Sodium alginate sponges with or without sodium hyaluronate: *In vitro* engineering of cartilage

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Abstract: Studies are underway to design biosystems containing embedded chondrocytes to fill osteochondral defects and to produce a tissue close to native cartilage. In the present report, a new alginate three-dimensional support for chondrocyte culture is described. A sodium alginate solution, with or without hyaluronic acid (HA), was freeze-dried to obtain large-porosity sponges. This formulation was compared with a hydrogel of the same composition. In the sponge formulation, macroscopic and microscopic studies demonstrated the formation of a macroporous network (average pore size, 174 μm) associated with a microporous one (average pore size, 250 nm). Histological and biochemical studies showed that, when loaded with HA, the sponge provides an adapted environment for proteoglycan and collagen synthesis by chondrocytes. Cytoskeleton organization was studied by three-dimensional fluorescence microscopy (CellScan EPR™). Chondrocytes exhibit a marked spherical shape with a nonoriented and sparse actin microfilament network. Type II collagen was detected in both types of sponges (with or without HA) using immunohistochemistry. In conclusion, the sponge formulation affords new perspectives with respect to the *in vitro* production of "artificial" cartilage. Furthermore, the presence of hyaluronate within the alginate sponge mimics a functional environment, suitable for the production by embedded chondrocytes of an extracellular matrix. © 2001 John Wiley & Sons, Inc. J Biomed Mater Res 57: 268–278, 2001

Key words: cartilage; alginate; sponge; hyaluronic acid; chondrocytes

INTRODUCTION

Tissue engineering is currently approached by using biomaterials that provide a temporary scaffold. The literature reports on a variety of biomaterials designed from natural[1–3] or synthetic polymers,[4,5] for cell transplantation or infiltration. Some of them are prepared as high-porosity networks, well adapted to the seeding of numerous cells and to the reconstruction of the tissue, *in vitro* as well as in the animal.[6,7] The design or selection of appropriate biomaterials depends on the type of tissue to be repaired. Porous matrices have been proposed for skin repair,[8–10] liver reconstruction,[11–13] bone formation,[14–16] and also for cartilage repair.[17–21]

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The chemical nature of biomaterials as well as the size and orientation of pores must be selected so as to allow the homogeneous distribution of cells within the scaffold, their adherence, and the conservation of their phenotype. The biomaterials must also be biocompatible and biodegradable. In addition, their mechanical properties must remain stable on the time scale of the repair process.

Macroporous scaffolds have been used for the *de novo* growth of cartilage. Among them, synthetic polymers, such as polylactic acid,[6,19,22] polyglycolic acid,[5] their copolymers (polylactic acid–polyglycolic acid), or natural polymers such as collagen[18,23] or polysaccharides[2,20,24] have proved to be suitable carriers for cell transplantation. The aim of the present work was to prepare and to study a new macroporous material able to act as a three-dimensional scaffold for the transplantation of chondrocytes. In this new material, sodium hyaluronate (HA), a matrix component of cartilage, is associated with calcium alginate gels.

In the literature, these two components have been studied either alone or associated with others poly-

mers. For instance, hyaluronic acid, which plays a critical role in the retention of proteoglycans in the cartilaginous matrix,[25] has been recently developed as a cell carrier.[8,17,20,21] The choice of alginate results from its biocompatibility and hydrophilicity, both characteristics widespread and successfully used for three-dimensional cell culture.[2,11] However, this polysaccharide is commonly used for cell transplantation, in mixtures with other components such as fibrin,[26] demineralized bovine bone matrix,[27] or powdered glass-ceramic.[28] Recently, Fragonas et al.[29] used Ca^{2+}-alginate-chondrocyte implants to repair cartilaginous defects created in rabbit knees. Qualitative observations of the new tissue provided evidences of a successful repair.

Thus, our goal was both to investigate the influence of a macroporous network on the biochemical properties of the extracellular matrix synthesized *in vitro* and to determine the potential interest of adding hyaluronate in the alginate biomaterial.

MATERIALS AND METHODS

Isolation of chondrocytes

Chondrocytes were isolated from femoral head caps of male Wistar rats (10 weeks old, average weight 285 g) (Charles River, Saint Aubin les Elbeuf, France). Rats were euthanized by cervical dislocation after general anesthesia and the knee joints were dissected. Cartilage from four animals was pooled, cut in small pieces, and washed three times with 0.9% NaCl (B. Braun, Melsungen, Germany) supplemented with gentamicin (GIBCO BRL, Paisley, UK). Cartilage chips (100 mg) were digested in 5 mL of pronase, issued from *Streptomyces griseus* (Sigma-Aldrich, St. Quentin, Fallavier, France) (2 mg/mL in 0.9% NaCl), for 2 h at 37°C, 5% CO_2. After centrifugation (1200 rpm, 10 min), the resulting samples were rinsed three times with 0.9% NaCl and were treated with 5 mL of collagenase B, issued from *Clostridium histolyticum* (Boehringer-Mannheim, Germany) (1.5 mg/mL in Dulbecco's modified eagle's medium (DMEM)/Nut mix F-12 medium (GIBCO BRL) supplemented with gentamicin). After 12 h at 37°C, 5% CO_2, the cell suspension was centrifuged (1200 rpm, 10 min). The cell pellet was finally resuspended in DMEM F12 culture medium.

Cell culture

The cell culture was performed in DMEM F-12 medium supplemented with glutamin (12:1000 v/v), gentamicin (8:1000 v/v), fetal calf serum (FCS) (110:1000 v/v), and amphotericin B (1:1000 v/v), all purchased from GIBCO BRL. Throughout the following text, this mixture will be called "complete medium." In the present study, this complete medium was supplemented with 3 m*M* $CaCl_2$ (Merck, Darmstadt, Germany) to maintain the structural integrity of alginate-based materials.

Preparation of alginate sponges with or without HA

Sodium alginate (Alg) (medium viscosity, M = 250,000 g/mol, guluronic residue/mannuronic residue ratio = 30:70) issued from *Macrocystis pyrifera* (Sigma-Aldrich) and HA (M = 480,000 g/mol) from bacterial origin (Acros Organics, Geel, Belgium) were used. An Alg solution (20 g/L) or a mixture containing Alg (20 g/L) and hyaluronate (6.6 g/L) was prepared in double-distilled water. A 100-m*M* $CaCl_2$ solution was dripped under gentle magnetic stirring in these polysaccharide solutions (20 mL). After 30 min, the excess of calcium chloride solution was discarded and the sponge-shaped biomaterial was washed twice with 0.9% NaCl, frozen at −20°C, and freeze-dried at −60°C under vacuum (Fisher Heto FD3 freeze-dryer, Elancourt, France) during 48 h. Before cell culture, the resulting sponges were punched with a biopsy pen (5 mm diameter; Stiefel, Offenbach, Main Germany) and autoclaved at 121°C for 20 min. This sterilization procedure results in some decrease of the polysaccharides' molecular weights (110,000 g/mol for Alg and 240,000 g/mol for HA, measured by size exclusion chromatography–multi-angle laser light scattering). After sterilization, the sponges proved to remain stable in the culture medium for at least 3 months.

The punched sponge pieces were placed in 24-well plates (Corning, New York, USA) (one punch per well) and then moistened with 20 μL of complete medium containing 50,000 cells. The plates were incubated (37°C, 5% CO_2) for 1 h to promote chondrocyte adhesion within sponges. Then, 2 mL of complete culture medium supplemented with 3 m*M* $CaCl_2$ were added in each well. Identical sponges, but containing no cells, were used as a control.

Preparation of alginate beads with or without HA

Alginate beads were chosen as references. Alg and HA were autoclaved (121°C, 20 min) before solubilization. Solutions of Alg, loaded or not with HA, were prepared by dissolution of autoclaved polymers in sterile 0.9% sodium chloride at the same concentrations as sponges (see above). Cell pellets was suspended at the desired concentration (4×10^6 cells/mL) in the polymer solutions. The mixtures were dripped in 100 m*M* $CaCl_2$, using the method described by Guo et al.[2] Resulting beads containing 50,000 cells/bead were placed in 2 mL of complete medium supplemented with 3 m*M* $CaCl_2$. Identical Alg and Alg–HA beads, but containing no cells, were used as a control.

Transmission electronic microscopy (TEM)

The porous network of the sponges was observed by using TEM with an analytical electronic microscope (Zeiss 902A, 80 Kvolts high tension, Jena). Sterilized freeze-dried sponges were rehydrated with 0.9% NaCl, then punched with a 2-mm diameter biopsy pen and fixed for 12 h at 4°C with a cacodylate buffer (100 m*M*, pH 7.2) containing cacodylic acid (20 g/L) (Sigma-Aldrich), alcian blue (2 g/L) (Searle, High Wycombe Bucks, UK), ruthenium red (2 g/L)

(Touzart et Matignon, Courtaboeuf, France), and 3 m*M* $CaCl_2$, mixed in equal proportions with 5% glutaraldehyde. After washing fixed punches with cacodylate buffer, a second fixation of samples was performed with a mixture containing an equal volume of cacodylate buffer and 80 m*M* osmic acid. The samples were washed with distilled water and then dehydrated using acetone/water mixtures of increasing acetone concentration (20, 40, 60, 80, 95, and 100% acetone/water). After drying, the samples were treated with increasing concentrations of Epon 812 resin in acetone, as described by Oerther et al.[30] Samples were cut in 0.1-μm-thick sections with a diamond knife and observed in TEM.

An identical procedure was used with Alg and Alg–HA sponges seeded with chondrocytes, and cultured for 2 weeks in complete medium supplemented with 3 m*M* $CaCl_2$.

Cytoskeleton organization of chondrocytes embedded in sponges or beads

F-actin filament organization in alginate sponges or beads was observed using optical sections of chondrocytes. Analyses were performed with a CellScan EPR™ optical scanning acquisition system (IPLab-Scanalytics, Billerica, Fairfax, VA, USA) equipped with an Olympus IX-70 epi-fluorescence inverted microscope (Bionis Instruments Scientifiques, Clamart, France) and a 60×/1.25 NA oil immersion apochromatic objective (Olympus, Rungis, France). Scanning along the optical axis was performed by a piezo-electric z-axis focus device (at a z spacing of 0.25 μm). Images of biological samples were collected on a cooled 12 bits Charge-Coupled Device camera (Princetown Instruments Inc., Evry, France). Fixation and permeabilization of cells as well as staining of F-actin microfilaments were performed as described by Dumas et al.[31]

Histological evaluation

Alg and Alg–HA sponges were fixed for 4 h at 20°C using a 4% paraformaldehyde solution (pH 7.4) (Sigma-Aldrich) containing 100 m*M* cacodylic acid and 10 m*M* $CaCl_2$. Samples were dehydrated, embedded in paraffin, cut in sections (5-μm thickness), and then stained with HES (hematoxylin, eosin, safran) for the observation of cell density and morphology, alcian blue for the evaluation of proteoglycan matrix deposition, and Sirius red for the observation of both collagen matrix deposition and, under polarized light, of network organization. Observations of 10× and 40× were performed using a photonic microscope (Nikon type 104, Tokyo, Japan). Using a semiquantitative scale presented in Table I, a histological score was obtained, for each sponge, after 10, 20, and 40 days of culture.

DNA assay

DNA was assayed according to the fluorometric method of Labarca and Paigen.[32] The titration was achieved using the Hoechst Dye 33258 probe (Interchim, Montluçon, France). Beads and sponges were dissolved in a 150 m*M* sodium chloride solution containing 55 m*M* sodium citrate (GIBCO BRL) and 50 m*M* EDTA (Merck), adjusted to pH 6.8. The dissolution was followed by an enzymatic digestion with papain (Sigma-Aldrich), as described by Petit et al.[33]

The fluorescence intensity was read on a Hitachi F2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan) (broadcast = 458 nm and excitation = 356 nm). The calibration curve was obtained using calf thymus DNA solutions (Sigma-Aldrich) in the 0.4 to 8 μg/mL range.

TABLE I
Semiquantitative Scale Used for Histological Evaluation of Alg and Alg–HA Sponges

Coloration	Parameter	Observation	Score
HES	Cell density	High	3
		Medium	2
		Poor	1
		No cells	0
	Cell morphology	Chondrocytic aspect	2
		Chondrocytic and fibroblastic aspect	1
		Fibroblastic aspect	0
	Cluster formation	Present	1
		Absent	0
Alcian blue	Pericellular blue stained matrix deposition	High	3
		Medium	2
		Poor	1
		No deposition	0
Sirius red	Pericellular red stained matrix deposition	High	3
		Medium	2
		Poor	1
		No deposition	0
	Polarized light	Positive	1
		Negative	0
	Fiber organization under polarized light	Normal	2
		Partly organized	1
		None	0

Cell proliferation and proteoglycan synthesis

The study was performed by dual labeling of three-dimensional cultures with ^{3}H-thymidine and $Na_2{}^{35}SO_4$ to evaluate cell proliferation and proteoglycan synthesis, respectively. Before radioactivity incorporation, sponges or beads, with or without cells, were first cultured for 24 h with 2 mL of DMEM medium (glutamin, antibiotics, and 3 m*M* $CaCl_2$ as described above) containing 2% of FCS, then for a further 24 h with the same medium but containing no FCS. Thereafter, this latter medium was replaced by a radioactive one containing ^{3}H-thymidine (5 μCi/mL) and $Na_2{}^{35}SO_4$ (5 μCi/mL). The incubation was performed for 24 h.

Sponges or beads were rinsed five times with 0.9% NaCl to get rid of the nonspecific radioactivity. After dissolution and hydrolysis of the samples (as described for DNA assay), the incorporated radioactivity was measured by liquid scintillation (Beckman LS 3801; Fullerton, CA, USA).

Immunohistochemistry of type II collagen

Sponges seeded with chondrocytes were embedded in paraffin and sectioned, as previously described for histological evaluation. The immunohistochemical technique for visualization of type II collagen has been reported by Petit et al.[33] and Chang and Poole.[34] Briefly, after enzymatic digestion by testicular hyaluronidase (Sigma-Aldrich) and two washes in phosphate buffered saline, Tween 20 (2 g/L), nonspecific antibody-binding sites were blocked by incubation at 20°C for 1 h in a solution containing phosphate buffered saline, Tween 20 (2 g/L), and bovine serum albumin (30 g/L) (Sigma-Aldrich) (solution "A"). The samples were incubated at 4°C in a wet chamber for 12 h in solution "A" containing a 1:10 diluted anti-type II collagen antibody (rabbit anti-rat; Calbiochem Corporation). An FITC-labeled anti-rabbit antibody (Dako, France) diluted 50-fold in solution "A" was used for detection. Control samples were prepared as described above, except for the addition of anti-type II collagen antibodies which was omitted. Qualitative distribution of type II collagen was examined using three-dimensional fluorescence microscopy (Cellscan™) as previously described for cytoskeleton organization.

Statistical analysis

One-way analysis of variance followed by Student's comparison test were used for statistical analyses in this study. Differences were considered significant if $p < 5\%$.

RESULTS

Porosity of sponges

Macroscopic observation of sponges provided evidence of an interconnected network of channels. The observation by light microscopy enabled distinction of a macroporous network comprised of large pores (174 ± 98.6 μm evaluated from four different materials with $n = 20$ pores) (Fig. 1). This result was obtained for sectioned, fixed, and HES-stained sponges which were maintained in DMEM medium for 10 days in order to test the stability of their three-dimensional structures in culture conditions. The observation by TEM evidenced an association between a macroporous and microporous network, localized in channels [Fig. 2(a)]. The mean pore size of the microporous network, evaluated by TEM, was 250 nm ± 73 (for $n = 21$ pores) [Fig. 2(b)]. TEM observations performed on sponges seeded with chondrocytes showed that cells adhere and grow in these channels (Fig. 3).

Cytoskeleton organization of chondrocytes embedded in sponges and beads

Figure 4 shows the volume rendering of F-actin microfilaments of a chondrocyte entrapped in a bead [Fig. 4(a)] or in a sponge [Fig. 4(b)]. This result was obtained by projection on an axis of a stack of two-dimensional optical sections, collected at sequential focal planes through the chondrocyte. Images were deblurred by deconvolution image processing using the optical transfer function of the microscope.

In both types of formulations, chondrocytes exhibited a marked spherical shape with a nonoriented and sparse network of actin microfilaments.

Histological evaluation

The histological score (Table I, maximum total of 15 points) is plotted versus the culture duration (10, 20,

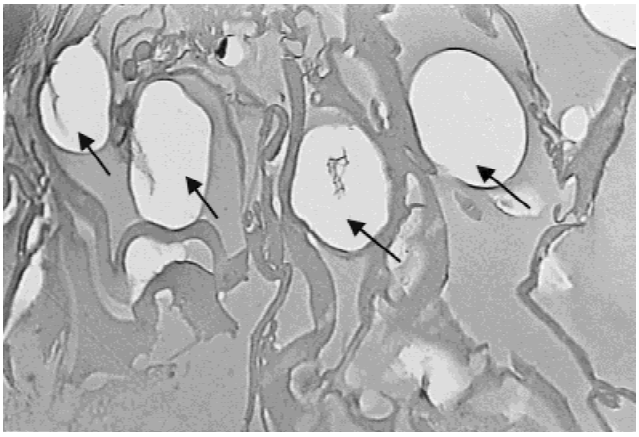


Figure 1. Macroporous pores in an alginate sponge (arrows). Observation was performed on 5-μm-thick sections, after HES coloration, using a photonic microscope (Nikon type 104) equipped with a 10× objective. The average pore size, evaluated with $n = 20$ pores of four different sponges was 174 ± 98.6 μm.

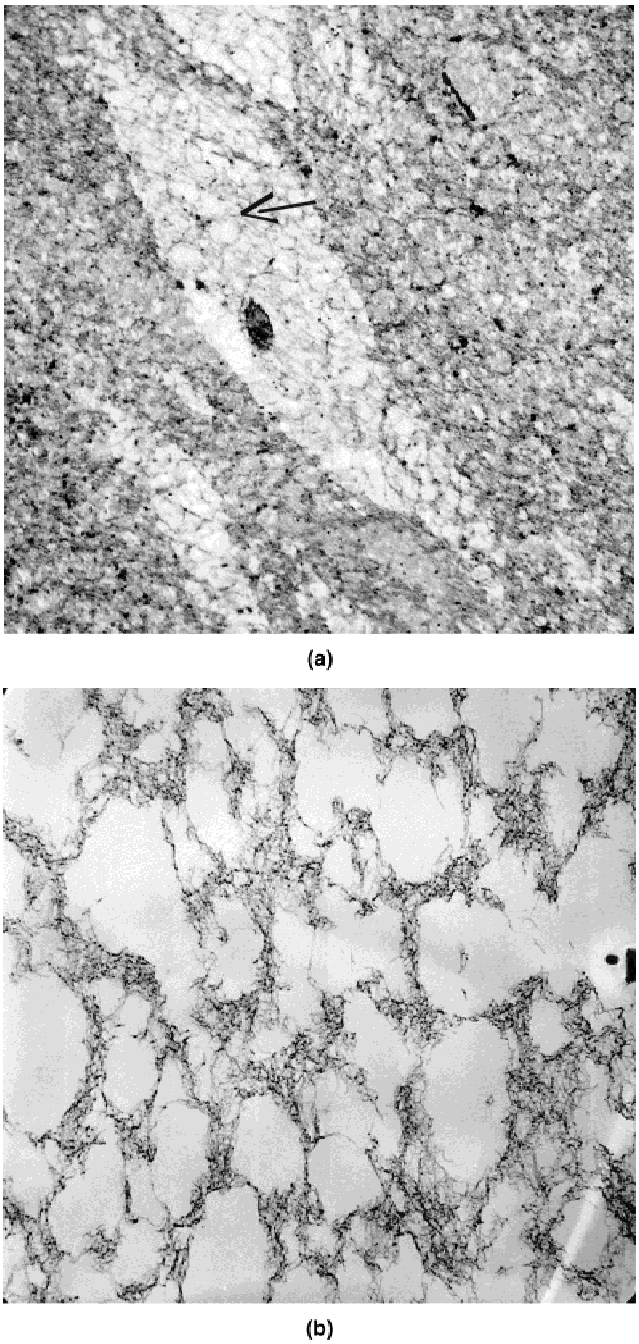


Figure 2. Small macroporous channels [(a) arrow; original magnification ×7000] and details of the microporous network [(b) original magnification ×30,000] in an alginate sponge loaded with sodium hyaluronate. The average pore size of the microporous network, determined by TEM, was 250 ± 73 nm. Observations were performed with an analytical electronic microscope (Zeiss 902A of 80 Kvolts high tension).

or 40 days) (Fig. 5). A statistical comparison of the score obtained for Alg and Alg–HA sponges shows a significant increase of this score with time ($p < 0.01$ for Alg sponges for day 10 versus day 40 and $p < 0.025$ for Alg–HA sponges for day 10 versus day 40) (Fig. 5). However, the presence of hyaluronate does not modify significantly the global score at day 40 (8.2 ± 1.6 for Alg sponges and 9.5 ± 2.3 for Alg–HA sponges).

HES coloration made possible the observation of the density and morphology of cells. Most of them had a normal spherical shape. Chondrocytes, localized in channels, were numerous after 20 days in both types of sponges and started forming clusters at day 40 [Fig. 6(a)]. Cell density increased with time in both Alg and Alg–HA sponges and no significant difference was observed between these two materials.

At days 20 and 40, cells were surrounded by a characteristic blue coloration (alcian blue staining) reflecting the presence of a proteoglycan-rich pericellular matrix [Fig. 6(b)].

At day 40, the extracellular collagen-rich matrix deposition was clearly visualized (Sirius red coloration) in both materials [Fig. 6(c)]. Under polarized light, collagen fibers not only appeared, but the network started being organized [Fig. 6(d)] with a dominance of orange and green colorations, characteristic of a birefringent organization.

On this qualitative basis, these materials seem to act as favorable environments both for cellular growth and for deposition of an extracellular matrix rich in proteoglycans and in collagens. However, no clear-cut conclusions can be drawn on possible differences between these three-dimensional systems, loaded or not with hyaluronate.

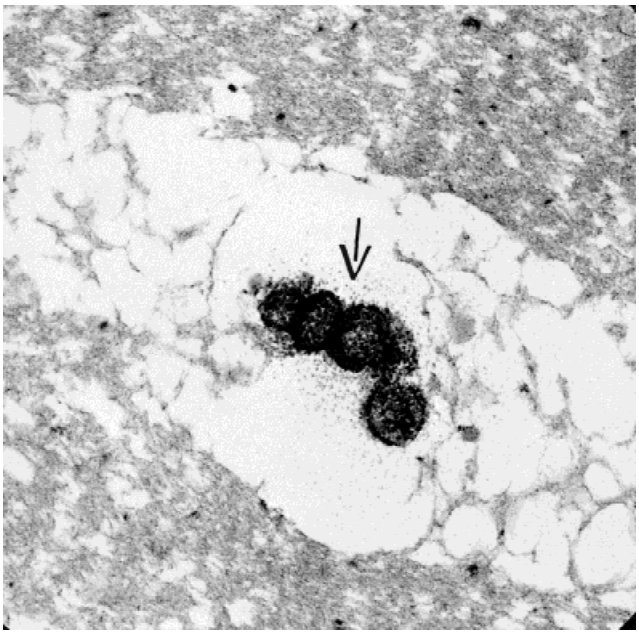


Figure 3. Chondrocytes (arrow) in a channel of an alginate sponge, 2 weeks after initial incubation. Observation was performed with an analytical electronic microscope (Zeiss 902A of 80 Kvolts high tension, original magnification ×12,000).

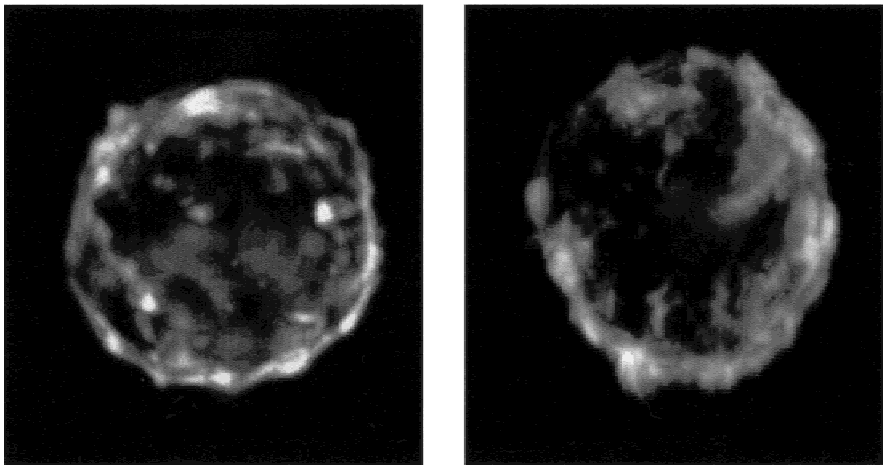


Figure 4. Three-dimensional aspect of chondrocytes. Volume rendering of a chondrocyte within a bead (a) or a sponge (b) was obtained by projection on an axis of a stack of two-dimensional optical sections. F-actin microfilaments were stained by Rhodamine-Phalloidine. Details are given in Materials and Methods.

Cell proliferation

Cell proliferation in beads or sponges was evaluated at different times (7, 14, and 21 days), from both total cellular DNA in the materials and ^{3}H-thymidine incorporation by living cells. Results (^{3}H dpm per microgram of total DNA) are presented in Figure 7(a) for beads and in Figure 7(b) for sponges.

From day 14, in beads with or without HA, the significantly lower radioactive signals indicate that the number of cells able to incorporate ^{3}H-thymidine decreases ($p < 0.05$ for day 7 versus day 14 and for day 7 versus day 21) [Fig. 7(a)]. This phenomenon did not occur with sponges, for which a constant progression of living cells at the different times is observed, leading after 21 days, to a significant increase of cell proliferation ($p < 0.001$ for day 7 versus day 21) [Fig. 7(b)].

It is noteworthy that, after 7 days, the total cellular DNA was always higher in sponges than in beads (data not shown), even though the initial seeding was performed using a same cellular density in both formulations (50,000 cells/piece).

However, comparative to the beads, the ^{3}H dpm/total DNA values for sponges demonstrate that a limited number of cells is able to incorporate ^{3}H-thymidine since the beginning of the culture. But, after day 14, the radioactivity signal in sponges continues to increase, whereas the cells embedded in beads seem to reach a constant state of proliferation.

The presence of hyaluronate in materials significantly improved cell proliferation at day 14 for beads ($p < 0.025$) and at days 7 and 21 for sponges ($p < 0.05$ and $p < 0.001$, respectively).

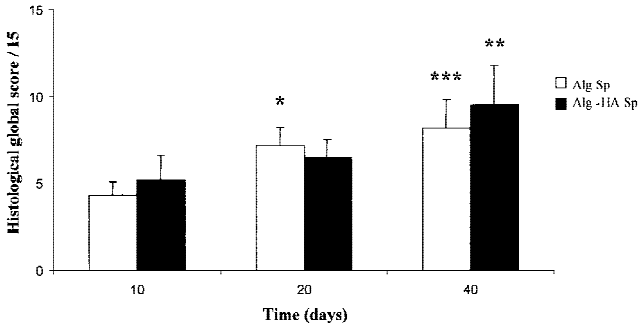


Figure 5. Histological global score (maximum total of 15 points) (means ± SD) of sponges using a semiquantitative scale (histological scoring details are given in Materials and Methods). Observations were performed on histological sections (5-μm thickness) from three different sponges examined blindfold and independently by two observers at days 10, 20, and 40. HES, Alcian blue, and Sirius red staining techniques were used. For each material, the significance p was expressed for day 10 versus day 20 and for day 10 versus day 40. Differences were considered significant if $p <$ 5% (*), $p < 2.5\%$ (**), and $p < 1\%$ (***).

Proteoglycan synthesis

The use of ^{3}H/^{35}S dual labeling made possible the evaluation of both cellular proliferation and proteoglycan synthesis activity of chondrocytes embedded within the materials.

A high ^{35}S incorporation was observed in beads at day 7 [Fig. 8(a)]. The values were 20- to 40-fold higher than in sponges. However, at day 14, the synthesis activity in beads decreased significantly ($p < 0.001$ for Alg beads for day 7 versus day 14 and $p < 0.025$ for Alg–HA beads for day 7 versus day 14). This loss of activity was 78% in alginate beads and 52% in beads loaded with hyaluronate.

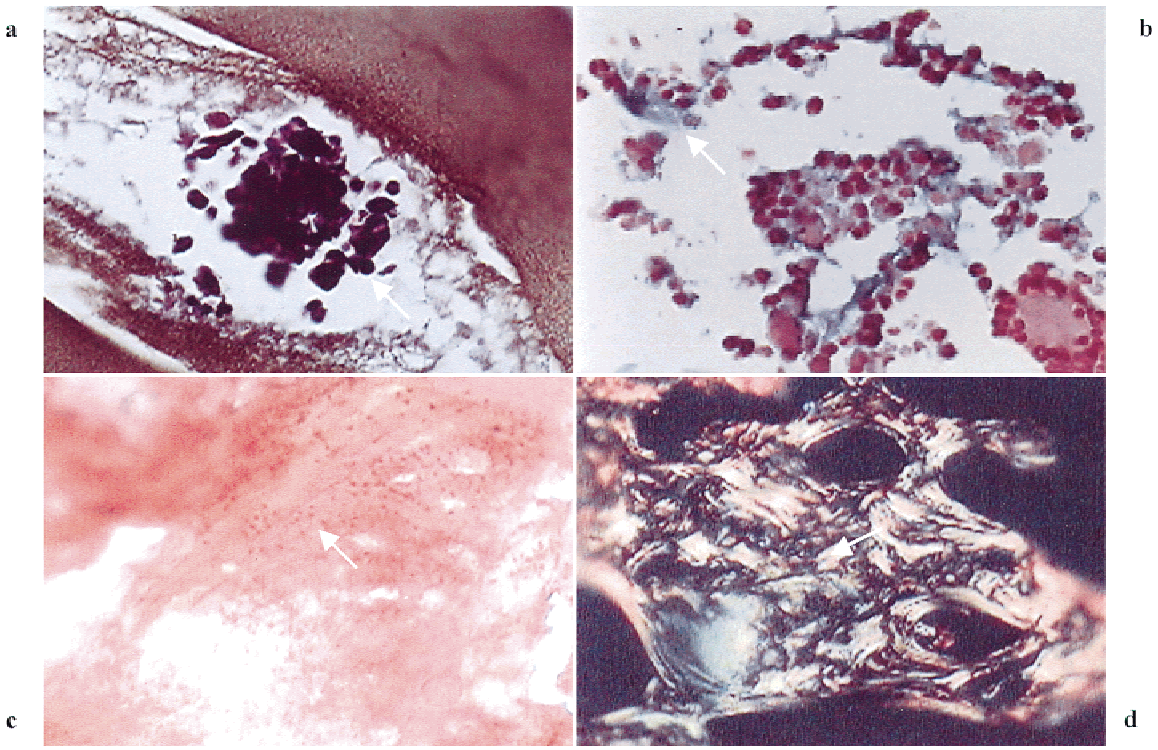


Figure 6. *In vitro* histological survey at day 40 of chondrocytes cultured in alginate sponges loaded with hyaluronate. Observation of chondrocytes (arrow) was performed with the HES staining technique (a). Alcian blue staining was used for the observation of proteoglycan-rich matrix deposition (arrow) (b). Extracellular collagen-rich matrix deposition (arrow) was observed using Sirius red under nonpolarized light (c) or under polarized light to visualize collagen fiber organization (arrow) (d). Observations were performed on 5-μm-thick sections using a photonic microscope (Nikon type 104) with a 40× objective. Images were collected on a color CCTV Panasonic WVCL350 camera.

However, the ^{35}S signal increased from day 14 to day 21 for alginate beads (1201 ± 338 dpm^{35}S/μg DNA to 2554 ± 617 dpm^{35}S/μg DNA, respectively), whereas values obtained for Alg–HA beads remained stable in the same period, 3717 ± 497 dpm^{35}S/μg DNA at day 14 and 3941 ± 1627 dpm^{35}S/μg DNA at day 21.

For both Alg and Alg–HA sponges [Fig. 8(b)], proteoglycan synthesis significantly increased with time ($p < 0.001$ for day 7 versus day 21) to reach at day 21, values (2311 ± 856 dpm^{35}S/μg DNA and 4681 ± 856 dpm^{35}S/μg DNA, respectively) close to those obtained with Alg and Alg–HA beads.

The presence of hyaluronate in the biomaterials led to a significant increase of dpm^{35}S/μg DNA values (1.4- to 3.3-fold), at day 7 and day 14 for beads ($p < 0.01$ and $p < 0.01$, respectively) and at day 7 and day 21 for sponges ($p < 0.05$ and $p < 0.01$, respectively).

Detection of type II collagen in sponges

Type II collagen immunostaining was performed at days 10, 20, and 40 of three-dimensional culture. When cultured in sponges, cells stained positive (Fig. 9), suggesting that chondrocytes synthesized this cartilage specific protein. Collagen production was qualitatively observed since day 10 for Alg and Alg–HA sponges, but no significant increase of the staining with time was observed.

The immunodetection of type I collagen was also performed (data not shown). At all times, the sponges were never qualitatively devoid of this protein.

DISCUSSION

Alginate sponges, with or without HA, exhibit a macroporous and interconnected network comprising numerous pores and channels. In opposition with observations performed on beads,[30] the addition of HA to alginate does not increase the pore size of the sponges. Several phenomena could explain the macroporous structural organization of these sponges.

First, the interconnected and macroporous network has an average pore size in agreement with the observations of Shapiro and Cohen.[35] These authors correlated the sponge porosity with the size of the ice crystal formed during the freezing process. The pore walls instantaneously gel at the impact area of $CaCl_2$ drops with the alginate solution surface. The "well" struc-

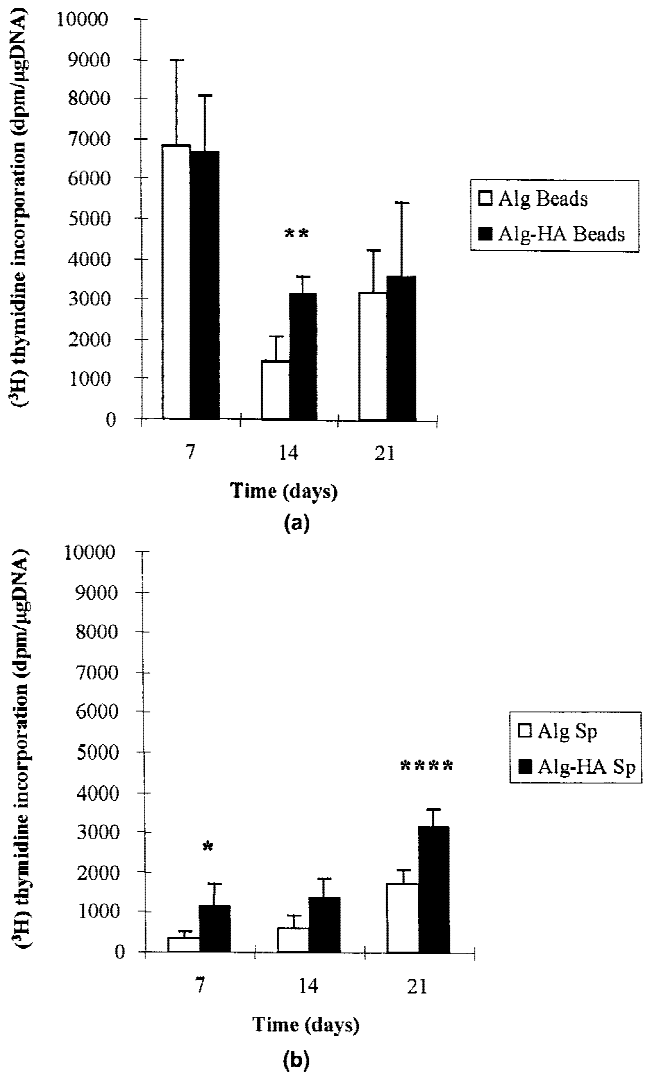


Figure 7. Evolution with time of ^{3}H-thymidine incorporation (dpm^{3}H/µg DNA) in beads (a) and in sponges (b) with or without HA. Statistical analysis was performed, at each time, for Alg beads versus Alg–HA beads and Alg Sp versus Alg–HA Sp (means ± SD, $n = 3$ different materials evaluated for each time and each formulation). Differences were considered significant if $p < 5\%$ (*), $p < 2.5\%$ (**), and $p < 0.1\%$ (****).

ture formed could be paralleled with that of "hollow" beads described by Guo et al.[2] The porosity thus obtained is appropriate for the migration of cells inside the network. In fact, the formation of a repair tissue via infiltration of environmental cells of the lesion has already been reported.[36]

Second, the formation of channel structures could be paralleled to the creation, during the gelation process, of an alginate gradient that limits the further inward diffusion of calcium ions, as previously described by Smidsrod and Skjak-Braek[37] and Skjak-Braek et al.[38] for alginate gels. It is clear that the presence of these channels could promote cell diffusion into the core of the biomaterial. Such a phenomenon has been described by Kim et al.[39] for the continuous flow culture of hepatocytes in a three-dimensional material having this type of interconnected channels network, and also by Peretti et al.[40] for the colonization of a devitalized cartilage matrix. In this latter example, the scaffold had narrow channels into which isolated chondrocytes could start repopulating.

A microporous network is commonly observed in calcium alginate gels.[36] However, its association with

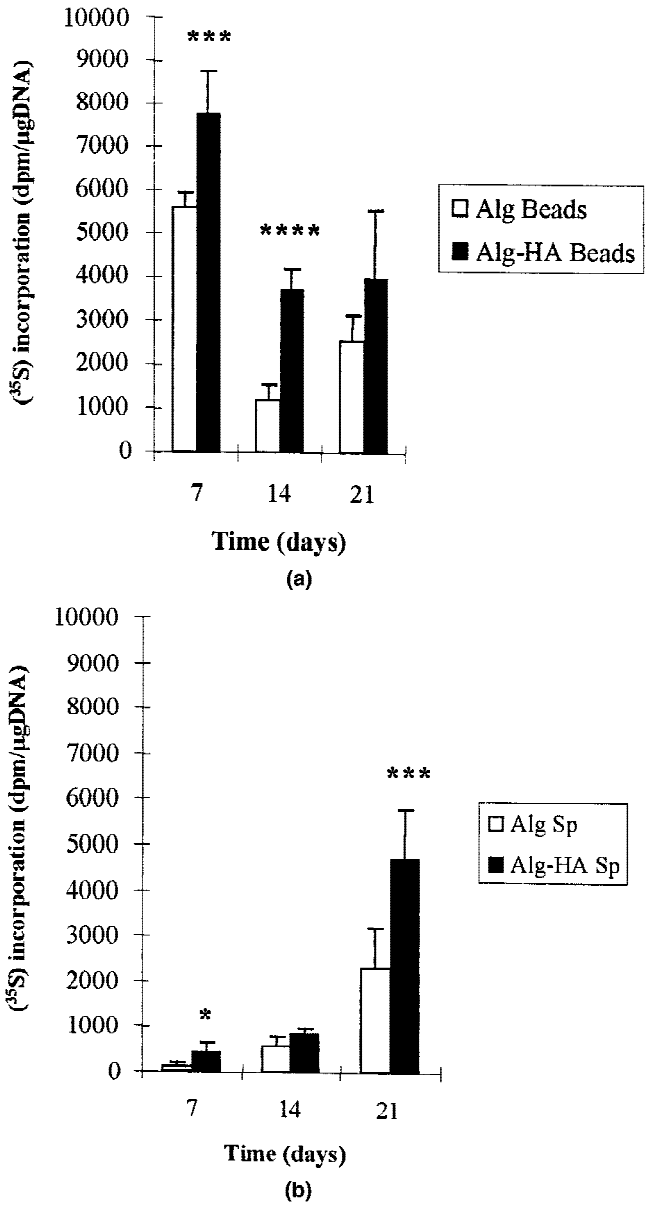
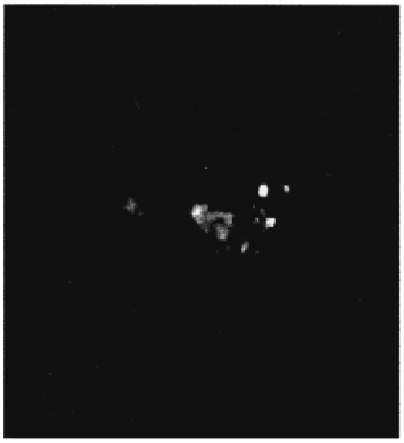
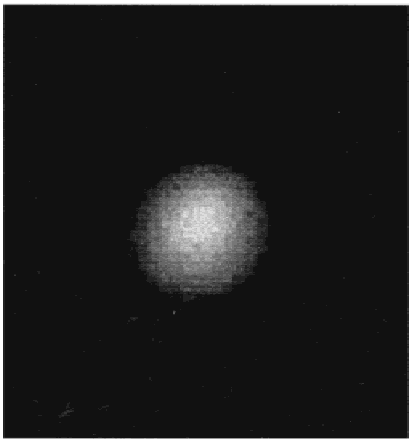


Figure 8. Evolution with time of $Na_2{}^{35}SO_4$ incorporation (dpm^{35}S/µg DNA) in beads (a) and in sponges (b) with or without HA. Statistical analysis was performed, at each time, for Alg beads versus Alg–HA beads and Alg Sp versus Alg–HA Sp (means ± SD, $n = 3$ different materials evaluated for each time and each formulation). Differences were considered significant if $p < 5\%$ (*), $p < 1\%$ (***), and $p < 0.1\%$ (****).

a



b



c

Figure 9. Immunohistochemistry of type II collagen for chondrocytes in Alg sponges at day 40 (a) and Alg–HA sponges at day 20 (b). The control (c), in which the addition of the anti-type II collagen antibodies was omitted, was used for comparison. The observation was performed on 5-μm-thick sections using an Olympus IX-70 epi-fluorescence inverted microscope, equipped with a 60×/1.25 NA oil immersion apochromatic objective. Images were collected on a cooled 12 bits Charge-Coupled Device camera.

a macroporous structure could turn out promising, especially in terms of cellular adhesion. Recently, Chen et al.[41] pointed out the interest of a hydrophilic microporous network associated with a macroporous structure, in terms of cell seeding and attachment.

In sponges, chondrocytes preserve their phenotype. The cells have a spherical shape and an organization of sparse actin filaments [Fig. 4(b)]. According to Mallein-Guerin et al.,[42] this organization of actin filaments is characteristic of non-dedifferentiated chondrocytes able to synthesize proteoglycans and type II collagen,[18,19] in opposition with dedifferentiated cells (fibroblast-like cells) which synthesize type I collagen and have well-defined large actin cables.[41] Cell culture in sponges is therefore as efficient as that in beads, well known to preserve[43,44] or to reestablish the phenotype of chondrocytes.[45] Further evidence of the non-dedifferentiation of chondrocytes is provided by the qualitative detection of type II collagen (Fig. 9). As previously described for beads,[33] chondrocytes are able to synthesize this cartilage specific protein within the sponges. Collagen fibers are bi-refringent[46] under polarized light and they start being organized at day 40.

The histological survey provides qualitative evidences that sponges are a favorable environment for cell growth as well as for deposition of a proteoglycan-rich matrix. Even though the beneficial effect of hyaluronate on both cell proliferation and proteoglycan synthesis cannot be demonstrated by histological observations, it is quantitatively evidenced by the incorporation of ^{3}H-thymidine and $Na_2{}^{35}SO_4$.

The stimulatory effect of hyaluronate could be explained by both a physicochemical process influencing cellular activity and a biologic effect, already described in the literature for hyaluronate or its oligosaccharides. In fact, in the presence of hyaluronate, the resulting less tight network[30] provides more free room for the formation of a new tissue via cell proliferation and extracellular matrix synthesis. However, the diffusion of nutrients is made easier. Also, the high hydrophilic character of hyaluronate[47] may lead to some increase of the hydrostatic pressure within the sponge loaded with this polymer. Cellular proliferation and synthesis activity could then be enhanced, as previously reported in numerous studies with chondrocyte-like cell lines.[48,49]

Some authors have shown that hyaluronate exerts a stimulatory effect on cellular proliferation and proteoglycan synthesis (particularly aggrecan) according to various mechanisms. Kawasaki et al.[50] have evidenced a dose-dependent effect of HA on chondroitin sulfate synthesis by chondrocytes cultured in a collagen gel. In a recent study performed by Lindenhayn et al.,[51] human and porcine chondrocytes exhibited a more important proliferation when cultured in hyaluronan-containing alginate gels, as compared with that in HA-

free alginate beads. Hyaluronate modifies the metabolism of chondrocytes[52] and it has been shown to suppress the release of proteoglycans from the cell matrix layer.[51] Moreover, in chondrocyte cultures, hyaluronan has been found to reduce cell injury and proteoglycan resorption induced by oxygen-derived free radicals arising in culture media due to respirative processes.[53] At last, it has been proved recently that HA induces protein kinase activity through CD44 and activates a cytoplasmic signal transduction pathway.[54] This pathway may include mitogen-activated protein activation, resulting in nuclear translocation of extracellular signal-regulated kinase 1 to stimulate cell proliferation.[52]

CONCLUSION

Alginate formulations, sponges or beads, with or without HA, proved efficient for the *in vitro* culture of chondrocytes. The addition of hyaluronate improved both cell proliferation and proteoglycan synthesis. In either type of sponges, specific cartilage proteins, such as type II collagen, have been detected and collagen fibers proved to be organized. In agreement with previous studies, no dedifferentiation of chondrocytes was noted in either type of alginate formulations.[44] Van Susante et al.[55] have suggested that, in alginate gel, the conservation of chondrocyte phenotype and the gradual increase of proteoglycan synthesis are favorable factors for the *in vitro* formation of a neocartilage. However, the diffusion rate of nutrients into the relatively dense porous structure of alginate gels was a limiting factor. The large porosity of sponges as well as the presence of HA are two promising factors in the perspective of obtaining an extracellular matrix close to native cartilage. To complement this *in vitro* study, biocompatibility and mechanical properties of such hybrid alginate–hyaluronate sponges should be investigated in the near future.

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References

1. Hauselmann HJ, Masuda K, Hunziker EB, Neidhart M, Mok SS, Michel BA, Thonar EJ. Adult human chondrocytes cultured in alginate to form a matrix similar to native human articular cartilage. Am J Physiol 1996;271:742–752.
2. Guo J, Jourdian GW, McCallum DK. Culture and growth characteristics of chondrocytes encapsulated in alginate beads. Connect Tissue Res 1989;19:277–297.
3. Wakitani S, Kimura T, Hirooka A, Ghi T, Yoneda M, Yasui N, Owaki H, Gno K. Repair of rabbit articular cartilage with allograph chondrocytes embedded in collagen gel. J Bone Joint Surg 1989;71B:74–80.
4. Kim BS, Mooney DJ. Development of biocompatible synthetic extracellular matrices for tissue engineering. Trends Biotechnol 1998;16:224–230.
5. Lohmann CH, Schwartz Z, Niederauer GG, Carnes DL Jr, Dean DD, Boyan BD. Pretreatment with platelet derived growth factor-BB modulates the ability of costochondral resting zone chondrocytes incorporated into PLA/PGA scaffolds to form new cartilage *in vivo*. Biomaterials 2000;21:49–61.
6. Freed LE, Marquis JC, Nohria A, Emmanual J, Mikos AG, Langer R. Neocartilage formation *in vitro* and *in vivo* using cells cultured on synthetic biodegradable polymers. J Biomed Mater Res 1993;27:11–23.
7. Newman AP. Articular cartilage repair. Am J Sports Med 1998; 26:309–324.
8. Choi YS, Hong SR, Lee YM, Song KW, Park MH, Nam YS. Studies on gelatin-containing artificial skin. II. Preparation and characterization of crosslinked gelatin-hyaluronate sponge. J Biomed Mater Res 1999;48:631–639.
9. Orgill DP, Straus FH II, Lee RC. The use of collagen-GAG membranes in reconstructive surgery. Ann NY Acad Sci 1999; 888:233–248.
10. Mikos AG, McIntire LV, Anderson JM, Babensee JE. Host response to tissue engineered devices. Adv Drug Delivery Rev 1998;33:111–139.
11. Glicklis R, Shapiro L, Agbaria R, Merchuk JC, Cohen S. Hepatocyte behavior within three-dimensional porous alginate scaffolds. Biotechnol Bioeng 2000;67:344–353.
12. Mayer J, Karamuk E, Akaike T, Wintermantel E. Matrices for tissue engineering-scaffold structure for a bioartificial liver support system. J Controlled Release 2000;64:81–90.
13. Kneser U, Kaufmann PM, Fiegel HC, Pollok JM, Kluth D, Herbst H, Rogiers X. Long-term differentiated function of heterotopically transplanted hepatocytes on three-dimensional polymer matrices. J Biomed Mater Res 1999;47:494–503.
14. Ohgushi H, Caplan AI. Stem cell technology and bioceramics: From cell to gene engineering. J Biomed Mater Res 1999;48: 913–927.
15. Corden TJ, Jones IA, Rudd CD, Christian P, Downes S, McDougall KE. Physical and biocompatibility properties of poly-epsilon-caprolactone produced using *in situ* polymerisation: A novel manufacturing technique for long-fibre composite materials. Biomaterials 2000;21:713–724.
16. Radice M, Brun P, Cortivo R, Scapinelli R, Battaliard C, Abatangelo G. Hyaluronan-based polymers as delivery vehicles for bone-marrow-derived mesenchymal progenitors. J Biomed Mater Res 2000;50:101–109.
17. Angele P, Kujat R, Nerlich M, Yoo J, Goldberg V, Johnstone B. Engineering of osteochondral tissue with bone marrow mesenchymal progenitor cells in a derivatized hyaluronan-gelatin composite sponge. Tissue Eng 1999;5:545–554.
18. Nehrer S, Breinan HA, Ramappa A, Shortkroff S, Young G, Minas T, Sledge CB, Yannas IV, Spector M. Canine chondrocytes seeded in type I and II collagen implants investigated *in vitro*. J Biomed Mater Res 1997;38:95–104.
19. Gugala Z, Gogolewski S. *In vitro* growth and activity of primary chondrocytes on a resorbable polylactide three-dimensional scaffold. J Biomed Mater Res 2000;49:183–191.
20. Solchaga LA, Dennis JE, Goldberg VM, Caplan AI. Hyaluronic acid-based polymers as cell carriers for tissue-engineering repair bone and cartilage. J Orthop Res 1999;17:205–213.
21. Brun P, Abatangelo G, Radice M, Zacchi V, Guidolin D, Daga Gordini D, Cortivo R. Chondrocyte aggregation and reorganization into three-dimensional scaffolds. J Biomed Mater Res 1999;46:337–346.
22. Vacanti CA, Langer R, Schloo B, Vacanti JP. Synthetic polymers

seeded with chondrocytes provide a template for new cartilage formation. Plast Reconstr Surg 1991;88:753–759.
23. Grande DA, Halberstadt C, Naughton G, Schwartz R, Manji R. Evaluation of matrix scaffolds for tissue engineering of articular cartilage grafts. J Biomed Mater Res 1997;34:211–220.
24. Lahiji A, Sohrabi A, Hungerford DS, Frondoza CG. Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes. J Biomed Mater Res 2000; 51:586–595.
25. D'Souza AL, Masuda K, Otten LM, Nishida Y, Knudson W, Thonar EJ. Differential effects of interleukin-1 on hyaluronan and proteoglycan metabolism in two compartments of the matrix formed by articular chondrocytes maintained in alginate. Arch Biochem Biophys 2000;374:59–65.
26. Perka C, Schultz O, Spitzer RS, Lindenhayn K, Burmester GR, Sittinger M. Segmental bone repair tissue-engineered periosteal cell transplants with bioresorbable fleece and fibrin scaffolds in rabbits. Biomaterials 2000;21:1145–1153.
27. Marijnissen WJCM, van Osch GJVM, Aigner J, Verwoerd-Verhoef HL, Verhaar JAN. Tissue-engineered cartilage using serially passaged articular chondrocytes: Chondrocytes in alginate, combined *in vivo* with a synthetic (E210) or biologic biodegradable carrier (DBM). Biomaterials 2000;21:571–580.
28. Loty S, Sautier JM, Loty C, Boulekbache H, Kokubo T, Forest N. Cartilage formation by fetal rat chondrocytes cultured in alginate beads: A proposed model for investigating tissue–biomaterial interactions. J Biomed Mater Res 1998;42:213–222.
29. Fragonas E, Valente M, Pozzi-Mucelli M, Toffanin R, Rizzo R, Silvestri F, Vittur F. Articular cartilage repair in rabbits by using suspensions of allogenic chondrocytes in alginate. Biomaterials 2000;21:795–801.
30. Oerther S, Le Gall H, Payan E, Lapicque F, Presle N, Hubert P, Dexheimer J, Netter P. Hyaluronate-alginate gel as a novel biomaterial: Mechanical properties and formation mechanism. Biotechnol Bioeng 1999;63:206–215.
31. Dumas D, Gigant C, Presle N, Cipolletta C, Miralles G, Payan E, Jouzeau JY, Mainard D, Terlain B, Netter P, Stoltz JF. The role of 3D-microscopy in the study chondrocyte-matrix interaction (alginate bead or sponge, rat femoral head cap, human osteoarthritic cartilage) and pharmacological application. Biorheology 2000;37:165–176.
32. Labarca C, Paigen K. A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 1980;102:344–352.
33. Petit B, Masuda K, D'Souza AL, Otten L, Pietryla D, Hartmann DJ, Morris NP, Uebelhart D, Schmid TM, Thonar EJ. Characterization of crosslinked collagens synthesized by mature articular chondrocytes cultured in alginate beads: Comparison of two distinct matrix compartments. Exp Cell Res 1996;225:151–161.
34. Chang J, Poole CA. Sequestration of type VI collagen in the pericellular microenvironment of adult chondrocytes cultured in agarose. Osteoarthritis Cartilage 1996;4:275–285.
35. Shapiro L, Cohen S. Novel alginate sponges for cell culture and transplantation. Biomaterials 1997;18:583–588.
36. Oxley HR, Corkhill PH, Fitton JH, Tighe BJ. Macroporous hydrogels for biomedical applications: Methodology and morphology. Biomaterials 1993;14:1064–1072.
37. Smidsrod O, Skjak-Braek G. Alginate as immobilization matrix for cells. Trends Biotechnol 1990;8:71–78.
38. Skjak-Braek G, Grasdalen H, Smidsrod O. Inhomogeneous polysaccharide ionic gels. Carbohydr Polym 1989;10:31–54.
39. Kim SS, Utsunomiya H, Koski JA, Wu BM, Cima MJ, Sohn J, Mukai K, Griffith LG, Vacanti JP. Survival and function of hepatocytes on a novel three-dimensional synthetic biodegradable polymer scaffold with an intrinsic network of channels. Ann Surg 1998;228:8–13.
40. Peretti GM, Randolph MA, Caruso EM, Rossetti F, Zaleske DJ. Bonding of cartilage matrices with cultured chondrocytes: An experimental model. J Orthop Res 1998;16:89–95.
41. Chen G, Ushida T, Tateishi T. A biodegradable hybrid sponge nested with collagen microsponges. J Biomed Mater Res 2000; 51:273–279.
42. Mallein-Gerin F, Garrone R, Van der Rest M. Proteoglycan and collagen synthesis are correlated with actin organization in dedifferentiating chondrocytes. Eur J Cell Biol 1991;56:364–373.
43. Bonaventure J, Kadhom N, Cohen-Solal L, Ng KH, Bourguignon J, Lasselin C, Freisinger P. Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Exp Cell Res 1994;212:97–104.
44. Hauselmann HJ, Aydelotte MB, Schumacher BL, Kuettner KE, Gitelis SH, Thonar EJ. Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads. Matrix 1992;12:116–129.
45. Binette F, McQuaid DP, Haudenschild DR, Yaeger PC, McPherson JM, Tubo R. Expression of stable articular cartilage phenotype without evidence of hypertrophy by adult human articular chondrocytes *in vitro*. J Orthop Res 1998;16:207–216.
46. Dharmavaram RM, Liu G, Tuan RS, Stokes DG, Jimenez SA. Stable transfection of human fetal chondrocytes with a type II procollagen minigene. Arthritis Rheum 1999;42:1433–1442.
47. Balazs EA. Use of hyaluronic acid in eye surgery. Annee Ther Clin Ophtalmol 1982;33:95–110.
48. Takano-Yamamoto T, Soma S, Nagakawa K, Kobayashi Y, Kawakami M, Sakuda M. Comparison of the effects of hydrostatic compressive force on glycosaminoglycan synthesis and proliferation in rabbit chondrocytes from mandibular condylar cartilage, nasal septum, and spheno-occipital synchondrosis *in vitro*. Am J Orthod Dentofacial Orthop 1991;99:448–455.
49. Takahashi K, Kubo T, Kobayashi K, Imanishi J, Takigawa M, Arai Y, Hirasawa Y. Hydrostatic pressure influences mRNA expression of transforming growth factor-beta 1 and heat shock protein 70 in chondrocyte-like cell line. J Orthop Res 1997;15:150–158.
50. Kawasaki K, Ochi M, Uchio Y, Adachi N, Matsusaki M. Hyaluronic acid enhances proliferation and chondroitin sulfate synthesis in cultured chondrocytes embedded in collagen gels. J Cell Physiol 1999;179:142–148.
51. Lindenhayn K, Perka C, Spitzer RS, Heilmann HH, Pommerening K, Mennicke J, Sittinger M. Retention of hyaluronic acid in alginate beads: Aspects for *in vitro* cartilage engineering. J Biomed Mater Res 1999;44:149–155.
52. Knudson W, Casey B, Nishida Y, Eger W, Kuettner KE, Knudson CB. Hyaluronan oligosaccharides perturb cartilage matrix homeostasis and induce chondrocytic chondrolysis. Arthritis Rheum 2000;43:1165–1174.
53. Larsen NE, Lombard KM, Parent EG, Balazs EA. Effect of hylan on cartilage and chondrocytes cultures. J Orthop Res 1992;10:23–32.
54. Slevin M, Krupinski J, Kumar S, Gaffney J. Angiogenic oligosaccharides of hyaluronan induce protein tyrosine kinase activity in endothelial cells and activate a cytoplasm signal transduction pathway resulting in proliferation. Lab Invest 1998;78: 987–1003.
55. Van Susante JLC, Buma P, Homminga GN, van den Berg WB, Veth RPH. Chondrocyte-seeded hydroxyapatite for repair of large articular cartilage defects: A pilot study in the goat. Biomaterials 1998;19:2367–2374.