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Chondrocyte-alginate bioconstructs: An nuclear magnetic resonance relaxation study

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Abstract: Proton nuclear magnetic resonance (NMR) relaxometry can give informations about hydrogel scaffold properties. As these properties can be modified with culture time and conditions according to scaffold biodegradability and new tissue biosynthesis, the aim of this research was to test the efficiency of this noninvasive NMR technique in the follow-up of 3D cultures for tissue engineering. The distributions of proton relaxation times T_1 and T_2 have been measured on cylindrical gel samples of different types of alginate, in the presence or absence of hyaluronate, in gels or bioconstructs with encapsulated chondrocytes cultured for 30 days in normal or reduced weight conditions. It was found that T_2 increases with the mannuronate/guluronate ratio in alginate samples and with the presence of hyaluronate. The distributions of both T_1 and T_2 result wider for bioconstructs cultured in normal gravity than for those cultured in reduced weight conditions. Neither cell growing nor collagen production but only GAG neosynthesis have been demonstrated in our experimental conditions. In conclusion, T_2 is sensitive to the gel properties (possibly to the rigidity of macromolecular components). The homogeneity of bioconstructs can be monitored by the distribution of T_1 and T_2. We propose that nonspatially resolved NMR relaxometry can efficiently be used in monitoring tissue development in a biodegradable scaffold for tissue engineering. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 83A: 345–353, 2007

Key words: alginate gels; chondrocyte 3 D cultures; bioconstructs; ^{1}H NMR relaxometry; normal and reduced weight conditions

INTRODUCTION

Alginates, a polysaccharide family of unbranched copolymers of (1 → 4) linked residues of β-D-mannuronic and α-L-guluronic acids, can form three-dimensional gels when their aqueous solutions are treated with Ca^{2+} or other multivalent cations. The electrostatic interactions between Ca^{2+} ions and the guluronic residues of alginates are the driving forces for gel formation.[1] The properties of the resulting gels (i.e., mechanical strength, porosity, biological properties, etc.) are highly dependent on the monomer composition (guluronic/mannuronic molar ratio, guluronic (Gul), or mannuronic (Man) block frequency), on the concentration of alginate in the gel and on the concentration and the nature of the cations used for gelation.[2,3]

Dilute gels (0.5–2% w/v) of Ca^{2+}-alginate have been frequently used for cell encapsulation[4] as 3D scaffolds for *in vitro* and *in vivo* immobilization systems in tissue engineering.[5–11] Encapsulation processes generally produce bioconstructs in the shape of beads of a diameter ranging from 0.1 to 1 or 2 mm. In other situations, like *in vivo* implant of cell/alginate solutions jellified *in situ* by addition of $CaCl_2$[12] or for use in specific bioreactors, the use of cylindrical bioconstructs of comparatively large size (5–15 mm in diameter and 3–6 mm in height) may turn out to be more advantageous than that of beads.

The chemical and physical characteristics of alginate types and those of the beads have been extensively investigated by SRIXE (synchrotron radiation induced X-ray emission)[13] and by nuclear magnetic resonance (NMR) techniques.[14,15] Similar investigations have not yet be done on gels and bioconstructs of macroscopic size in which homogeneity, porosity, and strength

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may be strongly modified, if compared with the bead situation, by the type of alginate used, by the concentration and diffusion properties of the jellifying ions.

NMR relaxometry is the study of the time evolution of the longitudinal and transverse components of the ^1H nuclei magnetization.

The present investigation was aimed at the evaluation of the NMR relaxometry method as a noninvasive procedure for the follow-up of cell cultures in 3D hydrogel scaffolds. The potential of this technique was tested by measuring the MR parameters (i.e., T_1 and T_2) on cylindrical alginate gels, on bioconstructs with chondrocytes entrapped in the gels, before and after a 30-day period culture, and in conditions of both normal and reduced weight as obtained in a rotary vessel system. Biochemical measurements have also been performed to quantify the biosynthetic activity of immobilized cells in different culture conditions.

MATERIALS AND METHODS

Humeral-scapular joints of mature pigs were collected at a slaughterhouse and transferred in ice to the laboratory. Articular cartilage was aseptically removed from the humeral proxymal head within 2 h from the sacrifice. Cells were then isolated by enzymatic digestion from thin slices of the tissue as already described[16] and kept in 10 m*M* HEPES, 140 m*M* NaCl, 5 m*M* KCl, 5 m*M* glucose, pH 7.4, without $CaCl_2$ until the moment of bioconstruct casting. Two sterile lots of alginate have been used in this study: SLM100 and SLG100 provided by FMC Biopolymer (Drammen, Norway). The former was characterized by a comparatively low guluronic acid content (44% of residues; $F_{GG} = 0.20$; $F_{MM} = 0{,}37$; $F_{GM+MG} = 0.43$, where F_{GG} is the frequency of blocks of guluronate, F_{MM} the frequency of mannuronate blocks, and F_{GM+MG} is the frequency of blocks of alternating guluronate and mannuronate residues), the latter by a higher guluronic acid content (64% of residues; $F_{GG} = 0.51$; $F_{MM} = 0{,}19$; $F_{GM+MG} = 0.30$), respectively; they both displayed a high apparent viscosity (183–187 mPa s). Alginate solutions were prepared dissolving the lyophilized powders to a 2 mg mL^{-1} concentration in a sterile physiological buffer (10 m*M* HEPES, 140 m*M* NaCl, 5 m*M* KCl, 5 m*M* glucose, pH 7.4).

Gels and bioconstructs were prepared at a final alginate concentration of 15 mg mL^{-1} by addition of suitable amounts of the dissolving buffer described above or by addition of a chondrocyte suspension in the same buffer. A concentration of 2×10^6 cells $\times$ (mL gel)$^{-1}$ have been used throughout this investigation.

In some reference gels, high M_w hyaluronate (M_w: 1,500,000) was added (at 0.1% w/v) to alginate solutions before gelation.

Gelation procedure was carried out by dialysis exposing the alginate solutions or the alginate–chondrocyte suspensions to 100 m*M* $CaCl_2$ in a 10 m*M* HEPES buffer pH 7.4, for 1 h at room temperature, in sterile conditions. In some experiments, gelation was performed utilizing dialysis tubing with a diameter of 6 mm and with an approximate protein cutoff of 12 kDa (Sigma Chemical, Steinheim, Germany). The gel rods obtained by this procedure were then regularly cut to obtain a series of small cylindrical samples (∅ 6 mm, height about 6 mm). For some experiments, when gels or bioconstructs of the same volume were needed, gelation was accomplished by exposing identical volumes (170 μL) of alginate or alginate-cell suspensions, to the $CaCl_2$ solutions in glass tubes (∅ 6 mm) sealed at one extremity by a dialysis membrane.

The alginate gels or the bio-constructs were kept, throughout the preparation and growth experiments until the NMR measurements or the biochemical analysis, in a culture medium (DMEM supplemented with 10% FCS, 2% glutamine, 500 μg/mL streptomycin, and 500 U/mL penicillin). When long-lasting experiments were carried out, the medium was changed for every 3 days.

Gels and bioconstructs were kept in reduced-weight conditions in a rotary cell–culture system (RCCS 4D; Synthecon, Houston). Up to 10 gels or bioconstructs, each of a 170-μL volume were hosted in the standard 50-mL disposable vessels. A 15-rpm rotation speed was adopted to keep the bioconstructs in free-fall conditions (reduced weight conditions).

Biochemical analysis

At the end of the culture time (30 days), the DNA, GAGs, and hydroxyproline content of each construct were quantified.

The standard procedure of Enobakhare et al.[17] was used for the determination of sulfated GAGs in the presence of alginate by 1,9-dimethylene blue dye. Cell number was estimated by the Hoechst 33258 DNA assay as reported by Rao and Otto.[18] Hydroxyproline was determined according to Hoemann et al.[19]

Histology

At the end of the experiments, the bioconstructs were initially incubated at ambient temperature for 30 min in a 5% BSA solution in HEPES buffer pH 7.4 containing 100 m*M* $CaCl_2$ and then fixed with neutral formalin for 4 h at 4°C. The pretreatment with BSA prevented the shrinking of the gels during the standard dehydration procedures. A final embedding in immuno-bed (Electron Microscopy Sciences, Washington) was done according to the manufacturer instructions. Toluidine blue and silver impregnation staining, for GAGs and collagen identification, were finally performed on 5-μm thick sections.

NMR relaxometry

Proton relaxation measurements were performed at 20 MHz (0.47 T) at 25°C by means of a home-built relaxometer equipped with NMR data station (Stelar, Pavia, Italy). The measurements began after the temperature of the sample had equilibrated with that of the probe. Add/subtract phase-cycled sequences were used in all the measurements, and measurements were accumulated for signal averaging. Relaxation delay was adequate for full magnetization to be reached after each sequence. Longitudinal relaxation data

TABLE I
Proton Relaxation Times T_1 and T_2 in Cylindrical Gels

Gel Composition	T_1 (ms)	T_2 (ms)
1.5% Alginate (64% gul)	1769 ± 77	99 ± 4*
1.5% Alginate (64% gul) + 0.1% hyaluronate	1820 ± 14	119 ± 10*,**
1.5% Alginate (44% gul)	1745 ± 84	78 ± 10*,**
1.5% Alginate (44% gul) + 0.1% hyaluronate	1784 ± 11	103 ± 3**

Proton relaxation times T_1 and T_2 in cylindrical gels (6 mm Ø × 6 mm height) of alginates at high (64%) or low content (44%) of guluronate residues with or without added hyaluronic acid (0.1%, M_w = 1,500,000). Data are the mean ± SD of, at least, four samples (differences between * or ** are significant, $p < 0.05$).

(relaxation time T_1 process) were taken by inversion-recovery (IR) sequences, and for each of 64 IR times the free induction decay (FID) signal was sampled and recorded at 2-μs intervals from 10 to 266 μs. The shortest IR time is usually 1 ms, with successive times increased by fixed factors, giving a maximum recovery time of 10 s. Preliminary measurements with the shortest available IR time demonstrated that 1 ms could be used without loosing any short T_1 component. Transverse relaxation data (relaxation time T_2 process) were taken by Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence at echo time TE = 200 μs and TE = 1 ms. No significant differences were observed.

Quasi-continuous relaxation time distribution analysis of the data was performed by UPEN,[20] which is based on a Uniform-PENalty (UPEN) inversion algorithm, using a regularizing or smoothing coefficient varying with relaxation time and determined by iterative negative feedback in such a way that the smoothing penalty, rather then the coefficient is roughly uniform. This algorithm reduces the generation of peaks not required by the data to be separated, which might be misinterpreted as physically meaningful resolved compartments.

The performances of the relaxometer and of the data processing were tested by repeating the whole set of measurements for doped water samples.

NMR microscopy (μMRI)

The μMRI experiments were performed at 24°C with a Bruker AM 300 console equipped with a standard microimaging unit and coupled to a Spectrospin vertical wide bore (WB) superconducting magnet, operating at 7.05 T (300 MHz for ^{1}H). High resolution images were obtained using a modified spin-echo sequence.[21] The repetition time between phase encoding steps was 1.5 s and an echo time of 34 ms led to the predominance of T_2 contrast. The matrix size of 128 × 128 and the field of view of 6 mm resulted in an in-plane resolution of 47 μm.

Statistical analysis

Statistical analysis was performed using a student t test. Values are expressed as mean ± standard deviation. Differences at $p \leq 0.05$ were considered statistically significant.

RESULTS

The influence of the difference in monomer composition of alginate hydrogels on the proton relaxation times T_1 and T_2 was tested in preliminary experiments.

T_1 and T_2 have been measured on gels prepared with two different lots of alginate: one characterized by a high (64%) and the other by a low (44%) guluronate residues content. In addition, a second series of measurement was carried out on the same types of gels but after addition of 0.1% of high M_w hyaluronate. For all samples, the quasi-continuous distributions of T_1 and T_2, not presented here, showed single peaks. To represent the distributions by a single parameter, the geometric mean relaxation times were computed, as $T_{ig} = \exp(\langle \ln T_i \rangle)$, with $i = 1, 2$ for longitudinal and transverse relaxation time, respectively. In the following, we shall refer to these compact average relaxation times simply as T_1 and T_2.

Results are summarized in Table I. The molecular differences on gel composition (i.e., the type of residues and the presence or absence of hyaluronate) do not affect significantly T_1 relaxation times. On the contrary, T_2 appears to be significantly increased upon an increase of the Gul/Man ratio in the alginate sample and by the addition of 0.1% hyaluronate to the gels.

In the following experiments, SLM100 alginate (at low Gul content) was used for the preparation of gels or bioconstructs with entrapped chondrocytes. This

TABLE II
DNA, Collagen, and GAG Concentration in Bioconstructs

Time and Type of Culture	DNA (μg/gel mL)	GAGs (mg/gel mL)	Hydroxyproline (μg/gel mL)
Control	79.4 ± 17.9	n.d.	0.75 ± 0.21
30 days, stationary	55.8 ± 12.3	1.87 ± 0.09*	1.09 ± 0.19
30 days, rotary vessels	62.18 ± 26.4	2.43 ± 0.29*	0.56 ± 0.19

DNA, collagen, and GAG concentration in bio constructs before culture (controls) and after 30 day of culture in normal weight (stationary) conditions and reduced weight (rotary vessels) conditions. Data are reported as average ± SD of at least seven determinations; n.d. = not detectable (* are significant differences, $p < 0.05$).

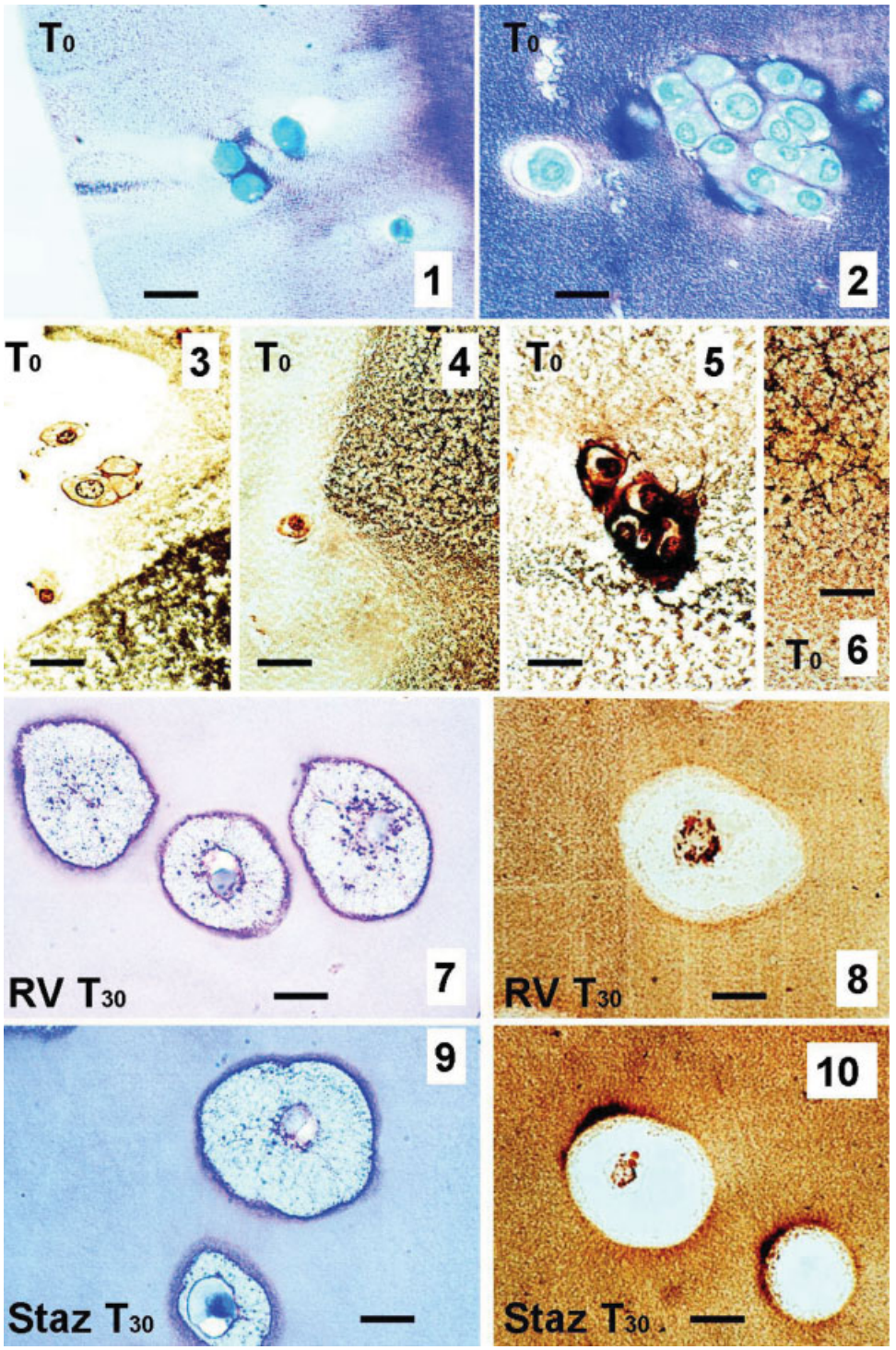

Figure 1. Histology of bioconstructs before and after 30 days of culture in normal and reduced weight conditions. Toluidine blue and silver impregnation staining were performed for evidencing GAGs and collagen in controls, before culture,[1–6] and after 30 days of culture in reduced weight conditions (RV, rotary vessels, 7,8) and in stationary conditions (Staz, 9–10)[1–6]: chondrocytes entrapped in alginate appear isolated or in groups of 2 or more elements. Alginate has a non-homogeneous appearance both in toluidine-blue or in silver-impregnation staining[7,8] and [9,10] correspond to RV and stationary cultures, respectively. Chondrocytes has carved a hole into the alginate gel, which was incompletely filled with proteoglycans.[7,9] Only alginate seems to be present outside. Chondrocytes show many cytoplasmic inclusions as a clear signal of phagocytic activity against alginate. In silver-stained sections,[8,10] collagen II is not detectable outside the cells, lending to conclude that it is not produced; the extracellular staining is due to the silver-alginate nonspecific reaction. Bars in Figure 1[1–10] represent 10 μm.

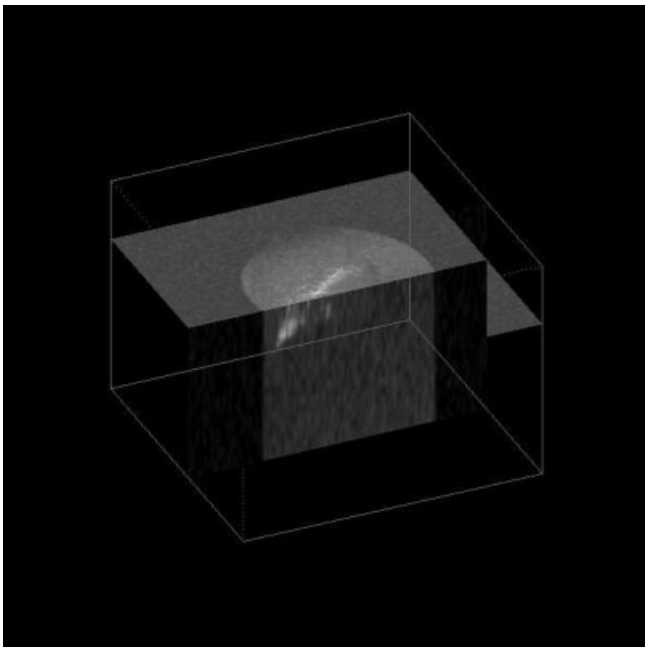

Figure 2. 3D MR microimage at 7 Tesla of a cylindrical bioconstruct cultured for 30 days in normal weight conditions (stationary cultures). The hyperintense signal present into the gel is given by a developing cartilagelike nodule. This type of nodule was not observed when bioconstructs were cultured in reduced weight conditions in rotary vessels.

choice was dictated by the well known property of alginates at high guluronic acid content to give stiff and brittle gels, which frequently hinder the growth and survival of encapsulated cells. As described in the Materials and Methods section, the proton relaxation times have been determined on gels, both with and without entrapped chondrocytes, under those conditions: (a) immediately after the gel formation, (b) after 1 month culture in stationary conditions (normal weight conditions), and (c) after 1 month culture in rotary vessels in free fall conditions (reduced weight conditions).

DNA, GAGs, and hydroxyproline (from collagen) content of bioconstructs was also measured before and after 30 days in culture. Results are reported in Table II. During the culture period, in our experimental conditions, the DNA content of gels does not change significantly, suggesting absence of proliferation. The GAG concentration increases more in reduced condition than in normal weight conditions; moreover, in reduced weight conditions, the collagen biosyntesis is blocked while a slight increase can be observed in normal weight conditions.

GAG production and failure of collagen biosynthesis under reduced weight conditions were demonstrated also by histology (Fig. 1).

A common finding in the cylindrical bio-constructs cultured for 30 days in normal weight conditions was the formation of cartilagelike tissue nodules of relevant size (up to 2–3 mm in lenght). 3D micro images thereof, collected at 7 Tesla, produced a hyper intense signal (Fig. 2). This type of nodule was never observed when bioconstructs were cultured in reduced weight conditions in rotary vessels.

It is long known that macromolecular protons can contribute to the FID signal from biological tissues,[22] and particularly, in normal cartilage,[23,24] and in bone.[25] In the latter, they contribute with a "solidlike" component that can be approximated by the form $\exp[-[1/2](T/T_{GC})^2]$, where T is time after the end of the 90° pulse and T_{GC} is the Gaussian decay time. Despite the fact that the FID signal could not reliably be used before 10 μs, in our previous investigations on cartilage and bone samples, we were able to observe the approximate Gaussian component, which was interpreted due to the protons of the fibers of mature collagen.[24,25] On the contrary, in the bioconstructs of this study, we never observed the "solidlike" component, so confirming that mature collagen fibers were either not present or present at a concentration too low to be detectable.

The relaxation times T_1 and T_2 of gels and bioconstructs (cast with an alginate of the same type by from a batch different from that used to obtain the data reported in Table I) have been measured and their distributions have been determined by quasi-continuous distribution analysis. The geometric mean times of the distributions have also been calculated and are collected in Table III. T_1 and T_2 of the gels show only minor variations after 1 month of culture both in normal and in reduced weight condi-

TABLE III
Relaxation times T_1 and T_2 as measured on gels and bioconstructs

		Before Culture	30 Days in Normal Weight Conditions	30 Days in Reduced Weight Conditions
Gels	T_1 (ms)	2350 ± 100	2540 ± 30	2490 ± 80
	T_2 (ms)	195 ± 20	207 ± 8	203 ± 4
Bio constructs	T_1 (ms)	2280 ± 30	2190 ± 30	2380 ± 30
	T_2 (ms)	170 ± 20	170 ± 20*	208 ± 8*

Relaxation times T_1 and T_2 as measured on gels and bio constructs: before and after 30 days culture period at in normal and reduced weight conditions.Data are reported as average ± SD of at least seven determinations; * are significant differences, $p < 0.05$.

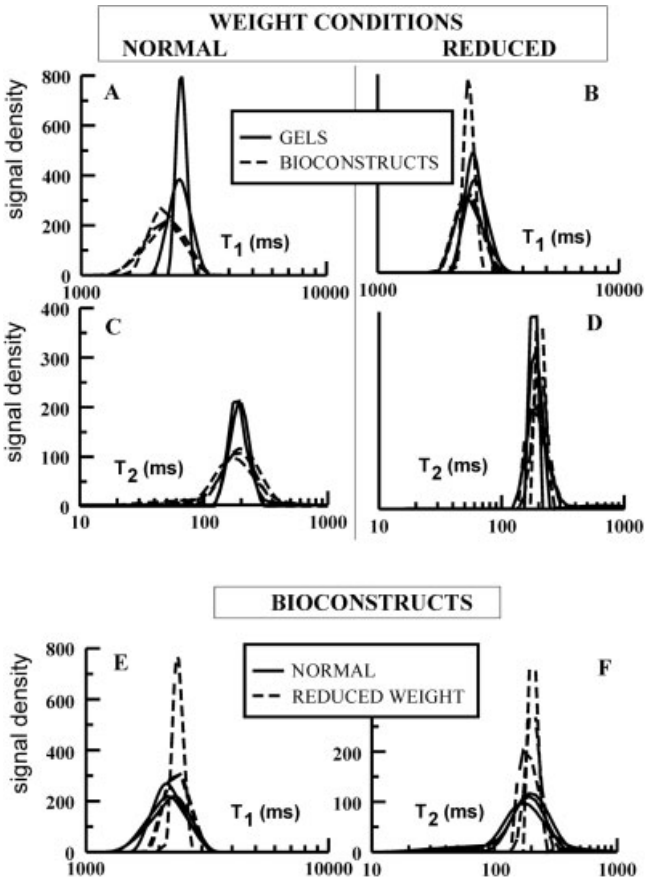

Figure 3. Relaxation time distributions obtained by UPEN for gels (solid lines) and bioconstructs (dashed lines) after 30 days: (a) T_1 in normal weight conditions, (b) T_1 in reduced weight conditions, (c) T_2 in normal weight conditions, and (d) T_2 in reduced weight conditions. All distributions are normalized to unit area. The widths of the distributions for bioconstructs after 30 days in normal weight conditions are wider than the widths due to the noise.

tions (in Table III, T_1 of the gels after 30 days in normal weight conditions is the average value of only two data, so the differences have no statistical significance). On the contrary, a clear effect appears for T_2 of bioconstructs, while T_1 show a very modest decrease (if any) after 30 days of culture of bioconstructs in normal weight conditions and a similar modest increase (if any) after the same period in reduced weight conditions, T_2 remains unchanged after culture in normal weight conditions but increases by about 20% after 30-day culture in rotary vessels.

The distributions of the relaxation times are reported in Figure 3. After culture in normal weight conditions [Fig. 3(A,C)], both the T_1 and T_2 distributions for bioconstructs appear wider then for gels alone. On the contrary, after culture in reduced weight conditions [Fig. 3(B,D)], the distributions of T_1 and T_2 of bioconstructs and gels appear similar. The differences observed for bioconstructs is better seen in Figure 3(E,F) and the peaks of both T_1 [Fig. 3(E)] and T_2 [Fig. 3(F)] of bioconstructs kept in normal weight conditions (stationary cultures) appear wider than those of bioconstructs cultured in reduced weight conditions (rotary vessels). Moreover a shift for longer T_2 times is observed expecially for T_2 in reduced gravity, when compared with normal gravity conditions (in agreement with the data of Table III).

DISCUSSION

Polysaccharide hydrogels are widely used for the preparation of bioresorbable scaffolds for tissue engineering [e.g., Refs. 26 and 27]. Alginate gels, in particular, have been employed for encapsulation and culture of chondrocytes *in vitro* and for *in vivo* implantation in defective cartilages.[12,,28,29] The *in vitro* or *ex vivo* growth of new tissue was monitored in the past also by NMR microscopy.[12,28]

The present investigation was undertaken to evaluate the possibility of a follow-up of tissue regeneration by a noninvasive NMR method based on the spatially nonresolved relaxation of ^{1}H nuclei. The efficiency of the method was tested by performing a first characterization of the NMR properties of alginate gels of different composition (i.e., different Gul/Man ratio or ± hyaluronic acid), and then of those of the bioconstructs obtained by entrapment of chondrocytes in the gels, before and after a 30-day culture in conditions of both normal and reduced weight as obtained in a rotary vessel system. Longitudinal and transverse relaxation times (T_1 and T_2) of the various preparations have been measured and evaluated. Biochemical measurements have also been performed to quantify the biosynthetic activity of cells immobilized within the constructs.

In principle, the main events expected to occur during the long periods (months) required for the *in vitro* development of a new tissue appear to be a progressive degradation of the supporting gels, possibly because of the concurrent action of chondrocyte enzymes and by mechanical stresses, and an increase of the number of cells and the accompanying biosynthesis and excretion of matrix components, namely collagen type II and proteoglycans.

Is it possible to use relaxometry to obtain supporting evidence of gel degradation?

At low alginate concentration (1.5%), significantly higher T_2 values have been recorded from the gels formed using alginates at high-guluronic acid content respect to those formed with alginate at low-guluronic acid content. The addition of high M_w hyaluronate at a concentration similar to that existing in cartilage (0.1%) to give interpenetrated gels, also

promotes an increase of T_2 with respect to alginates alone (20–30%) with both types of alginates.

As well known, several factors can affect relaxation time. Both T_1 and T_2 are sensitive to water-macromolecules interactions and exchanges, to molecular dynamics, to bound/free water ratio, and to surface-to-volume ratio of the pore space where water molecules can diffuse before relaxing. Our results are in line with those of Simpson et al.[30] who suggested that guluronic acid-rich alginate generates gels of pores larger than those formed by using mannuronic acid-rich alginates. In our opinion, the compositional differences between the two polysaccharide samples (high G or high M alginates) are able to induce a different behavior with divalent cations and could hence lead in the case of high M alginate to a more compact-gel structure.

This fact may become more relevant when, as in our experiments, high viscosity (i.e., high Mw) alginates are utilized for gel formation in the presence of high Ca^{2+} concentration (100 m*M*). The rigidity of high M_w structures, the number of Ca^{2+}-alginate (guluronate) interactions influence the three-dimensional architecture of the macromolecular network. On the basis of this interpretation, the behavior of T_2 also suggests that a component of large hydrodynamic volume and negative charge, as hyaluronan, might, to some extent, impair calcium binding by alginates modifying the resultant structure of the final gels. Whether this fact only stems from an increase of the dimensions of the pores or from a different type of 3D architecture of the gel because of the additional polymer polymer interactions is still to be ascertained.

In any case, T_2 appears to be a useful parameter to follow changes of the scaffolds.

The follow-up of low guluronate-alginates gels (without encapsulated cells) for 1 month in normal weight conditions or in rotary vessels by measuring T_1 and T_2, both unchanged after this period, indicates that the scaffolds remain essentially unchanged showing a very limited degradation, if any, in a physiological culture medium.

In the same culture period, gels with encapsulated chondrocytes (bioconstructs) show minimal variations in their T_1 values both in stationary and in reduced weight conditions (rotary vessel). In addition, while the T_2 values measured on bioconstructs maintained in normal weight conditions of culture remain constant, those obtained for bioconstructs cultured in rotary vessels are significantly increased (+20%). The distribution of both T_1 and T_2 of bioconstructs after 30 days of culture in reduced weight conditions show a narrower distribution than after the same period of time in normal weight conditions.

The biochemical and histochemical investigation on the cultured bioconstructs gave the unexpected information that in this type of hydrogels (high-mannuronic alginate of high Mr and high initial viscosity) chondrocytes are unable to duplicate nor are stimulated to produce collagen. In addition, they produce more GAGs in rotary vessels cultures than in normal weight conditions. Similar results have been reported by Pei et al.[31] for GAG production in rotary vessels using various scaffold materials (Hyaff 11® and PGA, in different physical forms). At variance with alginate, in this case, chondrocytes synthesized significant amount of type II collagen.

High mannuronic alginate are reported as supporting βTC3 cell growth.[32–34] In our experiments, the chondrocyte growth is hindered. This may be because of differences in cell biology or, more probably, to the fact that the high M_w alginates (of high initial viscosity) can give stiff hydrogels of low degradability unable to favor cell growth.

A possible explanation of the observed increase of the T_2 values in reduced weight condition of culture (rotary vessels) can be suggested. Chondrocytes present many cytoplasmic inclusions as a clear signal of an active phagocytic activity possibly against alginate (Fig. 1). The cutting of critical structures and a modest loss of jellifying Ca^{2+} ions can lead to reduced gel stiffness. The holes that the cells carved into the alginate matrix were only incompletely, albeit homogenously, filled with glicosaminoglycans while the lack of collagen synthesis hindered the building of a physiological matrix. The homogeneity of this situation is indicated by the distributions of the relaxation times T_1 and T_2, which are narrow for samples grown in reduced weight conditions (Fig. 3).

The fact that the mean T_2 of bioconstructs kept in normal weight conditions (stationary cultures) remains unchanged after a 1-month culture can be the accidental consequence of an anisotropic distribution of material in the constructs. In fact, the phagocytic activity of chondrocytes was accompanied by a partial filling of the lacunae with glycosaminoglycans, which were also produced in a lower amount, when compared with what happens in the rotary vessels. In addition, this type of culture developed an opalescent accumulation of the produced GAGs in specific zones of the constructs (Fig. 2). The consequence of an irregular scaffold structure and of the local concentration of neosynthesized material is the likely explanation of the wider relaxation time distributions (both T_1 and T_2) observed in normal weight conditions, when compared with the narrower distributions observed for the more homogeneous rotary vessels cultures (Fig. 3).

In conclusion, the following information have been obtained:

> Spatially nonresolved NMR relaxometry can be an efficient noninvasive technique useful in the study of biodegradable scaffolds in tissue engineering science.

NMR relaxometry can give information about the 3D structure of hydrogels and about the possibility to modify them by the addition of quantitatively minor components (as high M_w hyaluronic acid).
In conditions of reduced weight, GAGs neosynthesis is enhanced when compared with normal weight condition.
The lack of a significant collagen production in both situations may be because of the fact that in stiff alginate scaffolds the synthesis of collagen can be blocked. Alternatively, 1 month of culture can be a too short period to observe *in vitro* the formation of a correct extracellular matrix.

It may be supposed that in long-term experiments, as *in vitro* maintenance for few months, the formation of a developing tissue can be monitored from a change of T_2 relaxation time parallel to the development of the new hydrogel. The latter, that is, the neosynthesized extracellular matrix, will be characterized by a different chemical composition. In addition, if the expected physiological collagen synthesis occurs, then also a FID component of "solidlike" protons will possibly be observed. Watrin-Pinzano et al.[28] observed only after 60 days from *in vivo* regenerating rat patellae, a collagen network organization by means of zonal variations in T_2 maps.

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References

1. Grant GT, Morris ER, Rees DA, Smith PJC, Thom D. Biological interactions between polysaccharides and divalent cations: The egg-box model. FEBS Lett 1973;32:195–198.
2. Stokke BT, Draget KI, Yuguchi Y, Urakawa H, Kajivara K. Small angle X-ray scattering and rheological characterization of alginate gels. Macromol Symp 1997;120:91–101.
3. Martinsen A, Skjåk-Bræk G, Smidsrod O. Alginate as immobilization material. I. Correlation between chemical and physical properties of alginate gel beads. Biotechnol Bioeng 1989;33:79–89.
4. Smidsrod O, Skjåk- Bræk G. Alginate as immobilization matrix for cells. Trends Biotechnol 1990;8:71–78.
5. Eiselt P, Yeh J, Latvala RK, Shea LD, Mooney DJ. Porous carriers for biomedical applications based on alginate hydrogels. Biomaterials 2000;21:1921–1927.
6. Marijnissen WJ, van Osch GJ, Aigner J, van der Veen SW, Hollander AP, Verwoerd-Verhoef HL, Verhaar JA. Alginate as a chondrocyte-delivery substance in combination with a non-woven scaffold for cartilage tissue engineering. Biomaterials 2002;23:1511–1517.
7. Chung TW, Yang J, Akaike T, Cho KY, Nah JW, Kim SI, Cho CS. Preparation of alginate/galactosylated chitosan scaffold for hepatocyte attachment. Biomaterials 2002;23:2827–2834.
8. Wang L, Shelton RM, Cooper PR, Lawson M, Triffitt JT, Barralet JE. Evaluation of sodium alginate for bone marrow cell tissue engineering. Biomaterials 2003;24:3475–3481.
9. Hsu SH, Whu SW, Hsieh SC, Tsai CL, Chen DC, Tan TS. Evaluation of chitosan-alginate-hyaluronate complexes modified by an RGD-containing protein as tissue-engineering scaffold for cartilage regeneration. Artif Organs 2004;28:693–703.
10. Ueng SW, Yuan LJ, Lee N, Lin SS, Chan EC, Weng JH. In vivo study of biodegradable alginate antibiotic beads in rabbits. J Orthop Res 2004;22:592–599.
11. Sambanis A, Papas KK, Flanders PC, Long RC Jr., Kang H, Costantinidis I. Towards the development of a bioartificial pancreas: immunoisolation and NMR monitoring of mouse insulinomas. Cytotechnology 1994;15:351–363.
12. 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.
13. Thu B, Skjåk- Bræk G, Micali F, Vittur F, Rizzo R. The spatial distribution of calcium in alginate gel beads analysed by synchrotron-radiation induced X-ray emission (SRIXE). Carbohydr Res 1997;297:101–105.
14. Grasdalen H. High field ^{1}H NMR spectroscopy of alginate: sequential structure and linkage conformations. Carbohydr Res 1983;118:255–260.
15. Potter K, Balcom BJ, Herrod NJ, Carpenter TA, Hall LD. The gelation of sodium alginate with calcium ions studied by magnetic resonance imaging (MRI). Carbohydr Res 1994;257: 117–126.
16. Grandolfo M, D'Andrea P, Paoletti S, Martina M, Silvestrini G, Bonucci E, Vittur F. Culture and differentiation of chondrocytes entrapped in arginate gels. Calcif Tissue Int 1993;52:42–48.
17. Enobakhare BO, Bader DL, Lee DA. Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures by use of 1,9-dimethylmethylene blue. Anal Biochem 1996;243:189–191.
18. Rao J, Otto WR. Fluorimetric DNA assay for cell growth estimation. Anal Biochem 1992;207:186–192.
19. Hoemann CD, Sun J, Chrzanowski V, Buschmann MD. A multivalent assay to detect glycosaminoglycans, protein, collagen, RNA, and DNA content in milligram samples of cartilage or hydrogel-based repair cartilage. Anal Biochem 2002;300:1–10.
20. Borgia GC, Brown RJS, Fantazzini P. Uniform-penalty inversion of multiexponential decay data. II. Data spacing, T2 data, systematic data errors, and diagnostics. J Magn Reson 2000;147:273-85. ibid. 1998;132:65–77.
21. Hsu EW, Schoeninger JS, Bowtell R, Aiken NR, Horsman A, Blackband SJ. A modified imaging sequence for accurate T2 measurements using NMR microscopy. J Magn Reson Ser B 1995;109:66–69.
22. Edzes HT, Samulski ET. The measurement of cross-relaxation effects in the proton NMR spin-lattice relaxation of water in biological systems: Hydrated collagen and muscle. J Magn Reson 1978;31:207–229.
23. Lattanzio P-J, Marshall KW, Damyanovich AZ, Peemoeller H. Macromolecule and water magnetization exchange modeling in articular cartilage. Magn Reson Med 2000;44:840–851.
24. Tessier JJ, Dillon N, Carpenter TA, Hall LD. Interpretation of magnetization transfer and proton cross-relaxation spectra of biological tissues. J Magn Reson Ser B 1995;107:138–144.
25. Fantazzini P, Bortolotti V, Brown RJS, Camaiti M, Garavaglia C, Viola R, Giavaresi G. Two ^{1}H NMR methods to measure internal porosity of bone trabeculae: By solid–liquid signal separation and by longitudinal relaxation. J Appl Phys 2004;95:339–344.
26. Aufderheide AC, Athanasiou KA. Comparison of scaffolds and culture conditions for tissue engineering of the knee meniscus. Tissue Eng 2005;11:1095–1104.
27. Levesque SG, Lim RM Shoichet MS. Macroporous interconnected dextran scaffolds of controlled porosity for tissue-engineering applications. Biomaterials 2005;26:7436–7446.

28. Watrin-Pinzano A, Ruaud JP, Cheli Y, Gonord P, Grossin L, Bettenbourg-Brault I, Gillet P, Payan E, Guillot G, Netter P, Loeuille D. Evaluation of cartilage repair tissue after biomaterial implantation in rat patella by using T2 mapping. MAGMA 2004;17:219–228.
29. Miyamoto K, An HS, Sah RL, Akeda K, Okuma M, Otten L, Thonar EJ, Masuda K. Exposure to pulsed low intensity ultrasound stimulates extracellular matrix metabolism of bovine intervertebral disc cells cultured in alginate beads. Spine 2005;30:2398–2405.
30. Simpson NE, Grant SC, Blackband SJ, Costantinidis I. NMR properties of alginate microbeads. Biomaterials 2003;24:4941–4948.
31. Pei M, Solchaga LA, Seidel J, Zeng L, Vunjak-Novakovic G, Caplan AI, Freed LE. Bioreactors mediate the effectiveness of tissue engineering scaffolds. FASEB J 2002;16:1691–1694.
32. Costantinidis I, Rask I, Long RC Jr, Sambanis A. The effects of alginate composition on the metabolic, secretory, and growth characteristics of entrapped β TC3 mouse insulinoma cells. Biomaterials 1999;20:2019–2027.
33. Stabler CL, Wilks K, Sambanis A, Costantinidis I. Effects of alginate composition on encapsulated β TC3 cells. Biomaterials 2000;22:1301–1310.
34. Stabler CL, Sambanis A, Costantinidis I. Effects of alginate composition on the growth and overall metabolic activity of beta TC3 cells. Ann NY Acad Sci 2002;961:130–133.