ARTICLE

Evaluation of Alginate and Hyaluronic Acid for Their Use in Bone Tissue Engineering

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Abstract In this study, we compared the structural and physicochemical properties of different concentrations of alginate and high molecular weight hyaluronic acid (HA) hydrogels and their biocompatibility and bioactivity after long-term culture with MC3T3-E1 cells. Both hydrogels were biocompatible and supported long-term viability and cell proliferation. Alginate induced higher alkaline phosphatase (ALP) activity levels than HA. Calcium content was increased in concentration dependent manner in cells cultured with alginate compared to control. Culture with HA hydrogels reduced alkaline phosphatase (*Alp*), bone sialoprotein (*Bsp*) and osteocalcin (*Oc*), while alginate increased *Oc* mRNA levels. Unmodified alginate hydrogels supported osteoblast differentiation better than HA hydrogels, suggesting that alginates are more suitable for biomaterial applications in bone tissue engineering.

1 Introduction

Hydrogels have been used in a wide variety of tissue engineering applications [1]. Hydrogels show excellent biocompatibility, probably due to their structural similarity to the macromolecular-based components in the body [2]. Its high, tissue-like water content and porous structure allows the influx of low molecular weight solutes and nutrients crucial to cellular viability, as well as the transport of cellular waste out of the hydrogel [3]. As biomaterials, the use of injectable hydrogels also allows the

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administration of the material through minimally invasive techniques, to fill any area with a good physical integration into the defect, to incorporate cells or various therapeutic agents (e.g., growth factors) with a facile and homogenous distribution within any defect [4–7]. Therefore, hydrogels have been identified to be suitable as bone and cartilage repair materials because they can be carriers for growth and morphogenetic factors to exert host cell chemotaxis, proliferation, differentiation and new tissue formation at the site of injury or defect [8].

A variety of synthetic or natural polymers have been used in bone tissue engineering as delivery vehicles for cells or growth/morphogenetic factors [e.g., transforming growth factors, bone morphogenetic proteins (BMPs)] [8–10]. Among them, sodium alginate and hyaluronic acid (HA) have been widely used for tissue-engineering approaches, due to excellent biocompatibility and biodegradability [11–13].

Alginate is a linear unbranched polysaccharide composed of 1,4-linked β-D-mannuronic acid (M-block) and α-L-guluronic acid (G-block) [12], extensively studied in tissue engineering, including the regeneration of skin, cartilage, bone, liver and cardiac tissue [11]. It has previously been reported from in vitro studies that modified alginate hydrogels with RGD-sequences [14], with immobilization of osteogenic peptides [15], with BMP-2 [16] or in combination with other polymers [17] support cell attachment, cell proliferation, osteogenic differentiation and mineral deposition [14, 15, 17]. Further, in vivo studies have shown that chitosan–alginate gel alone or encapsulating MSCs and BMP-2 and that alginates with modified-peptides allow cell differentiation and an early calcification in vivo [15, 17, 18].

HA is a natural linear polysaccharide consisting of repeating D-glucuronic acid-β-1,3-*N*-acetyl-D-glucosamine-β-1,4 units

[13, 19]. HA is one of the main components of the extracellular matrix present in all connective tissues [20] and involved in a variety of biological functions [5, 21], including direct receptor-mediated effects on cell adhesion, growth and migration [22], as well as acting as a signaling molecule in cell mortality [23], inflammation [24] or wound healing [25]. HA hydrogels have been used as vehicle for delivery of BMP-2 in vitro [5, 8] and in vivo [21], as well for the delivery of biphosphonates [21]. In vitro studies using derivatized hyaluronic acid (Hyffa-11) have shown to support cell attachment and growth, and to induce ALP activity and osteocalcin expression in a murine fibroblast cell line when containing rhBMP-2 [8]. Moreover, when containing bFGF, HA hydrogels enhance calcium deposition, osteopontin and BSP expression and decrease alkaline phosphatase in rat bone marrow stromal cells [26]. In vivo, HA hydrogels containing BMP-2 showed increased bone formation [19, 27].

Although several modified sodium alginate and HA hydrogels have been used as osteogenic bone substitutes, a direct comparison of the effect on osteoblast differentiation of high molecular weight HA and alginate hydrogels has never been reported. The aim of this study was to compare the structural and chemical properties of two natural polymers at different concentrations and their biocompatibility and bioactivity in the pre-osteoblastic cell line MC3T3-E1. The present work demonstrates that alginate hydrogels support osteoblast differentiation better than high molecular weight hyaluronic acid hydrogels, pointing to alginates as a more suitable polymer for biomaterial applications in bone tissue engineering.

2 Materials and Methods

2.1 Preparation of the Hydrogels

Alginate (FMC Biopolymers, Protanal LF200M, Norway) and hyaluronic acid (HA) (Bioibérica, F002103, Mw 800–1,200 kDa, Spain) hydrogels were prepared overnight at 25 °C in Phosphate Buffer Saline (PBS) (240–320 mOsmol/kg) at 1, 2 and 3 % (w/v). Preliminary studies evaluating different media [water, saline solution 0.9 % (w/v) (308 mOsmol/kg), Phosphate Buffer Saline (PBS) (240–320 mOsmol/kg) and culture media] and several alginate concentrations [1, 2, 3, 6 % y 10 % (w/v)], showed that 1, 2 and 3 % alginate dissolved in PBS were the most promising formulations for their use in tissue engineering due to their pH (pH 7.0) and their viscosity values (0.3, 0.6 and 6.8 Pas at 1, 2 and 3 % of alginate concentration respectively) (data not presented).

To evaluate long term stability of the resulting hydrogels, freeze-drying studies were performed. More specifically, 1 mL of polymeric hydrogels (alginate or HA) were frozen at −80 °C, and then freeze-dried during at least 72 h. Final freeze-dried products were dissolved in water by simple agitation with vortex, and their macroscopic aspect evaluated.

2.2 Characterization of the Hydrogels

The effect of polymer concentration, pH and temperature on viscosity of the hydrogels was studied. Viscosities were determined using a R5 spindle and stirring at 200 rpm by using a Visco Star R viscosimeter (JP Selecta, Spain). The studies were performed three times and each sample analyzed in triplicate (n = 9).

The equilibrium swelling ratio (ESR) of the hydrogels was evaluated as follows: 1 mL of alginate or HA hydrogel at different concentrations was incubated in PBS at 37 °C. At prefixed time points (0.5, 1, 3 and 24 h) each sample was centrifuged at $16,000 \times g$ for 15 min. The supernatants were discarded, and the wet gels immediately weighted (Ws). Then, hydrogels were frozen at −80 °C and freeze-dried during at least 72 h. Lyophilized products were weighted again (Wd) in order to determine ESR values following the formula showed below. The experiment was performed in triplicate (n = 3). The swelling ratios of the resulting gels were determined using the following equation: ESR = (Ws − Wd)/Wd.

Qualitative determination of the hydrogel structure at each time point of incubation was carried out by scanning electron microscope (SEM, Hitachi S-3400N, Hitachi High-Technologies Europe GmbH, Krefeld, Germany) to evaluate the changes in the gel network and the effects of polymer concentration on the network structure (pore size and pore distribution). Briefly, after prefixed time points, the hydrogels were frozen at −80 °C and freeze-dried during at least 72 h. Samples were then frozen in liquid N_2 to allow an accurate transversal section using a sharp scalpel and observed at 15 kV, 40 Pa and $100\times$ of magnification. Pore size was measured along the largest axis of the pore by using Hitachi S-3400N software in at least two different gels, and two images per gel were scanned.

2.3 Cell Culture

The mouse osteoblastic cell line MC3T3-E1 (DSMZ, Braunschweig, Germany) was maintained in α-MEM (PAA Laboratories GmbH, Pasching, Austria), which contains ascorbic acid (45 μg/ml) and sodium dihydrogen phosphate (140 mg/l), and supplemented with 10 % fetal bovine serum (FBS) (PAA Laboratories GmbH, Pasching, Austria) and antibiotics (100 IU penicillin/ml and 100 μg streptomycin/ml) (PAA Laboratories GmbH, Pasching, Austria) under standard cell culture conditions (at 37 °C in a

humidified atmosphere of 5 % CO_2). Cells were subcultured 1:10 before reaching confluence using PBS and trypsin/EDTA.

Cells were seeded onto polyethylene terephthalate (PET) membrane inserts with a pore size of 1 μm (1×10^4 cells/membrane) (Millipore Corporation, Billerica, MA, USA) and were maintained in α-MEM supplemented with 10 % FBS and antibiotics (Fig. 1). After cells reached confluence, alginate or HA hydrogels at different concentrations were added on cells and culture media was added at the basolateral side. In the control group, PBS was added on cells growing on PET membrane inserts instead of hydrogel. Media was changed every 2–3 days in the basolateral side. After 24 h, culture media was collected to test cytotoxicity (LDH activity). Cells were harvested at day 21 and the number of cells, gene expression of osteoblast differentiation markers, calcium content and ALP activity were analysed.

2.4 Cell Morphology by Confocal Microscopy

To validate viability of cells growing in contact to alginate, cell morphology after 24 h of incubation with alginate hydrogel was observed by confocal microscopy (Leica TCS SPE Microsystems Wetzlar GmbH, Wetzlar, Germany). Briefly, 0.5 mL of cell suspension (20,000 cells/well) were seeded on 24 well plates, and cultured for 24 h before treatment. Then, culture media was replaced by a mixture (50:50) of culture media/1 % or 2 % (w/v) alginate hydrogel in PBS, and incubated for additional 24 h. Next, the monolayers were stained with FITC-phalloidin (P5282, Sigma Aldrich, St. Quentin Fallavier, Germany) and DAPI (F6057 St. Quentin Fallavier, Germany).

2.5 Cell Viability Determination

LDH activity in the culture media was used as an index of cell toxicity. The activity of the cytosolic enzyme was
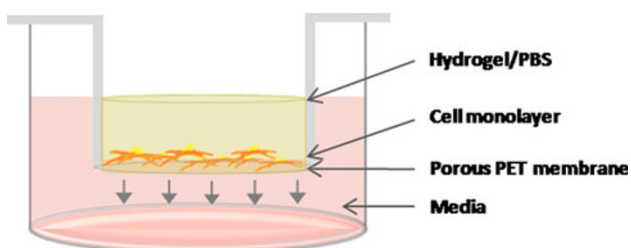

Fig. 1 Schematical drawing showing experimental setup with the MC3T3-E1 cells seeded onto polyethylene terephthalate (PET) membrane inserts and the addition of hydrogel or PBS to the different experimental groups analyzed in the study. Also indicated is the outer compartment with the cell culture media that was changed every 2–3 days in the basolateral side

estimated according to the manufacturer's kit instructions (Roche Diagnostics, Mannheim, Germany), by assessing the rate of oxidation of NADH at 490 nm in presence of pyruvate. Results from all the samples were presented relative to the LDH activity in the medium of cells treated with PBS (low control, 0 % of cell death) and of cells treated with PBS containing 1 % Triton X-100 (high control, 100 % cell death). The percentage of LDH activity was calculated using the following equation:
Cytotoxicity (%) = (exp.value − low control)/ (high control − low control) × 100.

2.6 ALP Activity Determination

In order to compare the effect of different hydrogels on osteoblast differentiation, ALP activity was quantified from cell monolayers after 21 days of cell culture. Briefly, hydrogels were discarded and cells were washed twice in PBS and solubilised with 0.1 % Triton X-100. Then, samples were incubated with an assay mixture of p-Nitrophenyl Phosphate (pNPP). Cleavage of pNPP (Sigma, Saint Louis, Missouri, USA) in a soluble yellow end product which absorbs at 405 nm was used to assess ALP activity. In parallel to the samples, a standard curve with calf intestinal alkaline phosphatase (CIAP) (Promega, Madison, USA) was constructed; 1 μl from the stock CIAP was mixed with 5 ml of alkaline phosphatase buffer (1:5,000 dilution), and subsequently diluted 1:5.

2.7 Calcium Content Determination

Total calcium content was quantified after 21 days of cell culture. Cells were washed twice in PBS and solubilised with 0.1 % Triton X-100. Lysates were also treated with 0.5 N hydrochloric acid overnight, followed by centrifugation at $500 \times g$ for 2 min for the subsequent determination of Ca^{2+} content in the supernatant by inductively coupled plasma optical emission spectrometer (ICP-OES) Optima 5300 DV (PerkinElmer, Massachusetts, USA). Data were compared with $CaCl_2$ standards included in the assay.

2.8 Cell Number Determination

To assess the effect of the hydrogels on cell number, the DNA content after 21 days of cell culture was determined. DNA content was isolated using Tripure® (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. DNA pellets were dissolved using TE buffer and quantified at 260 nm using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The number of cells was calculated taking into account that 5.4 μg of DNA are equivalent to 1×10^6 murine cells.

2.9 RNA Isolation and RT-PCR Analysis

The effect of different types of hydrogels on gene expression was studied after 21 days of culture on pre-osteoblast MC3T3-E1 cells.

Briefly, hydrogels were discarded and total RNA was extracted using Tripure®, following the manufacturer's protocol. Total RNA was quantified at 260 nm using a nanodrop spectrophotometer and 350 ng of RNA were reverse transcribed to cDNA at 37 °C for 60 min using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA), according to the protocol of the supplier. Aliquots of each cDNA were frozen (-20 °C) until the PCR reactions were carried out.

Real-time PCR was performed in the Lightcycler 480® (Roche Diagnostics, Mannheim, Germany) using SYBR green detection. Real time PCR was done for two reference genes [18S and glyceraldehyde-3-phosphate dehydrogenase (Gapdh)] and three target genes [bone sialoprotein (Bsp), alkaline phosphatase (Alp) and osteocalcin (Oc)].

The primer sequences were as follows: 18 s rRNA-F: 5′-GTAACCCGTTGAACCCCATT-3′; 18 s rRNA-R: 5′-CCATCCAATCGGTAGTAGCG-3′; Gapdh-F: 5′-ACCCAGAAGACTGTGGATGG-3′; Gapdh-R: 5′-CACATTGGG-GGTAGGAACAC-3′; Bsp-F: 5′-GAAAATGGAGACGGCGATAG-3′; Bsp-R: 5′-ACCCGAGAGTGTGGAAAGTG-3′; Alp-F: 5′-AACCCAGACACAAGCATTCC-3′; Alp-R: 5′-GAGAGCGAAGGGTCAGTCAG-3′; Oc-F: 5′-CCGGGAGCAGTGTGAGCTTA-3′; Oc-R: 5′-TAGATGC-GTTTGTAGGCGGTC-3′.

Each reaction contained 7 µl Lightcycler-FastStart DNA MasterPLUS SYBR Green I (containing Fast Start Taq polymerase, reaction buffer, dNTPs mix, SYBRGreen I dye and MgCl2), 0.5 µM of each, the forward and the reverse specific primers and 3 µl of the cDNA dilution in a final volume of 10 µl. The amplification program consisted of a preincubation step for denaturation of the template cDNA (10 min 95 °C), followed by 45 cycles consisting of a denaturation step (10 s 95 °C), an annealing step (8–10 s 60 °C, except for Alp that was 8 s 65 °C) and an extension step (10 s 72 °C). After each cycle, fluorescence was measured at 72 °C (λex 470 nm, λem 530 nm). A negative control without cDNA template was run in each assay.

Real-time efficiencies were calculated from the given slopes in the LightCycler 480 software using serial dilutions, showing all the investigated transcripts high real-time PCR efficiency rates, and high linearity when different concentrations are used. PCR products were subjected to a melting curve analysis on the LightCycler and subsequently 2 % agarose/TAE gel electrophoresis to confirm amplification specificity, Tm and amplicon size, respectively.

Relative quantification after PCR was calculated by dividing the concentration of the target gene in each sample by the mean of the concentration of the two reference genes in the same sample using the Advanced relative quantification method provided by the LightCycler 480 analysis software version 1.5 (Roche Diagnostics, Mannheim, Germany).

2.10 Statistical Analyses

Data are presented as mean values ± SEM or mean values ± SD. A Kolmogorov–Smirnov test was done to assume parametric or non-parametric distributions for the normality tests; differences between groups were assessed by Mann–Whitney-test or by Student t test depending on their normal distribution. SPSS® program for windows (Chicago, IL), version 17.0 was used. Results were considered statistically significant at p values $\leq$ 0.05.

3 Results

3.1 Characterization of the Hydrogels

Table 1 shows the effect of temperature and concentration of the alginate and HA hydrogels on viscosity values. Both polymers studied showed a significant increase on viscosity values in parallel to higher polymer concentrations. In addition, a significant reduction of the viscosity values was observed with higher temperatures, except for alginate 2 % where, although a decrease in viscosity was obtained at 37 °C compared to 25 °C, data did not reach statistical significance. Similar pH values were obtained in alginate hydrogels either at 1 and 2 % of polymer concentration; significant lower pH values were found for higher polymer concentrations for all the HA hydrogels tested in this study.

3.2 Swelling Studies of Alginate and HA Hydrogels

Quantitative ESR and pore size results of freeze-dried 1 and 2 % alginate and HA hydrogels are shown in Fig. 2a. Swelling happened immediately after dilution in PBS at 37 °C for any of the polymers and for any of the concentrations studied. The ESR capacity was higher in 1 % alginate compared to 1 % HA at any of the time points studied. However, when comparing differences on ESR capacity between both hydrogels at a concentration of 2 %, only after 3 h of incubation with PBS the ERS was significantly higher in 2 % alginate compared to 2 % HA. Further, whereas no differences in ERS were observed when comparing alginate polymer concentration at the different time points tested, an increase in HA polymer concentration was associated with significantly higher swelling capacity at any of the time points tested.

Table 1 Characterization of alginate and hyaluronic acid (HA) hydrogels

Polymer concentration	Alginate		HA	
	1 %	2 %	1 %	2 %
pH	7.10 ± 0.053	7.06 ± 0.049	7.30 ± 0.017	6.72 ± 0.032^a
Viscosity at 25 °C (Pas)	0.324 ± 0.061	0.639 ± 0.080^a	0.107 ± 0.020	0.966 ± 0.238^a
Viscosity at 37 °C (Pas)	0.186 ± 0.040^b	0.507 ± 0.099^a	0.071 ± 0.019^b	$0.680 \pm 0.152^{a,b}$

pH values of different hydrogels obtained in PBS at 25 °C (n = 3) and viscosimetric measures of alginate and HA hydrogels at 1 and 2 % (w/v) obtained in PBS, at 25 °C and at 37 °C (n = 9). Values represent the mean ± SD. Differences between groups were assessed by Mann–Whitney-test or by Student t test depending on their normal distribution. Results were considered statistically significant at p values ≤ 0.05 for each polymer

[a] 1 versus 2 %

[b] 25 versus 37 °C

a   Polymer

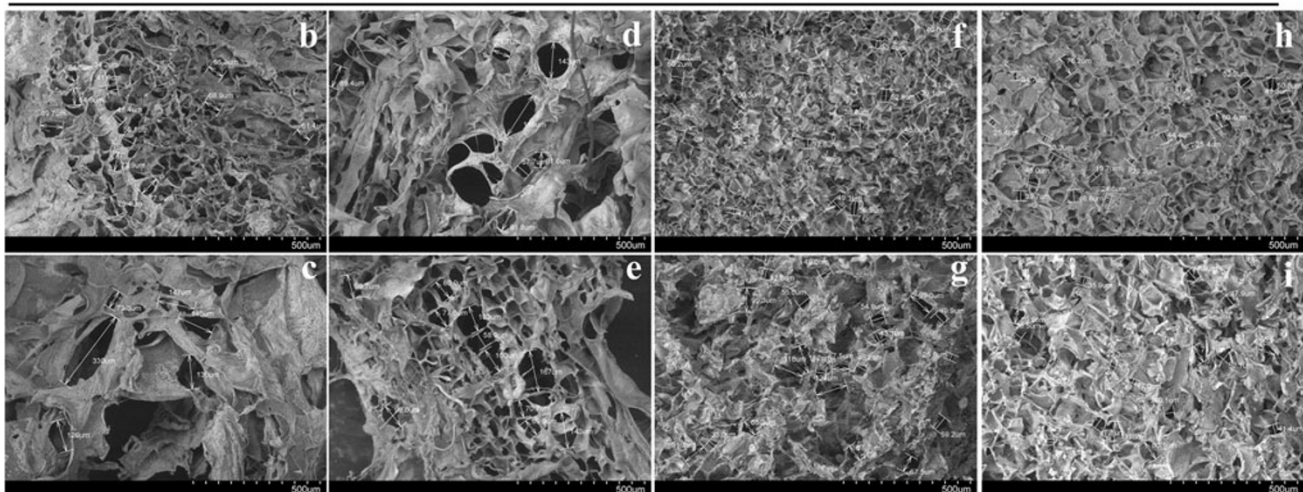|	Alginate				Hyaluronic acid			
	1%		2%		1%		2%	
Time (h)	ESR	Pore Size (µm)	ESR	Pore Size (µm)	ESR	Pore Size (µm)	ESR	Pore Size (µm)
0,5	$0,909 \pm 0,112$	$74,5 \pm 12,44$	$0,878 \pm 0,091$	$74,4 \pm 15,63$	$0,193 \pm 0,108^a$	$45,8 \pm 10,31^a$	$0,891 \pm 0,053^b$	$31,2 \pm 9,18^{ab}$
1	$0,896 \pm 0,053$	$75,1 \pm 13,50$	$0,875 \pm 0,226$	$96,5 \pm 7,79^b$	$0,084 \pm 0,040^a$	$61,2 \pm 18,48$	$0,864 \pm 0,155^b$	$48,2 \pm 7,33^a$
3	$0,894 \pm 0,026$	$77,7 \pm 27,31$	$0,862 \pm 0,061$	$69,2 \pm 17,58$	$0,157 \pm 0,074^a$	$78,5 \pm 23,20$	$0,556 \pm 0,087^{ab}$	$48,1 \pm 5,41^{a\,b}$
24	$0,857 \pm 0,038$	$120,7 \pm 30,00$	$0,846 \pm 0,062$	$79,5 \pm 11,28^b$	$0,149 \pm 0,045^a$	$90,7 \pm 22,73^a$	$0,726 \pm 0,074^b$	$52,9 \pm 15,67^{a\,b}$

Fig. 2 Equilibrium of swelling ratio of 1 or 2 % of alginate and hyaluronic acid hydrogels obtained after incubation in PBS at 37 °C. **a** This table represents the evolution of ESR and pore size of 1 and 2 % (w/v) alginate and HA hydrogels after incubation in PBS at 37 °C for 0.5, 1, 3 and 24 h and freeze-dried during at least 72 h. Values represent the mean ± SD. Significant differences were assessed by Student t test; $p < 0.05$ **a** alginate versus HA, **b** 1 versus 2 %. **b–i** These images show the microscopic structure of alginate (**b–e**) and HA (**f–i**) hydrogels at different concentrations (1 and 2 %) after 0.5 h (**b, d, f, h**) and 24 h (**c, e, g, i**) of incubation in PBS. Observation by SEM was done at 15 kV, 40 Pa and ×100 of magnification

Lower pore diameters were observed for HA compared to alginate freeze-dried hydrogels (Fig. 2b–i), though statistical significance was only reached for 1 % concentrations after 0.5 and 24 h and for 2 % concentrations at any of the time points of incubation studied. Further, an increase in the concentration of polymer was associated with a decrease in pore size for both hydrogels, although no differences were observed
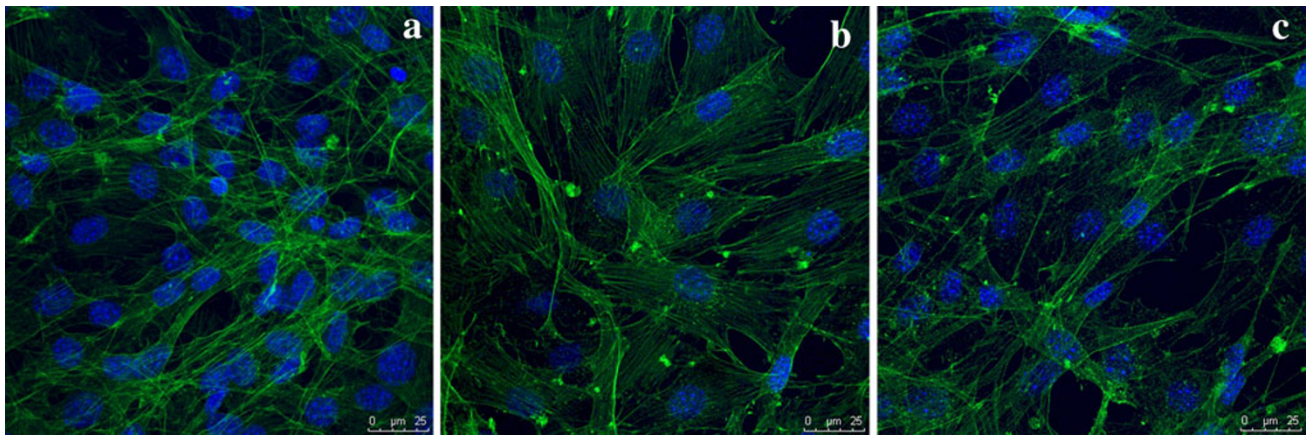
Fig. 3 Confocal images of MC3T3-E1 monolayers after 24 h of incubation with **a** culture media, **b** 1 % alginate hydrogel, and **c** 2 % alginate hydrogel

between alginate at 1 and 2 % after 0.5 and 3 h of incubation.

3.3 Cell Morphology by Confocal Microscope

The cytoskeleton organization of cells seeded with either 1 or 2 % alginate hydrogel and control cells was examined after 24 h of attachment. Representative confocal images of actin staining of osteoblasts cultured in different conditions are shown in Fig. 3. While control cells appeared elongated and with actin filaments haphazard orientated, cells cultured with alginate hydrogel presented the actin bundles aligned in the same direction and in osteoblasts cultured with alginate at 2 % more stretched actin filaments were observed. The formation of stretched and roughly parallel actin filaments may indicate the development of organized actin filaments in the form of stress fibers in cells cultured with alginate hydrogels.

3.4 Effect of the Hydrogels on Cell Viability

In order to determine the effect of the different hydrogels on cell viability, the LDH activity in the culture media was measured after 24 h of culture. As shown in Fig. 4, none of the hydrogels tested had a toxic effect on MC3T3-E1 cells. Further, a significant increase on cell viability was observed when comparing 1 % HA with 1 % alginate hydrogels.

3.5 Effect of the Hydrogels on ALP Activity

Cells cultured with HA hydrogels showed significantly lower ALP activity levels compared to control cells and to 2 and 3 % alginate hydrogels, respectively. In addition, a significant increase on ALP activity was found when comparing cells cultured on 2 % HA compared to 1 % HA (Fig. 5).
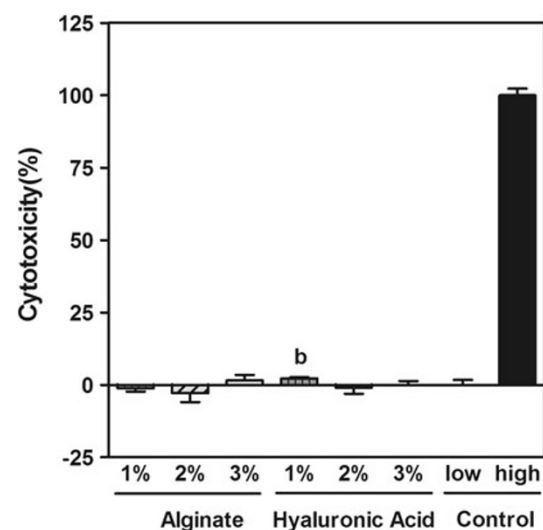

Fig. 4 LDH activity measured from culture media collected 24 h after exposure of MC3T3-E1 cells to alginate or hyaluronic acid hydrogels at different polymer concentrations (1 %, 2 % and 3 %). High control (100 % cytotoxicity) was cell culture media from cells seeded on polyethylene terephthalate (PET) membrane inserts and incubated with PBS containing 1 % Triton X-100. Low control (0 % cytotoxicity) was cell culture media from cells seeded on PET membrane insert and incubated with PBS. Values represent the mean $\pm$ SEM. Significant differences were assessed by Student t test: $p < 0.05$ *b* alginate versus HA

3.6 Effect of the Hydrogels on Calcium Content

Calcium content in the cell monolayer was quantified after 21 days of culture. As seen in Fig. 6, higher values of calcium content were found on cells cultured with alginate compared to control cells, and rising amount in calcium content were found as the polymer concentration increased. Unfortunately, calcium content in cells cultured in direct contact with HA could not be determined, since an interference of HA on the calcium determination method used could
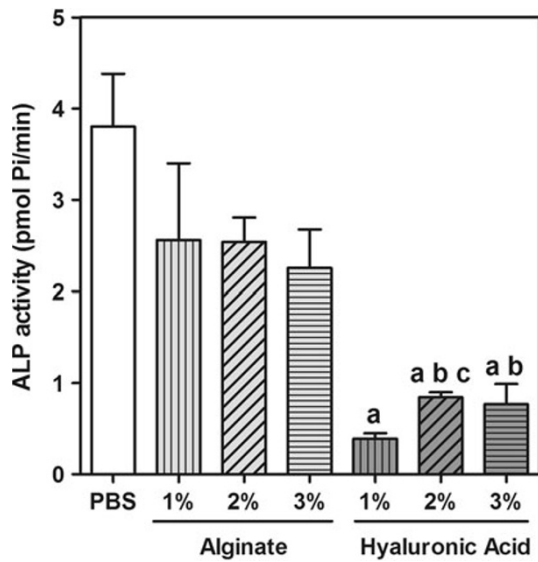
Fig. 5 Alkaline phosphatase (ALP) activity in MC3T3-E1 cells cultured for 21 days with alginate or hyaluronic acid at different polymer concentrations (1, 2 and 3 %). Values represent the mean ± SEM. Significant differences were assessed by Student *t* test: p < 0.05 **a** versus control PBS, **b** alginate versus HA, **c** between polymer concentrations
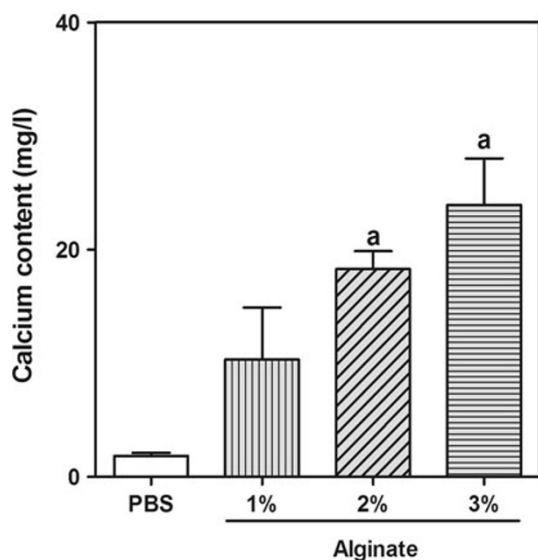

Fig. 6 Calcium content in MC3T3-E1 cells cultured for 21 days with alginate at different polymer concentrations (1, 2 and 3 %). Values represent the mean ± SEM. Significant differences were assessed by Student *t* test: p < 0.05 **a** versus control PBS

be confirmed by previous studies, where high molecular weight HA hydrogels alone incubated with cell culture media without cells gave high values of calcium content, and similar to HA hydrogels with cells (data not shown).

Based on ALP activity and calcium content results, 2 and 3 % polymer concentrations were selected for further experiments.
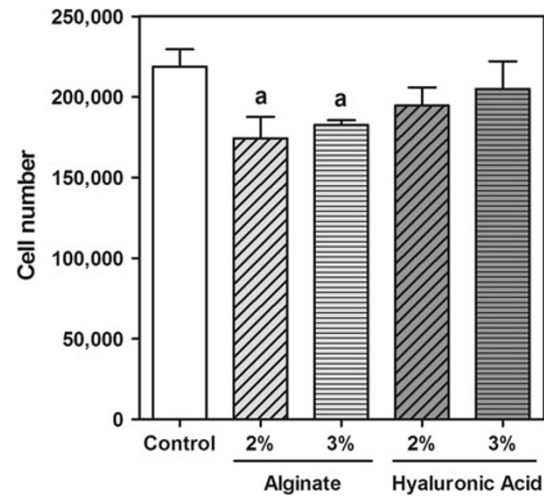

Fig. 7 Quantification of cell number in MC3T3-E1 cells cultured for 21 days with alginate or hyaluronic acid at different polymer concentrations (2 % and 3 %). Values represent the mean ± SEM. Significant differences between groups were assessed by Student *t* test: p < 0.05 *a* versus control PBS

3.7 Effect of the Hydrogels on Cell Number

As shown in Fig. 7, after 21 days of culture no differences in cell number were observed between control cells (PBS) or cells seeded with HA either at 2 or 3 %. However, when cells were cultured with 2 or 3 % alginate hydrogels a significant decrease on cell number was found compared to untreated cells. No differences on the cell number among polymers were found after 21 days of culture.

3.8 Effect of the Hydrogels on the Expression of Osteogenic Related Genes

The effect of the different polymers on osteoblast cell differentiation was also assessed at gene expression levels of several markers (Fig. 8). Higher mRNA expression levels of the different markers studied were found on cells cultured with alginate hydrogels compared to those cells cultured with the high molecular weight HA hydrogels used in this study.

Significantly higher expression levels of *Bsp* mRNA levels were found in cells cultured with 2 % alginate hydrogels compared to cells cultured with 2 % HA hydrogels. While no differences were observed in *Alp* mRNA levels between cells cultured with alginate hydrogels and control cells, a down-regulation of *Alp* mRNA levels was found in cells cultured with HA compared to control, and this down-regulation was also dependent on the concentration of HA. Osteocalcin mRNA expression levels were significantly higher in cells cultured with alginate hydrogels compared to control and to cells
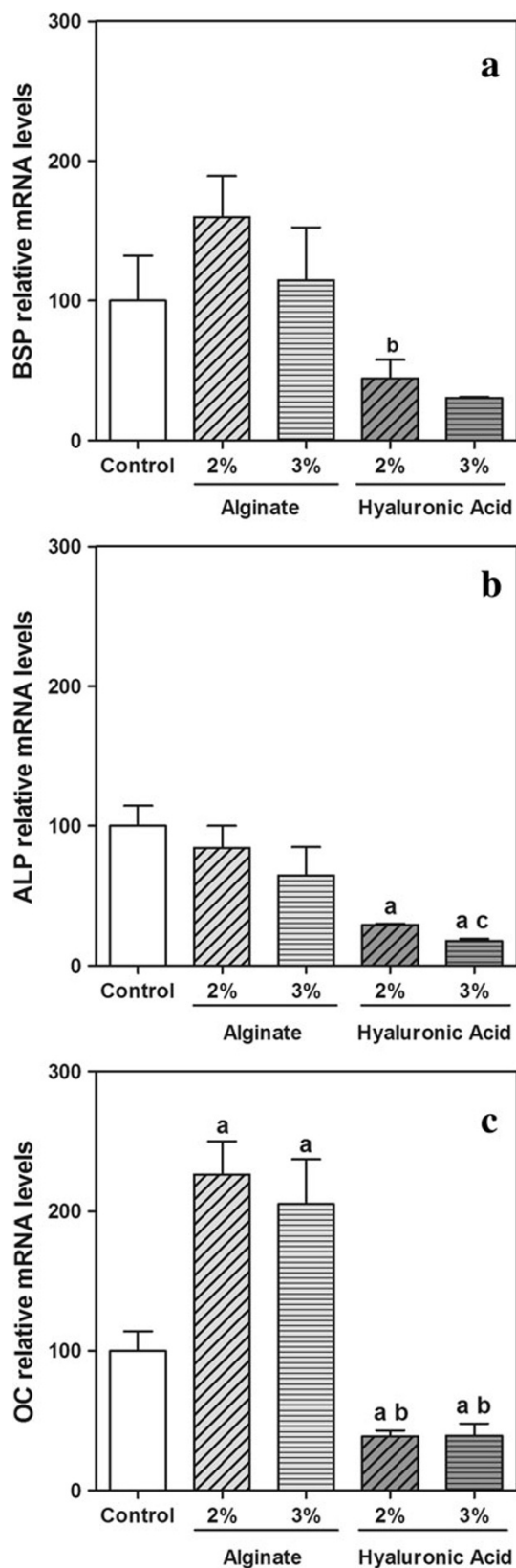
◄ **Fig. 8** Expression of osteoblast differentiation related genes in MC3T3-E1 cells cultured for 21 days with alginate or hyaluronic acid at different polymer concentrations (2 and 3 %). Data represent relative mRNA levels of target genes normalized with reference genes, expressed as a percentage of untreated cells (PBS), which were set to 100 %. Values represent the mean ± SEM. Differences between groups were assessed by Student t test: $p < 0.05$ **a** versus control PBS, **b** alginate versus HA, **c** between polymer concentrations

4 Discussion

The use of hydrogels for bone regeneration has recently been reviewed [28], suggesting that the use of hydrogels offers an option for bone-tissue engineering and that further research is needed to identify the biological and physical properties of hydrogels. Sodium alginate and hyaluronic acid are natural polymers that have widely been used for several applications in tissue engineering; however, to the best of our knowledge, the direct comparison of the effect on osteoblast differentiation of those two polymers has never been reported. Here, we report the structural and physicochemical properties of different concentrations of these two polymer hydrogels and their biocompatibility and bioactivity after long-term culture of MC3T3-E1 cells.

Hydrogels are used in tissue engineering as scaffolds that provide structural integrity to tissue constructs, as control drug and protein delivery to tissues and cultures, and as adhesives or barriers between tissue and material surfaces [29]. The pH of the environment, viscosity of the hydrogel matrix, the swelling behavior and the pore size of the microstructure will determine the suitability of the material for their different applications on tissue regeneration. Under the preparation conditions described in the present study for both polymers, alginate and high molecular weight HA formed reversible hydrogels with a weak structure, which could be related with the interactions between the polymer chains, as a consequence of their proximity and the polymer concentration used. In this context, we cannot discard the existence of some solvation processes, at least for the HA hydrogels used in this study. In fact, further modifications of the original polymer have been proposed in order to significantly increase hydrogels robustness and to improve their mechanical properties [30].

Viscosity is a decisive parameter for controlling the scaffold structure. The analysis of the different variables involving polymer solution viscosity, leads to the identification of the optimal conditions for polymer scaffold preparation [31]. The viscosity of the hydrogel is governed by the pH, the temperature, molecular weight and polymer concentration [32, 33]. Actually, it has been reported the role of temperature on the distribution of the ionic charges along the polyelectrolyte chain, which is related with the coiled or extended conformation of the polymer chain and

cultured with HA hydrogels. In addition, cells cultured with HA hydrogels showed a significant down-regulation of *Oc* mRNA expression levels compared to control cells.

its final viscosity solution [31]. Therefore, the viscosity of a solution can be modulated depending on the requirements of specific applications. For example, for the application of enamel matrix derivative (EMD) onto denuded root surfaces, an initial viscous formulation of polyethyleneglycol alginate (PGA) containing EMD at room temperature allowed easy application of the solution to the site of defect, which further, at physiological conditions (higher temperature and neutral pH), decreased its viscosity allowing the complete coating of the defect to be treated [33]. In the present study, both alginate and high molecular weight HA hydrogels were prepared in PBS (pH $\sim$ 7.0) to be used for tissue culture, and changes in viscosity were analyzed. Any of the polymer concentrations tested permitted its easy application and shape to the specific site due to a decrease in the viscosity associated to an increase of temperature when used in cell culture conditions. Additionally, this temperature dependency is an attractive approach in the drug delivery field. More specifically, high viscous hydrogels are interesting during drug loading; whereas a decrease of hydrogel viscosity allows drug release. Thus, previous studies performed in our laboratory showed the ability of those hydrogels to associate and deliver active molecules in vitro (unpublished results).

When a hydrophilic matrix is placed in an aqueous medium, the hydrophilic colloid components swell to form a gelatinous surface layer. This then controls the diffusion of water into the matrix [32]. In the present study, the water uptake happened in the first 30 min for both alginate and high molecular weight hyaluronic acid hydrogels at any of the polymer concentrations tested. The capacity of swelling obtained indicates the weakness for both polymer hydrogels in wet state as a result of their low cross linking grade and of the hydrophilic nature of these polymers. It is interesting to note that for HA an increase in polymer concentration was related to an increase in the water uptake. As regards to pore size, it should be expected that it differs in hydrated and dried hydrogels, in fact, while in wet state both hydrogels display an homogenous, uniform and continuous nonporous solution, due to weakness and hydrophilic properties of both polymers (data not shown), either alginate or HA yielded a porous three dimensional structure with a pore diameter in the range of 53–121 μm in a swollen freeze-dried state.

Once we confirmed the development of hydrogels with expected properties close to physiological pH and at adequate polymer concentration to achieve easy handling, we investigated the effect of those polymers on the biological response of osteoblasts. In agreement with previous reports [8, 34, 35], biocompatibility of alginate and hyaluronic acid hydrogels at any of concentrations evaluated on osteoblast cells was confirmed.

The ability of these natural polymers to achieve osteoblast proliferation and differentiation when they are placed in direct contact with pre-osteoblast cells was also studied. Previous studies have demonstrated that modified alginate and hyaluronic acid hydrogels induce bone formation in vitro [8, 14, 15, 17, 26, 36, 37] and in vivo [15, 17, 19, 27]. Here, we demonstrate that unmodified hydrogels also support osteoblast differentiation, though alginate hydrogels induced a higher degree of differentiation than the high molecular weight hyaluronic acid hydrogels used in the present study.

ALP activity is often used as a marker for increased osteoblastic metabolic activity and an indicator of osteoblastic differentiation [15]. Cells cultured in direct contact with alginate hydrogels showed higher ALP activity levels than those cultured in contact with hyaluronic hydrogels at 2 and 3 % of polymer concentrations. Previous studies have described upregulation of ALP activity by alginate microbeads [36, 38] or HA in a dose-dependent manner at different dosages [39]. Intracellular calcium content was also measured as an index of cell differentiation for alginate hydrogels. In contrast to the ALP activity profile, cells cultured with alginate showed increased calcium content compared to control cells, where raising amounts in calcium were related to higher polymer concentrations, suggesting that alginate induce matrix mineralization which is related with increasing polymer concentration. In agreement with previous reports that show an induction of mineral nodule formation by modified alginate hydrogels [40].

Taking all these into account, 2 and 3 % of polymer concentration were selected to further evaluate its effects on cell proliferation and on the mRNA expression levels of markers related to osteoblast differentiation. The effect of the hydrogels on cell number was investigated by DNA content quantification after 21 days of culture. Although a significant decrease in cell number was observed when cells were cultured with alginate compared to control cells, the DNA results indicate long-term viability and the support of the hydrogels for cell proliferation, as the number of cells at day 21 surpasses the initial seeding density. Supporting these data is the cytoskeleton organization of cells cultured with alginate hydrogels as seen by the confocal images. Actually, Hong and coworkers reported the relationship between the enhanced actin fiber density and an early stage of osteoblast differentiation [41].

Finally, expression of markers related to late stages of osteoblast cell differentiation was determined. The acquisition of an extracellular matrix competent for mineralization is governed by the expression of markers related with maturation and organization of the bone matrix. In the present study, on one hand, and consistent with the decrease of ALP activity induced by HA hydrogels, the

mRNA expression levels of *Alp* were also markedly decreased in MC3T3-E1 cells cultured with HA compared to both, control cells and cells cultured with alginate. Cells cultured with alginate showed increased mRNA levels of both, *Bsp* mRNA levels—a component of the extracellular matrix which has been described to bind to hydroxyapatite crystals [42]—and *Oc* mRNA levels—an extracellular matrix protein synthesized and secreted exclusively by osteoblastic cells in the late stage of maturation and considered an indicator of osteoblasts differentiation and mineralization [43]—indicating a higher degree of cell differentiation than cells cultured with HA. In agreement with these results, cells encapsulated into alginate microcapsules enhanced mRNA expression levels of osteocalcin when compared to monolayer cultures over the course of 21 days [38], while a decrease in OC secretion with HA has been reported in osteoarthritic osteoblast cells [44]. However, in vitro [8] and in vivo [5] studies using modified HA hydrogels with BMP-2 have reported high OC expression, thus, reinforcing the importance of the specific HA used.

On the other hand, it is interesting to note that even if cell number was lower when cells were cultured with alginate hydrogels compared to control cells, cytoskeleton organization, calcium content and gene expression levels indicate that cells cultured with alginate showed a significant increased osteogenic activity.

5 Conclusion

In conclusion, in this study we have compared for the first time the effect of different natural polymers that are widely used for tissue engineering in terms of osteoblast viability, proliferation and differentiation. The biocompatibility of hyaluronic acid and alginate hydrogels has been validated, and we have shown that alginate hydrogels might be more suitable for bone tissue engineering applications than high molecular weight hyaluronic acid hydrogels.

Conflict of interest No competing financial interests exist.

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