–Dawley rats, 6 weeks old and weighing 150–170 g, were used as a source for bone marrow stromal cells (Torun Kose *et al*., 2003). Briefly, following euthanasia by diethyl ether inhalation, femurs were aseptically excised and the soft tissue cleaned off, then washed in Dulbecco's modified Eagle's medium (DMEM) containing 1000 units/ml penicillin and 1000 units/ml streptomycin. The marrow was flushed out with 5 ml primary medium [DMEM containing 20% fetal bovine serum (FBS), 100 units/ml penicillin and 100 units/ml streptomycin], then centrifuged at 400 $\times$ *g* for 10 min. The resulting cell pellets were resuspended in 12 ml primary medium and plated in T-75 flasks (cells from two femurs per flask) and incubated in an incubator under 5% CO_2 at 37 °C. The haematopoietic stem cells and other cells were excluded from the flasks by washing with phosphate-buffered saline (PBS, 500 mM, pH 7.4; Invitrogen, Darmstadt, Germany). DMEM–high glucose with 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml

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penicillin–streptomycin (Biochrom, Berlin, Germany) was added and the cells were incubated in a CO_2 incubator at 37 °C under 5% CO_2 and the medium was refreshed every other day. When the cells reached confluence they were detached with trypsin (0.25%)–EDTA (Invitrogen) treatment for 5 min at 37 °C. Total cell number was determined using a C-reader Automatic Cell Counter (INCYTO, Chungnam, Korea) by using C-Reader chips and cell viability stain solutions. The cells were determined using a flow cytometer (BD FACSCalibur, USA) to be mesenchymal stem cells (MSCs; data not shown).

2.2. Preparation of growth factor-loaded PLGA and PNIPAM nanoparticles

Bovine serum albumin (BSA) was used as a model protein in place of the growth factors during investigation of the release kinetics from nanoparticles. In order to encapsulate IGF-I (or the model compound BSA) in PLGA (50:50), 10% w/v nanocapsules, water-in-oil-in-water (w/o/w) system developed by Yilgor *et al* (2009) was used. Briefly, an aqueous solution of the IGF-I (100 μl, 0.1 mg/ml) was dispensed in a dichloromethane solution of PLGA (600 μl, 10 %w/v; Boehringer, Ingelheim, Germany) by probe sonication for 15 s at 50 W. This w_1/o emulsion (700 μl) was added into an aqueous solution of PVA (4% w/v, 2 ml) and sonicated (50 W, 15 s) to form the ($w_1/o/w_2$) emulsion. This double emulsion was then added into more PVA (0.3% w/v, 50 ml) and the medium was vigorously stirred overnight. The nanocapsules were collected by centrifugation (15 000 × *g*, 10 min), washed twice with Tris–HCl, pH 7.4, resuspended in distilled water and lyophilized after freezing at −80 °C.

PNIPAM polymers were prepared via free radical polymerization. *N*,*N*-methylene bisacrylamide (MBA; 0.44% w/v) and *N*-isopropylacrylamide monomer (1 g, 10% w/v) were dissolved in distilled water (10 ml). The polymerization was carried out at 65 °C for 16 h in the oven by using ammonium persulphate (APS; 0.3 ml from 5% APS stock solution) as the initiator and *N*,*N*,*N*,*N*-tetramethyl ethylenediamine (TEMED; 0.3 ml from 10% TEMED stock solution) as the accelerator under a nitrogen atmosphere. Before polymerization, N_2 gas was bubbled through the solution for a few minutes and then the system was sealed. After the completion of the reaction, the polymer was washed several times with ethanol and distilled water.

N,*N*-Methylene bisacrylamide (MBA; Merck, Darmstadt, Germany)-crosslinked PNIPAM (Sigma-Aldrich, Taufkirchen, Germany) nanoparticles carrying TGF-β1 (or the model compound BSA) were prepared by nanoprecipitation (Bayyurt, 2009). TGF-β1 was loaded into these nanoparticles by equilibrium partitioning. PNIPAM nanoparticles (10 mg) were added to an aqueous solution of TGF-β1 (0.2 ml, 5 μg/ml TGF-β1 in 4 mM HCl and 0.1% BSA). The suspension was cooled to 4 °C to allow the PNIPAM particles to absorb and was maintained in this state for 24 h. The PNIPAM container was then brought to 37 °C to get PNIPAM above its LCST and to entrap TGF-β1. The particles were centrifuged at 13 500 rpm for 20 min and the nanoparticle pellet was dried at 37 °C overnight.

2.3. Encapsulation efficiency

The encapsulation efficiency of PLGA nanocapsules was determined by dissolving the particles with dichloromethane, followed by repeated extraction with water. The protein content was then quantified using the standard Bradford assay.

Encapsulation efficiency in PNIPAM nanoparticles was calculated from the difference between the input and the unabsorbed protein in the loading medium, using the Bradford assay (Bayyurt, 2009).

2.4. Nanoparticle shape and size determination

An aqueous suspension of PLGA nanoparticles (50 μl) was added onto double sided adhesive carbon tapes. Scanning electron microscopy (SEM) stubs and the morphology of the nanoparticles were investigated by SEM (QUANTA 400 F Field Emission SEM, The Netherlands) after sputter-coating with gold; high vacuum (HV), 20.00 kV; magnification, ×50 000; working distance (WD), 9.3 mm).

The size of the particles was determined by using Image J (NIH, USA) and SEM micrographs. Size distributions were determined using a Malvern Nano ZS90 (UK) system (Yilgor *et al*., 2010).

The physical appearance of the PNIPAM particles (roundness, smoothness and formation of aggregates) was studied by SEM. Samples were prepared by spreading concentrated nanoparticle dispersions over SEM stubs. NPs were coated with gold under vacuum and were observed in a Quanta 400 F field emission SEM (FE–SEM). The size and distribution of particles were determined using the image analysis software Image J (NIH, USA) and SEM micrographs.

2.5. GF release studies

In an earlier study (Yilgor *et al*., 2009), BSA and BMPs were released from PLGA nanocapsules and the encapsulation efficiency and release kinetics were found to be similar, so in the present study the nanocapsules were expected to behave as before, presenting a similar release kinetics for the IGF-I and the BSA; therefore, in the current study, only the release kinetics of BSA were studied because of their convenience.

PLGA nanocapsules (5 mg) were placed in PBS (1 ml, pH 7.4) in Eppendorf tubes and incubated at 37 °C. At various time points (3 h and 1, 2, 4, 6, 8, 10, 15 and 21 days) the samples were centrifuged, and the released protein in the supernatant was determined using the Bradford assay, as described by the manufacturer. Briefly, 150 μl of the sample was put into a 96-well plate and 150 μl Bradford reagent was added to the wells. After 10 min at room

temperature, the absorbance at 595 nm was determined using a plate reader (Molecular Devices, USA). The absorbance was correlated with the protein concentration, using a calibration curve. The nanocapsules were then resuspended in 1 ml fresh PBS solution and incubation was continued.

The same method (Bradford assay) was used to detect BSA content and the release kinetics of the PNIPAM nanoparticles. After obtaining pellets containing BSA-loaded PNIPAM nanoparticles, as described above, supernatant was collected to determine the unloaded BSA amount, which was used to calculate the encapsulation efficiency and the loading of the PNIPAM nanoparticles. The BSA release of PNIPAM nanoparticles was investigated by placing BSA-loaded PNIPAM nanoparticles (5 mg) in PBS (1 ml, pH 7.4) in Eppendorf tubes and incubating at 37 °C. The supernatants were collected at determined time points and the protein concentrations were determined by comparing the absorbance of the solutions with a standard curve, as described above.

2.6. *In vitro* studies on TCPS with BMSCs

Cartilage differentiation medium (1 ml/well, DMEM-high glucose; Sigma-Aldrich, Taufkirchen, Germany) with 100 unit/ml penicillin-streptomycin, 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenic acid (ITS; Invitrogen, Darmstadt, Germany), 5.33 μg/ml linoleic acid, 40 μg/ml proline, 100 μg sodium pyruvate, 1.25 mg/ml BSA (Sigma-Aldrich), 50 μg/ml L-ascorbic acid and 100 nM dexamethasone (AppliChem, Darmstadt, Germany) were put into 24-well plates containing BMSC (25 000 cells/well). The effect of the growth factors on the cells was studied, using their different combinations (Table 1).

In order to direct the BMSC stem cells to cartilage differentiation, samples with cells only (OC) were incubated in cartilage differentiation medium as controls. As blanks of IGF-I-loaded PLGA nanocapsules (IP) and TGF-β1-loaded PNIPAM nanospheres (TN), growth factor-free (empty) PLGA nanocapsules (EP) and empty PNIPAM nanospheres (EN) were used. A control without nanoparticles and growth factors (OC) was also tested, along with the nanoparticle carrying samples. Each study was carried out in triplicate on days 1, 7 and 14. Final concentrations of 10 ng/ml TGF-β1 and 100 ng/ml IGF-I in the nanoparticles were achieved in the media by using appropiate amounts of nanoparticles.

Table 1. Medium contents of cell-seeded samples in a 24-well plate

Sample	PLGA nanocapsules	PNIPAM nanospheres
OC	–	–
EP	+ Empty	–
EN	–	+ Empty
IP	+IGF-I	–
TN	–	+ TGF-β1
IPTN	+IGF-I	+ TGF-β1

2.7. Determination of cell numbers

At predetermined times, the MTS test (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, WI, USA) was used to determine the number of cells (Torun Kose *et al*., 2003). MTS/PMS reagent (100 μl) was added to each well of the 24-well plate and incubated for 140 min at 37 °C in a CO_2 incubator. Absorbance was determined at 490 nm, using an Elisa Plate Reader (Bio-Tek, ELx400, USA). All experiments were repeated three times. An absorbance vs cell number calibration curve was used to calculate the cell numbers.

2.8. Determination of collagen deposition using hydroxyproline sssay

Determination of collagen deposition by the BMSCs was carried out by determining the amount of hydroxyproline, according to Pratta *et al*. (2003). The cell culture medium in which the samples incubated was discarded, 50 μl 12 M HCl was added and then the samples were incubated for 18 h at 100 °C. The hydrolysate was further incubated overnight to dry the samples in a dessicator with NaOH pellets. The residue was dissolved in 150 μl water and dried in a fume hood. Water (60 μl) was then added to each sample, followed by 20 μl assay buffer (1-propanol: water:pH 6 buffer; 3:2:10 ratio). Chloramine T reagent (40 μl, 50 mM) was added and samples were shaken for 15 min at room temperature. After the addition of DMBA reagent (2 g dimethylamino benzaldehyde, 1.25 ml 1-propanol, 2.75 ml perchloric acid), the samples were incubated for 20 min at 70 °C, then allowed to cool. Absorbances were measured at 570 nm, using a Thermo LabSystems Multiscan Spectrum microplate reader (Model 1500, USA). The data were converted to the amount of hydroxyproline (g), using a hydroxyproline standard curve. The DNA content of the samples was also determined, using Hoechst 33258, and converted to μg using a DNA standard curve, then the hydroxyproline results were normalized according to the DNA content of each sample.

2.9. Determination of glucosaminoglycan (sGAG) deposition in the scaffold-free system

Determination of sGAG deposition as an indicator of the ECM produced by the cells in culture was carried out using 1,9-dimethylmethylene blue (DMMB) assay (Müller and Hanschke, 1996). Cells (25 000 cells/well) were introduced to 1.5 ml polypropylene tubes and the samples were digested in 300 μg/ml papain in 20 mM sodium phosphate, pH 6.8, 1 mM EDTA and 2 mM dithiothreitol at 60 °C for 1 h. Digested samples (100 μl) were mixed with 200 μl DMMB solution (16 mg/l in glycine, NaCl and HCl, pH 3.0) and the absorbance was measured at 525 nm in the microplate reader. The data were converted to the amount of chondroitin sulphate, using a

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chondroitin sulphate standard curve. DNA content of the samples were also determined using Hoechst 33258 and converted to μg using the DNA standard curve, and then the sGAG results were normalized according to the DNA content of each sample.

2.10. Detection of collagen type II and aggrecan deposited in the scaffold-free system

Detection of ECM produced by the BMSCs was made using immunohistochemistry. First, the samples described in Section 2.6 were fixed in 3.7% formaldehyde solution for 1 h. For collagen type II, samples were washed with PBS and stored in 3% FBS/PBS for 10 min. The solution was removed and collagen type II mouse monoclonal primer antibody IgG_{2b} (Ab, 1.5% FBS/PBS 1:100; Santa Cruz Biotechnology, Heidelberg,Germany) was added. After incubation at room temperature for 6 h, the samples were washed with PBS. Goat anti-mouse antibody IgG–FITC (1% FBS/PBS 1:200; Santa Cruz Biotechnology) was added as the secondary Ab after washing and then the samples were incubated at 37 °C for 45 min. Unbound antibody was removed by washing with PBS.

The other ECM component, aggrecan, was stained with aggrecan rabbit polyclonal IgG Ab solution (1.5% FBS:PBS 1:200, Santa Cruz Biotechnology) after incubation in it for 6 h at room temperature. The samples were washed with PBS and secondary Ab solution, anti-rabbit AlexaFluor® 647 (1% FBS:PBS 1:200, Invitrogen) was added to each sample and the samples were incubated at 37 °C for 45 min. After the incubation, the samples were washed with PBS.

The stained samples were investigated using a confocal microscopy (Leica TCS SP2 Laser Scanning Spectral Confocal System, Wetzlar, Germany).

2.11. Preparation and characterization of PLGA scaffolds

PLGA (50:50) was dissolved in dichloromethane (0.08 g/ml). NaCl crystals (300–500 μm) were introduced and air-dried. The scaffolds were then dialysed against distilled water, frozen at −20 °C and lyophilized. The pore sizes were determined using the SEM micrographs. Degradation of the foam was carried out for 60 days by immersing the PLGA scaffolds in PBS (pH 7.4, 0.1 M, room temperature; data not shown).

2.12. Cell proliferation on PLGA scaffolds

PLGA scaffolds (7 mm diameter × 1.9 mm thickness) were placed in 24-well plates and 250 μl chondroitin sulphate (CS; 1% sodium salt from bovine trachea, ca. 70%, Sigma-Aldrich) was added to each scaffold (except the control) and then forced into the pores of the scaffold by the application of vacuum–pressure cycles with 30 s intermissions.

Different combinations of nanoparticles (PLGA, PNIPAM or both, growth factor-loaded or -free) were added to the PLGA–CS scaffolds according to Table 2 and a vacuum–pressure cycle was applied to insert the particles into the scaffolds. Each scaffold was seeded with 25 000 cells and the samples were incubated for 2 h in a CO_2 incubator (37 °C, 5% CO_2). After cell seeding, alginic acid (500 μl, 2%; Sigma-Aldrich) was introduced to the scaffolds to entrap the nanoparticles under a coat of alginic acid inside the 3D matrix, which was crosslinked with Ca^{+2}.

The scaffolds carrying chondroitin sulphate and alginate (CSA) were washed with PBS and 1 ml cartilage differentiation medium was added into each well. CSA was the control for both EP and EN. The plates were incubated under 5% CO_2 at 37 °C. Cell numbers were determined as described in the scaffold-free system.

2.13. Analysis of the total RNA with real-time PCR

Total RNA was isolated from the wells on days 1, 7 and 14 of incubation, using an RNeasy Mini Kit (Qiagen, Düsseldorf, Germany). First-strand cDNA synthesis was performed using Sensiscript Reverse Transcriptase. Sequences used for real-time PCR were; *β*-actin, forward 5′-TTCTACAATGAGCTGCGTGTG-3′, reverse 5′-GCTGGGGTGTTGAAGGTC-3′ (125 bp); collagen type II, forward 5′-TGAACAACCAGATCGAGAGCA-3′, reverse 5′-CCAGTCTCCATGTTGCAGAAG-3′ (175 bp); aggrecan, forward 5′-TTGTGACTCTGCGGGTCATC-3′, reverse 5′-GTCCCTAGGAGGGCCTTCAG-3′ (112 bp). Aggrecan primer sequences were taken from a study by Zheng *et al*. (2007) and the other primers were designed in our laboratory. All the primers were purchased from Invitrogen.

For each sample, 12.5 μl Maxima SYBR Green qPCR Master Mix (×2; Fermentas, Vilnius, Lithuania), 0.5 μl forward and reverse primer for each (from 10 μM), a 2 μl template and 9 μl distilled water were used in an iCycler™ real-time system (Bio-Rad, CA, USA). The range of relative gene dosage of aggrecan and collagen type II was determined from $2^{-\Delta\Delta Ct}$.

2.14. Detection of sGAG produced on PLGA scaffolds

sGAG production by the cells in the matrix were studied using an Alcian blue staining kit (Bio-Optica, Italy). PLGA

Table 2. Cell-seeded PLGA sponges with different contents

Sample	Chondroitin sulphate	PLGA nanocapsules	PNIPAM nanospheres	Alginate
OC	–	–	–	–
CSA	+	–	–	+
EP	+	+ Empty	–	+
EN	+	–	+ Empty	+
IP	+	+ IGF-I	–	+
TN	+	–	+TGF-*β*1	+
IPTN	+	+ IGF-I	+TGF-*β*1	+

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scaffolds were washed with distilled water and 10 drops of reagent A of the kit were added and incubated for 30 min. The solution was drained and 10 drops of reagent B were added. After 10 min the samples were washed with distilled water and 10 drops of reagent C were added. The final washing step with distilled water was followed by an examination under a light microscope (Nikon, Tokyo, Japan) and scored by at least two investigators according the matrix staining (Alcian blue stain) histological grading scale; no stain, 0; faint staining, 1; reduced staining, 2; normal staining, 3.

2.15. Statistical analysis

Statistical significance was assessed using the Mann–Whitney non-parametric U-test. Significant difference was statistically considered at the level of $p \leq 0.05$. Data analyses were performed using SPSS 19.0.

3. Results and discussion

In this study, two growth factors, IGF-I and TGF-β1, were entrapped in synthetic polymeric nanoparticles of PLGA and PNIPAM and were tested either as such or after loading into macroporous PLGA scaffolds to study their effect on tissue-engineered cartilage production through BMSC proliferation and differentiation into chondrocytes.

3.1. Nanoparticle properties

3.1.1. Nanoparticle size and distribution

PLGA nanocapsules were observed to have smooth surfaces (Figure 1a), with an average diameter of 327 ± 42 nm and a particle size range of 190–615 nm. Their wall thicknesses were measured from the SEMs to be ca. 50–70 nm.

The average particle size of PNIPAM nanospheres was 225 ± 17 nm and they had a particle size range of 205–256 nm (Figure 1b). They were also spherical but aggregation of the particles due to the drying process in SEM preparations was observed in the micrographs.

3.1.2. Encapsulation efficiency and loading

In order to determine the encapsulation efficiency, loading and release kinetics, BSA was used in place of the growth factors because this allowed us to use larger amounts in quantification and thus have higher accuracy in the detection of the protein than that of growth factors.

The encapsulation efficiency (amount loaded/initial amount) of PLGA nanocapsules was 84.75% and the loading was 0.67 μg/mg. For TGF-β1-loaded PNIPAM nanoparticles, the encapsulation efficiency and loading were 13.97% and 0.014 μg/mg, respectively.

3.1.2. BSA release from the nanoparticles

The release of BSA from PLGA and PNIPAM nanoparticles could be better represented with Higuchi kinetics. It was observed with the same PLGA nanocapsules that their release behaviour fitted the Higuchi model, with a k value of 0.0908 (Yilgor *et al*., 2009). For PNIPAM nanospheres, the Higuchi kinetic constant k was 0.155, indicating a faster release than from PLGA.

3.2. Influence of growth factor release under *in vitro* conditions on TCPS

3.2.1. Cell proliferation

The influence of simultaneous release of two growth factors from the nanoparticles (IPTN) was compared with those from nanoparticles with single growth factor (IP or TN).

The cell numbers on day 1 were generally regarded as an indicator of the level of cell adhesion on a carrier. Samples were seeded with cells (10 000 cells/well) and on day 1 they all had approximately the same number of cells (10 000–20 000 cells/well), with no indication of an influence of growth factors or the nanoparticles (Figure 2).

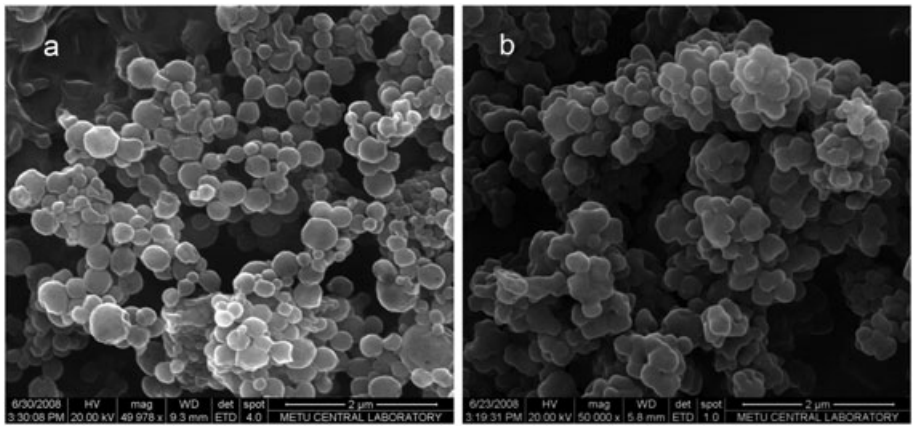

Figure 1. SEM micrographs of nanoparticles: (a) PLGA nanocapsules; (b) PNIPAM nanospheres (magnification × 50 000)

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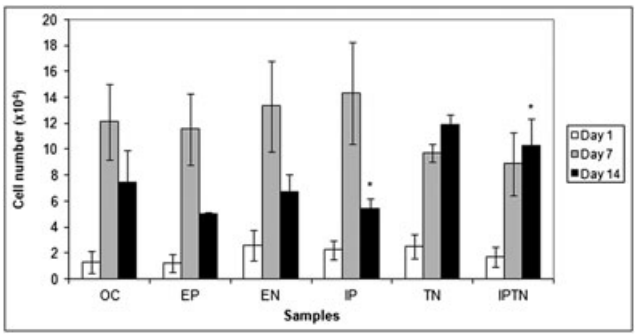

Figure 2. Cell growth determination using different growth factor combinations in the culture medium at the end of 1, 7 and 14 days of incubation, by MTS assay. Initial cell number was 10 000 cells/well. Statistical analysis was carried out for the comparison of IP and TN with EP and EN, respectively, and IPTN with IP and TN. OC, only cell; EP, empty PLGA; EN, empty PNIPAM; IP, IGF-I in PLGA; TN, TGF–β1 in PNIPAM; IPTN, IGF-I in PLGA and TGF–β1 in PNIPAM. *Statistically significant difference between IP and IPTN. Statistically significant differences are labelled for $p < 0.05$ level ($n = 3$)

The trends changed on the following days. Cell numbers on empty or IGF-I-loaded nanoparticles (OC, EP, EN and IP) reached a maximum on day 7, but all decreased towards day 14, possibly due to overgrowth, contact inhibition and detachment of the cells. The numbers were very close.

On the other hand, TGF-β1 loaded alone or with IGF-I (TN and IPTN) led to steady increase in the cell numbers throughout the 14 days of incubation. TGF-β1 is known to have an indirect mitogenic effect for stromal MSCs and is a stimulator of ECM deposition (Moses *et al*., 1991; Kay *et al*., 1998; Lee *et al*., 2006). This suggests that TGF-β1 in the medium encourages differentiation in both TN and IPTN samples, which may lead to slower cell growth than the others and does not become inhibited by overgrowth. A statistically significant difference between IPTN and IP was observed on day 14.

3.2.2. Total collagen formation

This assay was carried out to determine the amount of collagen secreted by the cells under the influence of the various combinations of the growth factors. After 1 day of incubation, all the samples except IP showed very low collagen production. The highest collagen formation was observed on day 7 for all samples throughout 14 days of incubation, except for IP (Figure 3). Some increase in collagen formation in the TN-containing samples and in the control (OC) was also detectable. Collagen formation on IP was higher than the others at day 7 and continued to increase until day 14. On the other hand, collagen production decreased substantially in all samples except IP by day 14.

The collagen production levels of both growth factor-carrying samples were significantly higher than their GF-free controls but lower than IP (single growth factor IGF-I). This was expected, because IGF-I is known to stimulate collagen production (Sonal, 2001; Olesen *et al*., 2007). Worster *et al*. (2001) also found that collagen type II deposition was evident in IGF-I-treated mesenchymal stem cell progenitor cultures.

3.2.3. sGAG determination

The sGAG production by bone marrow stem cells' growth in medium supplemented with different growth factors was determined by DMMB assay. On day 1, all samples except the control showed some degree of sGAG formation (Figure 4). After 7 days in culture, there was an increased sGAG formation by cells in OC-, EN-, IP- and IPTN-treated

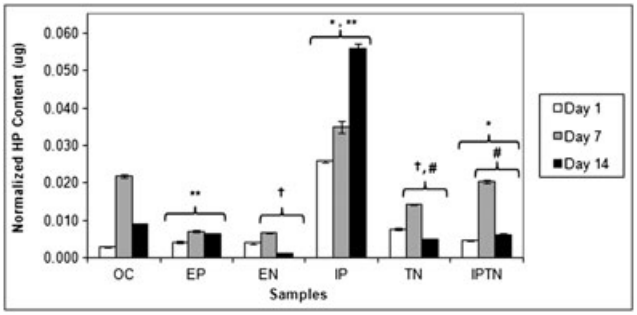

Figure 3. Total collagen production by cells in the presence of different growth factors at the end of 1, 7 and 14 days of culture, by hydroxyproline assay. Statistical analysis was carried out for the comparison of IP and TN with EP and EN, respectively, and for the comparison of IPTN with IP and TN. OC, only cell; EP, empty PLGA; EN, empty PNIPAM; IP, IGF-I in PLGA; TN, TGF–β1 in PNIPAM; IPTN, IGF-I in PLGA and TGF-β1 in PNIPAM. Statistical analysis was carried out for the comparison of EP–IP, EN–TN, IP–IPTN and TN–IPTN, which are denoted **, †, * and #, respectively. Significant differences are labelled for $p < 0.05$ level ($n = 3$)

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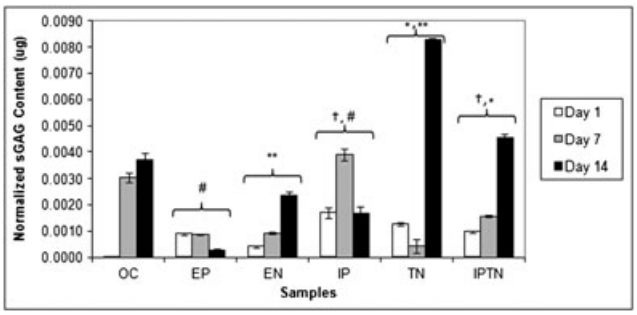

Figure 4. sGAG formation by cells in the presence of different growth factors at the end of 1, 7 and 14 days, by DMMB assay. Statistical analysis was carried out for the comparison of IP and TN with EP and EN, respectively, and IPTN with IP and TN. OC, only cell; EP, empty PLGA; EN, empty PNIPAM; IP, IGF-I in PLGA; TN, TGF–β1 in PNIPAM; IPTN, IGF-I in PLGA and TGF–β1 in PNIPAM. Significant differences are labelled for $p < 0.05$ level ($n = 3$). #, **, † and * indicate statistically significant differences between EP–IP, EN–TN, IP–IPTN and TN–IPTN, respectively

samples. In the following week (14 days), however, the sGAG production level was substantially improved in samples EN, TN and IPTN, with TN showing the highest value.

IGF-I- or TGF-β1-added samples showed statistically significant higher sGAG production than their GF-free controls.

The highest levels of sGAG formation were observed in TGF-β1-carrying TN and IPTN samples. There have been several publications reporting a similar effect of TGF-β1 on aggrecan deposition (Kudo *et al*., 2001; Yamanishi *et al*., 2002). Worster *et al*. (2001) found that medium GAG content in MSC and chondrocyte monolayer cultures was significantly (1.9–3.3-fold) increased above controls by TGF-β1 treatment in 6 day cultures.

In the present study, the empty nanoparticles showed the same behaviour on differentiation as in the case of hydroxyproline assay. The presence of sGAG in the absence of growth factors in the OC samples was probably due to incubation in the cartilage differentiation medium.

3.2.4. Confocal microscopy for collagen type II and aggrecan

ECM deposition by BMSC was determined by confocal microscopy of samples double-stained for both collagen type II and aggrecan. Articular cartilage isolated from rat knee joint was used as the positive control. On day 7, IP, TN and IPTN exhibited more collagen type II and aggrecan deposition than the only cell control, OC (Figure 5b–e). On day 14, collagen type II and aggrecan secreted by OC, IP, TN and IPTN were higher (Figure 5f–i), whereas TN and IPTN had still higher deposition than OC.

It was observed that collagen type II was deposited around the nucleus in the positive control, which was rat articular cartilage (Figure 5a). The negative control (OC) (Figure 5b, c) showed high collagen type II expression, as was also found in the hydroxyproline assay (Figure 3), but the collagen deposition around the nucleus was not the same as it was in the positive control (Figure 5a). On the other hand, TN and IPTN showed similar accumulation patterns of collagen type II around the nucleus with articular cartilage cells. Moreover, TN showed high amounts of aggrecan deposition, which supports the data of the DMMB assay. Johnstone *et al*. (1998) reported the stimulation of cartilage-specific proteoglycan production by TGF-β1. Connelly *et al*. (2008) also reported the promotion of aggrecan gene expression and sGAG accumulation of BMSCs by TGF-β1.

3.3. *In vitro* studies of cells on scaffolds

3.3.1. Properties of the PLGA scaffolds

The scaffolds were made with PLGA, chondroitin sulphate and alginic acid, with the main frame being PLGA. The porosity and degradation rate of PLGA scaffolds were studied before starting the cell-seeding experiments. The pore size and distribution are quite important parameters for cell growth inside the scaffolds. The pores of the PLGA scaffold were evenly distributed and their sizes were in the range 200–400 μm, large enough for cell penetration and growth. An *in situ* degradation study performed on a cell-free scaffold in PBS at pH 7.4 showed that at the end of 40 days of incubation, $>$ 90% (by weight) of the PLGA foams were degraded (data not shown). Degradation was accompanied by a pH decrease to 2.6, probably due to the release of lactic acid, glycolic acid and their oligomers.

Chondroitin sulphate introduced into the scaffold is also an essential component of natural cartilage tissue. According to Hardingham (1998), its presence in the scaffold might lead to differentiation. Thus CS could help the differentiation of MSCs into chondrocytes. The final component, alginic acid, was chosen because of its biocompatibility. Thus, the three-component system, with its degradability, biocompatibility and large pores, was an appropriate carrier for MSCs.

3.3.2. Cell proliferation on PLGA scaffolds

The cell number on day 1 was lower in all the samples than the initial cell-seeding density (25 000 cells/well), indicating that not all cells had adhered (Figure 6). There was no significant increase in cell numbers in the control OC throughout the 14 days of incubation, probably due to the medium being a cartilage differentiation medium. The amount of cells on CSA and IP increased throughout 7 days of incubation and then decreased towards day 14.

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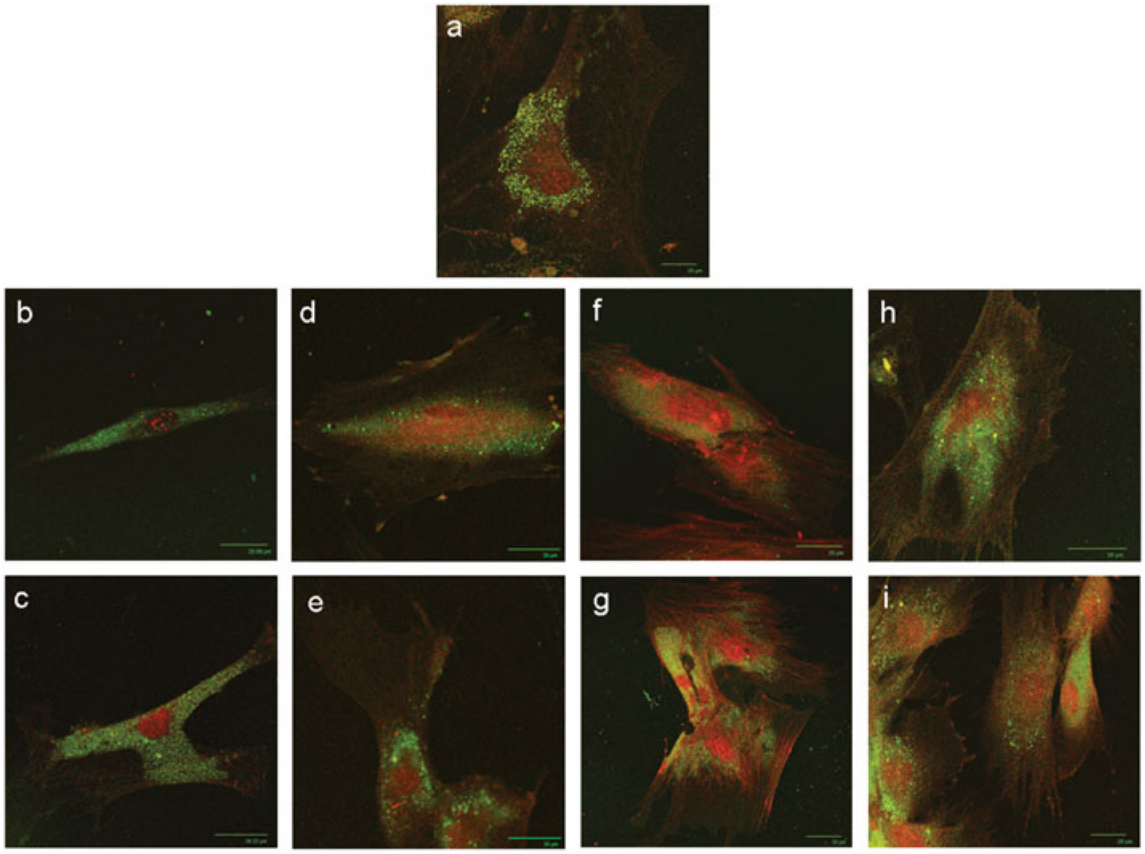

Figure 5. ECM deposition by cells grown in different culture media. Collagen type II (green) and aggrecan (red) double staining: (a) rat knee joint articular cartilage; (b, d, f, h) OC, IP, TN, IPTN at the end of 7 days of incubation; (c, e, g, i) OC, IP, TN, IPTN at the end of 14 days of incubation (magnification × 63)

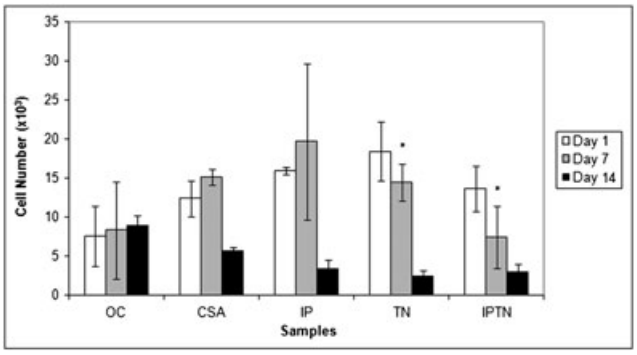

Figure 6. Cell growth determination in different culture media at the end of 1, 7 and 14 days of incubation on PLGA scaffolds, by MTS assay. OC samples do not contain CSA. Initial cell number was 25 000 cells/well. OC, only cell; CSA, chondroitin sulphate and alginate added control; EP, empty PLGA; EN, empty PNIPAM; IP, IGF-I in PLGA; TN, TGF–β1 in PNIPAM; IPTN, IGF-I in PLGA and TGF–β1 in PNIPAM. *Statistical significance difference between TN and IPTN. Significant differences are labelled for $p < 0.05$ level ($n = 3$)

A similar trend was observed in cell proliferation on TCPS. However, TN and IPTN showed a continuous decrease through days 7 and 14. This observation is not in agreement with the results on TCPS (Figure 2), where both TN and IPTN had shown continuous increase throughout the 14 day incubation. A reason for this decrease could be the effect of chondroitin sulphate (CS), which was not included in the OC and TCPS samples, where no carrier, and therefore no CS, was used. CS in known to encourage the differentiation of cells and differentiation is known to decrease proliferation. It was reported that the addition of chondroitin sulphate (CS) in to a polyethylene glycol (PEG)-based hydrogel increased chondrogenic differentiation of goat MSCs (Choi *et al*., 2010). Sechriest *et al*. (2000) also showed that CS in the scaffold promoted the secretion of proteoglycan and type II collagen, both of which are indicators of differentiation.

3.3.3. Collagen type II and aggrecan expression

Real-time PCR was used to investigate the expression levels of cartilage differentiation markers, such as

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collagen type II and aggrecan. IPTN exhibited the most collagen type II expression at all time points (Figure 7a); especially after 14 days, collagen type II expression of IPTN increased significantly.

For aggrecan, real-time PCR showed that higher aggrecan expressions were observed on days 1 and 14 (Figure 7b); especially IPTN had significantly higher expression levels at the end of 14 days. Thus, IPTN led to the highest expression of both collagen and aggrecan.

Collagen type II is the major component of hyaline cartilage (Pulkkinen *et al*., 2008) and aggrecan is a major proteoglycan in the articular cartilage, providing the scaffold with a hydrated gel structure that enables the cartilage to carry loads (Kiani *et al*., 2002). The highest collagen type II and aggrecan expressions were in IPTN samples. In the samples tested on TCPS, the IP had produced the largest amount of total collagen and TN produced the highest sGAG. However, when the 3D PLGA scaffolds were used, the IPTN group had the highest amount of both the collagen type II and aggrecan gene expression. Since the IPTN sample had both IP and TN, their synergic effect had apparently made a positive impact on differentiation of the stem cells.

In principle, the activities of chondrocytes are observed when they are placed in a proper 3D environment. During the development and growth of cartilage, the chondrocytes produce abundant ECM (mainly collagen type II and sGAG), encase themselves and are eventually separated from each other (Yamaoka *et al*., 2006). 3D scaffolds provide a suitable environment for such activities: they also allow for controlled local delivery of bioactive agents, such as polypeptides or chemical molecules, that stimulate cartilage-like tissue formation (Kuo *et al*., 2006). These are all absent in a 2D cell carrier, which is the scaffold-free TCPS system in this study.

3D scaffolds have some specific advantages, such as porosity for cell attachment and migration inside of the material. They provide a 3D framework to support the tissue or

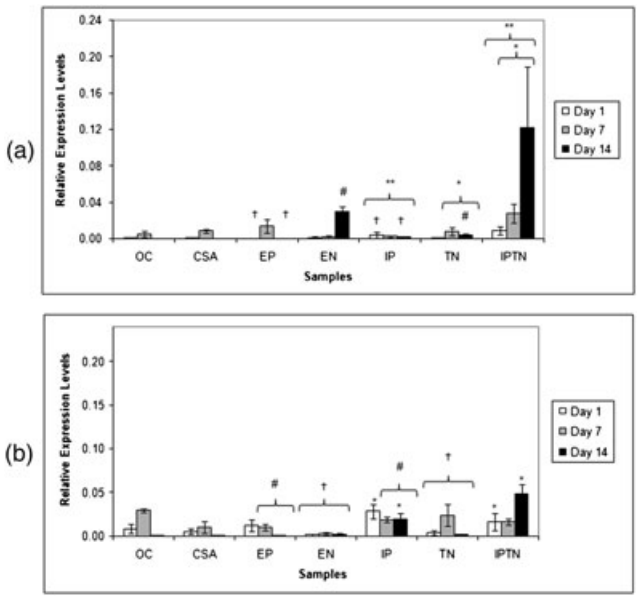

Figure 7. Real-time PCR results for samples on the matrix for three time points: (a) collagen type II; (b) aggrecan marker proteins. OC, only cell; CSA, chondroitin sulphate and alginate added control; EP, empty PLGA; EN, empty PNIPAM; IP, IGF-I in PLGA; TN, TGF–β1 in PNIPAM; IPTN, IGF-I in PLGA and TGF–β1 in PNIPAM. Statistical analysis was carried out for the comparison of EP–IP, EN–TN, IP–IPTN and TN–IPTN, which are denoted (a) †, **, ‡, # and *; (b) #, †, * and **, respectively. Significant differences are labelled for $p < 0.05$ level ($n = 3$)

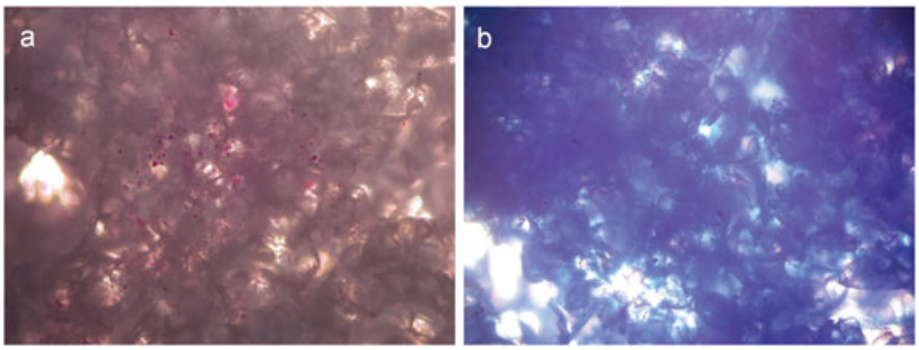

Figure 8. sGAG formation of IPTN samples in the PLGA scaffolds, as shown by Alcian blue (stains chondroitin sulphate blue) and nuclear fast red (stains the nucleus red) staining at the end of: (a) 1 day; (b) 14 days of incubation (magnification × 4)

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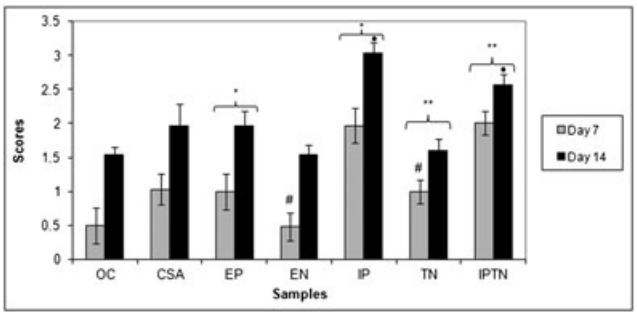

Figure 9. sGAG formation of cells with different culture media in PLGA scaffolds at the end of 7 and 14 days of incubation, by Alcian blue staining. OC, only cell; CSA, chondroitin sulphate and alginate added control; EP, empty PLGA; EN, empty PNIPAM; IP, IGF-I in PLGA; TN, TGF–β1 in PNIPAM; IPTN, IGF-I in PLGA and TGF–β1 in PNIPAM. Statistical analysis was carried out for the comparison of EP–IP, EN–TN, IP–IPTN and TN–IPTN, which are denoted *, #, • and **, respectively. Significant differences are labelled for the $p < 0.05$ level ($n = 3$)

cells. The scaffold not only provides mechanical support but must also supply critical nutrients and transport metabolites to and from the developing tissue (Fauci *et al*. 2008). Important scaffold properties vary, depending on the tissue, but typically include specific biomechanical properties, porosity, biocompatibility and appropriate surface characteristics for cell adhesion and differentiation.

3.3.4. sGAG localization in the cells

sGAG in the cells was studied using light microscopy. The cell body was stained blue with Alcian blue and the nucleus was stained red with nuclear fast red. On day 1, only the nuclei of all the samples were stained red, which meant that neither of the samples produced sGAG. After 7 days in culture there was sGAG production in all the samples. Figure 8 shows Alcian blue-stained cells on IPTN. Also, the highest sGAG content was observed on IP and IPTN samples (Figure 9).

4. Conclusion

In this study, the influence of growth factors IGF-I and TGF-β1 released from biodegradable nanoparticles was studied on TCPS and on PLGA scaffolds carrying these nanoparticles. It was seen that samples carrying both nanoparticles led to the best results with respect to cartilage differentiation results on the scaffolds. It thus appears that use of growth factor-loaded nanoparticles could have a significant contribution for cartilage tissue engineering.

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