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# Native and DPPA cross-linked collagen sponges seeded with fetal bovine epiphyseal chondrocytes used for cartilage tissue engineering

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## Abstract

Collagen-based biomaterials in the form of sponges (bovine type I collagen, both native and cross-linked by treatment with diphenylphosphorylazide, noted control and DPPA sponges respectively) were tested as three-dimensional scaffolds to support chondrocyte proliferation with maintenance of the phenotype in order to form neocartilage. Control and DPPA sponges were initially seeded with  $10^6$  or  $10^7$  fetal bovine epiphyseal chondrocytes and maintained for 4 weeks in culture under static conditions in RPMI/NCTC medium with 10% FCS and without addition of fresh ascorbic acid. Both supports were always present during the study and a partial decrease in size and weight was detected only with control sponges, both seeded and unseeded. Cell proliferation was only noted in the  $10^6$  cells-seeded sponges (4-fold increase after 4 weeks of culture). Specific cartilage collagens (types II and XI) were deposited in the matrix throughout the culture and traces of type I collagen were noticed only in the culture medium after 2–3 weeks and 4 weeks in the case of  $10^6$  and  $10^7$  cells-seeded sponges, respectively. Glycosaminoglycans accumulated in the matrix, up to 1.8 and 9.8% of total dry weight after one month with both seeding conditions, which was much lower than in the natural tissue. In the  $10^7$  cells-seeded sponges, mineral deposition, observed with unseeded sponges, was significantly decreased (2- to 3-fold). These in vitro results indicate that both collagen matrices can support the development of tissue engineered cartilage. © 2000 Elsevier Science Ltd. All rights reserved.

*Keywords:* Articular cartilage repair; Collagen-based biomaterial; Collagen synthesis; Glycosaminoglycan deposition; Mineral deposition

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## 1. Introduction

Mature articular cartilage does not repair adequately when injured as a consequence of trauma or diseases such as osteoarthritis. The integrity of the cartilage is maintained by the chondrocytes that both produce an extracellular matrix consisting mainly of collagens [1] and proteoglycans [2] and also control its composition and organization. These cells are ineffective in responding to injury as wounds limited to cartilage stimulate only a slight reaction in the adjacent chondrocytes [3,4]. Moreover, after a full-thickness injury the observed fibrocartilaginous response due to the subchondral bone is short lived and has inferior mechanical properties to those of natural cartilage [5]. During the past decade many investigations have pursued techniques to stimulate articular cartilage repair or regeneration (for

references see reviews [6–8]). Reports have described the replacement of damaged or lost cartilage with tissue grafts (periosteum, perichondrium or cartilage) [9,10] or with delivery of chondrocytes or mesenchymal stem cell suspensions [11–17]. Stimulation of cartilage formation by the local injection of factors with chondrogenic potential such as transforming growth factor beta-1, insulin growth factor, bone morphogenic proteins was also investigated [3,18,19]. Successful results of autologous chondrocyte transplantation have led to the commercial development of this protocol in young adults with localized cartilage defects [20,21]. Briefly, chondrocytes were harvested from an area of the patient's normal articular cartilage, grown in culture and injected in the defect under a flap of periosteum. This procedure has produced satisfactory results in more than 90% of patients but its application seems to be limited to patients younger than 40 and without osteoarthritic pathologies.

Another approach to introducing a new cell population into a cartilage defect was to deliver it in a scaffold to facilitate cell carrying and surgical handling for

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implantation and also to support cell growth and new matrix formation. These tissue engineering techniques [22] are based on the use of various biocompatible vehicles for grafting cartilage defects such as synthetic polymers: polyglycolic acid (PGA) [23–27], polylactic acid (PLA) [28], polyglycolic acid-poly-L-lactic acid copolymer (PGA-PLA) [29,30], polyethylene [31], polyethylene oxide [32]. Carrier materials of natural origin have also been used: alginate [33], fibrin [34–36], hyaluronic acid [37,38], gelatin [39], collagen gel [13,33,40], collagen spheres [41], collagen-glycosaminoglycan matrices [42] and collagen sponges [25,43–46].

The use of collagen sponges as scaffolds for culture is facilitated by the fact that they are mechanically stable, have a pore structure that allows cell colonization, have low immunogenicity and are easily configured into different shapes [47,48]. Collagen sponges (native type I collagen) can be chemically cross-linked by different techniques such as the acyl azide method using diphenylphosphorylazide (DPPA) developed in our laboratory [49,50]. This treatment results in collagen biomaterials with excellent biocompatibility, increased mechanical stability and decreased biodegradability, which are currently used as membranes for tissue guided regeneration [51]. We have recently described the behaviour of mouse and human fibroblasts in these native and DPPA-cross-linked sponges [52,53].

The objective of this study was to evaluate *in vitro* the ability of these three-dimensional matrices, seeded with fetal bovine epiphyseal chondrocytes, to support formation of a cartilage construct with a composition as close as possible to that of natural cartilage. In the current protocol of transplantation of autologous chondrocytes in humans the cells are propagated in monolayer culture on plastic where they undergo a rapid dedifferentiation. We have studied here the possibility of multiplying chondrocytes directly into the collagen sponges without changes in their initial phenotype. Fetal bovine epiphyseal chondrocytes, which we previously studied in long term culture at high density [54–56], were seeded on these supports at different initial densities and their metabolic activity and phenotype were followed during 4 weeks in culture. Total protein and collagen syntheses, proteoglycan content and mineral deposition were determined at different times in culture as well as the biochemical composition of the cartilage construct at the end of the experiment.

## 2. Materials and methods

### 2.1. Collagen sponges

Collagen sponges, manufactured by Coletica (Lyon, France), were made up of collagen extracted from the skin of young calves containing 90–95% (dry weight)

native type I collagen and 4–9% type III collagen. Sponges were prepared from a collagen solution (0.7% w/v) freeze-dried to produce a porous matrix with  $58 \pm 24 \mu\text{m}$  mean pore size (measured from 50 pores on SEM photographs as described by Matsuda et al. [57]) and 6 mm thickness. Individual discs were cut out with a 10 mm diameter punch before being sterilized with 15 kGy  $\beta$  radiation. Such sponges exhibited thermal transition temperatures measured by differential scanning calorimetry (Setaram, Lyon, France) of  $36.3 \pm 0.1^\circ\text{C}$  (onset) and  $44 \pm 0.5^\circ\text{C}$  (peak) and were referred to as control sponges. To increase their stability, some control sponges were cross-linked using the diphenylphosphorylazide (DPPA) technique described by Petite et al. (1994) [49]. Briefly, they were soaked in distilled water and then in dimethylformamide (DMF) and left for 24 h at room temperature in a solution of DMF containing 25  $\mu\text{l}$  of DPPA per 0.8 g of sponge. Such sponges, referred to as DPPA sponges, punched and sterilized like the control sponges, exhibited thermal transition temperatures of  $42.9 \pm 3.4^\circ\text{C}$  (onset) and  $49.7 \pm 2.2^\circ\text{C}$  (peak).

### 2.2. Chondrocytes

Chondrocytes were obtained by enzymatic digestion from the reserve zone of the distal femoral epiphyses of four-month-old bovine fetuses (six different animals were used in this study) after careful removal of the articular surface and the growth plate [54]. The isolated cells were kept in RPMI/NCTC (v/v) medium (Seromed®, Strasbourg, France/Sigma, Saint Quentin Fallavier, France) containing 10% foetal calf serum (Seromed®), 50 U/ml penicillin, 50  $\mu\text{g/ml}$  streptomycin and 1 ng/ml amphotericin. No fresh ascorbic acid was added during this study.

### 2.3. Cell culture

Before seeding, the sponges were placed in 24-well culture plates for 2 h at room temperature in complete culture medium. After removing the medium, each sponge was seeded with  $10^6$  or  $10^7$  chondrocytes deposited onto the sponge in a volume of 50  $\mu\text{l}$  and further incubated at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  for 2 h. After this, 2 ml of complete culture medium were added to each well. After 24 h in culture, to remove the cells present on the bottom of the well, especially in the  $10^7$  cells-seeded supports, the sponges were placed in new culture plates. The sponges were kept in culture for one month with changes of medium every 3 days. During the study, unseeded sponges were kept in culture as controls.

### 2.4. Cell proliferation

DNA content of the sponge was measured by the fluorimetric method of Lipman [58] on the cytofluor

4000 (Millipore, Saint-Quentin Yvelines, France) and with DNA calf thymus as standard. Cell number was calculated by using a converting factor of 8 pg DNA per chondrocyte [56].

### 2.5. *Sponge weight and surface area*

Each week, the weight of seeded and unseeded sponges was measured on an electric microbalance (Mettler-Toledo, Girolay, France) after rinsing and freeze drying. The growth of the sponge diameter (average size of the construct measured on at least three different sponges) was determined each week on photomicrographs.

### 2.6. *Histologic examination*

Samples were fixed at 4°C overnight with 2% paraformaldehyde in 0.1 M cacodylate. Sponges were dehydrated in a graded series of ethanol baths and then impregnated and embedded in Histo-Resin GMA (Leica, Bron, France) at 4°C according to the manufacturer's instruction. 4 µm thick sections were cut with a tungsten knife on a Reichert/Jung 1040 Autocut rotary microtome (Leica). Sections were stained with Azure II/methylene blue.

### 2.7. *Analysis of extracellular matrix components*

#### 2.7.1. *Cell metabolic labeling and total protein and collagen syntheses*

Total protein and collagen syntheses were measured by labeling the culture with 2 µCi per sponge of <sup>14</sup>C Proline (290 mCi/mmol, NEN, Le Blanc Mesnil, France) for 24 h. After labeling, medium and washed sponges were separately dialyzed against water to eliminate unincorporated tracers. The samples were frozen, lyophilized and submitted separately to limited pepsin digestion. Culture media were treated as previously described [56]. Neosynthesized proteins in the sponges were digested by pepsin A from porcine stomach mucosa (Sigma, Saint Quentin Falavier, France) for 24 h at 4°C with magnetic stirring and at a 1/10, w/w pepsin/protein ratio. More than 92% of radiolabeled proteins were extracted by this procedure. The content of dpm in the pepsin-treated samples gave the level of synthesis of total protein. Pepsin-resistant proteins, assumed to be collagenous, were obtained after dialysis of the samples. After lyophilization the samples were dissolved in Laemmli's buffer and counted for their radioactive content. Total neosynthesized collagen content was expressed as total dpm/DNA deposited in the cell layer and in culture medium, or as percentage of total protein synthesis after correction for the enrichment of proline in collagen with respect to the non-collagen proteins [59].

#### 2.7.2. *Collagen typing*

Neosynthesized pepsin-soluble collagens deposited in the cell layer and secreted in the culture medium after 24 h labeling were analyzed by SDS-PAGE in a 7% separating gel under non-reducing conditions. Extracts containing the same amount of dpm (10 000) were precipitated by ethanol, redissolved in electrophoresis buffer and loaded on each lane of the gel. The bands corresponding to type I collagen (heterotrimer composed of 2 α1{I} chains and one α2{I} chain), type II collagen (homotrimer composed of 3 α1{II} chains) and type XI collagen (heterotrimer composed of α1{XI}, α2{XI}, α3{XI} chains) were visualized by fluorography and identified by comparison with collagen standards and western blotting with specific antibodies as previously described [60].

#### 2.7.3. *Glycosaminoglycan content*

A modification of the dimethylmethylene blue (DMMB) method of Farndale et al. [61] was used to quantify sulphated GAG content in the cultured sponges. Briefly, the samples were digested in 0.1% papain + 0.1% proteinase K in 1 mM CaCl<sub>2</sub>, 10 mM Tris HCl, pH 8 at 60°C for 24 h with low stirring. A 100-µl aliquot of the digest solution was assayed for total GAG content by addition of 2.5 ml of DMMB solution. The absorbance was measured at 525 nm and the proteoglycan content calculated by comparison with shark chondroitin sulfate (Sigma) standard curve.

#### 2.7.4. *Glycosaminoglycans identification*

The separation of the different GAG contained in the sponges was performed using an electrophoretic technique described by Bertolotto et al. [62]. The samples were prepared as for GAG content analysis and Titan III Zip Zone cellulose acetate plates (Helena Laboratories, Saint-Leu la Forêt, France) and 0.1 M barium acetate, pH 5 were used. Bovine placenta dermatan sulfate (Coletica), shark cartilage chondroitin sulfate (Sigma) and hyaluronic acid (Bomann Laboratories, St Etienne de Crossey, France) were used as standards. Glycosaminoglycans were visualized after alcian blue (0.1%) staining followed by a destaining in acetic acid (10%) and methanol (50%). Electrophoretograms were scanned using a Personal Densitometer<sup>TM</sup> (Molecular Dynamics).

#### 2.7.5. *Amino acid analysis*

Sponges seeded with chondrocytes, collected after one month in culture, were freeze-dried and hydrolysed with 6 N HCl for 24 h at 110°C under vacuum in a Picotag<sup>TM</sup> work station (Waters). The amino acid content was established with an automatic System 6300 analyser (Beckman). The percentage of collagen was estimated from the hydroxyproline level. Results were reported as the collagen percentage on a dry weight basis.

## 2.8. Sponge mineralization

Calcium and phosphorus contents of the sponges after one week and four weeks in culture were determined on lyophilized samples by induction coupling plasma-atomic emission spectroscopy at the Service Central d'Analyse du CNRS (Solaize, France).

## 3. Results

### 3.1. Macroscopic characteristics of the sponge

When compared to their initial dry dimensions, a decrease in sponge diameter was observed in the first days of culture for control sponges ( $30.8 \pm 3\%$ ) either unseeded or seeded (Fig. 1A). During the rest of the culture, no additional changes in diameter were observed. Unseeded and seeded DPPA sponges did not present significant variations in comparison with their initial diameter. These variations were confirmed by histological analysis of the remnant network: its size decreased by 1/3 in the native sponges and was not modified in the DPPA-cross-linked sponges (Fig. 2).

After five days of culture the unseeded control sponges had lost 29% of their initial weight and this decrease reached 40% after 4 weeks in culture (Fig. 1B). In the presence of  $10^6$  cells, similar changes were observed over this time. A smaller decrease (20%) was measured after 4 weeks in the  $10^7$  cells-seeded sponges. DPPA sponges, either unseeded or seeded, exhibited at each measurement larger variations in weight than control sponges but did not decrease in weight after one month in culture.

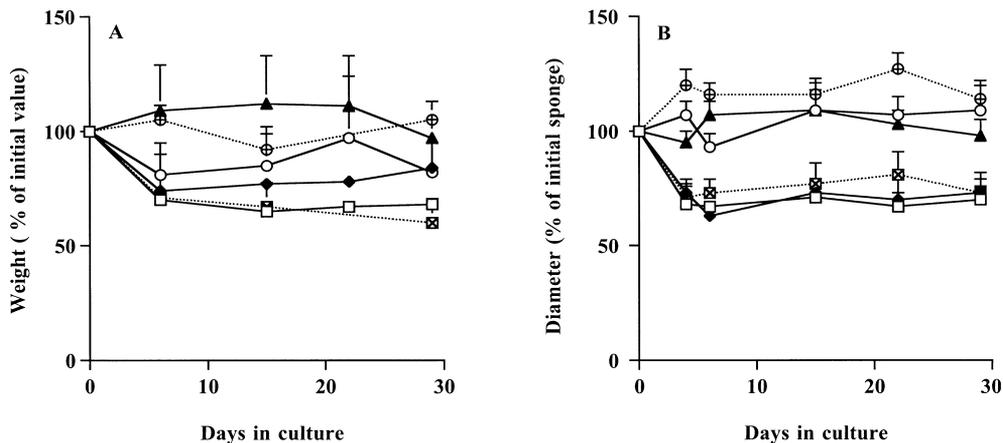


Fig. 1. Changes of dry weight (A) and diameter (B) (expressed as percentage of initial dry value) of sponges during 1 month of culture. Unseeded control (■) and DPPA sponges (⊕). Cells were seeded in either control sponges (□ for  $10^6$  cells and ◆ for  $10^7$  cells) or in DPPA sponges (○  $10^6$  cells and ▲ for  $10^7$  cells). Data are presented as the mean  $\pm$  s.d. of triplicate samples.

## 3.2. Histological analysis

In Fig. 2, we report the results with DDPA sponges only, as the same differences were obtained with the two types of support seeded with  $10^6$  and  $10^7$  cells, respectively. Over the 4 week time period the pore sizes ( $58 \pm 24 \mu\text{m}$ ) were constant in the unseeded sponges (Fig. 2a and b), cultured at  $37^\circ\text{C}$ . At the start of the culture both supports showed a progressive penetration of predominantly elongated cells throughout the collagen network (Fig. 2c and e). After 1 week in culture, chondrocytes remained flat and attached to the walls of the sponge in the center of the  $10^6$  cells seeded sponges (Fig. 2c). The cells became round and isolated and were surrounded by a new intensely stained extracellular matrix after 1 week in culture in the  $10^7$  cells-seeded sponges (Fig. 2e) and after 4 weeks in the  $10^6$  cells-seeded sponges (Fig. 2d). On the sponge surface many cells became larger and round (Fig. 2d and f) and formed a 3–4 -cells thick layer after 4 weeks of culture. With time in culture a dense extracellular matrix was formed in both types of sponge with cell density decreasing from the sponge surface to the center.

### 3.3. Cell proliferation

Chondrocyte proliferation, depending on the initial number of cells seeded in the sponges, was not affected by the type of sponge throughout the present study as shown in Fig. 3. A decrease in cell number ( $30 \pm 4\%$ ) was observed during the first week in culture for the sponges initially seeded with  $10^7$  chondrocytes. This corresponded to the detachment of cells from the sponge probably due to the high cell number/sponge volume ratio. Thereafter, a constant cell number ( $7 \pm 0.4 \times 10^6$ ) was

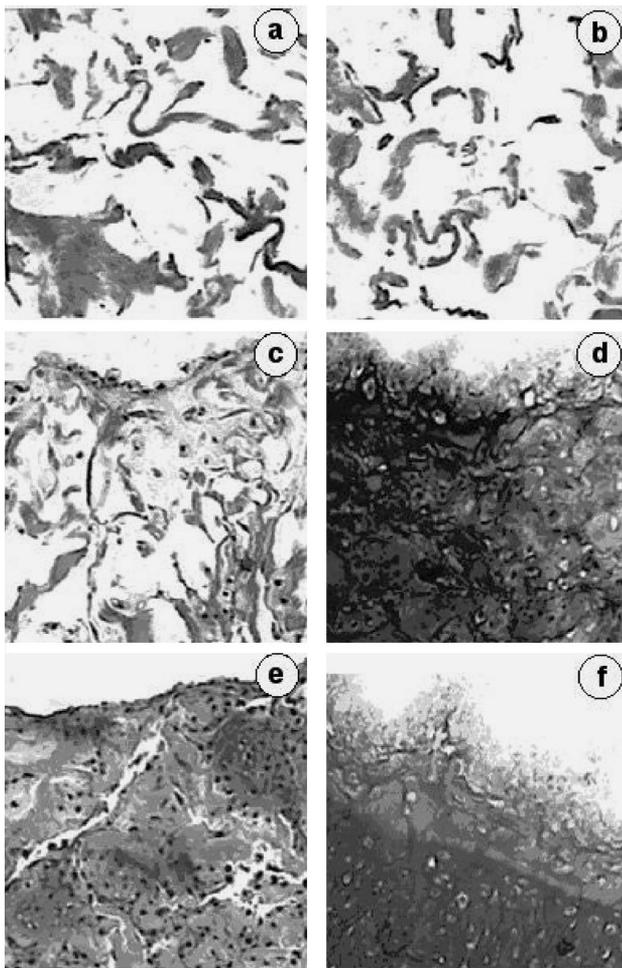


Fig. 2. Light microscopic analysis of collagen DPPA sponges grown in culture. (a) and (b) sponges without cells; (c) and (d) sponges seeded with  $10^6$  chondrocytes; (e) and (f) sponges seeded with  $10^7$  cells. (a), (c) and (e) after 1 week of culture and (b), (d) and (f) after 4 weeks of culture. Histo-Resin GMA embedded section stained with Azure II/methylene blue. Bar = 100  $\mu$ m.

observed throughout the culture. When the initial seeding was  $10^6$  chondrocytes, the cell number consistently increased reaching  $4 \pm 0.4 \times 10^6$  cells after 4 weeks in culture.

### 3.4. Analysis of matrix components

#### 3.4.1. Collagen synthesis

Collagen synthesis after 24 h labeling, expressed per cell or as a percentage of total protein synthesis, presented slight variations throughout the study in the two types of support and for the two seeding conditions (Table 1). The deposition in the cell layer of neosynthesized pepsin-resistant material increased with time in the  $10^6$  cells-seeded sponges in parallel with the increase in cell number. In the  $10^7$  cells-seeded sponges this material was deposited constantly as there was no change in cell density.

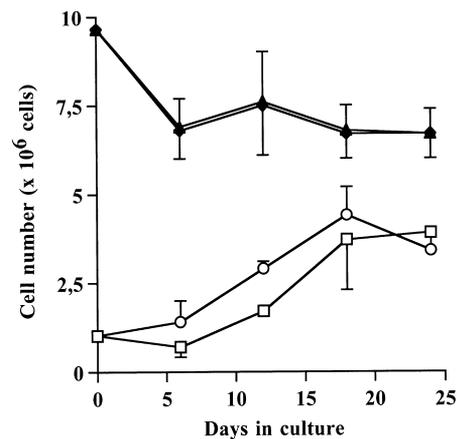


Fig. 3. Proliferation of bovine chondrocytes seeded in collagen sponges. Cells were seeded in either control sponges (□ for  $10^6$  cells and ◆ for  $10^7$  cells) or in DPPA sponges (○ for  $10^6$  cells and ▲ for  $10^7$  cells). Cell numbers were measured by the DNA content of the sponges every week during the culture. Data are presented as the mean  $\pm$  S.D. of triplicate samples.

Table 1

Changes in the level of neosynthesized pepsin-resistant proteins (24 h-labeling) by bovine chondrocytes during one month in culture<sup>a</sup>

Days	7	13	20	27
<i>Total pepsin-resistant proteins (deposited in the sponge and secreted in the culture medium) per cell (dpm/<math>\mu</math>g DNA <math>\times 10^{-3}</math>) and as a percentage (%) of total protein synthesis</i>				
<i>Control sponge</i>				
$10^6$ cells	4.3 (8%)	3 (11%)	1.8 (8%)	1.3 (7%)
$10^7$ cells	2.4 (5%)	1.8 (7%)	2.6 (5%)	2.4 (4%)
<i>DPPA sponge</i>				
$10^6$ cells	6.3 (6%)	1.2 (4%)	2.3 (12%)	2.1 (13%)
$10^7$ cells	4.2 (6%)	1.4 (5%)	2.4 (5%)	1.6 (3%)
<i>Pepsin-resistant protein deposited in the sponges (dpm <math>\times 10^{-3}</math>)</i>				
<i>Control sponge</i>				
$10^6$ cells	9.8	21.3	46.6	32.8
$10^7$ cells	82.5	58.9	117.3	115.7
<i>DPPA sponge</i>				
$10^6$ cells	11.6	17.3	72.7	48.5
$10^7$ cells	60.3	35.3	117.4	82.7

<sup>a</sup>Values represent the data from one representative single experiment. Comparable results were obtained with chondrocytes isolated from another fetus.

#### 3.4.2. Collagen typing

When the neosynthesized pepsin-resistant and -soluble collagens of the sponge compartment were examined by SDS-PAGE analysis, type II collagen was the most abundant protein extracted from the two types of sponge seeded with  $10^6$  or  $10^7$  cells (Fig. 4a–d). Type XI was detected in small amounts both in the sponge compartment and in the culture medium. Traces of type I collagen were present in the culture medium (Fig. 4e–h) only

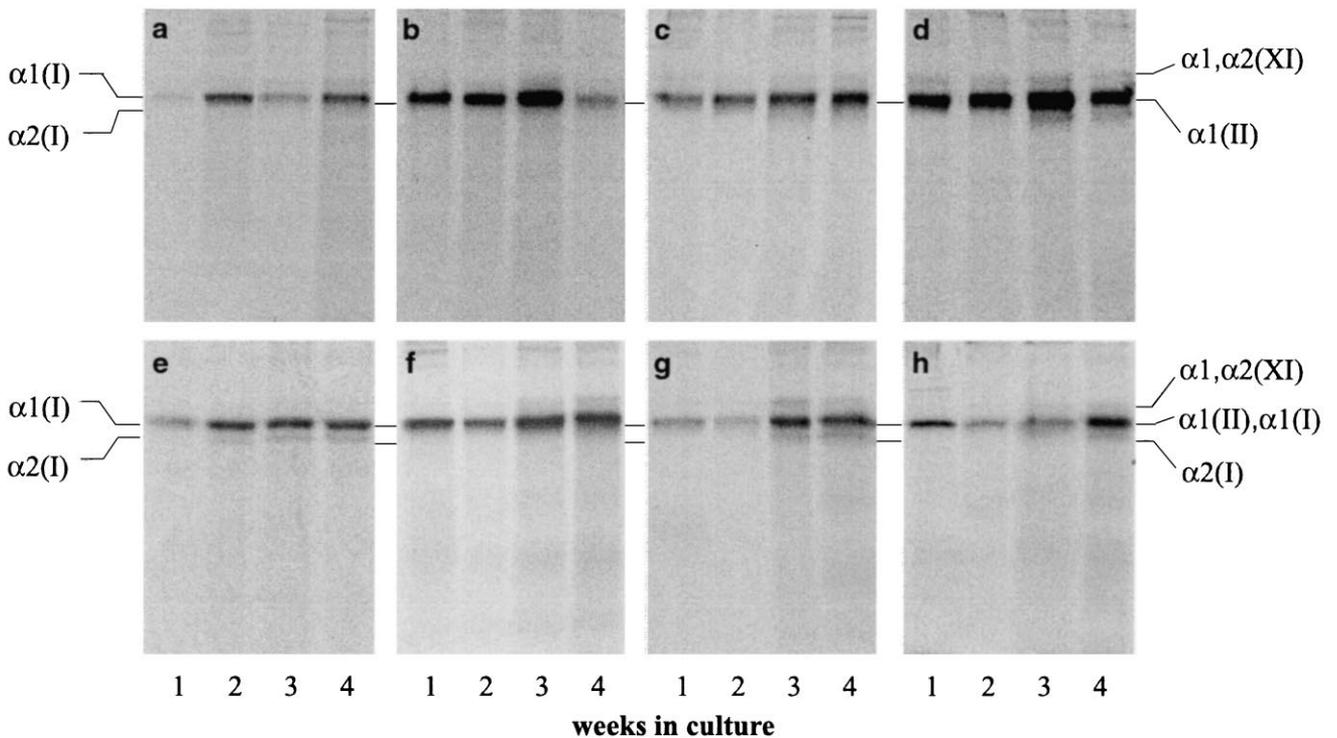


Fig. 4. Fluorographs of  $^{14}\text{C}$ -labeled pepsin-soluble collagens neosynthesized by bovine chondrocytes for the last 24 h, deposited in the cell layer (a–d) and secreted in the culture medium (e–h), after 1, 2, 3 and 4 weeks of culture in sponges and separated by SDS-PAGE. Control sponges (a and e:  $10^6$  cells; b and f:  $10^7$  cells) and DPPA sponges (c and g:  $10^6$  cells; d and h:  $10^7$  cells). Data are representative of three separate experiments.

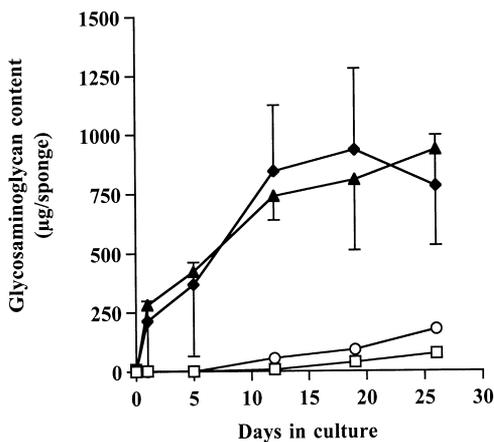


Fig. 5. Total sulphated glycosaminoglycan (GAG) content in collagen sponges seeded with bovine chondrocytes grown during 4 weeks of culture. Cells were seeded in either control sponges (□ for  $10^6$  cells and ◆ for  $10^7$  cells) or in DPPA sponges (○ for  $10^6$  cells and ▲ or  $10^7$  cells). GAG content was measured by a colorimetric method with dimethylmethylene blue. Data are presented as the mean  $\pm$  S.D. of triplicate samples. All values are on a dry weight basis.

after 2–3 weeks in culture in sponges seeded with  $10^6$  cells and only after 4 weeks with the  $10^7$  cells-seeded sponges.

### 3.4.3. GAG content

Sulfated glycosaminoglycan content was similar in both control and DPPA sponges and was dependent on

the initial cell density, as shown in Fig. 5. Amounts in both types of sponge seeded with  $10^7$  cells reached a maximum of 900  $\mu\text{g}$  per sponge in 2 weeks and remained stable from day 14 to day 25. In the sponges seeded with  $10^6$  cells, the amount of glycosaminoglycan had reached only 150  $\mu\text{g}$  per sponge at day 30.

### 3.4.4. GAG identification

Glycosaminoglycans, extracted from the different constructs and from fetal bovine cartilage, were separated by electrophoresis on cellulose acetate (Fig. 6). Chondroitin sulfate and hyaluronic acid, the major GAGs of cartilage, were present in the two types of sponge seeded with  $10^6$  and  $10^7$  cells after 4 weeks of culture. Traces of dermatan sulfate were detected in fetal bovine cartilage and after 4 weeks of culture in DPPA sponges seeded with  $10^6$  and  $10^7$  cells (Fig. 6, lanes 5, 9 and 10).

### 3.5. Sponge mineralization

The initial amounts of Ca and P were 10 times less in DPPA sponges than in control sponges due to the long washing step performed at the end of the DPPA treatment (Table 2). In the unseeded sponges, the mineral content increased with time in culture to reach similar values in both types of sponge. In the presence of  $10^6$  cells, the Ca and P values were no different from the values found in the unseeded sponges. When seeded with

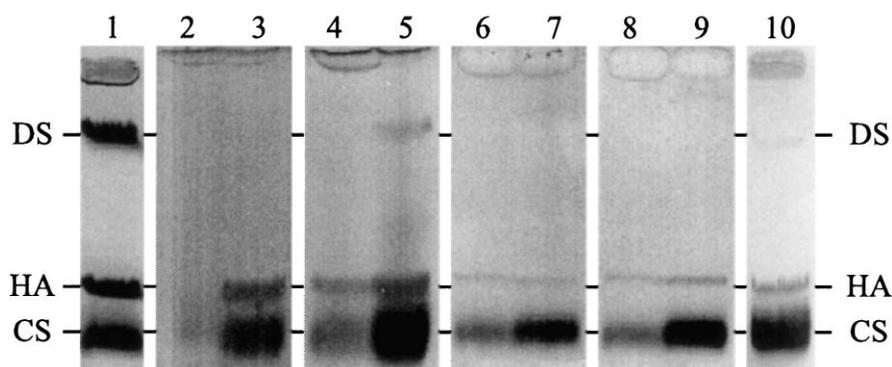


Fig. 6. Electrophoretogram of GAG contained in collagen sponges seeded with bovine chondrocytes. GAG were extracted by papain digestion of the sponges and the cartilage pieces and visualized after Alcian blue staining as described in Methods. Control sponges (lanes 2 and 3: seeded with  $10^6$  cells; lanes 6 and 7: seeded with  $10^7$  cells) and DPPA sponges (lanes 4 and 5: seeded with  $10^6$  cells; lanes 8 and 9: seeded with  $10^7$  cells) after 2 days (lanes 2-4-6-8) and 27 days (lanes 3-5-7-9) in culture. For comparison lane 1 received a mixture of dermatan sulfate (DS), chondroitin sulfate (CS) and hyaluronic acid (HA) and lane 10 received the GAG extracted from fetal bovine articular cartilage. Data are representative of three separate experiments.

Table 2  
Calcium and phosphorous content in of control and DPPA sponges seeded with bovine chondrocytes<sup>a</sup>

	Calcium content (%)		Phosphorous content (%)	
	Control	DPPA	Control	DPPA
Initial content of sponges	$0.5 \pm 0.1$	$0.05 \pm 0.01$	0.26	0.02
Unseeded sponges				
Day 7	3.1	2	1.6	1.8
Day 27	$4.9 \pm 0.7$	$3.6 \pm 1.7$	$2.5 \pm 0.3$	$1.8 \pm 0.8$
Cell-seeded sponges (Day 27)				
$1 \times 10^6$	$6.5 \pm 0.5$	$2.8 \pm 0.4$	$3.5 \pm 0.3$	$1.6 \pm 0.2$
$1 \times 10^7$	$2.4 \pm 0.5$	$1.2 \pm 1.3$	$1.6 \pm 0.3$	$0.9 \pm 0.5$

<sup>a</sup>The values in % (w/w) represent the mean  $\pm$  S.D. for  $n = 3$

$10^7$  cells a 2–2.5 fold decrease in the mineral content was observed in the two types of sponge. The Ca/P ratios measured in the different conditions varied between 1.5 and 2.

### 3.6. Biochemical characteristics of the sponges

The composition of the cartilage constructs after one month in culture is presented in Table 3 in comparison with natural bovine fetal epiphyseal and neo-natal articular cartilage. Amino acid analysis indicated that, with time in culture, the protein content of the sponges decreased towards that of natural cartilage. This was also true for the collagen level (including both sponge collagen and neosynthesized collagens), especially for the  $10^7$  cells-seeded sponges. GAG content in the 4-week constructs was dependent on cell number and represented 25% (for the  $10^7$  cells-seeded sponge) of natural bovine cartilage. Cellularities were comparable to the value found in natural articular new-born cartilage.

## 4. Discussion

In this study, sponges, made of native and diphenylphosphorylazide cross-linked collagen, were tested as scaffolds to maintain bovine chondrocytes in in vitro culture and to determine the best culture conditions for the production of a functional cartilage construct. We demonstrated that fetal bovine epiphyseal chondrocytes were able to proliferate, to populate and to deposit an extracellular matrix without disintegration of the support during one month in culture in these scaffolds. The variability in response was dependent mainly on the number of cells initially seeded in the sponges and not on the composition of the support, i.e. native or cross-linked sponges. Indeed the main difference observed between these two matrices was a rapid partial drop in weight and size of the control sponges (unseeded or seeded) after 5 days incubation at  $37^\circ\text{C}$ . This probably corresponded to partial denaturation of the collagen triple helix as the onset denaturation temperature of these control sponges was below  $37^\circ\text{C}$  as compared to  $43^\circ\text{C}$  for the DPPA sponges. We have previously demonstrated that fibroblasts were not able to contract these same sponges [52] in contrast to what was usually observed with free-floating collagen gels.

Under our culture conditions, characterized by the absence of the addition of fresh ascorbic acid to the culture medium and static flasks, and when seeded, with a low initial cell number ( $10^6$ ), chondrocytes proliferated in the sponges over time, showing a 4-fold increase after 4 weeks, with maintenance of a differentiated phenotype. Indeed the neosynthesized collagen deposited in the sponge was composed essentially of type II collagen and traces of type I collagen were only detected in the culture medium. With an initial cell number 10 times higher ( $10^7$ ), chondrocytes populated the sponges without proliferation over time but with the rapid formation of an

Table 3  
Biochemical characterization of tissue engineered constructs after one month in culture<sup>a</sup>

	Amino acid (% dry weight)	Collagen (% dry weight)	S-GAG (% dry weight)	Cell <sup>b</sup> (% dry weight)	Calcium (% dry weight)
Control sponge					
Unseeded	90 ± 19	88 ± 5	0	0	4.9 ± 0.7
10 <sup>6</sup> cells	60 ± 10	48.6 ± 6	1.3	3.9 ± 0.5	6.5 ± 0.5
10 <sup>7</sup> cells	64 ± 4	49 ± 5.8	9.8	6.7 ± 0.7	2.4 ± 0.5
DPPA sponge					
Unseeded	92 ± 17	87 ± 3	0	0	3.6 ± 1.7
10 <sup>6</sup> cells	89 ± 10	74.6 ± 3	1.8	3.4 ± 0.4	2.8 ± 0.4
10 <sup>7</sup> cells	74 ± 9	54.4 ± 7.4	6.4	6.7 ± 0.7	1.2 ± 1.3
Bovine cartilage					
fetal epiphyseal	53 ± 2	36.4 ± 4.8	40 ± 9	2.1 ± 0.6	0.061 ± 0.001
articular new born [26]	ND <sup>c</sup>	51 ± 5	34 ± 3	5 ± 0.5	ND <sup>c</sup>

<sup>a</sup>Data represent mean ± S.D. and *n* = 3.

<sup>b</sup>The dry weight of one chondrocyte was estimated to 10<sup>-10</sup> g [26].

<sup>c</sup>ND: not determined.

abundant extracellular matrix composed of type II collagen and glycosaminoglycans. Thus, differentiated chondrocytes seeded into a type I collagen matrix maintain their phenotype if the culture conditions are appropriate, indeed the addition of fresh ascorbic acid to the culture medium led to a rapid dedifferentiation of the cells (unpublished data). Similar observations were obtained with the same cells and the same culture medium composition (with or without ascorbic acid) in long-term culture at high density on plastic [56]. Therefore, it does not seem necessary to use type-II collagen matrices as proposed by Nehrer et al. [42], who showed that chondrocytes, dedifferentiated after proliferation in a monolayer culture, presented a higher level of redifferentiation after seeding in type-II versus type-I collagen sponges. Furthermore, the arthritogenic potential of type-II collagen does not favor its clinical use in humans.

After 4 weeks, cells in both scaffolds presented a round shape but with a higher density at the periphery than at the center of the sponges, certainly reflecting the access to nutrient and gas transfer as also reported using collagen disks seeded with horse chondrocytes [43] and PGA disks seeded with new-born bovine chondrocytes [25].

As the presence of mineral may be a problem for an optimum biofunctionality of cartilage constructs and as acellular apatite deposition in our collagen sponges incubated in different cell culture media was recently reported [52,63], we measured the variation in the Ca and P content in the different sponges, seeded and unseeded with chondrocytes, during the culture period. Mineral deposition in the unseeded collagen supports was previously reported to be related firstly to the sponge residual P content and secondly to Ca and P concentration in the incubation medium [63]. The culture medium used in this study (NCTC/RPMI) contains a low calcium concentration (1.18 mM) and in its presence we observed after

4 weeks in culture, a calcium content in the unseeded control and DPPA sponges (4.9–4.6%) much lower than the content (18–14%) previously [52] reported after incubation in DMEM (Ca concentration : 1.96 mM). This mineralisation was significantly decreased (2- to 3-fold) for the sponges seeded with a high cell density but in the 4-week constructs the calcium content (1.2–2.4%) was still much higher than in natural cartilage. Our findings demonstrated that this parameter, not usually considered by other authors, should not be neglected.

Comparison of the global biochemical composition of our cartilage constructs, obtained after 4 weeks of culture, with that of natural articular cartilage, demonstrated the presence, in the extracellular matrix, of the same individual components (collagens, glycosaminoglycans) but with differences in their relative proportions, especially too low a glycosaminoglycan content.

It is interesting to compare these results with those described by the MIT group using polyglycolic acid as a scaffold [17,24,26,27,64–66]. These constructs prepared under optimized conditions [27,66] more closely approximated cartilage with respect to composition and function but still without quite reaching the properties of the natural tissue (for example, a lower collagen concentration and cross-link content) [26].

These results suggest several possibilities to improve the quality of our cartilage construct, for example, by the use of a rotating bioreactor instead of static flasks, by increasing the culture time or by addition of specific factors such as TGF β1 [3,67,68], FGF-2 [17,46] or BMP-2 [19] whose mitogenic and/or chondrogenic effects were previously tested for regeneration of articular cartilage. Characterization of the exact nature of the proteoglycans synthesized and of the mechanical properties of the construct also appears necessary for a better evaluation of the biofunctionality of the final product.

Parallel studies are also performed with mature human chondrocytes to obtain neocartilage of potential use in clinical human application.

In conclusion, these results and encouraging preliminary findings (unpublished results) in the repair of a partial thickness cartilage defect in rabbit knee with DPPA cross-linked collagen sponges seeded with allogenic rabbit chondrocytes, demonstrate the suitability of collagen-based scaffolds for cartilage tissue engineering.

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